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REVIEW

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The β cell transcription factors and development of the pancreas

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Abstract The pancreatic β cell is the major source of circulating insulin in adult mammals. In the multistep process of insulin synthesis it is initiation of transcription that restricts insulin synthesis to the β cell since all subsequent steps can be performed by other cell types. Many of the transcription factors that bind to the insulin promoter and activate insulin gene transcription have been isolated. Some of these factors are restricted in their expression pattern, but so far no truly β cell-specific transcriptional activator has been found. Since different transcription factors synergize to activate insulin gene transcription, cell-specific transcription of insulin is probably realized through the interactions of a unique set of regulatory proteins in the β cell. The same transcription factors that regulate insulin gene transcription in the adult β cell are involved in determining cell differentiation during pancreatic development. The endocrine and exocrine pancreas form from the gut endoderm as a dorsal and a ventral bud which later fuse to build a single organ. The homeodomain protein PDX-1, an insulin gene transcription factor, is uniformly expressed in the early pancreatic bud, and null mutation of PDX-1 in mice results in a failure of the pancreatic bud to grow and differentiate. Other transcription factors, such as the helix-loop-helix protein Beta-2 and the homeodomain protein Nkx 6.1, show a restricted pattern of expression during embryogenesis and in the mature islet. Those proteins may serve a dual role for the organism: during embryogenesis they may determine islet cell differentiation and in the adult they may ensure tissue-specific expression of the islet cell hormones. A better understanding of the factors involved in insulin gene transcription and islet cell differentiation will ultimately provide the basis for novel therapy of diabetes.

Key words Pancreas · Islet · β Cell · Insulin · Transcription · PDX-1 · Development

Abbreviations *bHLH* Basic helix-loop-helix · *HNF* Hepatic nuclear factor · *NRE* Negative regulatory element · *PP* Pancreatic polypeptide

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Introduction

The β cells in the pancreatic islets of Langerhans synthesize and secrete insulin, the key regulator of glucose ho-

meostasis. Decreased insulin secretion or impaired insulin action leads to one of the major world health problems, diabetes mellitus. Diabetes mellitus causes early death and prolonged ill health from severe complications including kidney, cardiovascular, and eye disease. Because of its central role in metabolic control, tight regulation of insulin production and release is critical.

In adult mammals insulin production is limited to β cells, which account for the majority of the islet cells. The islets of Langerhans are unique in that each of the four phenotypically distinct cell types produces a distinct hormone. α, δ, and pancreatic polypeptide (PP) cells secrete glucagon, somatostatin, and PP, respectively. With the possible exception of very low level expression in the central nervous system [1], the β cell is the only tissue in adults that synthesizes preproinsulin mRNA, but any cell, if altered to allow transcription of the insulin gene, can produce proinsulin [2, 3]. Other endocrine cells, similarly altered, can even process, package, and secrete mature insulin in a regulated fashion [4]. Since non-β cells can perform all subsequent steps, it is initiation of transcription that restricts insulin production to the β cell.

The combined efforts of a number of laboratories have provided important clues regarding the control of insulin gene transcription. Several models have been proposed to explain the mechanisms for β cell specific expression of the insulin gene. However, little is known thus far about the developmental processes that lead to differentiation of islet precursor cells into mature β cells. We are just beginning to understand that the key transcriptional regulators of the insulin gene in mature β cells are also involved in producing the differentiated phenotype during development. With the advances in islet cell transplantation as a potential treatment of diabetes mellitus, interest in the factors controlling pancreatic growth and differentiation has been renewed.

Insulin gene expression

A cDNA copy of the preproinsulin mRNA was first cloned by Ullrich and colleagues [5] from a rat islet cDNA library. Since then, insulin genomic and cDNA clones have been isolated from a variety of animal species from human to sponges [6–8]. The human insulin gene is located on chromosome 11p15.5, between the tyrosine hydroxylase (TH) and insulin-like growth factor-2 (IGF2) genes [9]. The human insulin gene contains three exons and two introns. The final spliced mRNA transcript is 446 bp in length and codes for the preproinsulin peptide. The structure of the insulin gene has been remarkably well conserved throughout evolution. Most animals have a single copy of the insulin gene, with the exception of rat and mouse which carry a duplication of the gene [10].

Evolutionary conservation of the insulin gene extends to the promoter sequence, which is critically important for insulin expression, as it determines the transcription start site for RNA polymerase and regulates the rate of transcription initiation [11]. The promoter consists of all the 5′ flanking DNA necessary for appropriate initiation of transcription. The exact 5′ end of the promoter is not well defined, but it is known that sequences at least 4 kb upstream contribute to the regulation of transcription [12, 13]. The insulin gene promoter and the transcription factors binding to this region largely determine cell-specific expression of the insulin gene in the β cell of the pancreas.

When isolated from the remainder of the gene and linked to a reporter gene, such as the bacterial gene for chloramphenicol acetyl-transferase (CAT), 400 bp of the insulin promoter direct expression of CAT exclusively to the β cell [14, 15].

The promoter also functions in a distinctly cell-type specific manner in transgenic mice. By linking the rat insulin II promoter to the coding sequence for the simian virus 40 large T-antigen, Hanahan [16] developed a transgenic mouse line in which the insulin promoter directed expression of this viral oncogene. These animals express T antigen exclusively in their β cells and develop insulin-producing islet cell tumors, demonstrating the cell-specific function of the insulin promoter in vivo.

What are the mechanisms that allow expression of the insulin gene exclusively in the β cell and prevent transcription in any other cell type? The insulin gene promoter contains multiple sequence elements that act as recognition sites for DNA binding proteins. Figure 1 shows the relative positions of some of the *cis*-acting elements along the insulin promoter and some of the transcription factors that bind to these elements. The nuclei of β cells contain numerous distinct protein complexes that bind specific sequences within the insulin promoter. Some of these complexes are restricted to β cells or a small subset of cell types. However, accumulating evidence suggests that no single protein complex alone accounts for cell-specific expression. It is more likely that the specific combination of transcription factors in the β cell is unique, and that their interactions are required to activate transcription of the insulin gene.

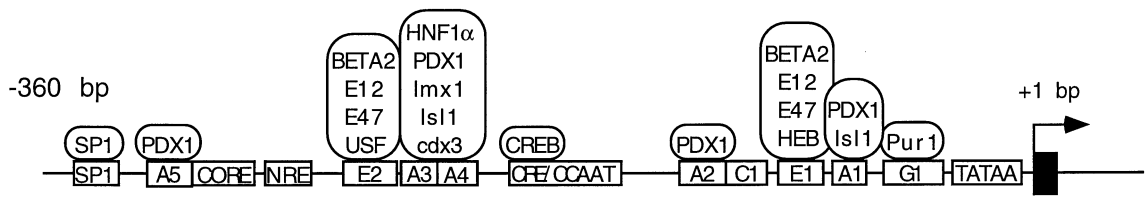

Fig. 1 A composite insulin promoter with its known *cis*-acting elements and binding factors. The boxes represent the DNA sequence elements. *Circle (above the promoter)*, cloned binding proteins

Table 1 Transcription factors expressed in islet cells

Class	Transcription factor	Tissue distribution	Insulin promoter binding site
bHLH	E12/E47	Ubiquitous	E box
	HEB	Ubiquitous	E box
	Beta-2	Islet, brain	E box
	Beta-3	Islet, lung, kidney, brain	
Homeodomain	PDX-1	Islet, duodenum	A box
	Cdx-3	Islet, intestine, testis	A box
	HNF1α	Islet, liver	A box
	Nkx 6.1	β cell	
	Nkx 2.2	Islet, pancreas, brain	
	alx3	Islet, pancreas, testis	
	Pax-6	Embryo: islet, CNS, eye, nose	
	Prox 1	Embryo: pancreas, liver, heart, CNS	
	Hox11	Embryo: pancreas, spleen, CNS	
LIM homeodomain	Lmx-1	Islet, limb bud	A box
	Isl-1	Islet, motor neurons	A box
	Lim1	Islet, pancreas, brain, liver, testis	
Zinc finger	Pur-1	Ubiquitous	G1 element

E box elements

Two sequence elements, E1 and E2, within the rat insulin I promoter are crucial for transcriptional activity of the whole promoter. Mutation of either of the two elements results in a 90% loss of promoter activity when transfected into a hamster insulinoma tumor cell line. A double mutation of the two elements effectively abolishes transcription [17]. These two elements coincide with identical 8 basepair sequence motifs GCCATCTG at –105 and –231 bp of the rat insulin I promoter. The more proximal motif is well conserved within the insulin promoter of various mammals [11, 18], and its importance has also been demonstrated for the rat insulin II [19, 20] and the human insulin promoters [21, 22].

This sequence element belongs to the class of regulatory sites known as E boxes, which contain the consensus CANNTG and are implicated in the tissue-specific regulation of genes in a variety of tissues [23–25]. E boxes bind factors belonging to the basic helix-loop-helix (bHLH) family of transcription factors. The HLH motif is defined by two amphipathic helices that act as dimerization domains, separated by a nonconserved loop and adjacent to an aminoterminal basic region necessary for DNA binding [26, 27].

The E box sequences bind a specific nuclear protein complex that is found in α cells and pituitary cells as well as β cells but is absent from a variety of non-endocrine cells. [28–32]. This insulin E box binding complex contains various members of class A bHLH proteins: E47/E12 [26, 31, 33–35] and HEB [36, 37]. E47 and E12 are differential RNA splicing products of the same gene, *E2A* [38]. Class A members of the bHLH proteins are ubiquitously expressed; therefore their presence in the insulin E box binding complex does not explain the tissue restriction of this protein complex. The bHLH proteins bind to DNA as dimers, and heterodimers between the ubiquitously expressed class A members and the tissue-restricted class B bHLH protein members function as potent transcriptional activators of tissue-specific genes. The cell-restricted class B members of bHLH proteins play an important role in specifying cell fate in a variety of different tissues [39, 40].

Despite intensive efforts by several laboratories to isolate cDNAs encoding putative islet-specific bHLH proteins, conventional expression screening strategies proved to be unsuccessful. Recently Naya et al. [41] succeeded in cloning an islet-specific class B bHLH factor, termed Beta-2, from a hamster insulinoma tumor cell cDNA library by using a modified yeast two-hybrid selection method. Beta-2 proved to be the same as the neural factor NeuroD [42]. Beta-2 expression is, apart from brain, indeed limited to pancreatic β and α cell lines and is a component of the native insulin E box binding complex (Table 1) [41]. Analogous to the induction of muscle cell differentiation by myogenic bHLH proteins [23], Beta-2 may play an important role in neural and islet cell development and differentiation. Construction and analysis of mice deficient for Beta-2 will undoubtedly give valuable insight into the function of this factor in vivo.

A elements

In the rat and human insulin promoter, A-T rich elements, termed A elements, are juxtaposed to the E1 and E2 elements. The human insulin promoter contains an additional A site upstream of E2. Mutation of the rat insulin I A3/A4 site or the corresponding A3 site in the human insulin promoter leads to a 75% loss in promoter activity, whereas mutation of the A1 site only modestly affects insulin transcription [17, 43, 44].

The A elements all share the core sequence TAAT, a recognition motif for homeodomain proteins. Homeodomain proteins are a group of transcription factors that, among other functions, specify the body plan and regu-

late development of higher organisms [45]. They share a common 61 amino acid motif that forms a helix-turn-helix structure, the homeodomain, that binds to DNA and is highly conserved among different species.

β Cell nuclei contain several different protein complexes that bind to A sites [21, 46–48]. Several of these proteins have been cloned, and all of them are members of the homeodomain class of proteins (Table 1) [48–54].

PDX-1, also called IPF-1, IDX-1, or STF-1, binds to all of the A sites in the rat and human insulin promoter and can activate the insulin promoter in non-β cells [44, 51, 55] (Odagiri and German, unpublished data). In the adult, expression of PDX-1 is largely limited to the pancreatic β cell and the duodenal epithelium, with occasional expression in somatostatin producing cells [51, 53, 56].

Another islet cell-specific homeodomain protein, Lmx 1.1, has been isolated by our laboratory [48]. Lmx 1.1 belongs to the class of LIM homeodomain proteins, which contain two cystein-histidine rich LIM domains. LIM domains have been implicated in protein-protein interaction [57] and may inhibit binding of the homeodomain to DNA [58, 59] or inhibit the transcriptional activation domain [48]. As with PDX-1, Lmx1.1 can activate the insulin promoter in non-β cells [48].

Other homeodomain proteins that bind to the A sites show a less tissue-restricted pattern of expression. Cdx-3 can be found in intestine and testes [48], and hepatic nuclear transcription factor (HNF) 1α in liver as well as β cells [50]. Cdx-3 can also activate the insulin promoter in non-β cells. The LIM homeodomain protein Isl-1 binds to the A3 site with significantly lower affinity than Lmx-1.1 and is expressed in a variety of endocrine and nonendocrine cells and in the central and peripheral nervous system [60, 61]. Even though all of these proteins were isolated from β cells, their function does not seem to be limited to activation of insulin gene transcription. Other β cell and islet genes, including glucokinase [62], amylin [63], glucagon [64], and somatostatin [65], contain similar homeodomain protein recognition sites in their 5′ flanking region. Isl-1 has been shown to activate transcription of the somatostatin, glucagon, and amylin genes [65–67]. Similar to its effect on the insulin gene promoter, Cdx-3 can activate the glucagon promoter in non-islet cells [68].

Other homeodomain proteins have been cloned from β cells, but their ability to bind to the insulin A boxes and to transactivate the insulin gene promoter has not been studied to date [54, 69–71]. Expression of some of these genes is restricted to a limited number of cell types, such as Nkx 6.1, which has been detected only in β and α cell lines [54].

The relative contributions of the different homeodomain proteins to β cell-specific transcription of the insulin gene are unclear, but it appears unlikely that one protein alone confers the specificity. Most likely, several transcription factors contribute to insulin gene transcription, and these contributions may change depending on the stage of development or physiologic setting.

Negative regulation of insulin transcription

In addition to transcriptional activation by *cis*-acting elements in β cells, the concept of negative regulation of insulin expression has been explored in non-β cells. Such repressors of insulin gene transcription may contribute to the maintenance of tissue specificity. *Cis*-acting negative regulatory elements that function in non-β cells have been identified in the human [72], the rat I [12, 73] and the rat II insulin promoter [20]. The E1 box in particular seems to bind factors that repress activity in non-β cells [35].

The purpose of a negative regulatory element that functions in β cells is more obscure. Deletion analysis of the human insulin promoter by Boam et al. [21] have shown that the region between –279 and –258 bp acts as a negative regulator of transcription in a β cell tumor line. Since the same region can also repress transcription when linked to a heterologous promoter [21, 74], the authors postulated a negative regulatory element (NRE) in the distal part of the human insulin promoter. When Walker et al. [14] transfected similar truncations of the human insulin promoter in β cells tumor lines, they found no rise in transcriptional activity. Also in contrast to Boam et al. [22], we have noted that deletion of the NRE results in a marked loss of promoter activity in primary-cultured rat islet cells. To resolve this contradiction we constructed a minienhancer that contained five copies of the NRE linked to a heterologous promoter. In agreement with the findings of Boam et al., this minienhancer acts as a negative regulator of transcription in a variety of β cell and non-β cell tumor lines and in primary non-β cells. By contrast, the NRE functions as potent activator of transcription in primary cultures of rat β cells [75]. These conflicting results demonstrate that tumor cells may not always reflect normal islet physiology.

Other *cis*-acting elements

At least one other element that is well conserved within the insulin promoter of different species may contribute to β cell specific transcription of the insulin gene. The C1 element lies between the A2 and E1 elements, and mutation of this element in the context of the rat insulin II promoter causes a drastic loss of activity [19, 76]. The C1 site binds a unique nuclear complex that is absent from non-islet cells and even from α cells [31] (Odagiri and German, unpublished data). However, the proteins binding to the C1 site have not yet been cloned.

Several other binding sites have been identified. Some of them, such as the Sp1 site, are not well conserved among species. The importance of others, including the cAMP-regulatory element and the core element, for insulin gene transcription has not been well established.

Mechanisms specifying transcription

The simplistic view of an enhancer as a chain of elements with transcription factors independently activating

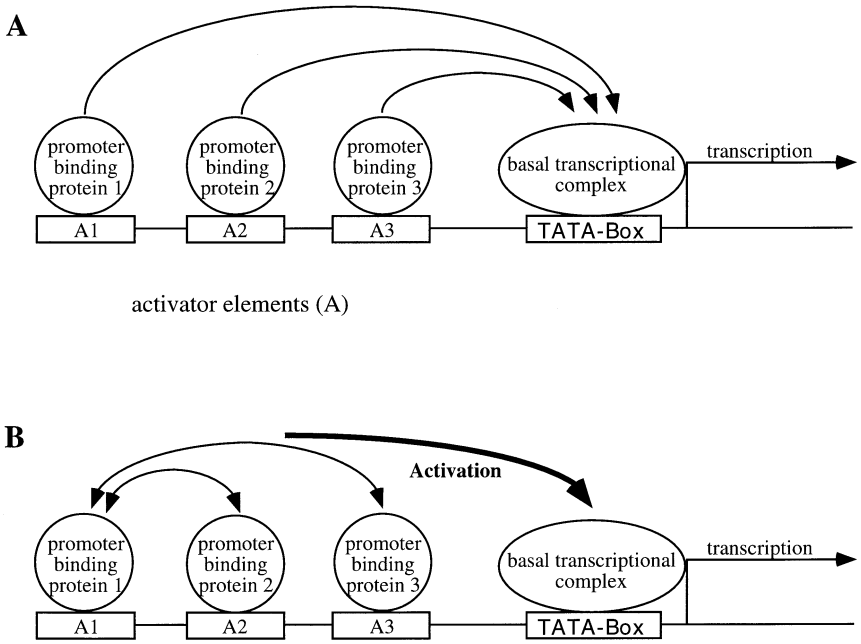

Fig. 2A, B Two possibilities for the way in which eukaryotic genes are activated. **A** "Bar code" model of a promoter/enhancer. The gene is activated by a number of transcription factors that bind to the promoter/enhancer (*elements A1–A3*) and independently activate the basal transcriptional machinery and allow RNA polymerase to attach and begin transcription. **B** "Allosteric model" of a promoter/enhancer. The transcription factors that bind to the promoter/enhancer interact with each other to activate the basal transcriptional machinery

gene transcription proved to be an insufficient model for explaining the tissue-specific activation of genes. To this end, no truly β cell specific transcription factor has been identified. PDX-1 is also expressed in the duodenum [52, 77, 78]. Lmx-1 proteins, which in the pancreas are restricted to β cells, are expressed in the limb bud during development and mediate dorsalization of the developing limb [79, 80]. Likewise, Isl-1 serves as a marker for motor neurons in the spinal cord [60]. Beta-2, apart from α and β cells, is also expressed in brain and may participate in the terminal differentiation step during vertebrate neuronal development [42]. Why is insulin, or other islet-cell specific genes, not expressed in the duodenum, motor neurons, or the developing limb bud?

Studies on other genes have taught us that cooperative effects of several activators with restricted expression patterns determine the subset of genes turned on in a given cell type [81, 82]. Therefore the promoter should not be viewed as a simple sum of independent elements but as a unit of interacting factors that activate transcription synergistically (Fig. 2). This model aids in explaining the exquisite cell-type specificity of the insulin promoter since deletion or substitution of any of these factors drastically reduces promoter activity.

To study synergy of adjacent elements we chose a small fragment of the rat insulin I promoter that contains the E2/A3/4 elements. A small minienhancer containing five copies of the E2/A3/4 element linked to a heterologous minimal promoter functions as a strong β cell-specific transcriptional enhancer [43, 63, 83]. However, deletion of either one of the elements renders the minienhancer inactive. The minienhancer is silent in a fibroblast cell line. Upon cotransfection of either the E1 site binding protein E47 or the A3/4 site binding protein Lmx-1.1, little activation of the promotor can be observed in fibroblasts. However, combined transfection of E47 and Lmx-1.1 activates minienhancer-driven transcription about 1000-fold [48]. Other LIM homeodomain proteins, such as Isl-1, are not able to activate the minienhancer synergistically. Recently we were able to show in a yeast two hybrid system that E47 and Lmx-1 interact directly via the Lim2 domain of Lmx-1.1 and the HLH domain of E47. Consistent with the transfection experiments, no interaction between E47 and Isl-1 can be observed [84]. These data provide a paradigm for how tissue specificity of gene expression may be realized.

The principle of synergy extends to other sequence elements and binding proteins of the insulin promoter. The homeodomain protein PDX-1 synergizes with E47 in a similar fashion to Lmx-1.1 [55]. Recently we demonstrated synergy between the E and A elements in the human insulin promoter, indicating that synergistic activation is a conserved mechanism [22]. In the rat insulin II gene, binding of the E47/Beta-2 complex requires the presence of the β cell-specific C1 binding complex to activate insulin gene transcription [41, 85].

Synergy of two adjacent elements is not the only mechanism specifying transcription. Homeodomain proteins are known to establish pattern formation during development. Distinct homeotic genes within a segment serve a gene regulatory function and act as transcription factors that regulate target genes in a precise spatial and temporal pattern. In contrast to their distinct activities in vivo, however, most homeodomain proteins indiscriminately bind to the consensus TAAT in vitro. In *Drosophila melanogaster* a cofactor, termed extradenticle, has been shown to promote target gene selection by enhancing the DNA binding specificity of certain homeodomain proteins [81, 86]. Recently Peers et al. [87] demonstrated that PDX-1 binds cooperatively to DNA with Pbx, the mammalian homologue of *extradenticle.* Cooperative binding and synergistic activation of transcription was

limited to one specific A site within the somatostatin promoter and could not be demonstrated for the A1 site of the insulin promoter. The results illustrate how target gene selection may be specified in the endocrine pancreas.

Role of transcription factors in pancreatic development

Most of the studies on the transcriptional control of islet hormone expression have focused on the mature islet cell. Our increasing knowledge of the control of expression of cell-type-specific genes in the adult pancreas is contrasted by a limited understanding of the development of differentiated cells in the pancreas. With the development of powerful new molecular and genetic tools and the availability of the β cell and pancreatic transcription factor genes, several questions in pancreatic development can now be approached.

Pancreatic morphogenesis

In the mouse at approximately embryonic day 9.5 (e9.5), the dorsal pancreatic bud first appears as a bulge in the primitive gut endoderm, near the junction of the foregut and midgut in the area that will become the duodenum [88]. Shortly thereafter the ventral pancreatic bud arises. As the stomach and duodenum rotate, the ventral bud and hepatopancreatic orifice move around until they come into contact, and around e16–17 fuse with the dorsal bud [89]. As the buds grow, they rapidly form new folds leading to a highly branched structure. Acini and ducts become clearly distinguishable as histologically distinct structures by about e14.5 in the mouse. Even though endocrine cells can be detected in the forming pancreas from the earliest stages (see below), islets, with the characteristic distribution of insulin-expressing cells in the center and non-insulin-producing cells in the periphery, do not form until the end of gestation, at about e18.5 in the mouse [90]. Neogenesis of islets continues throughout neonatal life but ceases shortly after weaning [91, 92]. Thereafter no additional islets form, and islet cells do not exhibit appreciable growth during adult life [93]. Between birth and weaning, insulin gene expression in the β cell increases, and the ability to sense glucose and to regulate insulin secretion becomes established.

While this sequence of pancreatic development is well established (Fig. 3), the signals that drive these developmental events remain a mystery. Progress has been hampered by the lack of a suitable model system that would allow in vitro manipulations and monitoring of subsequent developmental decisions. We list below the key questions in pancreatic development and differentiation.

Intrinsic signals: what determines the cells that will become pancreas?

Formation and differentiation of the pancreas from gut endoderm requires a series of distinct signals. A particular set of cells within the gut endoderm may be competent to develop into pancreatic cells, but growth and differentiation does not occur without additional extrinsic signals from adjacent tissues. On the other hand, other regions of the gut cannot develop into pancreatic cells, when given the same extrinsic signals [88]. Therefore both intrinsic and extrinsic signals are necessary, but neither is sufficient to cause gut endoderm to develop into pancreas. Wessells and Cohen [88] showed that by e8.5 in the mouse the region of the embryonic foregut from which the pancreas develops has acquired the ability to give rise to a differentiated pancreas, when explanted and cultured in vitro. Formation of the pancreatic bud was observed only when the foregut was cocultured with mesenchyme. These experiments indicate that dorsal gut endodermal cells are committed towards a pancreatic fate before the appearance of the first terminal differentiation products. mRNA for pancreatic genes can be detected before the first morphological evidence of pancreas development [94], and it cannot be excluded that the future pancreatic endodermal cells are subdivided into an endocrine and an exocrine compartment even before the bud forms.

Presumably, the fate of the individual endodermal cells is determined by an intrinsic “epigenetic code” established by the expression of a distinct set of transcription factors in the cells of the future pancreatic anlage. An example of how transcription factors define the future fate of a set of cells comes from flies, where expression of the homeodomain protein engrailed in one half of the wing imaginal disk determines a future posterior fate of these cells and renders them unresponsive to the secreted protein hedgehog [95, 96]. The factors that establish the “epigenetic code” of the gut endoderm, however, are unknown. HNF-3 α, β, and γ are members of the forkhead domain family of transcription factors and were first identified as transcriptional regulators of hepatocyte specific genes [97, 98]. During development they show a nested pattern of expression in the definitive endoderm, suggesting that HNF-3 proteins are involved in pattering of the primitive gut endoderm [99–101]. None of the HNF-3 class proteins, however, is expressed exclusively in the future pancreatic region of the foregut, indicating that additional factors may be required to establish prepatterning of the endoderm. Any of the pancreatic or gut transcription factors are potential candidates for this intrinsic signal, although it seems likely that a unique set of factors is required.

Extrinsic signals: what initiates pancreatic bud formation?

The extrinsic signals that initiate outgrowth of the pancreatic bud from the endoderm are unknown, and it is

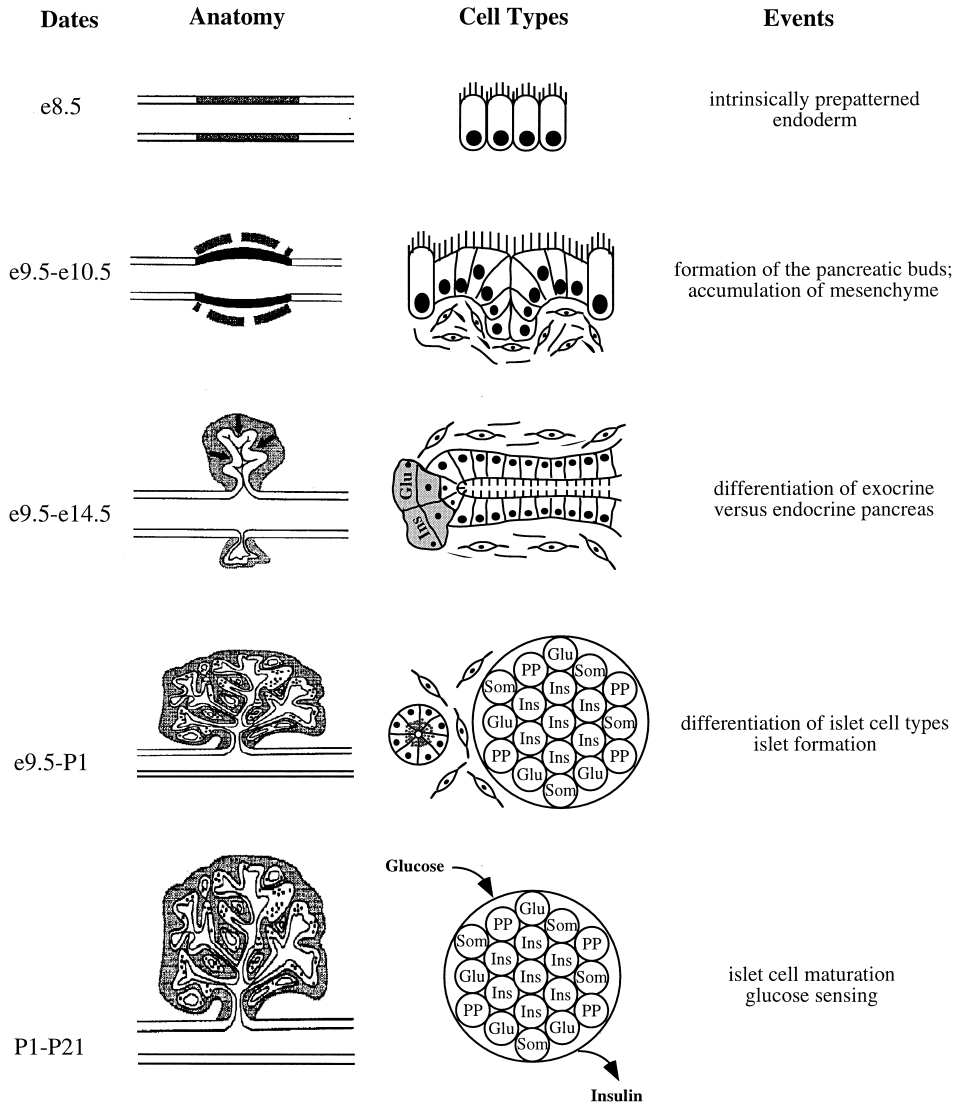

Fig. 3 Scheme of the developing mouse pancreas. *e8.5*, The endodermal region that gives rise to the future pancreas carries the "epigenetic code" to become pancreas. *e9.5–e10.5*, The dorsal and ventral pancreatic bud form. Mesenchymal cells (*light gray*) accumulate around the dorsal gut epithelium. *e9.5–e14.5*, Exocrine and endocrine pancreas differentiate. Epitheliomesenchymal interactions promote growth and differentiation of the pancreatic anlage. Acini and ducts are histologically differentiated by e14.5 in the mouse; amylase becomes detectable by immunostaining at the same time. Early endocrine cells are associated with the pancreatic ducts, but islets have not yet formed. *e9.5–P1*, The islet cell types differentiate; islets become morphologically distinct from the exocrine tissue by e18.5. *P1–P21*, The islet cells mature during the first 3 weeks of life. *Ins*, Insulin; *Glu*, glucagon

still unresolved which tissue provides the extrinsic signal for initiation of bud formation. Two sources of an extrinsic signal have been proposed: the notochord and the pancreatic mesenchyme. The notochord is known to be the source for other extrinsic signals in patterning of the developing embryo: it induces patterning of the mesoderm and the neural tube; both events are most likely mediated by the protein sonic hedgehog [102–104]. Early dorsal gut endoderm is in direct contact with the notochord, leading to the hypothesis that the inductive signal for bud formation arises from the notochord [105]. To date, the hypothesis that notochord produces an inductive signal for outgrowth of the pancreatic anlage still lacks support by experimental evidence, and a mesenchymal origin of such signal cannot be excluded.

At the same stage that the notochord abuts the dorsal gut endoderm the lateral sides of the gut are surrounded by mesenchyme. When the notochord separates from the dorsal gut at around e9 the mesenchyme starts to accumulate on the dorsal site of the gut. The exact timing of mesenchyme accumulation in the area of the dorsal pancreatic duct is controversial: either prior to or simultaneous with bud formation [88, 89, 94] or immediately following bud formation [105]. Determination of exact timing, however, is important. Mesenchyme induces pancreatic growth and differentiation in culture (see below) and therefore could be the inducer of bud formation if it is present at the appropriate time. The identification of factors that are required for bud formation will help to answer this question.

Differentiation: what are the signals for differentiation into endocrine, exocrine and ductal cells?

Growth and cytodifferentiation of the pancreas is a classic example of mesenchymal-epithelial interaction [106]. Pancreatic mesenchyme is made of loose cells of mesodermal origin. By stimulating growth and differentiation of undifferentiated epithelium mesenchyme induces the initial steps of organogenesis in a number of tissues, including salivary gland, lung, and kidney. When grown in

culture, pancreatic epithelial rudiments fail to proliferate and differentiate in the absence of mesenchyme [107]. It is not clear whether the factors produced by mesenchyme are diffusable or require cell-cell contact [106, 108]. The identity of these mesenchymal factors remains elusive. Sanvito et al. [109] showed that epithelial growth factor promotes ductal development, and that transforming growth factor β1 promotes development of the endocrine cells in pancreatic bud cultures. However, it is unknown whether these proteins are expressed in the mesenchyme surrounding the developing pancreas; therefore their function for pancreatic growth in vivo remains to be determined.

Differentiation of the three major pancreatic cell types, ductal, exocrine and endocrine, may be induced by different factors. Gittes and coworkers [108] separated pancreatic epithelium of 11-day mouse embryos from its surrounding mesenchyme and cultured it under various conditions. When cultured under the renal capsule, the rudiments gave rise to mature islets only; in a basement membrane rich gel ductal structures formed; acinar structures, however, developed only in the presence of mesenchyme. The results indicate that multiple factors are required for the various steps of pancreatic cytodifferentiation. The identification of the transcription factors required for differentiation will help in understanding the timing and ultimately the signals that induce differentiation.

Differentiation: what determines the lineage of islet cells?

Although many authors have developed models about islet cell lineage, mostly based on coexpression studies of the different hormones during development, the origin of the four islet cell lineages is still uncertain.

The appearance of the cell-type-specific hormones has been studied by reverse transcriptase-polymerase chain reaction. Somatostatin mRNA can be detected before formation of the pancreatic anlage as early as e8; insulin and glucagon mRNA expression can be observed from e9, PP from e10, and amylase mRNA from e12 [90, 94]. The first hormones that appear in the primordial pancreas are glucagon and insulin at e9.5, followed by somatostatin around e15 and PP at birth [110–112]. During development immature endocrine cells coexpress a "pp-fold" peptide, most likely peptide YY, but only a portion of the adult islet cells continue to coexpress peptide YY in addition to their principle product [112]. Coexpression of insulin and glucagon has been observed in the earliest endocrine cells, leading to the proposition that these cells are precursors for the endocrine cells [113] (Fig. 4a). However, coexpression does not prove a lineage relationship, since particular genes may be turned on and off during development. A cell that expresses insulin during early development may later switch off insulin gene expression and never develop into a mature β cell.

To obtain more definite results about cell lineage of the different islet cells, several groups have used a transgenic approach, driving expression of a reporter gene with the promoters of different islet cell hormone genes. All these approaches are based on the assumption that a short promoter expresses the transgene with the same tissue specificity as the endogenous gene. However, more then 10 years of experience with transgenic animals has taught us that correct expression of a particular gene often requires elements that are located far up- or downstream of the actual coding region. In addition, integration of the transgene at random positions in the genome may result in an altered expression pattern. Therefore, the transgenic studies on islet cell lineage need to be interpreted with caution. Alpert et al. [113] used the

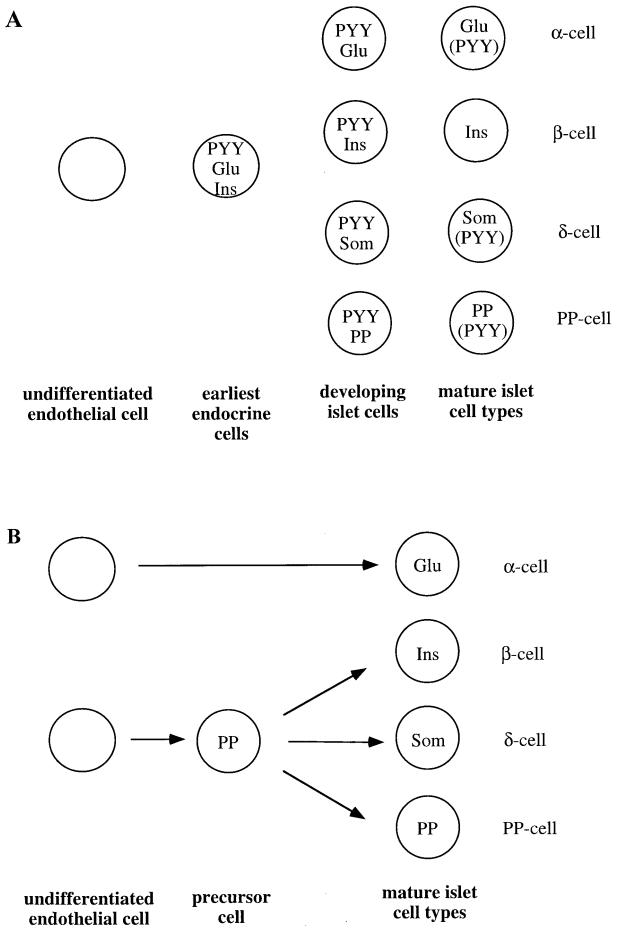

Fig. 4A, B Schematic diagram of the pancreatic islet cell lineage. **A** Model derived from coexpression studies during development. The earliest endocrine cells in the mouse appear at e9.5. It is unclear whether all endocrine cells originate from an endocrine stem cell. Early in fetal development endocrine cells coexpress different islet hormones and peptide YY (*PYY*). A direct lineage relationship between the early progenitors coexpressing glucagon and insulin has yet not been established. Mature islet cells coexpress peptide YY only in a small subset of cells. **B** Model derived from cell ablation studies with diphtheria toxin driven transgenes. Glucagon and insulin promoter driven toxin constructs only ablated their own cell type, while expression of the toxin driven by the PP promoter in addition to its own cell type also ablated somatostatin and insulin expressing cells. *Ins*, Insulin; *Glu*, glucagon; *Som*, somatostatin; *PP*, pancreatic polypeptide

rat insulin promoter to drive expression of T-antigen and during embryogenesis found coexpression of T-antigen with all four islet cell hormones. In adult animals T-antigen was detected only in β cells. The same approach was taken by Upchurch et al. [112] using the peptide YY promoter. Coexpression of T-antigen with three of the four islet hormones was found during development but very few islet cells expressed T-antigen in adult mice. Both studies support the idea of islet progenitor cells that coexpress different hormones (Fig. 4a). The data also indicate that promoter function does not become fully restricted to one islet cell type until mature islets have formed.

Diphtheria toxin A chain causes individual ablation of cells expressing the toxin and has been widely used to study cell lineage relationships [114]. Its use, however, is limited by poor penetrance of toxin-encoding transgenes. Herrera et al. [115] used a transient transgenic approach to drive expression of diphtheria toxin by the insulin, glucagon, or PP promoter. Toxin expression driven by the insulin or glucagon promoter only led to ablation of the targeted cell type, suggesting that a hormone coexpressing cell is not required for α or β cell development. By contrast, the PP promoter driven transgene led to a loss not only of PP-expressing cells but also of insulin- and somatostatin-producing cells (Fig. 4b). Unfortunately, no prior studies were performed to determine the specificity of the arbitrarily chosen PP promoter, leading to the possible explanation that the limited promoter shows less specificity than the native gene, and functions in any islet cell.

Without the ability to trace the fate of individual cells over time the exact islet cell lineage remains to be determined.

Maturation: what signals maturation of glucose sensing in the islet cells?

Unlike adult β cells, insulin secretion is not stimulated by glucose in fetal β cells, although fetal β cells do respond to other insulin secretagogues. Glucose sensitivity is acquired after birth, and the response to glucose reaches maturity after weaning [116–119]. The mature β cell senses glucose through its catabolic products; therefore β cell glucose sensing depends on the rate of glycolysis and specifically on the rate-limiting step for glycolysis in the β cell, glucokinase [120, 121]. We do not know which genes are responsible for the insensitivity of fetal β cells to glucose, but both glucokinase and glucose transporter 2, which allows the uptake of glucose into the β cell, are expressed in fetal as well as adult β cells (Hayes-Jordan, Kalamares, German, unpublished data) [122]. The expression pattern of other genes involved in glucose metabolism and glucose sensing during islet development will help elucidate the mechanism of islet cell maturation.

Transcription factors in pancreatic differentiation

The intrinsic capacity of specific cells in the early gut endoderm to become pancreas shows that these cells are phenotypically distinct from the cells in neighboring segments of the gut that lack this capacity. This phenotypic distinction is presumably determined by the expression of a unique set of transcription factors. The expression of these transcription factors allows these cells to respond to the signals from the pancreatic mesenchyme or other surrounding tissues that induce pancreatic growth and cell differentiation. We are only beginning to understand the molecular signals and secondary nuclear events that trigger the various steps in pancreatic differentiation. Understanding these signals should also help us outline the islet cell lineages.

In adult animals, expression of the homeodomain protein PDX-1 is limited largely to the pancreatic β cell and the duodenal mucosa [53, 56, 78]; and it is known to transactivate the insulin gene promoter [44, 51, 55]. In mouse embryos PDX-1 expression precedes insulin and glucagon expression and is first detected at e8.5 in the dorsal gut endoderm. One day later in development PDX-1 can be detected in all of the cells of the dorsal and ventral pancreatic buds and in the duodenal endoderm between them [56]. Because of the timing of its expression and because of its location in the future pancreatic region, PDX-1 was thought to be an intrinsic signal determining the region of the gut endoderm that would become pancreas [123]. When PDX-1 is removed from mice by targeted mutagenesis, the embryos fail to develop a pancreas [78, 124]. Interestingly, the pancreatic buds form in PDX-1-deficient mice, but subsequent morphogenesis and differentiation is arrested [78, 105], arguing against a role for PDX-1 in these early patterning events. The phenotype of the PDX-1 deficient mice demonstrates the requirement for PDX-1 for growth and full differentiation of the pancreatic buds. However, despite the absence of exocrine gene products and islet-like structures, insulin- and glucagon-positive cells can be detected in these early pancreatic rudiments, indicating that PDX-1 is not essential for insulin gene transcription [105]. The transcription factors that establish the intrinsic signal, or “epigenetic code,” of the future pancreatic gut endoderm still need to be found, and careful studies of the time and pattern of expression of transcription factors present in gut endoderm will reveal potential candidate genes.

Given the importance of transcription factor interactions in gene expression, other transcription factors must play equally important roles in pancreatic development. A more complete picture of the hierarchy of developmental signals will not arise until the expression patterns of these factors during development and the phenotype of the mice deficient in these factors have been characterized. Examples of potentially important factors include the homeodomain protein Nkx 6.1 that was cloned from a hamster β tumor cell library [54]. Similar to PDX-1, Nkx 6.1 is expressed in the pancreatic bud, but

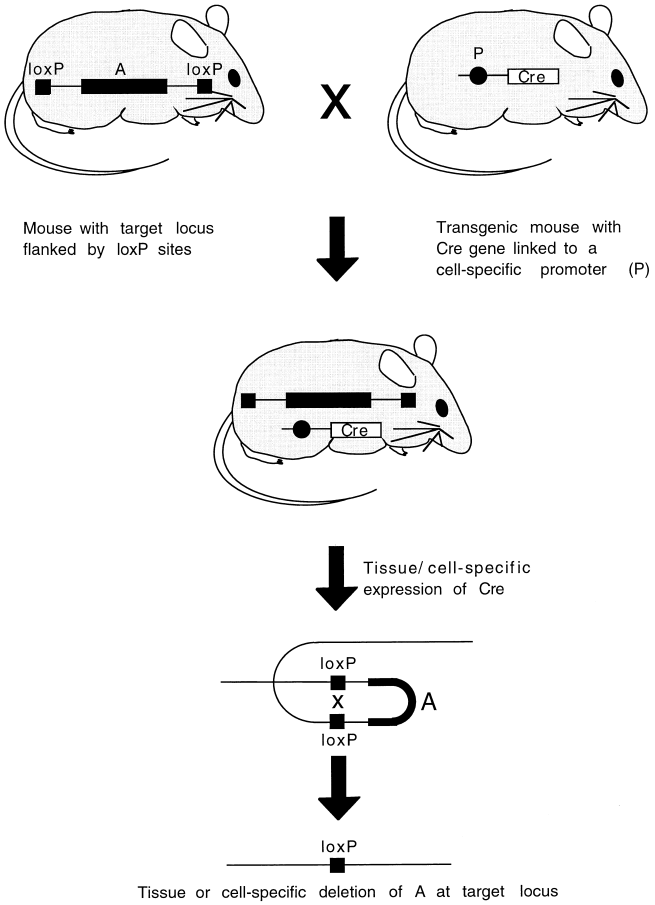

Fig. 5 Gene targeting using the Cre-loxP recombination system can be used to inactivate a gene in a desired cell type. The mouse with two loxP sites flanking the target locus A (typically a small gene or an internal exon which if deleted causes a frameshift mutation) is constructed by a standard homologous recombination method using mouse ES cells. Such mice can be mated with previously constructed transgenic mice which carry an integrated construct consisting of the Cre recombinase gene linked to a tissue-specific promoter (*P*). Offspring which contain both the loxP-flanked target locus plus the Cre gene express the Cre gene in the desired tissue type, and the resulting recombination between the loxP sites in these cells results in tissue-specific inactivation of the target locus

later expression is restricted to the pancreatic β cell. In contrast to PDX-1, Nkx 6.1 is expressed only in a subset of cells in the early pancreatic bud, and early on is restricted to insulin positive cells, pointing towards a role for Nkx 6.1 in β cell differentiation and function (Hayes-Jordan and German, unpublished data) [125]. Another recently cloned protein, the bHLH protein Beta-2, has also a very restricted expression pattern. In adult tissue Beta-2 expression is limited to brain and islet cells [41]. Its pattern of expression during embryogenesis remains to be studied. Other homeodomain proteins that are expressed in the early developing pancreas, but also in other parts of the embryo, are Pax-6, Prox-1, and Hox11 (Table 1) [69–71]. Many other transcription factors are expressed in mature islet cells, and for several of them a contribution to insulin gene transcription has been demonstrated [126]. Their expression pattern during development and their role in islet cell differentiation remains to be studied.

How do we determine which factors mediate each of the steps in development outlined above? The construction and analysis of mutant mice deficient for the various pancreatic transcription factors will provide new insights into the molecular mechanisms of islet cell development. Since many of these transcription factors may possess multiple functions at different time points in development, disruption of the genes at defined stages of development or in specific tissues of the developing embryo could provide a powerful tool to tackle those questions. The most promising approach to realize such conditional knockouts takes advantage of the bacteriophage Cre/loxP recombinase system (Fig. 5) [127]. Cre is an enzyme that excises the DNA between two recognition sequences, termed loxP. Two independent strains of mice need to be constructed. One strain must be modified by the introduction of two loxP sites flanking the gene of interest. The second strain carries a transgene that expresses Cre under the control of an inducible or developmentally regulated promoter. When these two strains are crossed with each other, progeny are deficient for the gene only in those cells that expressed Cre during development [128, 129]. Use of the various cell-type-specific promoters of the pancreatic islet genes to express Cre will provide important insight into the role of different transcription factors in determining islet cell lineage.

Conclusions

The data reviewed here demonstrate that transcription factors binding to the islet cell specific genes not only determine cell specific gene expression in mature islets but also dictate developmental decisions in the differentiation of pancreatic cell lineages. Therefore, our increasing knowledge of transcriptional regulation contributes to the understanding of the molecular events in developmental decisions. Since development of techniques to study the molecular basis of developmental processes has progressed rapidly over the past few years, we may expect answers to many of the outlined questions within a relative short period of time.

Our growing understanding of pancreatic development and β cell differentiation will be applied to the development of new therapies for diabetes. To date the β cell loss in insulin-dependent diabetes is terminal and cannot be reversed by regeneration of islets. As we learn more about the function of these transcription factors, we may apply this knowledge to the engineering of new β cells in vitro that could be transplanted into patients. Alternatively, we may be able to provoke β cell regeneration from precursor cells by gene therapy approaches.

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