An endocrine-specific element is an integral component of an exocrine-specific pancreatic enhancer

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We have analyzed the function of individual elements of the elastase I transcriptional enhancer in transgenic animals. This pancreas-specific enhancer comprises three functional elements, one of which (the B element) plays a dual role. Within the context of the enhancer, the B element contributes to appropriate acinar cell expression. However, when separated from the other enhancer components, the B element selectively directs transcription in islet cells of transgenic animals. This islet-specific activity is normally suppressed by an upstream repressor domain. The B element binds a novel islet-specific factor, and similar B-like elements are present in other pancreatic genes, both exocrine and endocrine specific. We suggest that a principal role of this transcriptional element and its associated factors is to activate many pancreatic genes as part of the program of pancreatic determination prior to the divergence of the acinar and islet cell lineages.

[Key Words: Transgenic mice; transcription element; transcription factor; cell specificity; islet of Langerhans; elastase gene]

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Organogenesis requires the differentiation of numerous cell types and the morphogenesis of multiple tissues within an organ in a coordinated fashion. The mammalian pancreas, for example, consists of both endocrine and exocrine tissues derived from a common pool of precursor cells (Pictet and Rutter 1972; Le Dourain 1988). The acinar cells of the exocrine tissue, which comprise ~85% of the organ, synthesize massive amounts of digestive enzymes, such as amylase, trypsins, carboxypeptidases, and elastases. The endocrine tissue, organized into islets of Langerhans, comprises ~2% of the pancreas and synthesizes several polypeptide hormones, such as insulin, glucagon, and somatostatin, involved in energy homeostasis. Each of these pancreatic hormones is synthesized by a different islet cell type. Development of the pancreas requires the selective activation of the genes for digestive enzymes and polypeptide hormones in separate cell types and the organization of those cells into two distinct tissue compartments.

Although much is understood about the transcriptional activation of cell-specific genes, little is known of the genetic and molecular mechanisms that coordinate the genesis of new cell types with the transcriptional activation of cell-specific genes during terminal differentiation. In some instances, the key transcription factors required for activation of genes that encode the cell-specific products characteristic of the differentiated state may also be required for early decisions that specify cell type (Lai and Darnell 1991; Weintraub et al. 1991). The transcriptional regulatory regions, including transcriptional enhancers, of cell-specific genes generally comprise multiple short elements, each of which binds a protein or protein complex (Maniatis et al. 1987; Mitchell and Tjian 1989). The multiple DNA elements with their bound factors often appear to perform redundant or overlapping duties (e.g., Veldman et al. 1985; Kadesch et al. 1986; Zenke et al. 1986; Schaffner et al. 1988). These gene-specific DNA elements and their associated transcription factors form a nucleoprotein complex in which one or more of the binding proteins is specific for the cell type and necessary for the formation of an active transcription complex. It is crucial to understand the role that individual elements and their transcription factors play in the formation of a functional cell-specific transcription complex and how their interplay determines the overall strength, specificity, and timing of transcriptional activation.

The transcriptional enhancer of the rat elastase I gene spans the nearby 5'-gene flanking sequences from -72 to -205 and is both necessary and sufficient for pancreas-specific transcription (Hammer et al. 1987). The region downstream of -72 contains a promoter that does not contribute to specificity but can respond to transcriptional elements specific for a variety of cell types (Ornitz...
et al. 1987, 1991). The enhancer consists of three functional elements, A (nucleotides -96 to -115), B (-146 to -160), and C (-166 to -195), identified by scanning mutagenesis [Kruse et al. 1988; Swift et al. 1989; F. Kruse, G.H. Swift, and R.J. MacDonald, unpubl.]. Any combination of two enhancer elements directs pancreas-specific expression in transgenic animals [Swift et al. 1989]; consequently, at least two must contain information for pancreatic transcription. To understand how this complex enhancer functions in animals to direct correct tissue-specific transcription, we have defined the roles of the individual elements and their associated transcription factors.

In this paper we describe a bifunctional element within the pancreas-specific transcriptional enhancer of the acinar cell-specific elastase I gene and its role in the complex regulatory strategy of this gene. By analysis of individual enhancer elements in transgenic animals, we show that although derived from an exocrine-specific enhancer, this element can selectively activate transcription in endocrine tissue of the adult pancreas. These results are evocative of the common developmental origin of these two pancreatic tissues and suggest a role for this element and its associated transcription factors in the early development of the exocrine and endocrine tissues of the pancreas.

Results

The B element directs pancreas-specific expression

To determine the role that each element and its associated factor play in directing cell specificity within the pancreas, we tested individual elements by generating homomultimers of A, B, and C linked to the elastase I promoter truncated at -92 and driving the human growth hormone (hGH) reporter gene. Although homomultimeric repeats of transcriptional elements are artificial, this organization satisfies the requirement for multiple elements to generate a functional enhancer. Any unexpected activities uncovered can be verified within the normal context of the enhancer. This approach has been successful for characterizing the binding sites for single transcription factors within complex viral [Yeldman et al. 1985; Ondek et al. 1987; Schirm et al. 1987] and cellular [Meister et al. 1989] enhancers by cell transfection and for pattern-forming transcriptional elements reintroduced into Drosophila embryos [Vincent et al. 1990]. In addition, the interleukin-2 gene element that mediates transcriptional induction by the nuclear factor of activated T cells (NFAT-1) has been shown to be active as a homomultimer in transgenic mice [Verweij et al. 1990]. Transgenesis provides the most comprehensive and rigorous test of cell specificity for transcriptional elements. However, dissection of a complex tissue-specific enhancer through the reintroduction of individual multimerized elements back into the genome of mammals has not been reported.

Our initial attempts to analyze the expression of homomultimeric constructs of the A, B, and C elements by transfection into cultured pancreatic acinar tumor cell lines had been unsuccessful: None of the constructs were active in either of two different acinar cell lines tested. Because transgenic animals had been more permissive for the expression of crippled elastase I enhancer constructs [Swift et al. 1989], we tested the homomultimer constructs in vivo. The homomultimers of the B \( B_{6} \) (Fig. 1) and A \( A_{6} \) [S. Rose, F. Kruse, G.H. Swift, R.J. MacDonald, and R.E. Hammer, unpubl.] elements directed pancreatic expression in transgenic mice, whereas \( C_{6} \) was inactive in this assay. This report focuses on the unique regulatory properties of the B element in transgenic animals.

The synthetic B element used to construct the homomultimer repeat comprises 21 bp of the mutation-sensitive B region of the elastase I enhancer (Fig. 1A). Of 12 independently derived transgenic founder mice bearing from 3 to 120 \( B_{6}.E_{6}.hGH \) transgenes, 8 expressed hGH mRNA in the pancreas (Fig. 1B). In contrast, transgenes with the elastase I promoter truncated at -71 were inactive [Hammer et al. 1987]. The level of expression of the \( B_{6} \) transgene in all mice was low; hGH transgene mRNA levels in the eight expressing mice varied from 0.2% to 6% of the level expected for a transgene containing an unaltered elastase I enhancer/promoter region from -205 to +8 [cf. lane w.t. of Fig. 1B, which represents 1%]. Therefore, most of the transgenic mice expressed the \( B_{6} \) homomultimer transgene, although expression levels were far below that of an enhancer restored to full activity.

To determine whether the \( B_{6}.E_{6}.hGH \) transgene was expressed in a pancreas-specific manner, we searched for the presence of hGH mRNA in 3 or 4 nonpancreatic tissues of six founder mice (Fig. 1C) and more extensively in 21 nonpancreatic tissues in two additional, independent transgenic lines (Fig. 1D). \( B_{6}.E_{6}.hGH \) expression was highly selective for the pancreas; expression in nonpancreatic tissue was found only once, with an aberrantly sized RNA detected in the thymus. This pattern of tissue-specific expression is even more restricted than that of the endogenous gene; elastase I is also normally expressed, although at lower levels, in the stomach, parts of the intestine, and bladder (Fig. 1D).

To verify that the elastase I promoter region from -92 to +8 did not contribute to this tissue specificity, we analyzed the expression of a transgene containing a \( B_{6} \) homomultimer linked to the herpes simplex virus thymidine kinase (HSV tk) promoter driving the hGH reporter gene \( B_{6}.t.k.p.hGH \). Of 11 mice bearing from 1 to 130 transgenes, 4 expressed hGH mRNA in the pancreas at levels 0.1-1% expected of an unaltered enhancer/promoter [data not shown]. Eighteen tissues were surveyed for two independently derived founder mice bearing 5 and 19 copies of the \( B_{6}.t.k.p.hGH \) transgene. hGH mRNA was present only in the pancreas of each mouse and at a lower level in the brain of one mouse (Fig. 1E). This tissue specificity of the B element, independent of all other elastase I gene sequences, demonstrates the remarkable selectivity that a single transcriptional element may exhibit in vivo.
The B element is specific for β cells of the islets of Langerhans

The pancreatic cell type expressing the B homomultimer transgene was identified by immunocytochemical localization of hGH. Surprisingly, hGH was not detectable in acinar cells; rather, the B element directed expression selectively to the islets of Langerhans (Fig. 2A). All four independent transgenic mouse lines examined had islet-specific expression of the B₅.ELp.hGH transgene (Table 1). hGH antigen, however, was present only in a subpopulation of islet cells (Fig. 2A). Thus, the low transgene mRNA levels were the result of high expression in a very few cells. The immunostained cells were large and often in the center of the islets—two characteristics of β cells. The hGH-positive cells also immunostained for insulin; however, not all insulin-positive cells contained hGH (Fig. 2A). Consequently, not all β cells expressed the transgene (even in second generation transgenic mice that cannot be mosaic for the transgene).

The poor expression penetrance in β cells is most likely the result of the inability of the multimerized B element to form a sufficiently strong enhancer to activate expression in all β cells of an animal. The fraction of β cells that expressed the transgene varied among founder mice (data not shown), possibly owing to differential chromosomal position effects on a weak regulatory element. That this may be the case was shown when we combined the B element with the C element from the El enhancer. Although the C element was required for enhancer activity in transfected cells (Kruse et al. 1988) and for optimal activity in transgenic mice (Swift et al. 1989), it was inactive as a homomultimer in both transfected cells and mice (data not shown). To determine whether the C element contributes to enhancer strength, we tested its effect on B element-mediated expression in a transgene containing three repeats of synthetic B and C elements linked in tandem, (BC)₃.ELp.hGH. For two independently derived founder mice examined, combining the C element with the B element increased the number of expressing islet cells manyfold without affecting expression in other pancreatic cell types (Fig. 2C).

Figure 1. Northern blot analysis of the expression of B-element multimer transgenes. The B₅.ELp.hGH construct contained five tandem copies of the 21-bp double-stranded B element linked to the elastase I gene promoter at −92 fused at +8 to the hGH reporter gene at +3. All lanes contained 10 μg of tissue RNA except where noted. Lanes labeled w.t. and Standard contained the equivalent of 100 hGH mRNAs per cell, representing 1% of the level of expression directed by a complete elastase I enhancer. (A) Schematic of the known regulatory elements of the enhancer and the promoter of the rat elastase I gene. The sequence of the double-stranded oligonucleotide for the B element used in the multimer construction is shown. (B) hGH mRNA levels in the pancreas of 12 founder mice bearing the B₅.ELp.hGH fusion transgenes. (C) Survey of the expression of the transgene in several nonpancreatic tissues for six of the founder animals. (D) Extended surveys of B₅.ELp.hGH expression in progeny of the 625-5 and 617-2 mice and of the endogenous elastase I gene in normal mice. The lane labeled pancreas for the elastase I survey contains only 0.1 μg of RNA. (E) The multimerized B element activates the HSV tk promoter in a pancreas-specific manner. The transgene construct B₅.tkp.hGH contained six tandem copies of the B element in their normal orientation linked to the HSV tk promoter fragment (−105 to +51 relative to the transcriptional start site) fused to the hGH gene at +3. (Founder 692-3) 5 transgene copies; (founder 693-1) 19 transgene copies.
Figure 2. Immunocytochemical identification of the pancreatic cell types expressing elastase–hGH fusion transgenes. All magnifications are 209 ×. (A,B) Immunocytochemical staining of hGH (A) and insulin (B) in adjacent pancreatic sections from a mouse of line 617-2 containing the Bṣ.Elī.hGH transgene. (C) Islet staining of hGH for a mouse of line 701-5 containing four copies of the B-plus-C element transgene [BC]₃₆.Elī.hGH. (D,E) Double immunocytochemical staining of pancreatic sections from a Bṣ.Elī.hGH mouse of line 617-2 for hGH (blue) and glucagon (red-brown) in D or somatostatin (red-brown) in E. (F,G) Immunocytochemical localization of hGH in both acinar and islet cells of a transgenic mouse bearing three copies of the –205Elī.hGH transgene (F) or only in acinar cells of a transgenic mouse bearing nine copies of the –500Elī.hGH transgene [G].
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Table 1. Expression of EIp.hGH fusion transgenes in exocrine and endocrine compartments of the pancreas

| Construct          | hGH detected by immunocytochemistry |
|--------------------|-------------------------------------|
|                    | immunoocytochemistry | acinar cells | islet cells |
| B₃.EIp.hGH         | 4                      | 0            | 4           |
| -205EIp.hGH        | 9                      | 9            | 6           |
| -500EIp.hGH        | 6                      | 6            | 1           |

*Number of independently derived founder mice examined for hGH by immunocytochemistry.

Double immunostaining for the B₃.EIp.hGH transgene product and either glucagon (Fig. 2D) or somatostatin (Fig. 2E) established that expression of the transgene was not detectable in either α or δ cells of islets. However, if the same [low] fraction of α and δ cells as β cells expressed the transgene, infrequent doubly stained cells may not be detected readily. Thus, we are unable to exclude rigorously the possibility that a very small number of α or δ cells also expressed the B multimer transgene. In summary, expression appeared selective for the β cells of islets, although expression penetrance in β cells was low and variable among the different transgenic lines. Neither acinar nor ductal cells expressed the transgene. The use of transgenic animals was crucial for detecting the unanticipated activity of the elastase B element in β cells.

Expression in β cells of islets, especially in the absence of acinar cell expression, is unexpected, because the endogenous elastase I gene is acinar cell specific. Moreover, fusion transgenes with extensive (4.5 kb) elastase I 5′-flanking sequences are expressed only in acinar cells, not in islets (Ornitz et al. 1985). To investigate whether truncation of the 5′ regulatory region of the elastase I gene might uncover the islet-specific activity of the B element, we examined the cellular localization of hGH in transgenic mice bearing hGH fusion transgenes with the shortened clastase enhancer/promoter region from +8 to −205 [construct −205EIp.hGH]. This transgenic construct has been shown previously to be expressed selectively in the pancreas (Ornitz et al. 1985; Hammer et al. 1987); in all, 26 of 29 independently derived transgenic mice examined [including 11 new founder mice generated for this study] expressed the −205EIp.hGH transgene to high levels in the pancreas.

Immunocytochemical localization of the hGH product detected expression in both acinar cells and β cells [Fig. 2F] for six of the nine −205EIp.hGH founder animals examined [Table 1]. The majority of acinar cells stained, whereas only a fraction of β cells stained, which was also characteristic of the multimerized B element. These results demonstrate that the β cell specific activity is an inherent property of the B element in situ and not a consequence of the homomultimeric organization of the element in the B₃.EIp.hGH transgene. This activity was revealed only when the minimal enhancer/promoter (−205 to +8) was separated from other elements farther upstream [see below].

As an independent verification of the activity of the elastase B element, we investigated its specificity by transfection of cultured rat tumor cell lines derived from β cells [insulinoma RIN1046-38 (RIN-38), Philippe et al. 1987], acinar cells [AR4-2J], Jessop and Hay 1980], and nonpancreatic cells [Rat2 fibroblasts]. The RIN-38 cell line expresses insulin selectively, but not glucagon and somatostatin, which are characteristic of other islet cell types. At low passage, the RIN-38 cells used in this study retain many characteristics of differentiated β cells, including selective expression of moderate levels of insulin mRNA [S. Hughes and C. Newgard, pers. comm.] and glucose-induced insulin secretion [Clark et al. 1990]. The AR4-2J tumor cell line expresses acinar-specific genes such as elastase I, trypsin I, chymotrypsin B, and amylase [Boulet et al. 1986; Swift et al. 1989]; and the Rat2 fibroblast line is unrelated to the pancreatic lineage. Deletion of all elastase I gene flanking sequences down to −92 created a truncated promoter that was expressed poorly [Fig. 3]. The lack of significant activity of this −92 truncation in these cell lines is consistent with the absence of expression in animals of a similar transgene truncated at −72 [Hammer et al. 1987]. Extending the 5′-flanking region out to −205 to include the three elements of the enhancer increased the activity of the −92 promoter nearly 30-fold in the acinar cell line but had no effect in the islet or fibroblast lines. Thus, as in transgenic animals, the −92 to −205 region provides the information for acinar cell expression. However, in this in vitro assay the islet-specific activity of a single B element within the enhancer was not detected in the cultured islet insulinoma cells. Two or five tandem copies of the B element linked to the −92 promoter [the B₃.EIp.hGH or B₃.EIp.hGH constructs], however, directed 7- and 26-fold greater expression, respectively, than the −92 promoter in the islet cell line, whereas no enhancement is observed in the acinar and fibroblast cell lines [Fig. 3].

To show that the B element activity was a general property of β-cell tumor cell lines, we tested expression in βTC3 cells, a well-differentiated mouse insulinoma cell line with high insulin mRNA levels [Efrat et al. 1987].

Figure 3. Differential expression of hGH fusion gene constructs in an insulin-producing islet β-cell line [RIN-38], a pancreatic acinar cell line [AR4-2J], and a fibroblast line [Rat2]. The level of expression of the transfected fusion genes was determined by the level of hGH production, corrected for transfection efficiency by monitoring the activity of a cotransfected RSV-CAT gene construct. The activities reported are relative to that of Elp.hGH and are the average of two to four separate transfections.
Expression of \( B_2 \cdot Elp.hGH \) in \( \beta TC3 \) cells was 35-fold greater than the \( -92 \) promoter construct \( [Elp.hGH] \) (data not shown), similar to the expression observed in transfected RIN-38 cells. Furthermore, to establish that the hGH reporter gene did not contribute to \( \beta \) cell specificity, we demonstrated that the \( B \) element still enhanced expression in a RIN cell-specific manner when a chloramphenicol acetyltransferase (CAT) reporter gene was used instead of the hGH gene. Thus, addition of \( B \) elements to an elastase I promoter-CAT construct (creating \( B_5 \cdot Elp.CAT \)) increased expression in RIN-38 but not in Rat2 cells to an extent similar to that of the \( B_5 \cdot Elp.hGH \) construct over \( Elp.hGH \) (data not shown). The selective action of the \( B \) element in the insulinoma cells is consistent with its specificity for \( \beta \) cells in transgenic animals.

Repression of \( \beta \)-cell expression by upstream elements

The ability of the truncated enhancer but not more extensive 5' regions to direct expression to \( \beta \) cells suggests the presence of a repressor activity upstream of the enhancer. In an attempt to confirm the presence of a region that restricts cell-type expression and to localize it, we assayed the expression of the transgene \( -500EI.hGH \), which contains an additional 295 bp upstream of \( -205 \). Six founder mice bearing 2 to 57 copies of this transgene had high levels of hGH mRNA in the pancreas as expected (data not shown). In contrast to the presence of hGH in islet cells for six of the nine founder mice with the truncated \( -205EI.hGH \) transgene, five of six \( -500EI.hGH \) mice (Table 1) had expression limited to \( \beta \) cells [Fig. 2G]. These results indicate the presence of a negative transcriptional regulatory element between \( -206 \) and \( -500 \) capable of selectively silencing expression in islet \( \beta \) cells in vivo. Thus, the \( \beta \) cell-specific control region contains two domains: the pancreas-specific minimal enhancer that acts in a positive manner to direct expression to both endocrine and exocrine compartments and a nearby repressor domain within \( -206 \) to \( -500 \) that selectively suppresses endocrine expression.

To investigate this repressor activity further, we tested the effect of the putative repressor region from \( -206 \) to \( -500 \) on the activity of the \( B_5 \cdot Elp.hGH \) construct in vitro. The presence of this region completely suppressed the activity of the \( B_5 \) multimer in transfected insulinoma cells [Fig. 3, construct \( -500/-206.B_5 \cdot Elp.hGH \)], confirming the presence of a negative element that represses the activity of the \( B \) element in \( \beta \) cells.

**The \( B \) element plays a role in both \( \alpha \)acinar and \( \beta \) cells**

The cumulative evidence dictates that the \( B \) element plays a role in both \( \beta \) and acinar cells. First, as a multimer, it is sufficient to direct \( \beta \) cell specific expression. Second, it is required for optimal activity of the enhancer in acinar cells [Kruse et al. 1988; Swift et al. 1989] and, therefore, must have an acinar cell function independent of its \( \beta \) cell-specific activity. The first role implies the presence of a unique DNA-binding transcription factor present only in \( \beta \) cells that mediates its cell-specific action there. The second role implies a different binding factor in acinar cells. Because the \( B \) element does not appear to contribute to acinar cell specificity, its acinar cell-binding factor may be a common factor not restricted to acinar cells. To test these predictions, we searched for a unique \( B \) element-binding activity limited to islet cells and a distinct binding activity in acinar cells also present in nonpancreatic cells.

Nuclear extracts from the insulin-producing RIN-38 line, the rat acinar tumor cell line AR4-2J, the pancreatic tumor cell line AR4-IP [derived from the same tumor as AR4-2J, but lacking expression of acinar cell genes [Jessep and Hay 1980]], and the Rat2 fibroblast cell line were tested for their ability to form sequence-specific nucleoprotein complexes with the elastase \( B \) element [Fig. 4]. Multiple mobility shift complexes were seen for each cell line; some of the complexes were shared among the cell lines, indicating the presence of common binding factors that may mediate the \( B \)-element activity in acinar cells. In addition, a complex of unique mobility [Fig. 4, arrow] was formed only with the RIN-38 cell extract. This complex was not observed with or competed by other double-stranded oligonucleotides including those bearing the elastase A and C elements [data not shown]. Therefore, this binding activity [designated \( \beta \)-cell transcription factor-1 \( [\beta TF-1] \)] represents a sequence-specific \( B \)-element factor selectively present in cells derived from
β cells of the endocrine pancreas. The unique electrophoretic mobility indicates that the βTF1 activity is either a cell-specific protein factor or a specific modification of a factor present in other cell types.

To test whether βTF1 could be responsible for the activity of the B element in β-cells, we compared the effects of B-element mutations on βTF1 binding and on transcriptional activation in RIN-38 cells. The effects of 4-bp transversion mutations across the B element on the transfection expression levels were congruent with the effects on competition for βTF1 binding (Fig. 5). Thus, the two outside 4-bp mutations, which affected expression slightly or not at all, also did not affect the ability to effectively compete for βTF1 binding. However, the two inner mutations decreased expression 13- to 33-fold and abolished the ability to compete for βTF1 binding. This correlation did not occur for the two other B-element binding activities observed in the RIN-38 cell extracts [bands a and b in Fig. 5].

Functional βTF-1-binding sites in other pancreatic genes

Because the B element directed islet-specific expression in transgenic mice, we searched for similar sequences in the regulatory regions of insulin, glucagon, and somatostatin genes to ask whether equivalent elements may play a role in the transcription of endocrine-specific genes. Sequences similar to an 11-base core of the B element were found in the nearby 5′ transcriptional regulatory region of each of these genes (Fig. 6). A B-like sequence adjacent to an enhancer core (GTGGAAAA; Weiher et al. 1983) is conserved among the human, two mouse, and two rat insulin genes. The B-like and core elements are contained within the 48-bp insulin gene E1 regulatory domain, which binds one or more islet-specific nuclear factors (Ohlsson and Edlund 1986; Philippe et al. 1988) and is necessary for optimal expression in cultured insulinoma cells (Karlsson et al. 1987) and in transgenic mice (Fromont-Racine et al. 1990). The regulatory region of the glucagon gene contains a potential B element that overlaps the distal part of the 40-bp G1 region, which is critical for specific expression of the glucagon gene (Philippe et al. 1988). For the somatostatin gene, the B-like sequence overlaps a DNase I footprint domain (−80 to −110) that binds islet-specific nuclear proteins (Powers et al. 1989). A similar sequence is present in the regulatory region of the mouse amylase 2.1 gene, an exocrine-specific gene expressed similarly to elastase I. In each instance, the significance of the presence of the B-like element is supported by its position within a narrow region shown previously to be important for appropriate expression.

Consistent with this proposal for βTF1-binding sites in other pancreas-specific genes, the insulin, somatostatin, glucagon, and amylase elements also bind βTF1. Each of these potential βTF-1 sites can form two different complexes [Fig. 7A] with nuclear extracts pre-enriched for binding activity by affinity binding to the elastase B element. One complex has the mobility of βTF1, is β cell-specific [data not shown], and therefore appears to be βTF1. A second complex with a slower mobility also forms with each of the B-like elements including that of elastase but is not cell-type specific [see Fig. 4]. The B-like elements from the insulin, glucagon, somatostatin, and amylase genes compete effectively for the binding of βTF1 to the B element [Fig. 7B], indicating further that each is capable of binding the same βTF1 protein complex present selectively in pancreatic endocrine cells. βTF1 appears to be a novel transcription factor, its binding site is distinct from that of the other insulin gene transcription factors so far described (Ohlsson et al. 1988; Karlsson et al. 1990).

To test whether any of these potential B elements

![Figure 5](image.png)
Figure 6. The presence of B element-like sequences (shaded ovals) in nearby upstream transcriptional regulatory regions of several pancreas-specific genes. [Left] The positions of previously identified transcriptional regulatory elements [open ovals] for elastase I, insulin, glucagon, somatostatin, and amylase genes and the relative positions of B-like elements. For the rat elastase I gene, the three enhancer elements [A-C], a mutation-sensitive transcriptional element at the enhancer/promoter boundary [P] that binds a common sequence-specific protein [Knepel et al. 1988], and the TATA box are indicated. The nearby 5'-flanking sequences of the rat insulin I gene contain four principal transcription elements [Karlsson et al. 1987, 1989]: one containing an enhancer core [EC] sequence originally identified in the SV40 enhancer by Weihl et al. (1983), two [IEB1 and IEB2] that share an 8-bp sequence similar to the μ elements of the immunoglobulin enhancers [Moss et al. 1988] and bind an islet-specific nuclear protein [Ohi et al. 1988] that appears to comprise a heteromeric complex of helix-loop-helix E2A proteins [German et al. 1991], and a binding site [Is1-1] for a cloned pancreatic endocrine-specific transcription factor [Karlsson et al. 1990]. The proximal 5'-flanking sequences of the rat glucagon gene contain a cAMP responsive element [CRE] [Kneple et al. 1990a] and three mutation-sensitive transcriptional elements [G1-G3] that bind sequence-specific DNA-binding proteins [Philippe et al. 1988; Kneple et al. 1990b]. For the rat somatostatin gene [Powers et al. 1989], the proximal upstream region contains a consensus CRE coincident with the transcriptional element that confers islet specificity and an islet-specific protein-binding site [FP], undefined elements in the region -65 to -250 limit transcription to somatostatin-producing cells. The proximal upstream region of the mouse amylase 2 gene contains a PTF-1-binding site [Poulet et al. 1986; Cockell et al. 1989] equivalent to the A element of the elastase I gene and a sequence-specific protein binding site [II], the PTF-1 binding region of the amylase gene also confers insulin regulation [Keller et al. 1990]. [Right] The 11-nucleotide core sequences of the putative B elements positioned at left are compared.

Discussion

These results demonstrate that homomultimers of the 21-bp B element of the elastase enhancer can direct islet-specific expression in transgenic mice independent of other enhancer elements and that this activity is not dependent on linkage to its cognate promoter. We have observed expression only in β cells; however, we cannot rule out expression in a small number of other islet cells. Although it has become generally accepted that the activity of cell-specific transcriptional regulatory regions is the result of the cumulative contributions of multiple transcription factors bound to individual recognition elements, few tests have been made of individual elements to verify their proposed role in animals and their independence of other adjacent elements. Our results indicate that an oligonucleotide comprising the binding site for a single transcription factor can direct cell-specific expression in animals consistent with its role in the context of the enhancer from which it is derived.

Although the B element directed expression to islet β cells, only a fraction of the β-cell population expressed the transgene. This incomplete expression penetrance suggests either that the β cells are heterogeneous in their ability to express the transgene or that the B multimer does not reconstitute an enhancer sufficiently strong to enforce expression in all β cells. In the latter instance, the expressing β cells may reflect a certain probability of expression; if the B-element activity could be strengthened, we would predict that the proportion of expressing cells would increase. We favor this explanation for two reasons. First, some enhancers do function by increasing the probability of whether a gene is transcriptionally active rather than modulating the number of RNA polymerases transcribing the gene. For example, the probability of transcription of individual rRNA transcription units depends on the number of enhancer elements in an rDNA repeat unit [Reeder 1984]. In another example, the 100-fold increase in activity obtained by the addition of the SV40 enhancer to the SV40 early promoter is the result of an increase in the fraction of transfected cells that express without an increase in the level of expression in each expressing cell [Moreau et al. 1981; Weintraub 1988]. Second, when the B element of the elastase enhancer is combined with the C element (which appears to augment activity nonspecifically), the level of transgene expression in islet cells increased ~10-fold over the levels of the construct with the B element alone.
Regulatory strategy for the elastase I gene

We can delineate the transcriptional regulatory strategy of the elastase I gene from the properties of the B element reported here, the properties of the other two transcriptional elements, A and C [Fig. 1A], and the properties of their binding proteins. The region from −92 to the start of transcription contains a functional promoter that does not contribute to cell specificity but can be activated by its cognate enhancer [Hammer et al. 1987] as well as other transcriptional enhancers from unrelated genes [Omitz et al. 1987, 1991].

The A element, common to all pancreatic serine protease genes [Swift et al. 1984], as well as other pancreatic secretory enzyme genes [Boulet et al. 1986, Cockell et al. 1989], binds an acinar cell-specific transcription factor [PTF-1 or XPF-1; Cockell et al. 1989; Roux et al. 1989; Weinrich et al. 1991] and is the sole positively acting element responsible for acinar cell transcription {S. Rose, F. Kruse, G.H. Swift, R.J. MacDonald, and R.E. Hammer, in prep.].

The C element, required for optimal activity in cell transfection [Kruse et al. 1988] and in animals [Swift et al. 1989], does not contribute to cell specificity and is unable to activate transcription on its own. The C element appears to augment the activity specified by the other elements, probably by binding a general transcription factor. In transgenic animals the C element increases the activity of the B element in islets.

The B element performs a binary function. First, either in the context of the enhancer truncated at −205 or alone as a homomultimer, the B element directs β-cell expression in the adult. This activity may be mediated by a transcription factor [ɛTF-1] present in the adult animal selectively in β cells. Second, within the context of the enhancer, mutations in the B element affect acinar cell expression [Kruse et al. 1988; Swift et al. 1989]; consequently, it is also active in acinar cells. Because the multimerized B-element transgenes are not active in acinar cells, the role of the B element in acinar cells may be similar to that of the C element, which is to augment the activity of the enhancer specified by the A element. Therefore, in acinar cells the B element may act in a non-cell-specific manner by binding ubiquitous transcription factors. Consistent with this proposal is our identification of a protein complex from acinar cells, and also present in other cell types, that appears to mediate the activity of the B element in acinar cells (Fig. 4; data not shown).

The difference between the action of the β-cell-specific factor and the factor acting on the B element in acinar cells points to a fundamental mode of organization of cell-specific enhancers. These transcriptional enhancers comprise a combination of binding sites for both cell-type-specific factors, which are crucial for transcription, and general factors that augment, rather than activate, transcription. This organizational scheme ensures that transcription of a given cell-specific gene will be initiated only in the presence of the appropriate cell-specific factors. Thus, the B-element homomultimer can activate transcription in β cells through the binding of the β-cell-specific factor ɛTF-1, whereas the ubiquitous B element-binding factor is insufficient to activate transcription in acinar cells.

Finally, a repressor activity located between −205 and −500 quenches β-cell expression in vivo without affecting acinar cell expression.

An endocrine-specific element in the regulatory region of an exocrine-specific gene

The presence of potential B-element equivalents in other pancreatic genes, both endocrine and exocrine specific, suggests that the element and its associated transcription factor ɛTF-1 may play a general role in pancreatic
gene regulation. To explain its presence in the regulatory regions of endocrine- and exocrine-specific genes, we suggest that the B element may play a regulatory role in the early stage of pancreatic development when the endocrine and exocrine compartments share a common precursor lineage. It is generally accepted that both endocrine and exocrine cells of the pancreas are derived from the same small population of precursor cells that emerge from the primitive foregut (Pictet and Rutter 1972; Pictet et al. 1976; Le Douarin 1988). It is possible that part of the process of determination that specifies which few cells of the primordial gut will become pancreas cells will be the activation of the pancreas-specific transcription factor βTF-1 that binds the B elements present in many or all pancreatic genes, regardless of whether they will become endocrine or exocrine specific in the adult. Binding of the factor would either activate these genes to a low "protodifferentiated" level or, perhaps, create a preactivated transcription complex in the chromatin of these multipotent cells that facilitates activation in the appropriate cell lineages later in pancreatic development.

The presence of a protodifferentiated state in early pancreatic development was first proposed by Rutter and colleagues (Rutter et al. 1968; Pictet and Rutter 1972), and was based on the presence of very low levels of pancreas-specific gene products during a 2- to 3-day period before the rapid accumulation of the secretory products and the appearance of morphologically recognizable acinar cells. The detection of small amounts of several acinar cellular mRNAs also supports the low level activation of many pancreatic genes during this early period of pancreatic differentiation (Han et al. 1986).

The subsequent divergent differentiation of the endocrine and exocrine compartments from the protodifferentiated cells would be mediated by the appearance of additional cell-specific transcription factors. Differentiation of the acinar cell lineage would involve activation of the acinar cell-specific A element-binding transcription factor, PTF-1 (Cockell et al. 1989; Petrucco et al. 1990) or XPF-1 (Weinrich et al. 1991), which selectively increases expression of the acinar genes. The appearance of PTF1 at 15 days of gestation (Petrucco et al. 1990), at the onset of the rapid accumulation of mRNAs for the acinar cell enzymes (Han et al. 1986), is consistent with this role for PTF1. The continued involvement of the B element in the elastase I enhancer in acinar cells is mediated by the common transcription factors found in acinar cells as well as other cell types and not the β-cell βTF1. The presence of repressor regions, such as the one upstream of the elastase I enhancer, would help to silence acinar-specific genes in islet cells.

Differentiation of the islet lineage would involve the continued presence of βTF-1 and the activation of additional transcription factors for insulin, glucagon, somatostatin, and pancreatic polypeptide genes. Coexpression of the polypeptide hormones in individual cells of the developing pancreas (Alpert et al. 1988) and in cloned cell lines derived from islet tumors (Philippe et al. 1987) indicates a common precursor for the individual differentiated cell types that selectively express separate hormones later in development. In our scenario, the distribution of βTF1, initially in all immature pancreatic cells, becomes more restricted during development until it is present only in the β cells of the adult animal. The absence of detectable expression of the β6.Elp.hGH transgene in any other pancreatic cell type of adult transgenic mice indicates that βTF-1 is likely present solely in β cells. At least one of its roles in β cells might be to act on the B-like element in the upstream region of the insulin gene. We will examine the appearance and changes in the distribution of βTF-1 during development of the exocrine and endocrine pancreas as a first step in assessing its role.

Materials and methods

Generation and analysis of transgenic mice

The fusion transgenes were assembled by standard recombinant DNA techniques (Sambrook et al. 1989). The β6.Elp.hGH transgene was constructed by creating an EcoRV restriction endonuclease site at −92 of the elastase 5′-flanking region through site-directed mutagenesis (Zoller and Smith 1984) of the elastase–hGH fusion gene E–GH 3 (Hammer et al. 1987) in pUC119. By digestion with EcoRV and SalI, the elastase gene sequences upstream of −92 were removed and a B element pentamer, polymerized from the 21-bp monomer (shown in Fig. 1 with T4 DNA ligase and isolated by polyacrylamide gel electrophoresis, was inserted. (B) Elp.hGH was constructed by inserting a 56-bp synthetic double-stranded oligonucleotide comprising adjacent C (EI gene nucleotides −196 to −163) and B (nucleotides −162 to −141) elements upstream of the Elp.hGH reporter gene. β6.tkp.hGH was constructed by inserting into the +3 B amplified site of the hGH gene in pUC119 a BamHI–BglII fragment from the HSV tk linker scanning mutant LS−115/−105 (McKnight and Kingsbury 1982) spanning the thymidine kinase promoter from −105 to +51. The fusion construct was then linearized with BamHI at −105 of thymidine kinase and a B element hexamer was inserted. The complex −500/−206, Elp.hGH was constructed by inserting the −500/−206 elastase HindIII–SalI gene fragment into a HindIII site in β6.Elp.hGH immediately upstream of the B element pentamer. β6.Elp.hGH was constructed by inserting a hexamer of the double-stranded oligonucleotide (coding strand, 5′-TTTCTGG-GAAATGAGGTGttc-3′) containing the insulin gene B-like sequence (uppercase) with three additional nucleotides (lowercase) to make a repeat unit of 21 bp. Transgenes free of plasmid sequences were obtained by digestion with restriction endonucleases that cut at the ends of the transgenes, and purification was done by preparative agarose gel electrophoresis. The construction of the transgenes −205EI.hGH, containing elastase I gene sequences from −205 to +8, and −500EI.hGH, containing elastase I sequences from −500 to +8, was reported previously (Ornitz et al. 1985).

Transgenic mice were generated by microinjection of fertilized mouse eggs and identified by quantitative dot-blot hybridization of tail DNA as described previously (Brinster et al. 1985), with an hGH cDNA hybridization probe (Seeburg 1982) provided by P. Seeburg. All activities involving mice were reviewed and approved by our institutional committee in accordance with current National Institutes of Health policy.

Hybridization analysis of RNA

The presence of hGH transgene mRNA and endogenous...
elastase I in tissue RNA samples isolated by guanidine thiocyanate extraction (MacDonald et al. 1987) from transgenic mice was assayed by Northern blot hybridization of RNA resolved by electrophoresis in the presence of methyl mercury [Bailey and Davidson 1976], transferred to Zeta-probe membrane, and hybridized with a CDNA probe either for hGH [Seeburg 1982] or mouse elastase I [Davis 1989]. The quality of each tissue RNA preparation was verified by staining the agarose gel with ethidium bromide and following the transfer of the ethidium bromide–RNA complex to the hybridization membrane.

**Immunohistochemical analysis**

Pancreatic tissue was fixed in Carnoy’s fixative and embedded in paraffin. Immunocytochemical analysis was performed under standard conditions using the streptavidin/biotin/peroxidase method (Shi et al. 1988). Double immunohistochemical analysis [Van der Loos et al. 1987] was performed using both the streptavidin/biotin/peroxidase and the streptavidin/biotin/alkaline phosphatase [Yam et al. 1988] procedures. Hydrogen peroxide/AEC [Zymed Laboratories Inc.] was used as substrate–chromogen for peroxidase, producing a red–brown deposit, and AP-Blue [Zymed] was used for the alkaline phosphatase conjugate, producing a blue color. Primary antibodies were rabbit anti-hGH (DAKO), guinea pig anti-human insulin (Linco), rabbit anti-human glucagon (DAKO), and rabbit anti-human somatostatin (DAKO). After antibody staining, the tissue sections were counterstained with hematoxylin or methyl green.

**Cell transfection**

hGH fusion gene constructs cloned in pUC119 were transfected into cultured RINr1046-38 cells by lipofection (Felgner et al. 1992) as modified by Bassel-Duby et al. (1992). The mutant B elements tested by transfection in RIN-38 cells comprised 21 bp (shown in Fig. 1A) with 4-bp transversion mutations within the central 16 bp region and were synthesized with 2-bp complementary staggered ends to facilitate multimerization. Hexamers of the mutant B elements were linked to the EI promoter at -92 driving the hGH reporter gene as described by Chodish et al. (1986).

The mobility shift binding reactions and electrophoresis were performed as described by Knebel et al. (1990b), with minor modifications. Binding reactions [20 μl] with crude nuclear extracts contained 0.5 μg of poly[d(I-C)]/poly[d(I-C)], whereas binding reactions with enriched B-element-binding activity contained 0.05 μg of poly[d(I-C)]/poly[d(I-C)], and 0.025% Nonidet P-40.

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**References**

Alpert, S., D. Hanahan, and G. Teitelman. 1988. Hybrid insulin genes reveal a developmental lineage for pancreatic endocrine cells and imply a relationship with neurons. *Cell* 52: 295–308.

Bailey, J.M., and N. Davidson. 1976. Methylmercury as a reversible denaturing agent for agarose gel electrophoresis. *Anal. Biochem.* 70: 75–85.

Bassel-Duby, R., M.D. Hernandez, M.A. Gonzalez, J.K. Krueger, and R.S. Williams. 1992. A 40-kilodalton protein binds specifically to an upstream sequence element essential for muscle-specific transcription of the human myoglobin promoter. *Mol. Cell. Biol.* 12: 5024–5032.

Boulet, A.M., C.R. Erwin, and W.J. Rutter. 1986. Cell-specific enhancers in the rat exocrine pancreas. *Proc. Natl. Acad. Sci. USA* 83: 3599–3603.

Brinster, R.L., H.Y. Chen, M.E. Trumbauer, M.K. Yagle, and R.D. Palmiter. 1985. Factors affecting the efficiency of introducing foreign DNA into mice by microinjection eggs. *Proc. Natl. Acad. Sci. USA* 82: 4438–4442.

Chodish, L.A., R.W. Carthew, and P.A. Sharp. 1986. A single polypeptide possesses the binding and transcription activities of the adenovirus major late transcription factor. *Mol. Cell. Biol.* 6: 4723–4733.

Chu, G., H. Hayakawa, and P. Berg. 1987. Electroporation for the efficient transfection of mammalian cells with DNA.
Islet-specific element in an exocrine gene

Keller, S.A., M.P. Rosenberg, T.M. Johnson, G. Howard, and M.H. Meisler. 1990. Regulation of amylase gene expression in diabetic mice is mediated by a cis-acting upstream element close to the pancreas-specific enhancer. Genes & Dev. 4: 1316–1321.

Knepeh, W., J. Chaftitz, and J.F. Habener. 1990a. Transcriptional activation of the rat glucagon gene by the cyclic AMP-responsive element in pancreatic islet cells. Mol. Cell. Biol. 10: 6799–6804.

Knepeh, W., L. Lepeal, and J.F. Habener. 1990b. A pancreatic islet cell-specific enhancer-like element in the glucagon gene contains two domains binding distinct cellular proteins. J. Biol. Chem. 265: 8725–8735.

Kruse, F., C.T. Komro, C.H. Michnoff, and R.J. MacDonald. 1988. The cell-specific elastase I enhancer comprises two domains. Mol. Cell. Biol. 8: 893–902.

Lai, E., and J.E. Darnell Jr. 1991. Transcriptional control in hepatocytes: A window on development. Trends Biochem. Sci. 16: 427–430.

Le Douarin, N.M. 1988. On the origin of pancreatic endocrine cells. Cell 53: 169–171.

MacDonald, R.J., G.H. Swift, A.E. Pribyl, and J.M. Chirgwin. 1987. Isolation of RNA using guanidinium salts. Methods Enzymol. 152: 219–227.

Maniatis, T., S. Goodbourn, and J.A. Fischer. 1987. Regulation of inducible and tissue-specific gene expression. Science 236: 1237–1245.

McKnight, S.L. and R. Kingsbury. 1982. Transcriptional control signals of a eukaryotic protein-coding gene. Science 217: 316–324.

Meister, A., S.L. Weinrich, C. Nelson, and W.J. Rutter. 1989. The chymotrypsin enhancer core. Specific factor binding and biological activity. J. Biol. Chem. 264: 20744–20751.

Mitchell, P.J. and R. Tjian. 1989. Transcriptional regulation in mammalian cells by sequence-specific DNA binding proteins. Science 245: 371–378.

Moreau, P., R. Hen, B. Wasylik, R. Everett, M. Gauh, and P. Chambon. 1981. The SV40 72-base pair repeat has a striking effect on gene expression both in SV40 and other chimeric recombinants. Nucleic Acids Res. 9: 6047–6068.

Moss, L.G., J.B. Moss, and W.J. Rutter. 1988. Systematic binding analysis of the insulin gene transcription control region: Insulin and immunoglobulin enhancers utilize similar transactivators. Mol. Cell. Biol. 8: 2620–2627.

Nielsen, D.A., T.-C. Chang, and D.J. Shapiro. 1989. A highly sensitive, mixed-phase assay for chloramphenicol acetyltransferase activity in transfected cells. Anal. Biochem. 179: 19–23.

Ohlsson, H. and T. Edlund. 1986. Sequence-specific interactions of nuclear factors with the insulin gene enhancer. Cell 45: 35–44.

Ohlsson, H., O. Karlsson, and T. Edlund. 1988. A beta-cell-specific protein binds to the two major regulatory sequences of the insulin gene enhancer. Proc. Natl. Acad. Sci. 85: 4228–4231.

Onderko, B., S. Shepard, and W. Herr. 1987. Discrete elements within the SV40 enhancer region display different cell-specific enhancer activities. EMBO J. 6: 1017–1025.

Ornitz, D.M., R.D. Palmer, R.E. Hammer, R.L. Brinster, G.H. Swift, and R.J. MacDonald. 1985. Specific expression of an elastase-human growth hormone fusion gene in pancreatic acinar cells of transgenic mice. Nature 313: 600–603.

Ornitz, D.M., R.E. Hammer, B.L. Davison, R.L. Brinster, and R.D. Palmer. 1987. Promoter and enhancer elements from the rat elastase I gene function independently of each other and of heterologous enhancers. Mol. Cell. Biol. 7: 3466–
Differential requirements for cell-specific elastase I enhancer domains in transfected cells and transgenic mice. Genes & Dev. 3:687–696.

Van der Loos, C.M., P.K. Das, and H.J. Houthoff. 1987. An immunoenzyme triple-staining method using both polyclonal and monoclonal antibodies from the same species. Application of combined direct, indirect and avidin-biotin complex (ABC) technique. J. Immunochim. Cytocim. 35:1199–1204.

Veldman, G.M., S. Lupton, and R. Kamen. 1985. Polyomavirus enhancer contains multiple redundant sequence elements that activate both DNA replication and gene expression. Mol. Cell. Biol. 5:649–658.

Verweij, C.L., C. Guidos, and G.R. Crabtree. 1990. Cell type specificity and activation requirements for NFAT-1 [nuclear factor of activated T-cells] transcriptional activity determined by a new method using transgenic mice to assay transcriptional activity of an individual nuclear factor. J. Biol. Chem. 265:15788–15795.

Vincent, J.-P., I.A. Kassis, and P.H. O’Farrell. 1990. A synthetic homeodomain binding site acts as a cell type specific, promoter specific enhancer in Drosophila embryos. EMBO J. 9:2573–2578.

Weber, H., M. Konig, and P. Gruss. 1983. Multiple point mutations affecting the simian virus 40 enhancer. Science 219:626–631.

Weinrich, S.L., A. Meister, and W.J. Rutter. 1991. Exocrine pancreas transcription factor 1 binds to a bipartite enhancer element and activates transcription of acinar genes. Mol. Cell. Biol. 11:4985–4997.

Weintraub, H. 1988. Formation of stable transcription complexes as assayed by analysis of individual templates. Proc. Natl. Acad. Sci. 85:5819–5823.

Weintraub, H., R. Davis, S. Tapscott, M. Thayer, M. Krause, R. Benezra, T.K. Blackwell, D. Turner, R. Rupp, S. Hollenberg, Y. Zhuang, and A. Lassar. 1991. The myoD gene family: Nodal point during specification of the muscle cell lineage. Science 251:761–766.

Yam, L.T., A.J. Ianckila, and C.-Y. Li. 1988. The immunokalline phosphatase methods. In Advances in Immunohistochemistry [ed. R.A. DeLellis], pp.1–29. Raven Press, New York.

Zoller, M.J. and M. Smith. 1984. Oligonucleotide-directed mutagenesis: A simple method using two oligonucleotide primers and a single-stranded DNA template. DNA 3:479–488.
An endocrine-specific element is an integral component of an exocrine-specific pancreatic enhancer.

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