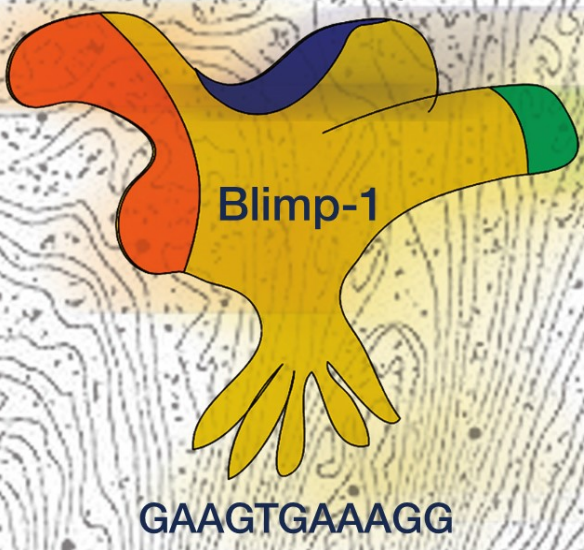
H. Singh R. Grosschedl (Eds.)

Molecular Analysis of B Lymphocyte Development and Activation



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H. Singh and R. Grosschedl (Ed.)

Molecular Analysis of B Lymphocyte Development and Activation

With 28 Figures, 15 in Color

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Preface

The B lymphocyte lineage represents a leading system for exploring molecular mechanisms that underlie cell fate specification, differentiation and cellular activation. In the past five years major advances have been achieved in the analysis of early B cell development, AID dependent class switch recombination as well as somatic hypermutation and Blimp-1 regulated plasma cell differentiation. Many of these findings and their implications are covered in this volume. Two emergent areas of research that are included in the contributions focus on the pre-BCR and Ikaros-family proteins. The pre-BCR is an unusual molecular device that is used to execute a critical developmental checkpoint in the B lineage. Its mechanism of action in relation to the pre-TCR and the mature antigen receptors (BCR, TCR) is of considerable interest. Ikaros-family proteins appear to function via recruiting target genes to domains of centromeric heterochromatin in the nucleus. Initially discovered in lymphocytes, they represent a novel system of gene regulation via nuclear compartmentalization. Finally, the volume includes a chapter on *Wnt* signaling in lymphopoiesis. Analysis of this evolutionally conserved pathway which regulates cellular proliferation and differentiation in diverse developmental contexts benefited enormously from the discovery of the LEF/TCF family of factors in lymphocytic lineages.

This volume is dedicated to the memory of Eugenia Spanopoulou, a colleague and a highly valued member of our scientific community. It contains a chapter on the biochemistry of V(D)J recombination that Eugenia co-authored with David Schatz.

Obituary

Eugenia Spanopoulou was an extraordinary person and scientist. Her enormous intelligence and energy sparked important scientific discoveries, and vaulted her to a point of breathtaking potential—a potential abruptly erased at the age of 37 with the crash of Swissair flight 111 on September 3, 1998. Lost with her were her husband, Andrew Hodtsev, and young son, Platon.

Eugenia was passionately interested in understanding the molecular basis of development and chose as her model system the development of B and T lymphocytes. She began by studying the transcriptional regulation of the T cell-specific gene, Thy-1, as a graduate student in Frank Grosveld's lab at Mill Hill, London. Thereafter, she turned her attention to the topic of V(D)J recombination, first as a postdoctoral fellow with David Baltimore at the Whitehead Institute in Cambridge, Massachusetts and subsequently in her own laboratory at Mount Sinai School of Medicine in New York City. Eugenia made fundamental discoveries concerning the biochemical mechanism of this reaction, and how defects in its central enzymatic components, RAG1 and RAG2, can lead to a human severe combined immunodeficiency disorder, known as Omenn syndrome. At the time of her death, she had been a Howard Hughes Medical Institute investigator for less than a year, but had already assembled a laboratory of 15 people working on an extensive array of topics in early lymphocyte development and V(D)J recombination.

Eugenia lived each moment of her life with an intensity fitting for the city in which she lived. Like Manhattan, she slept little. She expected a great deal of herself, and only a little less from those with whom she worked. Eugenia also had high expectations for the scientific process and was outspoken in her praise or condemnation of those who met or fell short of those expectations. She was quick to form an opinion and devoted herself ferociously to friendships and hypotheses.

Perhaps the greatest testimony to Eugenia is the affection and reverence felt for her by the members of her lab. While this stemmed in no small part from predictable sources—her keen mind and broad knowledge—it found its deepest source in Eugenia's ability to transmit, by example and word, her love of a life of learning and exploration. Sandro Santigata, a graduate student in Eugenia's lab, captured this eloquently at the memorial service for Eugenia and Andrew, when he said:

"If you have ever been spellbound by a great statue, enraptured by its strength and vitality, enthralled by its purity, purpose and grace and uplifted by the sense of hope that it sparks within your soul, then you have already understood why I adored Eugenia. She simply embodied the idealistic principles that form the core of a dedicated graduate student's heart. And to see these values materialized in the form of one's mentor can be nothing short of inspirational."

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List of Contributors

(Their addresses can be found at the beginning of their respective chapters.)

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Gene Regulatory Networks Orchestrating B Cell Fate Specification, Commitment, and Differentiation

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Abstract The B cell developmental pathway represents a leading system for the analysis of regulatory circuits that orchestrate cell fate specification, commitment, and differentiation. We review the progress that has been achieved in the identification and characterization of regulatory components of such circuits, including transcription factors, chromatin modifying proteins, and signaling molecules. A comprehensive developmental model is proposed that invokes sequentially acting regulatory networks which dictate the generation of B cells from multipotential hematopoietic progenitors.

1 Introduction

Cell fate specification in the hematopoietic system appears to involve the resolution of multi-lineage programs of gene expression and is enabled by the concomitant activation and silencing of lineage-specific subsets of genes. Signaling molecules, chromatin modifying complexes and transcription factors can be regarded as components of gene regulatory networks, which orchestrate cell fate specification, commitment and differentiation. Two cytokine receptors (Flk2/Flt3 and IL-7R) and six transcription factors (PU.1, Ikaros, E2A, Bcl11a, EBF, and Pax-5) are critical for the development of B cell precursors (Georgopoulos et al. 1994; Scott

et al. 1994; Bain et al. 1994; Zhuang et al. 1994; Nutt et al. 1997; Peschon et al. 1994; Lin and Grosschedl 1995; Mackarenhtschian et al. 1995; Liu et al. 2003). A recent study has also demonstrated a role for a microRNA in the generation of B cells from stem cells (Chen et al. 2004). It is well established that B cell development proceeds through an ordered set of intermediates that can be identified on the basis of cell surface markers and the expression of a characteristic set of B lineage genes encoding components of the B cell antigen receptor. Accompanying these developmental transitions are alterations in the nuclear compartmentalization and chromatin structure of immunoglobulin (Ig) heavy and light chain loci resulting in their stage-specific DNA rearrangements. In this review we attempt to assemble molecular circuits that regulate key transitions in B cell development, integrating the roles of signaling molecules, transcription factors, and chromatin-modifying proteins.

2
Specification and Commitment to the B Cell Fate

All blood cells are derived from a rare population of hematopoietic stem cells (HSCs). Considerable progress is being made in analyzing the molecular circuitry that instructs stem cell self-renewal vs differentiation. Importantly, HSC express at low levels many lineage-specific genes reflecting a developmentally poised state (Terskikh et al. 2003). The differentiating progeny of the HSC is the multipotential progenitor (MPP) or short-term repopulating cell. In contrast to the HSC, the MPP has only limited ability to generate all of the blood lineages (Wiesmann et al. 2000). Expression of CD27 within the stem cell compartment correlates with loss of long-term stem cell function in vivo and permits resolution of MPPs from stem cells. Single-cell RT-PCR analyses demonstrate that both HSC and MPPs exhibit wider multilineage gene expression patterns than their progeny, the lymphoid and myeloid progenitors, which express more restricted sets of genes (Miyamoto et al. 2002). The molecular mechanisms by which multipotential progenitors fine tune their gene expression patterns resulting ultimately in specification of distinct cell fates is an area of intense research in developmental biology.

Results from several studies have recently established that the molecular circuitry driving the lymphoid vs myeloid cell fate decisions is activated within the MPP population. One set of studies stemmed from the observation that a small fraction of cells within the MPP population were uniquely sensitive to a negative regulator of B and T lymphopoiesis, es-

trogen (Medina and Kincade 1994). Expression of terminal deoxynucleotidyl transferase (TdT), the enzyme responsible for addition of non-template encoded nucleotides during IgH recombination, was found to be associated with the estrogen-sensitive subset of CD27+ MPPs (Medina et al. 2001). Subsequent functional analysis of a similar population using a Rag-1/GFP knockin mouse revealed that CD27+ Rag-1/GFP+ MPPs could give rise to B, T and NK cells but had dramatically reduced erythroid and myeloid differentiation potential in vitro and in vivo (Igarashi et al. 2002). Furthermore, these cells expressed lymphoid-specific genes and evidenced D-J IgH gene rearrangements, unlike their CD27+ GFP– counterparts. This population is now designated ELP, for early lymphoid progenitor. Another study found that expression of the receptor tyrosine kinase Flk2/Flt3 within the MPP population correlated with loss of stem cell and myeloid lineage potential, but sustained lymphoid reconstitution in vivo (Adolfsson et al. 2001). Targeted inactivation of the Flk2/Flt3 gene results in defects in MPPs with a particularly severe deficiency in the generation of B-lineage progenitors (Mackarenhtschian et al. 1995). Consistent with the requirement for Flk2/Flt3 signaling in the development of B-lineage progenitors is the significant decrease in lymphoid progenitors observed in mice deficient in the Flk2/Flt3 ligand (Sitnicka et al. 2002). Taken together, these results strongly suggest that specification of the B-lymphoid cell fate initiates within the MPP population. B cell fate determination within the MPP compartment is likely to be initiated by the transcription factors E2A and EBF (see below) and appears to be accompanied by the downregulation of the erythromyeloid transcription factors GATA-1 and GATA-2 (Igarashi et al. 2002).

The signaling pathway through which Flk2/Flt3 selectively favors the generation of B-lineage progenitors is unknown, but in vitro data suggest that ligation of this receptor promotes expression of the interleukin-7 receptor (IL-7R) (Borge et al. 1999). However, as IL-7R+ progenitors (CLPs) give rise to T lineage and NK precursors in addition to B cell precursors, Flk2/Flt3 and IL-7R signaling must selectively activate the B cell developmental program. Interestingly, two recent studies revealed that combined loss of Flk2/Flt3 and IL-7R results in the failure to develop B-lineage cells both during fetal and adult hematopoiesis (Sitnicka et al. 2003; Vosshenrich et al. 2003). These results raise the possibility that combinatorial signaling through these receptors may activate the expression or modulate the activity of key transcriptional regulators such as E2A or EBF, which are required for specification of B cell fate. Analysis of the regulation of Flk2/Flt3 and IL-7R gene expression is likely to pro-

vide insight into the earliest circuitry underlying B cell development. Interestingly, Ikaros-null or Ikaros DN$^{-/-}$ hematopoietic progenitors are deficient in expression of Flk2/Flt3 (Nichogiannopoulou et al. 1999). Furthermore, PU.1$^{-/-}$ fetal liver hematopoietic progenitors also exhibit reduced Flk2/Flt3 transcripts (DeKoter et al. 2002). In addition to the defect in Flk2/Flt3 expression, PU.1$^{-/-}$ fetal liver hematopoietic progenitors are impaired in expression of IL-7R (DeKoter et al. 2002; Medina et al. 2004). PU.1 is implicated in directly regulating the transcription of the IL-7R alpha chain gene (DeKoter et al. 2002; Medina et al. 2004). Thus the severe reduction in B-lymphoid progenitors caused by the *PU.1*$^{-/-}$mutation is likely due to failed expression of both the Flk2/Flt3 and IL-7R receptors (Medina et al. 2004). Collectively, these data suggest that expression of Flk2/Flt3 in MPPs may depend on the concerted activities of Ikaros and PU.1, whereas PU.1 functions with other regulator(s) in promoting IL-7R expression in lymphoid progenitors (see model in Fig. 1).

Specification of B cell fate is characterized by the expression of genes encoding components of the B cell antigen receptor (mb-1, B29, λ5, and VpreB) and rearrangement of the immunoglobulin heavy-chain locus (IgH) (Hardy et al. 1991; Li et al. 1993). The transcription factors E2A and EBF are required for specification of B cell fate. The E2A gene encodes two basic helix-loop-helix proteins, E12 and E47, generated by differential splicing, whose activities are induced during B cell development (Murre et al. 1989; Sun and Baltimore 1991). EBF is an atypical helix-loop-helix zinc finger protein that is expressed exclusively in the B lineage within the hematopoietic system (Hagman et al. 1993). Targeted inactivation of the E2A or EBF gene results in a block in B cell development prior to the onset of early B-lineage gene expression and the initiation of D-J rearrangements at the IgH locus (Bain et al. 1994; Zhuang et al. 1994; Lin and Grosschedl 1995). The observation that either E2A or EBF can induce specific D-J_H rearrangements in a non-B cell line when ectopically expressed with the recombinase activating genes rag-1 and rag-2 suggests direct roles for these transcription factors in IgH recombination (Romanow et al. 2000). Regulation of D-J rearrangements in the IgH locus is likely dependent on E2A binding sites in the intronic enhancer Quong et al. 2002). Targeted deletion of the E2A gene results in severely diminished transcription initiated within the intronic enhancer as well as loss of EBF and RAG gene expression (Bain et al. 1994). Recently it was shown that restoration of EBF expression in E47$^{-/-}$ fetal liver hematopoietic progenitors promotes the generation of B cell precursors (Seet et al. 2004). These results support the contention that expres-

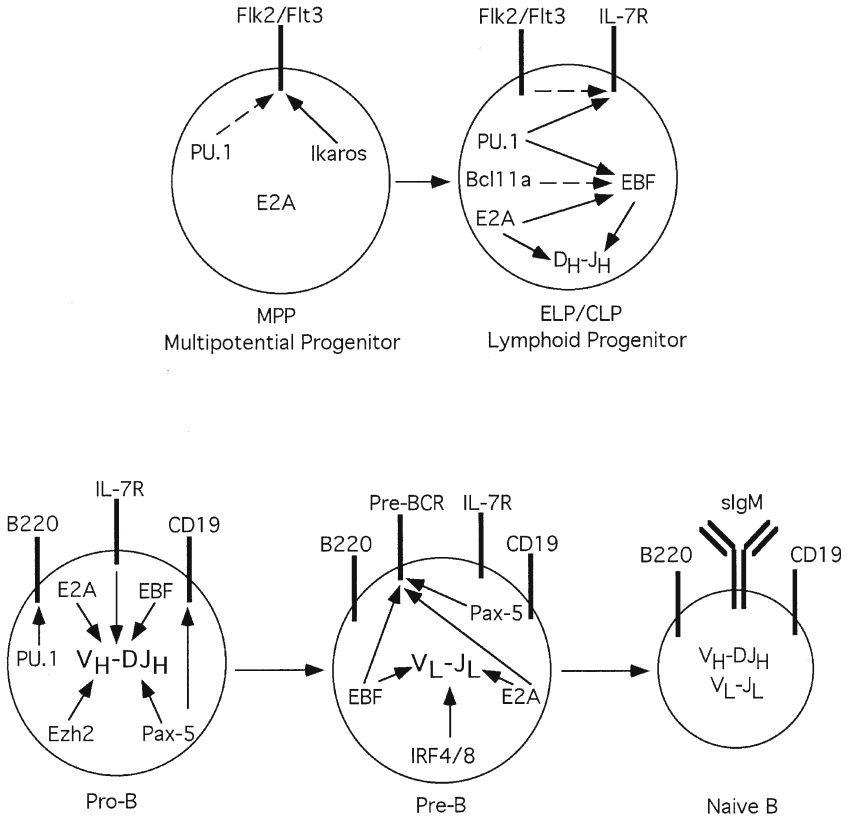

Fig. 1 Regulatory circuits orchestrating the generation of B cells. Five developmental states are depicted. Each state is dictated by a distinctive combination of regulatory molecules (transcription factors, chromatin modifying proteins, and cytokine receptors). Regulatory connections (*hatched* or *solid arrows*) are based on experimental evidence described in the literature. The surface phenotypes of the MPP, the ELP/CLP, the Pro-B, the Pre-B, and Naïve B are CD45+ c-kit^{hi} CD27+ AA4.1+ Flk2/Flt3+, CD45+ c-kit+ CD27+ AA4.1+ Flk2/Flt3+ IL-7R+, CD45R+ CD19+ CD43+ IL-7R+, CD45R+ CD19+ CD43- IL-7R+ $c\mu$+, CD45R+ CD19+ sIgM+, respectively. Within each state, essential developmentally regulated events are executed: MPP expression of the Flk2/Flt3R; ELP/CLP expression of EBF and the IL-7R; Pro-B expression of Pax-5 and V_H-DJ_H recombination; Pre-B expression of the Pre-BCR followed by V_L-J_L recombination; Naïve B surface expression of IgM. Only components of molecular circuits that orchestrate these key molecular events and enable transitions in developmental states are depicted

In addition to PU.1 and E2A, another transcriptional regulator, Bcl11a, has been implicated in regulation of EBF. Bcl11a is a Kruppel-related zinc finger protein expressed in multiple lineages, including hematopoietic progenitors (Saiki et al. 2000). The Bcl11a gene was originally identified as an oncogene which is translocated in a variety of B cell malignancies (Liu et al. 2003; Satterwhite et al. 2001). Targeted-inactivation of Bcl11a revealed that it is dispensable for the generation of myeloid or erythroid lineages, but is required for the development of B-lineage progenitors and thymocyte maturation (Liu et al. 2003). Consistent with the profound B cell developmental defect in Bcl11a$^{-/-}$ embryos, analysis of fetal liver hematopoietic progenitors revealed absence of EBF, Pax-5 and IL-7R transcripts. Conversely, the expression of genes required for early T-cell development was not impaired in Bcl11a$^{-/-}$ fetal thymocytes, including the IL-7R. The Bcl11a mutant phenotype in the lymphoid compartment is similar to the Flk2/Flt3 receptor or Flk2/Flt3 ligand knockout phenotypes. It remains to be demonstrated whether these mice have altered numbers of Flk2/Flt3+ fetal liver multipotential progenitors or a defect in production of Flk2/Flt3 ligand. Furthermore, the means by which Bc11a participates in the regulation of the EBF gene remains to be explored.

Once expressed, EBF, together with E2A, activates expression of the early program of B-lineage gene expression, including mb-1, λ5, VpreB, and Pax-5. The collaborative functions of E2A and EBF have been demonstrated by generating E2A/EBF compound mutant heterozygotes, which display a more severe defect in the developmental of B lymphocytes than the single heterozygous animals (O'Riordan and Grosschedl 1999). In contrast to E2A and EBF, Pax-5 is not required for specification of B cell fate. Pax-5$^{-/-}$ pro-B cells express early B-lineage genes and undergo D_H-J_H, and proximal V_H-DJ_H gene rearrangements (Nutt et al. 1997). Interestingly, Pax-5$^{-/-}$ pro-B lines exhibit extensive developmental plasticity, which is suppressed upon restoration of Pax-5 expression (Nutt et al. 1999, 2001). Not only is Pax-5 required for commitment to the B lineage and suppression of alternative cell fates, but it is also needed for maintenance of the B cell identity (Mikkola et al. 2002). Pax-5 target genes include mb-1, CD19, and BLNK (Nutt et al. 2001).

Taken together these studies reveal that the molecular circuitry driving specification of B cell fate involves the sequential as well as combinatorial activities of the transcription factors PU.1, Ikaros, E2A, Bcl11a, EBF and Pax-5 and the cytokine receptors Flk2/Flt3 and IL-7R (see Fig. 1). PU.1 and Ikaros are required for expression of Flk2/Flt3. Flk2/Flt3 signaling in concert with PU.1 induces IL-7R. Combinatorial signal-

ing through the Flk2/Flt3 and IL-7R is proposed to induce E2A, which in turn is directly implicated in regulating expression of the EBF gene along with PU.1. Once expressed, EBF, in concert with E2A, activates the early program of B-lineage gene expression as well as D-J_H recombination specifying B cell fate. In addition to promoting specification of B cell fate, EBF induces the expression of Pax-5, which blocks alternative lineage options, thereby ensuring commitment. Pax-5 also functions coordinately with EBF to reinforce the B-lineage program of gene expression. Importantly, Pax-5 function, in the differentiation of pro-B cells, is contingent on EBF (Medina et al. 2004).

Lineage commitment is generally considered an irreversible process or at the very least one that cannot be overridden readily. Recently, this concept has been challenged by the demonstration that enforced expression of C/EBPα/β transcription factors in bone marrow derived $CD19^+$ B cells in culture leads to their reprogramming into macrophages (Xie et al. 2004). This "lineage conversion" was accompanied by rapid and efficient downregulation of CD19 and upregulation of the macrophage marker Mac-1. It was suggested that C/EBP factors antagonize Pax-5 activity and thereby reverse the committed state. Interestingly, C/EBP factors appear to directly regulate the *Id1* gene (Saisanit and Sun 1995, 1997). Id1 negatively regulates E2A DNA binding activity through dimerization, thus inhibiting the expression of E2A target genes (Sun et al. 1991). Expression of an Id1 transgene driven by Ig regulatory elements has been shown to induce a profound block to early B cell development (Sun 1994). Thus, the "reprogramming" of B-lineage cells into macrophages observed after enforced expression of C/EBPα/β could also involve dysregulated Id1 expression. We note, however, that expression of C/EBP factors in committed, pre-B and B cells efficiently induces CD19 downregulation and upregulation of Mac-1 (Xie et al. 2004); this study did not unambiguously identify, through clonogenic assays, the precursors that give rise to macrophages upon ectopic expression of C/EBP factors. Such cells could be the CD19+B220- bipotent B/macrophage progenitors previously demonstrated in adult bone marrow (Montecino-Rodriguez et al. 2001). Thus it remains possible that C/EBP factors divert bipotential progenitors along the macrophage differentiation pathway rather than reprogram differentiated B cells into macrophages.

3
Regulation of Ig Gene Rearrangement and Pre-B Cell Differentiation

Coinciding with commitment to the B lineage, pro-B cells execute the second phase of IgH locus recombination, namely, V_H-DJ_H rearrangement. The IgH locus is large, spanning over three mega-bases (Mb) of DNA. The diversity (D), joining (J), and constant (C) region gene segments are in relative close proximity (Schatz et al. 1992). However, the variable (V) region heavy-chain gene segments, comprising more than 130 members, span greater than 2 Mb of DNA (Chevillard et al. 2002). Thus, V_H DNA segments positioned at a distance must not only be made accessible to the Ig recombinase (Rag1/2), but in addition be spatially juxtaposed with D-J_H segments. Recently Ig loci have been shown to undergo developmentally regulated compartmentalization in the nucleus as well as compaction. The latter may promote juxtaposition of widely separated V_H and D-J_H segments prior to recombination (Kosak et al. 2002). In addition to the discovery of these large-scale changes, new insights have emerged from the analysis of localized changes in chromatin structure, which determine the accessibility of specific Ig gene segments.

Fluorescence in situ hybridization (FISH) was used to demonstrate that IgH and Igκ loci undergo changes in nuclear positioning during the development of B-lineage precursors from multipotential progenitors (Kosak et al. 2002). Germline Ig loci in various non-B-lineage cells or IL-$7R^{-/-}$B220+ cells are localized at the nuclear periphery. In contrast, both the heavy and kappa light chain loci are centrally disposed in nuclei of committed B cell precursors. Nuclear repositioning occurs prior to and in the absence of recombination. The inactive Ig loci appear to associate with the nuclear lamina at the periphery, which may represent a novel repressive sub-compartment of the nucleus, precluding access by the transcription and recombination machinery. Using two-color FISH, the IgH locus was also found to undergo large-scale compaction in pro-B cells, in the absence of recombination, suggesting a mechanism for bringing distal V_H segments into proximity of rearranged D-J_H loci for recombination. A recent study has shown that the transcription factor Pax-5, which is required for rearrangement of distal V_H genes (Hesslein et al. 2003), induces compaction of the IgH locus in the absence of recombination (Fuxa et al. 2004). The regulator(s) involved in repositioning the Ig loci from the nuclear periphery to a central domain remains to be identified.

Accessibility of Ig gene segments appears to be regulated by specific histone modifications. V_H gene segments undergo an increase in histone

acetylation prior to recombination (Johnson et al. 2003). IL-7 signaling has been shown to induce acetylation of histones H3 and H4 associated with the distal (J558) V_H gene segments (Chowdhury and Sen 2001, 2003). Another histone modification resulting in altered chromatin structure is methylation. Ezh2, a polycomb group protein, regulates both basal and IL-7-induced methylation of histone H3 (lysine 27) in the V_H J558 gene cluster (Su et al. 2003). Targeted-inactivation of Ezh2 has a profound effect on distal V_H J558 gene rearrangements, resulting in the diminished generation of pre-B cells. In non-B-lineage cells, V_H genes are methylated at lysine 9 on histone H3, a mark of inactive chromatin (Johnson et al. 2004). Intriguingly, Pax-5 appears to facilitate removal of this inhibitory modification by a mechanism involving histone exchange. Thus the accessibility of V_H gene segments to the recombinase seems to be regulated by inhibitory and activating histone modifications. Transcription factors, such as Pax-5, may function to target histone-modifying complexes to Ig gene segments.

Successful recombination of IgH gene segments leads to expression of the heavy chain (Mu) protein. A Mu protein that can pair with the surrogate light chains is assembled into a Pre-B receptor and Pre-BCR signaling leads to expansion of these precursors. After several rounds of amplification, the cells exit the cell cycle and initiate Ig light-chain rearrangement. The molecular basis for control of cell cycle exit remains to be determined. This likely involves cessation of Pre-BCR signaling and is accompanied by the downregulation of surrogate light-chain gene expression. A unique role for the related interferon regulatory transcription factors IRF-4/8 has been recently uncovered in regulation of the pre-B to B cell transition (Lu et al. 2003). IRF4/8$^{-/-}$ mice exhibit a profound block to B cell development. IRF4/8$^{-/-}$ B lineage cells are blocked at the cycling pre-B cell stage, express high levels of the pre-BCR and fail to downregulate the surrogate light-chain genes. These results suggest that IRF-4/8 act as negative regulators of pre-BCR signaling, coordinating exit from the cell cycle and Ig light-chain gene rearrangement. IRF-4 binds in vitro and in vivo to functionally important sites in the kappa 3' and lambda gene enhancers. In addition to IRF-4/8, the transcription factors E2A and EBF also appear to directly regulate Ig light-chain gene recombination. Ectopic expression of E2A or EBF, along with the RAG proteins, can activate recombination of the kappa or lambda loci in non-B-lineage cells and generate a diverse Ig repertoire (Romanow et al. 2000; Goebel et al. 2001). Successful Ig light-chain rearrangement results in the generation of a naïve B cell expressing the BCR and the culmination of the antigen independent phase of B cell development.

4 Conclusions and Perspectives

The B cell developmental pathway represents a leading system for the analysis of regulatory circuits that orchestrate cell fate specification and commitment. Enormous progress has been achieved within the past decade in the identification and characterization of various regulatory components. These include transcription factors, chromatin-modifying complexes and signaling molecules. It is now possible to initiate the assembly of molecular circuits that underlie cell fate choice and specific developmental transitions. Our model (see Fig. 1) attempts to delineate such control circuits that operate in MPPs, ELPs, pro-B and pre-B cells. The connectivity of various components in each circuit remains to be extended and rigorously tested. As these circuits are experimentally refined, it will be of considerable interest to examine the design principles on which they are based to determine if shared architecture is used in the generation of lymphocytes as well as other hematopoietic cells.

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Helix-Loop-Helix Proteins in Lymphocyte Lineage Determination

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Abstract The cells of the lymphoid system develop from multipotent hematopoietic stem cells through a series of intermediate progenitors with progressively restricted developmental options. Commitment to a given lymphoid lineage appears to be controlled by numerous transcriptional regulatory proteins that activate lineage-specific gene expression programs and extinguish expression of lineage-inappropriate genes. In this review I discuss the function of transcription factors belonging to the helix-loop-helix protein family in the control of lymphoid cell fate decisions. A model of lymphocyte lineage determination based on the antagonistic activity of transcriptional activating and repressing helix-loop-helix proteins is presented.

1 Introduction

One of the current aims of developmental biology is to understand the mechanisms that direct multipotent cells to commit to a single cell fate. The hematopoietic system provides an ideal model to study this question owing to the sustained development of lineage-restricted cells from multipotent hematopoietic stem cells (HSCs) throughout life. The development of mature lymphoid and myeloid cells occurs through the progressive restriction of the developmental options of HSCs. Before committing to a particular lineage, HSCs differentiate into multipotent com-

mon myeloid progenitors (CMP) or common lymphoid progenitors (CLP) that lack the extensive self-renewal capacity of the parental HSC population (Akashi et al. 2000; Prohaska et al. 2002). Bone marrow-derived CLPs can differentiate into B and T lymphocytes, NK cells, and dendritic cells, whereas fetal liver-derived CLPs give rise to these cell types as well as a subset of macrophages (Mebius et al. 2001). CLPs may also be the precursors of $CD3^{-}CD4^{+}IL7R\alpha^{+}$ cells (called lymphoid tissue initiating cells, LTIC) that are essential for formation of multiple lymphoid organs including lymph nodes (LN), Peyer's patches (PP), and nasal-associated lymphoid tissue (NALT) during embryonic and neonatal life (Mebius 2003).

The mechanisms that promote the development of lineage-restricted lymphoid cells from CLP are beginning to be understood at the molecular level and include the activity of numerous transcriptional regulatory proteins, many of which are members of the helix-loop-helix (HLH) protein family (Bain et al. 2004; Zhuang et al. 1994, 1996; Yokota et al. 1999). There are seven classes of HLH proteins that are characterized by the presence of the HLH motif, which mediates protein dimerization (Murre et al. 1989). Class I and most class II HLH proteins are transcriptional activators that utilize a basic stretch of amino acids adjacent to the HLH motif to bind DNA (for a review of HLH protein structure, see Massari and Murre 2000). The class I HLH proteins E2A, HEB and E2–2 play essential roles in B and T lymphocyte development as homodimers or heterodimers (Bain et al. 1993, 1997; Shen and Kadesch 1995), and potentially as heterodimers with class VI HLH proteins Hes-1 and Hes-5 (Fisher and Caudy 1998a). The class V HLH protein Id2 plays an essential role in the development of NK cells, dendritic cells, and LTICs, presumably through its ability to antagonize the activity of the Class I HLH proteins (Yokota et al. 1999; Fukuyama et al. 2002). HLH protein expression and activity can be regulated by multiple signaling pathways that are active during lymphocyte lineage determination; however, the relationship between these pathways and HLH activity during the commitment process is currently not known. In this review we will discuss the HLH proteins that are known to function in lymphocyte lineage commitment.

2 E2A Proteins

The *E2A* gene codes for two class I HLH proteins, E12 and E47, that differ only in the exon coding for the basic region and HLH domains (Murre and Baltimore 1992). The E2A proteins, and the other class I HLH proteins, HEB and E2–2, are referred to as E-proteins because they bind the canonical E-box motif (CANNTG) originally identified in the immunoglobulin (Ig) heavy (H) and light (L) chain enhancers (Massari and Murre 2000). Like all class I HLH proteins, E2A proteins are expressed in many cell types and form DNA binding homodimers or heterodimers with class I, II, or VI HLH proteins. The E2A proteins are the mammalian homologues of the Drosophila gene *daughterless (da)*, which is known to function in multiple cell fate decisions (Garrell and Campuzano 1991). During Drosophila sex-determination, heterodimers of da and the X-linked class II HLH protein Scute (Sc/SisB) are required for activation of the *Sex-lethal* (*Sxl*) gene, which produces an RNA splicing protein essential for the development of female offspring (Yang et al. 2001). Similarly, during development of sensory bristles, da is required as a heterodimer with the class II HLH proteins encoded by the achaete-scute (Asc) for specification of the sensory organ precursor (Van Doren et al. 1991). In mammals, the E-proteins have been implicated, as heterodimers with class II HLH proteins, in cell fate decision in multiple developmental processes including myogenesis, neurogenesis, and pancreatic islet cell development (Sabourin and Rudnicki 2000; Chu et al. 2001; Helms and Johnson 2003).

In most mammalian tissues, E2A proteins are found as heterodimers with class II HLH proteins; however, in B lymphocytes, homodimers of E2A proteins are the major E-box-binding complexes (Bain et al. 2003; Shen and Kadesch 1995). Given the absence of class II HLH proteins in B lymphocytes, and the low level of expression of other E-proteins, it is not surprising that $E2A^{-/-}$ mice display a severe defect in the development of early B lineage cells. The bone marrow and fetal liver of $E2A^{-/-}$ mice lack cells that express the majority of cell surface proteins found specifically on early B lineage progenitors such as CD19 and BP-1/6C3 (Bain et al. 1994; Zhuang et al. 1994). In addition, no cells can be found that have rearrangements of the *IgH* loci, although low levels of μ^o (IgH germline) transcripts can be detected by RT-PCR. In addition, few B lineage-associated genes are expressed, with the exception of the signal-transducing molecule B29 (Igβ).

The defect in B lymphocyte development in E2A$^{-/-}$ mice is believed to occur at the time of B lineage specification, that is, initiation of the B lineage differentiation program from the CLP. The degree to which E2A affects the development and function of CLPs has not been carefully addressed. If the CLP is a required intermediate in the development of all lymphoid cell types then CLPs must be present in E2A$^{-/-}$ mice since T lymphocytes, NK cells, and dendritic cells develop in these animals. However, CLPs may not be completely "normal" in E2A$^{-/-}$ mice since these cells do express E2A proteins and some E2A target genes (Tudor et al. 2000) (and unpublished data). In addition, mice that lack both E2A and the receptor for interleukin 7 (IL-7Rα) show lymphoid defects consistent with partially redundant roles for these proteins in CLPs (Kee et al. 2002). Regardless of this potential function in CLPs, it is evident that E2A proteins are required for the emergence of committed B lineage progenitors from the CLP population.

E2A proteins function in a transcriptional hierarchy that is required for commitment to the B lymphocyte lineage. The essential target of E2A in this transcriptional cascade is early B cell factor (EBF) (Kee and Murre 1998; Smith et al. 2002). EBF is a transcription factor that also has a HLH dimerization motif, although it is not known to interact with other HLH proteins, and can bind DNA as a monomer or homodimer (Hagman et al. 1993, 1995). EBF is required for B lymphopoiesis at the same stage of development as E2A (Lin and Grosschedl 1995). EBF, either alone or in synergy with E2A, is essential for the expression of Pax-5, a paired-domain transcription factor that is essential for repression of non-B lineage differentiation programs (Kee and Murre 1998). While EBF is clearly an important target of E2A at this early stage of B lymphocyte development, EBF and E2A cooperatively control the expression of many B lineage genes that function at later stages. Many of these genes are required for the function of the pre-B cell receptor (pre-BCR) such as the surrogate light chain proteins λ5 and Vpre-B and the signal-transducing molecule mb-1 (Igα) (Sigvardsson et al. 1997, 2002). The cooperative regulation of B lymphopoiesis by E2A and EBF was demonstrated using mice heterozygous for both proteins. E2A$^{+/-}$;EBF$^{+/-}$ mice develop committed B lineage cells but the number of cells at later stages of B cell development are dramatically reduced, and these mice almost completely lack late-stage pro-B lymphocytes (fraction C by the designation of Hardy et al. 1991 [Hardy et al. 1991; O'Riordan and Grosschedl 1999]). The E2A and EBF target genes that are required for this transition have not yet been identified, although one possible target is Pax-5. Interestingly, E2A$^{-/-}$ and EBF$^{-/-}$ mice lack B lineage cells at an earlier stage than

that observed in the double heterozygous mice, implying a distinct function for E2A or EBF at the time of B lineage commitment. It is possible that the essential function of E2A in B lineage commitment is the induction of EBF and that EBF is a required protein for B lineage commitment. Alternatively, both E2A and EBF may have distinct essential functions during the commitment process.

The E-proteins HEB and E2–2 are also expressed in B lineage cells but constitute a minor portion of E-box-binding proteins, are not essential for B cell development, and cannot compensate for the loss of E2A proteins during B lineage commitment. However, $HEB^{-/-}$ and $E2–2^{-/-}$ mice do have a mild impairment in the development of B lineage progenitors in the fetal liver, as do all double heterozygous combinations of E2A, HEB, and E2–2 (Zhuang et al. 1996). These findings suggest that the level of E-protein activity may be the central determinant of B lineage commitment and differentiation. Consistent with this idea, forced expression of HEB can rescue B cell development in $E2A^{-/-}$ mice (Zhuang et al. 1998).

$E2A^{-/-}$ mice also show defects in T lymphocyte development, with an arrest of differentiation at the $CD4^{-}CD8^{-}CD44^{+}CD25^{lo}$ stage, where cells are thought to undergo lineage commitment (Bain et al. 1997). However, E2A is not absolutely required for T lymphopoiesis, as mature T cell numbers are decreased by only fivefold in these mice. In contrast, E-protein activity is absolutely required for T lineage commitment, since ectopic expression of Id proteins, either in a transgenic mouse model or by retroviral infection of multipotent progenitors, can completely abolish T cell development (Heemskerk et al. 1997; Kim et al. 1999). Therefore, the leakiness of the $E2A^{-/-}$ T cell defect is likely due to the expression of HEB, which forms a major component of E-box binding complexes in T lymphocyte progenitors and is required for T cell development beyond the $CD4^{-}CD8^{-}$ stage (Zhuang et al. 1996). The essential targets of E-proteins during T lineage commitment remain to be identified. Interestingly, the absence of E2A, or partial inhibition of E-protein activity by Id proteins, leads to the emergence of T cell lymphoma, indicating the E-proteins also function as tumor suppressor in T lymphocyte progenitors (Bain et al. 1997; Yan et al. 1997; Kim et al. 2002).

3
Class VI HLH Proteins: Hes-1/5

One of the major determinants of the T lymphocyte fate in the thymus is signaling through the Notch-1 transmembrane receptor (reviewed in Allman et al. 2002). Binding of Notch ligands, such as Delta-like-1 (DL1), to Notch results in activation of multiple intracellular signaling pathways, one of which induces expression of the hairy/enhancer of split related proteins Hes-1 and Hes-5. Hes-1 has been shown to be required for the development of T lymphocytes (Timita et al. 1999). Hes proteins can function as transcriptional repressors in part by recruitment of Groucho-related proteins through a conserved WRPW motif in the C-terminal portion of the protein (Fisher and Caudy 1998a). Groucho-related proteins can repress transcription by recruiting histone deacetylation complexes that modify chromatin structure (Fisher and Caudy 1998b). Hes proteins form homodimers that bind to a DNA sequence (CACNAG) called an N-box, which is distinct from the E-box sequence bound by class I HLH proteins (CANNTG) (Sasai et al. 1992). Therefore, Hes proteins are able to repress a distinct set of genes from those activated by E-proteins. Hes proteins can also form heterodimers with E-proteins and thereby interfere with the activation of E-protein-dependent genes through sequestration of E-proteins from E-box sites (Fisher and Caudy 1998a). Since the DNA binding specificity of E2A:Hes heterodimers has not been characterized extensively, it is also possible that Hes-1 could actively suppress E-protein-dependent genes by binding directly to the E-box site with E2A and recruiting Groucho-related proteins. Identification of the target genes and mechanism of action of E2A and Hes-1 that are critical for T cell development will be essential for understanding the genetic basis of T lineage commitment.

4
Id Proteins

The class V HLH proteins, Id1–4 in mammals, can form heterodimers with all class I and a subset of class II HLH proteins. The Id proteins function as antagonists of HLH transcription factors because they lack the basic region required for binding to DNA and therefore form non-functional heterodimers (reviewed in Rivera et al. 2001). Id2 and Id3 can be detected in multipotent hematopoietic progenitor populations and at low levels in B and T lymphocytes. Id3 is required for optimal prolifera-

tion of activated B lymphocytes and negative selection of a subset of developing T cells; however, no defects in antigen-independent stages of lymphocyte development have been reported (Pan et al. 1999; Rivera et al. 2000). In contrast, $Id2^{-/-}$ mice display multiple defects in the development of cells arising from CLPs (Yokota et al. 2002). $Id2^{-/-}$ mice have a severely reduced number of NK cells and subsets of dendritic cells, particularly Langerhan cells (Yokota et al. 1999; Ikawa et al. 2001; Hacker et al. 2003). In addition, these mice fail to develop lymph nodes, Peyer's patches and nasal-associated lymphoid tissue (NALT), presumably because of a failure to develop progenitors of LTIC (Fukuyama et al. 2002). These findings suggest that Id2 may play a pivotal role in lymphoid cell-fate decisions by antagonizing E-proteins.

The expression of both Id2 and Id3 can be induced by activation of the Erk-MAP Kinase pathway or by transforming growth factor (TGF)-β (Bain et al. 2001); Kee et al. 2001). The Erk-MAP Kinase pathway is activated in response to signals initiated from the BCR and T cell receptor (TCR) and from both the pre-BCR and pre-TCR. Transient inhibition of E-protein activity in response to pre-TCR signaling may be a necessary and sufficient requirement for transition of $CD4^{-}CD8^{-}$ thymocytes to the $CD4^{+}CD8^{+}$ stage (Engel et al. 2001). Id proteins may function in a similar capacity downstream of the pre-BCR during the pro-B to pre-B cell transition; however, this remains to be demonstrated. Many signaling pathways utilize the Erk-MAP Kinases and may have the ability to transiently induce Id protein expression during lymphopoiesis. We have shown that TGF-β1 can induce both Id2 and Id3 expression in pro-B lymphocytes through the activation of Smad transcription factors (Kee et al. 2001). TGF-β, and the related bone morphogenic proteins (BMP), may play a role in maintaining HSC proliferation and the HSC gene expression program via modulation of *Id* gene expression; however, the role of Id2 and Id3 in these processes remains to be determined (Bhatia et al. 1999; Pierelli et al. 2002).

5 Conclusions and Future Directions

The observed necessity for HLH proteins during the development of distinct lymphoid lineages suggests a hypothetical model for lymphoid lineage commitment from multipotent progenitors in which E-proteins and their antagonists play a critical role (Fig. 1). The level of functional E-protein dimers in multipotent progenitors, such as CLPs, is not

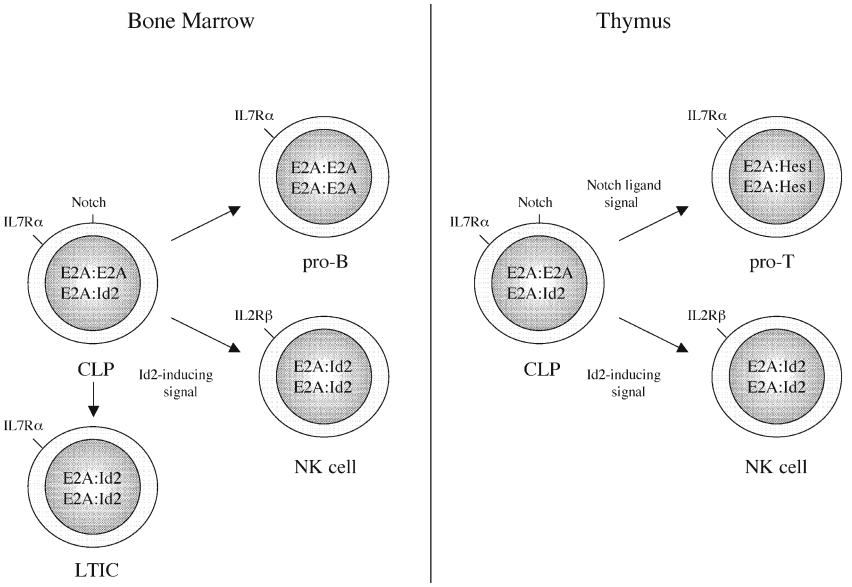

Fig. 1 A model of HLH protein regulation during lymphoid lineage commitment from common lymphoid progenitors. A hypothetical model is presented in which common lymphoid progenitors (*CLP*) are depicted as giving rise directly to pro-B lymphocytes, NK cells, or lymphoid tissue initiating cells (*LTIC*) in the bone marrow or pro-T lymphocytes and NK cells in the thymus. It is proposed that CLP express a significant, although low, level of functional E-protein dimers (here represented as E2A homodimers) that are kept at a low level by the antagonistic activity of Id2. An increase in E2A homodimers, potentially due to a decrease in Id2 expression, would lead to the induction of the E2A target gene EBF and subsequent commitment to the B lymphocyte lineage. In contrast, an increase in Id2 expression, due to an unidentified Id2 inducing signal, would cause a further decline in functional E2A homodimers and differentiation toward the LTIC or NK cell fate. The decision to follow the LTIC or NK cell fate may be related to the overall level of Id2 expression or may be dependent on the activation of distinct lineage-determining factors. In the thymus CLP may interact with Notch ligands, which are known to be essential for T lineage commitment. Activation of the Notch receptor leads to induction of Hes-1 and Hes-5, which alone or as heterodimers with E2A are required for commitment to the T lymphocyte lineage. In contrast, the failure to activate Notch, or interaction with an Id2-inducing signal, would cause a decrease in the available E2A and subsequent differentiation toward the NK cell fate

known; however, E-protein is expressed in these cells (unpublished data). In the model shown in Fig. 1, we have assumed that some functional E-protein complexes (here represented at E2A homodimers) exist but that these are kept at a low level by the antagonistic activity of Id

proteins. In this model we are considering only Id2 protein expression, although other Id proteins may play a role in setting the level of E-protein complexes in CLPs. In the fetal liver or bone marrow (and perhaps in the thymus as well), an increase in Id2 protein levels would further antagonize E-protein activity, resulting in the loss of expression of E-protein target genes and commitment of the CLP to the NK cell or LTIC fate. How the NK cell and LTIC cell fates are distinguished is currently not known. One possibility is that different levels of Id2 expression, resulting in different levels of E-protein activity, may distinguish the two cell types (i.e., LTICs might require less Id2 than NK cells). Alternatively, there is some evidence to suggest that LTICs may be the progenitors of NK cells (Mebius 2003). Therefore, the increase in Id2 may promote the development of LTIC and subsequent changes in gene expression, or a further increase in Id2, result in the emergence of NK cells. The nature of the signal that would elevate Id2 expression in CLPs is not known. A decrease in Id2 or other E-protein antagonists in the CLP would result in increased E-protein activity and the induction of EBF and the B lymphocyte differentiation program.

In the thymus a resident CLP, which expresses the Notch 1 transmembrane receptor, will interact with a Notch ligand (most likely delta-like-1) thereby inducing the expression of Hes-1 and Hes-5 and consequently T cell development. As mentioned above, Hes proteins may form heterodimers with E-proteins and repress the expression of E-box-dependent genes, either directly or indirectly. In contrast, an increase in Id2 would result in commitment of the CLP toward the NK cell fate. This model of lymphoid lineage commitment implies active regulation of HLH protein complex composition during the development of distinct lymphoid cell fates. One major objective of future research will be to identify the extracellular and intracellular molecules that modulate the expression, or activity, of HLH complexes in multipotent progenitors. In addition, our understanding of the mechanisms of lineage commitment will be greatly facilitated by identification of the targets of the HLH transcription factors that that are required for B and T lineage commitment and those that antagonize the development of LTIC and NK cells. Identification of these target genes will also provide insight into the mechanisms by which loss of E-protein activity leads to immune deficiency and T cell lymphoma.

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Ikaros-Family Proteins: In Search of Molecular Functions During Lymphocyte Development

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Abstract The regulatory steps that lead to the differentiation of hematopoietic cells from a multipotential stem cell remain largely unknown. A beginning to the understanding of these steps has come from the study of DNA-binding proteins that are thought to regulate the expression of genes required for specific developmental events. Ikaros is the founding member of a small family of DNA-binding proteins required for lymphocyte development, but the members of this family differ from other key regulators of lymphopoiesis in that direct target genes have not been conclusively identified, and reasonable support has been presented for only a few potential targets. Therefore, the molecular mechanisms that Ikaros uses for regulating lymphocyte development remain largely unknown. Current data suggest that, in some instances, Ikaros may function as a typical transcription factor. However, recent results suggest that it may function more broadly, perhaps in the formation of silent and active chromatin structures. In this review, our current knowledge of the molecular functions of Ikaros will be discussed.

1 Introduction

The regulatory steps that lead to the differentiation of hematopoietic cells from a multipotential stem cell remain largely unknown. A beginning to the understanding of these steps has come from the study of DNA-binding proteins that are thought to regulate the expression of genes required for specific developmental events (Clevers et al. 1993; Shortman and Wu 1996; Glimcher and Singh 1999). In most cases, these DNA-binding proteins appear to activate the transcription of target genes by binding critical regulatory elements within their promoters and enhancers. The importance of these proteins for hematopoiesis has been demonstrated by gene disruption experiments in mice (Orkin 1995; Clevers and Grosschedl 1996; Singh 1996; Ting et al. 1996; Georgopoulos 1997; Cortes et al. 1999; Glimcher and Singh 1999; Georgopoulos 2002). The defects observed in these experiments have elucidated the first developmental step where the respective transcription factor is required.

Ikaros is like many of these DNA-binding proteins in that it is required for lymphocyte development (Georgopoulos et al. 1997; Nichogiannopoulou et al. 1998; Cortes et al. 1999; Georgopoulos 2002), but it differs in that direct target genes have not been conclusively identified, and reasonable support has been presented for only a few potential targets. In fact, a role for Ikaros as a direct regulator of transcription has not been conclusively established. Therefore, the molecular mechanisms that Ikaros uses for regulating lymphocyte development remain largely unknown. Current data suggest that, in some instances, Ikaros may function as a typical transcription factor. However, recent results suggest that it may function more broadly, perhaps in the formation of silent chromatin structures. In this review we will summarize our current knowledge of Ikaros and discuss possible molecular functions.

2 Discovery

Ikaros was first discovered as a murine transcription factor that binds in vitro to critical regulatory elements in the CD3δ (Georgopoulos et al. 1992) and TdT genes (Lo et al. 1991; Hahm et al. 1994). Since then, Ikaros has been cloned from divergent vertebrate species including trout and *Xenopus* (Hansen et al. 1997), chicken (Liippo and Lassila 1997),

and human (Molnár et al. 1996). Interestingly, even though Ikaros binds to the CD3δ and TdT elements with high affinity, its precise role in the regulation of these genes has not been firmly established. In the case of TdT, Ikaros binds to a critical promoter element called D'. However, within the D' element there is an overlapping binding site for an Ets family protein that is most likely Elf-1 (Ernst et al. 1993). Fine mutational analysis of the D' element showed that binding of Ikaros did not fully correlate with transcriptional activity, whereas binding of Elf-1 did correlate (Ernst et al. 1996). Therefore, Elf-1 is most likely the transcriptional activator that functions through D'. More recent results have established that Ikaros competes with Elf-1 for occupancy of the D' element and that nucleotides that are selectively contacted by Ikaros are required for the downregulation of TdT transcription during the differentiation of a double-positive thymocyte line containing stably integrated TdT promoter–reporter plasmids (Trinh et al. 2001). Ikaros-binding sites were similarly required for downregulation of the murine λ5 gene during B-cell differentiation (Sabbattini et al. 2001), suggesting that Ikaros is important for the developmental silencing of both genes. Confirmation from alternative assays that Ikaros is a direct regulator of these genes is required, but has not yet been obtained. Reasonable evidence has been provided for a third direct target of Ikaros, the murine CD8α gene (Harker et al. 2002). In this case, Ikaros appears to activate transcription, with the current data suggesting that Ikaros contributes to activation by inducing chromatin modifications.

3
Structure and Family Members

The structure of Ikaros is shown in Fig. 1. A distinguishing feature of Ikaros is that it contains two separate regions of zinc fingers. This basic structure is conserved in the *Drosophila* Hunchback protein, and the Ikaros and Hunchback zinc fingers show a high degree of sequence homology (Georgopoulos et al. 1992; Hahm et al. 1994). Within Ikaros, the four zinc fingers in the amino half of the protein facilitate sequence-specific DNA binding (Hahm et al. 1994; Molnár and Georgopoulos 1994). The first three fingers are of the classical C2-H2 type, and the fourth has a C3-H1 structure. Ikaros binds to purine-rich sequences, and site selection experiments have found that the highest affinity sites contain 2–3 guanosines followed by 2–4 adenosines (Molnár and Georgopoulos 1994; Hahm et al. 1998). At the C-terminus are two additional zinc fin-

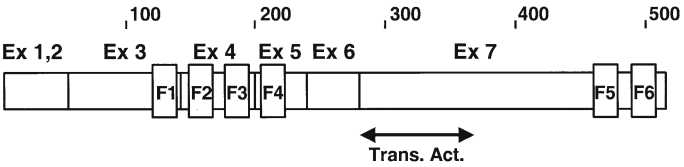

Fig. 1 Structural representation of Ikaros. *Numbers at the top* denote the amino acid number. Exon boundaries are delineated by *vertical lines* and the exon number is shown *above* (Georgopoulos et al., 1997). The six zinc finger domains are represented by the *large boxes* with the finger number (*F1–6*) shown *inside*. Fingers 1–4 mediate DNA binding, and fingers 5 and 6 mediate association between Ikaros proteins. The boundaries of the putative transcriptional activation domain are shown *below*. See text for details

gers that do not bind to DNA but rather facilitate protein–protein interactions (Sun et al. 1996; McCarty et al. 2003; Westman et al. 2003). Through this domain Ikaros proteins can form dimers or perhaps multimers. Lastly, between the two zinc finger domains, from residues 283 to 362, is a domain that directs transcriptional activation when fused to the DNA-binding domain of Gal4 (Sun et al. 1996). However, it is not known if this domain can promote transcriptional activation in the context of the intact Ikaros protein.

Complicating the study of Ikaros is the fact that there are many different protein isoforms generated by alternative splicing of the mRNA (Hahm et al. 1994; Molnár and Georgopoulos 1994). All isoforms contain the first two coding exons and the last, but they vary in the number of central coding exons, and thus, in the number of zinc fingers in the DNA-binding domain. DNA binding minimally requires the presence of fingers 2 and 3, but high-affinity DNA binding requires in addition finger 1 or 4 (Hahm et al. 1994; Molnár and Georgopoulos 1994; Cobb et al. 2000). The most commonly found isoforms are the full-length protein (isoform VI, ik-1) and one lacking exon 3, which contains finger 1 (isoform V, ik-2). Both of these isoforms are capable of high-affinity DNA binding and usually are expressed at roughly equivalent levels. Isoforms lacking fingers 2 and 3 do not bind DNA, but because they contain the C-terminal zinc fingers, they are capable of binding the largest isoforms. Such heterodimers have been reported to be greatly inhibited in their ability to bind DNA (Sun et al. 1996), which gives the smaller isoforms the potential to act as dominant negative inhibitors of Ikaros function.

Further complicating the study of Ikaros is the existence of Ikaros-related proteins. Two additional family members are enriched in hematopoietic cells, Aiolos (Morgan et al. 1997) and Helios (Hahm et al. 1998;

Kelley et al. 1998). Two final family members that are expressed more broadly have been isolated, Eos and Pegasus (Honma et al. 1999; Perdomo et al. 2000). No other Ikaros family members were uncovered in the murine and human genome sequences. All family members have the same basic structure as Ikaros, with zinc finger DNA binding and protein–protein interaction domains. The sequence homology in these domains is very high and all family members have the potential to form heterodimers or heteromultimers with one another. Furthermore, with the exception of Pegasus, all bind similar DNA sequences. Outside of the zinc finger domains, there are only a few small regions with substantial homology. Interestingly, one of these regions is in the putative transcriptional activation domain. With the many family members and isoforms that have been observed, a large number of distinct Ikaros complexes may exist; however, the functions of these different complexes remain unknown.

4 Genetics

Gene disruption experiments have shown that Ikaros is a critical factor in lymphocyte development. A true null phenotype was obtained by targeting exon 7, which encodes the C-terminus (Wang et al. 1996). In these mutant mice, no Ikaros isoforms appeared to be expressed. The mice were viable, but they exhibited severe defects in lymphocyte development. Both fetal and adult B cells and all B cell progenitors were absent. In addition, fetal T cells, NK cells, some gamma delta T cell subsets, and dendritic antigen-presenting cells were absent. In contrast, adult T cells were present, but an increase in the CD4 population was apparent. These defects were found to be intrinsic to the progenitor cells containing the mutation, as bone marrow reconstitution studies in mice showed that these defects were not affected by the environment in which development occurred (Wu et al. 1997).

A second gene disruption of Ikaros targeted the first three zinc fingers of the DNA-binding domain, disrupting exons 3 and 4 (Georgopoulos et al. 1994). In these mutant mice, smaller isoforms lacking the DNA-binding domain were still expressed, and the effect on lymphocyte development was more severe. As in the null mutation, B and NK cells were not found; however, this mutation also caused a complete loss of all T cells. Even in the heterozygous mice, abnormalities were observed (Winandy et al. 1995). These mice exhibited normal lymphocyte populations, but

they displayed a T cell lymphoproliferation defect, which led to leukemia and lymphoma within the first 3 months of life. The transformed cells were of clonal origin and had deleted the wild type allele of the gene. These results suggest that Ikaros may be involved in growth control in later stages of T cell development.

More recently, two additional mouse strains containing Ikaros mutations have been described. One strain contains a β-galactosidase cassette integrated within the Ikaros locus, creating a hypomorphic allele (Kirstetter et al. 2002; Dumortier et al. 2003). This mutation does not completely disrupt B cell development and has been used to characterize specific events in the B cell pathway that are defective when Ikaros is expressed at abnormally low concentrations (Kirstetter et al. 2002). This hypomorphic strain has also been used to establish an important role for Ikaros during neutrophil differentiation (Dumortier et al. 2003). The second strain contains a point mutation in one of the critical zinc fingers within the DNA-binding domain (Papathanasiou et al. 2003). The hematopoietic phenotype of this mutant strain is more severe than the phenotype of the mutation that deletes the exons encoding the DNA-binding zinc fingers, resulting in embryonic lethality and a severe defect in erythrocyte development.

A simple model to explain the phenotypic differences is that in the null mutation, B cell development is blocked because Ikaros function cannot be compensated by the expression of other Ikaros family members. However, Aiolos and Helios may have the capacity to compensate for the loss of Ikaros during adult T cell development. When the DNA-binding domain of Ikaros is deleted, the short Ikaros isoforms that are expressed may prevent compensation by other Ikaros family members by forming non-functional dimers or multimers with these proteins. The Ikaros point mutation appears to be even more detrimental to the hematopoietic pathway because it competes more effectively for dimer formation with wild type Ikaros family members than does the mutant protein that entirely lacks the Ikaros DNA-binding domain (Papathanasiou et al. 2003).

In contrast to the severe defects caused by the disruption of the Ikaros gene, a null mutation of Aiolos caused only slight defects during early stages of hematopoiesis (Wang et al. 1998). The normal complement of B and T cells was present, but the B cells had a lower threshold for activation through the antigen receptor. Additionally, there was an increase in the number of germinal centers in the spleens, serum levels of IgG and IgE were increased and auto-antibodies and B cell lym-

phomas were frequently seen. Therefore, Aiolos may be involved in the signaling pathways that lead to an effector state in B cells.

It is important to note that more recent studies of the Ikaros mutant strains have revealed reduced activation thresholds during various stages of lymphocyte development, similar to the reduced thresholds that were originally observed with the Aiolos-deficient mice (Nichogiannopoulou et al. 1999; Winandy et al. 1999; Avitahl et al. 1999; Kirstetter et al. 2002). This general function of Ikaros family members in maintaining appropriate thresholds for cell activation represents one of the most intriguing general discoveries to emerge from the phenotypic analyses of Ikaros- and Aiolos-deficient mice. Unfortunately, a clear molecular explanation for this function has not yet emerged from studies that have explored the mechanism by which Ikaros family members regulate intranuclear processes.

Lastly, the functional significance of Helios in lymphocyte development will be interesting to examine because of its T cell restricted expression. However, the functional significance awaits the completion of the gene disruption experiments.

5
Localization to Centromeric Heterochromatin

Immunolocalization experiments provided some of the earliest clues regarding potential functions of Ikaros. The initial experiments showed that Ikaros is unusual among cell-specific DNA-binding proteins in that, in interphase cells, it is found primarily within regions of centromeric heterochromatin. Confocal immunostaining experiments of B cells showed that Ikaros exhibits a distinctive staining pattern in the nucleus that overlaps with dense DAPI staining or heterochromatic DNA regions (Brown et al. 1997). Consistent with this, immunogold electron microscopy experiments in B cells have shown that Ikaros is preferentially localized to heterochromatic regions (Klug et al. 1998). Furthermore, when an immuno-FISH technique was used that involved simultaneous staining with both Ikaros antibodies and DNA probes derived from the γ-satellite repeat sequence that is abundant at the pericentromeric regions of all murine chromosomes, Ikaros was found to co-localize with these pericentromeric DNA sequences (Brown et al. 1997). DNA-binding studies and an analysis of the localization of Ikaros mutant proteins revealed that the targeting of Ikaros to pericentromeric heterochromatin is due to the direct binding of Ikaros to the γ-satellite repeats (Cobb et al.

2000). Pericentromeric localization of Ikaros was found during both G1 and G2, but during S phase Ikaros became dispersed throughout the nucleus (Brown et al. 1997). During mitosis, Ikaros staining was completely masked, and it only reappeared late in anaphase at a region surrounding the kinetochores.

The presence of a key developmental regulator at pericentromeric regions was surprising given the most obvious function of the centromere in chromosome segregation. However, an additional finding by Brown et. al. (1997) provided a potential explanation for the localization of Ikaros to foci of pericentromeric heterochromatin. Namely, in G1/G2 B cells, silent lymphocyte-specific genes were found to co-localize with Ikaros to the pericentromeric foci. This finding demonstrated that gene silencing during development is often accompanied by the physical movement or repositioning of silent genes to pericentromeric foci. Since the majority of Ikaros is located at these same pericentromeric foci, Ikaros may play a role in developmental gene silencing (Smale and Fisher 2002).

Interestingly, an additional study found that nuclear organization in resting mature B cells is very different from that found in interphase cycling B cells (Brown et al. 1999). In resting cells, Ikaros was expressed at much lower levels and was primarily localized in the cytoplasm. Also, silent genes did not co-localize with the pericentromeric heterochromatin. However, upon activation of the B cells and entry into the cell cycle, the organization of the nucleus changed, with Ikaros and the silent genes moving toward the pericentromeric foci with slow and similar kinetics.

The localization of Ikaros to foci of pericentromeric heterochromatin appears to provide support for the hypothesis that the functions of Ikaros extend beyond a potential function as a simple transcription activator. The studies described above that have suggested a requirement for Ikaros-binding sites during the developmental silencing of the murine TdT and $\lambda 5$ genes provide additional support for this hypothesis (Trinh et al. 2001; Sabbattini et al. 2001). Interestingly, both the TdT and $\lambda 5$ genes are among the list of genes that become repositioned to pericentromeric foci when silenced during lymphocyte development (Brown et al. 1997, 1999). Further support for a function in gene silencing has come from the discovery that Ikaros interacts with a variety of protein complexes involved in gene repression and silencing, most notably the NuRD histone deacetylase complex (Kim et al. 1999; Koipally et al. 1999; Koipally and Georgopoulos 2000).

A function for Ikaros as a transcriptional activator certainly cannot be excluded, however; Ikaros has been reported to act as a transcription-

al activator in transient transfection assays (Georgopoulos et al. 1997) and has recently been implicated in the activation of the CD8α gene during T cell development (Harker et al. 2002). It is possible that Ikaros acts as a transcriptional activator for some genes under certain circumstances and as a repressor for other genes. Such a model has been proposed for the *Drosophila* Hunchback protein which, as stated above, possesses the same zinc finger organization as the Ikaros proteins. Hunchback has been proposed to function as a transcriptional activator in the early stages of *Drosophila* development and then as a transcriptional silencer at later stages of development, perhaps by recruiting Polycomb-group proteins to induce the formation of heterochromatin (Zhang and Bienz 1992; Poux et al. 1996). Further experiments are needed to clarify the possible transcriptional activator and repressor functions of Ikaros. However, since an understanding of the function of Ikaros will undoubtedly require an understanding of its functions at the pericentromeric heterochromatin, a brief examination of the structure and functions of centromeres will be beneficial.

6
Centromere Structure and Function

The DNA sequence of the centromere shows tremendous variability between species, despite having a conserved role as the position of kinetochore formation and spindle attachment during cell division. In the budding yeast, centromeres are a short (~250-bp) and relatively simple sequence of DNA containing binding sites for specific proteins that organize the kinetochore. In most other eukaryotes, centromeres are larger and, in most species, remain poorly defined (Pluta et al. 1995). Vertebrate centromeres are made up of short, highly repetitive elements that are not found elsewhere within a chromosome (Karpen and Allshire 1997; Murphy and Karpen 1998; Wiens and Sorger 1998). In humans, the centromere is made up of a repetitive sequence called the alpha satellite, whereas in the laboratory mouse, *Mus musculus,* the centromere contains two types of repetitive sequences, the major or gamma satellite and the minor satellite. Here, centromere function is thought to be more closely tied to the minor satellite sequences because in metaphase spreads, gamma satellite sequences extend farther out into the chromosome from the primary constriction that defines the kinetochore. In contrast, the minor satellite sequences are found only at the primary constriction (Wong and Rattner 1988).

There are several reasons to believe that the specific sequence of the centromeric DNA is not important for centromere function. First, the sequences of the satellite repeats are not conserved, even in closely related species (Karpen and Allshire 1997; Murphy and Karpen 1998; Wiens and Sorger 1998). Second, in humans, only a portion of the alpha satellite DNA is bound by kinetochore proteins (Warburton et al. 1997). Third, no sequence-specific DNA-binding proteins have been found that consistently bind to centromeres in different species or even to all the centromeres within one species (Kipling and Warburton 1997). Fourth, the location of the centromere has some degree of plasticity, as shown below in certain chromosomal translocations (Karpen and Allshire 1997; Murphy and Karpen 1998; Wiens and Sorger 1998).

In rare instances, translocations in humans resulting in the loss of the original centromere can still leave a stably propagated chromosome. These chromosomes are stable because they contain a new or neocentromere at a region that does not function as a centromere in the original chromosome. The neocentromere does not contain alpha satellite sequences, nor does it appear to be structurally altered from the original chromosome. Additionally, its position is stably maintained in all cells of the individual; therefore, apparently unrelated sequences can be recruited to function as a centromere.

Translocations also exist where the resulting chromosome contains two centromeres. Such dicentric chromosomes would normally be unstable because the two centromeres would at some point lead to attachment of the same sister chromatid to both spindle apparatuses, thereby leading to chromosome breakage. Stable dicentric chromosomes are found, however, and in these instances only one centromere maintains function. The non-functional centromere exhibits no gross rearrangements, and the choice of which centromere maintains function appears to be random because different individuals with the same translocation can have either centromere retain function. Once the choice is made, however, the active centromere does not appear to change in an individual; therefore, the active centromere is somehow marked so that its function can be maintained through multiple rounds of cell division.

The above data have led to models in which the location of the centromere is determined by epigenetic means. The primary sequence of the centromere may be important only for determining a specific chromatin structure. For example, one feature of repetitive DNA that may facilitate its function as a centromere is its inclusion into heterochromatin. Maintaining the centromeric DNA in a heterochromatic state appears to be important for centromere function because mutations in *S. pombe*

that disrupt heterochromatin formation result in the instability of chromosome segregation during cell division (Karpen and Allshire 1997). Other features may also play a role in centromere formation, such as replication timing, the methylation state of the DNA, and modifications of the N-terminal tails of core histones. Clearly, many possible mechanisms can be envisioned.

Specific proteins undoubtedly play a role in defining the centromere. Many proteins have been found to be located in the kinetochore. Of particular interest are the CENP proteins (CENP-A—CENP-F) (Pluta et al. 1995). Microinjection of antibodies to CENP-C and CENP-E have shown that they are critical for kinetochore function (Bernat et al. 1990, 1991; Yen et al. 1991; Tomkiel et al. 1994); however, little is known about their molecular functions. More is known about CENP-A, which is related to histone H3 (Sullivan et al. 1994). In humans, CENP-A is found in nucleosomal structures and the DNA with which it associates is highly enriched in alpha satellite sequences (Vafa and Sullivan 1997). Immunostaining experiments have shown that CENP-A is found only at the kinetochore and that it co-localizes with a portion of the alpha satellite DNA in the kinetochore. Additionally, in dicentric chromosomes, CENP-A is found only at the active kinetochore, and in chromosomes with a neocentromere, CENP-A is found at the new kinetochore location (Warburton et al. 1997). Therefore, an attractive model is that the centromere is marked by the incorporation of CENP-A, which normally occurs at the satellite repeats but can occur elsewhere. Once CENP-A is localized at a particular site, that location would be stable because some mechanism would allow CENP-A to be incorporated at the same position in the daughter chromatids. Interestingly, peak synthesis of CENP-A occurs in G2, which is after the peak of histone synthesis in S phase. Ectopic expression of CENP-A throughout S phase causes nonspecific incorporation throughout the chromosome and is toxic to cells (Shelby et al. 1997). Thus, the late synthesis of CENP-A appears to be important for its specific incorporation into centromeres. Whatever the means of CENP-A incorporation at the centromere, its incorporation might be one of many features that promotes kinetochore formation.

7
Centromere Role During Interphase

Most information regarding the function of the centromere concerns its role in kinetochore formation during mitosis. Far less is known about

its function during interphase. Here, centromeres and the pericentromeric regions are organized into discrete foci of heterochromatin (Pluta et al. 1995; Karpen and Allshire 1997). The CENP proteins are still associated with the centromeres (Masumoto et al. 1989) and microinjection of antibodies against CENP proteins during interphase disrupts the subsequent mitosis (Bernat et al. 1990, 1991; Tomkiel et al. 1994). Therefore, kinetochore formation appears to require events prior to mitosis. Beyond the centromere, the whole nucleus has a dynamic organization during interphase (Lamond and Earnshaw 1998). FISH staining using probes covering one entire chromosome have shown that chromosomes are located in patches that are separated by interchromosomal domains (Schardin et al. 1985). Additionally, the DNA of a chromosome appears to be organized into discrete regions. Actively expressed genes appear to be located on the surface of the chromosome (Kurz et al. 1996; Wansink et al. 1996). Moreover, late replicating DNA regions, including the centromeric and pericentromeric heterochromatin, are found primarily at the periphery of the nucleus, with early replicating regions found in the interior (Ferreira et al. 1997). As stated above, genes that are silenced during lymphocyte development become repositioned to foci of pericentromeric heterochromatin (Brown et al. 1997), suggesting that the organization of this region plays an important role in the regulation of gene expression.

8
Potential Ikaros Functions at Pericentromeric Heterochromatin

Three general functions can be envisioned for Ikaros at the pericentromeric heterochromatin: (1) direct regulation of gene expression, (2) indirect contributions to gene regulation, and (3) functions that are distinct from gene regulation. We must also consider the possibility that the pericentromeric heterochromatin serves as a reservoir for inactive Ikaros, which may instead function only when located at other regions of the nucleus.

Localization of Ikaros to pericentromeric heterochromatin, combined with the data supporting a role in gene silencing, suggests the intriguing possibility that Ikaros could play a major role in the pericentromeric repositioning of silent genes. One model is that Ikaros dimers bound to regulatory sequences in specific genes could promote gene repression by recruiting histone deacetylase complexes, and subsequently promote repositioning to foci of pericentromeric heterochromatin by forming mul-

timeric structures with Ikaros bound to the pericentromeric repeat sequences (Trinh et al. 2001). One caveat of this hypothesis is that Ikaros is abundantly expressed in all lymphocytes, yet the genes shown to localize to the centromeric heterochromatin are active at some stages of development and inactive at others. Furthermore, Ikaros-binding sites are found in the regulatory regions of many genes expressed in lymphocytes, and the expression patterns of these genes vary dramatically during lymphocyte development despite the continuous presence of Ikaros. Clearly, Ikaros cannot solely be responsible for repression and repositioning. The various Ikaros isoforms or Ikaros family members would need to act at distinct stages of development to repress distinct sets of genes. Alternatively, the Ikaros proteins would need to act in concert with other developmental stage-specific factors.

A second general model is that Ikaros may not directly contribute in the regulation of specific target genes by binding to control elements within those genes. Instead, it could perform a more global role of establishing and maintaining chromatin structure at the pericentromeric regions. This could be achieved through its association with histone deacetylase complexes. This hypothesis is perhaps less attractive than a role in regulating specific genes during development because it is not immediately obvious why a lineage-specific protein would be responsible for the general function of establishing and maintaining constitutive heterochromatin. In addition, if Ikaros is dedicated to this general function, another mechanism would be needed for the pericentromeric recruitment of silent genes. One possibility is that silent chromatin at developmentally silent genes possesses an intrinsic affinity for the constitutive heterochromatin at the pericentromeric foci, resulting in repositioning.

The above hypotheses have focused on potential roles for Ikaros that involve gene regulation and chromatin structure. However, other functions can also be envisioned. For example, Ikaros could contribute to the regulation of DNA replication at specific replication origins, such as the origins within pericentromeric heterochromatin that stimulate DNA synthesis at late stages of S phase. Other possibilities include a role in maintaining nuclear structure or in marking the centromere for subsequent kinetichore formation during mitosis. An initial step toward addressing this latter possibility would be to perform high-resolution studies to determine whether Ikaros truly localizes to the kinetichore or is restricted to the pericentromeric regions of murine chromosomes. Again, one weakness of these hypotheses is that it is difficult to understand why these functions would become dependent on lineage-specific

proteins in hematopoietic cells. Until conclusive evidence is provided that Ikaros carries out an important function at the pericentromeric foci, one must also consider the possibility that the pericentromeric regions simply act as a reservoir for inactive Ikaros. The important functions of Ikaros could be carried out solely by the small quantities of Ikaros that are located within the euchromatic regions of the nucleus. Alternatively, Ikaros could carry out important functions during S phase and mitosis, when it is not restricted to the pericentromeric heterochromatin. It may be worth noting that, if pericentromeric heterochromatin is indeed used primarily as a storage site for inactive Ikaros, localization to this site may still be very important, as it may provide an effective means of regulating euchromatic activities of this factor. One observation that supports the hypothesis that the pericentromeric localization of Ikaros is important for at least one function of Ikaros is that this property is evolutionarily concerned, at least between mice and humans (B.S. Cobb et al., unpublished data).

9
Future Directions

The discovery that Ikaros and transcriptionally inactive genes co-localize to centromeric heterochromatin has established a new approach toward the molecular functions of Ikaros. The next step will be to design experiments to elucidate the functions of Ikaros at this location. The discovery that Ikaros associates with histone deacetylase complexes is an important beginning for future mechanistic studies (Kim et al. 1999; Koipally et al. 1999; Koipally and Georgopoulos 2000). However, it will also be important to design experiments to rigorously determine whether Ikaros is required for the pericentromeric repositioning of silent genes. The evidence that Ikaros is essential for the transcriptional inactivation of target genes prior to their repositioning to pericentromeric foci makes this a particularly difficult question to address. One possibility is to selectively disrupt the formation of Ikaros multimeric structures during developmental gene silencing. Ikaros dimers may retain the capacity to inactivate the transcription of its target genes, but, if Ikaros is truly important for the subsequent repositioning event, repositioning may be disrupted if multimer formation is prevented.

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Biochemistry of V(D)J Recombination

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Abstract The genes that encode immunoglobulin and T cell receptor proteins are assembled from component gene segments in a reaction known as V(D)J recombination. The reaction, and its crucial mediators RAG1 and RAG2, are essential for lymphocyte development and hence for adaptive immunity. Here we consider the bio-

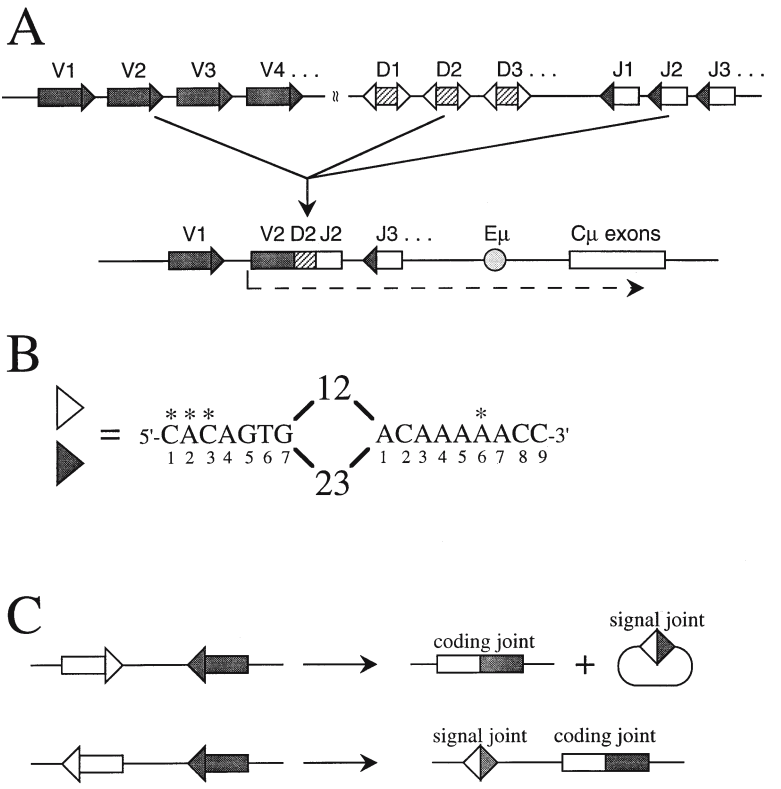
chemistry of this reaction, focusing on the DNA transactions and the proteins involved. We discuss how the RAG proteins interact with DNA and how coordinate cleavage of the DNA at two sites might be achieved. Finally, we consider the RAG proteins and V(D)J recombination from an evolutionary point of view.

1 Introduction

The ability of the immune system to recognize and respond to an enormous number of foreign antigens is mediated by clonotypic receptors expressed on the surface of B cells (immunoglobulin, Ig) and T cells (T-cell receptor, TCR). The genes encoding these antigen receptors are assembled by rearrangement of their composite segments in a reaction termed V(D)J recombination (Tonegawa 1983). The variable portion of the Ig heavy chain gene and the TCR β and δ genes are assembled from three gene segments termed V (variable), D (diversity) and J (joining) (Fig. 1A), while the Ig light chain genes and TCR α and γ genes use only V and J elements. The gene segments for a particular Ig or TCR locus are located on a single chromosome and can be scattered over hundreds of kilobases of DNA (reviewed in Lewis 1994).

During lymphoid differentiation, the pattern of V(D)J recombination events is tightly regulated (reviewed in Schlissel and Stanhope-Baker 1997; Spanopoulou 1996; Willerford et al. 1996). The order of events is controlled in part by checkpoints that ensure that only lymphoid cells expressing a functional antigen receptor chain are allowed to proceed in development. Initiation of V(D)J recombination at a particular locus is demarcated by an "opening" of its chromatin structure. Several transcriptional enhancers present in the Ig and TCR loci have been implicated in this process. In the B-cell lineage, Ig heavy chain (IgH) D-to-J rear-

Fig. 1 **A** A schematic representation of the Ig heavy chain gene locus (not to scale). V, D, and J gene segments are represented as *shaded*, *hatched* or *open rectangles*, respectively, while 12- and 23-bp recombination signals are represented as *open* and *shaded triangles*, respectively. The assembly of the variable portion of the heavy chain gene occurs in two steps. First, one of approximately 15 D gene segments is joined to one of four J gene segments. Thereafter, one of several hundred V gene segments is joined to the DJ complex to generate the fully assembled gene. Transcription (*dashed line*) is directed by a promoter upstream of V (not shown) and an enhancer downstream of the J gene segments ($E\mu$). The VDJ exon is spliced to the con-



stant region exons ($C\mu$) to generate the mature heavy chain mRNA. Because of the imprecise nature of the recombination reaction, approximately two-thirds of all assembled genes are out of frame. The leader exon upstream of the main V gene segment and the individual constant region exons are not indicated. **B** The V(D)J recombination signal. The consensus sequence of the heptamer and nonamer is shown, and each residue is numbered. *Asterisks* indicate the most highly conserved residues. Note that the *wide side of the triangle* representing the recombination signal corresponds to the heptamer end of the signal. Endogenous signals typically differ at one or more positions from the consensus shown, but the consensus sequence is the most active for recombination (Hesse et al. 1989). The spacer between the heptamer and nonamer is either 12±1 or 23±1 bp, and shows sequence conservation at some positions (Ramsden et al. 1994). **C** Topology of the V(D)J recombination reaction. In the standard V(D)J recombination reaction, the signals become joined to form a signal joint and the coding segments are joined to form a coding joint. Deletion occurs if the two signals are in opposite orientation to one another (*top*); in the example shown, the signal joint is deleted and the coding joint is retained on the chromosome. Inversion occurs if the two signals are in the same orientation (*bottom*), and in this case both joints are retained on the chromosome

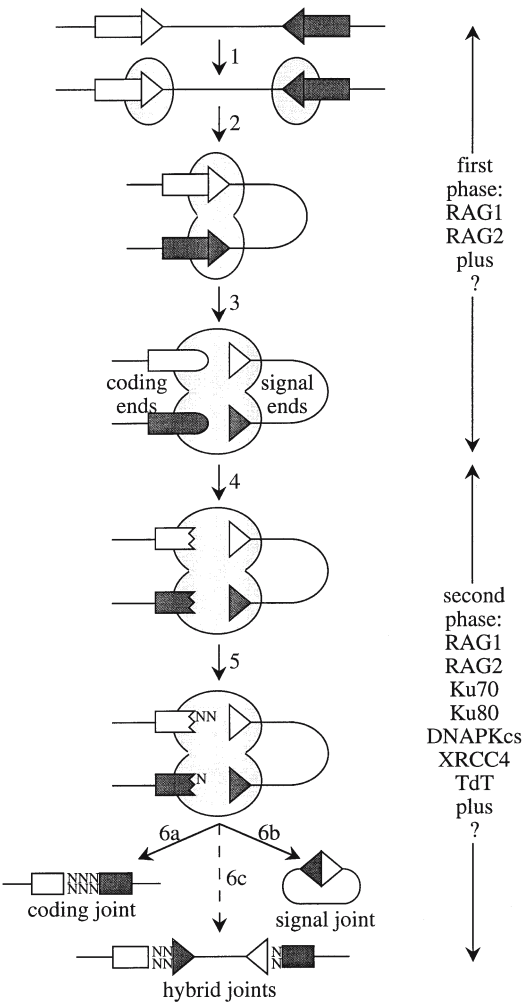


Fig. 2 A summary model for V(D)J recombination. Coding segments are represented as *rectangles*, signals as *triangles*, and the recombination machinery as *shaded ovals*. The first phase of the reaction consists of sequence-specific recognition of the recombination signals by RAG1 and RAG2 (*step 1*), synapsis of the signals (*step 2*), and DNA cleavage (*step 3*). One or more other factors, including the DNA bending proteins HMG-1 and HMG-2 (Sawchuk et al. 1997; van Gent et al. 1997), probably act to facilitate this phase of the reaction. The second phase of the reaction begins with opening of the hairpin CEs; the open ends are then frequently subject to further nucleotide loss (*step 4*). It has been proposed that base loss is a consequence of multiple nicks of the hairpin structure (Lieber 1991; Nadel and Feeney 1995). TdT can, if present, then add nontemplated nucleotides (*N*) to the 3′ ends of the coding regions

rangement precedes that of V-to-DJ, which in turn typically precedes rearrangement of the κ light chain (Igκ) locus. Despite having two heavy chain alleles, most B cells express only a single heavy chain on their surface, a phenomenon known as allelic exclusion. This appears to be accomplished by feedback inhibition of V-to-DJ rearrangement mediated by a receptor complex containing the expressed μ heavy chain (reviewed in Rajewsky 1996). Similar regulatory mechanisms dictate ordered recombination and development in the T-cell lineage (reviewed in von Boehmer 1995). The general rule appears to be that both initiation and termination of V(D)J recombination are governed by signal transduction cascades originating from cell surface receptors that often contain antigen receptor polypeptides.

2 DNA Transactions in V(D)J Recombination

The many different antigen receptor gene segments that serve as potential targets for rearrangement must be recognized by the same recombination machinery. This is achieved by highly conserved recombination signals that flank each gene segment and determine the site of reciprocal recombination (Fig. 1B). Each signal consists of a highly conserved 7-base-pair (bp) sequence (the heptamer) and a moderately conserved, A-T rich 9-bp sequence (the nonamer), separated by a spacer of either 12±1 or 23±1 bp (12-signal and 23-signal, respectively), whose sequence is not well conserved (Tonegawa 1983). In vivo, efficient recombination occurs only between a 12- and a 23-signal, a restriction referred to as the 12/23 rule (Tonegawa 1983). A 12/23 pair of signals are the necessary

(*step 5*). Finally, the processed CEs are repaired and ligated to form a coding joint (*step 6a*), and the SEs are ligated to form a signal joint (step 6b). Signal joints are usually precise fusions of the heptamers, and their formation need not be coupled to coding joint formation (see text). Alternatively, SEs can be joined to CEs to yield hybrid joints (*step 6c*), or open and shut joints (not shown). The second phase of the reaction is thought to be mediated by a group of DNA repair factors, including Ku70, Ku80, DNAPKcs and XRCC4, and it seems likely that RAG1 and RAG2 also play a role in this phase. The model is based on that proposed by Lewis and Gellert (1989), modified to include hairpin CEs, as proposed by Lieber (1991) and Gellert (1992)

and sufficient DNA elements for recombination of artificial test substrates (reviewed in Lewis 1994a).

V(D)J recombination results in the formation of two new DNA junctions: a coding joint, formed by fusion of the two coding gene segments, and a signal joint, formed by fusion of the heptamers of the two signals (Fig. 1C). While signal joints are typically precise (with no bases lost or added at the junction), coding joints are frequently imprecise, with bases of the coding segments lost, and novel bases added (reviewed in Lewis 1994a). The added bases are generated by two mechanisms. P, or palindromic, nucleotides, which form a palindrome with one or more bases of the coding end originate from opening of the hairpin structure (Roth et al. 1992a; Laffaile et al. 1989). N, or non-templated, nucleotides, which are added at random (although with a G/C bp bias) by the enzyme terminal deoxynucleotidyl transferase (TdT).

The relative orientation of the two signals on the DNA determines the topological outcome of the reaction. The most common configuration, with the signals in opposite orientation, results in retention of the coding joint on the chromosome (or plasmid), and deletion of the signal joint on a circular DNA molecule (Fig. 1C, top). However, if the signals are in the same orientation (as can occur in the Ig κ locus, and the TCR β and δ loci), then recombination results in inversion of the intervening DNA and retention of both joints on the chromosome (Fig. 1C, bottom). A model for the basic DNA transactions in V(D)J recombination is shown in Fig. 2, and described in detail in the legend.

3
Proteins That Mediate V(D)J Recombination

Rearrangement of antigen receptor loci is mediated by a number of lymphoid-specific and ubiquitous activities that execute the sequential stages of the process and link it to other cellular functions such as DNA repair and cell cycle control. DNA recognition and cleavage (phase 1) are mediated principally by lymphoid-specific factors, while end processing and joining (phase 2) are mediated by ubiquitous DNA repair activities (Fig. 2).

3.1
The Recombination Activating Proteins RAG1 and RAG2

V(D)J recombination is dependent on the activity of two lymphoid-specific proteins, RAG1 and RAG2. The *RAG1* and *RAG2* genes were identified by virtue of their ability to activate V(D)J rearrangement of an artificial recombination substrate in fibroblast cells (Schatz and Baltimore 1988; Schatz et al. 1989; Oettinger et al., 1990). Subsequently, they were identified in a variety of vertebrate species and in all cases were found to reside immediately adjacent to one other in the genome. Both RAG proteins show remarkable sequence conservation throughout evolution (for review see Thompson 1995).

RAG1 and RAG2 are indispensable for V(D)J recombination. In humans, point mutations that reduce or eliminate the recombination activity of RAG1 or RAG2 lead to severe immunodeficiencies due to the absence of antigen receptors and hence mature lymphocytes (Schwarz et al. 1996). Similarly, RAG1- or RAG2-deficient mice exhibit an identical phenotype in which lymphoid development is arrested precisely prior to the rearrangement of the antigen receptor loci (Mombaerts et al. 1992; Shinkai et al. 1992; Spanopoulou 1996). However, lymphoid development is fully reconstituted if rearranged Ig or TCR genes are provided as a transgene (Mombaerts et al. 1992; Shinkai et al. 1992; Spanopoulou et al. 1994; Young et al. 1994), suggesting that the RAG proteins are required solely for the rearrangement of antigen receptor genes. No rearrangements were initiated in the $RAG^{-/-}$ cells, suggesting that RAG1 and RAG2 act at the initial stages of recombination (Schlissel et al. 1993).

3.1.1
Physical and Structural Properties of the RAG Proteins

RAG1 and RAG2 are nuclear phosphoproteins that partition mostly in an insoluble nuclear fraction. RAG1 is a very labile protein, whereas RAG2 appears to be relatively stable, and it seems likely that both proteins are under translational and/or posttranslational control (Lin and Desiderio 1993; Leu and Schatz 1995; Spanopoulou et al. 1995). The two proteins can be co-immunoprecipitated, indicating that they exist in a physical complex. These complexes contain three to five molecules of RAG2 for each molecule of RAG1 (Leu and Schatz 1995; Spanopoulou et al. 1995). Curiously, the purified RAG proteins do not interact detectably (McBlane et al. 1995), suggesting that perhaps co-precipitation of the RAG proteins is mediated by another cellular factor, which is unlikely to

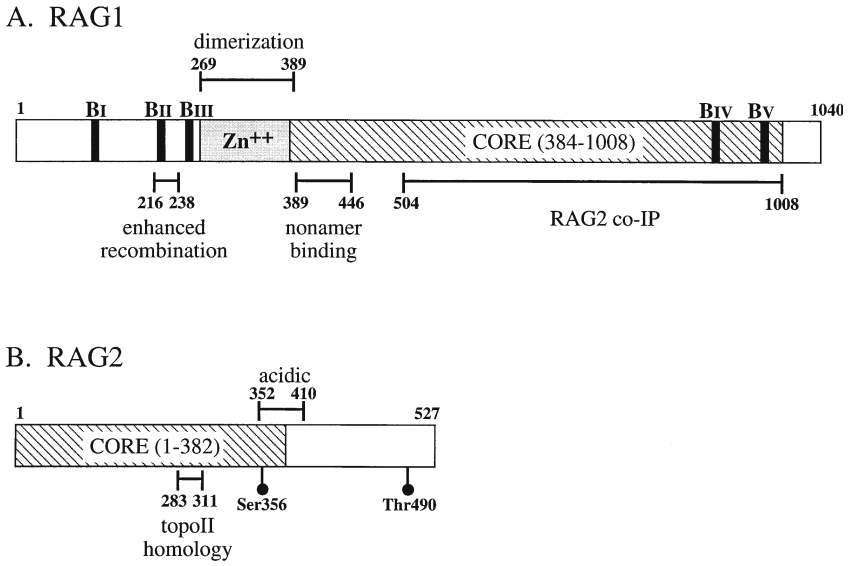


Fig. 3A, B Domains and structural features of the RAG proteins. The full length murine RAG1 **A** and RAG2 **B** proteins are represented schematically, with the endpoints of various domains indicated *above* and *below* with *bars*. *Numbers* refer to murine RAG1 and RAG2 amino acids (Schatz et al. 1989; Oettinger et al. 1990). The core regions are indicated as *hatch marked rectangles*, basic motifs involved in nuclear transport as *black rectangles*, and the zinc-binding homodimerization motif as a *lightly shaded rectangle* (see text for additional details). Two important sites of phosphorylation of RAG2 are indicated with *black circles* on stems: phosphorylation of Ser 356 regulates activity, while phosphorylation of Thr 490 regulates protein stability (Lin and Desiderio 1993)

be DNA or RNA (Leu and Schatz 1995). (This was later shown to be incorrect. There is now abundant evidence for a direct interaction between RAG1 and RAG2, that the interaction can occur prior to DNA binding, and that it substantially enhances the affinity and specificity of DNA binding [Gellert, 2002].)

Several domains of the two proteins have been determined based on their primary sequence and subsequent deletional analysis (Fig. 3). The latter has defined the core sequences of RAG1 (amino acids 384–1008) and RAG2 (amino acids 1–382) that are sufficient to carry out V(D)J recombination of an extrachromosomal substrate in fibroblasts (Sadofsky et al. 1993; Silver et al. 1993; Cuomo and Oettinger 1994; Sadofsky et al. 1994). Interestingly, the core sequence does not include parts of RAG1

and RAG2 that have been highly conserved during evolution. For example, RAG1 contains adjacent Ring and zinc fingers immediately N-terminal to the core (Fig. 3A). This Ring/zinc finger domain binds multiple Zn^{2+} atoms and mediates homodimerization of RAG1 (Rodgers et al. 1996). No function in recombination can yet be ascribed to this domain, since its deletion has little or no effect on recombination in standard transfection assays (Sadofsky et al. 1993; Silver et al. 1993). It is tempting to speculate that dimerization of RAG1 plays a role in recombination of endogenous gene segments, perhaps by facilitating synapsis of gene segments that lie far from one another on the chromosome.

RAG1 contains five basic motifs at the N- and C-terminus of the protein (Fig. 3A). The three N-terminal motifs (BI, BII and BIII) have multiple roles. They possess the potential to interact with the nuclear transport proteins SRP-1 and Rch-1 (Cortes et al. 1994; Spanopoulou et al. 1995; F-H. Wang and E. Spanopoulou, unpublished data), both of which were shown to interact with RAG1 using the yeast two hybrid system (Cortes et al. 1994; Cuomo et al. 1994). The three motifs can also drive nucleolar localization of overexpressed RAG1, indicating that the N-terminus of RAG1 has the potential to bind RNA or single-stranded DNA (Spanopoulou et al. 1995). One of these basic motifs, BII, appears to play a role in recombination beyond that of simply facilitating nuclear transport. Nearly full-length RAG1 is roughly tenfold more active than the RAG1 core in performing recombination of extrachromosomal substrates, and the BII motif is critical for this effect (McMahan et al. 1997). [Other studies also demonstrated that the N-terminal region of RAG1 enhances recombination (Roman et al. 1997; Steen et al. 1999), although the region responsible for this effect was mapped to different residues in the N-terminus (Roman et al. 1997)]. BII does not mediate its effect simply by increasing protein expression or enhancing nuclear localization, and its precise function remains to be determined. Basic motif IV at the C-terminus of the protein contributes to the nuclear localization of RAG1 and interacts with a truncated but not full -engtth form of Rch-1 (Spanopoulou et al. 1995; F-H. Wang and E. Spanopoulou, unpublished data). Basic motif V is indispensable for RAG1 activity, but its functional role remains to be determined. Although it had been suggested that BV contributes to nuclear localization of RAG1 (Spanopoulou et al. 1995), it was subsequently found that mutations within this motif unstructure RAG1, leading to degradation and cytoplasmic localization of the mutant protein (McMahan 1996; F-H Wang and E. Spanopoulou, unpublished data).

It has also been suggested that RAG1 contains a domain with sequence similarity to the catalytic domain of retroviral integrases (Bernstein et al. 1996) and homology to topoisomerase I at the C-terminus (in particular to the yeast protein HPR1) (Wang et al. 1990). The latter is indispensable for the function of RAG1, but its precise contribution to V(D)J recombination remains unknown. Mutations within the integrase-homology region of RAG1 had no effect on the recombination efficiency of the protein (C. Roman and D. Baltimore, personal communication). Finally, at the N-terminus of its active core, RAG1 contains a domain with homology to the DNA binding domain of the Hin-family of invertases and homeodomain proteins (Figs. 3A, 5). This constitutes the domain of RAG1 that mediates recognition of the nonamer, as described in detail below.

RAG2 shares limited homology to topoisomerase II (Silver 1993) and contains an extended acidic domain (Fig. 3B). Mutations within the topoisomerase II homology region inactivate the function of RAG2 (C. Roman and D. Baltimore, personal communication). Deletion of most of the acidic domain has no effect on the catalytic function of the protein (Cuomo and Oettinger 1994; Sadofsky et al. 1994). The best characterized functional domain of RAG2 is the ten amino acid region beginning at Thr 490, which controls cell cycle-dependent degradation of RAG2 by directing phosphorylation of Thr 490 in the S, G2 and M phases of the cell cycle (Lin and Desiderio 1993; Li et al. 1996). RAG2 phosphorylation is also important in controlling its activity. One of the major forms of RAG2 is phosphorylated at Ser 356, and mutation of this position to alanine substantially reduces the activity of RAG2 (Lin and Desiderio 1993).

3.2
Terminal Deoxynucleotidyl Transferase

Template-independent addition of nucleotides to coding joints (N-regions) is mediated by the lymphoid-specific enzyme TdT (for review, see Lewis 1994a). Co-expression of RAG1, RAG2, TdT and a recombination substrate in fibroblastoid cells results in the appearance of N-regions at coding joints, indicating that TdT is able to participate in V(D)J recombination even in a nonlymphoid environment (Kallenbach et al. 1992). Its function in junctional diversity has been confirmed by the absence of N-regions in coding joints from mice with targeted ablations of the TdT gene (Gilfillan et al. 1993; Komori et al. 1993). TdT shows an intriguing pattern of expression (reviewed in Lewis 1994a). The TdT gene

is not activated before neonatal day 3–5, while V(D)J recombination starts in the fetus. Thus, both fetal and neonatal repertoires lack N-region diversity. The significance of this delayed onset of TdT expression is currently not known.

3.3 Ubiquitous DNA Repair Activities Mediate the Second Phase of V(D)J Recombination

Initial evidence that the second phase of V(D)J recombination requires the involvement of ubiquitous DNA repair activities came from the analysis of *scid* lymphoid cells (Bosma et al. 1983; Schuler et al. 1986). Coding, but not signal, joint formation is disrupted by the *scid* mutation (Lieber et al. 1988). Coding ends (CE) fail to resolve and as a result, hairpin CEs accumulate in *scid* lymphoid precursors (Roth et al. 1992). *Scid* cells are not only deficient in V(D)J recombination but also in the repair of DNA double-strand breaks (DSBs) (for review, see Lewis 1994a). It was subsequently found that the *scid* deficiency is due to a point mutation in the kinase domain (Blunt et al. 1996; Danska et al. 1996; Araki et al. 1997) of the catalytic subunit (cs) of the DNA-dependent protein kinase DNA-PK (Blunt et al. 1995; Kirchgessner et al. 1995; Lees-Miller et al. 1995). DNA-PKcs is a 460-kD serine/threonine kinase that becomes activated by the presence of damaged DNA such as DSBs. Its binding to DNA and subsequent activation are stimulated by the DSB-binding component of DNA-PK, a heterodimer composed of the Ku70 and Ku80 proteins (for review, see Jackson and Jeggo 1995; Weaver 1995). DNA-PKcs is capable of phosphorylating a number of substrates in vitro, including those involved in transcription, cell cycle control, and DNA repair, but the in vivo phosphorylation targets are unknown.

The Ku proteins have been implicated in multiple functions in different species. Studies from several laboratories have suggested that in both yeast and mammalian cells, Ku recognizes altered DNA structures such as DSBs, nicks and hairpins and perhaps protects them from degradation by exonucleases (for review, see Jackson and Jeggo 1995; Smider and Chu 1997). Ku70 in yeast has been implicated in DNA repair and maintenance of chromosomal integrity (Boulton and Jackson 1996; Jackson 1996), while a Ku70 homologue in Drosophila has been found to be essential for P-element transposition (Beall et al. 1994). $Ku80^{-/-}$ mice exhibit an arrest of lymphoid development and growth retardation (Nussenzweig et al. 1996; Zhu et al. 1996), and Ku80-deficient fibroblasts exhibit arrest at the G2/M phase of the cell cycle, suggesting a role for

Ku80 in several pathways of DNA repair and cell cycle regulation (Nussenzweig et al. 1996).

While DNA-PKcs is critical for coding joint formation, Ku is important for both coding and signal joint formation. Ku80$^{-/-}$ mice are immunodeficient due to the absence of antigen receptors and both coding and signal joint formation are diminished (Nussenzweig et al. 1996; Zhu et al. 1996), reiterating the phenotype of Ku80$^{-/-}$ deficient cell lines (Pergola et al. 1993; Taccioli et al. 1993). However, unlike RAG$^{-/-}$ mice, both *scid* and Ku80$^{-/-}$ mice exhibit detectable levels of V(D)J recombination (Lewis 1994a; Nussenzweig et al. 1996; Zhu et al. 1996). This "leakiness" could be due to the fact that a low percentage of CEs and SEs are processed by the appropriate enzymatic activities despite the defect in DNA-PK. Alternatively, it is possible that a "bypass" pathway is activated in these cells and substitutes for the absence of DNA-PK. Consistent with this latter possibility, γ-irradiation of *scid* mice results in a burst of normal TCR gene rearrangement (Danska et al. 1994; Livak et al. 1996). It is therefore conceivable that an alternate DNA repair pathway is activated after DNA damage and permits processing of V(D)J CEs.

Although it is clear that DNA-PKcs and Ku70/80 are essential for efficient V(D)J recombination, their precise functions in the reaction remain to be determined. Based on the fact that in Ku$^{-/-}$ cell lines V(D)J recombination intermediates undergo large deletions, it was initially suggested that binding of Ku protects the DNA ends from the action of exonucleases that would otherwise degrade them (Taccioli et al. 1993). However, recent evidence indicates that DNA-PKcs/Ku70/80 have a more direct role in the reaction. In Ku80$^{-/-}$ lymphoid cells, both SEs and CEs can be detected, at least at some antigen receptor loci (Nussenzweig et al. 1996; Zhu et al. 1996). Furthermore, SEs and hybrid joints are generated at significant levels from extrachromosomal V(D)J substrates when RAG1 and RAG2 are expressed in a Ku-deficient cell line (Han et al. 1997). Interestingly, exogenously introduced blunt and hairpin DSBs can be processed normally in *scid* (Lewis 1994b) and Ku$^{-/-}$ cell lines (Zhu et al. 1996). These observations suggest that Ku or DNA-PKcs deficiency blocks V(D)J recombination because it renders SE and CE intermediates inaccessible to the activities that normally process them (Zhu et al. 1996). Components of DNA-PK have been found in a stable post-cleavage complex with the RAG proteins and SE/SE DNA intermediates (Agrawal and Schatz 1997). It is therefore conceivable that RAG1/RAG2/Ku70/80 and DNA-PKcs remain engaged in a post-cleavage complex that holds together CEs and SEs and regulates their processing. It has also

been proposed that Ku plays a role in the reaction before the completion of the cleavage phase of the reaction (Smider and Chu 1997).

Processing of SEs requires disassembly of this stable complex. In this context Ku70/80 could mediate events that promote the disengagement of RAGs from the SEs (Zhu et al. 1996). For example, Ku70/80 have a weak helicase activity (Tuteja et al. 1994), which could promote reconfiguration the protein/DNA complex. Alternatively, they might recruit or activate a chaperone that would remodel the complex, by analogy with the action of ClpX in Mu transposition (Levchenko et al., 1995; Kruklitis et al. 1996).

Processing of CEs follows a different pathway. CEs are resolved more rapidly than SEs (Ramsden and Gellert 1995 and references therein) and thus far have been found associated with components of DNA-PK but not the RAG proteins (Agrawal and Schatz 1997). CE processing requires opening of the covalently sealed hairpin ends, and conceivably, DNA-PK is involved in recruiting the required endonuclease. DNA-PKcs could also phosphorylate components of the post-cleavage complex, triggering an ordered array of events that permits progression to the final, ligation step of the reaction. The DNA-PK holoenzyme could serve additional functions, for example by coupling V(D)J recombination and DNA repair with control of the cell cycle (Jackson and Jeggo 1995; Weaver 1995).

The XR-1 cell line represents a distinct complementation group from that of *scid* and Ku80, and is defective in both DNA DSB rejoining and V(D)J recombination (for review, see Jackson and Jeggo 1995). The gene affected in XR-1, termed *XRCC4*, was recently isolated (Li et al. 1995) by a complementation strategy analogous to that used to identify RAG1 (Schatz and Baltimore 1988; Schatz et al. 1989; Oettinger et al. 1990). *XRCC4* is ubiquitously expressed and the predicted protein product does not contain significant sequence similarity to other known proteins (Li et al. 1995). Its function in V(D)J recombination and DSB repair remain unknown. [XRCC4 was subsequently shown to interact with DNA Ligase IV, the essential ligase for V(D)J recombination and NHEJ, and to play a critical role in stabilizing and activating the ligase (Gellert 2002.)]

4
Recent Insights from In Vitro Cleavage Studies

4.1
The Role of the RAG Proteins in the First Phase of V(D)J Recombination

The recent development by van Gent and others in the Gellert laboratory of an in vitro system for performing DNA cleavage at recombination signals (van Gent et al. 1995) has substantially accelerated progress in the study of the mechanism of V(D)J recombination. Of particular significance was the subsequent finding by the Gellert and Oettinger groups that the RAG1 and RAG2 proteins are necessary and sufficient to perform signal-dependent DNA cleavage (McBlane et al. 1995). In this study, highly purified core RAG proteins were shown to perform cleavage of oligonucleotide substrates containing an isolated signal in a two-step process (Fig. 4). In the first step, a nick is introduced on the "top" strand (5′ of the heptamer) to yield a 3′ hydroxyl group and a 5′ phosphate. In the second step (strand transfer), this 3′ hydroxyl becomes covalently linked to the corresponding phosphate group of the bottom

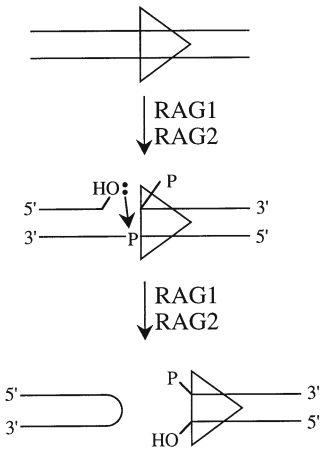


Fig. 4 DNA cleavage by RAG1 and RAG2. The purified RAG proteins are capable of performing cleavage at an isolated recombination signal. A nick is first introduced immediately 5′ of the heptamer to yield a 3′ hydroxyl group and a 5′ phosphate. In the subsequent strand transfer reaction, the hydroxyl group serves as the nucleophile for attack on the labile phosphate bond on the bottom strand to yield a covalently sealed, hairpin CE and a blunt, 5′ phosphorylated SE. This figure is based on the data and figures from McBlane et al. (1995) and van Gent et al. (1996a)

strand to yield a blunt, 5′ phosphorylated SE and a hairpin CE. Both steps of the cleavage reaction required a recombination signal, both RAG proteins, and Mn^{2+}.

These results allowed a number of important conclusions to be drawn. First, they provided definitive proof that the RAG proteins play a direct role in V(D)J recombination. Second, they demonstrated that the RAG proteins are responsible for sequence-specific recognition of the recombination signal. And third they showed that together the RAG proteins constitute a site-specific endonuclease, capable of both DNA strand scission and strand transfer. While the active site(s) of the RAG proteins have not yet been identified, further insight into the cleavage process was obtained using a substrate containing a chiral phosphorothioate group at the site of bottom strand cleavage to demonstrate that strand transfer/hairpin formation occurs by direct S_N2 nucleophilic attack of the top strand 3′ hydroxyl group on the labile phosphate (Fig. 4) (van Gent et al. 1996a). A similar mechanism is used in the corresponding steps of phage Mu transposition and retroviral integration. This study also demonstrated that the resemblance between V(D)J recombination and retroviral integration extends to the ability of alcohols to serve as first-strand nucleophiles under appropriate conditions. The active site domains of MuA transposase and HIV integrase adopt strikingly similar structures and contain a triad of acidic amino acids (the "DDE motif") essential for divalent metal ion binding and the nicking and strand transfer steps of their respective reactions (for review, see Grindley and Leschziner 1995). It is tempting to speculate that the V(D)J active site of the RAG1/RAG2 complex contains an analogous, metal binding DDE motif.

4.2 The 12/23 Rule

One puzzling aspect of these initial in vitro studies was the observation that DNA cleavage occurred as efficiently at an isolated signal as with a 12/23 pair of signals (McBlane et al. 1995; van Gent et al. 1995), in violation of the 12/23 rule. A system that overcame this difficulty was established using crude extracts containing the core RAG proteins (Eastman et al. 1996). When such extracts were incubated with DNA substrates in the presence of Mg^{2+}, a 12/23 substrate was cleaved approximately 25 times more efficiently than a 12/12 substrate, and substrates with an isolated 12- or 23-signal were not cleaved detectably. Under these conditions, it could be shown that cleavage required synapsis of the two sig-

nals, that the cleavage events at the 12-signal and 23-signal are tightly coupled temporally, and that mutation of one or the other signal is sufficient to block cleavage at both. However, if Mn^{2+} was substituted for Mg^{2+}, coupled cleavage was disrupted and low levels of cutting at isolated signals was observed (Eastman et al. 1996). These findings have recently been extended to V(D)J cleavage in vivo, where again it was found that cleavage is predominantly (but not exclusively) coupled, and that mutations at one signal exert effects on cleavage at both (Steen et al. 1997).

The importance of the divalent cation on properly regulated cleavage was emphasized by the finding that, in the presence of Mg^{2+} but not Mn^{2+}, the purified RAG core proteins also exhibit a preference for a 12/23 substrate over a 12/12 substrate, although in this case it is only three- to fourfold (van Gent et al. 1996b). This demonstrates that the purified core RAG proteins are sufficient for coupled cleavage at two signals, but that this is substantially less efficient than if cleavage is performed in the presence of crude extract. This suggests that one or more other factors may facilitate coupled, 12/23-regulated cleavage. This idea received strong support from recent experiments that reconstituted 12/23 regulated cleavage using purified RAG proteins and crude extract (Sawchuk et al. 1997). Of particular interest were the findings that the nonspecific DNA binding and bending proteins HMG1 or HMG2 increase cleavage at the 23-signal (Sawchuk et al. 1997; van Gent et al. 1997), stimulate coupled cleavage with a 12/23 pair of signals (van Gent et al. 1997), and are components of a stable post-cleavage synaptic complex with the RAG proteins and cleaved recombination signals (Agrawal and Schatz 1997). Interestingly, combining HMG1 with purified RAG1 and RAG2 does not appear to yield properly regulated cleavage, suggesting that one or more additional factors contribute to enforcement of the 12/23 rule (Sawchuk et al. 1997).

Initial reports indicated that Mg^{2+} allows nicking but not substantial hairpin formation with a single signal (van Gent et al. 1996b; Hiom and Gellert 1997). Very recently, however, highly active preparations of purified RAG1 and RAG2 have been demonstrated to form hairpins at an isolated signal in Mg^{2+} (Sawchuk et al. 1997; S. Santagata and E. Spanopoulou, unpublished data), an activity that is suppressed by the addition of crude extract (Sawchuk et al. 1997). Under conditions where hairpin formation strictly obeys the 12/23 rule, nicking can occur at isolated signals and in an asynchronous fashion in substrates with two signals (Q.M. Eastman and D.G. Schatz, unpublished data). The nicking step of

the cleavage reaction is therefore less tightly regulated than second-strand cleavage to form the hairpin.

4.3 Distinct Roles for the Heptamer and Nonamer

The ability of the core RAG proteins to cleave at an isolated signal in either Mn^{2+} or Mg^{2+} has allowed a detailed analysis of the substrate requirements for uncoupled cleavage. In Mn^{2+}, an oligonucleotide substrate containing only a heptamer is able to direct properly targeted nicking and hairpin formation, albeit at a five- to tenfold reduced efficiency compared to an intact signal (Cuomo et al. 1996; Ramsden et al. 1996). However, in the presence of Mg^{2+}, the heptamer allows nicking but not the subsequent transesterification reaction, indicating that the nonamer element is critical for hairpin formation under these conditions (S. Santagata and E. Spanopoulou, unpublished data). The first two positions of the heptamer are particularly important for hairpin formation, while the last four residues make relatively little contribution (Ramsden et al. 1996). In contrast, if only a nonamer is present then nicking is slow and inefficient, and little or no hairpin formation occurs. Interestingly, nonamer-dependent nicking is inaccurate, occurring at multiple locations centered around the position where nicking would normally occur with an intact 12-signal. Cooperation between the heptamer and nonamer requires that they be on the same side of the DNA helix: in a substrate with an 18-bp spacer ($\approx$1.5 turns of the helix) the heptamer and nonamer appear to interfere with one another, resulting in less cleavage than with an isolated heptamer, while a 34-bp spacer ($\approx$3 turns) allows heptamer/nonamer cooperation (Cuomo et al. 1996; Ramsden et al. 1996). Overall, these data support a model in which the heptamer functions to specify the precise site of cleavage while the nonamer serves to anchor the RAG proteins on the DNA (Cuomo et al. 1996; Ramsden et al. 1996).

4.4 DNA Melting During V(D)J Cleavage

Recombination by a mutant RAG1 core protein, referred to as D32 (Sadofsky et al. 1994), is exquisitely sensitive to the sequence of the two coding nucleotides immediately flanking the heptamer (Sadofsky et al. 1995). Certain sequences (referred to as good flanks) allow recombination to proceed as efficiently with D32 as with the wild type RAG1 core,

while others (bad flanks) specifically reduce recombination by D32 by two orders of magnitude or more. Remarkably, the same bad-flank sequences that are recombined poorly by D32 in vivo are cleaved poorly by the wild type core RAG proteins in a Mn^{2+} buffer in vitro (Cuomo et al. 1996; Ramsden et al. 1996). Substrates containing a bad flank are nicked efficiently but hairpin formation is dramatically reduced. Even more remarkably, introducing mismatches at the two coding positions immediately flanking the heptamer restores efficient hairpin formation at bad flank sequences. Hairpin formation must be accompanied by distortion of the coding flank DNA and melting of the CE residues, and the data suggest that this is particularly difficult with bad-flank sequences, either for D32 in vivo or the wild type core RAG proteins in vitro. It was then necessary to explain why wild type RAG proteins are not defective in recombination of bad flanks in vivo. The answer was that coupling of cleavage at the two signals eliminates the effect of bad flanks (van Gent et al. 1996b). When substrates containing a 12/23 pair of signals are incubated with the core RAG proteins and Mg^{2+}, coupled cleavage occurs as efficiently with bad flanks as with good flanks. In Mn^{2+}, however, where productive synapsis does not occur, each signal acts independently and bad flanks inhibit hairpin formation (van Gent et al. 1996b). Thus, in some way that is not understood, formation of a synaptic complex enhances the ability of the RAG proteins to deform CEs into the structure required for hairpin formation.

The ability of the RAG proteins to form hairpin CEs and to interact with unpaired coding flanks suggests that one or both of the proteins has affinity for single-stranded DNA. Indeed, RAG1 has been shown to localize to nucleoli in vivo in an RNA dependent manner (Spanopoulou et al. 1995). Surprisingly, the capacity of the RAG proteins to interact with single-stranded nucleic acids extends to the ability to mediate hairpin formation on a substrate containing a single-stranded recombination signal in Mn^{2+} in vitro (Cuomo et al. 1996; Ramsden et al. 1996). This appears to be mediated by recognition of the single-stranded heptamer, since mutation of the nonamer had little effect on cleavage of such substrates (Ramsden et al. 1996). Interestingly, the single-stranded DNA binding affinity of RAG1/RAG2 is only evident in the presence of Mn^{2+}. If reactions are performed with Mg^{2+}, the RAG proteins fail to bind to a single stranded signal and consequently fail to mediate hairpin formation (S. Santagata and E. Spanopoulou, unpublished data).

5
Sequence-Specific Binding of the Recombination Signal by RAG1 and RAG2

Initial studies revealed a high nonspecific DNA binding activity of RAG1 and no detectable DNA binding affinity for RAG2 (Leu and Schatz 1995; Spanopoulou et al. 1996; P. Cortes and E. Spanopoulou, unpublished data). To circumvent the difficulties raised by the nonspecific DNA binding activity of RAG1, three different strategies were employed. A genetic approach was developed to study the DNA binding specificities of RAG1/RAG2 in vivo. A second approach employed the highly sensitive assay of surface plasmon resonance. The third approach was based on conventional electrophoretic-mobility shift assays (EMSA) using stringent conditions that diminished the nonspecific DNA binding properties of RAG1.

5.1
Genetic, One Hybrid Assay

To study recognition of the recombination signal by the RAG proteins in vivo, a reporter vector was constructed by placing the heptamer-nonamer motifs in front of a minimal promoter-driving expression of a luciferase gene. The promoter is poorly active unless a transcriptional activator is provided in trans. For that purpose, RAG1 and RAG2 were converted to transcriptional activators by fusing them to the acidic domain of VP16. Co-expression of the RAG proteins with the reporter construct revealed their ability to bind to the signal by measuring luciferase production. These studies uncovered several important points (Difilippantonio et al. 1996). First, the nonspecific DNA affinity of RAG1 observed in vitro was not evident in vivo, suggesting that this activity of the protein is highly regulated. Second, RAG1 was able to specifically recognize the nonamer motif, albeit relatively weakly, and binding was abolished by amino acid changes in the N-terminal portion of the core (a region with homology to the Hin-family of bacterial invertases and to homeodomains; see below). Third, in the presence of the nonamer, it was possible to detect a weak interaction of RAG1 with the heptamer. Fourth, binding of RAG1 to the signal mediated recruitment of RAG2, and this occurred efficiently only when the heptamer and nonamer motifs were separated by one turn of the helix (12-signal) but not two turns of the helix (23-signal). Since in vivo a 12-signal almost invariably recombines with a 23-signal, the DNA binding profile revealed that distinct roles for

the 12- and 23-signals could be imposed in part at the level of signal recognition. It should be emphasized that although these interactions were measured in vivo and take into account the potential contribution of other cellular factors, recognition of recombination signals in the Ig and TCR loci likely require the involvement of additional factors that regulate accessibility of the signals and that therefore modulate DNA binding by the RAG proteins.

5.2 Surface Plasmon Resonance

Surface plasmon resonance (SPR) methodology was employed because it allows the measurement of real-time kinetics and the use of a broad spectrum of experimental conditions (Panayotou et al. 1993). Purified RAG1 and RAG2 were tested for their ability to interact with the heptamer-nonamer motifs immobilized on a chip, with 0.25 M NaCl used to reduce nonspecific DNA binding by RAG1 (Spanopoulou et al. 1996). Purified RAG1 specifically recognized the nonamer motif, and this interaction was mediated by the Hin/homeodomain-homologous region of the protein. Point mutations within that domain abolished specific binding, and a polypeptide encompassing the homeodomain region of RAG1 was sufficient for specific recognition of the nonamer. Interaction of RAG1 with the nonamer allowed subsequent recognition of the heptamer motif, but both interactions were of low affinity. Mutations within the potential heptamer-interacting region of RAG1 (see below) or the Hin-homologous region altered binding of the protein to the nonamer once the heptamer was mutagenized. Moreover, a RAG1 variant with mutations within the homeodomain helix III homologous region (see below) showed increased binding to the nonamer compared to the wild type protein, and binding of this mutant was further enhanced when the heptamer was mutagenized. The DNA binding profile of these mutants revealed that RAG1 is capable of recognizing both the heptamer and nonamer. RAG2 alone did not show any specific binding to the signal. When both RAG proteins were added simultaneously, DNA cleavage was detected. Under these experimental conditions, specific cleavage was dependent on the presence of an intact heptamer motif (Spanopoulou et al. 1996).

5.3 Electrophoretic-Mobility Shift Assays

Recently, the interaction of RAG1 and RAG2 with the recombination signal was analyzed by electrophoretic-mobility shift assays (EMSA) after performing binding in 20% DMSO and 100 mM potassium acetate to reduce nonspecific DNA binding (Hiom and Gellert 1997). Under these conditions, the low affinity interactions of RAG1 with the heptamer-nonamer motifs (observed in the one-hybrid and SPR experiments) were diminished and only the specific binding of RAG1 and RAG2 as a stable complex could be observed. Coordinated recognition of the heptamer-nonamer elements was much more pronounced on a 12-signal, with binding to the 23-signal reduced sixfold, consistent with the observations from the one hybrid system (Difilippantonio et al. 1996). Mutations of the first three nucleotides of the heptamer, which are very important for DNA cleavage (Cuomo et al. 1996; Eastman et al. 1996; Ramsden et al. 1996) reduced binding by 2.5-fold, while mutation of the last four reduced binding by fourfold. Elimination of the entire heptamer motif, however, drastically reduced binding (by 25-fold), and elimination of the nonamer reduced total binding by tenfold. The effect on binding of mutations of heptamer and nonamer of the 23-signal were not examined and hence the basis of reduced binding to the 23-signal remains unclear.

DNA binding was observed in the presence of Mg^{2+}, Mn^{2+}, or Ca^{2+}, but the three metal ions supported cleavage to different extents: Mg^{2+} allowed nicking of the DNA, Mn^{2+} permitted nicking and hairpin formation, but neither cleavage nor nicking were observed in Ca^{2+} (Hiom and Gellert 1997). Importantly, the complex formed in the presence of Ca^{2+} could be made competent for nicking, or nicking and hairpin formation, by replacing Ca^{2+} with Mg^{2+} or Mn^{2+}, respectively. After cleavage of the DNA in the presence of Mn^{2+}, the proteins were released from the DNA template, indicating that RAG1–RAG2 fail to remain bound to the blunt-ended signal. This is in contrast to the pattern observed after coupled cleavage of a substrate with a 12- and 23-signal, where the RAG proteins remain stably bound to the signals (Agrawal and Schatz 1997).

5.4 A Working Model for Signal Recognition by the RAG Proteins

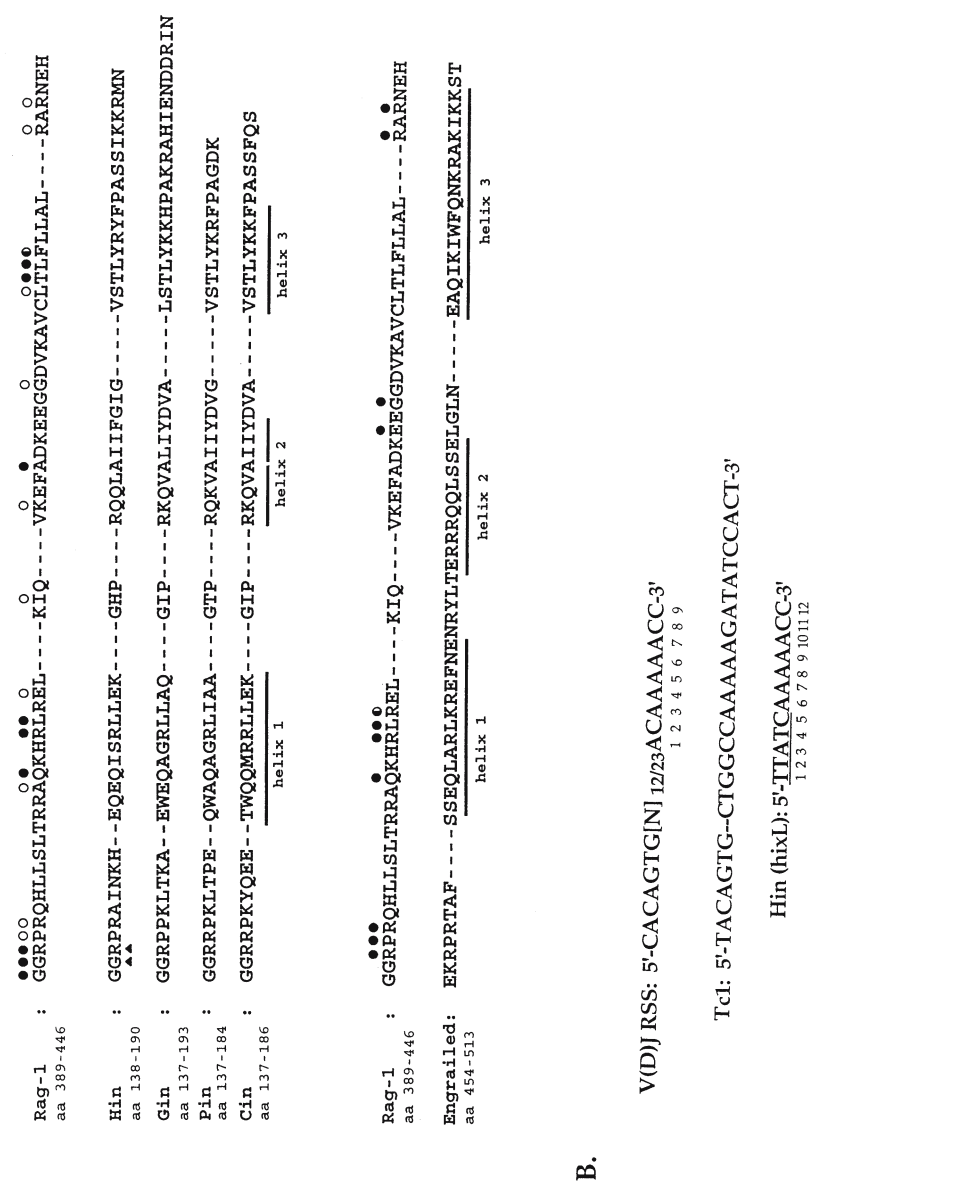
The above studies indicate that RAG1 has the ability to recognize the nonamer in the absence of RAG2, an interaction that may serve to an-

chor RAG1 on the signal and permit its subsequent interaction with the heptamer, at least in the case of the 12-signal. It is currently unknown whether the same molecule of RAG1 recognizes both the heptamer and nonamer, or whether recognition of the two elements is mediated by different RAG1 molecules. Binding of RAG1 to the signal would allow recruitment of RAG2, which enhances stability of the complex. This model and the existing data leave open the important question of whether RAG2 directly binds to DNA when complexed to RAG1, or whether it changes the conformation of RAG1 so that it can now recognize both elements with increased affinity. It also remains unclear whether, in vivo, formation of the RAG1–RAG2 complex precedes DNA binding, or if instead RAG1 binds first to the signal and subsequently recruits RAG2 to form a stable complex.

Several transposases recognize their target DNA in two phases, thereby increasing the steps at which binding and cleavage can be regulated (Derbyshire and Grindley 1992, and references therein). In the first phase, the transposase uses a DNA binding domain to anchor to the DNA adjacent to the site of cleavage. In the second phase, the cleavage site is recognized and cleaved. Such a mechanism facilitates formation of the proper productive complex and helps ensure that cleavage at the two ends of the transposon is coupled (Mizuuchi 1992). A similar mechanism can be envisioned to help establish the 12/23 rule in V(D)J recombination, with the first phase being anchoring of RAG1 on the nonamer. The different spacer lengths between the heptamer and nonamer would then dictate differential recruitment of RAG2 to the 12- and 23-signals, which in turn would influence formation of a productive synaptic complex. In vivo, DNA binding of the RAG proteins and enforcement of the 12/23 rule could be regulated, both positively and negatively, by the action of other proteins.

Perhaps the most interesting resemblance between V(D)J recombination and transposition stems from the similar modes of DNA recogni-

Fig. 5A, B Homology of RAG1 to the *Salmonella* Hin recombinase and to homeodomain proteins. **A** Homology of RAG1 (amino acids 389–442) to the DNA binding domains of the Hin family of invertases and to the engrailed homeodomain. *Closed circles* indicate conserved amino acid residues and *half-open circles* indicate conservative substitutions. *Open circles* show homology of RAG1 to other enteric invertases. **B** The cognate DNA binding half-site of Hin (*hix*) (Hughes et al. 1992), the 26-bp terminal nucleotides of transposon Tc1 (Vos and Plasterk 1994) and the consen-



sus nonamer motif of V(D)J recombination (Hesse et al. 1989; Ramsden et al. 1994). *Triangles* above the nonamer-homologous region of *hix* indicate the nucleotides contacted by Hin amino acids G_{139} and R_{140} (**A**) in the minor groove while underlining indicates nucleotides contacted by Hin helix III in the major groove (Feng et al. 1994)

tion by RAG1–RAG2 and by the nematode transposase Tc1A. It was previously recognized that the end sequences of the Tc1 transposon share striking similarity to the heptamer and nonamer (Fig. 5) (Dreyfus 1992). The *Caenorhabditis elegans* Tc1A transposase establishes high-affinity interactions with the nonamer-like motif using its N-terminal domain. Cleavage occurs at the heptamer-like site, yet no interactions could be detected between Tc1A and the heptamer-like motif (Vos et al., 1993; Vos and Plasterk 1994).

5.5 The RAG1 Homeodomain

RAG1 shares homology to the Hin family of bacteriophage recombinases, in particular the DNA binding domain of the *Salmonella* Hin invertase (Fig. 5), as well as to homeodomain proteins. The cognate DNA binding site of Hin, referred to as hix, is a bipartite structure of two motifs, TTAT, followed by CAAAAACC (Hughes et al. 1992). Strikingly, of the last nine nucleotides in hix, eight are identical to the consensus nonamer sequence (Fig. 5). In fact, it had previously been pointed out that the Hin binding site in the flagellin promoter not only contains a nonamer homologous sequence but is preceded by a heptamer-like motif 23 bp upstream of the nonamer sequence (Rathbun and Tucker 1987; Simon et al. 1980) (Fig. 5). The DNA binding domains of the Hin family of recombinases (Hin, Cin, Gin and Pin) share extensive homology (Fig. 5) and are functionally interchangeable (Van de Putte and Goosen 1992). These domains fold into a helix-turn-helix structure (Feng et al. 1994) and have extensive homology to the well-characterized DNA binding domain of homeodomain proteins (Affolter et al. 1991). Homeodomains recognize A/T-rich DNA sequences and constitute the DNA binding domain of proteins that act as gene regulators specifying cell differentiation and development of the organism (Gehring et al. 1994).

The X-ray structure of the 52-amino acid DNA binding domain of Hin bound to its cognate DNA site has been solved (Feng et al. 1994). The DNA binding specificity of Hin is mediated by interactions of residues G139/R140 with nucleotides A8T9 (of the opposite strand) of the nonamer-homologous sequence in the minor groove, and interactions of helix III with nucleotides T1T2A3T4C5 in the major groove (Fig. 5). In addition to its perfect identity with the GGRPR residues of Hin, the N-terminal portion of the RAG1 core has a high α-helical content when expressed in bacteria (K. Rodgers and J. Coleman, personal communication). However, RAG1 shares less homology to Hin in the region corre-

sponding to helix III (Fig. 5), suggesting that the two recognition helices determine different DNA binding specificities. Based on these observations, the Hin-homologous region of RAG1 was mutagenized and analyzed for recombination and DNA binding activity (Difilippantonio et al. 1996; Spanopoulou et al. 1996). Single point mutations of amino acids that are important for Hin DNA binding abolished the ability of RAG1 to either activate V(D)J recombination or to establish specific interactions with the nonamer motif. The Hin-homologous region of RAG1 was sufficient for specific binding to the nonamer, and strikingly, substitution of the DNA binding domain of Hin for the corresponding region of RAG1 produced a hybrid protein that could execute V(D)J recombination at levels 20% of wild type RAG1 (Spanopoulou et al. 1996). Interestingly, the Hin/RAG1 hybrid protein could mediate V(D)J recombination of a wild type recombination substrate but not of a substrate in which the second part of the Hin recognition site (TTAT) was placed adjacent to the nonamer motif to generate the sequence TTATCAAAAACC (S. Santagata and E. Spanopoulou, unpublished data). Presumably in this substrate, helix III of Hin interacts with the TTAT site, thus altering the conformation of the Hin/RAG1 hybrid protein.

The fact that RAG1 contains a DNA binding domain with homology to homeodomain proteins raises several issues concerning specificity of DNA binding by the RAG1/RAG2 complex. Compilation of the sequence of many recombination signals has indicated that the nonamer is not conserved to the same degree as the heptamer (Ramsden et al. 1994). The answer as to how the RAG proteins recognize signals with divergent nonamer sequences might lie with the homeodomain of RAG1. Homeodomains generally recognize AT-rich motifs and exhibit significant promiscuity in their interaction with target sites (Gehring et al. 1994). Given that homeodomain proteins achieve specificity of DNA binding by homodimerization or by forming heterodimers (White 1994), it is possible that the specificity of RAG1 DNA binding is modulated through its interaction with other proteins or by direct homodimerization. Therefore, while purified RAG1 exhibits a low affinity for the nonamer in vitro (Spanopoulou et al. 1996), heterodimerization of RAG1 with other proteins could increase (or perhaps change) its DNA target specificity in vivo. This is an important issue not only for uncovering the mechanism of the initial stages of V(D)J recombination, but also for understanding whether RAG1 could acquire altered DNA binding specificity. In several cases of chromosomal translocations, the action of the V(D)J recombinase has been implicated in the translocation of oncogenes (Rabbitts

1994). It is therefore relevant to elucidate the DNA binding potential of RAG1–RAG2 in vivo.

5.6 Recognition of the Heptamer

Initiation of V(D)J recombination requires recognition of both the heptamer and the nonamer by RAG1 and RAG2 (Hesse et al. 1989; Eastman et al. 1996;Hiom and Gellert 1997), but little is known about the domain(s) that recognize the heptamer. The SPR assay and one hybrid assay indicated that RAG1 alone has the ability to recognize both the nonamer and heptamer motifs (Difilippantonio et al. 1996; Spanopoulou et al., 1996), and several mutations within RAG1 altered its ability to recognize the heptamer site (Spanopoulou et al. 1996). The first indication that RAG1 might recognize the heptamer motif came from studies of the D32 mutant RAG1 protein described above, which exhibits sensitivity to the sequence of the coding nucleotides flanking the heptamer (Sadofsky et al. 1995). This mutation replaces six amino acids of the core (SEKHGS 606–611) with the sequence VD; mutation of His 609 to Leu was shown to have a similar effect (Roman and Baltimore 1996). As discussed above, the phenotype of these mutants can be explained by a deficiency in melting of the DNA at the heptamer/coding flank border. However, recent data suggest that the coding region flanking the heptamer might influence the reaction in additional ways. In cleavage substrates carrying a good flank (5′-TA-heptamer), if the 3′ bridging oxygen of the T-p-A phosphodiester bond is replaced by sulfur, the signal becomes a poor substrate for DNA recognition by the RAG proteins if EMSA is performed in the presence of Mg^{2+} rather than Mn^{2+} (S. Santagata and E. Spanopoulou, unpublished data). Since sulfur can properly coordinate Mn^{2+} but not Mg^{2+}, these data strongly suggest that the coding region flanking the heptamer is involved in coordination of Me^{2+} that is required for DNA recognition of the coding–heptamer region. Therefore, it appears that the coding region flanking the heptamer motif affects both DNA recognition and melting of the DNA during hairpin formation.

5.7 Evolution of V(D)J Recombination

The detailed study of prokaryotic recombination systems provides insight into the evolution of mechanisms that catalyze genomic rearrange-

ment and diversity. The clearest example is the striking parallels between bacteriophage integration/excision and retroviral integration. The homology of RAG1 to the DNA binding domain of bacterial site-specific recombinases and the presence of prototypic V(D)J recombination signals in the flagellin promoter suggest a potential evolutionary relationship. It has also been proposed that V(D)J recombination evolved from prokaryotic transposition systems (Thompson 1995). Although site-specific recombination and transposition are thought to be mechanistically distinct systems (Craig 1988), it is possible that they have evolved from interrelated processes. This notion is underlined by the function of the TnPR recombinases, which is encoded by the Tn3 transposon and mediates site-specific recombination of the IRS elements of Tn3 (Simon et al. 1980).

Based on the juxtaposition of the *RAG1* and *RAG2* genes and the organization of their coding information within one exon, it has been suggested that they represent the product of evolution of a single transposon (Thompson 1995). However, while *RAG1* from amphibians, reptiles, birds and mammals contains a single coding exon (Thompson 1995, and references therein), zebrafish and rainbow trout *RAG1* contain an intron that splits the active core coding information into two separate exons (Hansen and Kaattari 1995; K. Willet and L. Steiner, personal communication). Remarkably, the border of the two exons is at the position corresponding to amino acid 458 of the mouse RAG1 protein, thus separating the RAG1 Hin-homologous DNA binding domain from the rest of the protein. It is therefore possible that during evolution V(D)J recombination adopted DNA motifs and protein structures from several prokaryotic recombination systems.

6
Future Prospects

The extensive information accumulated from in vivo studies and the development of in vitro assays that can address the biochemistry of V(D)J recombination, together with the availability of powerful genetic techniques such as transgenic and knock out mice, establish the basis for studying the regulation and biochemistry of V(D)J recombination in the context of lymphoid differentiation. Crucial to our understanding of the regulation of V(D)J recombination is the identification of the second messenger pathways that lead from surface receptors to activation or suppression of recombination. Other major issues are the identification

of transcription factors that activate expression of *RAG1* and *RAG2,* and determining how transcriptional enhancers regulate rearrangement of Ig and TCR loci. In this latter context, a promising system was developed by Schlissel and colleagues in which accessibility of the Ig and TCR loci can be monitored in isolated nuclei and modulated by the addition of exogenous factors (Stanhope-Baker et al. 1996). This provides a powerful tool to identify regulatory factors and study their mode of action in a defined system.

At the initial stage of V(D)J recombination in vivo, RAG1 and RAG2 must specifically recognize two recombination signals that can be hundreds of kilobases apart. How is this recognition specificity determined, is it influenced by interactions of the RAG proteins with other factors, and as in prokaryotic systems, are DNA bending proteins involved? The identification of additional proteins that facilitate the formation of a productive synaptic complex should allow their characterization using the available in vitro assays. These studies could then address the nature and quality of protein–protein and protein–DNA interactions that establish the active complex. Since it has been shown that the RAG1–RAG2 complex contains endonuclease activity, important issues are the identification of the active center of the complex and the determination of how it relates to the active center of other known recombinases. It seems likely that structural studies of the RAG proteins will be of particular importance in answering such questions.

Cleavage of DNA by the RAG proteins leads to the engagement of the ubiquitous DNA repair proteins Ku70/80, DNA-PK and XRCC4. Hence, a major goal of future studies will be to determine the precise contribution of each of these proteins. The ongoing development, in several laboratories, of cell-free systems capable of performing the complete V(D)J recombination reaction offers a powerful tool for this analysis. Given that DNA repair is regulated by cell cycle processes, it will be of interest to determine whether DNA repair proteins provide a direct link between V(D)J recombination and the cell cycle.

7 Epilogue

This review was originally completed in mid-1997. One year later, on September 3, 1998, Eugenia Spanopoulou, her husband, Andrew Hodtsev, and their 1-year-old son, Platon, were killed in the crash of Swissair flight 111 near the coast of Nova Scotia. To the best of my

knowledge, this was the last review article Eugenia wrote. It has been left essentially unchanged from the form in which she left it so as to best reflect her thoughts and vision of the field. The review illustrates Eugenia's passion for a mechanistic understanding and her remarkable insight and intuition [e.g., concerning the mechanism of cleavage and the RAG active site (Kim et al. 1999; Landree et al. 1999; Fugmann et al. 2000), and the interaction of RAG1 with the heptamer (Peak et al. 2003)]. It also reports some observations made in her lab and never published.

In April of 1998, I had the opportunity to call and tell Eugenia that we had evidence that RAG1–RAG2 had transposase activity [published later that year in parallel with a paper from Martin Gellert's group (Agrawal et al. 1998; Hiom et al. 1998)]. I remember her sense of excitement, her unqualified pleasure in a colleague's discovery, and how her mind leapt to consider the implications of this new information. Indeed, in our last conversation, only days before her death, Eugenia was simply bursting with ideas and excitement about the field of V(D)J recombination and its potential for new and exciting advances.

Since this review was written, much has been learned about the RAG1 and RAG2 proteins, and the mechanism of V(D)J recombination. As noted above, the active site of the RAG1–RAG2 complex was identified and shown to contain one or more divalent metal ions (Kim et al. 1999; Landree et al. 1999; Fugmann et al. 2000). Using improved mobility shift assays, evidence for additional RAG–DNA complexes has been obtained (Hiom and Gellert 1998), and evidence for post-cleavage functions for the RAG proteins has emerged (Qiu et al. 2001; Schultz et al. 2001; Tsai et al. 2002). It has become clear that the 12/23 rule must be supplemented with a "beyond 12/23" rule, to accommodate the finding that not all 12/23 pairs of RSSs are compatible (Bassing et al. 2000), and new insights are emerging to help explain the mechanisms that regulate V(D)J recombination (Hesslein and Schatz 2001; Krangel 2003). Artemis has been discovered and demonstrated to be the gene defective in many cases of human SCID (Moshous et al. 2001), and strong evidence implicates the artemis protein (together with DNAPKcs) as the long-sought hairpin-opening factor (Ma et al. 2002). In addition, some insight has been gained into how RAG-mediated transposition, and its undesirable consequences, might be suppressed in vivo (Elkin et al. 2003; Tsai and Schatz 2003).

Finally, it is worth noting that at least two issues, identified in this review as critical for progress in the field, remain largely unresolved. The first is the function of RAG2. RAG1 contains a number of important activities and domains, but remains catalytically inert in the absence of

RAG2. How does RAG2 contribute to DNA binding affinity and specificity, and to catalysis? Evidence from mutagenesis studies implicate RAG2 in direct DNA binding and other functions (Fugmann and Schatz 2001; Qiu et al. 2001; Liang et al. 2002; Akamatsu et al. 2003), but to date, no detailed picture has emerged for the role of this enigmatic player. The second outstanding issue is that of the three-dimensional structure of the RAG proteins, in complex with one another and with DNA. Given that the RAG genes were discovered almost 15 years ago, it is surprising that such structural information remains elusive. One can't help but wonder how many of these major unresolved questions would already be answered had Swissair flight 111 remained aloft.

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The pre-B cell Receptor in B Cell Development: Recent Advances, Persistent Questions and Conserved Mechanisms

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Abstract B cell development is a process tightly regulated by the orchestrated signaling of cytokine receptors, the pre-B cell receptor (BCR) and the B cell receptor (BCR). It commences with common lymphoid progenitors (CLP) up-regulating the expression of B cell-related genes and committing to the B cell lineage. Cytokine signaling (IL-7, stem cell factor, FLT3-L) is essential at this stage of development as it suppresses cell death, sustains proliferation and facilitates heavy chain rearrangements. As a result of heavy chain recombination, the pre-BCR is expressed, which then becomes the primary determiner of survival, cell cycle entry and allelic exclusion. In this review, we discuss the mechanisms of B cell lineage commitment and describe the signaling pathways that are initiated by the pre-BCR. Finally, we compare pre-BCR and pre-TCR structure, signal transduction and function, drawing parallels between early pre-B and pre-T cell development.

1 Introduction

B cell development is a highly ordered and regulated process during which lineage-committed stem cells give rise to peripheral populations of diverse clonal B cells encompassing a broad repertoire of antigen specificities (Fig. 1). The driving event of B cell development is the sequential rearrangement of heavy and then light chain. Each recombination constitutes a discrete transition in which rearrangements capable of supporting expression of a surface receptor are selected for further development. Successful rearrangements also efficiently extinguish subsequent recombination, ensuring clonality. Subsequent to generating light chain, both negative and positive selection processes, as well as receptor editing, provide for populations of self-tolerant peripheral cells that have stereotypic responses to foreign ligands (Wang and Clark 2003; Wang et al. 2003). We, and other investigators, have recently summarized what is known about these latter-stage processes (Meffre et al. 2000; Hardy and Hayakawa 2001; Wang and Clark 2003). In this current review, we focus

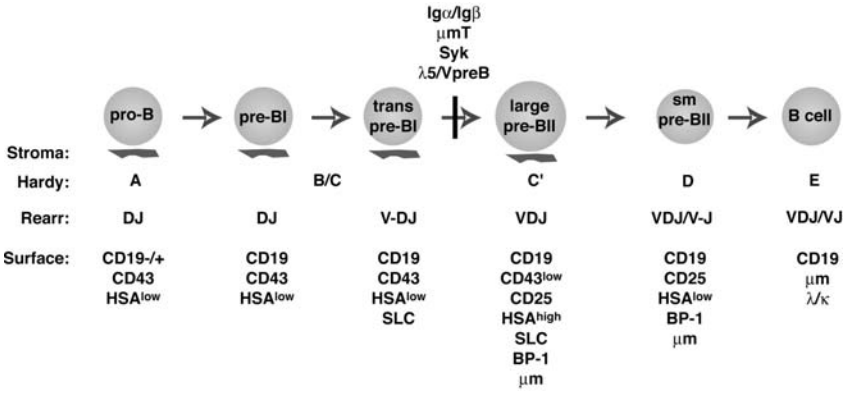

Fig. 1 B cell development. B cell development can be divided into a series of discrete stages each characterized by both the status of heavy (*VDJ*) and light chain rearrangement (*VJ*) and surface phenotype. There are two well-described schema for categorizing B cell development. The schema proposed by Melcher defines developmental stages with an emphasis on the status of heavy and light chain rearrangement describing B cell progenitors as either Pro- or Pre-B cells (*Melchens 1995*). The schema proposed by Hardy et al. (1991) defines each stage of development (*A through E*) based on surface markers and dependence on bone marrow stroma for growth ex vivo. Listed are markers that are readily detected on the cell surface at the indicated stages (*SLC*, surrogate light chain)

on the earlier stages of B cell development in which cells make the initial decision to become a B cell progenitor and at which heavy chain rearrangements are assembled into a pre-BCR and checked for competency. We then compare pre-BCR function to pre-TCR function to identify commonalities in early developmental mechanisms.

2 Commitment to the B Cell Lineage

Pro-B cells expressing CD19 are the first irrevocably committed B cell precursors (Hardy et al. 1991; Allman et al. 1999; Nutt et al. 1999). Surprisingly, $CD19^-$ progenitors expressing B220, Igα/Igβ and in which heavy chain DJ rearrangement has occurred still have multi-potentiality. Lineage commitment is determined by the transcription factor Pax5 (BSAP), which both activates B cell-specific genes (including *BLNK*, *CD19*, and *Igα*) and represses non-B lineage genes including *Notch1* (Nutt et al. 1999; Schebesta et al. 2002; Souabni et al. 2002). Pax5 expression in turn is controlled by a network of transcription factors including the E2A family transcription factors E12 and E47 (Bain et al. 1994), and the transcription factor EBF (Lin and Grosschedl 1995). In addition to upregulating Pax5 (Sigvardsson et al. 1997; Kee and Murre 1998; O'Riordan and Grosschedl 1999), these transcription factors regulate expression of the B cell-specific genes *λ5*, *VpreB*, *Igα*, and *Igβ*, as well as the recombinase-activating genes *RAG-1* and *RAG-2*. Although this network of transcription factors regulates the expression of several components of the pre-BCR, there is no evidence that this receptor plays a role in lineage commitment (Kitamura et al. 1992; Kraus et al. 2001; Pelanda et al. 2002). Rather, the reliance on transcription factors suggests that stochastic processes are important.

Commitment to the B cell lineage and the expansion of B cell progenitors is also influenced by the bone marrow microenvironment. Signaling through the IL-7 receptor upregulates Pax5 (Billips et al. 1995; Corcoran et al. 1998) and is required to generate $CD19^+$, $CD43^+$ pro-B cells (Miller et al. 2002). Signals through the IL-7 receptor also prime for subsequent V-DJ heavy chain rearrangement by opening the distal V locus (Corcoran et al. 1998).

3
The Development of Pre-B Cells

Progression past the pre-BI cell stage requires expression of a signaling-competent pre-BCR containing Igα, Igβ, an in-frame V-DJ rearrangement of heavy chain that can be expressed on the cell surface and a surrogate light chain complex containing λ5 and VpreB (Kitamura et al. 1991; Gong and Nussenzweig 1996; Pelanda et al. 2002; Shimizu et al. 2002). B cells lacking any component of the pre-BCR can undergo limited V-DJ recombination; however, B cells expressing successfully rearranged heavy chains are not selected for expansion and further development (Pelanda et al. 2002). The expression of the pre-BCR is transient; it is usually only detected on a small population of B cell precursors in the bone marrow (Burrows et al. 2002). However, this transient expression coordinates several discrete and temporally distinct processes required for B cell development.

The first, and probably the least understood process that occurs following successful heavy chain rearrangement is allelic exclusion (Kitamura and Rajewsky 1992; Papavasiliou et al. 1995). It has been proposed that chromosomal accessibility is a primary regulator of V-DJ recombination (Sleckman et al. 1998). This model is supported by several publications (Martensson et al. 2002), including recent observations that non-transcribed *IgH* alleles are associated with heterochromatin (Skok et al. 2001), that alleles destined for transcription and recombination occupy different positions within the nucleus (Kosak et al. 2002) and that histone acetylation correlates with recombination (Johnson et al. 2003).

Allelic exclusion ensures that monospecific B cells will be produced. It has been argued that monospecificity prevents autoreactive B cells from escaping to the periphery. However, it has recently been demonstrated that the majority of initial specificities generated in the bone marrow are autoreactive and that only a minority of B cells are selected into the periphery (Wardeman et al. 2003). If B cells were poly-specific, it is likely that almost all would be deleted, precluding a diverse peripheral repertoire (Schlissel 2002). Monospecificity also ensures that peripheral B cells will predictably discriminate between diverse ligands based on avidity.

Expression of the pre-BCR in transitional pre-B1 cells induces two to five cycles of cell division (Rolink et al. 2000) and transit to the large pre-BII stage. Proliferation does not equally expand all successfully rearranged heavy chains, as there is a skewing towards increased representation of distal Vh gene families. This may be due to better pairing of

these rearrangements with surrogate light chain and augmented proliferation (Martensson et al. 2002). During and after this proliferative burst, pre-B cells mature as manifested by the downregulation of several surface markers, including HSA and CD43, and the upregulation of CD25. Recent evidence indicates that IL-7 may contribute to the initial pre-BCR-mediated proliferative burst (Fleming and Paige 2001). However, maturing pre-BII cells rapidly become unresponsive to IL-7 and no longer require stromal cell contact for continued growth and survival. Noncycling small pre-BII cells then begin light chain rearrangement, first at the kappa locus and then at the lambda locus (Stoddart et al. 2000; Yankee and Clark 2000).

The general order of B cell development, and the principal markers of each stage have been known for some time (Hardy et al. 1991). However, the underlying complexities of how the pre-BCR mediates early B cell development are only becoming apparent. In the following sections, we provide a model for how the pre-BCR may transmit signals and for how these different signals may integrate to mediate early B cell development.

4 Initiating Signals Through the Pre-BCR

Specific genetic deletions in mice have demonstrated that many of the signaling molecules important for BCR signaling in peripheral B cells are also utilized by the pre-BCR to affect B cell development. These include the signaling components of the BCR, Igα, and Igβ as well as the tyrosine kinase Syk (Cheng et al. 1995), the Src family kinases, Fyn, Lyn and Blk (Saijo et al. 2003) and the linker molecule BLNK (Pappu et al. 1999). Cross-linking the pre-BCR complex on cultured pro-B cells induces robust intracellular calcium fluxes and the phosphorylation of cytosolic proteins. Therefore, it is likely the current biochemical model of BCR signaling derived from studies of peripheral B cells and B cell lines is relevant to the pre-BCR (Fig. 2).

However, there are several important differences between the signaling mechanisms utilized by the pre-BCR and peripheral BCR complexes. First, in peripheral B cells both Syk (Cornall et al. 2000) and BLNK (Ishiai et al. 1999; Kurosaki and Tsukada 2000) are obligatory for signaling through the BCR. In pre-B cells, functional homologs of both signaling molecules that are normally expressed exclusively in T cells, Zap-70 (Schweihoffer et al. 2003) and SLP76/LAT, respectively, are found in sig-

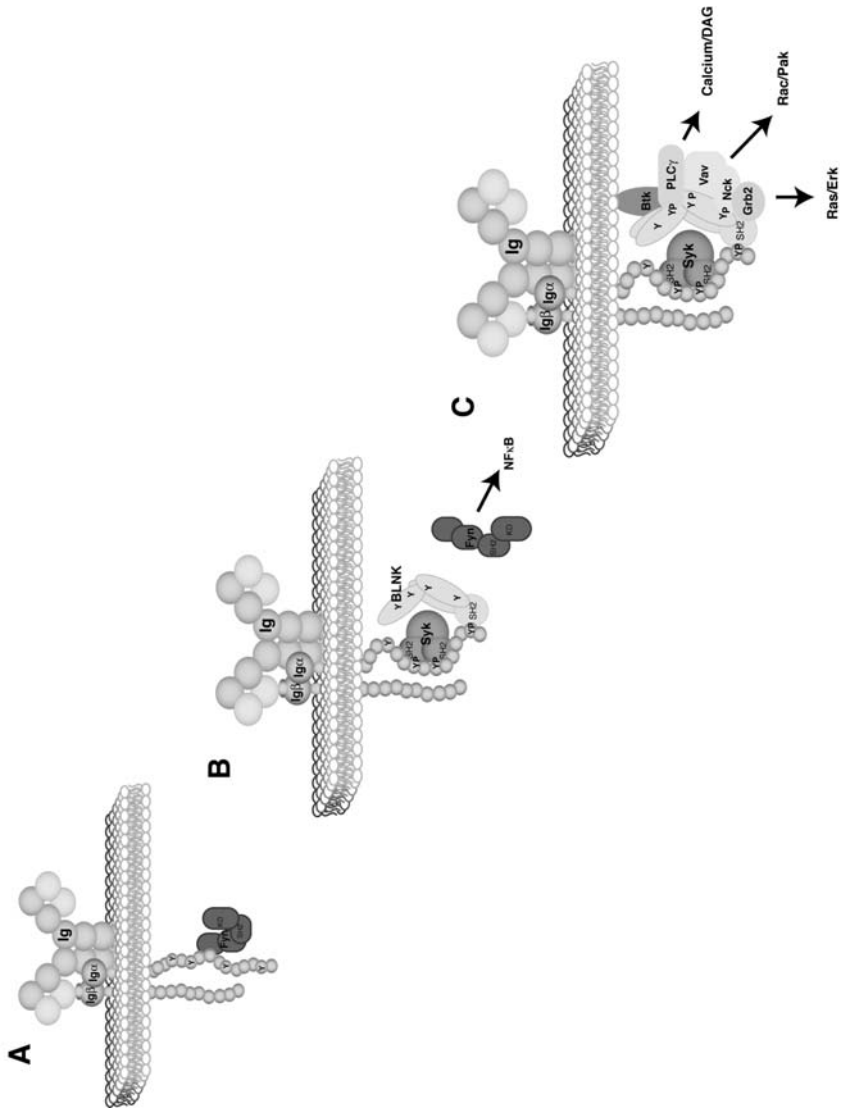

Fig. 2A–C Activation of BCR signaling pathways. **A** The resting receptor is assembled with inactive tyrosine kinases such as Fyn and probably Syk (Cambier et al. 1994; DeKoter et al. 2002). **B** Upon receptor activation these kinases become activate and phosphorylate both immunoreceptor tyrosine-based activation motif (*ITAM*) and non-ITAM tyrosines in the cytoplasmic tail of Igα (Campbell 1999; Kabak et al., 2002). Phosphorylation of the Igβ ITAM also occurs. The phospho-ITAM serves to recruit and activate Syk (Kong et al. 1995) while the carboxy-terminal phosphorylat-

nificant amounts (Su and Jumaa 2003). These homologs can partially compensate for a lack of their B cell-restricted counterparts. Permissive expression is probably a consequence of the recent multi-potentiality of these early B cell progenitors. Second, while the Igα ITAM tyrosines of the BCR are requisite for peripheral BCR function, they are largely dispensable for early B cell development. In both Igα and Igβ, ITAM tyrosine-independent domains within each tail are important (Kraus et al. 2001; Kraus et al. 1999; L.D. Wang, A.B. Cooper and M.R. Clark, manuscript in preparation). These observations suggest that signals may be initiated differently in the pre-BCR and peripheral BCR. Third, the pre-BCR is not antigen specific and therefore cannot respond to ligands in the same way as the peripheral BCR. The mechanisms by which the pre-BCR initiates signals has been an area of significant study.

One of the central questions in pre-BCR signaling is whether there is a selecting ligand. Stromal cell ligands have been described. The human pre-BCR can interact with galectin (Gauthier et al. 2002), while the murine pre-BCR binds heparan sulfate (Bradl et al. 2003). This latter interaction requires the non-immunoglobulin domain of lambda 5. However, the functional significance of these interactions is unclear. It is also possible that the pre-BCR self-aggregates on the cell surface. Arginine residues within the same non-immunoglobulin domain of lambda 5 that binds heparan sulfate allow the aggregation of the pre-BCR and the constitutive activation of BCR-dependent pathways (Ohnishi and Melchers 2003). The in vivo functional significance of lambda 5 aggregation is not clear, especially in light of recent evidence that B cell development can progress in the absence of lambda 5 (Schuh et al. 2003).

Beyond these investigations, most studies indicate that a selecting ligand for the pre-BCR is not required for B cell development. Indeed, several studies indicate that simple surface expression of the pre-BCR may be sufficient. Retroviral-mediated expression of a construct encoding the myristoylation domain of Lck and the cytoplasmic domains of Igα and Igβ in $RAG^{-/-}$ pro-B cells is able to rescue progression past the pre-B stage (Bannish et al. 2001). Furthermore, expression of a mu chain

ed non-ITAM tyrosine recruits the linker BLNK (Kabak et al. 2002). Fyn dissociates from the receptor and activates signaling cascades that regulate NFκB (Saijo et al. 2003). **C** Phosphorylation of BLNK occurs, which serves to recruit and coordinate multiple signaling molecules and initiate several signaling cascades (Chiu et al. 2002)

that cannot associate with surrogate light chains, or a mu chain in which a majority of the extracellular region has been replaced with an irrelevant protein (CD8) can still mediate transit to later developmental stages (Muljo and Schlissel 2002; Shaffer and Schlissel 1997) These studies suggest that the extracellular domains of the pre-BCR are largely irrelevant for mediating B cell development.

Even if surface expression of the pre-BCR is sufficient for early B cell development, it is likely that some aggregation of surface receptor complexes occurs. Aggregation could be an intrinsic property of the pre-BCR, as has been reported for the mature BCR (Schamel and Reth 2000). Alternatively, aggregation could occur through localization in circumscribed cholesterol- and sphingolipid-rich lipid microdomains (lipid rafts or GEMS). In human pre-B cell lines, approximately 30% of resting pre-B cell receptors accumulate in lipid rafts, whereas in mature B cell lines the BCR is excluded from the lipid rafts (Guo et al. 2000). Localization in lipid rafts would not only serve to concentrate receptor complexes but would bring them into proximity with other signaling molecules important in the activation of proximal signaling cascades including the src-family tyrosine kinases (Dykstra et al. 2001).

5 Different Signals for Different Functions

Recent careful analyses of mice deficient in BLNK indicate that the pre-BCR coordinates the activation of two or more signaling pathways, each with a different function, to affect B cell development. Deletion of BLNK, like the deletion of other signaling molecules in the pre-BCR pathway, leads to a significant block in pre-B cell development (Jumaa et al. 1999; Pappu et al. 1999). However, not all aspects of B cell development are equally affected. Deletion of BLNK inhibits the downregulation of RAG, $\lambda 5$, IL-7R and the upregulation of CD25 that normally occurs when pre-B cells mature (Hayashi et al. 2003). The pre-B cell proliferative burst is also inhibited. These defects are exacerbated if LAT (Su and Jumaa 2003) is also absent. Conversely, ectopic expression of BLNK in $Pax5^{-/-}$ μ^{+} cells, which are deficient in BLNK and arrest at a pre-B-like stage, rescue some aspects of pre-B cell receptor signaling and initiates a proliferative and developmental program (Schebesta et al. 2002). In vitro, pre-B cells from $BLNK^{-/-}$ mice proliferate robustly in response to IL-7 indicating that the defect is proliferation is specific to the BCR (Guo et al. 2000). The expression of surrogate light chain is not suppressed in

BLNK$^{-/-}$ mice and this is associated with an expansion of pre-B cells (Flemming et al. 2003).

The role of a putative downstream target of BLNK, Ras has been examined in B cell development. Expression of dominant negative Ras results in an overall reduction in B220^{+} cells. However, these cells are blocked developmentally at the B220^{+}, CD43^{+}, HSA^{+} stage, which is before the requirement for a pre-B cell receptor (Iritani et al. 1997). This suggests that Ras may have a role in signaling through the IL-7 or other cytokine receptors independent of BLNK. In contrast, expression of constitutively active Ras in Rag2$^{-/-}$ mice affords production of a unique population of cells that express markers of later B cell development (CD23^{+}, CD21^{+}, and CD35^{+}) (Shaw 1999b), indicating that downstream of the pre-BCR, Ras is an important mediator of developmental progression. Consistent with this conclusion, expression of constitutively active Ras in $J_H^{-/-}$ mice rescues kappa light chain rearrangement (Shaw 1999a). Ras may function, in part, by inducing expression of the pro-survival gene *Bcl-2* (Young 1997).

Although BLNK is important for many aspects of B cell development, heavy chain allelic exclusion in BLNK$^{-/-}$ mice is intact (Hayashi et al. 2003). The pathway(s) required for allelic exclusion are not known, although there are at least two possibilities. One is that an unidentified substrate of Syk is responsible for allelic exclusion (Schweihoffer et al. 2003). Another possibility is that substrates distal to the principal Src-family kinases expressed in B cells (Lyn, Blk, and Fyn) are required. Deletion of all three kinases results in a complete block in pre-B cell development (Saijo et al. 2003), while deletion of each kinase has little effect. Surprisingly, analysis if ex-vivo expanded pro-B cells from Lyn$^{-/-}$Blk$^{-/-}$Fyn$^{-/-}$ mice revealed that receptor-induced signaling was largely intact with readily detectable levels of Igα phosphorylation and intracellular calcium mobilization. There observations are congruent with recent studies of reconstituted BCR complexes in *Drosophila* cells, indicating that Syk can directly phosphorylate Igα and initiate signal transduction (Rolli et al. 2002). However, in the Lyn$^{-/-}$Blk$^{-/-}$Fyn$^{-/-}$ mice, there was a selective defect in NFκB activation. It is not known if allelic exclusion is aberrant in these mice.

The pre-BCR may not directly regulate allelic exclusion but may do so indirectly through regulation of the IL-7 receptor. During development, signals through the IL-7 receptor increase the accessibility of distal V genes (Corcoran et al. 1998), possibly by increasing histone acetylation (Chowdhury and Sen 2001). Expression of the pre-BCR can desensitize the IL-7 receptor (Smart and Venkitaraman 2000), leading to re-

versible deacetylation of the distal V genes and the termination of recombination (Chowdhury and Sen 2003).

6 The Pre-TCR and Pre-BCR: Conservation of Signaling Properties and Functions?

There are significant parallels between B cell and T cell development in both general organization and in the specific mechanisms that are evoked. Bone marrow (BM) or fetal liver-derived lymphoid progenitors seed the thymus and commit to the T cell lineage, in response to signals from the thymic microenvironment (Martin et al. 2003). Early double-negative (DN) $CD4^-CD8^-CD44^+$c-Kit^+ cells increase surface expression of IL-2Rα (CD25) and initiate rearrangement at the T cell receptor (TCR) β, γ and δloci (Borowski et al. 2002). Thymocytes with a productive rearrangement at the *TCR*β locus express a TCRβ chain, which associates with the invariant pre-TCRα (pTα) chain and CD3 signaling molecules to form the pre-TCR. As with the pre-BCR, the pre-TCR replaces cytokine receptor signaling in the regulation of immature T cell survival and proliferation (Di Santo et al. 1999). The receptor is expressed on the surface of immature $CD4^-CD8^-CD44^-CD25^+$ (DN3) and $CD4^-CD8^-CD44^-CD25^-$ (DN4) thymocytes (Fehling et al. 1995; von Boehmer et al. 1999). Expression of the receptor enables the cells to differentiate and reach the $CD4^+8^+$ (double positive, DP) stage of development. At the DP stage, cells expressing the $\alpha\beta$ antigen receptor (TCR) initiate positive and negative selection. Pre-TCR functions include induction of thymocyte proliferation, regulation of TCRβ allelic exclusion and commitment to the $\alpha\beta$ T cell lineage (Aifantis et al. 1997, 1998, 1999a, 1999b; Ardouin et al., 1998).

In contrast to the "mature" $\alpha\beta$TCR complexes—which visibly localize to lipid rafts in response to ligation (Viola et al. 1999), the pre-TCR localizes constitutively within these structures (Saint-Ruf et al. 2000). As is the case with the pre-BCR, localization in lipid rafts brings the pre-TCR within close proximity to numerous signaling molecules, including Lck, Zap-70 and the downstream adapter molecules LAT, SLP-76 and GADS. These proximal signaling molecules couple the pre-TCR to transcriptional regulators such as NFAT and NFκB, which are required for normal T cell development (Voll et al. 2000; Aifantis et al. 2001). Disruption of raft integrity blocks proximal signaling events, including the phosphor-

ylation of CD3 chains and Zap-70, which completely abrogates signal transduction (von Boehmer et al. 1999).

Multiple other signaling pathways have been proposed to be essential for pre-TCR signaling. The Raf-1/Ras^{-} and ERK-MAPK-mediated signaling pathways have been proposed to regulate proliferation of pre-T cells (Gartner et al. 1999; Iritani et al. 1999; Michie et al. 1999). Interestingly it seems that there is a division of labor downstream of the receptor as these pathways are not able to regulate allelic exclusion of the *TCRβ* locus. Additional signaling molecules, namely PKC and PKD kinases, have been shown to affect rearrangement of the *TCRβ* genes (Michie et al. 2001; Marklund et al. 2003). However, even if we have adequate information for membrane and cytoplasmic signaling events, genes and transcriptional targets of these pathways remain unknown.

Similar to the pre-BCR, mutant pTα and TCRβ chains lacking their extracellular domains can form a functional pre-TCR and induce T cell development (Irving et al. 1998), indicating that the pre-TCR does not have a selecting ligand. However, in contrast to the pre-BCR, the surrogate chain of the pre-TCR, pTα, contains a unique and significant cytosolic tail of 31 amino acids. Within this cytosolic tail are proline-rich regions with homology to sequences in the cytosolic tails of CD2 and CD28. In these co-stimulatory receptors, the proline-based motifs recruit downstream SH3 domain containing adapter proteins and kinases. It has recently been demonstrated that the proline-rich sequences of the pTα tail play an important role in promoting pre-TCR signaling and inducing T cell development (Aifantis et al. 2002).

7 Final Comments

It is becoming evident that the pre-TCR shares multiple properties and functions with the pre-BCR. The two receptors have evolved to enforce commitment of lymphoid progenitors to the T and B lineage, respectively. The receptors have similar structures, utilize parallel signaling pathways (localization in Rafts, utilization of downstream kinases and adaptor molecules, Ca^{2+} mobilization) and induce identical functions (proliferation, allelic exclusion, survival, IL-7R downregulation). They also differ from their "mature" counterparts (TCR and BCR) in both structure and function. However, there are some slight differences between them. The pre-TCR does not use a "V-pre-B-like" molecule, as the pTα, TCRβ and CD3 are the only known proteins comprising the receptor. Also, it

appears that the vast majority, and not only the 30% as in the case of the pre-BCR, of pre-TCR complexes are targeted to lipid rafts. Moreover, it appears that the pre-TCR does not select for specific TCRβ chains, as it was found that, unlike λ5, the pTα protein is able to pair equally well with all TCRβ chains tested. Finally, there is no ligand described for the pre-TCR; however, the search is still on.

These comparisons introduce an intriguing question: Do the pre-BCR and pre-TCR receptors function similarly or it is the developmental stage that dictates similar function? To answer this question one would replace in vivo the pre-TCR and pre-BCR with the "mature" TCR and BCR by expressing the latter at the same, early, developmental stage.

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Transcriptional Control of B Cell Activation

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Abstract The developmental program that commits a hematopoietic stem cell to the B lymphocyte lineage employs transcriptional regulators to enable the assembly of an antigen receptor complex with a useful specificity and with signalling competence. Once a naive IgM^+ B cell is generated, it must correctly integrate signals from

the antigen receptor with those from cytokine receptors and co-receptors delivering T cell help. The B cell responds through the regulated expression of genes that implement specific cell expansion and differentiation, secretion of high levels of high-affinity antibody, and generation of long-term memory. The transcriptional regulators highlighted in this chapter are those for which genetic evidence of function in IgM^+ B cells in vivo has been provided, often in the form of mutant mice generated by conventional or conditional gene targeting. A critical developmental step is the maturation of bone marrow emigrant "transitional" B cells into the mature, long-lived cells of the periphery, and a number of the transcription factors discussed here impact on this process, yielding B cells with poor mitogenic responses in vitro. For mature B cells, it is clear that not only the nature, but the duration and amplitude of an activating signal are major determinants of the transcription factor activities enlisted, and so the ultimate outcome. The current challenge is the identification of the target genes that are activated to implement the correct response, so that we may more precisely and safely manipulate B cell behavior to predictably and positively influence humoral immune responses.

1 Introduction

The developmental program that commits a hematopoietic stem cell to the B lymphocyte lineage engages the services of a number of transcriptional regulators (reviewed in the chapter by H. Singh and K. Medina, this volume) whose principal common goal is the assembly of an antigen receptor complex with a useful specificity and with signalling competence. Once this "virgin" IgM^+ B cell is generated, another (possibly overlapping) group of transcription factors must ensure that the cell can receive and correctly integrate signals from the antigen receptor with those from cytokine receptors, and with co-receptors delivering T cell help, in the course of an immune response. The B cell's response to these signals is determined by the activation of a subset of genes, whose activities implement the immune response, i.e., specific cell expansion and differentiation, secretion of high levels of high-affinity antibody, and generation of long-term memory. Many of the transcription factors implicated in gene regulation in mature B lymphocytes were first identified via their binding sites in regulatory regions of genes specifically expressed in B cells, such as the immunoglobulin (Ig) genes (see Staudt and Lenardo 1991). A very large body of work, using ectopic expression and transactivation of reporter constructs, has disclosed a potential for B cell-specific gene regulation for a number of these proteins. However, often only correlative data have been obtained, some of which has led to models that have since been proved incorrect by more definitive experi-

ments. Therefore, the transactivators highlighted in this chapter are those for which some genetic evidence of function has also been provided, often in the form of mice generated by specific gene targeting. Those transactivators that are required during early steps in B cell ontogeny may also play roles in mature cells, but this cannot be assessed in conventional null mutants; elucidation of mature cell functions relies on the generation of conditional mutations.

1.1 Definition of Lymphocyte Activation

The phrase "B cell activation" has a specific meaning, but I have broadened it for the purpose of this review. Cell activation can be specifically studied in vitro, but in many instances, immune cell function is measured in an intact animal. This really assesses the downstream consequences of B cell activation, as a physiologic, but pleiotropic response (see Melchers and Andersson 1984). B cell activation in vivo is achieved through antigen associating with sIg, and is enhanced by interactions with the surfaces of collaborating T cells or cells of the innate immune system, or with secreted products of these cells. These processes are dependent upon the B cell's capacity to migrate to the appropriate sites in the secondary lymphoid organs, and to make the correct associations (via surface adhesion) with other cells. A B cell that has received the right signal in the right place then divides rapidly, and matures to an antibody-secreting cell or to a high-affinity memory cell (see Rajewsky 1996). The net effect in vivo is the appearance of mature antibody-specific B cells in the periphery and antigen-specific antibody in the serum. Following an immune response, when the B cell's duty is finished, appropriate cell death signals must also be obeyed.

Some of these processes can be mimicked and measured in vitro, using polyclonal B cell activators such as lipopolysaccharide (LPS), anti-IgM or anti-CD40 antibodies on freshly isolated splenic B cells. Most splenic B cells in a healthy adult mouse are in a resting (G_0) state, so the first step in activation is entrance to the G_1 phase of the cell cycle, which is evident as a change in cell phenotype. The B cell enlarges, RNA synthesis is initiated and surface "activation" markers appear (including homing receptors and adhesion molecules). The polyclonal activators listed above can be used in vitro to assess the capacity of B cells to divide and their ability, under certain circumstances, to differentiate to antibody forming cells.

Recent improvements in our understanding of the continued maturation of virgin B cells during their transition from the bone marrow to the periphery have identified essential mediators of the positive selection that recruits immature cells into the long-lived, recirculating pool. The cells that emerge from the bone marrow to populate the spleen are designated transitional Btransitional B cells, and are distinguished by relative levels of particular surface markers (e.g., IgM, IgD, CD23, CD21, HSA) and by their sensitivity to negative selection (apoptosis) in response to B cell receptor (BCR) cross-linking (Allman 1992, 1993; Loder et al. 1999; Carsetti et al. 1995). Maturation requires both a BCR signal (Lam et al. 1997) and a signal from a newly described survival factor of the tumor necrosis factor (TNF) family, called BAFF (B cell activating factor of the TNF family; also called BlyS, TALL-1, zTNF4, THANK). In response to the combined signals of BCR and BAFF-receptor, B cells up-

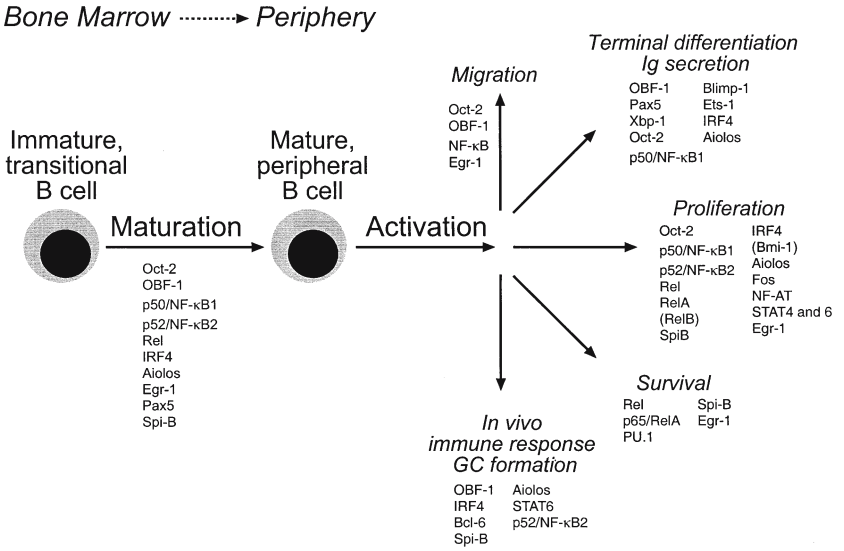

Fig. 1 Transcription factors that have been implicated in mature B cell functions. B cell activation and functional maturation have been artificially divided into several different phases in this figure. The divisions largely reflect the types of assays that can be used to assess B cell functions, rather than a sequential B cell differentiation program. The figure is meant to be guide, indicating transcription factors involved in specific phases of an overall process. The text provides more specific details as to the particular processes impacted by each transcription factor. RelB and Bmi-1 are enclosed in *parentheses* under the *Proliferation* heading, to indicate less dramatic effects than other genes in the same group

regulate cell survival factors of the Bcl-2 family (A-1 and Bcl-2; Tomayko and Cancro 1998; Carsetti et al. 1995; Batten et al. 2000) and become resistant to BCR-induced cell death. Indeed, the cells are now induced to divide and differentiate by BCR cross-linking. A number of transcription factors originally thought to influence mature B cell *activation*, are in fact critical for the *maturation* of transitional B cells to a cell that can respond positively to a BCR signal. In order to clearly understand the two processes, it is important to distinguish the molecular regulators that act in the two different contexts (see Fig. 1). It should be noted that responses to other mitogens, such as LPS and CD40 ligand, are equivalent in transitional and mature B cells (Allman et al. 1992; Sater et al. 1998).

Other aspects of B cell behavior must be measured in vivo, such as the distribution and phenotypes of B cell subpopulations in lymphoid organs, humoral responses upon immunization, or the ability to clear infections. For the purposes of this chapter, those transcription factors that regulate humoral immune responses through influences on B cell number, phenotype, proliferation, differentiation or cell survival have been included (see Fig. 1).

2
Octamer-Binding Proteins and Cofactors

Most Ig V_H and V_L gene promoters contain a highly conserved 8-bp motif, the octamer motif (Falkner and Zachau 1984; Parslow et al. 1984). The same motif also occurs in many Ig gene enhancers, and in the promoters of other genes specifically expressed in B cells. This DNA sequence was the bait that enabled isolation of the first B cell-restricted DNA binding protein, the Oct-2 transcription factor (Staudt et al. 1986) and subsequent cloning of the oct-2 gene (Clerc et al. 1988; Muller et al. 1988; Staudt et al. 1988; Scheidereit et al. 1988). Oct-2 joined Pit-1, Oct-1 and Unc-86 as founding members of the POU homeodomain family of transcription factors (Herr et al. 1988).

Expression of the oct-2 gene is largely B cell-restricted, with some myeloid and T cell lines also expressing the protein (Cockerill and Klinken 1990; Kang et al. 1992). Oct-2 is detectable from early stages of B cell differentiation and its level increases in mature lymphocytes upon activation. Oct-2 will activate octamer-containing promoters in B lymphocytes (see, e.g., Wirth et al. 1987; Junker et al. 1990; Jenuwein and Grosschedl 1991; Yuan et al. 1995). However, a strict dependence on

Oct-2 was called into question when it was noted that the ubiquitous Oct-1 protein displayed functional overlap with Oct-2 in vitro (Pierani et al. 1990; Kemler et al. 1991; Luo et al. 1992), and that in oct-$2^{-/-}$ B cells, Ig genes were expressed normally (Corcoran et al. 1993; Feldhaus et al. 1993; Corcoran and Karvelas 1994).

Subsequently, a B cell-specific coactivator, OBF-1 (Octamer binding factor-1; also called OCA-B or Bob-1), which interacts with and enhances the activity of either Oct-1 or Oct-2 in B cells, was discovered (Luo et al. 1995). However, OBF-1 is only able to potentiate transcription from Oct-1 or Oct-2 bound at a promoter proximal position (Pfisterer et al. 1995; Schubart et al. 1996b). Only Oct-2 can activate from a remote position, through a unique activation domain in its C-terminus and an additional B cell coactivator may be involved (see Wirth et al. 1995). The octamer motif remains an important regulatory element in B cells, but the chromosomal context of the motif and the proteins bound there will strongly influence its activity. Mice with null mutations in either the oct-2 or the OBF-1 gene, or both, have been generated, and the consequences for B cell physiology are discussed below.

2.1 Oct-2

The earliest defect observed in Oct-2 deficient mice is their death, consistently occurring a few hours after birth (Corcoran et al. 1993). The cause of death is not known, but during fetal development hematopoiesis occurred normally and in particular, B cell development was unaffected in oct-2-null animals. Only upon the emergence of IgM^+ B cells in the neonatal spleen was a problem observed, as mutants had less than half the number of IgM^+ B cells seen in control littermates. When LPS plus cytokine stimulation was used to enumerate the precursors of antibody-forming cells in neonatal liver and spleen, Oct-2-deficient cells showed a dramatically reduced capacity to differentiate to antibody-secreting cells. The few clones that were generated underwent isotype switching at a normal frequency. These observations and others indicated that Oct-2 plays a role exclusively at later stages of B cell differentiation.

Adoptive transfer of fetal liver stem cells into Rag-$1^{-/-}$ mice allowed examination of the behavior of oct-$2^{-/-}$ lymphocytes in adult mice (Corcoran and Karvelas 1994). In mice reconstituted using oct-$2^{-/-}$ fetal liver, serum Ig levels were depressed approximately eightfold. All classes were affected, with the curious exception of IgA. Immunization with either a

T cell-independent (TI) or T-dependent (TD) antigen induced a very poor immune response in mutant mice (Humbert and Corcoran 1997). In addition, oct-$2^{-/-}$ spleens harbored 10- to 15-fold fewer plasma cells than control spleens (unpublished observation) and in vitro assays confirmed that Oct-2-deficient B cells were defective in forming Ig secreting cells in response to LPS. The number of B cells in peripheral blood was reduced by approximately 50% and the long-lived, recirculating B cell compartment of the bone marrow was selectively reduced. Oct-2-deficient B cells were hyporesponsive in proliferation assays to LPS or anti-μ antibodies, but proliferated normally when stimulated through CD40. The defect was mapped to a point in the G_1 phase of the cell cycle, when cells make a commitment to progress to division. Early responses, such as calcium mobilization and upregulation of c-myc and cyclin D2 were intact. These observations all implied that Oct-2 regulates a gene(s) whose product relays specific proliferation signals. However, the phenotypes and behavior of Oct-2-deficient splenic B cells were shown subsequently to resemble immature transitional B cells from normal mice (Humbert and Corcoran 1997, and unpublished observations). Oct-2 was therefore found to be essential for the maturation of transitional B cells. The BAFF signalling pathway is not affected; instead BCR signalling appears to require Oct-2 (L.M. Corcoran, F. Batten, F. Mackay, in preparation). Other observations indicated that Oct-2 has a strong influence in vivo on the maintenance of the peritoneal B-1 lymphocyte lineage (Humbert and Corcoran 1997). In summary, Oct-2 is dispensable for antigen-independent aspects of B cell differentiation, but is required for normal proliferative responses, and for functional maturation.

These phenotypic consequences of the oct-2 mutation would not have been predicted based on the early assumption that Oct-2 was a major determinant of Ig gene transcription in B cells. In fact, the Ig genes do not require Oct-2 for their expression, at least in immature B cells. Other genes thought to be Oct-2-dependent in B cells, and whose deregulation would perturb signalling processes in mature B cells are B29, CD19, CD20, c-lyn and PU.1. However, all of these genes are expressed normally in mutant B cells (Corcoran and Karvelas 1994, and unpublished observations). Two genes, CD36 and crisp-3, have been shown to require Oct-2 for their expression in pre-B cells (Konig et al. 1995; Pfisterer et al. 1996), but the role crisp-3 might play in B cells has yet to be determined. CD36-null mice show normal responses in all B cell assessments performed (Corcoran et al. 2002). Wolf et al. (1998) have reported that the CXCR5/BLR-1 gene, which encodes the receptor for the chemokine that directs B cell migration to specific lymphoid compartments (Forster et

al. 1994) depends heavily on Oct-2, OBF-1 and NF-κB. Clearly an inability to activate the CXCR5 gene would impair the capacity of an activated B cell to migrate to sites where it could interact with helper T cells during an immune response. Other critical target genes that strictly rely on Oct-2 for expression in mature B cells, and contribute to phenotypic and functional maturation, are being pursued.

2.2 OBF-1/OCA-B/Bob-1

When the activity of the B cell-specific coactivator OBF-1 was first described (Luo et al. 1992), the mantle previously worn by Oct-2 as the main determinant for B cell-specific activation of Ig genes was passed to the new protein. OBF-1, an acidic, proline-rich protein without homology to known transactivators, was shown to be selectively expressed in B lineage cells. Through its N-terminus, it interacts specifically with the POU domains of Oct-1 and Oct-2, but not with other related POU family proteins (Gstaiger et al. 1995; Luo and Roeder 1995; Strubin et al. 1995) and brings an additional activating domain to the complex. Transcriptional enhancement may be achieved through the C-terminus of OBF-1 contacting the general transcription factors TATA binding protein (TBP) and TFIIB (Schubart et al. 1996b).

When OBF-1 contacts the POU domain of Oct-1 or -2, it influences the DNA binding specificity, and therefore the site selection, of the octamer binding protein (Gstaiger et al. 1996). The nature of the complex between a given octamer motif and Oct-1/Oct-1 determines whether OBF-1 can be recruited (Tomilin et al. 2000). Indeed, the promoter of the known Oct-2-dependent target gene CD36 cannot be activated by OBF-1 because of the nature of the octamer site (Shore et al. 2002). Qin et al. (1998) showed that OBF-1 was not detectable in resting primary B cells, but expression required the simultaneous receipt of signals from the antigen receptor and from helper T cells (in the form of IL-4 and CD40L). The protein is inducibly activated by phosphorylation in T cells and the same phosphorylation site is important for constitutive OBF-1 activity in B cells (Zwilling et al. 1997; Sauter and Matthias 1997). OBF-1 protein stability is regulated through interaction with the Siah1 ubiquitin ligase (Boehm et al. 2001; Tiedt et al. 2001).

Three groups have described the phenotypes of independently derived OBF-1 null mice (Kim et al. 1996; Schubart et al. 1996a; Nielsen et al. 1996). Again, early antigen-independent B cell development was largely spared, but maturation was perturbed. Significantly reduced

numbers of mature B cells in the periphery were reported (Schubart et al. 1996a; Nielsen et al. 1996; Hess et al. 2001), and the marginal zone B cell population was absent (Samardzic et al. 2002), but the B-1 compartment was spared. All groups noted abnormal serum Ig profiles: IgM was not affected, but all other isotypes were low, some severely so. Immunization with both TD and TI antigens provoked poor responses, with particularly low IgG titers. These observations prompted a look at germinal centers (GC), the sites of B cell maturation and Ig isotype switching. All three groups reported a failure of GC formation in OBF-$1^{-/-}$ mice, and Schubart et al. (1996a) showed that this was B cell autonomous. Kim et al. (1996) went on to show that isotype switching in fact occurred normally in the mutants, but that the switched Ig genes were not efficiently transcribed. Therefore one function of OBF-1 may be to enhance transcription of Ig genes other than IgM, perhaps through influences on the 3′ IgH enhancer (Stevens et al. 2000; Tang and Sharp 1999). These observations, along with a potential role in CXCR5 expression (see above), implicate OBF-1 in the migration of B cells to the correct microenvironment, and the subsequent integration of costimulatory signals during the formation of the GC.

A simplistic view is that Oct-2 and OBF-1 are functionally redundant in B cells. However, the promoter specificity and the more restricted site selection of OBF-1 predicted that the phenotypes of the two single mutants would overlap only partially, as has proven to be the case. Unlike Oct-2-deficient B cells, the proliferative responses of OBF-1^{-} B cells were relatively normal (Schubart et al. 1996a; Kim et al. 1996). The phenotypic profiles of bone marrow and splenic B cell populations of OBF-1- and Oct-2-null animals were almost identical (Corcoran et al. 1994; Nielsen et al. 1996; Humbert and Corcoran 1997), indicating that the two factors have similar influences on B cell maturation after bone marrow emigration. In contrast, B-1 cell development was OBF-1-independent, but Oct-2-dependent. In oct-2/OBF-1 double knockout B cells, the defects seen in single mutants were exacerbated, but no new abnormalities were seen (Schubart et al. 2001). This argues against strict functional redundancy between the two proteins. As double-mutant B cells still express normal levels of sIgM, Oct-2 and OBF-1 are not required for the activity of Ig gene promoters, despite the conserved octamer sites there.

3
Rel-NF-κB Family Proteins

NF-κB was one of the first DNA binding proteins identified in B cells, using DNA from the Igκ intronic enhancer as a probe (Sen and Baltimore 1986). Proteins of the Rel/nf-κB family share a domain implicated in DNA binding, dimerization and transcriptional activation, the Rel homology domain. There are at least five members of the family (p105/p50/NF-κB1, p100/p52/NF-κB2, p65/RelA, Rel and RelB) and many recent reviews describe their properties (see, e.g., Gugasyan et al. 2000; Li and Verma 2002; Ruland and Mak 2003). Briefly, the family members are widely expressed and can interact to form a variety of homo- and heterodimers. P50 and p52 are generated by proteolytic processing of p105 and p100 precursor proteins, respectively. The composition of a given dimer may determine its specificity and differential activity (Lernbecher et al. 1993). NF-κB dimers are generally retained in an inactive form in the cytoplasm, in complex with a member of the IκB family. Upon receipt of an activating signal, the IκB component of the trimeric complex is phosphorylated, marking it for destruction (see Finco and Baldwin 1995; Stancovski and Baltimore 1997). Phosphorylation takes place at the hands of IκB kinases that are part of a large protein complex that serves as a conduit in a growing number of signalling pathways (see Israel 2000). This phosphorylation releases the NF-κB protein dimer, which migrates to the nucleus. There it binds specifically to appropriate κB motifs and activates (or represses) the transcription of the adjacent gene.

NF-κB proteins have been implicated in the regulation of a large number of genes, most of which participate in immunological or inflammatory processes. These include genes for cytokines, Ig, major histocompatibility (MHC) genes, acute phase proteins, adhesion molecules. Autoregulatory loops have also been found: p65/RelA positively regulates IκB transcription (Scott et al. 1993; Sun et al. 1993), and the c-rel promoter is NF-κB dependent (Grumont et al. 1993). Both the transcriptional activation and the B cell-specific demethylation (Kirillov et al. 1996) of the Igκ locus were believed to be heavily dependent upon NF-κB. However, the NF-κB story is another example where abundant evidence from in vitro studies did not allow prediction of the phenotype of knockout mice. NF-κB has been shown to be dispensable for Igκ gene expression and for B cell generation. Components of the NF-κB complex become critically important in determining the functional capacity, and more recently, the growth and survival of mature B cells. In addition,

several important pathways in mature B cells proceed via NF-κB (including those initiated at the BCR, LPS/Tlr-4 and CD40). The consequences of targeted disruptions of genes encoding individual NF-κB subunits are discussed below.

3.1 p50 and p52

The p50 subunit is a principal component of NF-κB complexes in both pre B and mature B cells, where it predominantly pairs with p65 or Rel, respectively. The inducible NF-κB complex of mature B cells mainly consists of p50/Rel dimers (Grumont and Gerondakis 1994; Liou et al. 1994; Miyamoto et al. 1994). It was therefore surprising to learn that mutation of the NFKB1 gene (which encodes the p105 precursor of the p50 NF-κB subunit) did not affect normal development of B (or T) lymphocytes (Sha et al. 1995). Indeed, genes thought to be regulated by NF-κB in B cells, including Igκ and MHC, were expressed normally. However, mature B cell functions were significantly affected. In vitro, B cell proliferation upon LPS stimulation was ablated, and the response to anti-μ antibody stimulation was reduced. Serum Ig levels in naive $p50^{-/-}$ mice were altered, with IgM slightly elevated, and all other classes reduced. Mutant mice immunized with a TD antigen made poor primary responses generating low antigen-specific IgM titers, and even lower IgG titers. This was the first hint that IgH isotype switching may be dependent upon p50, and detailed studies on purified $p50^{-/-}$ B cells confirmed that this was the case (Snapper et al. 1996b). Germline transcription of some C_H genes was reduced, but this could not fully account for the serum Ig profile. In vitro activated $p50^{-/-}$ B cells proliferated normally after strong cross-linking of sIgD or CD40, and yet secreted abnormally low levels of Ig. An involvement of NF-κB in expression of B7–1, a receptor for activating signals from helper T cells (Zhao et al. 1996), in activation of germline Cγ1 and Cε gene transcription (Iciek et al. 1997; Cunningham et al. 1998) and in the activity of the 3′ IgH enhancer in plasma cells (Michaelson et al. 1996) might underlie at least some aspects of the $p50^{-/-}$ B cell phenotype. More recently, p50 has been shown to be required for both G_1 phase cell cycle progression and for survival in mitogen-activated B cells. p50-null B cells also turn over more rapidly than wild-type cells (Grumont et al. 1998). This phenotype is reminiscent of the immature transitional B cell described earlier, and hinted that Rel/nf-κB proteins also play a role during this critical maturation step.

p52 is a close relative of p50, and is usually co-expressed. Deletion of the gene for the p52 subunit of NF-κB compromised humoral immunity in mice, with a loss of germinal centers and abnormal splenic architecture (Franzoso et al. 1998; Caamano et al. 1998). However, the most striking effect on B cell development came with the p50/p52 double mutant, which makes no mature B cells, due to a block in B cell maturation coincident with exit from the bone marrow (Franzoso et al. 1997). BAFF-R signalling induces the processing of the inhibitory p100 NF-κB2 precursor to the active p52 form, which may explain the vital role of p52 in B cell maturation (Claudio et al. 2002; Kayagaki et al. 2002). CD40 and lymphotoxin-β signalling employ a similar mechanism for p100 processing to p52 in B cells (Coope et al. 2002; Dejardin et al. 2002). As has been seen in other instances, mutation of genes for individual NF-κB subunits reveals unique roles for each protein, but the greater severity of double mutants testifies to functional overlap as well.

3.2 Rel

As stated above, the Rel protein is the preferred partner of p50 in NF-κB complexes in mature B cells, and p50/Rel binds to the κB site in the κ intronic enhancer at least 20 times more stably than does the p50/p65 dimer (Miyamoto et al. 1994). Mice with a disrupted c-rel gene developed normally, and in particular showed no defects in hematopoiesis (Köntgen et al. 1995). However, like the $p50^{-/-}$ mice, both proliferation of mature lymphocytes and humoral immune responses were compromised by the c-rel mutation. Both B and T lymphocytes in $c\text{-}rel^{-/-}$ mice were shown to be hyporesponsive to mitogens; B cells did not proliferate in response to anti-μ or CD40L stimulation, and made a muted LPS response. While other serum Ig levels in unimmunized mutant mice were depressed a few fold, the titers of the T cell-dependent classes tested (IgG1 and IgG2a) were extremely low. This was reflected in the poor primary immune response upon immunization with a TD antigen. T cells exhibited a proliferation defect that could be overcome with exogenous IL-2, and it was shown that production of this cytokine by T cells requires Rel (Gerondakis et al. 1996). The defective TD humoral immune responses of Rel-deficient mice must in part reflect a B cell autonomous lesion, as CD40 signalling is faulty, but a significant part of the problem may be contributed by T cells that are unable to provide the cytokines necessary for appropriate B cell differentiation (including isotype switching) during an immune response.

The results suggest that Rel is critical for B cell mitogenesis in response to most stimuli, in contrast to p50, which has more restricted influences on proliferation. Once again, mature B cells were underrepresented in the Rel mutants (Tumang et al. 1998), and a closer look at the cells during stimulation showed that they were prone to G_1 arrest and to apoptosis upon surface Ig (sIg) cross-linking (Grumont et al. 1998). Critical Rel target genes have recently been shown to be the prosurvival proteins A1 and Bcl-x, both Bcl-2 family members (Grumont et al. 1999; Chen et al. 2000). A1 expression distinguishes long-lived, recirculating B cells from their less mature, "naïve" counterparts (Tomayko et al. 1998). Rel may be the means by which these prosurvival genes are activated during the transition that accompanies entry into the mature, long-lived B cell pool. Another new and important function for Rel was revealed when c-rel/NFKB1 double-mutant B cells were examined. Together, these proteins were required for mitogen-induced cell growth (cell enlargement that accompanies activation), through induction of c-myc transcription (Grumont et al. 2002).

3.3
3.3 RelA/p65

RelA deficiency causes embryonic lethality at day 15/16 of gestation, due to massive liver degeneration (Beg et al. 1995; Doi et al. 1997). Hepatocytes were the cell type most affected, while hematopoietic cells appeared to be normal in histological sections of degenerating liver. A general role for RelA in inducible, rather than basal transcription, of NF-κB-regulated pathways was deduced using $p65^{-/-}$ embryonic fibroblasts. The expression of IkBα and GM-CSF, two TNFα-responsive NF-κB target genes, was examined in these cells. Basal transcription was not affected, but induction in response to TNFα was lost in the mutants. Other work identified a role for RelA in particular (Beg and Baltimore 1996), and for NF-κB in general (Wang et al. 1996; van Antwerp et al. 1996) in protecting cells from apoptosis in response to TNFα or chemotherapeutics.

Adoptive transfer of fetal liver hematopoietic stem cells from $p65^{-/-}$ mice indicated that RelA is dispensable for B cell production and for Igκ expression (Horvitz et al. 1997; Doi et al. 1997), though mature B cell numbers were significantly reduced (Prendes et al. 2003) due to enhanced TNF-mediated cell death. RelA is therefore critical for regulating B cell survival by inhibiting TNFα cytotoxicity. However, RelA-null B cells were hyporesponsive to LPS and to anti-IgM treatment. This effect on B cell development and function was remarkably enhanced in the

p50/RelA double mutant, where lymphopoiesis failed entirely (Horwitz et al. 1997), and in c-rel/RelA double mutants, where mature peripheral B cells were virtually absent (Grossman et al. 2000). In the latter study, the combination of Rel and RelA was shown to be required for normal expression of the Bcl-2 cell survival gene, and ectopic bcl-2 expression in Rel/RelA-deficient B cells partially restored cell maturation and cell numbers.

3.4 RelB

The RelB protein is primarily expressed in lymphoid organs (Carrasco et al. 1993), where it exclusively complexes with p50 or p52. No RelB homodimers have been observed (Bours et al. 1992; Ryseck et al. 1992). Evidence suggests that RelB heterodimers participate in constitutive rather than in inducible binding to κB binding sites, thereby contributing more to the tissue-specificity than to the inducibility of NF-κB/Rel target genes (Lernbecher et al. 1993, 1994; Weih et al. 1994). Mice homozygous for a relB gene disruption suffer from multi-organ inflammation and myeloid hyperplasia (Weih et al. 1995). Although the B and T lymphoid compartments appeared grossly normal in the mutants, cellular immunity was impaired. A later assessment of B cell function in these mice (Snapper et al. 1996a) revealed a general but modest ($\leq$ fourfold) diminution in proliferative responses, using LPS, or IgD or CD40 cross-linking. Despite this defect, the RelB mutation had no effect on maturation to IgM-secreting cells in vitro or on switching to any downstream IgH isotype. Therefore, RelB appears to play a role in B cell expansion, but not maturation, consistent with its proposed participation in constitutive processes, rather than inducible ones.

The Rel/nf-κB family is therefore central to mature B cell integrity and function. Members of the family differentially mediate signals from the BAFF-R to induce pro-survival genes in maturing bone marrow emigrant B cells, enabling their persistence and selection into the functional, long-lived compartment. Later, through activating signals from the BCR or TLR-4, NF-κB activates c-myc expression, enabling cell growth and division, as well as enhancing mature cell survival. Finally, NF-κB influences Ig gene and cytokine/cytokine receptor gene expression during B cell differentiation.

4
Pax-5/BSAP

The pax-5 gene encodes Pax-5, the B-cell-specific activator protein (BSAP), a member of the paired-box family of DNA binding proteins. Pax-5 is expressed in the developing central nervous system, and in fetal liver, where timing of expression correlates with the initiation of B lymphopoiesis. Pax-5 is expressed in B lineage cells of all stages but the terminally differentiated plasma cell stage (Adams et al. 1992). The pax-5 gene has been disrupted, and mutant mice display both neurological and hematopoietic defects (Urbanek et al. 1994; Nutt et al. 1997). B cell differentiation was specifically affected, with a block at the pro-B cell stage. Pax-5 has been found to be critical for B lymphocyte lineage commitment, enabling B cell differentiation while restricting commitment to other hematopoietic lineages (Nutt et al. 1999). A limitation of traditional gene targeting approaches is that only the earliest aspects of a gene's function can be assessed. Insights into the functions of Pax-5 in late B cell stages were gained from mice with a conditional pax-5 mutation (Horcher et al. 2001).

Pax-5 activates the expression of critical genes early in B cell development that affect BCR integrity or signalling capacity (see the chapter by K.L. Medina and H. Singh, this volume). In addition, in mature B cells, Pax-5 releases the AID (activation-induced deaminase) gene from Id2-mediated repression (Gonda et al. 2003). AID is essential for the heavy chain class switching and somatic hypermutation that occurs in the GC (Muramatsu et al. 2000). As well as acting as an activator of transcription, Pax-5 also acts negatively, through association with corepressors of the Groucho family (Eberhard et al. 2000). A concentration-dependent mechanism for the coordinate activation or repression of Pax-5 target genes has been recognized, whereby the affinity of binding sites in genes activated by Pax-5 is significantly higher that the repressed sites (Wallin et al. 1998). At early stages of B cell development, Pax-5 coordinately represses genes involved in differentiation to the Ig-secreting plasma cell. Pax-5 binds to a site in the Ig 3′ α enhancer, repressing its activity (and those Ig genes most dependent upon it), possibly through the displacement of a positive acting factor, NF-αP (Singh and Birshtein 1993; Neurath et al. 1995a). In plasma cells, Pax-5 down-regulation releases this repression, promoting transcription of the associated IgH genes. Pax-5 also represses the gene for J chain, a mediator of Ig multimerization in plasma cells (Rinkenberger et al. 1996). Indeed, enforced overexpression of Pax-5 in a late B cell line inhibits Ig synthesis, expression of Blimp-1

(the B lymphocyte-induced maturation protein, essential for plasma cell differentiation) and differentiation to a plasma cell phenotype (Usui et al. 1997). The proliferation of splenic B cells activated with polyclonal mitogens such as LPS or anti-IgD antibodies could be reduced by treatment with oligonucleotides specifically designed to inhibit Pax-5 production, and the spontaneous proliferation of a B lymphoma cell line could be slowed with the same treatment. Conversely, overexpression of Pax-5 in activated splenocytes increased their proliferation (Wakatsuki et al. 1994). Therefore, it has been postulated that Pax-5 is a rate-limiting regulator of B cell proliferation.

These early studies were supported by genetic evidence from conditional loss of pax-5 expression in B cells in vivo (Horcher et al. 2001). In the absence of Pax-5, mature peripheral B cell numbers were specifically diminished, and the expression of many surface markers was reduced on the remaining cells (e.g., CD20, CD22, CD23, CD40, CD72, and class II). The proliferative response to LPS was muted, and terminal differentiation was abnormal; Blimp-1 and J chain were not induced, and IgG levels were significantly reduced. Genes that rely on Pax-5 in B cell precursors, such as CD19, mb-1/Igα and BLNK, were found to be Pax-5-dependent in mature B cells as well. It was therefore suggested that some of the phenotypic consequences of Pax-5 loss might be indirect, as a result of suboptimal signal transduction capacity. Nevertheless, it was concluded that Pax-5 is essential for mature B cell identity and function.

One means by which Pax-5 levels may be decreased physiologically in maturing B cells is through a signal from the activated B cell surface via OX40 ligand (OX40L). This signal is delivered from an activated T cell bearing OX40, a TNF receptor family member, on its surface. Cross-linking of OX40L on the surface of activated B cells induces antibody secretion (all classes tested), down-regulates Pax-5, and alters the protein binding pattern on the 3′ α enhancer (Stüber et al. 1995). Consequently, IgH gene transcription is enhanced. Rinkenberger et al. (1996) have also identified a role for IL-2 in the down-regulation of Pax-5. This provides a model whereby a helper T cell could specifically promote antibody secretion through down-modulation of Pax-5 levels in its cognate B cell.

5
Ets-1 Family Proteins

5.1
Ets-1

c-ets-1 was first identified as the cellular homologue of the v-ets retroviral oncogene, and has since become the founding member of a family of related genes. During fetal development in the mouse, the ets-1 gene is widely expressed, but from birth through adulthood, its expression is largely confined to the lymphoid organs (Kola et al. 1993). Based of the occurrence of the ets-1 binding site and functional assays, ets-1 has been implicated in the transcription of the IgH locus, the T cell receptor (TCR) α and β chain genes, and a number of other lymphoid restricted genes (see Clevers and Grosschedl 1996). Ets-1 has been shown to functionally interact with other proteins, often in a mutually dependent manner. For example, Ets-1 and PU.1 cooperate in binding to adjacent sites in the intronic IgH enhancer (Nelsen et al. 1993), thereby increasing chromatin accessibility (Nikolajczyk et al. 1999), and three members of the Ets family (Ets-1, Fli-1 and GABPα) are recruited by Pax-5 to the mb-1 promoter in vitro (Maier et al. 2003).

Two groups have assessed the behavior of ets-1-null lymphoid cells using the RAG-$2^{-/-}$ blastocyst complementation system (Bories et al. 1995; Muthusamy et al. 1995). Both noted differential effects on cells of the B and T lineages. T cell numbers were dramatically reduced, and in vitro T cells were hyporesponsive to mitogens, but highly susceptible to apoptosis. B cell numbers were not much affected, but surprisingly, ets-$1^{-/-}$ B cells in the spleen displayed an unusual phenotype consistent with accelerated maturation. Indeed, approximately ten times the normal number of IgM-secreting plasma cells was observed in mutant spleens, with an associated elevation in serum IgM levels. Levels of other Ig classes in serum were normal, indicating that class switching was not affected. B cell proliferation in response to mitogens was somewhat reduced, indicating either that Ets-1 plays a role in proliferative responses or that the unusual maturity of the B cells alters their sensitivity to the mitogens. Despite evidence for functional Ets-1 binding sites in the IgH and TCR α and β enhancers, these loci were transcribed normally in ets-$1^{-/-}$ lymphocytes. No mechanism has been proposed for the effects observed, but if, as some evidence suggests (Bhat et al. 1989), Ets-1 plays a role in maintaining lymphocytes in the resting state, its loss may direct

cells towards default pathways: death in the case of T cells and differentiation in the case of B cells.

5.2 PU.1/Spi-1

PU.1/Spi-1 is an Ets family member that is expressed in B and myeloid lineage cells. It has been strongly implicated in activation of the IgH enhancer, where it cooperates with Ets-1 (Nelsen et al. 1993). PU.1 also acts at the light chain enhancers, where in its phosphorylated form it directs the binding of IRF4/ Pip-1, a lymphoid-specific transactivator, to an adjacent position on the composite binding site (Eisenbeis et al. 1995). IRF4/ Pip-1 is not expressed in T cells, which may contribute to the B cell specificity of the Ig light chain enhancers. The promoters of the Btk kinase, J chain, and CD72 genes also contain functionally important PU.1 sites (Shin and Koshland 1993; Himmelmann et al. 1996; Ying et al. 1998). The biological significance of these observations could not be assessed using PU.1-deficient B lymphocytes, as mutation of the PU.1 gene blocks both lymphoid and myeloid differentiation (Scott et al. 1994; McKercher et al. 1996; Scott et al. 1997). However, using mice that were heterozygous for PU.1, Hu et al. (2001) discovered an important role for this factor. In cooperation with another Ets family member, Spi-B, PU.1 directly regulates expression of the c-rel gene, thereby indirectly promoting the survival of mature peripheral B cells. Upon terminal differentiation, PU.1 expression is lost because of repression by Blimp-1 (Shaffer et al. 2002). Conditional PU.1 mutant mice will clarify the specific role of PU.1 in mature B cells.

5.3 Spi-B

Spi-B, a close relative of PU.1, overlaps with PU.1 in several respects. Spi-B expression is restricted to B and T lymphocytes, with highest expression in B cells. It is able to bind in vitro to all known PU.1 sites, and can interact with IRF4/ Pip-1 (Su et al. 1996), so there is the potential for Spi-B and PU.1 to overlap functionally in B cells. Spi-B-deficient mice differ from PU.1-deficient mice, however, in that mature B and T cells were present in the former and absent in the latter, indicating a differential requirement for the two factors early and late in B cell development. The Spi-B-deficient B cells that were generated were functionally abnormal, in that they did not proliferate normally upon antigen receptor

cross-linking. Indeed they were killed by the treatment (Su et al. 1997) in a response reminiscent of that of immature, transitional B cells. In vivo, TD immune responses were severely muted, and GC formation and persistence were diminished. The molecular bases for these defects reflect the finding that Spi-B and PU.1 cooperate in activating c-rel transcription, and therefore promote the survival of maturing B cells (see above). The two factors are also required for the robust propagation of a BCR signal, presumably due to their combined actions on the promoters of genes for BCR signalling components (Garrett-Sinha et al. 1999).

5.4 IRF4/ Pip-1

Interferon regulatory factor 4 (IRF4)/ Pip-1 is not an ets-1 family member, but as mentioned above, it interacts functionally with PU.1 and Spi-B. IRF4 expression is largely restricted to lymphocytes, where it is induced by mitogenic activation (Matsuyama et al. 1995). Mice deficient in this protein possess mature B and T cells, but these cells are functionally impaired (Mittrucker et al. 1997). B cell development is blocked at a late stage of peripheral maturation. In vitro, IRF4-null cells responded poorly to LPS, normally to CD40L, but failed to proliferate in response to IgM cross-linking. Serum Ig was dramatically reduced, and immunization failed to provoke a humoral response. Not surprisingly, GCs and plasma cells were not found in the mice. This phenotype echoes that of the Spi-B mutant, but is more severe. The severity may reflect an influence on the activity of a number of transactivators that would normally function through recruitment of the IRF4/ Pip-1 cofactor. Regulation of light-chain gene transcription and rearrangement requires IRF4 in collaboration with the related IRF8 (Lu et al. 2003). Recently, mitogenic induction of IRF4 transcription in activated lymphocytes was shown to be absolutely dependent on the Rel protein of the NF-κB family (Grumont and Gerondakis 2000), identifying IRF4 as a direct downstream target of NF-κB in a number of activating signal pathways in B cells. IRF4 is also induced by IL-4 treatment (see Sect. 8.4). The ability of the IRF4 gene to be induced by a variety of signals (BCR, CD40, cytokines) suggests that IRF4 may play a role in integrating multiple responses for B cells in vivo.

6
Ikaros Family Proteins

The Ikaros family of transcription factors comprise a group of zinc-finger proteins that are expressed in hematopoietic cells and are important in early lymphoid differentiation (Georgopoulos et al. 1994). Aiolos is the family member most highly expressed in mature B cells. In Aiolos-null mice, B cell maturation was accelerated, with abnormally low numbers of immature, and high numbers of mature peripheral B cells (Wang et al. 1998). Mutant B cells were hyper-responsive to antigen receptor signalling in vitro, a potential cause of their accelerated maturation (Cariappa et al. 2001). In vivo, both serum Ig and GC numbers were elevated, even in the absence of immunization. In aged mice, splenomegaly was observed, autoantibodies accumulated, and lymphomas arose. More recently, aged Aiolos mice were found to succumb to a systemic lupus erythematosus (SLE)-like syndrome, characterized by autoantibodies and immune complex-mediated glomerulonephritis (Sun et al. 2003). Therefore, in normal B cells, Aiolos must exert a negative influence on antigen receptor signalling and consequent proliferation and differentiation.

Aiolos acts as a dimer with Ikaros, and careful sub-cellular localization has shown that the two proteins exist in macromolecular complexes that vary under different conditions. In interphase (resting) cells, the proteins co-localized specifically with heterochromatic chromatin, and with a number of transcriptionally silent, but not transcriptionally active, loci (Wang et al. 1988; Brown et al. 1997). This led to the notion of a higher order organization in the nucleus of centromeric domains of repressed genes, with Ikaros proteins associated. Upon mitogenic stimulation, Ikaros/Aiolos dimers moved to domains identified as replication foci, where they co-localized with the DNA replication machinery and associated methyltransferase. These observations, and evidence for chromosomal abnormalities in lymphomas from Ikaros- and Aiolos-deficient animals, have provoked the hypothesis that Ikaros proteins may help coordinate DNA replication and cell cycle progression. Indeed, in a novel Ikaros-targeted mouse that expresses only low amounts of Ikaros, B cell development was partially impaired, but mature B cells were shown to be hyperactive, with a lower activation threshold (Kirstetter et al. 2002).

7
NF-AT Family Proteins

Nuclear factor of activated T cells (NF-AT) is better known for its activities in T lymphocytes, where it regulates the expression of genes for a number of lymphokines and cytokines, notably IL-2 and IL-4 (see Schneider et al. 1995; Rao et al. 1994). NF-AT is a cytoplasmic component of a transcription factor complex that translocates to the nucleus upon lymphocyte activation and combines there with an AP-1 partner for the activation of target genes. Translocation is mediated by the calcium-dependent phosphatase, calcineurin. In T cells, NF-AT is activated by a TCR signal via a Syk/Vav complex (Deckert et al. 1996). NF-AT is also expressed in B lymphocytes and may be activated analogously through B cell receptor (BCR) cross-linking (Ma et al. 2001). There are four members of the NF-AT gene family. The proteins act similarly on target sequences, achieving specificity mainly through restricted tissue distribution. Genes for two members of the NF-AT family have been mutated in mice, NF-ATc and NF-ATp. Both mutations affected lymphokine production by T cells, with the expected consequences for humoral immune responses. Only the NF-ATc mutation appears to have a significant and direct effect on B cell function, while B cells in NF-ATp mutants behaved relatively normally in the limited assays performed (Hodge et al. 1996).

NF-Atc is expressed exclusively in lymphocytes and is induced upon activation. NF-ATc null mice died in utero, but RAG-2-deficient blastocyst complementation revealed a lymphoid system with T cell defects in activation and IL-4 secretion, and a loss of IL-4-driven Ig isotypes (Ranger et al. 1998). The effect on B cell function was not simply an indirect one, as a result of the lymphokine imbalance. It could be shown to be cell autonomous, as CD40- and IL-4-induced B cell proliferation and secretion of IL-4 dependent Ig isotypes were also diminished, albeit modestly, in vitro. Other work implicated low-level BCR-mediated activation of NF-ATc and NF-ATp in the maintenance of tolerance in B cells (Healy et al. 1997).

8
Other Transactivators Implicated in B Cell Activation

8.1
AP-1

Several studies have implicated AP-1 protein components in B lymphocyte activation. AP-1 is a generic name applied to heterodimeric protein complexes comprised of a Fos and a Jun family member (both subunits are bZip proteins). Mitogenic stimulation of B cells or B cell lines has been shown to induce rapid and transient increase of c-fos, fosB, fra-1 or junB activity, and in many cases, this induction has been shown to be protein kinase C (PKC) or mitogen-activated kinase (MAPK) dependent (see Foletta et al. 1998). Other work suggests that AP-1 may coordinate the activity of the IgH 3′ enhancer in response to signals from both sIg and CD40, via the induction of specific AP-1 components that then dimerize and bind to an AP-1/Ets site in the enhancer (Grant et al. 1996). AP-1 also relays signals from cytokine receptors to germline IgH loci, regulating the transcriptional activation that precedes switch recombination (Shen and Stavnezer 2001; Moon et al. 2001). Vasanwala et al. (2002) also recently proposed a role for specific AP-1 subunits in the regulation of blimp-1 expression.

B cells expressing c-fos transgenes have altered behavior. In one case, accelerated cell cycle progression upon LPS stimulation was observed (Phuchareon and Tokuhisa 1995), and in another, the response to IgM cross-linking was perturbed (Koizumi et al. 1994). c-jun is not an obligate member of B cell AP-1 complexes, as normal B cells are generated in the absence of c-jun (Chen et al. 1994). However, in two reports describing c-fos$^{-/-}$ mice, lymphocyte numbers were depressed. Johnson et al. (1992) noted a reduction in peripheral blood lymphocytes, with only marginal effects on myeloid cell numbers, and Wang et al. (1992) measured dramatic reductions in both B and T cell numbers in spleen and thymus, respectively. Therefore, c-fos may participate in B cell expansion in response to proliferative signals mediated through PKC.

8.2
Egr-1

Early growth response gene-1 (Egr-1) is a zinc finger protein that is rapidly and transiently induced in many cell types following activation, with kinetics that parallel c-fos induction. In B lymphocytes, egr-1 is induced following cross-linking of the antigen receptor, and this induction

is mediated by PKC (Klemsz et al. 1989; Seyfert et al. 1990). Antisense experiments have indicated that egr-1 induction is essential for B cell activation via sIg (Monroe et al. 1993). This activation proceeds through the mitogen activated protein kinase (MAPK) pathway. In vivo, overexpression of Egr-1 has been shown to accelerate maturation of pre B and immature bone marrow B cells (Dinkel et al. 1998). In the latter case, Egr-1 expression was directed to the B cell compartment by an IgH promoter and enhancer and resulted in an increased ratio of mature to immature B cells. There was no difference in the proliferation rates of these two subpopulations between transgenic and wild type cells, implying that Egr-1 may directly promote entry into the mature B cell pool independent of effects on proliferation. A part of this promotion may come in the form of enhanced cell survival, as Dinkel et al. (1997) have shown that Egr-1 overexpression can down-modulate CD95 (Fas/Apo-1) expression. Egr-1 up-regulation also may mediate changes in B cell migration and adhesion upon activation, as both the ICAM-1 and CD44 genes have been shown to be regulated by Egr-1 (Maltzman et al. 1996a, 1996b).

8.3 Bmi-1

The gene encoding the Bmi-1 proto-oncogene was discovered through its ability, when activated by nearby insertion of a Moloney MuLV provirus, to accelerate B lymphomagenesis in Eμ-myc transgenic mice (Haupt et al. 1991; van Lohuizen et al. 1991b). Subsequently, Bmi-1 was shown to be rapidly induced upon BCR cross-linking (Hasegawa et al. 1998). Bmi-1 is homologous to the posterior sex combs gene of the Drosophila polycomb group (van Lohuizen et al. 1991a), which regulates homeotic gene expression through transcriptional repression.

The bmi-1 mutation significantly influenced hematopoietic cell development, with progressive loss of white blood cells from the time of birth, culminating in the early death of bmi-null mice due to opportunistic infections (Vanderlugt et al. 1994). With respect to the B lymphocyte lineage, bmi-$1^{-/-}$ mice had reduced numbers of B lineage cells, an effect that became more pronounced in the mature populations. The reduction was in part the consequence of a failure of bmi-$1^{-/-}$ cells to respond to IL-7, thereby decreasing the size of the precursor B cell pool. This effect was cell autonomous. Mature B cells were generated, but their number in the spleens of young adults was less than one-fifth the normal number. In vivo, while B cell numbers were reduced, serum IgM levels were almost normal, and IgG levels were slightly elevated, indicating that

Bmi-1 is not required for Ig secretion or isotype class switching. LPS stimulation of bmi-$1^{-/-}$ splenocytes from adult mice elicited an eight- to tenfold lower response than bmi-$1^{+/+}$ cells, a poorer response than could be accounted for by the reduction in B cell number. Yet both B cell numbers and their response to LPS were relatively normal at birth. Bmi-1 was therefore postulated to participate in the mitogenic response directly, or indirectly by promoting differentiation to a responsive B cell. The requirement for Bmi-1 activity somehow increased as the animals matured.

A mechanism for a direct effect of Bmi-1 on the cell cycle has been elucidated (Jacobs et al. 1999). It was shown in primary embryonic fibroblasts (MEFs) that the ink4a gene was a critical target for repression by Bmi-1. Ink4a encodes two tumor suppressor proteins, p16 and $p19^{Arf}$, which restrict cell cycle progression and control replicative senescence. In the absence of Bmi-1, Ink4a expression was dramatically increased, the MEFs experienced a block in cell cycle progression, and went into premature senescence. Presumably the same occurs in bmi-$1^{-/-}$ B lymphocytes, explaining their low number and progressive loss in vivo, and their poor responses in proliferation assays. That ink4a was the critical target for Bmi-1's repressive effect was proven by a cross between bmi-1- and ink4a-null mice. Most phenotypic consequences of Bmi-1 loss were relieved by the simultaneous loss of ink4a (and thus p16 and $p19^{Arf}$). B cell function was not assessed in the double mutants, but cell numbers were indistinguishable from wild type mice. Therefore, Bmi-1 may contribute to B cell proliferation after Ig cross-linking by repressing a gene that encodes two major cell cycle inhibitors, thereby allowing passage through the G_1 restriction point and progression to S phase.

8.4 Signal Transducers and Activators of Transcription

Signal transducers and activators of transcription (STAT) proteins most commonly participate in signal transduction from cytokine receptors. After tyrosine phosphorylation of STAT proteins by receptor-associated Janus protein tyrosine kinases (JAKs), the STATs dimerize and migrate to the nucleus, where they bind to specific sites and activate transcription (see Ihle 1996). STAT6 is activated by IL-4, and not surprisingly, IL-4-mediated functions are severely depressed in STAT6-deficient mice (Shimoda et al. 1996; Linehan et al. 1998). For B cells, these include IL-4-induced surface marker expression and proliferation, and IgH class

switching to IL-4-dependent classes. IL-4-mediated STAT6 activation induces IRF4 transcription (see Pernis 2002). Therefore reduced IRF4 expression may contribute to the poor responses of $STAT6^{-/-}$ B cells. The diminished proliferative response of lymphocytes from STAT6 mutant mice is accompanied by a block in cell cycle progression (G_1 to S phase) that may be mediated by an abnormal accumulation of the cyclin-dependent kinase (cdk) inhibitor, $p27^{Kip}$; a similar phenomenon was seen with IL-4-stimulated $STAT4^{-/-}$ lymphocytes (Kaplan et al. 1998). Dominant-negative STAT3 and STAT5 proteins have been shown to inhibit expression of the c-myc and pim-1 genes, respectively (Mui et al. 1996; Kiuchi et al. 1999). c-myc and pim-1 have been implicated in B cell proliferation and transformation.

STAT proteins may also relay signals from non-cytokine receptors in other cells (see Ihle 1996) as well as B lymphocytes. Karras et al. (1996a, 1996b) have shown that cross-linking of sIg on primary B cells results in phosphorylation and nuclear localization of active STAT1, STAT5 and STAT6. The kinetics of STAT1 induction by sIg cross-linking were much slower than observed following cytokine treatment. Furthermore, STAT5 and -6 activation were found to be PKC-dependent. CD40 cross-linking can also induce phosphorylation of STAT6 and activation of a target gene (Karras et al. 1997a).

Peritoneal B-1 lymphocytes differ in a number of respects from conventional splenic (B-2) cells. Importantly, they are continuously cycling, as opposed to the majority of B-2 cells, which are in the resting (G_0) state in a healthy animal. Karras et al. (1997b) found that B-1 cells contain constitutively active STAT3, whereas in B-2 cells, STAT3 was undetectable unless cells were activated (in this case by BCR cross-linking). Kinetic and inhibitor studies implied that the coupling of STAT3 to the BCR was a novel one, differing from the classic STAT3-cytokine receptor interaction. These observations suggest that STAT proteins may lie in signalling pathways emanating from the B cell antigen receptor that are biochemically distinct from the cytokine signalling paths.

8.5 Transcriptional Mediators of Terminal Differentiation

Blimp-1, the B lymphocyte-induced maturation protein, is a zinc finger-containing transcription factor expressed at late stages of B cell differentiation concomitant with acquisition of a plasma cell phenotype (Turner et al. 1994). In primary cells and B cell lines, Blimp-1 is rapidly up-regulated when cells are induced to differentiate, and Blimp-1 overexpression

will drive the maturation of B cells to antibody secreting cells (Turner et al. 1994; Schliephake and Schimpl 1996). Among other changes, J chain gene transcription and surface expression of the plasma cell marker syndecan-1 are induced by Blimp-1 overexpression, and Blimp-1 represses c-myc expression in terminally differentiated B cells (Lin et al. 1997; Shaffer et al 2002). Mutation of the Blimp-1 gene causes early lethality, but a recent conditional Blimp-1 mutant mouse confirmed the essential role of Blimp-1 in plasma cell generation (Shapiro-Shelef et al. 2003).

In vivo, during the interaction of cognate B and T cells in the germinal center, the engagement of CD40 on the B cell surface has been shown to arrest differentiation before the stage of antibody secretion. This correlated with reduction in mRNAs for J chain, the secreted form of Ig, and significantly, Blimp-1 (Randall et al. 1998). Mechanistically, this is believed to be due to the repressive effects of the Bcl-6 proto-oncogene (see Calame et al. 2003). Bcl-6 is a POZ/zinc finger transcriptional repressor that is required for GC formation, and for the maintenance of the phenotype of B cells during the GC reaction (Ye et al. 1997; Shaffer et al. 2000). The model of terminal B cell differentiation is one of mutual repression between Bcl-6 and Blimp-1. Upon receipt of appropriate microenvironmental signals, Bcl-6 protein is degraded, and its repressive effect on blimp-1 expression is released. The induced blimp-1 then represses the transcription of several genes (including bcl-6) that determine the properties of mature B cells, and activates the plasma cell differentiation program (Shaffer et al. 2002). Another transcription factor that is essential for plasma cell differentiation is X-box binding protein (Xbp)-1 (Reimold et al. 2001). Xbp-1 is produced from an mRNA that is modified post-transcriptionally during the unfolded protein response (UPR), a physiological response to ER stress (Yoshida et al. 2001). In plasma cells, the extremely high rate of Ig production activates a classical Xbp-1-mediated UPR (van Anken et al. 2003). In addition, Xbp-1 controls production of IL-6, a cytokine that mediates plasma cell survival (Iwakoshi et al. 2003).

9 Concluding Remarks

In this review, rather than summarize a large amount of data showing correlative effects, and listing potential target genes by virtue of the occurrence of factor binding sites in promoters, I have tried only to mention work that has forged a causative link between a specific transactiva-

tor and a particular aspect of the B cell response to activating signals. The value of the genetic evidence discussed here is that it discriminates a small number of factors and target genes from the large number of candidates previously implicated by other approaches, thereby clarifying and simplifying the study of what is still a very complex biological response.

The shortcomings of targeted gene ablation have already been mentioned. Novel approaches are now being used to allow important functions to be uncovered, whether they occur early or late in a cell's (or an organism's) developmental program. Furthermore, as normal immune responses require functional interactions between several cell types, it will be important to establish the B cell autonomy of the phenotypes described here. It will also be important to distinguish whether particular gene products play a direct role in a signal transduction pathway, or an indirect one. In several cases mentioned here, mutant B cells were found to be hyporesponsive to a number of mitogens. However, the primary defect can now be mapped to the maturation of bone marrow emigrant, transitional B cells. Only after taking this step does a B cell become competent to respond positively to antigen receptor cross-linking (Allman et al. 1992, 1993).

We are beginning to understand some of the "upstream" events in B cell activation, starting at the cell membrane. It is now clear that not only the nature, but the duration and amplitude of an activating signal are major determinants in the transcription factor activities enlisted, and so the ultimate cellular outcome (Dolmetsch et al. 1997). In some cases, the activating signal's course to the nucleus has been charted (e.g., NF-κB, STATs, NF-AT). The major challenge now is the identification of the target genes that are activated to implement the correct response. The strategic use of cDNA microarrays analyses to measure the specific influences of individual transcriptional regulators will be a valuable starting point. Once such target genes are unambiguously identified, their products may be fitted into a biochemical context that will allow us to understand the signalling pathways that drive B cell activation, division and functional maturation. Quantitative in vitro analytical systems will help to identify more precisely the cellular behavior that is impacted by transcription factor loss or overexpression (Hasbold et al. 2003). With this knowledge, we may more precisely and safely manipulate B cell behavior to predictably and positively influence humoral immune responses.

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Expression of MHC II Genes

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Abstract Innate and adaptive immunity are connected via antigen processing and presentation (APP), which results in the presentation of antigenic peptides to T cells in the complex with the major histocompatibility (MHC) determinants. MHC class II (MHC II) determinants present antigens to $CD4^+$ T cells, which are the main regulators of the immune response. Their genes are transcribed from compact promoters that form first the MHC II enhanceosome, which contains DNA-bound activators and then the MHC II transcriptosome with the addition of the class II transactivator (CIITA). CIITA is the master regulator of MHC II transcription. It is expressed constitutively in dendritic cells (DC) and mature B cells and is inducible in most other cell types. Three isoforms of CIITA exist, depending on cell type and inducing signals. CIITA is regulated at the levels of transcription and post-translational modifications, which are still not very clear. Inappropriate immune responses are found in several diseases, including cancer and autoimmunity. Since CIITA regulates the expression of MHC II genes, it is involved directly in the regulation of the immune response. The knowledge of CIITA will facilitate the manipulation of the immune response and might contribute to the treatment of these diseases.

1 Innate and Adaptive Immunity

The immune system is composed of innate and adaptive branches, which are nonspecific and specific, i.e., they target common and unique parts of non-self antigens, respectively (Table 1). They discriminate between self and non-self, and activate appropriate effectors. Although innate and adaptive immunity appear independent, appropriate interactions between them are indispensable for the normal function of the immune response. Indeed, "mistakes" that happen in either branch can affect the organism as a whole.

Innate immunity is the first defense against invading pathogens and performs the function of immune surveillance (Calandra et al. 2003; Janeway 2001). Cells that constitute innate immunity are antigen-presenting cells (APCs), including DCs, macrophages and B cells. Innate immunity does not develop any antigen specificity because the discrimination between self and non-self is primarily based on pathogen-associated molecular patterns (PAMP), which are common components of many microorganisms and are not found in humans, i.e., lipopolisacharides (LPS). Ligation of PAMP with toll-like receptors (TLR) on the surface of APCs (Bachmann and Kopf 2002; Takeda et al. 2003) leads to phagocytosis and antigen processing and presentation (APP). APP results in the presentation of processed antigens to T cells (Cresswell and Lanzavecchia 2001) (Fig. 1) and the establishment of adaptive immunity (Kelly et al. 2002; Smyth et al. 2001).

The function of adaptive immunity is the elimination of non-self antigens and the creation of immune memory. Constituents of adaptive immunity are B and T cells. Adaptive immunity is unique and created only after the encounter with a specific non-self antigen, which is presented to them by APCs. Discrimination between self and non-self at the level of adaptive immunity is complex and involves the elimination or functional inactivation of self-reactive lymphocytes from the repertoire. Es-

Table 1 Properties of innate and adaptive immunity

	Innate	Adaptive
Tolerance to self	Yes	Yes
Specificity	Low	High
Diversity	Low	High
Memory	None	Yes
Cells	APC	B and T cells

APC, antigen-presenting cells.

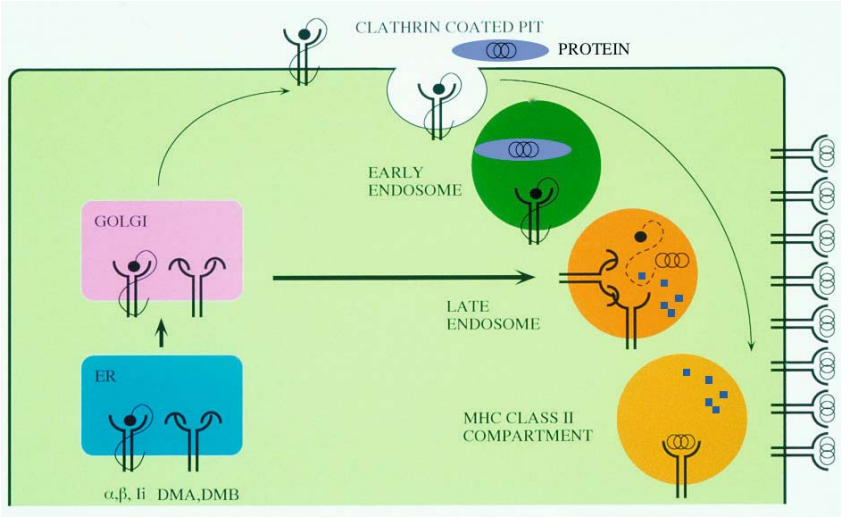

Fig. 1 Pathway of antigen processing and presentation (*APP*). HLA-DP, HLA-DQ, HLA-DR, which are in complex with Ii, and HLA-DM heterodimers are assembled in the endoplasmic reticulum (ER, depicted in *blue*). They travel to the *trans*-Golgi network (depicted in *pink*) and either fuse with the late endosome (depicted in *orange*) or continue to the cell surface. Degradation of phagocytosed antigenic peptides (depicted in *violet*) starts in an early endosome (depicted in *green*). In the late endosome, Ii is removed from the complex with HLA-DP, HLA-DQ, HLA-DR and replaced with antigenic peptides. Loaded with antigens, MHC II molecules travel from MHC class II compartment (MIIC, depicted in *yellow*) to the cell surface, where they activate T cells

tablishment of adaptive immunity is slow, but once it is established, it is memorized and able to respond faster to subsequent contacts with the same antigen. However, since innate immunity is faster, it keeps infections under control until the establishment of adaptive immunity (Smyth et al. 2003).

MHC determinants play a very important role in APP, which connects innate and adaptive immunity. Namely, processed antigenic peptides are presented to T cells only in the groove of MHC heterodimers.

1.1 Major Histocompatibility Complex Determinants

Genes that encode MHC determinants, also known as human leukocyte antigens (HLA), are located on the short arm of chromosome 6 and are extraordinarily polymorphic (Anonymous 1999). They are divided into

two classes: MHC I and MHC II determinants that present intracellular and extracellular antigens, respectively. MHC I determinants are expressed on most nucleated cells, whereas MHC II determinants only on APCs, mature B and activated T cells.

Three different MHC I determinants, namely HLA-A, HLA-B and HLA-C, are assembled and loaded with antigenic peptides, which are generated by protein degradation in the 26S proteasome, in the endoplasmic reticulum (ER) (Fruci et al. 2003; Saveanu et al. 2002). The complex between the MHC I heterodimer and antigenic peptide passes through the *trans*-Golgi network to the cell surface of APCs and activates $CD8^+$ T cells.

In humans, there are three classic MHC II determinants termed HLA-DP, HLA-DQ and HLA-DR, and two nonclassic determinants called HLA-DM and HLA–DO (Fig. 2). The former are cell-surface heterodimers that present antigenic peptides, whereas the latter are cytoplasmic oligomers that are involved in loading antigenic peptides. HLA-DP, HLA-DQ, HLA-DR and HLA-DM are composed of α and β chains, whereas HLA-DO form heterotetramers, composed of two α and two β chains (Fig. 2). Classic MHC II determinants are assembled in the ER in the complex with the invariant chain (Ii), which stabilizes them and prevents premature antigenic peptide loading. This complex is transported to the late endosome that contains degraded antigens. Antigenic peptide loading is a three-step process (Fig. 1). The first two steps, which take place in the late endosome, are the degradation and immediate replacement of Ii by the class II-associated Ii-chain peptide (CLIP). The third step occurs in the MHC class II compartment (MIIC) and is the exchange of CLIP with antigenic peptides. This exchange is mediated by HLA-DM and is regulated by HLA-DO. The complex between MHC II determinants and antigenic peptides then travels to the surface of APCs, where the antigen is presented to $CD4^+$ T cells, which are the main regulators of the immune response.

Since they present antigens to $CD4^+$ T cells, MHC II determinants are involved directly in the regulation of the immune response. Therefore, it is not surprising that the precise control of MHC II gene expression, which takes place at the level of transcription, is necessary for the equilibrated function of the immune system. Most of our knowledge about this control has been elucidated from studies of severe combined immunodeficiency called MHC II deficiency or the type II bare lymphocyte syndrome (BLS) (Table 2).

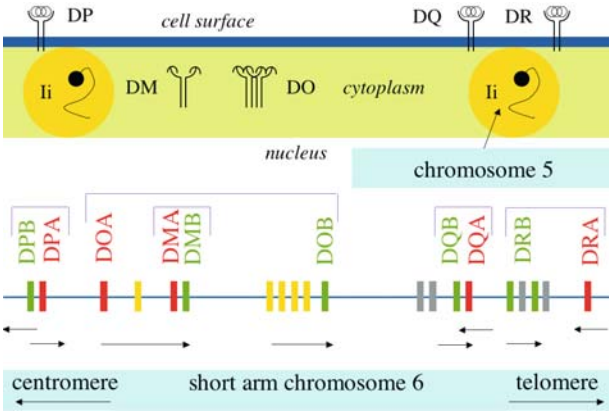

Fig. 2 The organization and direction of MHC II genes as well as sub-cellular localization of their proteins. Three heterodimers are transcribed from promoters that point in opposite directions (HLA-DPA and HLA-DPB, HLA-DQA and HLA-DQB, HLA-DRA and HLA-DRB), HLA-DO heterotetramer and HLA-DM heterodimer are transcribed from promoters that point towards the telomere. Genes for α and β chains are shown as *red* and *green bars*, respectively. Pseudogenes and MHC II-unrelated genes are shown as *grey* and *yellow bars*, respectively. *Black arrows* point in the direction of transcription. HLA-DP, HLA-DQ and HLA-DR heterodimers are found on the cell surface in complex with antigenic peptides. HLA-DM heterodimers and HLA-DO heterotetramers are cytoplasmic molecules, as well as invariant chain (*Ii*), all of which are also found in MHC class II compartments (MIIC, depicted as *yellow circles*). Genes coding for HLA-DP, HLA-DQ, HLA-DR, HLA-DM and HLA-DO are located on the short arm of chromosome 6, but gene coding for Ii is located on chromosome 5

Table 2 Properties of bare lymphocyte syndrome

Complementation group	A	B	C	D
MHC II expression	Absent	Absent	Absent	Absent
Enhanceosome	Formed	Not formed	Not formed	Not formed
Mutated gene	*MHC2TA*	*RFXANK/B*	*RFX5*	*RFXAP*
Mutated protein	CIITA	RFXANK/B	RFX5	RFXAP

MHC2TA, major histocompatibility class 2 trans-activator; RFXANK/B, regulatory factor X ANK/B; RFX5, regulatory factor X 5; RFXAP, regulatory factor X AP; CIITA, class II transactivator.

1.2 Bare Lymphocyte Syndrome

BLS is an autosomal recessive disease characterized by the lack of constitutive and inducible MHC II gene expression (Table 2) (reviewed in Reith and Mach 2001 and Nekrep et al. 2003). It is caused by mutations in factors that direct the transcription from MHC II promoters rather than in MHC II genes themselves.

More than 10 years ago, transcription of MHC II genes was known to require several unidentified proteins. The earliest studies performed to identify these proteins employed in vitro assays, which revealed many irrelevant DNA-protein interactions. However, only genetic studies of BLS finally resolved this puzzle. First, CIITA (Steimle et al. 1993) and regulatory factor X5 (RFX5) (Steimle et al. 1995) were identified, which rescued the expression of MHC II determinants in complementation group (CG) A and C, respectively. Subsequently, these findings led to the identification of regulatory factor X, which contains ankyrin repeats (RFXANK/B) (Masternak et al. 1998; Nagarajan et al. 1999) and regulatory factor X associated protein (RFXAP) (Durand et al. 1997), which rescued the expression of MHC II determinants in CGs B and D, respectively. These factors not only account for four CGs of BLS, but also represent all gene-specific *trans*-acting factors that bind *cis*-acting elements for MHC II transcription.

1.3 *cis*-Acting Elements

MHC II genes are transcribed from compact promoters (Fig. 3). They contain variable proximal promoter (PPS) and conserved upstream sequences (CUS) (reviewed in Nekrep et al. 2003; Reith and Mach 2001; Ting and Trowsdale 2002).

In HLA-DRA PPS are located from position −52 to the transcription start site. From the 5′ direction, they contain octamer-binding site (OBS) and initiator (Inr) sequences, but lack a functional TATA box. OBS binds the octamer-binding protein-1 (Oct-1), which recruits the B cell octamer-binding protein 1/Octamer-binding factor 1/Oct co-activator from B cells (Bob1/OBF-1/OCAB) (Matthias 1998), whereas the initiator, which represents the transcription start site, binds RNA polymerase II-associated transcription factor I ($TF_{II}I$) and TATA binding protein (TBP) -associated factor of 250 kDa ($TAF_{II}250$).

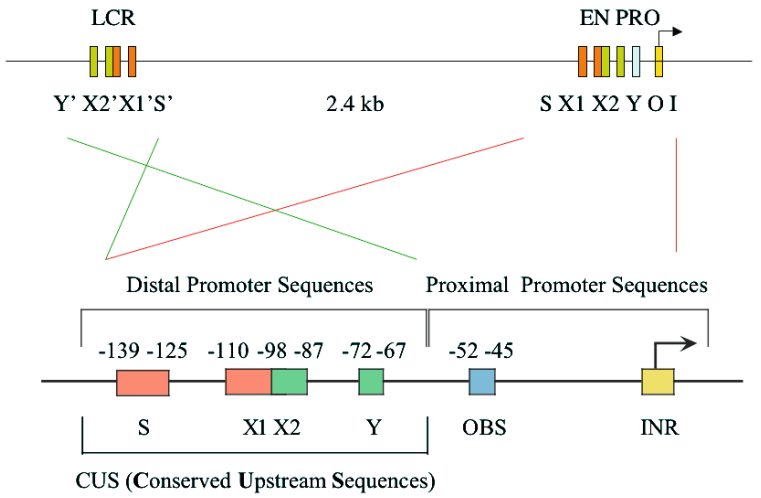

Fig. 3 *cis*-Acting elements in the DRA promoter. Locus control region (*LCR*), conserved upstream sequences (*CUS*), and proximal promoter sequences (*PPS*) are needed for the transcription from the DRA promoter. LCR is located 2.4 kb upstream from CUS. LCR and CUS contain Y′, X2′, X1′, S′ and S, X1, X2 and Y boxes, respectively. PPS contain the octamer-binding site (*OBS*) and the initiator (*INR*). The spacing between CUS and PPS is presented in the *lower panel*

CUSs are located from positions −139 to −67. From the 5′ direction, they contain S, X and Y boxes. The X box can be further divided into X1 and X2 boxes. Tight spatial constraints are preserved between these boxes (Jabrane-Ferrat et al. 1996), which bind different *trans*-acting factors. S and X1 boxes bind RFX (Fig. 4) (Jabrane-Ferrat et al. 1996; Ting and Trowsdale 2002); the X2 box binds X2 binding protein (X2BP), which can be c-AMP responsive element-binding protein (CREBP) or activator protein 1 (AP-1) (Moreno et al. 1997; Setterblad et al. 1997); and the Y box binds nuclear factor Y (NF-Y) (Fig. 4) (Maity and de Crombrugghe 1998; Mantovani 1999).

However, PPS and CUS are not sufficient for HLA-DRA gene expression. A distal locus control region (LCR), which is located approximately 2.4 kb upstream of the CUS, is needed for efficient transcription from the HLA-DRA promoter in the organism (Masternak et al. 2003) (Fig. 3). A LCR is a mirror image of CUS. It contains S', X1', X2' and Y' boxes, but in the opposite orientation from the promoter. Indeed, the LCR binds the same *trans*-acting factors as the CUS.

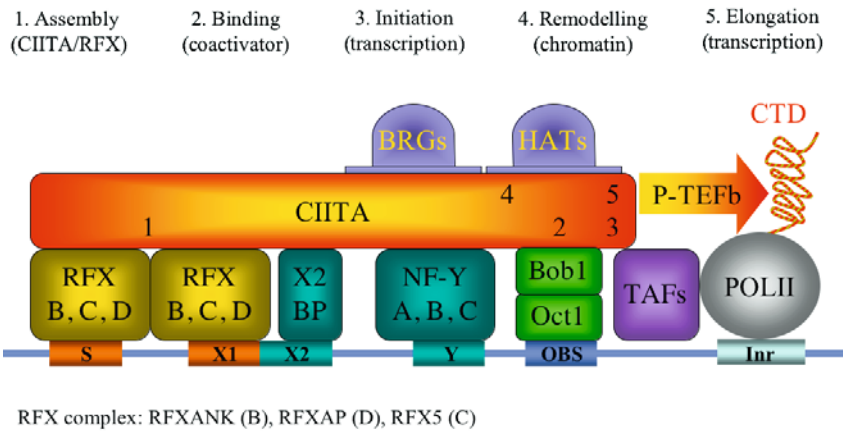

Fig. 4 *cis*-Acting elements and *trans*-acting factors on MHC II promoters. Conserved upstream sequences (*CUS*) contain S, X and Y boxes. X box is divided into X1 and X2 boxes. In the HLA-DRA gene, the octamer-binding site (*OBS*) and the initiator (*INR*) form the proximal promoter sequences (*PPS*). RFX, which contains RFXANK, RFXAP and RFX5, binds S and X1 boxes. X2 binding protein (*X2BP*) binds X2 box. NF-Y, which contains NF-YA, NF-YB and NF-YC, binds Y box. OBS binds octamer binding protein-1 (*Oct-1*), which recruits the B cell octamer-binding protein 1/Octamer binding factor 1/Oct co-activator from B cells (*Bob1/OBF-1/OCAB*). The initiator binds general transcription factors and positions RNA polymerase II (*RNAPII*) on the promoter. CIITA is recruited to the MHC II enhanceosome, which consists of RFX, X2BP and NF-Y on CUS. It binds histone acetyl-transferases (*HATs*, *CBP/p300*, *pCAF*) and brahma-related gene (*BRG*) proteins, which remodel the chromatin, Bob1/OBF-1/OCAB, GTFs and TAFs, which recruit RNAPII and initiate transcription, and positive transcription elongation factor b (*P-TEFb*), which phosphorylates the C terminal domain (*CTD*) of RNAPII and facilitates the elongation of transcription. These steps in the transcriptional process are given *above* the drawing

1.4 *trans*-Acting Factors

Several *trans*-acting factors are required for MHC II transcription (Fig. 4). Among them, RFX and NF-Y create the platform for other co-activators to access MHC II promoters.

NF-Y is composed of three subunits (reviewed in Matuoka and Yu Chen 1999). NF-YA contains 374 residues. At the N-terminus of NF-YA, there is glutamine-rich region, which is followed by a stretch of prolines, serines and threonines. Its subunit interaction and DNA-binding domains (DBD) are at the C-terminus.

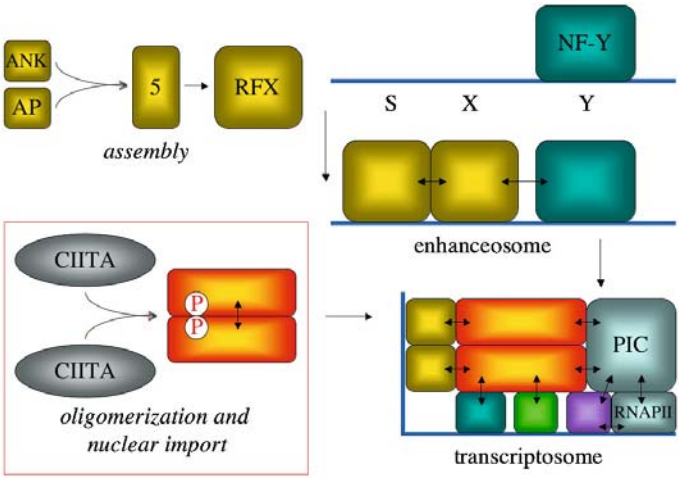

Fig. 5 Assembly of the MHC II enhanceosome and transcriptosome. RFXANK/B forms complex with RFXAP, which recruits the RFX5 oligomer. RFX binds S and X1 boxes, whereas NF-Y binds Y box. They form the MHC II enhanceosome. The phosphorylation of CIITA leads to its oligomerization and nuclear import. It binds to the MHC II enhanceosome and forms the MHC II transcriptosome, which forms the preinitiation complex (*PIC*) and recruits as well as modifies RNA polymerase II (*RNAPII*)

NF-YB contains 207 residues. They form a histone fold and TATA-binding protein (TBP) binding domains. The histone fold is similar to that of histones 2A and 2B (Mantovani 1999), which is responsible for their dimerization. This domain has the same function in NF-Y assembly.

NF-YC contains 335 residues. As with NF-YB, it contains a histone fold and TBP binding domains, and in addition a glutamine-rich region.

RFX is also composed of three subunits (Fig. 5) (reviewed in Reith and Mach 2001). RFXANK/B contains 260 residues that form an acidic domain and four ankyrin repeats, the source of its name.

RFX5 contains 616 residues, which form DBD and PEST domains. It is the fifth member of the RFX family that binds S and X boxes of MHC II promoters, hence its name.

RFXAP contains 272 residues. This protein has acidic, basic and glutamine-rich domains. Because it interacts directly with RFX5, it was named the RFX-associated protein.

Transcription from MHC II promoters starts after productive interactions between *cis*-acting elements and *trans*-acting factors, which lead to the formation of the MHC II enhanceosome and transcriptosome.

1.5 MHC II Enhanceosome and Transcriptosome

The MHC II enhanceosome is formed on CUS and LCR. Its assembly requires specific protein–protein and DNA–protein interactions. The formation of the enhanceosome starts off DNA (Fig. 5), where RFX and NF-Y bind loosely. Both RFX and NF-Y are formed in two steps. RFXANK/B forms a complex with RFXAP, which binds the RFX5 oligomer (Jabrane-Ferrat et al. 2002). NF-YB and NF-YC first form heterodimer via their histone folds and then bind NF-YA. RFX and NF-Y are linked via interactions between RFXANK/B, NF-YA and NF-YC (Jabrane-Ferrat et al. 2002; Nekrep et al. 2003; Ting and Trowsdale 2002). The binding of the enhanceosome to promoters occurs via RFX5, NF-YB and NF-YC DBDs. Each individual contact between the enhanceosome and DNA takes place on the same side of the double helix. As mentioned earlier, preserved spatial constraints are present in CUS and LCR. Completely invariant spacing between S and X boxes can be explained by the RFX5 oligomers, which bind each other next to their DBDs. These higher-order RFX complexes can occupy S and X boxes only if the exact spacing between them is preserved. Full helical turns are tolerated between X and Y boxes. This finding can be explained because their binding occurs far from their DBDs.

NF-Y not only interacts with RFX, but also with different histone acetyltransferases (HAT) that make chromatin accessible for other coactivators (Fontes et al. 1999; Harton et al. 2001). Moreover, NF-YB and NF-YC bind TBP in vitro. This interaction helps to attract general transcription factors (GTF) to promoters, which lack a TATA box. The X2 box is bound by CREBP or AP-1 (Moreno et al. 1995; Setterblad et al. 1997).

The very last step is the recruitment of CIITA to the MHC II enhanceosome, which converts it into the MHC II transcriptosome (Fig. 5). Once the transcriptosome is formed, MHC II genes can be transcribed.

2 Class II Transactivator

CIITA is the master regulator of MHC II gene expression. Except for CIITA, all *trans*-acting factors that are needed for the transcription of MHC II genes are expressed ubiquitously. Thus, the synthesis of MHC II determinants correlates directly with the presence of CIITA, which is expressed constitutively only in DCs and mature B cells and is inducible in most other cell types. After CIITA is recruited to the MHC II enhanceosome, it recruits the general transcriptional machinery.

CIITA contains 1,130 residues (Fig. 6). It can be divided into several domains, which are the N- terminal activation (AD), proline/serine/threonine-rich (PST) and GTP-binding domains (GBD), as well as the C-ter-

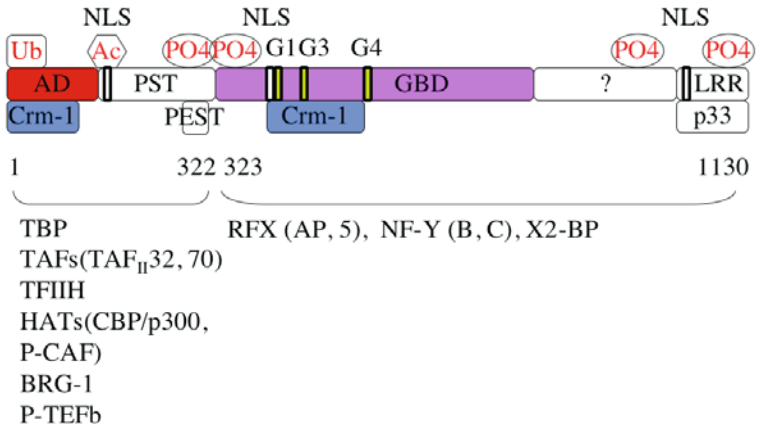

Fig. 6 The scheme of CIITA, its post-translational modifications and partner proteins. CIITA is divided into several domains, which are activation (*AD*), proline/serine/threonine-rich (*PST*), GTP binding (*GBD*) domains and leucine-rich repeats (*LRR*). Additionally, two nuclear localization signals (*NLS*) are located in the N-terminus and one in the C-terminus of the protein. Within PST domain there is a consensus proline/glutamine/serine/threonine-rich (*PEST*) sequence. GBD contains three GTP interacting sequences, designated by *G1*, *G3* and *G4*. Positions of post-translational modifications of CIITA, which are phosphorylation, acetylation and ubiquitylation, are given above the drawing in *red letters* as *PO4*, *Ac* and *Ub*, respectively. Chromosomal region maintenence-1 protein (Crm-1, depicted as a *purple box*) interacts with AD and GBD, and p33 (depicted as a *white box*) binds LRR. Transcriptional co-activators (*TBP, TAFs, HATs, BRG-1, P-TEFb*) as well as constituents of the MHC II enhanceosome (*RFXAP, RFX5, NF-YB, NF-YC* and *X2BP*), which interact with CIITA, are listed *under* the drawing

minal leucine-rich repeat (LRR). Additionally, CIITA contains three nuclear localization signals (NLS) and two putative nuclear export sequences (NES) (Fig. 6).

The optimal AD of CIITA spans the first 322 residues. Its amino terminal part is rich in aspartic and glutamic acids and resembles the classic acidic AD, similar to that of VP16. Indeed, it can be replaced by AD from VP16 (herpes simplex virus) and E1a (adenovirus) (Riley and Boss 1993). Between residues 145 and 322 is the PST domain. As its name implies, it contains several prolines, serines and threonines, which are targets for post-translational modifications. A consensus PEST sequence is located from positions 283 to 308, but it does not represent a degradation motif or degron (Schnappauf et al. 2003). Rather, degrons are located within the first 99 residues and from positions 230 to 260 of CIITA, respectively (Schnappauf et al. 2003).

A putative GBD resides downstream of AD, from positions 420 to 561. In contrast with the classic GBDs, which contain four GTP-interacting sequences, CIITA contains only three (Harton et al. 1999). These are Walker type A motif (G1), magnesium coordination site (G3) and a site that confers specificity for guanosine (G4). Indeed, although CIITA binds GTP, it does not hydrolyse it in vitro (Harton et al. 1999). This finding suggests that CIITA might be a constitutively active GTP-binding protein. As mutations of either motif reduce the activity of CIITA, the sole function of GTP binding to CIITA may be to alter its conformation.

Five or six consensus LRR are located from positions 988 to 1,097 (Harton et al. 2002). They bind a 33-kDa protein of unknown function (Hake et al. 2000). Certain mutations positioned in α helices of the LRR, but not in β sheets decrease the activity of CIITA. An additional LRR flanking the GBD might be involved in the aggregation of CIITA (Linhoff et al. 2001).

In CIITA, three NLS and two NES have been described. A bipartite NLS is located from positions 141 to 159, and two additional NLS were mapped from positions 405 to 414, next to G1 and 940 to 963 in the LRR (Cressman et al. 1999; Cressman et al. 2001; Nekrep et al. 2002; Spilianakis et al. 2000). NES have been poorly characterized. The first is located in the first 114 residues, the second from positions 408 to 550 (Kretsovali et al. 2001). However, none of these NLS and NES have been examined directly.

2.1 Regulation of CIITA Gene Expression

Since the transcription of MHC II genes is dependent on the presence of CIITA, it is not surprising that its expression is highly regulated. In general, there are two types of regulation of gene expression: genetic and epigenetic. CIITA employs both. Whereas specific activators dictate active transcription, epigenetic silencing occurs via chromatin condensation.

Transcription of CIITA can be initiated from up to three different promoters called PI, PIII and PIV (Fig. 7) (Muhlethaler-Mottet et al. 1997). Whereas in myeloid DCs, constitutive expression of CIITA is initiated from PI, in plasmacytoid DCs and B cells, it is initiated from PIII (Muhlethaler-Mottet et al. 1997). IFN-γ inducible expression of CIITA is mediated by PI in bone marrow-derived APCs and myeloid DCs and by PIV in somatic cells (Muhlethaler-Mottet et al. 1997; Waldburger et al.

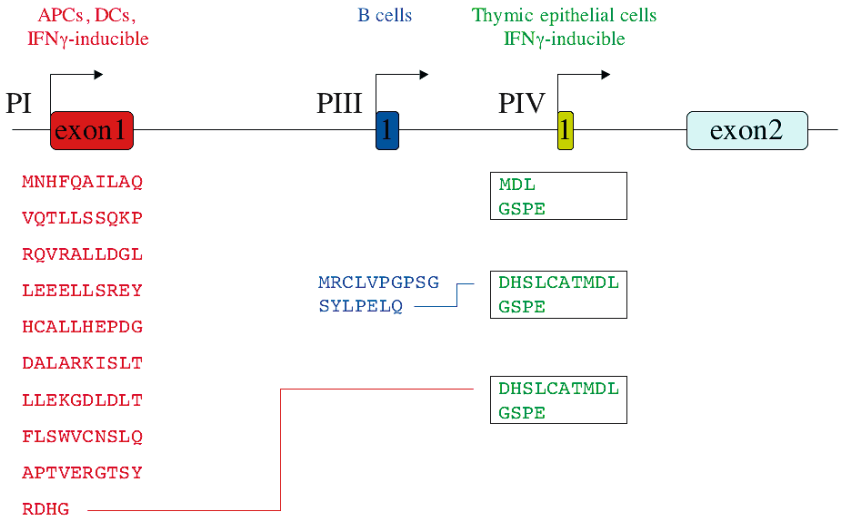

Fig. 7 CIITA promoters, their composition and specificity. CIITA is transcribed from three different promoters: PI, PIII and PIV with unique exons 1 (depicted as *red*, *purple*, and *green boxes* for PI, PIII, and PIV, respectively), which are spliced into common exon 2 (depicted as a *blue box*). The additional sequences in CIITA, transcribed from PI is given in *red letters* and from PIII in *purple letters*, while a common sequence from all promoters is given in *green letters*. Cell types and stimuli are given *above* the scheme. *IFN-γ*, interferon gamma, *APCs* antigen-presenting cells, *DCs* dendritic cells

2001). All promoters have a unique exon 1, which is spliced into the common exon 2 (Muhlethaler-Mottet et al. 1997). Translation of CIITA transcripts from PI and PIII starts from the first methionine in exon 1, whereas from PIV it begins from the first methionine in exon 2. This brings unique sequences to the N-terminus of CIITA (Muhlethaler-Mottet et al. 1997) and results in three isoforms (IF) of CIITA: IFI, IFIII and IFIV. IFI and IFIII contain additional 94 and 17 residues, respectively. The additional sequence in IFI bears homology with the caspase recruitment domain (CARD) (Nickerson et al. 2001) and most likely represents a new protein–protein interaction domain. It also has the highest transcriptional activity (Nickerson et al. 2001). Importantly, a single mutation of a conserved leucine in CARD abrogates the activity of CIITA (Nickerson et al. 2001).

Epigenetic regulation of CIITA gene expression is utilized for embryonic survival. Notably, immune responses against paternal antigens in the placenta cause fetal death. In these tissues, promoter hypermethylation and histone deacetylation are proposed mechanisms for silencing the transcription of CIITA (Holtz et al. 2003; Morris et al. 2000; van den Elsen et al. 2000). Derepression of CIITA transcription in trophoblasts after treatment with methylation inhibitors and trichostatin A with IFN-γ supports this mechanism.

In immature DCs, the expression of CIITA is observed from PI, but it does not lead to APP. Phagocytosis of an antigen leads to DC maturation, which is accompanied by the cessation of further phagocytosis and repression of de novo CIITA and MHC II transcription and up-regulation of APP (Cella et al. 1997; Landmann et al. 2001; Pierre et al. 1997; Turley et al. 2000). These events might be mediated by TLRs. Because only the specific population of encountered antigens are processed and presented to T cells, such regulation could represent the best way of establishing adaptive immunity. Since the expression of CIITA is shut down after the clearance of a foreign antigen, it also lowers the chance of DCs randomly presenting self-antigens.

In B cells, the constitutive expression of CIITA from PIII is accompanied by the presence of high levels of MHC II determinants on the cell surface. However, after B cells differentiate into highly specialized antibody-producing plasma cells, the expression of CIITA and MHC II determinants is lost (Chen et al. 2002). B lymphocyte inhibitory maturation protein 1 (BLIMP1) begins the process of this silencing (Piskurich et al. 2000).

CIITA gene expression can be induced with IFN-γ from PI and PIV. PIV contains gamma-activated sites (GAS), which bind the signal trans-

ducer and activator of transcription 1 (STAT1) and an interferon regulatory factor-1 binding site, which binds interferon regulatory factor-1 (IRF-1) (Muhlethaler-Mottet et al. 1998). Since PI is also responsive to IFN-γ, it could contain the same elements. After stimulation with IFN-γ, activated, phosphorylated STAT 1 translocates into the nucleus and binds GAS. Activated STAT 1 also induces the expression and accumulation of IRF-1. Binding of STAT 1 to GAS itself causes a weak acetylation of histones 3 and 4 in PIV (Morris et al. 2002). However, the hyperacetylation of histones, which makes transcription more efficient, occurs only after the accumulation and binding of IRF-1 to its site.

Thus, the regulation of transcription of the CIITA gene represents the first level of control of CIITA function. The second level consists of post-translational modifications of CIITA.

2.2 Post-Translational Modifications of CIITA

CIITA is post-translationally modified by acetylation, phosphorylation and ubiquitylation (Fig. 8). The effects of these modifications are complex and far from being clearly understood, but a general picture can be drawn from several studies. The fate of CIITA in the cell is not random, but each step, starting after its translation and ending with its possible degradation, is regulated precisely by the following modifications.

Phosphorylation is the first post-translational modification of CIITA. Two studies have shown that this modification increases the activity of CIITA. The first study mapped phosphorylation sites into the PST region from positions 253 to 321 (Tosi et al. 2002). The second study showed that protein kinase A (PKA) phosphorylates CIITA on one or more serines in the region between PST and GBD (Sisk et al. 2003). The phosphorylation of CIITA leads to its accumulation, oligomerization and nuclear translocation. Most likely, these latter events happen because the phosphorylation changes the conformation of CIITA and exposes its NLS.

After it translocates into the nucleus, CIITA is acetylated on lysines 141 and 144, by CBP/p300 and also by pCAF, which are located in the nucleus (Spilianakis et al. 2000). Acetylation of these residues keeps CIITA in the nucleus and increases the stability of the MHC II transcriptosome. Acetylation might also facilitate the subsequent ubiquitylation of CIITA. Indeed, recent data suggest that histone deacetylases (HDACs) are involved in the ubiquitylation of CIITA (Greer et al. 2003).

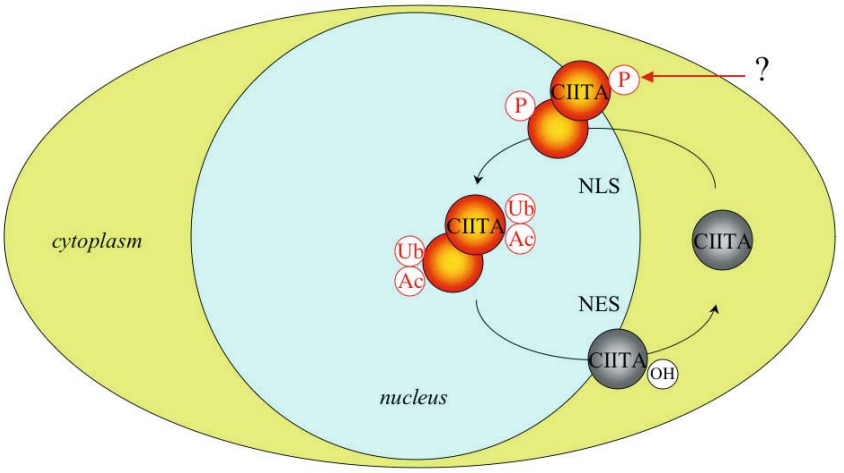

Fig. 8 Post-translational modifications of CIITA. After an unknown signal (depicted as the *red arrow*), CIITA is phosphorylated, which causes its oligomerization and nuclear translocation (*black arrow* directed from the cytoplasm into the nucleus). Acetylation and ubiquitylation increase CIITA transcriptional activity. Termination of transcription is accompanied by additional CIITA modifications, possibly phosphorylation that might result in the translocation of CIITA into the cytoplasm (*black arrow* directed from the nucleus into the cytoplasm) or in its degradation. CIITA monomers and oligomers are depicted as *gray* and *orange circles*. CIITA phosphorylation is represented as a *red P* attached to CIITA. CIITA acetylation and ubiquitylation are depicted as *red Ac* and *Ub*, respectively. *NLS* and *NES* stand for nuclear localization signal and nuclear export sequence, respectively

Ubiquitylation, which is a covalent modification of lysines, plays an important role in transcription. Monoubiquitylated transcription factors tend to be more potent activators, whereas polyubiquitylated proteins are destined for degradation by the 26S proteasome. The role of ubiquitylation of CIITA has been addressed in two studies, but clear conclusions on the function of ubiquitin cannot be reached. In the first study, monoubiquitin fused to CIITA prevented the degradation of the modified CIITA protein, but did not affect its transcriptional activity (Schnappauf et al. 2003). In the second study, CIITA co-expressed with ubiquitin had a higher activity than CIITA alone, which was even more pronounced if ubiquitin could not form oligomers (Greer et al. 2003). This effect of ubiquitin was on the stabilization of the MHC II transcriptosome rather than on transcription itself. Interestingly, the same study showed that HDAC prevented the ubiquitylation of CIITA.

Phosphorylation also completes the post-translational modifications of CIITA. Interestingly, PKA inactivates CIITA with phosphorylation of serines 834 and 1050. (Li et al. 2001). Does this modification influence the stability of the enhanceosome? Does it lead to the degradation of CIITA, or does it expose the NES and enable the export of CIITA from the nucleus?

2.3 CIITA Is a Shuttling Protein

Transcription factors and co-factors need to be transported into the nucleus to perform their function. Nucleocytoplasmic shuttling usually requires two types of functionally unique signals, NLS and NES. CIITA contains both, but none of them have been examined adequately (Fig. 6).

At steady state, CIITA is distributed equally between the nucleus and the cytoplasm. Mutant CIITA proteins, which do not follow this pattern, led to the identification of NLS. As mentioned earlier, two NLS are located at the N-terminus and one NLS in the C-terminus of CIITA (Fig. 6) (Cressman et al. 1999, 2001; Spilianakis et al. 2000). However, none of them has been examined directly, i.e., they have not been demonstrated to shuttle a heterologous protein. Moreover, binding to importins has not been investigated.

Two regions in the N-terminus of CIITA have been proposed to function as NES (Fig. 6). The only support comes from interactions between these regions with the chromosomal region maintenance-1 (Crm-1) protein (Kretsovali et al. 2001). Indeed, treatment with leptomycin B (LMB), which blocks Crm-1-dependent nuclear export (Kudo et al. 1998), leads to the nuclear accumulation of CIITA (Cressman et al. 2001; Kretsovali et al. 2001). However, no consensus NES can be found within these sequences. In addition, none of the five putative NES, three of which correspond perfectly to the consensus sequence [Lx(2,3)Lx(2,3)-LxL], functioned in a direct export or bound Crm-1 (G. Drozina, J. Kohoutek, B.M. Peterlin, unpublished observation).

All these results imply the presence of other transport mechanisms. Indeed, several additional regions of CIITA are involved in its nuclear localization, including GBD (Harton et al. 1999; Raval et al. 2003) and LRR (Hake et al. 2000; Harton et al. 2002; Towey and Kelly 2002). Moreover, some mutations in the LRR disrupt the interaction between p33 and CIITA, which interferes with its nuclear localization (Hake et al. 2000). Furthermore, it was suggested that NES from CIITA resemble the snur-

portin-1 NES, which is discontinuous (Paraskeva et al. 1999). In this case, only after a conformational change can Crm-1 bind and transport snurportin-1 from the nucleus to the cytoplasm (Paraskeva et al. 1999).

2.4 CIITA Is a Transcriptional Integrator

Once CIITA is modified appropriately and present in the nucleus, it can function. It binds to the MHC II enhanceosome, attracts several transcription factors and co-factors, and integrates initiation and elongation of transcription as well as chromatin remodeling into a process that finally results in the expression of MHC II genes. Moreover, CIITA might also be involved in the dissociation of the MHC II enhanceosome after the termination of transcription.

CIITA recruits RNA polymerase II (RNAPII) to MHC II promoters and increases transcription initiation and elongation rates. For the former function, interactions with TAFs (Fontes et al. 1997), TFIIB (Mahanta et al. 1997) and Bob1/OBF-1/OCAB (Fontes et al. 1996) might be necessary. For the latter, the interaction between CIITA and the positive transcription elongation factor b (P-TEFb), which phosphorylates the CTD of RNAPII, is needed (Kanazawa et al. 2000).

Not surprisingly, CIITA also recruits HATs and chromatin remodeling machinery to the HLA-DRA promoter. The AD of CIITA binds CBP/p300 (Fontes et al. 1999), pCAF (Spilianakis et al. 2000) and Brahma-related gene 1 (BRG-1) (Mudhasani and Fontes 2002). In addition, it has been suggested that CIITA possesses intrinsic HAT activity (Harton et al. 2001; Raval et al. 2001). This CIITA activity has been mapped into AD and bears homology with other HAT domains, e.g., in CBP/p300 (Harton et al. 2001). Interestingly, CIITA that lacks its AD is still able to mediate the acetylation of histone 4, but not of histone 3 (Beresford and Boss 2001). Thus, CIITA could contain another region with direct or indirect HAT activity.

After the termination of transcription, histone deacetylation, which is mediated by HDACs, is involved in chromatin condensation. Binding of CIITA to HDAC1 and HDAC2 decreases the activity of CIITA, most probably because histone deacetylation disrupts the MHC II enhanceosome (Zika et al. 2003). Thus, CIITA might play an active role in the regulation of its own function on MHC II promoters.

3 Conclusions and Future Directions

In the last few years, much research has resulted in greater understanding of the function and regulation of CIITA. Since CIITA plays a major role in the regulation of the immune response via MHC II determinants, this is not surprising. Moreover, if the rules of this regulation were understood this knowledge could be used to manipulate the immune system. However, many questions remain.

Why is CIITA transcribed from three distinct promoters? Are different CIITA IFs modified differently, thus influencing their activity and regulation?

Are post-translational modifications independent from each other or are they carefully orchestrated or sequential? CIITA phosphorylation increases or decreases CIITA activity, depending on phosphorylated residues. What signals regulate this phosphorylation that results in activation and inactivation of CIITA, and how? Why does the acetylated CIITA protein accumulate in the nucleus? What is the role of its ubiquitylation? Where is CIITA ubiquitylated? These and probably many more questions regarding the post-translational modifications of CIITA still need to be answered.

The shuttling of CIITA is a big puzzle. Since CIITA has not been shown to bind importins and since no NES can be found, how does CIITA shuttle? And why does it shuttle at all?

A lot is known about the *cis*-acting elements in the MHC II promoters, but the role of LCR needs more attention. What is the mode of action of these LCR? Do they also bring P-TEFb to RNAPII? How many LCR are there in the MHC cluster?

Once these questions have been answered, the manipulation of the immune system could become possible and eventually applicable in clinical medicine. Cancer and inadequate vaccination are examples of weak immune responses. Introduction of the constitutively active CIITA proteins could turn cancer cells into APCs. They would then present their own antigenic peptides to $CD4^+$ T cells to activate and direct immune response against transformed cells. Vaccination could also be more effective with the addition of CIITA. Autoimmunity is caused by the inappropriate responses to self-antigens. Dominant negative CIITA proteins could attenuate immune responses as well as the need for other immunosuppressive therapies. Thus, CIITA could become a new tool for immunotherapy in humans.

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Class Switch Recombination: An Emerging Mechanism

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Abstract Class switch recombination (CSR) has been the least well understood of the Ig gene DNA rearrangements. The discovery that activation-induced deaminase (AID) is a pivotal player in CSR as well as somatic hypermutation (SHM) and its variant, gene conversion, represents a sea change in our understanding of these processes. The recognition that AID directly deaminates ssDNA has provided a springboard toward the emergence of a model that explains the initiation of these events. Nonhomologous end joining (NHEJ), the main pathway for the repair of double-strand breaks in mammalian cells plays a key role in the resolution of CSR transactions. Mediators of general double-strand break repair are also involved in CSR and

are mutated in several immunodeficiency diseases. A global picture of the mechanism of CSR is emerging and is providing new insights toward understanding the genetic events that underlie B cell cancers.

1 Introduction

Humoral immunity is dependent on the expression of immunoglobulin (Ig) to fend off pathogenic challenges. The humoral immune system has evolved to produce Ig with a broad repertoire of binding specificities and to avoid expression of autoreactive antibodies by using positive and negative selection strategies. Ig molecules are composed of two heavy (H) and light (L) chains, each with two functionally distinct domains. The highly diverse variable (V) domains bind to antigens, whereas effector functions are mediated by the constant (C) regions. Individual Ig molecules are remarkably specialized with highly restricted antigen-binding capacities. To attain the diversity of antigen binding required by the immune system, V genes are assembled from multiple gene segments, thereby parlaying a limited complement of genetic material into a vast potential for antigen binding through the process of V(D)J joining (reviewed in Hesslein and Schatz 2001). During the primary immune response, infectious agents stimulate and cause clonal expansion of a few B cells bearing Ig with low-affinity antigen. To produce effective immunity, Ig must have high-affinity antigen-binding specificity and be located in the appropriate tissues. Ig undergoes two additional genetic alterations, class switch recombination (CSR) and somatic hypermutation (SHM), which serve to refine and increase the specificity of the humoral immune response.

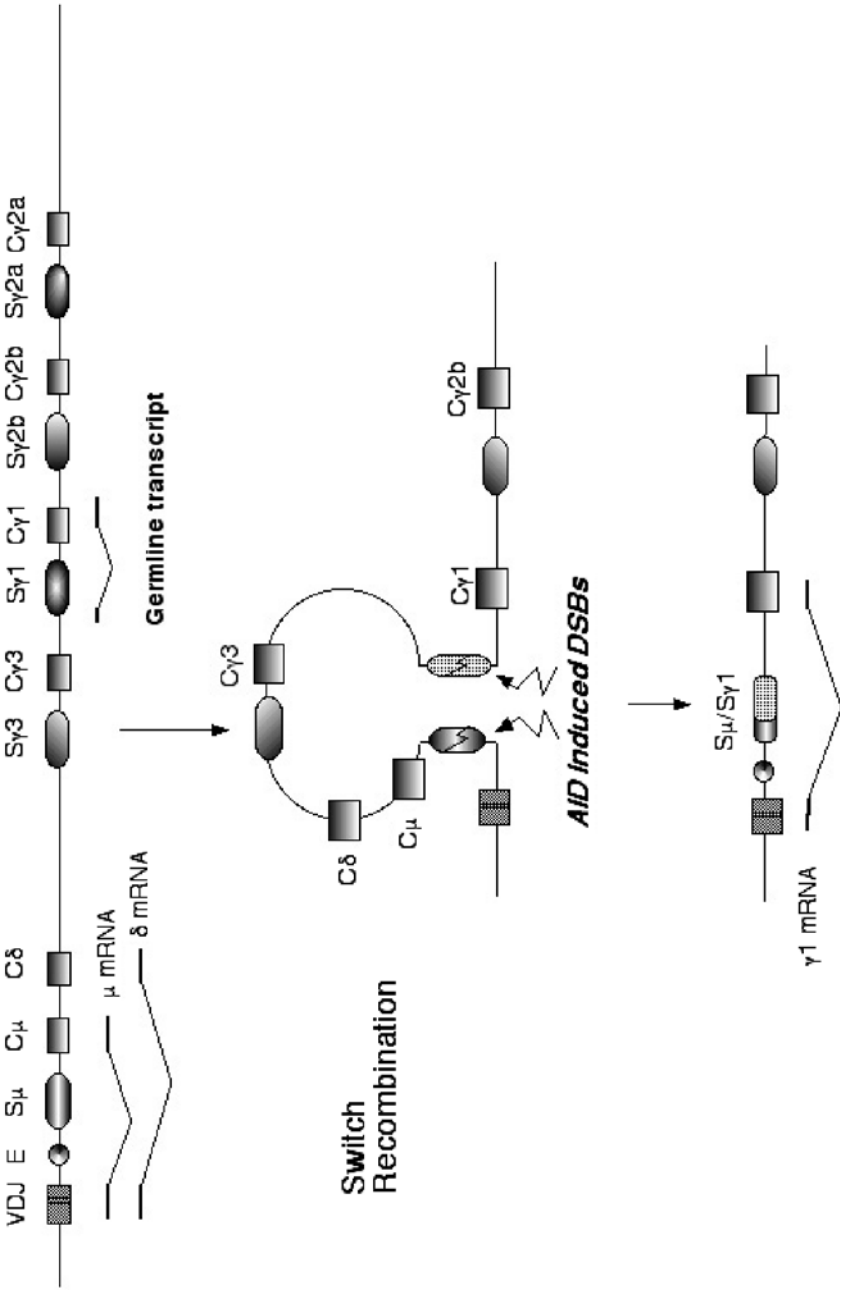
Successful V(D)J joining leads to assembly of H and L chain V regions and the expression of low-affinity Ig. These antibodies form the repertoire of the primary immune response and can bind antigen but cannot carry out all effector functions efficiently. Upon antigenic challenge, activated B cells undergo CSR to produce new effector functions and migrate to germinal centers, where their V regions become substrate for mutational events. SHM results in affinity maturation of the Ig repertoire, which increases the effectiveness of the humoral immune response. In chickens and rabbits, V regions are diversified by gene conversion that permits templated acquisition of sequences copied from parts of upstream pseudogenes. Although the molecular machineries which carryout CSR, SHM and gene conversion are quite different, excit-

ing new work based on the discovery of activation-induced deaminase (AID) indicates an unexpected link among these three processes. CSR has been the most enigmatic of the DNA rearrangement processes found in lymphocytes. In this review, I will focus on CSR and will present information that has led to a coherent model for the initiation of CSR, SHM and gene conversion and to important new mechanistic insights regarding CSR. I will concentrate mainly on the most recent findings, as CSR has been reviewed extensively (Honjo et al. 2002; Kenter 2003; Manis et al. 2002b; Stavnezer 2000).

2 Overview of CSR

CSR is used to attain diversity of Ig effector function and tissue localization. There are five classes of C_H region genes conserved in human, rat and mouse—IgM, IgD, IgG, IgE, and IgA—each having distinct effector activities. The murine IgH constant region locus is organized: 5′-V(D)J-Cμ-Cδ-Cγ3-Cγ1-Cγ2b-Cγ2a-Cϵ-Cα-3′. Isotype class switching involves an intrachromosomal deletional rearrangement that focuses on regions of repetitive switch (S) DNA located upstream of each C_H gene (with the exception of Cδ) (Fig. 1). S regions are comprised of 20- to 80-bp tandem repeats that are reiterated over 1–10 kb (Gritzmacher 1989). In CSR, composite Sμ-Sx junctions are formed on the chromosome, while the intervening genomic material is looped out and excised as a circle. The recombination breakpoints are found to fall largely but not exclusively within the S region tandem repeats (Dunnick et al. 1993; Lee et al. 1998). CSR does not conform to either of the two major classes of recombination, site specific and homologous recombination. No concensus sequence has been identified at the junction of recombined S/S DNA fragments, as would be expected in site specific recombination events. S regions are nonidentical to each other, making homologous recombination, with its requirement of DNA identity over extensive tracts of DNA, impossible. Thus, CSR is a unique region-specific form of recombination.

Work using mitogen and cytokines in activated primary B cell cultures indicates that CSR is targeted to specific S regions by the production of germline or sterile RNA transcripts (gts) (reviewed in Manis et al. 2002b; Snapper et al. 1997; Stavnezer 2000). Although the mechanism of CSR has not been fully elucidated, it is clear that this process requires the expression of activation-induced deaminase (AID) (Muramatsu et al.


VDJ E Sμ Cμ Cδ Sγ3 Cγ3 Sγ1 Cγ1 Sγ2b Cγ2b Sγ2a Cγ2a
μ mRNA
δ mRNA
Germline transcript
Switch
Recombination
Cγ3
Cδ
Cμ
Cγ1
Cγ2b
AID Induced DSBs
Sμ/Sγ1
γ1 mRNA

1999, 2000; Revy et al. 2000) in addition to gts through participating S regions. Mutations in the AID gene lead to deficits in CSR, SHM and gene conversion of IgV genes suggesting a mechanistic link among these three apparently unrelated processes (Arakawa et al. 2002; Buerstedde et al. 2002; Harris et al. 2002; Muramatsu et al. 2000; Revy et al. 2000).

3
A Model for the Initiation and Resolution Steps in CSR

3.1
AID Functions as a DNA Cytidine Deaminase to Initiate CSR, SHM and Gene Conversion

AID belongs to a family of proteins that includes APOBEC1, the apolipoprotein B mRNA editing catalytic subunit (Muramatsu et al. 1999). APOBEC-1 deaminates a cytosine in apoB mRNA to uracil to create a nonsense codon (reviewed in Smith and Sowden 1996). AID and Apobec proteins are also related to cytidine deaminase, an enzyme capable of deaminating cytidine monomers. Based on its homology to APOBEC-1, it was proposed that AID has an RNA editing function that led to the alteration of an encoded protein(s) involved with CSR, SHM and gene conversion (Muramatsu et al. 2000; Revy et al. 2000). Alternatively, AID might directly deaminate the relevant DNA, based on its homology with cytidine deaminase. The possibility that AID might act directly on DNA to deaminate dC residues was also suggested by a number of studies focused on the quality of mutations arising during SHM. In SHM, transition mutations occur more frequently in vivo than is predicted if muta-

Fig. 1 The looping-out and deletion model of CSR. A partial schematic map of IgH locus before CSR is shown at the *top*. The IgV gene is assembled early in B cell development by V(D)J joining. A productive V(D)J rearrangement is shown. IgM and IgD are transcribed from the same V_H promoter and are expressed by alternative RNA processing. Stimulation of B cells with antigen or mitogen induces germline transcription through the Sγ1-Cγ1 region prior to recombination. The gt must be expressed in *cis* to target switching. Intact Sμ and Sγ1 are separated by at least 80 kb. In the middle schematic, intrachromosomal deletion of genetic material between aligned Sμ and Sγ1 is shown. The *arrows* indicate AID-catalyzed DSBs, which are intermediates in the CSR reaction. In the *bottom diagram*, the IgH locus following $\mu \rightarrow \gamma$1 switching is shown

tions occur randomly (Golding et al. 1987). Higher mutation frequency of G/C base pairs located in hotspot motifs is also observed (Rogozin and Kolchanov 1992). Ectopic expression of AID in fibroblasts or hybridoma cells induced mutations at dC/dG bps, and most of these mutations were transitions (Martin and Scharff 2002; Yoshikawa et al. 2002).

Neuberger and coworkers sought to test the proposition that AID functions directly on DNA as a cytidine deaminase. They reasoned that if AID directly deaminates dC then expression of AID in *Escherichia coli* would produce C→T and G→A transition mutations. Ectopic expression of AID in *E. coli* led to increased mutation in several target genes and a shift towards transitions (Petersen-Mahrt et al. 2002). However, the incidence of AID-induced mutation in *E. coli* was very low and these studies did not address whether the cytidine deaminase activity was unique to AID. Subsequent studies demonstrated that cytidine deamination of DNA is associated with a number of APOBEC protein family members, thus not unique to AID (Harris et al. 2002). Nonetheless, only AID and not APOBEC-1 is capable of supporting SHM, suggesting that cytosine deamination of DNA alone is insufficient to facilitate this process (Eto et al. 2003).

The base excision repair (BER) pathway is the primary means by which cells remove uracil to avoid mutations arising from cytosine deamination (Lindahl 2000). In BER uracil is removed by the *E. coli* uracil-DNA glycosylase (UNG). This is followed by cleavage of the sugar–phosphate chain by apurinic apyrimidinic endonuclease (APE), excision of the abasic residue and local DNA synthesis and ligation. Perturbation of the BER pathway, by mutation of UNG, would force the rectification of G·U mismatches through DNA replication or mismatch repair (MMR). It was observed that in *E. coli*, AID-triggered mutations are synergistically enhanced by UNG deficiency, supporting the notion that deaminated dC residues in DNA are generated by AID (Petersen-Mahrt et al. 2002).

A key prediction of the deamination model is that UNG deficiency will lead to DNA replication over the dU lesion, resulting in transitions. In mouse, there are at least four uracil excision enzymes and any or all of them might be involved in CSR or SHM (Millar et al. 2002; Neddermann et al. 1996; Nilsen et al., 2000, 2001). Evidence suggests that the methyl-CpG binding domain 4 (Mbd4) glycosylase is not involved in either CSR or SHM (Bardwell et al. 2003). Analysis of CSR in UNG-deficient LPS-stimulated B cells showed that CSR was substantially, although not completely inhibited, implicating the BER pathway in the CSR reaction (Rada et al. 2002). Residual CSR in the mouse could be due to use of other dU glycosylases or engagement of the mismatch repair (MMR)

pathway. "Furthermore, the mutation spectra for SHM was also shifted in the UNG-deficient mouse, demonstrating that this enzyme is the major mouse DNA glycosylase involved in processing dU residues in V genes (Rada et al. 2002)". A similar pattern of deficit is found as a result of UNG deficiency in human. Recessive mutations of the human UNG gene lead to a form of hyper-IgM syndrome. Patients homozygous for these mutations express a profound impairment in CSR, with a partial disturbance of the SHM mutation pattern (Imai et al. 2003b). These studies demonstrate that UNG is the major glycosylase involved in processing uracil residues arising during CSR and SHM.

The mutagenic potential of uracil is very high since it can be efficiently replicated like normal thymine to yield C→T transition mutations (Lindahl 2000). Given that UNG is the primary uracil glycosylase involved in CSR and SHM, it might be predicted that UNG deficiency will lead to mutation accumulation and oncogenesis, particularly associated with B lymphocytes. Indeed, $UNG^{-/-}$ mice experience pathological changes in lymphoid organs, including lymphoproliferation and significantly increased incidence of B cell lymphomas (Nilsen et al. 2000, 2003). Together, findings in *E. coli*, chickens, mice and humans provide evidence for a physiological role of UNG downstream of AID. These studies support the DNA deamination model in which AID performs targeted deamination of cytosine within the S and V regions, thereby triggering CSR and SHM, respectively.

3.2
GL Sμ Mutations and Intra-S Region Deletions Represent Abortive CSR Events

The AID pathway for CSR could lead to the induction of mutations in S regions as well as to DSBs. Replication over G·U mismatches will produce transition mutations where dU could serve as a template for incorporation of an opposing dA residue (Petersen-Mahrt et al. 2002). Alternatively, when DNA polymerase encounters an abasic site, it will pause, and a translesion error-prone polymerase will replace it and insert any nucleotide opposite the abasic site and in the adjacent regions (reviewed in Friedberg et al. 2000). Finally, multiple DNA breaks could lead to intra-S region rearrangements and deletions without resulting in CSR.

Three laboratories have examined the germline (GL) Sμ to determine whether mutations accumulate prior to and during CSR. They found a high incidence of mutations in the regions flanking Sμ in wild-type but not in AID-deficient B cells, activated to switch, suggesting that the generation of mutations is integral to the process of CSR (Dudley et al.

2002; Nagaoka et al. 2002; Petersen et al. 2001; Reina-San-Martin et al. 2003). Mutations were not detected in resting B cells indicating that B cells must be induced for CSR for mutations to accumulate. Furthermore, mutations are specific to Sμ DNA since they were not found in the adjacent regions. Experiments showed that the mutations are predominantly single base changes in sequence motifs corresponding to SHM hotspots, RGYW and its complement WRCY (Lebecque and Gearhart 1990; Rogozin and Kolchanov 1992). Comparison of GL Sμ mutations arising in B cells stimulated with LPS+IL4 or LPS+anti-IgD crosslinked to dextran beads ($\alpha\delta$dex) shows focusing in hotspots and non-hotspots, respectively, suggesting that there is more than one mode of DNA lesion repair (Schrader et al. 2003a). These findings demonstrate that DNA lesions in Sμ DNA are detectable prior to CSR and imply that CSR might involve multiple lesions in Sμ, with successful CSR requiring similar lesions in the participating downstream S regions.

Intra-S region rearrangements of Sμ and downstream S regions have been observed coincident with activation of CSR (Alt et al. 1982; Bottaro et al. 1998; Gu et al. 1993; Hummel et al. 1987; Winter et al. 1987). The observation that internal Sμ region deletions and introduction of GL Sμ mutations are both dependent on AID expression demonstrates that these events are initiated through the same mechanism (Dudley et al. 2002) and most likely represent abortive CSR events.

3.3 S Region-Specific DSBs Are Recombination Intermediates in CSR

Production of excision circles as a direct by-product of CSR suggests that looping-out and deletion is a concerted mechanism in which DNA is broken and re-joined to form S/S recombinant junctions. Staggered DSBs could emerge following deamination of closely spaced dC residues located on opposing strands in the S region. Processing of dU residues through either the BER or mismatch repair (MMR) pathways would produce nicks or small gaps in the DNA, respectively (Rada et al. 2002; reviewed in Kenter 2003). Blunt DSBs could then be generated by processing the staggered ends with exonuclease and a B cell associated $3'\rightarrow5'$ exonuclease has been detected (Kenter and Tredup 1991). Blunt and staggered DSBs have been detected during mouse and human CSR (Catalan et al. 2003; Chen et al. 2001; Imai et al. 2003a; Wuerffel et al. 1997). In a crucially important finding, Sμ associated blunt DSBs found in activated human B cells are AID- and UNG-dependent as predicted by the AID deamination model (Catalan et al. 2003; Imai et al. 2003a,

2003b), directly demonstrating that blunt DSBs in S regions are intermediates in CSR. DSBs found in the downstream S regions have not yet been examined for AID or UNG dependency.

One of the first cellular responses to DSB formation is phosphorylation of histone H2AX, one of three H2A subfamily members involved in packaging DNA into nucleosomes (Rogakou et al. 1998). Phosphorylated H2AX, referred to as γ-H2AX, forms foci at the site of DSBs together with other DNA repair proteins (Celeste et al.,2003; Paull et al. 2000). CSR is significantly but not completely reduced in B cells isolated from H2AX-deficient mice, indicating that H2AX is an important facilitator of efficient CSR (Celeste et al. 2002; Petersen et al. 2001). In B cells activated to switch, γ-H2AX co-localized with the IgH locus in nuclear repair foci (Petersen et al. 2001) and co-localization was dependent on AID, indirectly confirming the presence of DSBs in the IgH locus.

These observations provide persuasive evidence that AID initiates CSR by creating DNA lesions in S regions that are processed into DSBs using elements of the BER and possibly the MMR pathways. The profound loss of CSR activity in UNG-deficient patients suggests that BER is the major pathway for DSB creation at Sμ in the human. The requirement for γ-H2AX, a mediator of DSB repair, in CSR provides additional genetic evidence that DSBs function as the recombination intermediates in CSR.

The involvement of DSBs as the initiating step in SHM has been extensively investigated (reviewed in Chua et al. 2002; Kenter 2003). DSBs in V region genes are not initiated by AID (Bross et al. 2000; Papavasiliou and Schatz 2000, 2002 Bross 2002), making it unlikely that these DSBs are intermediates in SHM.

3.4 CSR Is Resolved Through NHEJ

The structure of DSBs in DNA can be quite diverse. The detection of blunt DSBs in S regions and the inference of staggered ends in some instances of CSR illustrate this point (Catalan et al. 2003; Chen et al. 2001; Imai et al. 2003b; Wuerffel et al. 1997). Consequently the resection of broken DNA ends cannot simply be achieved through a simple ligation step. DSB repair can be mediated either by homologous recombination (HR) or by nonhomologous end-joining (NHEJ). Studies indicate that HR and NHEJ are the predominate pathways for DSB repair in yeast and in mammalian cells, respectively (reviewed in Lieber et al. 2003). The absence of extensive homology between the donor and acceptor S regions

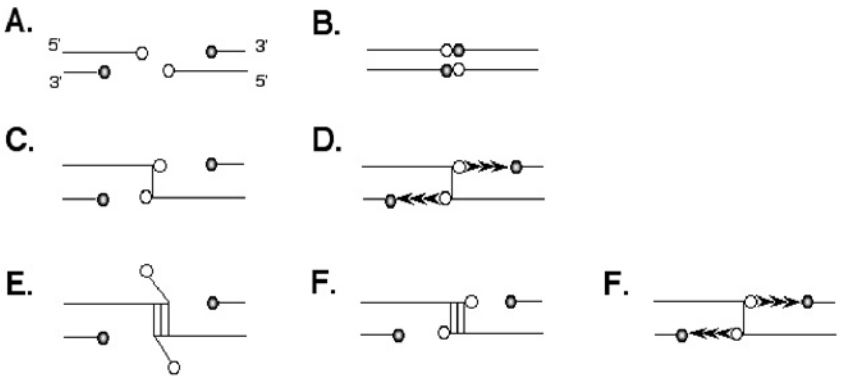


Fig. 2A–F Resolution of DSB intermediates by NHEJ and Msh2 during CSR. **A** Staggered DSBs derived from closely spaced deaminated dC residues located on opposing strands of S DNA. **B** Blunt-ended DSBs may be used directly in a ligation reaction. **C**, **D** Following a microhomology search, donor and acceptor ends are annealed either precisely at the DNA termini (**C**) or at an internal location (**E**). **D** When annealing occurs directly at the termini, gaps are filled in by an error-prone DNA synthesis event, as indicated by the *arrowheads*, followed by ligation. **E** When microhomologies are more internal, heteroduplex ends must be removed. **F** Flap removal is speculated to be an Msh2 dependent event. **F** The gaps at the processed ends are filled in by error-prone repair (as in **C**)

suggested that NHEJ would be the most likely modality of DSB repair in CSR. Examination of the switch junctions in recombinant S/S regions indicates features classically associated with NHEJ (Dunnick et al. 1993; reviewed in Kenter 1999). These characteristics include direct joining of blunt ends in about 50% of recombination events or microhomology of one to eight nucleotides in the balance of the events. The tendency for microhomology usage is seen in both general DNA end-joining as well as specialized physiological forms of DSB repair such as V(D)J joining (reviewed in Lieber et al. 2003). When DNA ends are aligned using short stretches of microhomology, there will often be either excess DNA beyond the point of alignment that must be removed by nucleases or there will be gaps that must be filled using polymerases (Fig. 2). When error prone polymerases are used to fill in the gaps, mutations will be introduced in DNA flanking the breakpoints. Mutations flanking the junctions are frequently observed (for example, see Dunnick et al. 1993; Schrader et al. 2003a and references therein).

Screening of X-ray-sensitive rodent cell lines led to the identification of genetic complementation groups with defects in both (NHEJ) DSB re-

pair and V(D)J joining (reviewed in Chu 1997; Smith and Jackson 1999). DNA-dependent protein kinase (DNA-PK) was found absent in several X-ray-sensitive and V(D)J-deficient cell lines and is capable of binding to DNA ends. Complementation studies demonstrated that DSB repair and V(D)J joining could be restored by expression of the DNA-PK subunits in deficient cells. DNA-PK is a complex composed of the Ku80 and Ku70 proteins and the DNA-PK catalytic subunit (DNA-PK_{cs}) (Chu 1997). The severe combined immunodeficiency disease (scid) mutation characterized by the absence of B and T lymphocytes is located in the DNA-PKcs unit (Chu 1997). Thus, the NHEJ form of DSB repair has both general and specialized functions in lymphocytes.

Direct examination of the constituents of the DNA-PK complex for their affect on CSR was complicated by the absence of B and T lymphocytes as a result of faulty V(D)J joining. To circumvent this difficulty, two groups complemented Ku80 (Casellas et al. 1998; Reina-San-Martin et al. 2003), Ku 70 (Manis et al. 1998a) and the DNA-PKcs-null mutation (Manis et al. 2002a) deficient cells with rearranged IgH and IgL genes introduced by targeted homologous recombination and found a marked dependency of CSR for these genes. Interestingly, the DNA-PK_{cs} null mutation led to loss of CSR to all isotypes except IgG1, suggesting the $\mu \rightarrow \gamma 1$ recombination is a special case (Manis et al. 2002a). In contrast, the scid mutation, which produces a protein truncation and loss of kinase function was permissive for CSR to all isotypes (Bosma et al. 2002), suggesting that DNA-PK_{cs} kinase activity is dispensable for CSR. Proliferation is impaired in Ku-deficient B cells, indicating that the CSR deficit could be indirect (Casellas et al. 1998; Manis et al. 1998a). However, B cells expressing the DNA-PK_{cs} null mutation proliferate normally, demonstrating that CSR is directly affected by this deficit. Furthermore, CSR was absent in $Ku80^{-/-}$ B cells that had been sorted for equivalent levels of proliferation, as in wild-type cells that had successfully switched (Reina-San-Martin et al. 2003). The dependency of CSR on DNA-PK indicates that DSBs in S regions are resolved through NHEJ.

In light of these observations, it is intriguing to consider the parallels and distinctions among the three DNA rearrangement processes in lymphocytes. CSR, like V(D)J joining, involves deletion of intervening DNA sequences and expulsion of the excised DNA as a circle. Furthermore, DNA DSB intermediates are part of both the CSR and V(D)J joining reactions, and both employ NHEJ processes to resolve the breaks. These reactions are distinct in that CSR occurs later in B cell development, is region-specific rather than site-specific and is independent of Rag1 and Rag2. CSR and SHM require AID, whereas V(D)J joining is independent

of AID. However, SHM, unlike CSR, is not dependent on DNA-PK, indicating that the NHEJ pathway does not contribute to this process.

3.5 Switch Junctions and DSB End Processing

Switch junctions are frequently flanked by mutations, insertions, and deletions, which are reminiscent of Ig V region SHM (Dunnick et al. 1993). Analysis of switch junctions derived from the I.29μ cell line, capable of inducible CSR, indicated that mutations were introduced during the CSR reaction (Dunnick and Stavnezer 1990; Dunnick et al. 1989). Several other groups noted mutations flanking both sides of the switch recombination breakpoints (Du et al. 1997; Nagaoka et al. 2002; Schrader et al. 2002, 2003b). The detection of mutations in GL Sμ and in switch junctions raises questions regarding the relationship and origins of these DNA alterations.

The mutations in S regions are most likely introduced by two mechanistically independent means. Mutations detected in GL Sμ arise during rectification of G·U mismatches from AID-catalyzed cytosine deamination through either the BER or MMR pathways (Rada et al. 2002). The source of mutations in switch junctions is more complex. CSR proceeds through DSB intermediates arising in Sμ and a downstream S region that must then undergo synapsis and resolution. Exonucleases can create blunt ends from staggered intermediates and provide an opportunity for direct end-to-end ligation (Fig 2A, B). In some cases, short stretches of DNA identity occur precisely at the ends of the staggered breaks, leaving internal gaps (Fig 2C). In other cases, synapsis is stabilized using microhomology at more internal locations and yielding heteroduplex DNA at the ends that must be removed. The heteroduplex flaps can be removed using a "flapase" and gaps can be filled in using a translesion error-prone polymerase (Fig 2E, F). This last method will produce mutations in proximity to the switch junction but not in other regions of the hybrid S/S regions.

Comparison of the frequency, distribution and nucleotide specificity of mutations in GL Sμ and in recombinant Sμ/Sγ3 junctions revealed striking differences (Schrader et al. 2003a). Mutations in recombined Sμ and Sγ3 regions focused to RGYW hotspots, whereas mutations in GL Sμ did not (Schrader et al. 2003a), suggesting that the lesions leading to these mutations are processed differently. Furthermore, the distribution of mutations in GL Sμ extends throughout the flanking region, whereas mutations associated with recombinant Sμ/Sγ3 junctions are located

within 150 bps of the breakpoint. This finding is consistent with the possibility that error prone polymerases fill in gaps and introduce DNA synthesis-dependent mutations.

Evidence indicates that in addition to its role in mismatch repair, the MMR heterodimers are involved in end processing of DSB intermediates during NHEJ. In Msh2, Mlh1- or Pms2-deficient mice, the frequency of CSR is diminished and structural alterations are observed in switch junctions (Ehrenstein and Neuberger 1999; Ehrenstein et al. 2001; Min et al. 2003; Schrader et al. 1999, 2002, 2003b). Specifically, knockout of different MMR genes leads to either extension or diminution of microhomology lengths and in some cases an increase of insertions at the recombination breakpoints. These observations indicate that MMR proteins have a role in the mechanics of CSR (Ehrenstein and Neuberger 1999; Ehrenstein et al. 2001; Martin et al. 2003; Min et al. 2003; Schrader et al. 1999, 2002; , 2003b).

The importance of S region tandem repeats (TRs) and Msh2 are highlighted when considered in the context of the $S\mu^{-/-}$ (Luby et al. 2001; Min et al. 2003). Deletion of the $S\mu$ TRs reduces CSR frequencies to levels comparable to MMR deficiencies (Luby et al. 2001). Analysis of the compound knockout, $S\mu^{-/-}$x$Msh2^{-/-}$ showed a profound reduction of CSR, indicating that in the absence of Msh2 the DSBs formed outside the SμTRs can not be readily processed (Min et al. 2003). RGYW hotspots may be preferential targets for cytosine deamination by AID, and the GAGCT motif, an RGYW variant, is present at high frequency in the SμTRs. Selsing and coworkers speculate that in the presence of GAGCT motifs AID will deaminate dC residues on opposite strands located in close proximity and produce staggered breaks with short 5′ or 3′ extensions (Fig. 2). In contrast, in the absence of the TRs, where the RGYW motifs are less dense, staggered DSBs will be formed with longer extensions, which are not easily processed by the NHEJ pathway. It is proposed that Msh2 may be able to recruit nucleases capable of processing long flaps (Fig. 2E). Precedence for MMR participation in DSB end processing comes from yeast where Msh2 is involved in removing heteroduplex flaps extending from zones of dsDNA and recruiting Rad1/XF and Rad10/ERCC1 to excise heterologous 3′ single-stranded tails (Paques and Haber 1997; Sugawara et al. 1997; reviewed in Kenter 1999).

3.6 Asymmetry in the CSR Transaction

Based on the forgoing observations it has been supposed that there is parity in the processing of DNA lesions in the Sμ (donor) and downstream (acceptor) S regions. Two new studies indicate asymmetry between donor and acceptor S regions during the CSR transaction. First, the frequency of mutations in GL Sμ was significantly higher than that found for GL Sγ3 and GL Sγ1. However, when another group sorted for B cells that had undergone five cell divisions, the mutation bias in the GL S regions was not evident (Reina-San-Martin et al. 2003). Cell division has been correlated with CSR (Deenick et al. 1999; Hodgkin et al. 1996; Tangye et al. 2002). It is possible that B cells which are most rapidly dividing are also the most likely to actively transcribe gts from downstream S regions, thus making these S regions substrate for AID-induced mutation. On average, however, the downstream S region does appear to acquire fewer DNA lesions. The bias in GL mutations represents an asymmetric hit rate for cytosine deamination in S regions and may function as the rate-limiting step in the CSR reaction.

Phosphorylated-H2AX was previously shown to be an important mediator of efficient CSR (Celeste et al. 2002; Petersen et al. 2001). It has now been shown that H2AX-deficient B cells activated to undergo CSR are fully capable of introducing mutations into GL Sμ and are proficient in producing internal Sμ deletions; however, these cells did not support intra-Sγ1 deletions (Reina-San-Martin et al. 2003). These studies demonstrate the involvement of γ-H2AX in the repair of DSBs in downstream S regions and suggest that γ-H2AX, specifically recruits downstream S region DSBs to the nuclear repair foci. Intra-Sμ deletions, by contrast, occur in the absence of H2AX, implying that another DNA repair factor specifically senses these breaks and escorts them to repair foci. The asymmetric recognition of the S region breaks may ensure that Sμ and the downstream S region breaks are located in the same repairosome, thus increasing the probability the successful recombination will ensue.

4 CSR and SHM Uncoupled

CSR and SHM are similar in that both require cytidine deamination to induce mutation. CSR has an additional requirement of synapsis between participating S regions to successfully complete recombination.

AID is the only B cell-derived factor required to induce CSR on integrated switch substrates (Okazaki et al. 2002), implying that it must itself be able to direct S region synapsis. Naturally occurring recessive mutations in the C-terminal domain of the human AID gene lead to loss of CSR but retention of SHM and *E. coli* mutagenesis(Ta et al. 2003; Zhu et al. 2003). Similarly, a structural variant of AID in which the C-terminal ten amino acids were deleted retained SHM and gene conversion function in $AID^{-/-}$ DT40 cells as well as the ability to generate mutations in UNG-deficient *E. coli* (Barreto et al. 2003). In $AID^{-/-}$ mouse B cells, the truncated AID protein induced mutations in GL Sμ with a mutation spectrum, distribution and hotspot preference similar to that of wild-type AID. In striking contrast, the deleted AID protein lost the capability to carry out CSR. These studies uncouple the cytosine deaminase and recombination functions in the AID protein. The deleted portion of the AID protein may be directly involved in promoting synapsis of the donor and acceptor S regions and/or involved in recruiting general DSB repair factors to S regions.

Evidence that additional factors are involved in successful CSR derived from a newly described group of human patients with hyper-IgM syndrome type IV (HIGM4) (Imai et al. 2003a). The HIGM4 patients carry normal AID and UNG genes and express normal levels of Sμ breaks but are unable to complete CSR (Imai et al. 2003a). It is likely that the C-terminus of AID recruits co-factors that function downstream of AID and UNG, such as γ-H2AX, to the appropriate S regions, thus stimulating synapsis. The requirement of de novo protein synthesis even in the presence of AID protein in CSR may stem from the need for general DSB repair proteins(Doi et al. 2003).

5 Genome Instability Syndromes and CSR

In the genome DSBs continuously arise from ionizing radiation, genotoxic stress, errors in replication and spontaneous chemical changes in DNA constituents. Ataxia-telangiectasia-mutated (ATM) protein, a nuclear PI3 family kinase, is the primary activator of the cellular response to DNA DSBs and a controller of the cell cycle (reviewed in Shiloh 2003). ATM activation, via autophosphorylation, is dependent on intact functional Mre11/Rad50/Nbs1 (M/R/N) complex (Uziel et al. 2003). The M/R/N complex contains DNA binding and nuclease activities and is involved in DSB end processing. Ataxia-telangiectasia (A-T) is a rare mul-

ti-system disorder characterized by cerebellar degeneration with ataxia, ocular and cutaneous telangiectasias, radiosensitivity, chromosomal instability, cancer predisposition and is caused by mutation of a single gene, ATM (Savitsky et al. 1995). A-T has been recognized as a primary immunodeficiency that encompasses both cellular and humoral immunity with recurrent infections (reviewed in Becker-Catania and Gatti 2001; Crawford 1998). IgA deficiency has been detected in up to 80% of patients and a subgroup of these expresses IgG deficiency, suggesting a defect in isotype switching as the underlying cause of the recurrent infections. Examination of the frequency of S/S recombinant junctions in B cells from A-T patients suggested that CSR was reduced as compared to controls (Pan et al. 2002). However, the PCR assay used in this study was not quantitative. Rare Sμ/Sα switch junctions amplified from AT patients are aberrant and are characterized by unusually long microhomologies and a severe reduction in mutations surrounding the breakpoints, indicating a defect in the repair mechanics of CSR (Pan et al. 2002; Pan-Hammarstrom et al. 2003). In contrast, SHM does not appear to be affected in A-T individuals (Pan-Hammarstrom et al. 2003).

A-T-like disease (A-TLD), a late onset variant of A-T, is caused by mutations in the MRE11 gene (Stewart et al. 1999). Hypomorphic mutations in the NBS1 locus lead to Nijmenegen breakage syndrome (NBS), which is a complex disorder expressing a combination of microencephaly, mental deficiency, immunodeficiency, radiation sensitivity, chromosomal instability, and cancer predisposition (Digweed et al. 1999; Tauchi et al. 2002). The NBS1 locus encodes the Nbs1 protein, a constituent of the M/R/N complex. Mutations in the RAD50 gene produce a variant of NBS (Shiloh 2003). The parallels between the pattern of immunodeficiency, radiation sensitivity, chromosomal instability, and cancer predisposition in NBS and A-T are striking (Wegner et al. 1999). Furthermore, the cellular phenotype of AT, A-TLD and NBS is also quite similar.

The similarity of the immunodeficiency phenotype in A-T and NBS patients stimulated examination of Sμ/Sα junctions derived from NBS patients (Pan et al. 2002). These studies showed usage of longer than average microhomologies, whereas the number of mutations in the recombinant switch junctions was comparable to controls. Thus, the NBS-derived Sμ/Sα junctions were similar to A-T junctions with regard to microhomology usage but different in the level of mutations found, suggesting that these proteins may have overlapping but nonidentical roles in CSR-specific DSB repair. It is important in this regard that ATM has significant DNA end-binding activity (Andegeko et al. 2001).

H2AX is phosphorylated by the PI3 kinases ATM, ataxia-telangiectasia-related (ATR) and DNA-PK in response to DNA DSB formation (Brown and Baltimore 2003; Burma et al. 2001; Furuta et al. 2003; Paull et al. 2000; Ward and Chen 2001) and Nbs1 is another substrate of ATM (reviewed in Shiloh 2003). This raises the possibility that the altered switch junctions in A-T patients arise from the inability of the B cells to generate γ-H2AX. Furthermore, in B cells activated to switch, co-localization of γ-H2AX with Nbs1 in nuclear repair foci was observed (Kobayashi et al. 2002; Petersen et al. 2001), implying that in the absence of NBS and therefore the absence of a functional M/R/N complex, γ-H2AX can not sense DSBs properly. Although H2AX deficiency had no effect on the quality of Sμ/Sγ3 junctions, its absence led to a significant attenuation of switching efficiency (Reina-San-Martin et al. 2003). This finding indicates that H2AX most probably does not function in DNA end processing. The findings that ATM, NBS1 and H2AX, genes which are involved in the general repair of DSBs, are also involved in CSR highlight how evolution has co-opted general DNA repair mechanisms for specialized usage in CSR.

6 Targeting Mechanisms in CSR

AID is a promiscuous mutator when ectopically expressed in *E. coli* (Petersen-Mahrt et al. 2002) and indiscriminately mutates other overexpressed genes, including itself in fibroblasts, a hybridoma cell line and in CHO cells (Martin and Scharff 2002; Yoshikawa et al. 2002). However, AID shows some targeting specificity since it mutates transgenic AID and V genes and endogenous V genes but not endogenous bcl6 or c-myc (Martin and Scharff 2002). These studies suggest that chromatin context may be a determinator for AID access to DNA targets. Promoter and enhancer elements collaborate to determine accessibility of genes to transcriptional machinery in order to regulate developmental states and establish tissue-specific gene expression patterns (for example, see Agalioti et al. 2000; Litt et al. 2001a, 2001b). Chromatin configuration has also been shown to determine the developmental regulation of Ig loci and V(D)J joining (reviewed in Krangel 2003; Mostoslavsky et al. 2003; Sleckman et al. 1996). It is therefore worthwhile to briefly review the location of the *cis*-acting elements that regulate CSR and SHM.

6.1 *Cis*-acting Elements Regulating CSR and SHM

Transcription through the S regions via the expression of gts plays a primary role in targeting CSR. Gts initiate from promoters located 5′ of the I exons, runs through the S region and terminates downstream of the C_H exons (reviewed in Manis et al. 2002b; Stavnezer 2000). Promoter integrity is required for efficient CSR as demonstrated by gene-targeting studies (Bottaro et al. 1994; Harriman et al. 1996; Jung et al. 1993; Seidl et al. 1998; Zhang et al. 1993). Efficient CSR requires elements in addition to the germline C_H promoters. Severely reduced CSR occurs when the intronic transcriptional enhancer (iEμ), located just 3′ of J_H, has been removed by gene targeting (Gu et al. 1993). Additional major control elements are located 3′ of the IgH locus and include four enhancers: HS3a, HS1,2, HS3b and HS4 (reviewed in Khamlichi et al. 2000). This region is conserved in humans and has locus control region (LCR) properties (Khamlichi et al. 2000; Madisen and Groudine 1994). Interestingly, HS3a and HS3b are nearly identical but their sequences are oriented in opposite directions. HS1,2 contain inverted repeats that are also oriented in opposite directions, whereas HS4 has a divergent sequence. The structure of these enhancers suggests that higher-order structures may collaborate to regulate gt transcription. Mutation analysis by insertion of pgk-neo^r cassettes into HS1,2 led to loss of gt expression for a series of C_H genes as well as CSR to those genes (Cogne et al. 1994). In contrast, a "clean" deletion of HS1,2 and HS3a by replacement with a loxP site had no effect on gt expression or CSR (Manis et al. 1998b). Additional insertion of drug-resistance cassettes into various positions in the IgH locus led to inhibition of gt expression from the upstream but not the downstream promoters (Qiu et al. 1999; Seidl et al. 1998). In contrast, insertion of the pgk-neo^r cassette just downstream of the HS4 has no obvious effect on CSR (Manis et al. 2003). Based on these findings, Alt and coworkers have suggested that the downstream regulatory regions, referred to as the IgH 3'RR, might operate by a promoter competition mechanism, although other mechanisms are also possible (Khamlichi et al. 2000).

Interestingly, SHM is also dependent on transcription of V genes and transcription determines the boundary for mutation. Mutations in V genes are found approximately 150 bps downstream of the transcription start site and extends 1.5–2 kb (Lebecque and Gearhart 1990; Rada et al. 1997). The mutation rate appears to be proportional to the rate of transcription (Bachl et al. 2001; Fukita et al. 1998). Furthermore, specific

transcriptional elements, including promoters and enhancers, are required for SHM (Betz et al. 1994; Peters and Storb 1996; Tumas-Brundage and Manser 1997).

6.2 Germline Transcripts May Have Two Functions in CSR

Cytokine-inducible promoters are located upstream of each S region and direct the production of RNA transcripts that terminate downstream of the C_H gene and are not translated into protein; hence the name germline or sterile transcripts (reviewed in Stavnezer 2000). Although CSR is critically dependent on the expression and appropriate splicing of gts, the function of these transcripts is unknown. It has been speculated that transcription of S regions changes the chromatin configuration in S DNA making them accessible to factors which mediate CSR (reviewed in Manis et al. 2002b; Stavnezer 2000).

Another possible function for gts is to directly alter the structure of S region DNA, thus, transforming them into substrate for AID. RNA–DNA hybrid structures have been shown to form along transcribed S regions in vitro (Daniels and Lieber 1995; Reaban and Griffin 1990; Reaban et al. 1994); however, their physiological importance and the precise base pairing between the RNA strand and the two DNA strands in the RNA–DNA hybrid remains unclear. Two possible structures to account for the RNA–DNA hybrids have been considered, an intermolecular triplex and an R-loop (Reaban et al. 1994; Tian and Alt 2000). In R-loops dsDNA is transformed into a RNA–DNA hybrid with the displaced nontranscribed strand of DNA in a single-stranded configuration. Lieber and coworkers, using sodium bisulfite as a structural probe for ssDNA demonstrated that R-loops are formed on the transcribed (C-rich) strand of plasmids and the endogenous Sγ3 and Sγ2b loci only when gts are produced (Yu et al. 2003). R-loop formation only occurred in stimulated but not in unstimulated B cells. However, the numbers of detected R-loop molecules was very low. Furthermore, transcription is also required for SHM, but R-loops do not form over the transcribed V regions. Nonetheless, this is an intriguing finding since AID has been shown to deaminate ssDNA but not dsDNA (Bransteitter et al. 2003; Chaudhuri et al. 2003; Dickerson et al. 2003; Pham et al. 2003; Ramiro et al. 2003) and dsS DNA can be deaminated by AID when the reaction is coupled to transcription (Chaudhuri et al. 2003; Pham et al. 2003; Ramiro et al. 2003). Notably, deamination occurs only when the dsDNA S region substrate is in the same transcriptional orientation as observed in the endogenous locus.

These findings suggest that gt expression provides for the generation of R-loops in S regions, thereby stabilizing ssDNA stretches and ensuring appropriate substrate for AID. Collectively, these findings show a direct functional link between transcription and substrate deamination by AID and provide a mechanistic explanation for the requirement of transcription in both CSR and SHM. The possibility that transcription also targets DNA for AID attack by imposing chromatin modifications is considered below. A study of V gene accessibility and SHM is cited since no studies on S regions are extant.

6.3 S Regions and V Genes Are Differentially Targeted During CSR and SHM

Mutations are targeted to the expressed V gene as opposed to the nearby germline V genes and S regions during SHM and reciprocally, to S DNA and not to V genes during CSR. One attractive explanation is that *cis*-regulatory elements, including promoters and enhancers, control the differential targeting of AID to V and S regions by regulating substrate accessibility. In support of this notion, studies demonstrate that expression of gts and V genes is required and proportional to the frequency of CSR and SHM, respectively (Bachl et al. 2001; Lee et al. 2001). However, both CSR and SHM should occur in cells that produce gts, transcribe the rearranged V gene and express AID. It is striking that LPS-activated splenic B cells transcribe gts and the rearranged V genes but undergo only CSR and not SHM (Manser 1987). Indeed, CSR and SHM are independently and differentially induced in the CL-01 cell line (Zan et al. 1999). Furthermore, I.29μ, a B cell line capable of CSR requires activation to switch despite constitutive expression of the appropriate gt and AID (Ma et al. 2002). These observations suggest that transcription of S regions or V genes is necessary but not sufficient to target AID to DNA substrates.

To probe this question further, Scharff and colleagues examined the relationship of transcription and histone acetylation, a measure of DNA accessibility, to SHM in the BL2 cell line (Woo et al. 2003). The BL2 cell line undergoes SHM following co-stimulation with anti-IgM and T helper cells (Faili et al. 2002b; Sale and Neuberger 1998), whereas Igμ transcription is unchanged by these stimuli (Woo et al. 2003). Co-stimulation led to V region restricted histone hyperacetylation, which occurred independent of AID and was correlated with SHM. Strikingly, treatment with Trichostatin A, a histone deacetylase inhibitor, or overexpression of AID led to extension of the region of histone hyperacetylation and mu-

tations into the C region (Woo et al. 2003). These findings demonstrate that histone hyperacetylation encompassing the expressed V gene promotes an AID-accessible region and suggests that B cells have developed a coherent strategy to exclude AID from genes by means of regulating chromatin states. Intriguingly, alteration of AID concentration can lead to histone hyperacetylation in V and C regions and constitutive mutation (Faili et al. 2002a; Woo et al. 2003), suggesting that AID itself is able to recruit chromatin modifiers to specific genes. This possibility is particularly provocative since it suggests new investigative directions for the study of AID targeting in B cells.

6.4 Isotype-Specific Targeting of S Regions in CSR

The absence of consensus recombination signal sequences in switch junctions has led to the suggestion that isotype targeting of CSR is achieved exclusively through production of gts and the expression of AID (reviewed in Manis et al. 2002b; Stavnezer 2000). This view may not encompass the full range of possible factors that contribute to CSR since other unidentified *trans*-acting factors may be important for CSR in addition to gts, AID and UNG. The Snapper laboratory devised a number of B cell activation protocols in which gts are induced for several isotypes but switching occurs only to a subset of them (Snapper et al. 1997). Furthermore, B cells lacking specific transcription factors maintain gt expression but switch only to restricted isotypes (Snapper et al. 1996; Wuerffel et al. 2001; Zelazowski et al. 1997). These findings do not support the minimalist notion that gt expression and AID are responsible for CSR because in this model all loci that express gts should switch.

To test the proposal that isotype-specific *trans*-acting factors contribute to CSR, Shanmugam and colleagues carried out transient transfection of isotype-specific switch substrates into a panel of mature B cell lines with well characterized switching phenotypes and into normal activated splenic B cells (Shanmugam et al. 2000). Evidence was found for individual switching activities that mediate $\mu \rightarrow \gamma 3$, $\mu \rightarrow \gamma 1$, $\mu \rightarrow \epsilon$ and $\mu \rightarrow \alpha$ CSR (Ma et al. 2002; Shanmugam et al. 2000). These studies also showed a strict correlation between the isotype specificity for endogenous and plasmid-based CSR providing a genetic basis for the interpretation of these observations. Switch plasmid recombination is dependent on the expression of AID, demonstrating the physiological relevance of the assay (Kenter et al. 2004). New studies indicate that a single tandem repeat is necessary and sufficient for plasmid switching, and a motif was

identified which conferred isotype specificity for $\mu \rightarrow \gamma 3$ recombination (Kenter et al. 2004). The motif is identical with an NF-κB p50-binding site and a p50-deficient mouse does not support either endogenous or substrate-based switching (Kenter et al. 2004). These findings strongly implicate S region sequence and *trans*-acting factors in the CSR reaction.

Given that AID directly deaminates dC residues in DNA, it is possible that the isotype-specific activities function as docking proteins that recruit AID to S regions. However, it is also plausible that these activities are suppressors of CSR that may preclude AID interactions with S regions and which are themselves repressible by combinations of B cell activators. Alternatively, the *trans*-acting activities may be involved in recruiting the recombination machinery to the broken S DNA ends to facilitate synapsis or to induce chromatin modifications at S regions. A number of DNA-binding proteins have been detected that are specific for S DNA, including NF-κB p50, which might be candidates for isotype specific factors (reviewed in Stavnezer 2000).

The Honjo laboratory reported that switch substrates assayed in stable transfection experiments could support $\mu \rightarrow \gamma 1$, $\mu \rightarrow \epsilon$ and $\mu \rightarrow \alpha$ switching in a subclone of the CH12 cell line (Kinoshita et al. 1998). They interpreted these results to mean that isotype specificity is not determined by the nucleotide sequence of the S region. Although the CH12 cell line switches predominantly $\mu \rightarrow \alpha$, it is also capable of occasional $\mu \rightarrow \gamma 3$ and $\mu \rightarrow \gamma 1$ switching, indicating that several switching activities can be expressed in these cells (Whitmore et al. 1991). Kinoshita and colleagues did not systematically test the specificity of their switch constructs in other switching cell lines with well-defined switching profiles. Therefore, the ability of several isotype-specific switch substrates to undergo recombination in CH12 cells may not be inconsistent with the conclusion that S DNA sequence contributes to isotype-specific targeting of CSR.

One prediction raised by the Shanmugam studies is that S region sequence is important to the recombination transaction. Deletion of the SμTRs led to a rather modest reduction of CSR (Luby et al. 2001). However, this mouse retained 13 copies of the GAGCT or GGGGT pentamers where the GAGCT is a potential hotspot for cytidine deamination by AID. The SμTRs are clearly required in the absence of Msh2, suggesting that the DNA ends are altered when DSBs occur outside the TRs (Min et al. 2003). These studies indicate that the structure of the Sμ TRs influence the structure of the DSBs, which function as CSR intermediates. Introduction of a random G-rich DNA sequence into the S$\gamma 1$ locus by gene

targeting led to a 93% reduction of $\mu \rightarrow \gamma 1$ switching (Shinkura et al. 2003). The residual recombination in this mouse could be due to motifs permissive for CSR in the inserted sequence. In addition, the inserted region probably remains a target for AID-induced mutations that are poorly consumed in the CSR. These studies indicate that the S region sequence is important to the CSR. It remains to be determined how isotype-specific factors interface with the switching reaction.

7 Concluding Remarks

The discovery of AID and the recognition that CSR, SHM, and gene conversion are mechanistically linked represents a paradigmatic shift in our understanding of these processes. The deamination model for the introduction of DNA lesions represents a powerful new insight that elegantly sets forth a role for AID. Direct biochemical determination that ssDNA is substrate for AID and the role of transcription in generating ssDNA regions in S regions provides a mechanistic explanation for the role of gt in switching. The recognition that DSBs are CSR intermediates, that the NHEJ pathway is involved in resolving breaks and the contribution of the MMR pathway in processing those ends provides the major outlines for the resolution phase of the CSR reaction.

It is clear that AID is a global mutator when ectopically expressed in a variety of cell types. Thus, very defined targeting mechanisms must exist to avoid unwanted mutations and consequent oncogenesis. Furthermore, the differential induction of CSR and SHM in B cells indicates that region-specific targeting of AID to S DNA or expressed V genes must occur. The next challenge is to define both the *cis*- and *trans*-regulatory elements governing the decision to switch or hypermutate. Definition of the targeting mechanisms that focus AID to appropriate templates has begun and includes regulation of chromatin status. Recognition of the importance of the S region sequence and isotype-specific *trans*-acting factors as components of isotype targeting is also emerging. Identification of the protein components of these activities, definition of their recognition motifs and characterization of their role in the recombination machinery will provide new perspectives on the specificity of CSR.

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Blimp-1; Immunoglobulin Secretion and the Switch to Plasma Cells

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Abstract The transcription factor Blimp-1 governs the generation of plasma cells and immunoglobulin secretion. Recent microarray experiments indicate that Blimp-1 regulates a large set of genes that constitute a significant part of the plasma cell expression signature. The variety of differentially expressed genes indicates that Blimp-1 affects numerous aspects of plasma cell maturation, ranging from migration, adhesion, and homeostasis, to antibody secretion. In addition, Blimp-1 regulates immunoglobulin secretion by affecting the nuclear processing of the mRNA transcript and by affecting protein trafficking by regulating genes that impact on the activity of the endoplasmic reticulum. Interestingly, the differentiation events that Blimp-1 regulates appear to be modulated depending on the activation state of the B cell. This modulation may be due at least in part to distinct regions of Blimp-1 that regulate unique sets of genes independently of each other. These data hint at the complexity of Blimp-1 and the genetic program that it initiates to produce a pool of plasma cells necessary for specific immunity.

1 Plasma Cell Biology

Immunoglobulins, or antibodies, are serum proteins produced by the B cell arm of the adaptive immune system and serve as a specific barrier to primary and secondary infections. B cells clonally produce antibodies capable of binding thousands, if not millions, of different entities. The process by which a B cell becomes an antibody-secreting plasma cell is regulated at multiple checkpoints throughout the lifetime of the differentiating B cell, in order to secure both specific immunity and to prevent the production of autoreactive B cells.

B cell differentiation is managed by a coordinated and reiterative system of signaling and transcriptional regulators that act in response to extracellular cues with the purpose of driving gene expression programs that incrementally promote B cells through successive steps of lymphoid homeostasis and immune response (Glimcher and Singh 1999). The homeostatic steps include the production of rearranged antibody genes, the quality control of the antibody molecule, the lifespan, and the migration of B cells throughout the lymphatics. The immune response steps include responding to T-independent or T-dependent antigen, clonal expansion, processing foreign antigen, interacting with T cells and dendritic cells, and modifying the antibody genes to increase antigen affinity and effector function, specialized processing of the Ig transcript, and finally generation of a highly efficient secretory apparatus to facilitate antibody secretion. Upon termination of the immune response, namely through antigen clearance, a small pool of high-affinity memory or long-lived secretory B cells are retained in anticipation of secondary infections. The latter is termed a plasma cell by virtue of the high secretory capacity of these cells, which is necessary to maintain the protective levels of serum immunoglobulin. Whereas many of the signaling and transcription factors that control the early phases of both the homeostatic and immune response phases of B cell differentiation are steadily being characterized, the characterization of the factors that contribute to the homeostatic and immune response aspects of the late phases of B cell differentiation are in their infancy.

Plasma cell malignancy, or multiple myeloma, is a disease with a high incidence of morbidity where treatment is not yet curable (Hideshima et al. 2003). Knowledge of the properties of plasma cells may aid in the development of better therapy. Additionally, understanding the mechanisms of plasma cell generation and maintenance may aid in vaccine design where humoral immunity plays a critical role.

1.1 The Secretory Switch

The immunoglobulin locus extends over 1 Mb and is a highly complex unit involving multiple gene and regulatory elements (Ernst and Smale 1995). The primary transcript is subject to alternative splicing and poly-adenylation site usage that produces two mature transcripts differing in their 3′ ends (Alt et al. 1980; Early et al. 1980; Rogers et al. 1980). The longer transcript encodes the membrane bound form of the protein and the shorter transcript encodes the secreted protein. Production of the membrane transcript is dependent on a splicing event that removes a portion of the secreted transcript's exon and the secretory poly-A site, and production of the secreted transcript involves preferential usage of the secretory poly-A site and arrest of alternative splicing (Peterson et al. 1991). 3′ end processing is developmentally controlled such that mature B cells produce each form in equal amounts and plasma cells primarily produce the secreted form, approaching a ratio of 100:1 (Edwalds-Gilbert et al. 1997). The position and strength of the *cis*-acting splice and poly-A sites are integral to regulated processing; however, Ig gene-specific signals are not necessary (Peterson 1994). Strikingly, in plasma cells, it appears that the differentiation-specific event is accounted for by the enhanced activity, binding, and recruitment of the poly-adenylation complex to the secretory poly-A site (Edwalds-Gilbert and Milcarek 1995). Expression levels of the RNA-binding and specificity factor of the poly-adenylation complex, CstF64, is implicated in mediating the differentiation-specific switch (Takagaki et al. 1996); however, differential expression is not observed in all experimental systems (Martincic et al. 1998; Sciammas and Davis 2004). Nevertheless, CstF64 and the core poly-adenylation complex are necessary for proper processing (Takagaki and Manley 1998). In addition, it has been proposed that levels of hnRNP F can influence this decision (Veraldi et al. 2001). Recent developments indicate that general control of 3′ end processing is integrated during transcript initiation and the by nature of the polymerase II complex (Proudfoot et al. 2002). Therefore, elucidation of the differentiation-specific mechanism may require analysis beyond the secretory polyA site.

1.2 The History of Blimp-1

Blimp-1 is a five-zinc-finger transcription factor of the C2H2 family whose expression is induced during the immune response phase of B cell activation and is maintained in plasma cells (Turner et al. 1994). Independent studies of the *Blimp-1* human orthologue (*PRDFI-BF1*) identified a DNA-binding site in the β-interferon promoter, which is mediated by the first two zinc fingers (Keller and Maniatis 1991a, 1991b). This report also indicated that Blimp-1 expression is inducible during the antiviral response of the fibroblastoid cell line HeLa. In addition, *Blimp-1* expression is inducible in terminal-differentiating macrophage cell lines (Chang et al. 2000). Lastly, expression is restricted to a few cell types, as determined from the EST sequencing efforts, and includes the lymphoid system, head and brain, and embryo. Preliminary studies indicate that Blimp-1-null mice exhibit embryonic lethality (S. Vincent, R. Sciammas, M.M. Davis, and E. Robertson, unpublished observations). This is an exciting finding as the *X. laevis* orthologue, *xBlimp-1*, has roles in embryonic development and head formation (de Souza et al. 1999; Chang et al. 2002). Although these data indicate that Blimp-1 has roles in multiple tissues and aspects of gene regulation, this review will focus on recent work that has focused on B cell maturation.

Initial experiments demonstrated that Blimp-1, when ectopically expressed in mature B cell lines, can induce several hallmarks of the plasma cell phenotype, especially the switch to Ig secretion (Turner et al. 1994; Schliephake and Schimpl 1996). Thus the creation of the acronym Blimp-1 (B lymphocyte-induced maturation protein-1). This observation indicated that Blimp-1 was a potential "master regulator" of B cell terminal differentiation. Subsequently, Blimp-1 was found to repress the expression of *c-myc*, a key determinant of cells exiting the cell cycle and regulating their telomeres during the process of cellular senescence and terminal differentiation (Lin et al. 1997). Notably, this aspect of Blimp-1 activity is not sufficient to induce other hallmarks of plasma cell differentiation, including antibody secretion (Lin et al. 2000). Additionally, the gene *C2ta*, known to be silenced in plasma cells, was found to be directly repressed by Blimp-1, indicating that it has roles in controlling diverse aspects of plasma cell differentiation, in this instance restricting antigen presentation (Piskurich et al. 2000). Both of these genes contain functional Blimp-1 binding sites in their promoters.

The primary structure of Blimp-1 suggests the existence of multiple functional elements. In fact, the proline-rich region in the middle of the

sequence is necessary for repression of both the *c-myc* and *C2ta* promoters in reporter constructs (Yu et al. 2000; Ghosh et al. 2001). This is accomplished via recruitment of both the Groucho family of corepressors and members of the Histone deacetylase family of chromatin remodelers (Ren et al. 1999; Yu et al. 2000). Second, the roles of the additional zinc fingers that do not contribute to the known DNA-binding specificity are unknown. Third, the carboxy terminus, following the zinc finger region, is enriched for acidic residues, an attribute of many transcriptional transactivators. Fourth, the PR domain of Blimp-1 exhibits striking homology to the recently characterized catalytic SET domains, which have roles in chromatin remodeling by site-specific histone methylation (Huang et al. 1998; Jiang and Huang 2000; Jenuwein 2001). The roles of these regions in the master regulator activity of Blimp-1 during B cell maturation remain to be discovered.

This review will focus on four aspects of Blimp-1 and plasma cell biology. First, we present evidence that supports a role for Blimp-1 in transcriptional regulation of Ig secretion. Second, we discuss recent studies cataloguing the nature of the genetic program driven by Blimp-1 activity and the comparison of this program with the expression "signature" of a differentiated plasma cell. Third, we describe recent experiments that show that execution of the maturational program is regulated by distinct modules of Blimp-1. Lastly, we present evidence supporting the role of Blimp-1 in regulating Ig secretion at the post-translational level. These results impact on our basic knowledge of plasma cells and thus may contribute to better therapies for multiple myeloma and more efficient vaccine development.

2
Transcriptional Regulation of Ig Secretion

When expressed in a number of B cell lines or in stimulated primary B cells, Blimp-1 induces Ig secretion. We sought to determine whether Blimp-1 exerted its effect during 3′ end formation of the Ig transcript (Sciammas and Davis 2004). This can be discriminated by assessing the production of both the membrane and secretory transcripts using Northern analysis. Indeed, a 10:1 ratio of secretory to membrane transcripts is observed in Blimp-1-transduced M12 B cells compared to a 1:1 ratio in controls (Fig. 1A). Interestingly, the abundance of total Ig message is increased in parallel, indicating that Blimp-1 simultaneously influences the production or stability of the message. As with previous ex-

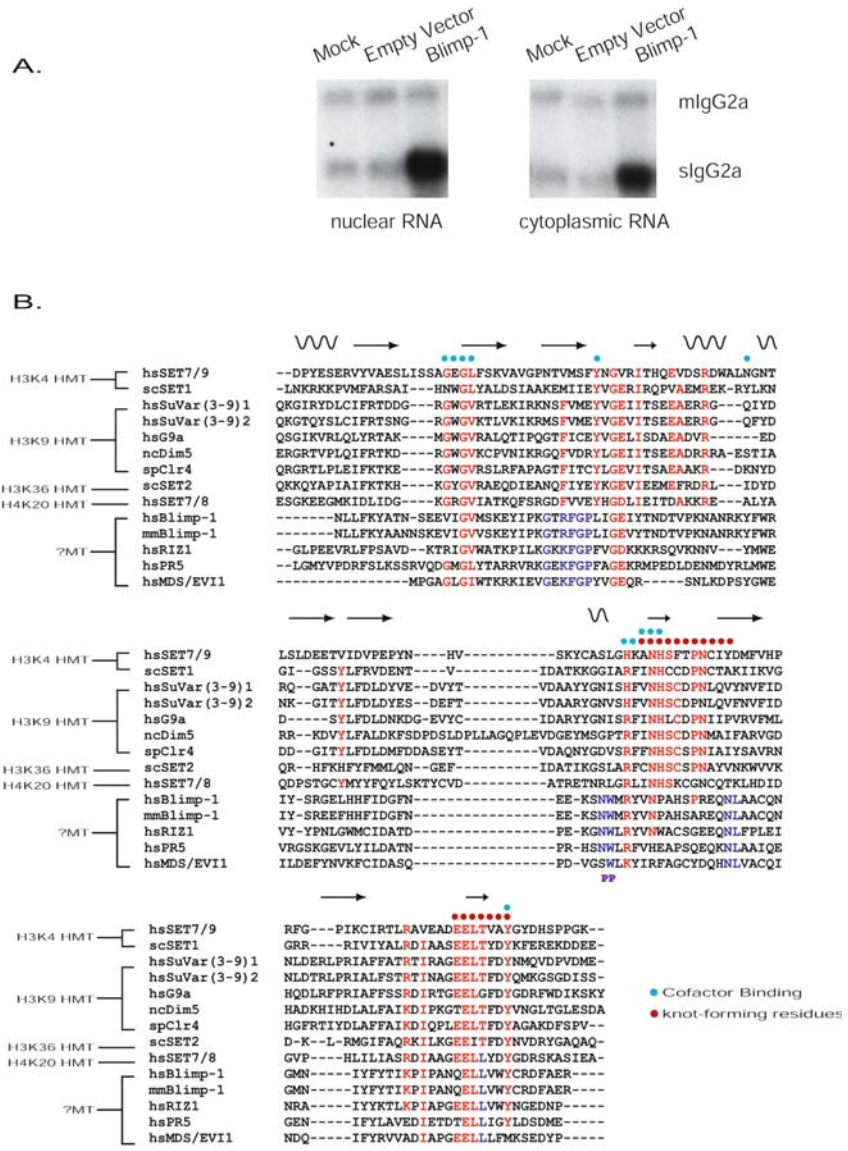


Fig. 1 **A** Blimp-1 regulates Ig secretion at the level of 3′ end processing of the Ig mRNA. Blimp-1 or control retroviruses were used to transduce the M12 lymphoma line. Nuclear or cytoplasmic RNA was prepared and used for Northern analysis with a probe for the IgG2a constant region. **B** Clustal W alignment of canonical SET domains and PR sequences. Sequences of canonical SET domains were used to align the PR domain proteins and the displayed layout is adapted from Jacobs et al.

periments, this step occurs in the nucleus since both the nuclear and cytoplasmic messages are similarly processed. This effect appears to be proximal to Blimp-1 activity since supernatant from Blimp-1-transduced cells does not stimulate Ig secretion in the heterologous B cell line, BCL_1 (R. Sciammas and M.M. Davis, unpublished observations). In addition, the effect is dependent on continued Blimp-1 expression, since transduced cells selected to discontinue Blimp-1 expression revert to unprocessed Ig production. Thus, Blimp-1 appears to induce Ig secretion by influencing the nuclear 3′ end processing events of the Ig message, the defining characteristic of plasma cell differentiation.

The mechanism of Blimp-1 induced Ig 3′ end processing and the relationship between Blimp-1 and CstF64-mediated processing was explored by using a dominant negative form of Blimp-1 consisting of the minimal zinc fingers. Interestingly, expression of this construct of Blimp-1 is able to attenuate cytokine-induced Ig secretion (Messika et al. 1998; Shaffer et al. 2002). Consistent with this, a mutant form of Blimp-1 lacking the first two zinc fingers fails to induce secretion when transduced in M12 cells (Sciammas and Davis 2004). This suggests that Blimp-1 regulates the expression of an intermediary gene that is involved in 3′ end processing. The expression levels of CstF64, an essential component of the poly-adenylation complex whose expression levels are reported to change upon acquisition of Ig secretory capacity (Takagaki et al. 1996), is static between control and Blimp-1-transduced M12 cells or between cytokine-treated and -untreated BCL_1 cells, negating it as a target of Blimp-1 regulation (Sciammas and Davis 2004). Secondly, CstF64 is not

(Jacobs et al. 2002). Annotations above the sequences represent tertiary structure configurations and dots above the sequences denote residues which were found to interact (*blue*) with the cofactor *S*-adenosyl-methionine or residues important for forming a unique knot structure (*red*) that are found in the SET 7/9 crystal. Residues in *red* are conserved in both the SET and PR families and residues in *blue* are conserved residues unique to the PR family. GenBank accession numbers for *Homo sapiens* (*hs*) SET7/9, P_040150; *hs* Su(Var)3–9H1, NP_003164; *hs* Su(Var)3–9H2, NP_078946; *hs* G9a, S30385; *Neurospora Crassa* (*nc*) Dim-5; AAL35215; Schizosaccharomyces pombe (*sp*) Clr4, O60016; *Saccharomyces cerevisiae* (*sc*) SET2, P46995; *hs* SET7/8, AAL40879; *hs* Blimp-1, NP_001189; *Mus musculus* (*mm*) Blimp-1, A53503; *hs* RIZ1, AAC50820; *hs* PR5, NP_061169; and *hs* translocation product MDS/EVI1, AAF04011 (MDS) spliced to AAB05840 (EVI1). The *PP below* the sequence denotes the residues in Blimp-1 that were mutated to proline-proline for functional characterization of this region in Ig secretion. (Sciammas and Davis 2004)

post-translationally modified by phosphorylation or proteolysis in Ig-secreting cells (Martincic et al. 1998). Since then, CstF64 has been shown to be genetically essential for secretory Ig mRNA processing (Takagaki and Manley 1998); perhaps Blimp-1 is regulating the transcription of a gene that functions to localize or augment the activity of CstF64. Recently, FY, an *Arabidopsis thaliana* protein that modulates the timing of flowering by affecting RNA processing, has been implicated as a poly-adenylation complex auxiliary factor. This observation establishes that differential poly-adenylation can be influenced by factors impacting on the core complex (Simpson et al. 2003), rather than by only the core complex itself, and that Blimp-1 may be regulating the plasma cell-specific expression poly-adenylation complex auxiliary factor.

The primary structure of Blimp-1 suggests the presence of multiple regulatory modules. To date the proline-rich region, which recruits transcriptional co-repressors, is the only one that has been extensively characterized. To determine the role of this or other regions of Blimp-1 in regulating Ig secretion, we transduced an allelic series of mutants in M12 cells (Sciammas and Davis 2004). Strikingly, in contrast to a deletion of the proline-rich or the carboxy-terminal regions of Blimp-1, the amino terminus deletion of 181 amino acids failed to induce secretion. Since this deletion removes half of the PR domain, a point mutant was employed to inactivate this domain. When compared to wild type Blimp-1, this point mutant failed to induce Ig secretion despite efficient protein expression. Therefore, the PR domain, in conjunction with the DNA binding domain, plays a critical role in Ig secretion.

The amino-terminus of Blimp-1 possesses a 130-amino acid region with strong homology to a growing family of genes (Jiang and Huang 2000). It was first noted by homology between the human genes *PRDI-BF1* (*Blimp-1)* and *RIZ* (Retinoblastoma-interacting zinc finger protein); hence the designation PR domain (Buyse et al. 1995). This region also exhibits striking similarity to SET domains (Su(var)39–1, Enhancer of Zeste, Trithorax), which are active sites for the catalysis of lysine methylation (Huang et al. 1998; Rea et al. 2000). Many, although not all, SET domains also contain pre- and post-regions of similarity, and these regions are thought to be important for histone substrate specificity (Jenuwein 2001). The PR domain, however, lacks these regions, suggesting a different specificity. In addition, the alignments of the PR domain with canonical SET domains are not perfect, throwing into question whether the PR domain also functions as a methyltransferase (Fig. 1B). However, recent X-ray crystallographic data of canonical SET domains has revealed both a unique knot structure and amino acid main- and side-

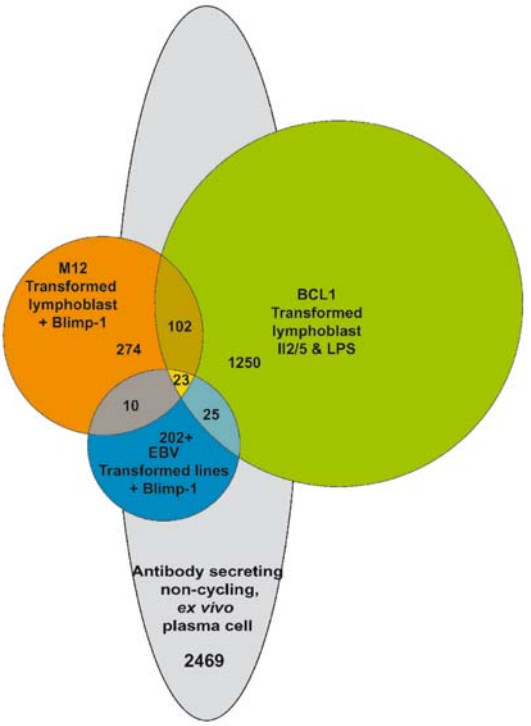
chain interactions with the obligate cofactor *S*-adenosyl-methionine (Jacobs et al. 2002; Trievel et al. 2002; Wilson et al. 2002; Zhang et al. 2002; Kwon et al. 2003; Xiao et al. 2003). It is these regions that are similar, suggesting, but not proving, that PR domains may also function as methyltransferases (Fig. 1B). To date, the SET type of methyltransferases are all lysine- and histone-specific and have roles in chromatin remodeling that, interestingly, affect transcription with both positive and negative outcomes (Zhang and Reinberg 2001). On the other hand, the six members of the PRMT class (protein arginine methyltransferase) are arginine-specific but have multiple protein targets and affect a range of functions, from roles in signaling to mRNA processing (Gary and Clarke 1998). PRMTs also bind the cofactor *S*-adenosyl-methionine; however, they use a different tertiary structure for catalysis (Fauman 1999). Therefore, post-translational modifications, via methionine, have multiple cellular activities and it is intriguing that Blimp-1 may regulate Ig secretion via a putative methyltransferase activity.

3
Blimp-1-Regulated Maturation Program

Two microarray studies have focused on the role of Blimp-1 in plasma cell differentiation and one has focused on the expression profile of terminally differentiated plasma cells (Shaffer et al. 2002; Underhill et al. 2003; Sciammas and Davis 2004). Strikingly, both similarities and substantial differences are seen between these studies, indicating that this maturation program is very plastic in nature (Fig. 2). Several different types of antibody-secreting cells exist in vivo that differ in proliferative capacity, migratory behavior or in the nature of the activating stimulus, and since all these cells appear to express Blimp-1, perhaps a differential expression program endows these cell types with specialized stage-specific properties (Turner et al. 1994; Angelin-Duclos et al. 2000; Martin and Kearney 2002). Interestingly, many of the differences are reconciled when analyzing the expression profile of terminal differentiated plasma cells.

With an interest in the mechanism of Ig secretion, we selected murine B cell lines with robust Ig secretory ability in order to track Blimp-1's effects on secretion (Sciammas and Davis 2004). Although the ability to secrete was consistently observed in different cell lines, the M12 cell line was the most dynamic. The M12 cell line was also pursued because of a lack of endogenous expression and a relatively tolerant phenotype in re-

A.



B.

M12 + Blimp-1 regulated genes compared to other plasma cell expression signatures

	Increased	Increased/ Decreased	Decreased/ Increased	Decreased	Number of genes in both sets / total
BCL1 + IL2/5	50	16	13	15	94 / 1400
BCL1 + LPS	49	19	12	21	101 / 1400
BCL1 + IL2/5 & LPS	35	13	9	11	68 / 1400
EBV Transformed cells + Blimp-1	4	19	1	9	33 / 260
Ex vivo Plasma cells	59	46	8	33	146 / 2469
Total genes validated in different cellular contexts	*92*			*36*	

Fig. 2 The Blimp-1 differentiation program is plastic in nature and constitutes a large fraction of the plasma cell expression signature. **A** Venn diagram of the microarray-based studies exploring the plasma cell genetic program induced by Blimp-1 compared to the expression signature of antibody secreting, nondividing, ex vivo murine plasma cells. The large quantity of nonoverlapping genes in the PC data is likely due to the contribution of the naïve B cell transcriptome used in the analysis

sponse to Blimp-1 toxicity. The retroviral expression strategy allowed a high enrichment of Blimp-1-expressing cells that could be analyzed at 72 h after transduction. When compared to control-transduced cells, a large number of differentially expressed genes are observed, 409, including both known and uncharacterized genes. Strikingly, the majority of these genes were induced, indicating an unanticipated ability of Blimp-1 in transcriptional upregulation. This possibility was tested in primary B cells transduced with Blimp-1-expressing retrovirus. Of 11 genes analyzed by real time PCR, eight were induced in both Blimp-1-transduced M12 and primary B cells, demonstrating Blimp-1's ability to activate gene expression cascades. In general, the differentially expressed genes have roles in B cell function, signaling, transcription, and homing, as well as genes involved in cell cycle control, as observed by Shaffer et al. (2002) (Fig. 3). However, a large set of genes involved in various aspects of secretion, including ER stress control, are upregulated in these cells, providing an unprecedented glimpse at the mechanism of Blimp-1 induced Ig secretion. The large number of differentially regulated genes combined with the transcriptional program associated with Ig secretion confirms the view of Blimp-1 as a master regulator of plasma cell differentiation.

In a separate study, the role of Blimp-1 was assessed by expressing it in various human EBV transformed cell lines (Shaffer et al. 2002). Calame and colleagues combined retroviral mediated transient expression of Blimp-1 with an inducible expression system. An inducible expression system was employed because they observed a high level of toxicity induced by Blimp-1 expression, as previously noted in B cell lines of an immature stage (Messika et al. 1998). Three cell lines were analyzed between these two systems and resulted in the differential regulation of

(Underhill et al. 2003). **B** Tabular display of the number of genes regulated in each condition shows the amount of both variability and overlap. The columns named Increased/Decreased or Decreased/Increased represent genes that, relative to M12 cells, are regulated in the opposite direction. The M12, BCL_1, and ex vivo plasma cell experiments were performed using Affymetrix MGU74 chips and can thus be easily compared. However, this comparison is not complete since the M12 and BCL_1 experiments include gene expression changes from all three chips of the MGU74 set and the ex vivo plasma cell experiments only include gene expression changes from the "A" chip. Comparisons with the EBV transformed cell experiments could only be estimated due to differences in the gene annotations between human and mouse

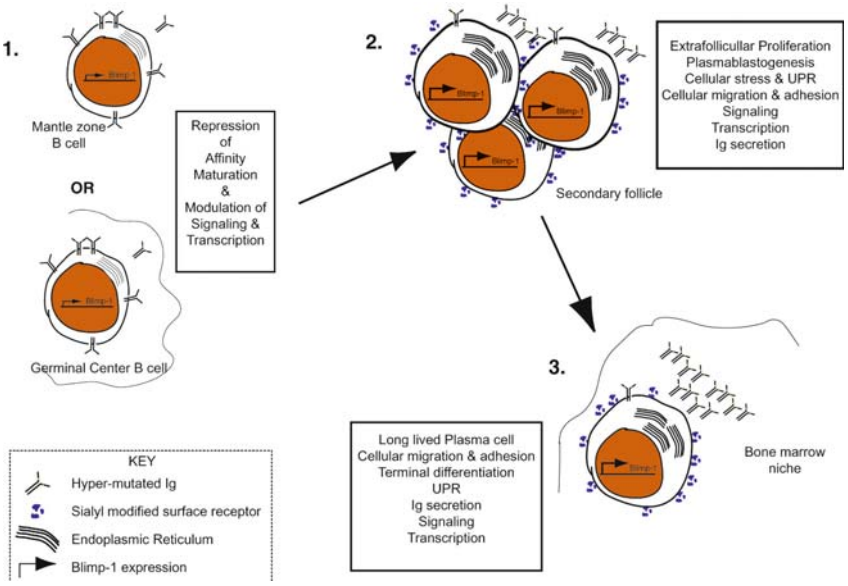


Fig. 3 Schematic of the transition of an activated B cell to a plasma cell and the functional categories of genes that Blimp-1 regulates at each step. Throughout the transition, signaling and transcription factors are differentially expressed as needed to respond to extracellular cues. *1* Activated mantle zone B cells (predominantly T-independent responders) and activated germinal center B cells (predominantly T-dependent responders) begin to express Blimp-1, which downregulates genes involved in affinity maturation. *2* Blimp-1-expressing cells migrate to secondary follicles where they expand by rapid proliferation as plasmablasts. Interactions with plasma cell-associated dendritic cells contribute to this process and thus the adhesion molecules regulated by Blimp-1 become candidates for this interaction. Transcriptional-coupled processing of the Ig transcript and high-rate Ig secretion is initiated by Blimp-1 and genes involved in the secretory pathway and in the UPR. *3* The Blimp-1 expressing plasmablasts, which survive in the secondary follicles at the termination of the immune response, migrate to specialized niches in the bone marrow where they initiate and maintain important contacts with stromal cells. These cells continue to secrete Ig and genes in the secretory pathway and the UPR are regulated. Lastly, these cells are terminally differentiated and are maintained for long periods but are arrested in the cell cycle

over 260 genes (the authors do not include a set of differentially expressed- and unannotated transcripts in this tally). Interestingly, the vast majority of the reported genes were repressed, consistent with the early description of Blimp-1 activity at specific target genes and Blimp-1's ability to interact with transcriptional repressors (Ren et al. 1999; Yu, et

al. 2000). The differentially expressed genes have roles in B cell function, signaling, transcription, and homing, as well as genes involved in cell cycle control (Fig. 3). Many of these genes have been documented to be silenced in plasma cells. Strikingly, Blimp-1 failed to induce Ig secretion in any of these cell lines, indicating that something about the nature of the cells or transformation status is blocking this function of Blimp-1. Still, the large number of genes differentially regulated by Blimp-1 as well as the identification of a large cohort of genes known to be silenced in plasma cells supports the notion of its role as a master regulator of terminal differentiation.

Functionally, the genes identified as regulated by Blimp-1 between Calame and colleagues (Shaffer et al. 2002) and ourselves (Sciammas and Davis 2004), are similar; however, significant differences exist. First is the set of genes involved in antibody secretion, which is only seen in our experiments. Second, Shaffer et al. (2002) observed the repression of a large number of genes that modulate B cell functions, including cell surface receptors, signaling, and transcription factors. In fact, the important B cell-specific transcription factors included in this set, *Ebf* (early B cell factor) and *Pax5,* may regulate many of these B cell-specific genes (Horcher et al. 2001). For reasons associated with activation or differentiation state, these two genes were not repressed by Blimp-1 in M12 cells. Third, the Blimp-1 maturational program in M12 cells is largely inductive in nature, indicating that a large fraction of activated genes is necessary for plasma cell differentiation. This conclusion is substantiated by the observation that many of the induced genes are also induced in Blimp-1-transduced primary B cells, fresh plasma cells (Underhill et al. 2003), and differentiating BCL_1 cells (Fig. 2 and described in Sect. 3.1). Whereas the M12 gene set is validated by the observed regulation of genes associated with antibody secretion (including the *Ig* genes themselves), the data of Shaffer et al. (2002) are validated by the repression of genes known to be silenced in plasma cells.

3.1
Differentiation-Specific Regulation

It is recognized that Blimp-1 expression peaks in rapidly cycling plasmablasts and is maintained throughout terminal differentiation, suggesting that the Blimp-1 transcriptional program is highly dependent on nuclear context. The reductionist approaches described by Shaffer et al. (2002) and Sciammas and Davis (2004) are very informative about the capacity of Blimp-1 as a differentiation agent; however, we still lack in-

sight into the nature of the complex regulatory networks that seem to impact on Blimp-1. To begin to address this issue, we analyzed the inducible B cell line, BCL_1, which, when stimulated with the cytokines IL2 and IL5 or by the B cell mitogen LPS, exhibits many of the physiological and genetic changes believed to occur during the transition of an activated B cell to a secreting plasmablast (Brooks et al. 1983; Blackman et al. 1986; Turner et al. 1994). These stimuli also induce Blimp-1 expression as an immediate early gene, indicating that the program (or subprogram) of differentiation is downstream of Blimp-1. A microarray-based, time-course experiment of gene expression changes following cytokine or LPS stimulation revealed a large set of genes with both stimulus-specific and temporal dynamics (Sciammas and Davis 2004). Genes with putative roles in differentiation, by virtue of first being differentially expressed during peak Blimp-1 protein expression (12 h) and second maintained at late time points when BCL_1 cells continue to express Blimp-1 and are secreting at a high rate (48 h), were identified from a large set of differentially expressed genes. Again, a large set of differentially expressed genes (606 at twofold and 1,400 at 1.5-fold) that affect B cell function, signaling, transcription, homing, mitogenesis, and secretion were identified.

Interestingly, a comparison of the BCL_1 data with the dataset derived from Blimp-1 expression in M12 cells revealed an overlap with genes in many of the functional categories associated with Blimp-1 activity. Indeed, these experiments validate the induction and repression of 64 and 25 genes, respectively (Fig. 2). Despite this functional overlap, the number of genes in common between the M12 and BCL_1 dataset is lower than expected (minimally 28%; a few genes that the microarrays failed to efficiently detect are shown by Northern analysis to be similarly regulated in both settings, suggesting that the microarray calculation is an underestimate). It is important to note that, in addition to differences between the cellular contexts of Blimp-1 expression in stimulated BCL_1 and genetically manipulated M12 cells, these two cell lines possess differences in transformation and lineage status that may also impact on Blimp-1 activity. Strikingly, however, these comparisons reveal that about one-third of the differentially expressed genes common between the BCL1 and M12 experiments exhibit unique stimulus- and temporal-expression characteristics. These results suggest that a subset of genes involved in the differentiation program can be influenced by extracellular cues and the nuclear milieu. Perhaps these genes are differentially required depending on the differentiation or activation state of the B cells. Indeed, different types of

antibody-secreting cells exist in vivo, providing a physiological context for these observations (Martin and Kearney 2002).

3.2 Blimp-1 as a Master Regulator

A very broad study has recently been published that describes the gene expression differences between murine resting B cells and nondividing, antibody-secreting plasma cells (Underhill et al. 2003). These plasma cells originate from a unique strain of mouse that presents with an unexplained pathology of hyper-gammaglobulinemia, cervical lymphadenopathy, and an inability to control skin infections. The cervical lymphadenopathy is in part contributed by plasma cells and therefore represents a tractable means to isolate a high number of "normal" plasma cells to perform microarray experiments. More than 2,400 genes were differentially expressed between these two cell types, hinting at the magnitude of gene expression changes associated with this transition. Strikingly, a share of these genes is downstream of Blimp-1 (Shaffer et al. 2002; Sciammas and Davis 2004), further supporting the concept of it as a master regulator. Importantly, many of the genes that are induced in the M12 expression system are represented in the untransformed plasma cell signature (Fig. 2), indicating that not all of Blimp-1's effects are repressive in nature. It is also important that many of the genes observed to change as reported by both Shaffer et al. (2002) and Sciammas and Davis (2004) are represented in the plasma cell expression profile. Many of the results of Shaffer et al. (2002) and Sciammas and Davis (2004) are represented in these experiments with "normal" plasma cells, which lends credence to the notion that different facets of the maturation program are differentially used in maturing B cells, but are ultimately resolved upon terminal differentiation. Indeed, this demonstrates that the set of differentially activated genes in the M12 cell experiments may be representative of different aspects or routes of the plasma cell state, and are thus not artifactual.

A multitude of the genes that are Blimp-1-responsive in both the EBV transformed lines and in the M12 lymphoblasts are components of the plasma cell expression signature as described by Underhill et al. (2003). This indicates that Blimp-1 plays a large part in managing this cellular transition. How large a part, however, cannot be fully discerned with these comparisons. It is likely that a large share of the 2,400 expression changes reflects on the nature of the naïve, resting B cell—of which Blimp-1 has no role. Lastly, the role of *Irf4,* a plasma cell expressed transcription factor with important roles in B cell maturation in this transition awaits analysis (Pernis 2002).

3.3 Modular Design

Preliminary experiments indicated that different mutants of Blimp-1 were both sufficient and deficient in regulating different sets of genes, suggesting a kind of autonomy of the different regulatory modules (Sciammas and Davis 2004). This notion was explored further by per-

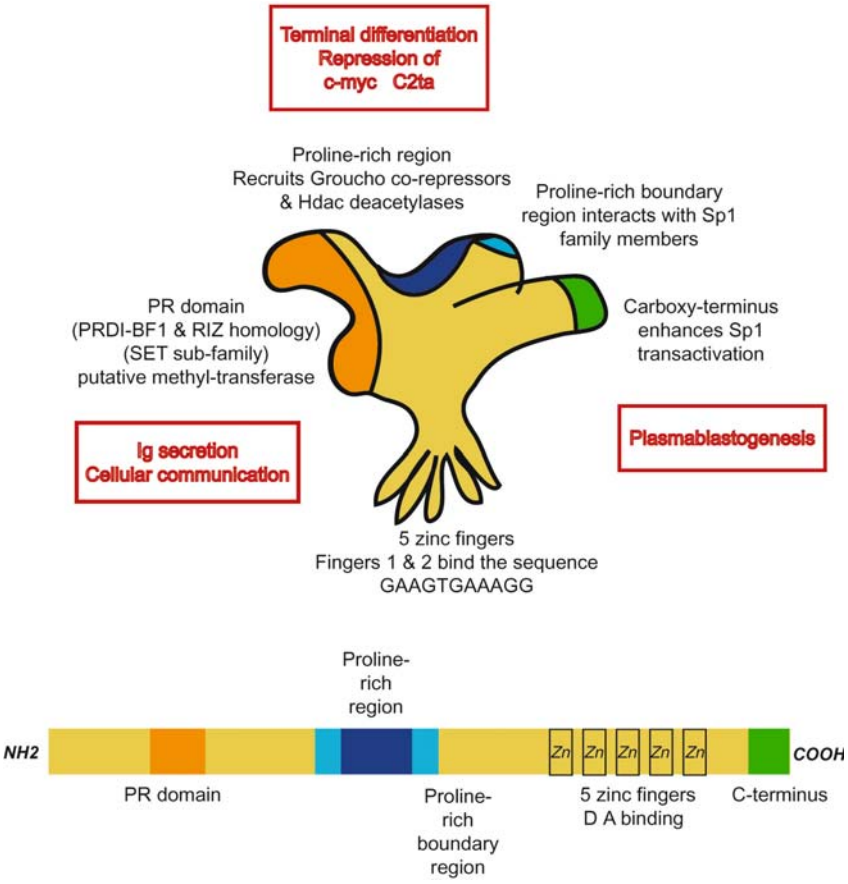


Fig. 4 Architecture of the primary and tertiary (imaginary) structure of Blimp-1 and the proposed functional roles of its modules. The linear arrangement of the color-coded modules from the primary structure is shown at the bottom and in the middle is the tertiary (imaginary) structure of Blimp-1. *Black text* describes the characteristics of the modules and the *red text* is the proposed function based on the types of types of differentially expressed genes dependent on that module

forming microarray experiments with select mutants of Blimp-1. Strikingly, when compared to either wild type or independent mutants of Blimp-1, two regions of Blimp-1 exhibit the ability to regulate a unique subset of genes (Fig. 4). Neither of these gene sets overlap, indicating that Blimp-1 operates at distinct regulatory regions or in mutually exclusive regulatory complexes to execute these changes. That the individual mutants did not alter the majority of genes indicates that the failure to observe differential expression is not a consequence of compromised stability, DNA binding, or expression level. The first mutant, disrupted by a double point mutation in the PR domain fails to induce Ig secretion as well as 30 other genes, the majority of which are destined for the extracellular space or are involved in the secretory pathway. The second mutant, a truncation of the carboxy terminus, fails to synergize with the Sp1 family of transcription factors and fails to regulate 13 genes (J-T Chi and MM Davis, manuscript in preparation) (Sciammas and Davis 2004). Interstingly, two of these genes have important roles in B cell activation. Mutation of the proline-rich region prevents repression at the *c-myc* and *C2ta* loci (Lin et al. 1997; Piskurich et al. 2000; Ghosh et al. 2001); however, information regarding the global role of this domain in Blimp-1 activity is lacking. Therefore, it appears that Blimp-1 diversifies the maturation program into separate pathways, presumably by interacting with different regulatory complexes. In addition, differential usage of the regulatory modules may contribute to stage-specific usage of the maturation program. To date, few transcription factors exhibit a diversification of its regulatory abilities, which may be a characteristic of proteins that sit at important regulatory junctions.

4 Post-translational Regulation of Ig Secretion

Subcellular analysis of plasma cells reveals a cytoplasm replete with a highly developed endoplasmic reticulum (ER), illuminating how plasma cells accommodate a high-level secretion of antibody (Wiest et al. 1990). These cytological changes occur following B cell activation; in parallel with the increase in production of secretory Ig transcript and the induction of Blimp-1 expression. Several recent observations suggest that staging of the plasma cell secretory machinery is a programmed pathway that involves the unfolded protein response (UPR). The UPR is a sensitive signaling pathway, conserved in all eukaryotic cells, which perceives malfolded proteins in the ER and signals to simultaneously arrest

protein translation and induce the expression of key chaperones and ER resident proteins that feedback to relieve the malfolded ER environment (Ma and Hendershot 2001).

Strikingly, it was found that B cells deficient in the transcription factor, Xbp1, fail to secrete Ig and, consequently, are severely immunocompromised (Reimold et al. 2001). Recently a connection between *Xbp1* and the UPR has been established, squarely implicating the UPR as a critical checkpoint in plasma cell development (Shen et al. 2001; Calfon et al. 2002). Ire1, an ER resident transmembrane protein, serves as an ER sensor. Interestingly, the cytoplasmic domain is an endonuclease that acts as an RNA-splicing enzyme whose target is the Xbp1 mRNA. Prior to stress, Xbp1 mRNA remains latent and poorly translated in the cytoplasm, and following ER stress, Ire1 splices a 26-bp intron, which converts the mRNA into a highly translatable form. Incidentally, the reading frame is altered and it produces an Xbp1 protein with both a different carboxy terminus and molecular weight. Experiments with Xbp1-deficient B cells suggest that UPR-dependent Xbp1 mRNA splicing is maintained throughout the life of the plasma cell and that nascent Ig continuously signals the UPR (Iwakoshi et al. 2003). Interestingly, regulation of the UPR and splicing of Xbp1 occur in two cell lines that are induced to secrete Ig by cytokines or LPS (R. Sciammas and M.M. Davis, unpublished observations) (Gass et al. 2002). Strikingly, UPR-dependent Xbp1 mRNA splicing leads to the transcription of IL6, a vital survival factor for plasma cells (Iwakoshi et al. 2003). This suggests an interesting feedback loop, which couples the quality control of secreted Ig with a survival signal. Importantly, *Xbp1*-deficient plasmablasts continue to express Blimp-1 and to produce high amounts of 3′ end processed Ig message, indicating that transcriptional and translational control of Ig secretion is regulated in parallel by Blimp-1 and Xbp1, respectively.

A broad-based proteomics approach has been applied to a temporal analysis of plasma cell differentiation and suggests that various aspects of the secretory apparatus and the UPR are regulated in two phases (van Anken et al. 2003). Several waves of protein changes were apparent following LPS stimulation, commencing with proteins involved in cellular metabolic competence and ER secretory capacity, which precedes the accumulation of Ig in the ER. In this analysis, the UPR—as defined by Xbp1 splicing—was initiated only coincident with the accumulation of Ig, indicating that the early phase of ER expansion occurs independently of a UPR signal.

The interface between Blimp-1, the ER, and the UPR is interesting. Shaffer et al. (2002) observed a slight increase in the total Xbp1 message

following ectopic Blimp-1 expression; however, the magnitude of this increase is small compared to plasmablasts, indicating that other factors are regulating Xbp1. On the other hand, in Blimp-1-expressing M12 cells that are induced to secrete Ig, a number of ER and UPR genes are induced. These include *Jaw1*, *Hsp70–1*, several DnaJs, *Erp44*, *Rer1*, several thioredoxin domain containing genes, several carbohydrate-modifying genes, and *Chop10*. This may in part be due to UPR-dependent processing of Xbp1, as two Xbp-1 target genes (*Edem* and *Dnajb9*) are upregulated in Blimp-1-transduced M12 cells. This processing is occurring at low levels since we fail to observe processed Xbp1 protein following Blimp-1 transduction (R. Sciammas and M.M. Davis, unpublished observations). Strikingly, upregulation of the two Xbp1 target genes is sensitive to the Blimp-1 PR mutation, indicating that the Xbp-1 arm of the UPR is downstream of Blimp-1 regulated 3′ end processing of the Ig mRNA (Sciammas and Davis 2004).

The overlap between the Blimp-1-induced ER genes and the LPS-induced ER proteins observed by van Anken et al. (2003) is not complete, indicating that Blimp-1 regulates a portion of the ER expansion that is pre-emptive of maximal Ig secretion. However, this is an imperfect comparison due to the limitations of drawing conclusions based on measuring different biochemicals, RNA and proteins, respectively. Nonetheless, these results suggest that Blimp-1 may be participating in the staging of the initial phase of ER expansion. Therefore, these data indicate that Blimp-1 regulates Ig secretion at the post-translational level, in addition to its role in regulating the post-transcriptional events that lead to antibody secretion.

5 Remarks and Future Directions

The identification of Blimp-1 as a major transcription factor involved in the transition from activated B cells to antibody-secreting plasma cells has opened new ways and insights towards understanding this critical differentiation phase. Additionally, the finding that Blimp-1, and specifically a putative methyltransferase domain in the amino-terminus (PR domain), plays a major role in regulating the mRNA processing events that affect efficient antibody secretion provides a new handle to tackling the mechanism of this long-standing question. Some of the issues regarding the molecular events involved during this critical stage of B cell maturation have been answered by analysis of the Blimp-1 regulated

genes. Differential expression of genes involved in affinity maturation, adhesion, migration, signaling, transcription, and secretion reveal mechanisms of how an activated B cell participates in the humoral response. Additionally, many of these genes are activated, indicating that the effect of Blimp-1 on differentiation is not strictly repressive in nature. Strikingly, these microarray experiments have identified a set of genes whose expression in the lymphoid system has not been previously noted, suggesting an unanticipated role in the immune system (Sciammas and Davis 2004). In addition, the large set of unannotated ESTs suggests the involvement of new players with roles in this maturation process.

Different aspects of this maturational program may be modulated by signaling- or activation-specific cues to endow different antibody-secreting cells with specialized functions. This plasticity is hinted at by the observation that a portion of the target genes observed in different Blimp-1-expressing cells, ranging from different transfected populations (M12 and Raji) and activated cells (BCL_1) to terminally differentiated plasma cells, can be regulated in different directions. These different contexts may be relating that plasma cell differentiation is more heterogeneous than previously anticipated.

Multiple domains of Blimp-1 subserve distinct functions and operate independently to execute different aspects of the maturation program. This property of Blimp-1 may also contribute to the plastic nature of differential gene expression observed under different conditions. The possibility that Blimp-1 may be a component of distinct protein complexes in these differentiation pathways is very interesting. It is intriguing to speculate that the different modules may regulate genes with related functional consequences. For instance, over half of the genes dependent on the PR domain are involved in secretion or traffic through the secretory pathway, and among the genes dependent on the carboxy-terminus are key regulators of plasmablastogenesis. The proline-rich region involved in repression of *c-myc* and *C2ta* suggests that this module may be important for maintaining the terminally differentiated phenotype (Fig. 4). Questions remain as to how these genetic programs are shuffled and/or regulated between different stages of B cell terminal differentiation.

As a whole, the differentiation program induced by Blimp-1 appears to be multilayered. Future work is needed to determine which of the genes are directly and indirectly regulated by Blimp-1. This is a high priority as it will provide information on the mechanisms of plasticity and will provide a blueprint for the networks of genetic regulation of B cell differentiation. Indeed, several transcription factors are required, up-

stream, downstream, and in parallel with Blimp-1. In this regard, there is accumulating evidence that there may be negative feedback regulation between different transcription factors to regulate the fate of B cell differentiation. First, Bcl6, an important transcription factor involved in affinity maturation, and Blimp-1 appear to antagonize the expression of each other (Shaffer et al. 2000; Shaffer et al. 2002; Sciammas and Davis 2004). Second, the observation that Il6 expression, an important plasma cell survival factor, may be tied into the integrity of the antibody-secreting cell, including Blimp-1 activity, since it is driving Ig secretion, indicates another checkpoint in this differentiation process (Iwakoshi et al. 2003). Finally, characterizing the role and potential interplay of Irf4 and these factors will be a stimulating area of investigation. Dissecting the regulatory network of and identifying the roles of the Blimp-1 regulated genes in plasma cell differentiation will surely provide a bounty of interesting surprises.

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Wnt Signaling in Lymphopoiesis

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Abstract Wnt signaling elicits changes in gene expression and cell physiology through β-catenin and LEF1/TCF proteins. The signal transduction pathway regulates many cellular and developmental processes, including cell proliferation, cell fate decisions and differentiation. In cells that have been stimulated by a Wnt protein, cytoplasmic β-catenin is stabilized and transferred to the nucleus, where it interacts with the nuclear mediators of Wnt signaling, the LEF1/TCF proteins, to elicit a transcriptional response. Loss-of-function and gain-of-function experiments in the mouse have provided insight into the role of this signaling pathway in lymphopoiesis. The self-renewal and maintenance of hematopoietic stem cells is regulated by Wnt signals. Differentiation of T cells and natural killer cells is blocked in the absence of LEF1/TCF proteins, and pro-B cell proliferation is regulated by Wnt signaling.

The ability of one cell to influence the physiology of another cell is achieved through cell–cell communication, termed cell signaling. These communication mechanisms depend on the production of extracellular

signal molecules by the signaling cells and the reception of the signal by responding cells that are located close by or at a distance. The communication between cells in developmental processes is highly coordinated and it involves multiple signaling pathways that interact in complex signaling networks. Over the past years, several signaling pathways have been elucidated, including the Notch, the sonic hedgehog, the bone morphogenetic protein (BMP), and the Wnt signaling pathways. Deregulation of any of these pathways often leads to disease, as for example cancer. In this review, we will discuss the function and role of Wnt signaling in lymphopoiesis.

1 Overview of the Different Wnt Signaling Pathways

Binding of Wnt proteins to receptors of the Frizzled family can elicit three cellular responses. First, the canonical Wnt pathway involves β-catenin and LEF1/TCF proteins, which act in the nucleus to regulate genes containing LEF1/TCF-binding sites (Hurlstone and Clevers 2002). In the canonical Wnt pathway, the Frizzled protein acts on the protein dishevelled (Dsh) to recruit the glycogen synthase kinase-3β-binding protein (GBP/Frat1), resulting in a block of β-catenin degradation. Second, in the planar cell polarity (PCP) pathway, Frizzled activates the small GTPases rho and cdc42, as well as the Jun-N-terminal kinase (JNK) via an interaction with the Daam1 protein (Weber et al. 2000). This pathway, which regulates the asymmetric cytoskeletal organization of cells and the polarization of cells within the plane of epithelial sheets, is independent of β-catenin and branches from the canonical Wnt pathway at the level of disheveled (David et al. 2002). The PCP pathway involves the cadherin-related transmembrane protein flamingo, the proteoglycan knypek and the strabismus protein (David et al. 2002). Third, in the Wnt/Ca^{2+} pathway (Kuhl et al. 2000; Wang and Malbon 2003), the Frizzled receptor appears to act through heterotrimeric guanine nucleotide-binding proteins and to activate phospholipase C and phosphodiesterase (Slusarski et al. 1997; Ahumada et al. 2002). The Wnt/Ca^{2+} pathway leads to decreased intracellular concentrations of cyclic guanosine monophosphate (cGMP) and to increased concentrations of free intracellular calcium, which can activate the phosphatase calcineurin to dephosphorylate the transcription factor NF-AT and translocate it into the nucleus.

2 The Canonical Wnt Signaling Pathway

Over the past decade, a large body of experiments has allowed for the elucidation of the canonical Wnt pathway (Fig. 1). The key player of the

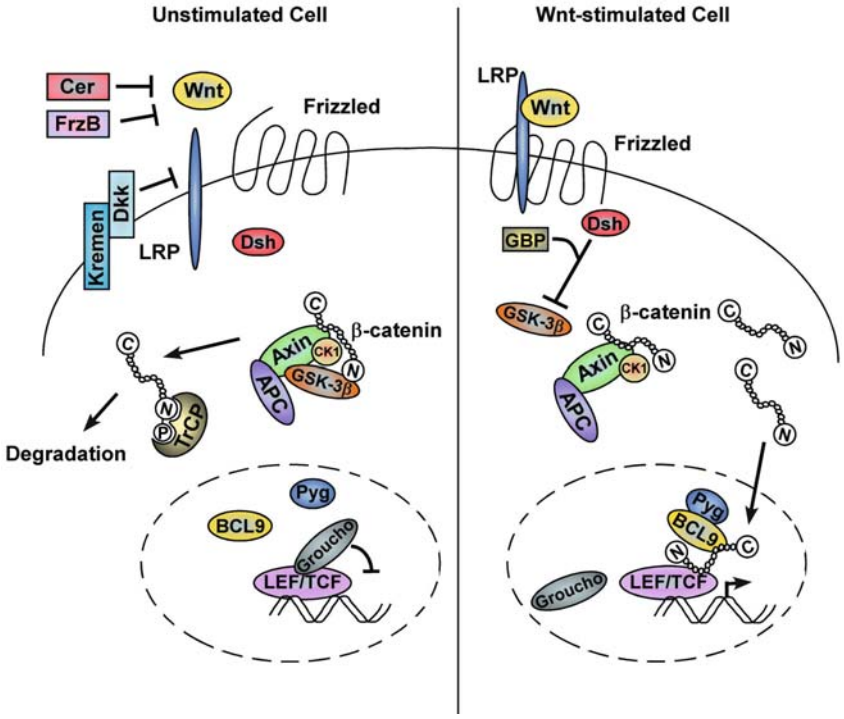

Fig. 1 The canonical Wnt signaling pathway. Scheme showing the components of the Wnt pathway in stimulated (*right-hand panel*) and unstimulated (*left-hand panel*) cells. In unstimulated cells, *β*-catenin is associated with a multiprotein complex that mediates phosphorylation and targeting of *β*-catenin to the proteasomal degradation pathway. In the nucleus, the activities of the Wnt-responsive transcription factors are repressed by association with co-repressors of the Groucho family. Wnt signals can also be antagonized by the Wnt antagonists Cerberus (*Cer*) and Frizzled-related protein (*FrzB*). The activity of the Wnt co-receptors, low-density lipoprotein receptor-related proteins (*Lrp-5/-6*), can be antagonized by Dickkopf (DKK). In Wnt-stimulated cells, Gycogen synthase kinase-3*β* (*GSK-3β*) is inhibited by Disheveled (*Dsh*), resulting in accumulation of *β*-catenin in the cytoplasm. *β*-catenin is translocated into the nucleus, where it associates with LEF1/TCF proteins to activate Wnt-responsive target genes. The nuclear activity of *β*-catenin can be augmented by the association of BCL9 and Pygopus with *β*-catenin

canonical Wnt signaling pathway, β-catenin, was initially identified as a component of specialized epithelial intercellular junctions (adherens junctions), which links the Ca^{2+}-dependent transmembrane protein E-cadherin to the cortical actin cytoskeleton (McCrea et al. 1991; Wheelock and Knudsen 1991). Subsequent biochemical and genetic experiments revealed the function of β-catenin in the Wnt signaling pathway (Hinck et al. 1994a; Hinck et al. 1994b) and in the transcriptional activation by LEF1/TCF proteins (Behrens et al. 1996; Huber et al. 1996; Molenaar et al. 1996; van de Wetering et al. 1997). In unstimulated cells, β-catenin is in a large cytoplasmic complex, containing the scaffold protein axin, tumor suppressor protein adenomatous polyposis coli (APC) and the constitutively active glycogen synthase kinase-3β (GSK-3β; Behrens et al. 1998; Ikeda et al. 1998; 2000; Kishida et al. 1998; 1999). This complex also recruits casein kinase I, which collaborates with GSK-3β to phosphorylate β-catenin at four N-terminal serine and threonine residues (Ikeda et al. 1998; Kishida et al. 1998). The phosphorylation marks β-catenin for recognition by the Slimb/β-transducin repeat-containing protein (TrcP), a component of an ubiquitin E3 ligase, which mediates ubiquitination of β-catenin and subsequent proteasomal degradation (Aberle et al. 1997; Jiang and Struhl 1998; Marikawa and Elinson 1998). This regulation ensures that the concentration of soluble β-catenin is maintained at a low level in absence of Wnt signals.

Wnt proteins are ligands of the Frizzled (Fz) family of serpentine receptors (Bhanot et al. 1996). Wnt proteins comprise a large family of secreted glycoproteins that have been shown to be associated with the cell surface or extracellular matrix of secreting cells, favoring a local activity (Bradley and Brown 1990; Papkoff and Schryver 1990). In addition, Wnt proteins are lipid-modified via an N-terminal cysteine by membrane-bound acyltransferases of the porcupine family that are required in the Wnt-producing cell (Willert et al. 2003). Lipid-modification of Wnt proteins is thought to influence transport between cells. The binding of Wnt proteins to the Frizzled receptors is augmented by the low-density lipoprotein receptor-related proteins (LRP)-5 and -6, which form a tripartite complex that initiates the Wnt signaling cascade (Pinson et al. 2000; Tamai et al. 2000). The initiation of Wnt signaling can also be inhibited by at least two mechanisms. Soluble Wnt proteins can be sequestered by the Wnt antagonists Cerberus (Cer) and FrzB. In addition, the interaction of LRP5/6 proteins with the Frizzled receptors can be impaired by Dickkopf (Dkk), which antagonizes Wnt signaling by blocking access of Wnt proteins to the LRP co-receptor and by inducing endocy-

tosis of LRP5/6 in cooperation with the protein Kremen (Mao et al. 2002, 2003).

Activation of the Wnt signaling cascade by the Wnt–Frizzled–LRP5/6 complex results in the displacement of GSK3β from the axin complex by the GSKβ-binding protein (GBP), which is recruited to the axin complex via disheveled (Noordermeer et al. 1994; Kishida et al. 1999; Smalley et al. 1999; Itoh et al. 2000). The displacement of GSK-3β from the axin complex and the modulation of phosphorylation by the protein phosphatase PP2A prevent the degradation of β-catenin (Ikeda et al. 2000). Stabilized β-catenin is transferred into the nucleus, where it can interact with the LEF1/TCF transcription factors and activate Wnt target genes (Behrens et al. 1996; Huber et al. 1996; Molenaar et al. 1996; Hsu et al. 1998). Transcriptional activation is facilitated by the interaction of β-catenin with the histone acetyl transferase CBP and with chromatin remodelling complexes of the Swi/Snf family (Hecht et al. 2000; Miyagishi et al. 2000; Takemaru and Moon 2000). In addition, β-catenin interacts with the BCL9 protein, which recruits the PHD-domain protein Pygopus to augment gene transcription (Belenkaya et al. 2002; Kramps et al. 2002; Thompson et al. 2002).

The activation of Wnt targets by LEF1/TCF proteins and β-catenin can also be antagonized by multiple mechanisms. LEF1/TCF proteins can associate with Groucho, a co-repressor that actively represses transcription via recruitment of a histone deacetylase (HDAC; Zhang et al. 2000; Brantjes et al. 2001). Likewise, some but not all LEF1/TCF proteins interact with the co-repressor CtBP (Korinek et al. 1998b; Brannon et al. 1999). In addition, binding of β-catenin to ICAT negatively regulates Wnt signaling (Tago et al. 2000; Daniels and Weis 2002; Graham et al. 2002). Finally, DNA binding of LEF1/TCF proteins can be antagonized by phosphorylation via NEMO-like kinases (NLK; Ishitani et al. 2003).

3
Biochemical and Biological Properties of LEF/TCF Proteins

The nuclear responses of canonical Wnt signaling pathway are mediated by members of the LEF1/TCF family of transcription factors (Fig. 2). The LEF1/TCF family of transcription factors includes four members in the mouse and in man: T cell factor-1 (*Tcf1*; Oosterwegel et al. 1991; van de Wetering et al. 1991), lymphoid enhancer factor (*Lef1*) (Travis et al. 1991; Carlsson et al. 1993) and T cell factor –3 and –4 (Korinek et al. 1998b). Proteins of the LEF1/TCF family share an 80 amino acid

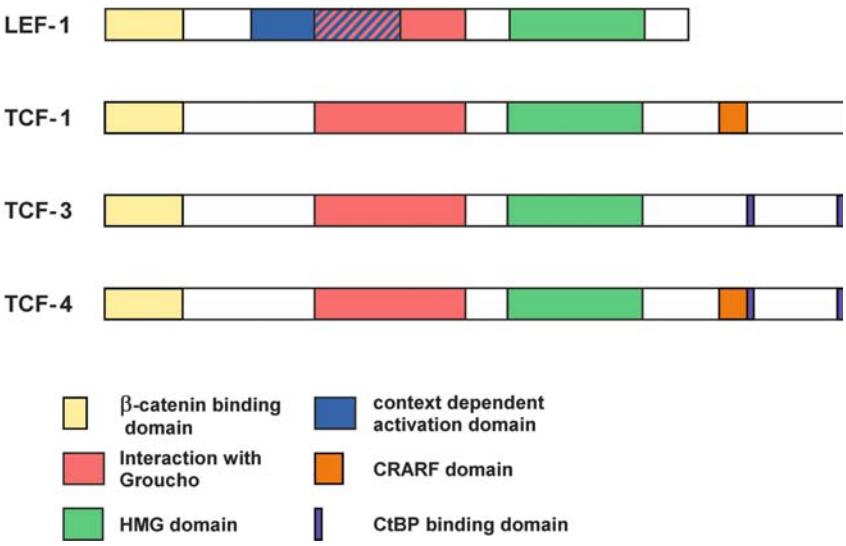

Fig. 2 Schematic representation of LEF1/TCF proteins and their conserved domains. In mouse and man, four members of the LEF1/TCF family of transcription factors share the *β*-catenin interaction domain (*yellow*) and the high-mobility group (*HMG*) domain (*green*), which mediates binding to DNA. All LEF/TCF proteins have an additional domain that interacts with co-repressors of the Groucho family. In addition, some protein domains are specific for members of the LEF1/TCF family. LEF-1 displays a context-dependent activation domain (*blue*) that interacts with the protein ALY. Finally, the long isoforms of the TCF3 and TCF4 have additional binding domain for the co-repressor C-terminal binding protein (*CtBP*) and the long isoforms of TCF1 and TCF4 have a CRARF domain, which appears to facilitate the activation of a subset of Wnt target genes

DNA-binding domain, termed high-mobility group (HMG) box, which mediates binding to the consensus sequence 5′CCTTTGAT through contacts in the minor groove (Giese et al. 1991; van de Wetering et al. 1991). LEF1/TCF proteins have been proposed to act as architectural proteins in the assembly of higher-order nucleoprotein complexes because they induce sharp bends in the DNA helix (Giese et al. 1992, 1995). LEF1/TCF proteins cannot regulate transcription by themselves: they require the interaction with cofactors. The amino-terminus of LEF1/TCF proteins contains a binding domain for *β*-catenin, a component of the Wnt signaling pathway (Behrens et al. 1996; Huber et al. 1996; Molenaar et al. 1996). Through this interaction, LEF1/TCF proteins mediate the nuclear response to Wnt signaling (van de Wetering et al. 1997; Hsu et al. 1998).

The central part of LEF1/TCF proteins can interact with members of the Groucho family of co-repressors (Bienz 1998; Cavallo et al. 1998; Levanon et al. 1998; Roose et al. 1998). Binding of Groucho confers repression on LEF1/TCF target genes by recruitment of histone deacetylases (Zhang et al. 2000; Brantjes et al. 2001). LEF1/TCF-Groucho complexes have been proposed to keep Wnt-responsive genes in a silent state in cells that have not received a Wnt signal. In addition to these common interaction motifs, some members of the LEF1/TCF family contain specific protein-binding domains. LEF1, but not the other TCF proteins, contains a context-dependent activation domain (CAD) that associates with the ubiquitous nuclear protein Ally of AML-1 and LEF-1 (ALY) (Carlsson et al. 1993; Giese and Grosschedl 1993; Bruhn et al. 1997). Aly, which has also been shown to regulate the coupling of transcription complexes with the mRNA export machinery (Zhou et al. 2000; Strasser et al. 2002), augment the activation of the T cell receptor α (TCRα) enhancer by LEF1 and AML1 (Bruhn et al. 1997). In addition, TCF-3 and TFC-4 contain a binding domain for the co-repressor CtBP in the C terminus. Finally, some isoforms of TCF1 and TCF4 comprise conserved CRARF motifs, which have been shown to augment the response of a subset of promoters with LEF1/TCF-binding sites to activation by β-catenin (Atcha et al. 2003; Hecht et al. 2003).

The proposed architectural role of LEF1/TCF proteins also appears to facilitate the integration of multiple signaling pathways at promoters and enhancers. For example, LEF1 has been found to mediate the synergy between Wnt signaling and Dpp signaling in Drosophila (Riese et al. 1997). The integration of Wnt signaling and other signaling pathways may also be enhanced by direct interaction of LEF1/TCF proteins with other nuclear mediators of signaling pathways. The HMG box of LEF1/TCF proteins has been shown to interact with Smad proteins, transcriptional activators that mediate a nuclear response to signals of the TGFβ family of growth factors, which include TGFβ, activin and BMPs (Labbe et al. 2000; Nishita et al. 2000). In addition, the HMG box of LEF1 has been found to mediate interaction with the intracellular domain of Notch1, which is translocated to the nucleus after proteolytic processing of the ligand-bound Notch1 receptor (Ross and Kadesch 2001).

The biological roles of LEF1/TCF factors have been elucidated by targeted gene inactivations. Targeted inactivation of LEF1 indicated a role of this transcription factor in the development of several organs, including teeth, hair follicles, mammary glands, the mesencephalus nucleus of the trigerminal nerve, and the dentate gyrus of the hippocampus (van Genderen et al. 1994; Galceran et al. 2000). The defects in tooth organo-

genesis in LEF1-deficient mice were found to affect the signaling from the epithelial to the mesenchymal tissues. In particular, LEF1 was shown to regulate the expression of the signaling molecule FGF4 in the epithelium of the tooth germ (Kratochwil et al. 2002). TCF1 knockout mice were shown to display a defect in differentiation of thymocytes, which will be discussed in detail (Verbeek et al. 1995; Staal et al. 2001). Although neither LEF1 knockout mice nor TCF1 knockout mice mimic the phenotypes of mutations in Wnt genes, the combined inactivation of LEF1 and TCF1 results in a defect in formation of the paraxial mesoderm that is vertical, identical to the defects found in Wnt3a-deficient mice (Takada et al. 1994; Galceran et al. 1999). Finally, targeted gene inactivation of the Tcf4 gene revealed defects in the proliferation of intestinal mucosa (Korinek et al. 1998a; Batlle et al. 2002). Therefore, LEF1/TCF proteins regulate multiple developmental processes, including cell fate decisions and proliferation and differentiation of cells.

4 Activation of LEF1/TCF Function by β-Catenin and Other Cofactors

The binding of stabilized β-catenin to LEF1/TCF proteins in Wnt-stimulated cells results in the transcriptional activation of Wnt target genes that contain LEF1/TCF-binding sites. The interaction between β-catenin and LEF/TCF proteins is mediated through the armadillo repeats 3–10 of β-catenin (Graham et al. 2000). Transcriptional stimulation is mediated by C-terminal activation domain of β-catenin that interacts with the TATA-box-binding protein (TBP) in vitro (van de Wetering et al. 1997; Hecht et al. 1999; Hsu et al. 1998). In addition, N-terminal sequences of β-catenin contain a transcription activation domain (Hsu et al. 1998). Wnt-dependent transcription was also shown to depend on the dephosphorylation of β-catenin. Clevers and co-workers found that the stabilization of β-catenin is necessary but not sufficient for transcriptional activation (Staal et al. 2002). Treatment of various cell lines with the proteasome inhibitor ALLN was found to result in increased levels of β-catenin, but not in the activation of a promoter carrying LEF/TCF binding sites. Using an antibody that recognizes specifically the dephosphorylated form of β-catenin, it could be shown that treatment of cells with Wnt-1 or LiCl, an inhibitor of GSK-3β activity, increased the levels of both total and dephosphorylated β-catenin, whereas treatment with ALLN increased the levels of total, but not dephosphorylated β-catenin (Staal et al. 2002).

β-Catenin-dependent transcription is also augmented by β-catenin-interacting proteins. Basler and co-workers identified BCL9/Legless as a new component of the Wnt signaling pathway (Kramps et al. 2002). BCL9, a putative oncogene that is overexpressed in some lymphoid tumors, binds β-catenin through its homology domain (HD)2. BCL9 binds also another protein, Pygopus (Pygo), through its HD3 domain and it has been proposed that the primary function of BCL9 consists in the recruitment of Pygopus to LEF1/TCF–β-catenin complexes. The principal role of BCL9 as an adaptor of Pygopus was shown by the ability of fusion protein, in which full-length Pygopus was linked with the HD2 domain of BCL9, to confer a proper Wnt response in Drosophila (Kramps et al. 2002). Pygopus is a PHD domain protein that acts genetically in the Wnt pathway and stimulates β-catenin-dependent transcription by an unknown mechanism (Belenkaya et al. 2002; Kramps et al. 2002; Parker et al. 2002; Thompson et al. 2002).

In addition, β-catenin associates with at least two protein complexes that act on chromatin. The histone acetyltransferase CBP/p300 augments β-catenin-dependent transcription in mammalian cells and *Xenopus* embryos (Hecht et al. 2000; Miyagishi et al. 2000; Takemaru and Moon 2000). CBP/p300 is recruited to Wnt-responsive promoters via the interaction with the armadillo repeats 10–12 and the C-terminal activation domain of β-catenin. Since the armadillo repeats 10–12 also interact with the inhibitory polypeptide ICAT, the mechanisms of inhibition may involve an antagonism with the recruitment of CBP/p300. The major function of CBP/p300 involves an associated histone acetyltransferase (HAT) activity, the transfer of acetyl groups to lysine residues of core histones. This modification alters the chromatin structure and makes it more accessible for the interaction with regulatory proteins. However, the HAT activity of CBP/p300 is not required for the enhancement of LEF1/TCF-β-catenin-dependent transcription (Hecht et al. 2000).

Chromatin-remodeling enzymes of the Swi/Snf family, which mobilize nucleosomes in an ATP-dependent manner (Logie and Peterson 1997), have also been shown to influence transcription of Wnt-responsive genes. In mammals, two SWI2 homologs, Brahma and Brahma-related gene-1 (Brg1), have been identified so far. Brg1 binds, via a conserved domain in the N-terminal half of the protein, to the armadillo repeats 7–10 of β-catenin (Barker et al. 2001). Brg-1 was found to enhance TCF/β-catenin-mediated transcription of target genes, whereas expression of a dominant negative form of Brg-1, an ATPase-dead mutant, repressed the TCF/β-catenin activity in transfection assays. In addition, Collins and co-workers were able to provide evidence for a role of Brah-

ma in Wnt signaling in Drosophila. A reduction in the levels of endogenous Brahma was found to result in more severe mutant phenotypes of Wnt signaling defects, whereas the overexpression of Brahma suppressed the mutant phenotypes (Collins and Treisman 2000). The effects of the chromatin-modifying and -remodeling complexes on Wnt-responsive transcription in vivo are consistent with the observation that stimulation of transcription by β-catenin in an in vitro-reconstituted system requires a chromatin template (Tutter et al. 2001).

Finally, the function of LEF1/TCF factors can be augmented by association with other transcription factors. In particular, LEF1 has been found to interact with TGFβ-responsive transcription factors of the Smad family, which allows for a synergistic activation of transcription targets containing binding sites for both LEF/TCF and Smad proteins (Labbe et al. 2000). This synergy allows also for the coupling of Wnt and TGFβ signaling.

5
Transcriptional Repression and Inhibition of LEF1/TCF Function

LEF1/TCF proteins can also actively repress transcription. Experiments in *Drosophila* and *Xenopus* provided evidence for a repression of *Ubx* and *Siamois* gene expression in the absence of a Wnt/Wg signal, which is abrogated by mutations in the LEF1/TCF-binding sites of the promoters (Brannon et al. 1997; Riese et al. 1997; Bienz 1998). Two proteins have been identified that associate directly with LEF1/TCF proteins to repress target genes. Groucho, a co-repressor that interacts with several DNA-binding proteins such as hairy, engrailed, and dorsal (Fisher and Caudy 1998), binds all LEF1/TCF proteins through a central protein domain (Cavallo et al. 1998; Levanon et al. 1998; Roose et al. 1998; Brantjes et al. 2001). The Groucho interaction domain of LEF1/TCF proteins does not overlap with the β-catenin interaction domain, which might also allow for a repression in the context of a Wnt signal. Consistent with this possibility, the repression of the E-cadherin promoter by LEF1 and Groucho was found to occur in the presence of β-catenin (Jamora et al. 2003). In addition, recent data indicate that the expression of Groucho is induced in cells of chick feather buds, in which active Wnt signaling occurs (Houghton et al. 2003). This observation raises the possibility that Wnt signaling and β-catenin stabilization mediate LEF1/TCF-dependent repression indirectly via an induction of Groucho. Thus, Groucho may confer repression upon LEF1/TCF target genes in both the absence and

the presence of Wnt signals. Moreover, some but not all LEF1/TCF proteins can repress transcription via an interaction with the co-repressor CtBP. XCtBP was shown by Moon and co-workers to interact with the C-terminal part of XTCF-4 and to mediate repression (Brannon et al. 1999). Clevers and co-workers found that the vertebrate TCF4 also interacts with CtBP and confers a trichostatin A-sensitive repression, indicating an involvement of histone deacetylases (Korinek et al. 1998b).

The function of LEF1/TCF proteins can also be antagonized by post-transcriptional modifications. First, LEF1/TCF proteins can be phosphorylated in the HMG domain by the NEMO-like kinase NLK (Ishitani et al. 2003). This modification impairs the DNA binding of a β-catenin–TCF4 complex, suggesting that NLK antagonizes all transcriptional effects of LEF1/TCF proteins. Another class of proteins that was shown to interact with LEF-1 and TCF4 is the protein inhibitor of activated STAT-y (PIASy) (Sachdev et al. 2001; Yamamoto et al. 2003). PIASy has been identified as an E3 ligase for the small ubiquitin-like modifier (SUMO), and the interaction of PIASy with LEF-1 results in the covalent modification of two lysines. In addition, the interaction with PIASy confers repression upon LEF1, which correlates with a sequestration of LEF1 into PML nuclear bodies.

6 Hematopoiesis

All mature blood cells in the body are generated from a relatively small number of hematopoietic stem cells (HSCs) and progenitors of cell lineages (Weissman 2000). In the mouse, a single HSC can reconstitute the entire hematopoietic system for the natural lifespan of the animal (Osawa et al. 1996). Two types of HSC have been identified, the long-term HSC, which is important for the self-renewal of the lineage, and short-term HSC, which differentiates into various hematopoietic lineages through a series of intermediate progenitors (Fig. 3). The common lymphoid progenitors (CLPs) give rise to B cells, T cells and natural killer (NK) cells, and the common myeloid progenitors (CMPs) generate monocytes, granulocytes, megacaryothytes, and erythrocytes (Kondo et al. 1997; Akashi et al. 2000). Differentiation of B and T cells involves multiple developmental stages that are defined by the expression of surface markers and the rearrangement status of the antigen receptor loci. In particular, pro-B cells differentiate into pre-B cells, which display the pre-B cell receptor on the surface and undergo immunoglobulin light

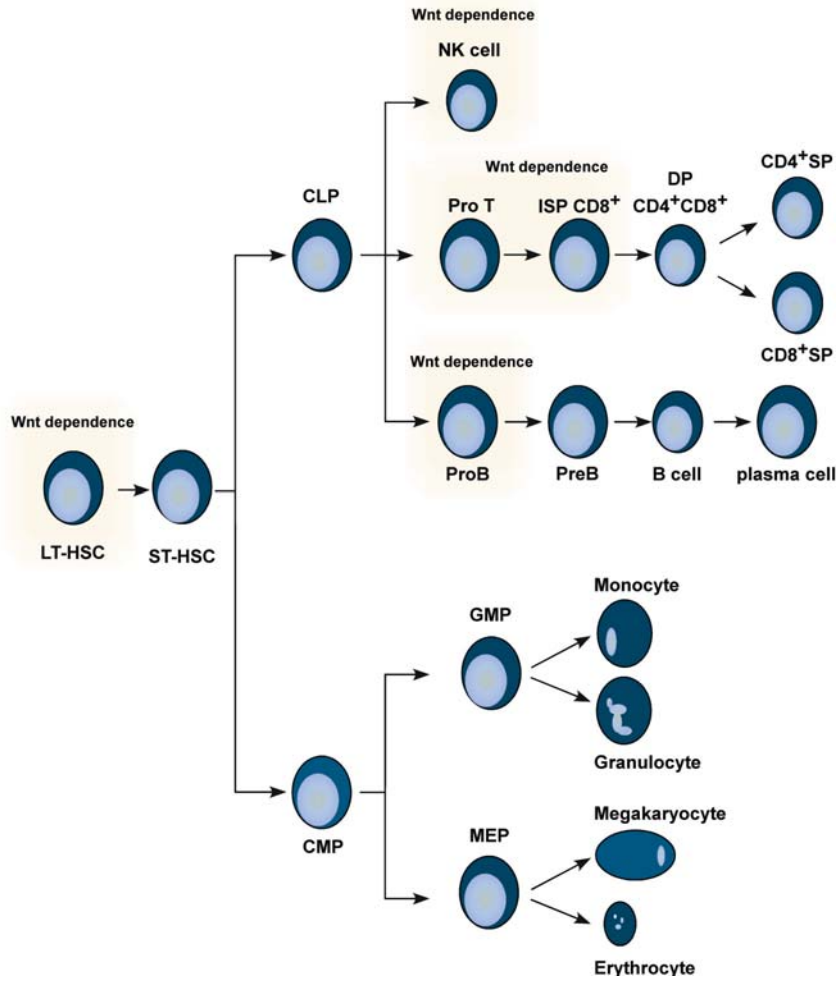

Fig. 3 Scheme of hematopoietic cell lineages. Long-term hematopoietic stem cells (*LT-HSCs*) differentiate into short-term hematopoietic stem cells (*ST-HSCs*). These cells give raise to two multipotent progenitor cells, the common lymphoid progenitor (*CLP*), and the common myeloid progenitor (*CMP*). CLPs further differentiate into natural killer cells (*NKs*), pro-T cells and pro-B cells. T cells develop via immature CD8 single-positive (*ISP*) cells into $CD4^+CD8^+$ double-positive (*DP*) cells and finally into $CD4^+$ single-positive (*SP*) or $CD8^+$SP cells. Pro-B cells differentiate into pre-B cells, mature B cells and finally into antibody-secreting plasma cells. Wnt signals appear to regulate the self-renewal of LT-HSCs, development of NK cells, the transition from pro-T to ISP $CD8^+$ cells, and the proliferation of pro-B cells. The differentiation of cells of the myeloid lineage, via granulocyte/monocyte progenitors (*GMP*), and cells of the megakaryocyte-erythrocyte lineage appear to be independent of Wnt signals

chain gene rearrangement. Subsequently, B cells are generated, which differentiate into antibody-secreting plasma cells when they encounter antigen. T cells that regulate the humoral immune response are also generated through a series of differentiation steps that occur in the thymus. Through an intermediary step, the immature CD8 single-positive cell, pro-T cells produce CD4CD8 double-positive cells that are selected into CD4 single-positive helper T cells or CD8 single-positive cytolytic T cells. Terminally differentiated lymphocytes cannot divide any longer and they undergo apoptosis after days or years, depending on the cell type. Myeloid progenitors generate more specialized progenitors, which are further restricted to the differentiation of specific cell lineages. The granulocyte/monocyte progenitors (GMPs) give raise to granulocytes and monocytes, and the megacarythrocyte/erythrocyte progenitors (MEPs) produce megacarythrocytes and erythrocytes (Akashi et al. 2000).

Hematopoiesis has been shown to depend on multiple developmental signaling pathways, including signaling by Wnt proteins, Shh and BMPs. In the next sections, we will discuss recent data that reveal an important role of Wnt signaling in the self-renewal of long-term HSCs and implicate Wnt signaling in the control of proliferation and differentiation of pro-B cells, T cells and NK cells.

7
Role of the Wnt Pathway in Hematopoietic Stem Cells

The hematopoietic stem cells (HSCs) are pluripotent cells that have the capacity to produce cells of all blood lineages. For the production of differentiated cells during the entire life span of animals, the HSCs need to balance the potential for cell specialization and the maintenance of the undifferentiated state by self-renewal. To date, the insight into the mechanism underlying the self-renewal of HSCs is limited; however, recent studies provided evidence for a role of Wnt signaling in this process. Wnt genes, such as Wnt5a and Wnt10b, are expressed in the murine yolk sac and other microenvironments of hematopoietic stem cells such as stromal cells of the fetal liver (Austin et al. 1997). In addition, forced expression of Wnt genes in HSCs was found to stimulate their proliferation (Austin et al. 1997; Van den Berg et al. 1998), Together, these findings suggested that the Wnt signaling pathway is involved in cell fate decisions and/or in the self-renewal of HSCs. Recently, Weissman and colleagues provided evidence for a role of Wnt signaling in the self-renewal

of the HSC (Reya et al. 2003). Purified HSCs from H2K-BCL-2 transgenic mice were infected with retroviruses that express β-catenin and green fluorescence protein (GFP), or GFP alone. The infected HSCs were sorted for GFP expression and analyzed for their proliferation potential in the absence of growth factors. The forced expression of β-catenin increased the proportion of proliferating HSCs by a factor of two, relative to the proliferation of control cells. In addition, the β-catenin-expressing cells proliferated for more than 4 weeks, whereas the control cells showed a modest proliferation after 2 weeks, suggesting that Wnt signaling confers long-term growth upon HSCs. No up-regulation of lineage-specific markers was observed in the β-catenin-expressing HSCs, showing that the majority of the cells maintained an undifferentiated phenotype. Consistent with the maintenance of an undifferentiated state, limiting numbers of in vitro propagated β-catenin-expressing cells were able to reconstitute both lymphoid and myeloid cell lineages in adoptive transfer experiments, whereas no reconstitution was observed with control cells.

Evidence for a role of endogenous LEF1/TCF proteins and intact Wnt signaling in HSCs was provided by experiments showing that HSCs that have been transduced with a GFP reporter containing multimerized LEF1/TCF-binding sites express the reporter gene efficiently. Moreover, a soluble form of the Frizzled cysteine-rich domain (CRD) that inhibits binding of Wnt proteins to the Frizzled receptor impaired proliferation of HSCs by a factor of four. Likewise, ectopic expression of axin, which enhances β-catenin degradation, decreased proliferation and cell survival. Reya et al. (2003) also found that the expression of the HoxB4 and Notch1 genes, which have been implicated in the self-renewal of HSCs, is augmented in β-catenin-expressing cells. Together, these findings indicate that Wnt signals regulate cell survival and proliferation of HSCs.

8
Role of Wnt Signaling in Pro-B cell Proliferation and Survival

B cell differentiation is characterized by the rearrangement status of the immunoglobulin genes and by the expression of different cell surface markers. During fetal development, B cells develop in the fetal liver, whereas in adults, B cell differentiation occurs in the bone marrow. The differentiation is a tightly regulated process that depends strongly on the coordinate expression of multiple genes. The earliest B cell precursors express the markers AA4.1, B220, and CD43 on their surface and consti-

tute the fraction A of pre-B cells, also called preBI cells (Hardy et al. 1991; Rolink and Melchers 1993; Li et al. 1996). They differentiate into fraction B cells, also termed pro-B cells, which express B220, CD43, and the heat-stable antigen (HSA). In this phase, the D-J_H rearrangement of the immunoglobin heavy chain locus occurs. At the next stage of differentiation (fraction C), cells express BP-1 on their surface and V_H-DJ_H gene recombination is initiated. Cells of fractions B and C express the immunoglobulin surrogate light chain genes, $\lambda 5$ and VpreB, which together with Ig heavy chains and the signal transduction components Igα (mb-1) and Igβ (B-29) form the pre-B cell complex (pre-BCR). At the immature B cell stage, Ig heavy chains are co-expressed with Ig light chains, forming the B cell receptor IgM on the surface. Immature B cells migrate to the periphery, express IgD and become mature B cells that are fully capable of responding to antigens.

Many genes that are involved in the process of B cell development have been studied extensively. The Wnt-responsive transcription factor LEF-1 is expressed in transformed pre-B cell lines but not in mature B cell lines (Travis et al. 1991; Reya et al. 2000). Insertion of the bacterial *lacZ* gene into the *Lef1* locus (Galceran et al. 2000), which allows for a visualization of *Lef1*-expressing cells also indicated that LEF1 is expressed during early B cell development in the fetal liver and adult bone marrow. The expression of LEF1 was found to be up-regulated in fraction B cells and expression could also be detected in fraction C cells. However, no LEF1 expression was detected in IgM-positive B cells. Initial evidence for a role of Wnt signaling in B cells came from the finding that some leukemic B cell lines overexpress a novel Wnt protein, Wnt 16 (McWhirter et al. 1999). In addition, a role for Wnt signaling in developing B cells was inferred from the analysis of LEF1-deficient mice (Reya et al. 2000). To test for a correlation between expression patterns and the function, fetal liver of *Lef1*$^{-/-}$ embryos and perinatal bone marrow were analyzed, as an analysis of older mice was not possible due to the early death of *Lef1*$^{-/-}$ mice. The number of B220$^+$ cells was found to be reduced by more than twofold and was even more obvious after excluding the dying and dead cells. Analysis of B220$^+$ cells from wild type and LEF1-deficient mice by TUNNEL assay and Annexin V staining showed that B220$^+$ cells of *Lef1*$^{-/-}$ mice die at an up to 20-fold higher frequency than wild type cells (Reya et al. 2000). As a cause for the reduced survival, Reya and co-workers analyzed the expression level of several genes known to be involved in apoptosis. Whereas the levels of Bcl-2, Bcl-x, and p53 remained unchanged in sorted pro-B cells (fraction B) of *Lef1*$^{-/-}$ mice relative to the levels in wild type mice, the expression of the *Fas*

and *c-myc* was elevated. A second defect that can contribute to the reduced size of the B cell compartment in *Lef1*$^{-/-}$ mice is the diminished proliferation of LEF1-deficient pro-B cells. By thymidine incorporation assays, it could be shown that proliferation of *Lef1*$^{-/-}$ pro-B cells is decreased, indicating that LEF1 also has an important role in the proliferation of pro-B cells. As LEF1 is a component of the Wnt pathway, the responsiveness of pro-B cells to external Wnt stimuli was tested. Addition of Wnt3a-conditioned medium increased the proliferation of wild type pro-B cells, but it did not alter the proliferation of LEF1-deficient pro-B cells. Moreover, LiCl, which acts as a surrogate Wnt signal, was found to stabilize β-catenin in pro-B cells. Taken together, these findings suggest a role of Wnt signaling and LEF1 expression for the proliferation and survival of pro-B cells.

9 Role of Wnt Signaling in T Cell Development

Differentiation of T cells in the thymus is a well-defined process that can be characterized by the expression of specific surface markers. The T cell precursors that migrate to the thymus express almost no surface markers that are typical for T cells (Wu et al. 1991). When the precursors start to differentiate, they first reach a stage termed the double negative (DN) stage, expressing neither CD4 nor CD8. These cells can be divided into four distinct differentiation stages, defined by the surface markers CD44 and CD25, starting up as CD44$^+$CD25$^-$, followed by CD44$^+$CD25$^+$. In the third phase, the cells down-regulate CD44 (CD44$^-$CD25$^+$), up-regulate the expression of the *Rag1* and *Rag2* genes and rearrange the T cell receptor (TCR) β, δ, and γ chains (Godfrey et al. 1994). Only if the cells succeed in a productive rearrangement of the β chain can they go to the next step, down-regulating CD25 (CD44$^-$CD25$^-$) and generating a pre-T cell receptor complex (Saint-Ruf et al. 1994). The formation of the pTα complex is essential for the generation of α/β T cells (Mombaerts et al. 1992). If the cells fail to rearrange the β chain and build the pTα complex but successfully rearrange the γ and δ chain, they can develop to γ/δ T cells. Differentiation of α/β cells continues with the up-regulation of CD8, generating immature single-positive (ISP) cells, followed by the up-regulation of CD4 to double-positive (DP) CD4$^+$CD8$^+$ cells. These double-positive cells down-regulate either CD4 or CD8, and generate mature single-positive (SP) CD4$^+$CD8$^-$ or CD4$^-$CD8$^+$ cells.

To investigate the role of Wnt signaling in T cell development, several different approaches have been used. One approach consisted in studying mice that carry targeted mutations in the *Lef1/Tcf* genes or in β-catenin. As the first gene of the LEF/TCF family members, LEF1 was inactivated by inserting a neo-resistance gene into an exon encoding the DNA-binding domain (van Genderen et al. 1994). For TCF1 two different knockout mice have been made, targeting either exon V ($Tcf1(V)^{-/-}$) or exon VII ($Tcf1(VII)^{-/-}$) of the *Tcf1* gene (Verbeek et al. 1995). The $Tcf1(V)^{-/-}$ knockout allows the production of a truncated but still functional TCF-1 protein, whereas the $Tcf1(VII)^{-/-}$knockout eliminates the production of a functional TCF-1 protein. Whereas in $Lef1^{-/-}$ knockout mice the T cell development is normal, a weak block of T cell development was observed in the two $Tcf1^{-/-}$ knockout mice. In particular, the null allele of *Tcf1* resulted in an incomplete block at the transition form immature CD8 single-positive cells to CD4CD8 double-positive cells (Verbeek et al. 1995). These mice also showed a defect in the expansion of thymocytes (Schilham et al. 1998). A complete block in thymocyte differentiation was observed in mice lacking LEF1 and carrying the hypomorphic allele of TCF1. Fetal thymic organ cultures from $Lef1^{-/-}Tcf1(V)^{-/-}$ mice showed a block of the T cell development after the immature CD8 single-positive stage, suggesting a redundant function of LEF1 and TCF1 (Okamura et al. 1998). However, the block in the differentiation of thymocytes in double knockout mice did not impair pre-TCR signaling because treatment of fetal organic cultures with anti-CD3ϵ antibody allowed for the appearance of double-positive cells (Okamura et al. 1998). Finally, this study confirmed that the T cell receptor-α gene is a genetic target of LEF1/TCF1 proteins because no TCRα transcripts were detected in ISP cells from $Lef1^{-/-}Tcf1(V)^{-/-}$ mice.

In an elegant study aimed at examining the dependence of the function of TCF1 on Wnt signaling, clevers and colleagues performed a rescue experiment with the $Tcf1(VII)^{-/-}$ knockout mouse (Ioannidis et al. 2001). Two different isoforms of *Tcf1* were tested for rescuing the TCF1 deficiency. A full length form *Tcf1*, including the β-catenin interaction domain, and a shorter isoform, missing the β-catenin interaction domain were introduced as transgenes into TCF1-deficient mice. Only the full-length of TCF1 could overcome the defects in T cell development, whereas the shorter form failed to rescue the knockout (Ioannidis et al. 2001). This result strongly suggests that at least some of the defects observed in $Tcf1(VII)^{-/-}$ mouse are attributable to the disruption of the Wnt signaling pathway. This study also identified the anti-apoptotic gene *Bcl-xl* as a target of TCF1, indicating that the apoptosis of CD4 CD8

double-positive cells in *Tcf1(VII)*$^{-/-}$ mice may be accounted for by the down-regulation of Bcl-xl (Ioannidis et al. 2001).

Additional support for a role of Wnt signaling in thymocyte differentiation was provided by the targeted inactivation of both *Wnt1* and *Wnt4* genes (Mulroy et al. 2002). The thymocyte phenotype of *Wnt1*$^{-/-}$*Wnt4*$^{-/-}$ mice is similar but not as severe as that of *Lef1*$^{-/-}$*Tcf1(V)*$^{-/-}$ mice. The mild defect in *Wnt1*$^{-/-}$*Wnt4*$^{-/-}$ mice is probably due to a redundancy of Wnt proteins because at least three other family members of Wnt proteins are expressed in thymocytes.

The role of Wnt signaling in thymocyte development was also addressed by two studies that involved the generation of mice with altered β-catenin levels. Boehmer and co-workers (Gounari et al. 2001) overexpressed a stabilized form of β-catenin in mice in which the third exon of β-catenin was deleted (β-cat$^{\mathrm{Thy}\Delta\mathrm{ex3}}$). Stabilization and overexpression of β-catenin in the thymus resulted in an overall lower number of thymocytes and in an alteration of thymocyte subpopulations. In particular, CD4$^+$CD8$^+$ double-positive cells were detected that lacked the expression of intracellular TCR-β chains. These findings indicated that a large proportion of thymocytes with stabilized β-catenin proceeded to the DP stage in the absence of pre-TCR and TCR$\alpha\beta$ signaling. Gounari et al. (2001) also tested the effects of the stabilized β-catenin in RAG-2$^{-/-}$ mice that normally fail to develop B and T cells as a result of their lack of TCR chains. Thymocytes of RAG-2$^{-/-}$ mice that have been crossed with the β-cat$^{\mathrm{Thy}\Delta\mathrm{ex3}}$ mice were able to overcome the block at the DN3 stage and could develop to DP and even SP cells. These findings were interpreted to suggest that the effects of the constitutive active β-catenin have to occur either in parallel and most likely independent of the pre-TCR signaling, as development is achieved in the absence of TCR chains. Finally, it could be shown that DN3 cells proliferate more extensively in β-cat$^{\mathrm{Thy}\Delta\mathrm{ex3}}$ mice as compared to wild type mice.

In another study, Sen and colleagues generated mice that have β-catenin conditionally deleted in thymocytes and splenic T cells. In the mutant mice, T cell development is blocked at the DN to DP transition, and it was shown that the DN3 to DN4 transition was impaired as a result of decreased proliferation of DN4 T cells (Xu et al. 2003). The Wnt responsiveness of splenic T cells was also examined and it was found that β-catenin translocated to the nucleus in response to anti-CD3 treatment and stimulation of purified CD4$^+$ T cells with phorbol 12-myristate 13-actetate.

The presumed role of Wnt signaling in hematopoiesis and T cell differentiation was challenged by a recent study of Radtke and colleagues

who showed that conditional deletion of β-catenin had no effect in hematopoietic cell lineages (Cobas et al. 2004). These authors used a different mutant allele of β-catenin that was deleted at the amino-terminus by an interferon-induced Cre activity. Based on the mortality of β-catenin-deficient mice, most experiments were conducted in competitive bone marrow chimeras that showed normal reconstitution of all hematopoietic cell lineages. Although the inactivated allele used in this study could in principle allow for the generation of a truncated form of β-catenin, the authors did not detect any smaller form of β-catenin. A possibility to account for the discrepancy of these results with those of others is a redundancy of β-catenin with the related plakoglobin protein, although in other developmental systems no evidence for such a redundancy could be observed. In addition, it is possible that only some effects of LEF1/TCF proteins are mediated by Wnt signals and conversely, that Wnt signals in hematopoietic cells can affect β-catenin-independent processes similar to those associated with the noncanonical Wnt pathways.

10 Role of LEF/TCF in NK Cell Development

NK cells are natural killer cells that kill target cells that lack some histocompatibility complex (MHC) class I molecules on their surface. MHC class I-specific inhibitory receptors are, for example, the class of mouse Ly49A receptors that currently comprises fourteen highly related genes (Wong et al. 1991; Karlhofer et al. 1992; Brennan et al. 1994; Smith et al. 1994; McQueen et al. 1998). The labs of Clevers and Held showed that a specific class of NK cells, the Ly49ANK cells, are dependent on TCF-1 expression (Held et al. 1999). They provided evidence that TCF-1 binds two LEF/TCF consensus sites in the Ly49 promoter region and they found that the promoter responds to TCF-1 overexpression. In mice deficient for TCF-1, the Ly49NK cells were missing and this defect was independent of the environment of the NK cells. Reintroduction of one functional *Tcf1* allele resulted in the appearance of Ly49NK cells, but at lower levels than in wild type mice, suggesting a dose dependence of TCF expression. In another study, Held and co-workers compared the relative contributions of LEF-1 and TCF-1 to NK cell development (Held et al. 2003). Notably, a reconstitution of lethally irradiated mice with cells from either $Lef1^{-/-}Tcf1(V)^{-/-}$ or $Tcf1(VII)^{-/-}$ mice showed a similar reduction in NK cells, whereas normal numbers were observed with cells

from *Lef1*$^{-/-}$ mice. This result was interpreted to suggest that TCF1 but not LEF1 regulates Ly49A and provides evidence for specific effects of different members of the LEF1/TCF family of transcription factors.

11 Concluding Remarks

Several studies have provided evidence for the importance of Wnt signaling and LEF/TCF proteins in the renewal of HSCs and the differentiation and proliferation of T, B and NK cells. However, many questions remain unanswered. An important issue will be to distinguish between Wnt-dependent and Wnt-independent effects of LEF1/TCF proteins because these proteins interact with multiple protein partners. Another issue will be the identification of functionally relevant target genes for LEF1/TCF target genes. Finally, it will be important to understand how Wnt signaling intersects with other signaling pathways and how LEF1/TCF proteins help to integrate multiple signals at transcriptional control sequences.

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