Developmental Regulation of the β-Globin Gene Locus

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Abstract The β-globin genes have become a classical model for studying regulation of gene expression. Wide-ranging studies have revealed multiple levels of epigenetic regulation that coordinately ensure a highly specialised, tissue- and stage-specific gene transcription pattern. Key players include *cis*-acting elements involved in establishing and maintaining specific chromatin conformations and histone modification patterns, elements engaged in the transcription process through long-range regulatory interactions, *trans*-acting general and tissue-specific factors. On a larger scale, molecular events occurring at the locus level take place in the context of a highly dynamic nucleus as part of the cellular epigenetic programme.

1 Introduction

Our picture of gene regulation at the transcriptional level, and particularly the functional relationships with higher-order chromatin and nuclear structure, remains largely incomplete. Any given gene is subject to many regulatory echelons that ultimately control when and where it is transcribed as well as the level of transcription. These include the transcription factor environment, regional and distal controlling elements, local chromatin conformation, chromatin modifications, as well as higher-order folding and nuclear organization. Many of these parameters encode or have the potential to be influenced by epigenetic information. Though the β-globin locus has long served as a paradigm in the study of many of these regulatory levels, the recognition of epigenetic control of developmental β-globin gene expression has only recently emerged. This chapter is not intended to be an exhaustive review of the literature on globin gene regulation, but will instead focus on some of the key elements of potential epigenetic control.

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2 The β-Globin Clusters and Their Ontogeny

The α- and β-like globin genes are very highly expressed in erythroid cells, making up approximately 90 % of the total poly(A) RNA in mature reticulocytes (Hunt 1974). This is due in part to the exceptionally high levels of globin gene transcription. The β-like genes are clustered on chromosome 7 in mice and chromosome 11 in humans. The temporal and spatial expression of the genes is tightly regulated during development, providing an appropriate and unique haemoglobin type for each stage. The structure and regulation of the mouse and human β-globin loci are similar in many aspects (Fig. 1).

The mouse β-globin cluster contains four genes (εy, βH1, βmaj, and βmin), while the human locus contains five genes (ε, ^{G}γ, ^{A}γ, δ, and β). In both cases the linear arrangement of the genes along the chromosome reflects the developmental order of expression. This phenomenon, often referred to as co-linearity, has been observed in other gene clusters such as the hox genes (Kmita and Duboule 2003). During human development ε-globin is expressed from 6–8 weeks of gestation in primitive nucleated erythroid cells derived mainly from the embryonic blood islands of the yolk sac (Collins and Weissman 1984). As the major site of erythropoiesis changes to the fetal liver, the ε gene is silenced and transcription of the γ-globin genes is switched on. At around the time of birth the main site of erythropoiesis changes again to the adult bone marrow concomitant with a further 'switch' in gene expression. Erythroid cells from the bone marrow express predominantly the β-globin gene (95 %); however, low levels of δ- and γ-globin expression are detectable. Though the γ genes are normally considered to be silenced in adult erythroid cells, low-level γ expression is the result of transient expression in the early stages of adult erythroid differentiation (Pope et al. 2000; Wojda et al. 2002;

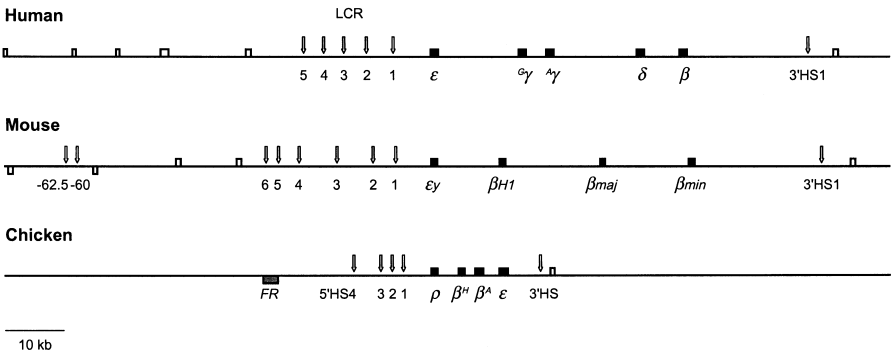

Fig. 1. Maps of the human, mouse, and chicken β-globin loci. Genes are presented as *boxes*: *black boxes* represent globin genes; *open boxes* olfactory receptor genes; *grey box* the chicken folate receptor gene. Genes transcribed in the sense direction, such as all globin genes, are located *above the line*; genes transcribed in the opposite direction are *below the line*. *Vertical arrows* indicate DNase-hypersensitive sites

Chakalova et al., unpubl. observ.). The δ-globin is normally expressed at low levels throughout adult life (2.5 % of the β-globin-like protein), which may reflect deficiencies in its promoter sequence.

In mice, red blood cell formation begins at day 8 in yolk sac blood islands and by day 9 primitive, nucleated erythrocytes are released into circulation from the yolk sac. These cells express primarily the embryonic εy and βH1 genes, although low levels of βmaj and βmin transcription are detectable (Brotherton et al. 1979; Trimborn et al. 1999). Interestingly, the transcriptional output of each gene is inversely proportional to its distance from the upstream locus control region (Trimborn et al. 1999; see also discussion below). The fetal liver becomes the main site of erythropoiesis after day 10, giving rise to definitive erythroid cells. The embryonic genes εy and βH1 are completely silenced and the adult βmaj and βmin genes are transcriptionally upregulated to full activity which persists into adult life (Brotherton et al. 1979; Trimborn et al. 1999).

The human β-globin locus has also been studied by inserting part, or all, of the locus into transgenic mice. Transgenic mice containing the entire human globin locus express ε-globin and γ-globin in primitive erythrocytes derived from the yolk sac (Strouboulis et al. 1992; Peterson et al. 1993). The switch to expression in the fetal liver occurs after day 10 and is accompanied by complete silencing of ε and persistent but reduced transcription of the γ genes, concomitant with increased δ- and β-gene transcription (Strouboulis et al. 1992; Peterson et al. 1993; Fraser et al. 1998). By day E16.5 γ-gene silencing is nearly complete, whereas δ- and β-gene transcription continues through adult life. Though there are differences that may be attributable to the dramatically shortened gestation period, the consistent observation that spatial and temporal globin switching of the human genes can be retained in a transgenic mouse highlights the degree of conservation between the human and mouse β-globin loci.

3
Models for Studying the β-Globin Locus

Over the years many experimental systems have been employed to study the β-globin genes. The best methods involve looking directly at the organism in question. To this end, chicken and mouse have been widely used as model systems to study developmental regulation. Though the literature is rich in the characterization of specific mutations that affect expression in the human locus (Stamotoyannopoulos and Grosveld 2001), developmental analysis presents more of a challenge for ethical and logistic reasons; so various alternatives have been sought. As described above, the human locus can be cloned and inserted into mice. In such transgenic mice many of the features of the β-globin locus, including tissue and developmental specificity, are retained. Thus, transgenic mice provide a reasonable and useful method to assay func-

tion in the human locus, although caution must be exercised when interpreting the results. An alternative to studying the organisms in vivo is to use cell lines. Cell lines have been isolated that appear to reflect some aspects of erythroid and developmental specificity. For example, murine erythroleukaemia (MEL) cells, immortalized with the Friend leukaemia virus (Friend et al. 1966; Marks and Rifkind 1978), are thought to represent a murine pro-erythroblast stage cell. They can proliferate in a predifferentiated state, or be induced to differentiate with a variety of chemical compounds into cells that mimic the adult stage of murine erythroid development, expressing βmaj and βmin. Erythroid cell lines from chicken, human, and mouse have been very useful in studying various aspects of globin gene regulation; however, it should be noted that none of them reproduces the magnitude of gene expression dynamics seen in the globin locus in vivo nor fully recapitulates the epigenetic changes associated with development.

More recently, attention has focused on manipulation of the endogenous mouse locus by homologous recombination as a means of functional analysis of the locus. This approach represents the most definitive system for functional analyses of a complex gene locus.

4 The LCR Is Required for High-Level Expression

The globin genes are transcribed at exceptionally high levels in erythroid cells. Initial attempts to characterize the DNA sequence elements responsible for this high-level activation concentrated on the regions immediately flanking the gene (Wright et al. 1984; Townes et al. 1985; Kollias et al. 1986; Behringer et al. 1987; Antoniou et al. 1988). Many β-globin transgenic mice were generated with various promoter and downstream sequences (Chada et al. 1986; Kollias et al. 1986, 1987). Though much useful information was gained regarding gene-proximal regulatory elements, the level of expression generated in such mice was highly variable, and in most cases one or two orders of magnitude lower than the endogenous mouse globin genes. The variability of expression in different transgenic lines was attributed to genomic position effects (PE) at the site of integration. PE can be either positive or negative presumably depending on the chromatin flanking the transgene. Transgene integration in or near constitutive heterochromatin such as pericentromeric regions is now generally recognized as one of the causes of some types of transgene silencing in mammals (Festenstein et al. 1996; Milot et al. 1996). Position effect variegation (PEV), which was initially recognized in yeast and *Drosophila* and has now been seen for a number of genes in vertebrates, results in clonal heritable silencing of a transgene in a subpopulation of cells of a tissue. PEV has also been observed in non-centromeric transgene integration sites (Savelier et al. 2003). For globin transgenes affected by PEV it is worth noting that whilst overall expression is low due to the reduced num-

ber of expressing cells, erythroid specificity and developmental timing is often retained (Milot et al. 1996). Collectively, these results suggest that the sequences immediately flanking the globin gene contribute to tissue- and developmental-specific expression but are insufficient to overcome the effects of the surrounding chromatin to ensure high-level expression in all cells.

Clues that distant elements were involved in globin gene regulation came from naturally occurring deletions in humans that led to deregulation of the β-globin genes. Patients with β-thalassaemia have severely reduced HbA (adult hemoglobin) due to downregulation of β-globin gene transcription. Several thalassaemia mutations have been identified and characterized; the most informative in this case are the large deletions involving regions upstream of the β gene such as the Dutch thalassaemia (van der Ploeg et al. 1980; Kioussis et al. 1983; Harteveld et al. 2003), English thalassaemia (Curtin et al. 1985; Curtin and Kan 1988), and Hispanic thalassaemia deletions (Driscoll et al. 1989; Forrester et al. 1990). In the search for potential distant regulatory elements, DNase I-hypersensitive sites (HS) in the β-globin locus were mapped. Several erythroid-specific HS were found in a 15-kb region upstream of the ε gene (Tuan et al. 1985; Forrester et al. 1986, 1987; Grosveld et al. 1987). Collectively known as the locus control region (LCR) each HS contains a core with several binding sites for erythroid and ubiquitous transcription factors (Philipsen et al. 1990; Talbot et al. 1990; Pruzina et al. 1991; Strauss and Orkin 1992; Furukawa et al. 1995; Goodwin et al. 2001). When the LCR was linked to the β-globin gene and inserted into transgenic mice, the level of β-gene expression was found to be equivalent to the endogenous mouse globin genes (Grosveld et al. 1987). The LCR was able to drive high-level expression of the gene in all transgenic mice regardless of the integration site, and the level of expression was directly proportional to the number of LCR–gene constructs integrated. Thus the LCR was functionally defined as a sequence that confers integration-independent, copy number-dependent, high-level expression upon a linked transgene. The LCR's ability to drive expression regardless of integration site was postulated to be due to a positive chromatin opening activity that allowed it to 'open' the chromatin of the adjacent transgene regardless of the site of integration. Indeed, analyses of a number of different LCRs linked to transgenes have demonstrated that even the repressive effects of pericentromeric heterochromatin can be overcome by a complete LCR (Festenstein et al. 1996; Milot et al. 1996; Kioussis and Festenstein 1997; Fraser and Grosveld 1998). The LCR was also shown to be capable of reprogramming heterologous genes, through its ability to drive erythroid-specific expression of non-erythroid genes (Blom van Assendelft et al. 1989). These findings led to suggestions that the LCR was responsible for opening the entire β-globin locus in erythroid cells. However, conclusive evidence showing that the LCR is not necessary for chromatin opening was provided by seminal experiments in which the mouse and human LCRs were deleted by homologous recombination (Epner et al. 1998; Reik et al. 1998; Alami et al. 2000). Globin gene expression was virtually abolished in the absence of the LCR in the human locus

whereas β-gene expression in the mouse locus was greatly reduced but still developmentally specific. However, the erythroid-specific open chromatin structure and histone hyperacetylation of the locus were maintained in the absence of the LCR (Schubeler et al. 2000). Collectively, these results show that the LCR is required for high-level transcription of the globin genes, but they also demonstrate that other sequence elements in the locus control opening and maintenance of an active chromatin structure.

5 The Role of Individual HS

The functional contribution of the different HS has been dissected in many experiments. The initial approaches assayed gene expression in small constructs containing one or all of the LCR HS(s) linked to a gene. As the technology for larger transgenes became available, individual HS deletions were assayed in what were often described as 'full locus' constructs. Human HS1, HS2, HS3, and HS4 can all increase the level of expression of a linked gene depending on the experimental system. Interestingly, HS2 is the only LCR HS with classical enhancer activity (Tuan et al. 1989; Moon and Ley 1991), which by definition is a sequence element that can enhance expression of a linked transgene in a transient transfection assay in either orientation, upstream or downstream relative to the gene. All of the other HS show little activity in this type of assay. However, when integrated into chromatin, as occurs in stably transfected cells, both HS2 and HS3 were able to drive increased expression levels, indicating that the sites are functionally distinct (Collis et al. 1990). In transgenic mice, Human HS1, HS2, HS3, and HS4 were able to significantly increase transgene expression levels depending on the developmental stage assayed (Ryan et al. 1989; Fraser et al. 1990, 1993; Jackson et al. 1996). HS2 and HS3 appeared to provide the bulk of the activity at most developmental stages (Fraser et al. 1990, 1993). In general, expression of linked genes increased in an additive rather than a synergistic fashion when multiple HS were included in the same construct (Collis et al. 1990; Fraser et al. 1990). Interestingly, in multi-gene constructs the developmental pattern of γ- versus β-gene expression varied depending on the HS used, suggesting distinct developmental specificities (Fraser et al. 1993). HS3 was the only site capable of driving γ-gene expression in the fetal liver, while HS4 activity peaked during β-gene expression in adult cells. Deletion of single HS from YAC transgenes appears to support the findings of developmental or gene specificities for some of the LCR HS in the human locus (Peterson et al. 1996; Navas et al. 1998, 2001, 2003). However, similar phenomena were not as apparent in knockouts of single HS in the endogenous mouse locus (Fiering et al. 1995; Hug et al. 1996; Bender et al. 2001). When the core of HS2, HS3, or HS4 was removed, expression of the globin genes was severely reduced (Bungert et al. 1995, 1999). Likewise, substitution of one HS core for another resulted in greatly reduced expression,

again suggesting that each HS plays a unique role. Deletion by homologous targeting of HS1, HS2, HS3 or HS4 lead to modest reductions in adult globin expression consistent with the additive effect on transcription as observed in transgene constructs (Fiering et al. 1995; Hug et al. 1996; Bender et al. 2001). Deletion of HS2 had the largest effect in adult erythroid cells, reducing gene expression levels to 65 % of wild type. On the other hand, deletion of HS5 and HS6 (Bender et al. 1998; Farrell et al. 2000) had little or no apparent effect on gene expression levels in adult erythroid cells. Collectively, these findings are consistent with the assertion that HS1–-4 all contribute to increasing gene expression levels but appear to achieve this via distinct mechanisms. Human HS2 can act as a classical enhancer, whilst HS1, HS3, and HS4 may increase the level of expression through chromatin-mediated, gene-specific, developmental or structural mechanisms.

6
Gene Competition and the LCR Holocomplex

An unusual phenomenon occurred when the adult β-globin gene was linked to the LCR in transgenic mice. Expression was seen in fetal and adult cells as expected, but abnormal expression of the β gene was also observed in embryonic cells (Enver et al. 1990; Hanscombe et al. 1991). Interestingly, placement of a γ gene between the β gene and LCR restored correct developmental expression of the β gene. In such LCR-γ/-β constructs γ was expressed normally in embryonic and early fetal cells while β was expressed in fetal and adult cells. Reversal of the gene order, i.e. LCR-β/-γ, led to co-expression of both genes in embryonic cells followed by fetal silencing of the γ gene. These experiments were interpreted as evidence of gene competition for the LCR and suggested a looping mechanism in which the LCR interacted preferentially with the nearest gene promoter in embryonic cells, thereby suppressing transcription of a downstream gene. One prediction of the gene competition hypothesis was that the LCR could activate only one gene in cis at any given moment. This was tested in single cells by RNA FISH (fluorescence in-situ hybridization) to detect γ- and β-primary transcripts in early fetal cells of transgenic mice carrying a single copy of a 70-kb construct (Wijgerde et al. 1995). At this stage nearly all cells co-express both the γ- and β-genes as shown by immunofluorescent detection of γ- and β-polypeptides (Fraser et al. 1993). However, RNA FISH showed that in the vast majority of cells only a single primary transcript signal was detected per locus. Since primary transcripts have a very short half-life, detection is indicative of ongoing or very recent transcription. Interestingly, a significant number of cells in homozygotes showed transcription of the γ-gene on one allele and transcription of the β-gene on the other, showing that each locus could respond independently and differently to the same *trans*-acting factor environment. Cells transcribing only the γ-gene were observed containing large amounts of the develop-

mentally late gene product β mRNA in the cytoplasm, demonstrating that transcription of the genes alternated or switched back and forth. These results supported the gene competition hypothesis and lead to the LCR flip-flop model in which it was proposed that the LCR formed semi-stable interactions with individual genes to activate transcription (Wijgerde et al. 1995). The competition mechanism was extended to suggest that developmental regulation of the entire cluster was accomplished through polar gene competition for the LCR. In the early stages, sequestration of the LCR by the ε- and γ-genes was implicated in the prevention of β-gene transcription. Silencing of the ε-gene in fetal cells would then allow the LCR to occasionally interact with the β-gene in competition with the γ-genes, and, finally, γ-gene silencing in bone marrow-derived cells led to sole expression of the β-gene by default. This scenario was further supported by transgenic experiments in which the ε-gene and flanking sequences were deleted and replaced with a marked β-gene (Dillon et al. 1997). In these mice the marked β-gene was transcribed in embryonic cells and partially suppressed γ-gene transcription. In fetal cells, γ expression was completely suppressed by the LCR-proximal β-gene as was fetal and adult transcription of the wild-type β-gene in its normal downstream position. Although it is clear from several experiments that gene competition for the LCR occurs, the suggestion that it is responsible for developmental regulation of the locus requires caution since in nearly every case the genes were removed from their normal epigenetic context (see below). Nevertheless these and other data led to the postulation of the LCR holocomplex theory (Ellis et al. 1996). This idea proposes that all the HS in the LCR interact to form a univalent nucleoprotein structure (the holocomplex) capable of interacting with and activating transcription of a single globin gene at one time.

7
The β-Globin Locus Resides in a Region of Tissue-Specific Open Chromatin

The β-globin loci of human and mouse are embedded in clusters of olfactory receptor genes (*Org*) that are transcriptionally silent in erythroid tissues (Bulger et al. 1999, 2000). The chicken β-globin locus is flanked on the 3′ side by *Org*s but has an erythroid-expressed folate receptor (*FR*) gene upstream (Litt et al. 2001a). General DNase I sensitivity of the β-globin locus has been assayed at various resolutions in the mouse, human and chicken. The chicken locus which is the most highly characterized in terms of DNase I sensitivity has four genes. The chicken LCR and globin genes lie in an approximately 30-kb region of open chromatin (Felsenfeld 1993; Litt et al. 2001b). Directly upstream of the LCR is a 16-kb stretch of relatively closed chromatin followed by the *FR* gene that is expressed in erythroid cells prior to β-gene activation (Prioleau et al. 1999). The human locus is also in a large region of open chro-

matin, but appears to be further divided into developmentally controlled subdomains of increased sensitivity to DNase I digestion (Gribnau et al. 2000). The LCR and active genes lie in regions of hyperaccessible chromatin, while the developmentally inactive globin genes are surrounded by chromatin of intermediate sensitivity compared to a non-erythroid gene. This pattern changes during development concurrent with the gene expression pattern. The mouse locus also resides in a relatively open chromatin region that spans approximately 150 kb from upstream of the –62.5 HS to downstream of 3′HS1 (Bulger et al. 2003). Some evidence from MEL cells suggests that the mouse locus is also divided into subdomains of differential sensitivity (Smith et al. 1984). The open region upstream of the mouse LCR contains a number of *Org*, which raises questions as to how these genes are kept silent in erythroid cells especially in view of their proximity to the LCR, which has been shown capable of activating heterologous genes.

8
The Role of Insulators

Insulators are sequence elements capable of enhancer blocking and/or chromatin barrier activity (Bell and Felsenfeld 1999). Several insulator elements have been described so far, but the most highly characterized vertebrate insulator is the chicken β-globin LCR element HS4. HS4 resides at the very 5′ end of the β-globin locus in chicken and marks the transition between the highly condensed chromatin upstream and the open chromatin of the globin locus (Hebbes et al. 1994; Prioleau et al. 1999). When HS4 flanks a transgene, the expression of that gene in transformed cells is stably maintained (Pikaart et al. 1998; Mutskov et al. 2002). In the absence of the insulators the transgene is silenced soon after transfection, a process associated with increased levels of H3/K9 methylation. Flanking HS4 insulators thus appear to possess a chromatin barrier function, which is thought to prevent the spread or influence of nearby repressive chromatin (Litt et al. 2001b; Mutskov et al. 2002). HS4 is also able to block the action of an enhancer when placed between the gene and enhancer in transfection assays. The enhancer blocking activity is mediated by the CTCF protein, and is separable from the chromatin barrier function (Recillas-Targa et al. 2002). Potential CTCF binding sites have been identified by sequence analysis in both human and mouse HS5 and 3′HS1 (Farrell et al. 2002). These sites have been assessed for CTCF binding in vitro and enhancer-blocking activity. Mouse 3′HS1 showed CTCF-binding activity comparable to chicken HS4, and only slightly lower enhancer-blocking activity (Farrell et al. 2002). Consistent with this data, it has been shown that the 3′ chromatin boundary of the mouse locus resides near 3′HS1 (Bulger et al. 2003). Human 3′HS1, and both human and mouse HS5, have an intermediate affinity for CTCF, and intermediate to low enhancer-blocking activity (Farrell et al. 2002). However, deletion of human and mouse HS5 from their endogenous positions

had little or no effect on chromatin structure, or globin, or *Org* expression (Reik et al. 1998; Farrell et al. 2000; Bulger et al. 2003), suggesting that these HS do not play a major role in locus organization or preventing LCR activation of the *Org* genes. Chromatin immunoprecipitation using antibodies against CTCF in mouse suggests that it may bind at the upstream –62.5 HS (Bulger et al. 2003). These HS did not show enhancer-blocking activity, but the transition of closed to open chromatin was mapped to a region closely upstream, suggesting they may be involved in the formation of a chromatin domain boundary (Bulger et al. 2003). Thus the role of insulators in the human and mouse globin loci is not entirely clear nor is the function of chicken HS4 in its endogenous position. It is possible that these loci may not be completely analogous and, therefore, it remains to be seen what role HS4 plays in the organization of the chicken β-globin domain and whether it prevents inappropriate activation of the upstream FR gene as suggested by its chromatin barrier and enhancer-blocking activities seen in transfection experiments.

9 Intergenic Transcription

Intergenic transcripts were first described in the β-globin locus by Imaizumi et al. (1973) as long RNA species encompassing both intergenic and gene sequences. These giant transcripts were interpreted as examples of eukaryotic polycistronic pre-mRNAs, similar to the prokaryotic polycistronic RNAs that had recently been discovered. The discovery of β-globin primary transcripts and intron splicing led to the rejection of the vertebrate polycistronic transcript theory, and intergenic transcripts were largely dismissed as an artefact. However, β-globin intergenic transcripts re-emerged in the 1990s (Tuan et al. 1992; Ashe et al. 1997; Kong et al. 1997; Gribnau et al. 2000) and were shown to be rare, long, erythroid-specific, nuclear-restricted RNA molecules, encompassing both the LCR and intergenic regions. The direction of intergenic transcription in the locus is the same sense direction as gene transcription (Ashe et al. 1997). Subsequent work showed that intergenic transcripts in the human β-globin locus are developmentally specific (Gribnau et al. 2000). At least three subdomains have been defined in the human β-globin locus in transgenic mice on the basis of intergenic transcript abundance. The LCR subdomain, which is devoid of genes, is transcribed throughout development, while transcription of the embryonic subdomain containing the ε- and γ-genes, is five- to ten-fold higher in embryonic cells compared to adult cells. Transcription of the adult subdomain encompassing the δ- and β-genes is very low in embryonic cells and five- to ten-fold higher in fetal and adult cells (Gribnau et al. 2000). The same adult stage-specific pattern of intergenic transcripts seen in the human transgene locus is also found in the endogenous human locus, in ex vivo cultured adult erythroid precursors (Goyenechea et al., unpubl. observ.; Miles and Fraser, unpubl. observ.). Similar developmental changes in

intergenic transcription are also detected in the mouse locus (Chakalova et al., unpubl. observ.). The most intriguing aspect of the intergenic transcription pattern was its precise correlation with areas of increased general DNase I sensitivity. Intergenic transcripts delineate the highly accessible chromatin domains that surround the active genes and the LCR HS (Gribnau et al. 2000). Recently, several groups have focused their attention on defining the start sites of intergenic transcription.

10 Intergenic Promoters

The intergenic transcription initiation site for the adult subdomain is located approximately 3 kb upstream of the δ-gene in the human β-globin locus in transgenic mice by 5′ RACE (Gribnau et al. 2000). The upstream region (referred to as the δβ promoter) does not contain a canonical TATAA element or typical gene promoter elements. The δβ promoter behaves like a weak promoter when linked to a promoter-less EGFP reporter gene in stable transfection assays (Debrand et al. 2004). The level of EGFP expression per cell is much lower with the δβ promoter compared to a gene promoter; however, a much larger percentage of cells express EGFP under the δβ promoter in comparison to a gene promoter. In the β locus, non-coding transcripts initiated from the δβ promoter presumably extend through the entire δβ domain since removal of the element leads to extinction of all intergenic transcription throughout the subdomain (Gribnau et al. 2000; Debrand et al. 2004).

Intergenic promoters in the εγ domain, active in embryonic red cells, have not yet been pinpointed. Initiation in the LCR subdomain appears to be much more complicated. Multiple sites within the LCR are capable of initiating transcription (Tuan et al. 1992; Kong et al. 1997; Leach et al. 2001; Routledge and Proudfoot 2002). Human HS2 gives rise to sense intergenic transcripts in erythroleukaemia K562 cells (Kong et al. 1997). Moreover, HS2 enhancer activity appears dependent on its ability to produce intergenic transcripts (Tuan et al. 1992). Intrinsic intergenic promoter activity has also been shown for human HS3 (Leach et al. 2001; Routledge and Proudfoot 2002). Upstream of the LCR, transcripts are initiated from a human endogenous retroviral LTR element located approximately 1 kb upstream of HS5 (Long et al. 1998; Plant et al. 2001). The properties of the LTR element have been partially dissected by transient transfection analysis. The LTR fires long non-coding transcripts that read through a downstream gene promoter, and that correlate with high-level synthesis of the coding gene mRNA. Reversal of the direction of intergenic transcription away from the gene drastically reduces initiation from the gene promoter (Long et al. 1998), showing that the LTR possesses an intrinsic, directional, enhancer-like activity associated with transcriptional sense. This conclusion was most elegantly demonstrated by experiments in YAC trans-

genic mice in which the LCR was inverted with respect to the genes by cre recombinase (Tanimoto et al. 1999). LCR inversion severely reduced expression of all the β-genes.

11 Histone Modification and Developmental Globin Gene Expression

In recent years, much attention has focused on the correlation between post-translational covalent modifications to the amino-terminal tails of histones and gene expression status. The first demonstration of a link between histone hyperacetylation and an 'open', DNase I-sensitive, chromatin structure was in the chicken β-globin chromosomal domain (Hebbes et al. 1994). Chromatin immunoprecipitation (ChIP) revealed a broad pattern of acetylation over the 33 kb of DNase I-sensitive chromatin containing the genes and LCR. Both the transcriptionally active and previously active globin genes were found to be acetylated; however, later analyses suggested that some of the developmentally early genes picked up histone H3 lysine 9 (H3/K9) dimethylation, indicative of inactive chromatin (Litt et al. 2001a). Using antibodies to a range of acetylated histone isoforms and cell lines representing different stages of erythroid maturation, changes in the pattern of acetylation during differentiation of the chicken β-globin locus and the neighbouring FR gene were demonstrated (Litt et al. 2001b). Condensed chromatin and developmentally inactive genes maintained the lowest acetylation, whilst activation of genes correlated with a dramatic increase in acetylation (Litt et al. 2001b). Using antibodies to dimethylated H3/K4 revealed a perfect correlation to the pattern of histone acetylation. Dimethylation of H3/K9, however, was inversely correlated.

Tissue-specific histone acetylation has also been demonstrated for the mouse and human β-globin loci. Chromatin immunoprecipitation of the murine β-globin locus in both primary tissue (fetal liver) and MEL cells revealed an enrichment of histone H3 and H4 acetylation over the locus control region (LCR) and transcriptionally active βmaj and βmin gene promoters, whilst the region encompassing the silenced embryonic genes εy and βH1 was relatively hypoacetylated (Forsberg et al. 2000). A higher resolution analysis of histone modification across the murine β-globin locus in adult anaemic spleen demonstrated the presence of subdomains of acetylation and histone H3/K4 dimethylation over the LCR and the active βmajor and βminor genes (Bulger et al. 2003). It was observed that in contrast to the chicken β-globin locus, the pattern of histone acetylation and H3/4 dimethylation did not precisely correlate with nuclease-sensitivity. There appeared to be a large 150-kb domain of nuclease sensitivity.

The histone H3 and H4 acetylation pattern of the human β-globin locus has, until recently, only been analysed on human chromosomes that have

been transferred into MEL cells (Schubeler et al. 2000). Assessing a limited number of sites, the authors detected basal H3 and H4 acetylation throughout the locus, with peaks of H3 acetylation at the LCR and the active β-globin gene promoter. The peak of H3 acetylation at the promoter was lost in a mutant locus with a deletion of HS2–5 of the LCR; however, the general pattern of H3 and H4 acetylation was maintained.

High-resolution ChIP analysis of the human β-globin locus during development in transgenic mice and in human adult primary erythroid cells has revealed subdomains of histone H3 and H4 acetylation and also H3 K4 di- and trimethylation (Miles and Fraser, unpubl. observ.). In embryonic blood the enriched domains comprise the LCR and the transcriptionally active ε- and γ-genes. These modifications correlate with the occurrence of intergenic transcription and increased DNase I general sensitivity. In adult cells two clear domains of modified chromatin exist surrounding the LCR and the δ- and β-genes, again matching precisely the pattern of intergenic transcription and increased general DNase I sensitivity. Thus a tight correlation between 'active' histone modifications, intergenic transcription, and nuclease sensitivity demarcate developmentally regulated subdomains in the human β-globin locus.

12
The Role of Intergenic Transcription

The question of the functional significance of intergenic transcription has been addressed by analyzing a number of deletions of the δβ promoter. A 2.5-kb deletion was first studied in transgenic mice (Gribnau et al. 2000). As expected, intergenic transcription in the adult domain is lost in the mutant locus. More importantly, the adult domain fails to open to a DNase I hyperaccessible structure in definitive erythroid cells, showing that the deleted region is essential for chromatin remodelling of the adult domain. Consequently, transcriptional activation of the β-gene is also abolished. In contrast, developmentally specific transcription of the ε- and γ-genes is normal, indicating that the defect is specific only for the adult domain. To exclude the possibility that other elements in the deleted region contribute to the observed phenotype, a specific 300-bp deletion was engineered with cre/lox technology that includes the minimal δβ promoter. The resultant phenotype exactly matches that of the 2.5-kb deletion. The ε- and γ-genes are highly transcribed in all embryonic erythroid cells, but β gene transcription in fetal and adult stages is dramatically reduced. In addition, the adult domain does not acquire the developmentally specific histone modifications normally present across intergenic and gene regions (H3/K4 di- and trimethylation, and H3 and H4 acetylation). These results show that domain-wide chromatin remodelling is dependent on the δβ promoter, strongly suggesting that transcription leads to remodelling. This concept is further strengthened by biochemical studies,

which show that ATP-dependent remodelling complexes, HATs, and histone methyltransferases are associated with the elongating RNA polymerase II complex (Orphanides and Reinberg 2000). Furthermore, transcription through chromatin by RNAP II has been shown to be highly disruptive, leading to partial disassembly of nucleosomes in the wake of the polymerase complex (Studitsky et al. 2004). This presents an ideal opportunity to modify existing nucleosomal tails and/or insert replacement histones associated with active chromatin. Thus intergenic transcription appears to be part of the mechanism of an essential, processive, remodelling machine, which disrupts chromatin fibres and modifies histones through the specific activities associated with the RNAP II complex.

13 The Cell Cycle Connection

Intergenic transcription is not continuous in erythroid cells. Approximately 15–25 % of globin loci show intergenic signals in a non-synchronized population of erythroid cells assessed by RNA FISH. A number of assays have been used to show that intergenic transcription is cell cycle regulated, occurring primarily in G1 phase cells and also in early S phase (Gribnau et al. 2000). Some evidence suggests that the G1 phase intergenic transcription occurs directly after cells exit mitosis, while S phase-specific intergenic transcripts are detectable on newly replicated globin alleles in early S phase. These are major potential control points in chromatin remodelling and reorganization in the cell cycle. In the first hour after mitosis, active gene loci are rapidly decondensed and correct nuclear positioning is established (Dimitrova and Gilbert 1999; Thomson et al. 2004). After DNA replication of the globin locus in early S phase, epigenetic marks need to be re-established on newly replicated daughter alleles. Thus cell type-specific chromatin states could be implemented and/or existing epigenetic conformations maintained through this process. A replication-independent chromatin assembly pathway has been proposed, which includes transcription-coupled histone replacement. This process is thought to occur in active chromatin through replacement of histone H3 with the variant histone H3.3 during transcription (Ahmad and Henikoff 2002a,b). Interestingly, H3.3 is highly enriched in modifications associated with transcriptionally active chromatin, such as methylated H3/K4, and deficient in heterochromatin-specific marks such as H3/K9 methylation (McKittrick et al. 2004). In *Drosophila* cells nearly all H3/K4 methylation is found on H3.3, suggesting that detection of H3/K4 methylation (as seen in intergenic transcription domains in the globin locus) is indicative of the replication-independent chromatin assembly pathway. Nucleosome replacement also provides a mechanism for the activation of domains that are silenced by presumably stable histone methylation marks.

14
The Corfu Deletion

Over the years the many naturally occurring mutations in the β-globin locus have provided a rich source of information regarding the effects of various sequence elements on globin gene regulation (Collins and Weissman 1984; Stamatoyannopoulos and Grosveld 2001). The Corfu mutation involves a 7.2-kb deletion that removes the 5′ part of the δ-globin gene and several kilobases of upstream sequence (Kulozik et al. 1988) including the δβ intergenic promoter responsible for transcription of the adult subdomain (Fig. 2). This is the smallest known deletion which removes a critical 1-kb region that appears to be the minimal region of difference between deletions that cause HPFH (hereditary persistence of fetal haemoglobin) or β thalassaemia (Collins and Weissman 1984). HPFH is a relatively benign condition characterized by elevated pancellular expression of fetal haemoglobin (HbF; product of the γ genes) in adult cells, while β thalassaemia can be a life-threatening anemia resulting from abnormally low β-gene expression coupled with normal low expression of the γ genes (Stamatoyannopoulos and Grosveld 2001). Deletions that remove the δ- and β-genes result in thalassaemia, whereas similar mutations that also delete the critical 1-kb region, located approximately 3 kb upstream of the δ-gene, often result in an HPFH phenotype. The Corfu mutation has interesting phenotypic consequences depending on gene dosage. Corfu heterozygotes have moderately reduced β-gene expression and normal low-level γ expression (~2 %). Paradoxically, Corfu homozygotes exhibit greatly elevated HbF (80–90 %) and barely detectable HbA (product of the β-gene). We have shown that the Corfu deletion which removes the intergenic δβ promoter results in high-level transcription of the fetal γ-genes on the affected chromosome in both heterozygotes and homozygotes by RNA FISH (Chakalova et al., unpubl. observ.). Post-transcriptional regulation of γ mRNA appears to be responsible for the low level of γ expression in heterozygotes

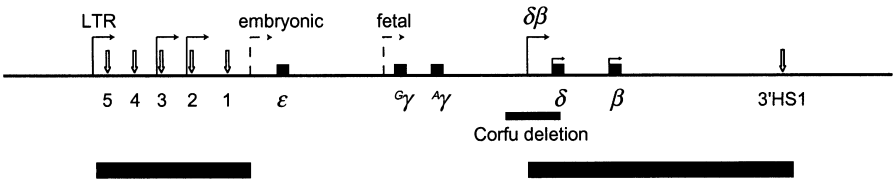

Fig. 2. Features of the human β-globin locus in adult erythroid cells. Active chromatin subdomains are represented by *large black boxes*. These domains show high levels of intergenic transcription, nuclease hyperaccessibility, and histone modifications indicative of active chromatin, such as H3 and H4 hyperacetylation, H3/K4 methylation. Active δ- and β-gene promoters are denoted by *small arrows*. *Large arrows* represent intergenic promoters across the locus: LTR, HS3, HS2 and δβ promoter. Putative embryonic and fetal intergenic promoters are shown as *dashed arrows*. The Corfu deletion is shown as a *thick line below the map*

which seems to be de-repressed in homozygotes. Intergenic transcription analysis by RNA FISH reveals that intergenic transcripts in the δβ domain of the deleted allele appear to initiate approximately 16 kb upstream of the breakpoint, 5′ of the γ-genes (Goyenechea et al., unpubl. observ.). This entire 16-kb region which is normally in a closed conformation and unmodified in adult cells exhibits increased DNase I sensitivity, hyperacetylation of H3 and H4, as well as increased H3/K4 trimethylation. Thus it appears that deletion of the normal 3′ boundary of the γ domain results in a fusion of the fetal and adult domains and persistent activation of the intergenic promoter upstream of the fetal γ-genes. This persistent intergenic transcription through the γ domain is thought to unmask the γ-genes, allowing greater access to the adult transcription factor environment, and perhaps equally importantly recognition and interaction with the upstream LCR leading to high-level γ-gene transcription in adult cells in competition with the β-gene.

15
Higher Order Folding and Long-Range Regulation

High-level transcription of the globin genes is absolutely dependent on the LCR. Although much indirect evidence suggested direct physical contact between the LCR and genes as the mechanism of transcriptional activation, direct evidence was lacking. Recently, two different techniques have been used which have come to the same general conclusion: the LCR functions through direct contact with the globin genes. The first method, RNA TRAP (tagging and recovery of associated proteins), is a modification of the RNA FISH method (Carter et al. 2002). Briefly, hapten-labelled RNA FISH probes are hybridized to nascent transcripts of an actively transcribing globin gene. Antibodies coupled to horseradish peroxidase (HRP) recognize and bind the haptens on the RNA FISH probes, concentrating HRP activity to the site of the transcribing gene. The HRP is used to catalyze the activation of a biotin-tyramide molecule, which covalently attaches and thus deposits biotin on chromatin proteins in the immediate vicinity of the transcribing gene. Biotinylated proteins and associated DNA are then purified and the enrichment of various DNA sequences measured. RNA TRAP directed towards either the βmaj or βmin genes reveals that the classical enhancer component of the LCR, HS2, is in proximity to both genes when they are actively transcribed. The other HS (HS2, HS3 and HS4) appear to be more peripheral, or only transiently associated with the gene, suggesting a major role for HS2 in transcription. This is consistent with individual HS deletion studies, showing that deletion of HS2 leads to the largest drop in expression compared to the other HS (Fiering et al. 1995; Hug et al. 1996; Farrell et al. 2000; Bender et al. 2001). The only HS found in proximity to the active genes are HS1–4, totally consistent with functional studies, showing that individual deletion of these HS has an effect on gene expression (Fiering et al. 1995; Hug et al. 1996; Bender et al. 2001). Deletion of HS5 and HS6, which

were not found in proximity to the genes by RNA TRAP, have been shown to have no measurable effect on adult β-globin expression when deleted (Farrell et al. 2000). The HS at –62, –60 and 3′HS1 also were never found in proximity to the transcribing gene, suggesting no direct role in transcription. The spatial arrangement of the different HS may give insight into their function in vivo. For example, the proximity of HS2 to the active genes may represent a direct interaction with the transcriptional apparatus, whilst the more peripheral positions of HS1, HS3 and HS4 could represent a different role in adding to the level of transcription, perhaps by modulating chromatin or stabilizing the interaction through binding to flanking sequences. However, it is also possible that HS1, HS3 and HS4 are closely associated with the active gene (as is HS2) but are only required transiently for activation.

The CCC (chromosome conformation capture) method involves cross-linking chromatin in cells followed by restriction digestion and ligation under dilute conditions, which favour intramolecular ligation (Dekker et al. 2002). Distant genomic regions that are often in contact, and thus have high cross-linking frequencies, are then detectable by PCR across the novel ligation junctions. Though the resolution of CCC is somewhat poor compared to RNA TRAP, the active β-globin genes were seen to ligate to the LCR at a higher frequency than the inactive genes, again providing direct evidence for LCR–gene contact (Tolhuis et al. 2002). However, unlike RNA TRAP, the CCC data suggested contact between many other sequence elements such as the HS at –62 and –60 (approximately 35 kb upstream of the LCR) as well as HS5 and HS6 of the LCR and 3′HS1 located approximately 25 kb downstream of the βmin gene. These data were interpreted as evidence of an active chromatin hub consisting of a cluster of all the HS over the 150-kb region studied playing a role in initiating and maintaining a structure conducive to transcription.

16
Nuclear Organization

As is the case with other genes, nuclear compartmentalization and organization appears to impact β-globin gene regulation. Both the human locus on chromosome 11 and the mouse locus on chromosome 7 reside in regions of high gene density. Bickmore and colleagues have demonstrated a correlation between the gene density of regions in which a particular gene resides and its nuclear position relative to the chromosomal territory (Mahy et al. 2002b). They found that genes on human chromosome 11 and mouse chromosome 7, in the gene-rich region containing the globin genes, tend to be located on the edge of the territory, or indeed outside the territory, presumably looped out from the intensely stained territorial mass. In contrast, genes located in relatively gene-poor regions tend to reside within the bulk of the territory. Although transcription may be able to occur within the central regions of the territory, extraterritorial positioning appears also to relate to transcriptional

status or potential (Mahy et al. 2002a). Treatment of cells with transcriptional inhibitors results in movement of extraterritorial regions to more territory-proximal positions. Whereas the β-globin locus has been observed in a slightly internal position relative to the chromosome territory and adjacent to centromeric heterochromatin in lymphoid cells (Brown et al. 2001), it is often in an extraterritorial position in MEL cells (Ragoczy et al. 2003).

The LCR is likely to contribute to functional positioning of the β-globin locus. The human locus does not adopt an extraterritorial position in MEL cell hybrids when the LCR has been deleted from chromosome 11 (Ragoczy et al. 2003). Furthermore, the Hispanic thalassaemia deletion, which removes the LCR and approximately 30 kb of upstream sequences including the LTR, causes the locus to locate to centromeric heterochromatin (Schubeler et al. 2000), whereas loci with deletions that remove only parts of the LCR remain positioned away from centromeric heterochromatin. This suggests that positioning away from repressive chromatin environments may be linked to sequences outside the LCR.

17 Summary Model

Obviously, there is still much to learn regarding the gene activation pathway, and though there are many gaps we are not deterred from proposing a general hierarchy of events. The initial pre-activation of the β-globin locus domain during erythroid differentiation in human and mouse most likely involves chromatin modifications over a region of approximately 150 kb beginning tens of kilobases upstream of the LCR and extending down to 3′HS1. This could probably best be described as a poised conformation in which the entire locus is relatively decondensed compared to a non-erythroid locus, but not quite as open as an active subdomain during gene expression. The HS at –62 and –60 in mouse and the potential human homologue at approximately –110 kb may play a role in this initial opening along with other elements in the locus (Bulger et al. 1999; Palstra et al. 2003). Intergenic transcription may be involved in setting up this initial domain through very long, S phase-specific intergenic transcription through the entire region. These changes may be involved in preventing the globin locus from associating with repressive centromeric heterochromatin. As erythroid differentiation proceeds and the globin genes become activated, non-S-phase intergenic transcription in the LCR and the developmentally appropriate active gene subdomains may promote histone replacement with variant histone H3.3 and hyperacetylation of H3, H4 and H3/K4 methylation. These modifications may allow increased factor access and additional modifications of histones in promoter regions, for example. This would promote LCR recognition of, and dynamic interaction with, the appropriately modified gene(s) while ignoring intervening, distal, or upstream genes lacking the proper conformation or modifications. The

LCR–gene complex may promote extension of the locus beyond the confines of the chromosome territory as a result of, or in order to facilitate, highly efficient transcription of the globin genes.

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