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Cooperation between Elements of an Organ-Specific Transcriptional Enhancer in Animals

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The elastase I gene enhancer that specifies high levels of pancreatic transcription comprises three functional elements (A, B, and C). When assayed individually in transgenic mice, homomultimers of A are acinar cell specific, those of B are islet specific, and those of C are inactive. To determine how the elements interact in the elastase I enhancer and to investigate further the role of the C element, we have examined the activity of the three possible combinations of synthetic double elements in transgenic animals. Combining the A and B elements reconstitutes the exocrine plus endocrine specificity of the intact enhancer with an increased activity in acinar cells compared with that in the A homomultimer. The B element therefore plays a dual role: in islet cells it is capable of activating transcription, whereas in acinar cells it is inactive alone but greatly augments the activity specified by the A element. The C element augments the activity of either the A or B element without affecting their pancreatic cell type specificity. The roles of each element were verified by examining the effects of mutational inactivation of each element within the context of the elastase I enhancer. These results demonstrated that when tested in animals, the individual enhancer elements can perform discrete, separable functions that combine additively for cell type specificity and cooperatively for the overall strength of a multielement stage- and site-specific transcriptional enhancer.

The elastase I (EI) gene is one of the pancreatic digestive enzyme genes which are activated in a stage- and site-specific manner during development of the mammalian gut. EI mRNA is present at very high levels in pancreatic acinar cells, equivalent to approximately 1% of the mRNA population, and at 100- to 1,000-fold-lower levels in other parts of the gastrointestinal tract including the stomach, duodenum, and colon (16, 26). EI mRNA first becomes detectable in the pancreas at about embryonic day 14 in rats (9), about 4 days after the appearance of the pancreatic bud on the developing gut. The transcriptional regulatory regions that confer this organ- and stage-specific expression lie immediately upstream of the EI structural gene (14, 22). This region includes a 134-bp enhancer (−72 to −205) that is both necessary and sufficient for the spectrum of gastrointestinal transcription (8, 26) and a TATA box-containing promoter (+8 to −71) that does not contribute to organ specificity (21, 26). Although organ specific, the isolated enhancer directs expression inappropriately to islet cells as well as acinar cells (14). The acinar cell specificity notable for the endogenous gene is enforced by a negative regulatory region immediately upstream of the enhancer (−206 to −501) that selectively suppresses islet cell expression.

The EI transcriptional enhancer contains three functional elements (A, B, and C) of 12 to 25 bp each (26, 35). When multimerized, the A and B elements can direct the activation of a naive reporter gene in the pancreas of transgenic mice (14, 26). The C element, although necessary for full enhancer activity (13, 34), is inactive as a homomultimer in mice. The A element directs transgene expression to the pancreas, stomach, and intestine, largely recapitulating the organ-specific transcription of the EI gene. The A element binds the pancreatic transcription factor PTF1, which appears to mediate the acinar cell activation of the entire complement of genes for the pancreatic digestive enzymes (5, 26). The B element plays a dual role. In acinar cells, it binds a heteromeric factor (35) and contributes to the strength of the enhancer. However, as a homomultimer and within the context of the isolated (134-bp) enhancer, it activates expression in β cells of the islets of Langerhans (14). In β cells, the B element binds an islet-specific factor distinct from the heteromeric factor of acinar cells (14). The upstream repressor region is responsible for suppressing this islet-specific activity of the B element.

In this report, we show that the properties of the three enhancer elements are additive within the complex physiology of animals by analyzing the activity and specificity of synthetic pairwise combinations in transgenic mice. Moreover, we demonstrate that the elements retain their specific regulatory functions within the context of the enhancer by analyzing in mice the effects of enhancer mutations that inactivate each element in turn.

MATERIALS AND METHODS

Construction of transgenes and analysis of transgenic mice. Transgenes were constructed by standard recombinant DNA procedures (28). Plasmids 6A.EIp.hGH, $6A_{26/32}$.EIp.hGH, and 6A.tkp.hGH (26); 5B.EIp.hGH, 6B.tkp.hGH, and 3(CB).EIp.hGH (14); and −205EIp.hGH (22) were described previously. The sequences of the double-stranded oligonucleotides used to create the tandem repeats of the single elements are given in Fig. 1. Plasmids 6C.EIp.hGH, 3(BA).EIp.hGH, and 3(CA).EIp.hGH were constructed by fusing six tandem repeats of the C domain oligonucleotide (−196 to −165; Fig. 1) or three tandem repeats of the BA or CA oligonucleotides to the EI promoter at −92 of the EIp.hGH plasmid (14). The sequence of the double-stranded BA oligonucleotide (B from −163 to −141 joined at / to A from −120 to −92) was

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ATATCAGATAAATGAGTTGACTT/TTCATGTCACCTGTGCTTTTCCCTGCCTT
  TAGTCTATTTACTCAACTGAA/AAGTACAGTGGACACGAAAAGGGACGGAATA
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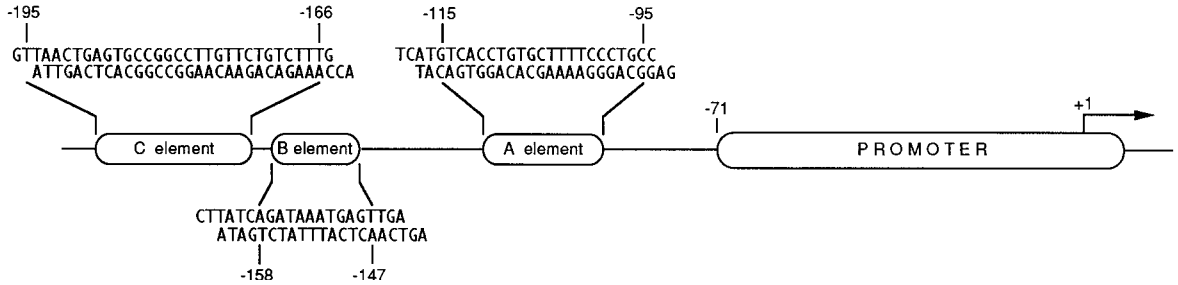

FIG. 1. Schematic of the three regulatory elements of the rat elastase I enhancer. The boundaries for each enhancer element are denoted by the numbers. The double-stranded oligonucleotide sequence used to construct multimers of each element is shown. Each double-stranded oligonucleotide had a 2-bp 5′ overhang for head-to-tail multimerization. The double-stranded oligonucleotide sequences used to construct multimerized combinations of EI enhancer elements are given in Materials and Methods.

The CA oligonucleotide (C from −196 to −165 joined at / to A from −121 to ^{1}−92) was

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GTTAACTGAGTGCCGGCCTTGTTCTGTCTTTG/TTTCATGTCACCTGTGCTTTTCCCTGCCTT
  ATTGACTCACGGCCGGAACAAGACAGAAAC/AAAGTACAGTGGACACGAAAAGGGACGGAACA
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the herpes simplex virus (HSV) thymidine kinase promoter (tkp from −105 to +51) fused to the hGH reporter gene (14, 26). Figure 2 summarizes the results of Northern blot analyses of A, B, and C homomultimeric transgenes for an extended set of independently derived transgenic founder mice. Six tandem copies of the A element (6A.EIp.hGH) or five tandem copies of the B element (5B.EIp.hGH) directed pancreatic expression of hGH mRNA in a fraction of the mice. In contrast, pancreatic hGH mRNA could not be detected in 21 independent transgenic mice bearing 6C.EIp.hGH transgenes (Fig. 2), demonstrating that neither the C element nor the EI promoter is capable of directing pancreatic expression. The EI promoter from nucleotides −72 to +8 without additional regulatory sequences was previously shown to be inactive in transgenic mice (8). Pancreatic expression of the A and B homomultimers driving the HSV tkp (transgenes 6A.tkp.hGH and 6B.tkp.hGH) verified that the activities of the A and B elements were independent of the EI promoter (Fig. 2) (14).

The strength of natural or reconstructed transcriptional regulatory regions can be measured in transgenic mice by two independent parameters: expression penetrance, which is the fraction of independently derived founder mice that express the transgene, and the level of transgenic mRNA in tissues. The former is a measure of the probability of gene activation, and the latter is an indirect measure of transcription rate. Although the A and B elements can activate pancreatic expression, transgenes with homomultimeric A or B elements have a much lower expression penetrance and much lower mRNA levels than do transgenes with the three-element enhancer (Fig. 2). Transgenes containing the intact three-element enhancer with the EI promoter (−205EIp.hGH) were expressed in 90% of independently derived mice (26 of 29 founders tested [reference 8 and data not shown]). In contrast, transgenes containing the multimerized A element were expressed in only 25% of founders (Tables 1 and 2). This analysis included approximately equal numbers of mice bearing three different A-element homomultimeric transgenes (Fig. 2): 6A.EIp.hGH, which has a 26-bp A-element repeat; $6A_{26/32}$. EIp.hGH, which has the spacing of the 26-bp region in the repeat altered by half a helix turn; and 6A.tkp.hGH, which has the EI promoter replaced by the HSV tkp (26). Similarly,

The A element from −115 to −95 was mutated by site-directed mutagenesis of the −205EIp.hGH plasmid so that the −115 to −95 template strand had its sequence backward; i.e., the normal sequence was replaced with its sequence read 3′ to 5′ (not an inversion). The mutant A element sequence was finally altered to 5′-CGTCGCTATTCGTGTCCACTG-3′ by eliminating a potential E box motif that arose (CANNTG, which is part of a functional A element) by transversion mutations. The B and C elements were disrupted by 10-bp substitution mutations between nucleotides −151 and −160 and nucleotides −181 and −190, respectively (13).

Transgenic mice were created by microinjection of fertilized mouse eggs, and the transgene copy number was determined by quantitative dot-blot hybridization as described previously (3), with a human growth hormone (hGH) cDNA hybridization probe (30). Each animal examined in this study was either an independently generated founder mouse or a progeny of another independent founder mouse.

Northern blot hybridization of RNA. Total RNA was isolated from all tissues except pancreas by RNA STAT-60 (Tel-Test "B," Inc., Friendswood, Tex.) (4, 12). Pancreatic RNA was isolated by the guanidine thiocyanate method (17). RNA samples were electrophoresed in 1.5% agarose gels containing methyl mercury (1), transferred to Zeta-probe membrane (Bio-Rad), and hybridized with an hGH cDNA hybridization probe (30). The quality of all RNA preparations was verified by ethidium bromide staining of the agarose gels prior to transfer to the hybridization membrane and again after transfer by examining the ethidium bromide-RNA complexes bound to the Zeta-probe. The level of pancreatic hGH mRNA was quantified with a Betagen Analyser and adjusted for differences in sample load following stripping of the Northern (RNA) blots and rehybridization with an oligonucleotide probe for 18S rRNA.

Immunohistochemical analysis. Pancreatic tissue was fixed in Carnoy's fixative, embedded in paraffin, and sectioned (2- to 5-μm sections) onto polylysine-coated slides for immunolocalization of hGH protein. Immunohistochemical analysis was performed under standard conditions with the biotinylated secondary antibody with streptavidin-linked peroxidase (31). AEC (3-amino-9-ethylcarbazole; Zymed Laboratories Inc.) was used as the substrate chromogen, producing a red-brown deposit. Optimal dilutions of rabbit anti-hGH antibody (DAKO) were between 1:2,000 and 1:4,000 for pancreatic samples from all the transgenic lines except those from mice containing either the 6A.EIp.hGH (1:500) or 5B.EIp.hGH (1:8,000) transgene. In certain cases, immunostaining adjacent sections for synaptophysin was used to identify islet cells (rabbit anti-synaptophysin antibody was a generous gift of Thomas Sudhof).

RESULTS

EI enhancer elements in pairwise combinations are more active than individual elements. Previously, we analyzed the role of the three EI enhancer elements (A, B, and C; Fig. 1) separately by testing the activity of homomultimeric repeats of each driving either the EI promoter (EIp from −92 to +8) or

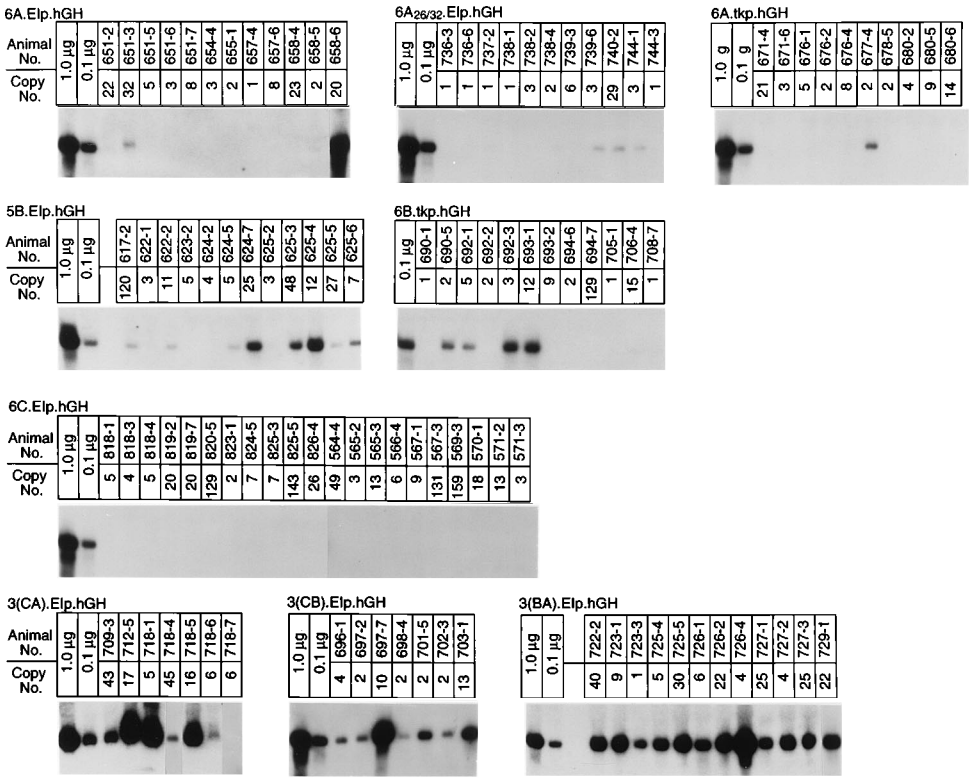

FIG. 2. Northern blot analyses of pancreatic RNA from transgenic founder mice bearing hGH transgenes driven by EI enhancer elements. See Materials and Methods for details of the construction of each transgene. As an example of the single-element transgene nomenclature, the 6A.EIp.hGH transgene contained six tandem copies of the 26-bp doubled-stranded oligonucleotide encompassing the A element (shown in Fig. 1) linked to the EI gene promoter at −92 fused at +8 to the hGH reporter gene at +3. In the 6A.tkp.hGH and the 6B.tkp.hGH constructs, the EI promoter was replaced by the HSV tkp. The $6A_{26/32}$.EIp.hGH transgene is the same as the 6A.EIp.hGH transgene, except that three additional non-EI bases were added to each end of the 26-bp oligonucleotide containing the A element. As an example of the double-element transgene nomenclature, the 3(CA).EIp.hGH transgene contained three tandem copies of a 62-bp double-stranded oligonucleotide containing the C element fused to the A element. All lanes except standards contained 10 μg of pancreatic RNA. Lanes labeled 1.0 μg and 0.1 μg contained the equivalent of 1,000 and 100 hGH mRNAs per cell, respectively, representing 10 and 1% of the average level of expression directed by an intact three-element EI enhancer. Copy no. indicates the number of transgene copies present in each founder animal.

transgenes with B-element homomultimers were expressed in only 54% of founders (Tables 1 and 2).

In addition to poor expression penetrance, levels of transgenic hGH mRNA in the pancreas were approximately 2 orders of magnitude lower for A and B homomultimers than for the intact enhancer (Fig. 2). Among the A multimer animals, the presence of a single, high-expressing animal (6A.EIp.hGH mouse 658-6) was presumably due to the integration of the weak regulatory region into a particularly favorable chromosomal localization. Transgenic mRNA levels remained low despite attempts to optimize expression by changing to the HSV tkp (6A.tkp.hGH) or by changing the junction sequences and the phasing of the tandemly linked A elements by half a helix turn ($6A_{26/32}$.EIp.hGH) (see above). Similarly, expression of the B-element homomultimer with either the EI or the tkp was also about 1% that of the full enhancer (Fig. 2).

To test whether combinations of the elements would overcome the poor expression penetrance and low transgenic mRNA levels, we assembled mixed pairs of enhancer elements linked to the EI promoter (from −92 to +8) and driving the hGH reporter gene. The three paired-element constructs, 3(CA).EIp.hGH, 3(CB).EIp.hGH, and 3(BA).EIp.hGH, each have a total of six regulatory elements, which matches the number generally present in the A, B, and C homomultimeric repeats. Although the A element is separated from the B and C elements in the normal enhancer, the synthetic BA and CA heteromultimers contain the elements directly linked. The transgenes were expressed in 6 of 7 founders for the CA heteromultimer, 7 of 7 for CB, and 12 of 12 for BA (Fig. 2). Therefore, in contrast to the homomultimeric A- or B-element transgenes, mixed pairs of elements increased the expression penetrance to that of the intact EI enhancer (Tables 1 and 2).

Moreover, the double-element constructs expressed approximately 10-fold more pancreatic hGH mRNA than did the homomultimeric A or B constructs (Tables 1 and 2). To ensure the significance of comparisons between mice bearing different transgenes, we analyzed large numbers of independently derived founder mice (Fig. 2). Largely because of unique chromosomal integration sites and gene copy numbers for individual transgenic mice, the expression of the same transgenic construct can vary manyfold among independently derived founders. Nonetheless, combining enhancer elements could be shown to increase transgene expression significantly (Table 2). These data were compared without adjusting for the transgene copy number of each animal. The differences between the homomultimeric and heteromultimeric transgenes were equally significant statistically when the data were compared as mRNA level per transgene (i.e., mRNA per cell per transgene copy number). Addition of the C element, inactive as a homomultimer, to the A element increased average transgene mRNA levels eightfold. Similarly, combining the C and B elements increased mRNA levels sevenfold over the average level of expression of homomultimeric B-element transgenes. The A and B elements together interact synergistically to in-

TABLE 1. Pancreatic expression of fusion transgenes with EI enhancer elements

Transgene	Mean gene copy no. (range)	Expression penetrance (%)	Avg hGH mRNAs/cell[a] (SEM)
6C	37 (2–159)	0 (0/21)	0[b]
6A[c]	12 (1–32)	25 (8/32)	32 (30)
5-6B[d]	23 (2–120)	54 (13/24)	30 (11)
3(CA)	22 (5–45)	86 (6/7)	260 (100)
3(CB)	5 (2–13)	100 (7/7)	210 (150)
3(BA)	16 (1–40)	100 (12/12)	650 (240)

[a] Including nonexpressing mice as 0 mRNAs per cell.
[b] Below detection (<2 mRNAs per cell).
[c] Includes 6A.EIp.hGH, $6A_{26/32}$.EIp.hGH, and 6A.tkp.hGH.
[d] Includes 5B.EIp.hGH and 6B.tkp.hGH.

TABLE 2. Comparison of hGH mRNA levels for different transgenes

Comparison	Fold increase in hGH mRNA level[a]	Two-tailed *P* value
3(CA) vs 6A	8	0.0018
3(CB) vs 5-6B	7	0.0220
3(AB) vs 6A	20	<0.0001
3(AB) vs 5-6B	22	<0.0001

[a] Derived from Table 1.

crease expression levels approximately 20-fold compared with homomultimeric A and B elements, respectively. The combination of A and B elements gave hGH mRNA levels approaching 10% that of transgenes with the complete three-element enhancer.

The cell type specificity of individual elements is additive. As detailed above, when either the A or B elements are separated from the enhancer, they retain the necessary sequence information to activate transcription in the pancreas. However, the two elements do not direct expression in the same pancreatic cell type. Immunocytochemical analysis demonstrated that the A element recapitulated the appropriate acinar cell-specific expression pattern of the endogenous EI gene in the pancreas (Fig. 3, panel A) (26). Expression was not detectable in all acinar cells of the A homomultimer mice, as was the case for mice containing the complete three-element enhancer (data not shown). In contrast, the B element directed expression selectively to the islets of Langerhans in 5B.EIp.hGH mice (Fig. 3, panel B) (14), a site where the endogenous EI is not expressed. Expression was limited to approximately 10% of the β cells (14). hGH protein was not detected in pancreatic samples from mice carrying 6C.EIp.hGH transgenes (Fig. 3, panel C), consistent with the absence of hGH mRNA as shown by Northern blot analysis (Fig. 2).

Although addition of the C element increased the level of activity of A-element transgenes, the acinar cell specificity of the A element was retained (Fig. 3, panel CA). For most mice with the A homomultimeric transgenes, the level of hGH expression, presumably distributed throughout the acinar tissue, was below the level of immunohistochemical detection (26), whereas mice with CA transgenes generally had readily detectable expression that was acinar cell specific for all four independently derived mice and lines examined (Table 3). Consequently, the increased expression due to the C element appears restricted to acinar cells.

The addition of the C element to the B element also produced a stronger transcriptional activator than the homomultimeric B element and did not alter the specificity, because hGH was still selectively localized in islets (Fig. 3, panel CB). For both independently derived 3(CB).EIp.hGH lines examined, the number of β cells expressing hGH increased manyfold over that in 5B.EIp.hGH mice. Because we have not analyzed the colocalization of the hGH reporter and pancreatic polypeptide hormones other than insulin, it is possible that hGH is expressed in other islet cell types of 3(CB).EIp.hGH mice, for example, in α and δ cells.

For transgenic mice containing 3(BA).EIp.hGH transgenes, hGH was present in both acinar and β cells (Fig. 3, panel BA), which is the sum of the specificities of the A and B elements. The B element appears to affect the A-element activity by augmenting acinar cell expression, evident in two ways: the hGH mRNA level was increased 20-fold (Tables 1 and 2), and the fraction of mice with acinar hGH levels detectable by immunohistochemistry was increased (Table 3). Although the transgenic mRNA in the pancreas has both endocrine and exocrine components, the increase must be principally from the acinar cells, because they account for approximately 50-fold more of the pancreatic mass than do islets.

Effects of combining elements on nonpancreatic expression. The homomultimeric A element is active in the stomach and intestine as well as the pancreas (26). This expression pattern is consistent with that of the endogenous EI gene and transgenes directed by the three-element enhancer (26). In contrast, the homomultimeric B element activates transgenes only in the pancreas (14). The multimerized C element, inactive in the pancreas (Fig. 2), was also inactive in all 18 nonpancreatic tissues examined for two different founder mice (Fig. 4, bottom two panels). On the basis of these findings, we predicted that the tissue distribution of hGH mRNA from mice bearing either 3(BA).EIp.hGH or 3(CA).EIp.hGH transgene constructs would closely mimic the gastroenteropancreatic pattern specified by the A element. That this is the case was demonstrated by Northern blot analysis of RNA isolated from 19 different tissues of one BA-multimer mouse and two independently derived CA-multimer mice showing transgene expression in the pancreas, stomach, duodenum, jejunum, and ileum (Fig. 4, top three panels). The BA-multimer mouse also had hGH mRNA in the colon and kidney, while one of the CA-multimer mice (mouse 718-5-6) expressed hGH mRNA in the brain. This limited amount of inappropriate expression is probably due to integration site position effects on the transgenes.

Because the activity of the B-element homomultimers was restricted to the pancreas, we predicted that the site of the 3(CB).EIp.hGH expression would be specified by the B element and thus would be limited to the pancreas. However, hGH mRNA was present in a diverse array of tissues including the brain, submaxillary and sublingual glands, thymus, heart, lung, pancreas, and all the gut tissues except the colon and was present weakly in the skeletal muscle, uterus, and testes (Fig. 4). The expression pattern for the 3(CB).EIp.hGH transgene was nearly identical for two independently derived mice, indicating that the transgene and not the chromosomal integration site determined which tissues expressed hGH mRNA. It is unlikely that this nonpancreatic expression pattern is due to a general activity of the C element, because the C homomultimer is inactive (Fig. 4, bottom). Moreover, the simple interaction between the B and C elements does not produce these results, because such wide-ranging expression does not occur with the wild-type EI enhancer, which has the C and B elements in the same linkage. One possible explanation is that multimerization of the CB oligonucleotide fortuitously created a junction sequence recognized by a transcription factor(s)

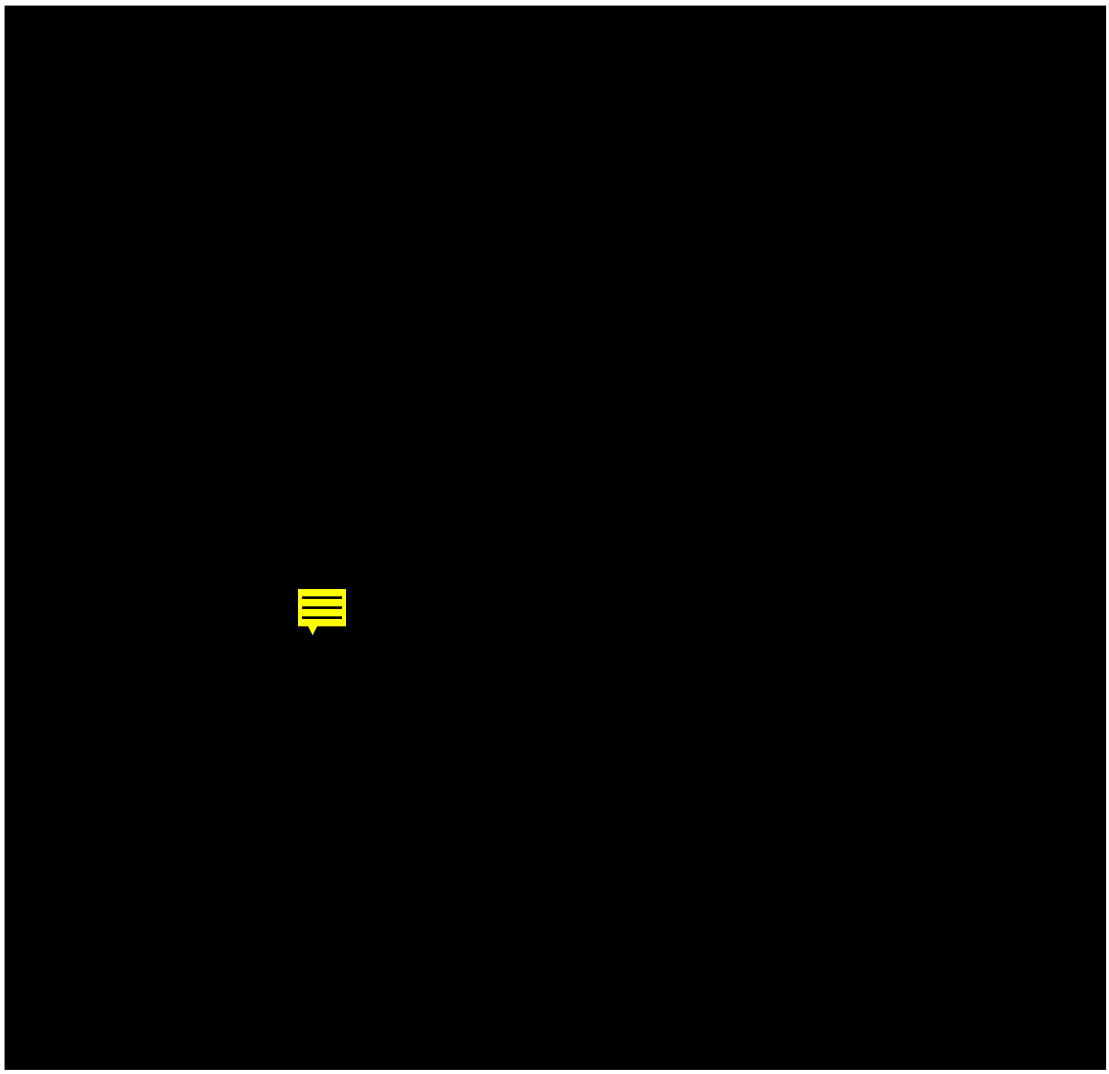

FIG. 3. Immunolocalization of hGH in pancreatic cell types of transgenic animals expressing single- and double-element-hGH fusion transgenes. The reporter hGH was detected by immunocytochemical staining in some pancreatic acinar cells for the 6A.EIp.hGH transgene (a mouse from line 658-6 is shown) (A) and the 3(CA).EIp.hGH transgene (mouse 718-1) (CA). hGH was detected in islet cells for 5B.EIp.hGH (line 617-2) (B) and 3(CB).EIp.hGH (line 701-5) (CB). hGH was detected in both acinar and islet cells for 3(BA).EIp.hGH (mouse 726-4) (BA). There was no staining for hGH in pancreatic cells for the 6C.EIp.hGH transgene (mouse 567-3) (C). Arrowheads point to the edge of the islet(s) in each panel. Bars, 20 μm.

common to a large number of tissues. Nevertheless, in spite of the widened pattern of expression of the CB transgene in tissues outside the pancreas, the expression in the pancreas was still restricted to the islets.

Mutational tests of the role of each element within the context of the enhancer. The properties of EI enhancer elements singly and in combination led to the regulatory strategy proposed in Fig. 5 for the transcriptional specificity of the EI gene in the pancreas. The A element is the sole activating element for acinar cell transcription and plays no detectable role in islet cells. This is demonstrated by the ability of the A element alone as a homomultimer to selectively activate transcription in acinar cells but not islet cells; none of the other elements have this ability. The B element has a role in both the acinar and islet cells of the pancreas: it is responsible for the selective activation in β cells, whereas in acinar cells it augments the activity of the A element. The C element, which cannot activate transcription on its own, augments the activities of the A element in acinar cells and the B element in islet cells without affecting cell type specificity. Finally, a repressor activity located immediately upstream of the EI enhancer suppresses the B-element-mediated EI gene expression in islet cells without silencing acinar expression (14).

To test the function of each element within the context of the enhancer, we constructed three −205EIp.hGH transgenes, each containing a mutation that inactivated either the A, B, or C element (see Materials and Methods). The A-element mutation greatly lowered enhancer activity in transgenic mice (data not shown), consistent with inactivation of the key element responsible for acinar cell transcription. Mutating the B or C elements had a much smaller effect on transgene mRNA levels; the principal effect was increased variation in pancreatic levels of hGH mRNA which was independent of transgene copy number and was probably indicative of increased susceptibility to chromosomal position effects (data not shown).

Because the A element has a low level of activity in the stomach, duodenum, and colon, we examined two independent founder mice for the effect of mutating the A element on

TABLE 3. Expression of EI enhancer element-hGH fusion transgenes in exocrine and endocrine compartments of the pancreas

Transgene	No. of mice examined[a]	No. of mice in which hGH was detected by immunocytochemistry in:	
		Acinar cells	Islet cells
6A.EIp.hGH[b]	1	1	0
5B.EIp.hGH[c]	4	0	4
6C.EIp.hGH	2	0	0
3(CA).EIp.hGH	4	4	0
3(CB).EIp.hGH[d]	2	0	2
3(BA).EIp.hGH	7	4	7

[a] Number of independently derived founder mice or independent lines examined for hGH by immunocytochemistry.

[b] Results summarized from reference 26. Additionally, four independent $6A_{26/32}$.EIp.hGH mice (the low expressors shown in Fig. 2) were examined for hGH, and in all cases hGH was below immunocytochemical detection.

[c] Results summarized from reference 14.

[d] Immunocytochemistry for one of the 3(CB).EIp.hGH lines was reported previously (14).

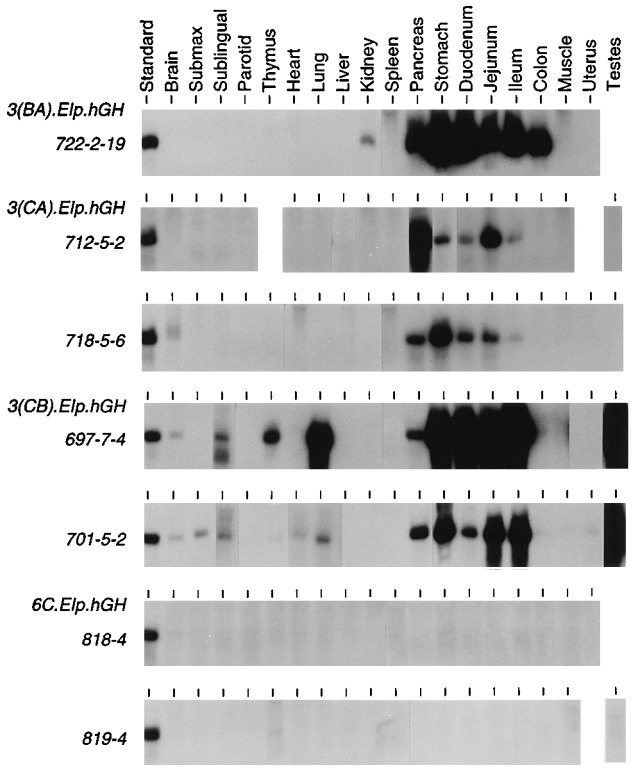

FIG. 4. Northern blot analyses of hGH mRNA in tissues of transgenic mice harboring either EI enhancer double-element multimer transgenes or the multimerized C-element transgene. Tissues from progeny of the 722-2 line were analyzed for expression of the 3(BA).EIp.hGH transgene. Tissues from progeny of two independent transgenic mouse lines were examined for expression of the 3(CA).EIp.hGH (712-5 and 718-5 lines) and the 3(CB).EIp.hGH (697-7 and 701-5 lines) transgenes. Tissues from two founder animals were analyzed for 6C.EIp.hGH activity. All lanes contained 10 μg of total RNA. The standard lane contained the equivalent of 100 hGH mRNAs per cell, representing 1% of the average level of expression directed by a complete EI enhancer.

expression in these tissues. hGH mRNA was not detectable in these three gut tissues of one mouse and was significantly decreased in the duodenum and colon, but retained in the stomach, of the second mouse (data not shown). These results are generally consistent with the proposed role of the A element in the low-level expression in the nonpancreatic tissues of the gut as well as the high-level expression in the pancreas.

To determine the effects of the A, B, and C mutations on expression in individual pancreatic cell types, we examined the distribution of the hGH reporter by immunohistochemistry (Fig. 6). Specific predictions for the effects of each mutation on expression in the exocrine and endocrine compartments of the pancreas that are diagnostic for the model are given in the left-hand portion of Fig. 6. The predictions are based on the qualitatively different roles proposed for the three elements and on the requirement for a minimum of two elements for EI enhancer activity in transgenic mice (34). The results for several independent mice bearing each mutant construct are summarized in Table 4, and representative staining is shown in the right-hand portion of Fig. 6. In the absence of the upstream repressor region, the complete three-element enhancer directs expression to both the acinar and islet tissue compartments (Fig. 6, top panel). hGH protein was detected in both acinar cells and β cells for six of the nine founder mice examined that contained this wild-type −205EIp.hGH transgene.

As predicted, mice with A mutant transgenes lacked acinar cell staining for hGH, because the activating element for acinar cells has been inactivated in the transgene. However, because the A element plays no role in islets, expression in islets was unaffected; the activating element for islets, the B element, and an augmenting element, the C element, were intact and sufficient.

Also as predicted, mice with B mutant transgenes lacked islet staining, because the sole element specifying islet expression was inactivated. However, these mice retained acinar cell staining, because the acinar cell-specific A element and the augmenting C element remained intact.

The most diagnostic test of this model was the inactivation of the C element. In acinar cells, the combination of A and B elements alone should be sufficient for enhancer activity. However, because there is no role for the A element in islet cells, inactivation of the C element leaves only the B element active. Even though the B element contains the information for islet specificity, as a single element it is not sufficient (34). Indeed, four of the five independent C mutant mice examined expressed transgene mRNA in acinar cells, whereas none expressed in islets.

DISCUSSION

The enhancer of the EI gene is a compact and relatively simple eukaryotic transcriptional on-off switch. It activates transcription to high levels in the pancreas and to low levels in other developmentally related gut organs but is inactive elsewhere. It comprises only three mutation-sensitive elements within a span of 100 bp. To understand the role of each element in the natural context of the animal, we have analyzed their activity in transgenic mice. Each of the three elements has distinct regulatory properties (Fig. 5). These separable functions are mediated by discrete DNA-binding transcription factors, and the activity of the complete enhancer is a simple compilation of the properties of the individual elements.

When the elements are tested in pairs, the combinations acquire properties consistent with the expanded action of the intact enhancer in animals. For example, when the acinar cell-specific A element and the islet-specific B element are linked, both cell activities are expressed. Consequently, these two elements alone recapitulate the exocrine plus endocrine specificities of the minimal enhancer. In determining the strength of the enhancer, the actions of the elements are cooperative.

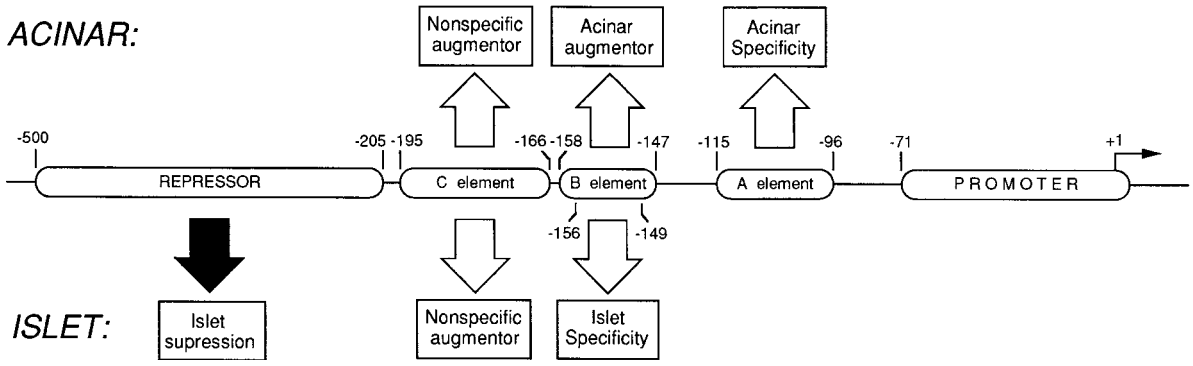

FIG. 5. Regulatory strategy for the expression of the rat EI gene in the pancreas. This model proposes the role of each EI enhancer element and a repressor that cooperate for the selective expression of the EI gene to high levels in the acinar cells of the pancreas. Details are discussed in the text.

When the B element, inactive alone in acinar cells, was combined with the A element, the activity in acinar cells increased manyfold. Similarly, the C element, inactive alone, increased the activity of either the A or B element nearly 10-fold without affecting their cell specificity.

The principal effect of adding the C element to the B element was a striking increase in the number of islet cells that express the transgenes; whereas the homomultimeric B element was active in only about 1 in 10 islet cells, the CB combination was active in nearly all islet cells. This result suggests that the nearly 10-fold-greater activity of the CB combination is principally due to an increased number of expressing cells rather than an increased level of transcription in individual cells. Consequently, adding the C element to the B element appears to increase the probability that the promoter will be active in a given cell, but the rate of transcription, once activated, is not affected. Although it is generally thought that the effect of regulatory sequences on promoter activity is to alter the RNA polymerase load on individual transcription units, some enhancers affect the probability of promoter activation without affecting the polymerase load of an active promoter (25a, 37). The increased expression of the CB transgene compared with the homomultimeric B transgene may be an in vivo manifestation of this kind of enhancer mechanism.

Although increased recruitment of cells with activated transgenes may also occur for the addition of B or C elements to the A element, the expression of A-homomultimer transgenes was generally characterized by hGH reporter levels below immunocytochemical detection, whereas the combination of A with B or C raised acinar cell expression to readily detectable levels. This effect is more consistent with an increased expression in individual cells rather than with the recruitment of greater numbers of expressing cells. In addition to increasing the level of expression in individual transgenic animals, combining elements invariably increased the expression penetrance in mice, so that virtually all animals, rather than a small fraction, expressed the transgenes.

The cooperative association of the three elements in the EI enhancer provides the attributes necessary for useful gene expression in complex animals, i.e., the assurance that all appropriate cells of all individuals transcribe the gene to appropriate (in this case, high) levels. Although generally assumed from the properties of enhancers examined in transfected cells, this has not previously been shown to occur in animals. These results also illustrate the potential to mix and match individual transcriptional elements with distinct regulatory features to create control regions with a wide range of precisely defined regulatory properties for use in animals.

Regulatory functions of the enhancer elements. The model in Fig. 6 (top panel) proposes the molecular basis for the regulatory features of the EI enhancer and is based on the genetic properties of the elements and the biochemical properties of their DNA-binding factors summarized below.

(i) The A element. As a homomultimer or in combination with the C element, the A element directs expression of the transgene in the pancreas selectively to the acinar cells. Mutational inactivation of the A element in the EI enhancer abolishes enhancer activity in cultured acinar tumor cells (13, 26) and in pancreatic acinar cells of transgenic mice (Fig. 6). These results demonstrate that the A element is responsible for the acinar cell type specificity of the EI gene.

An equivalent transcriptional element, previously termed the pancreas consensus element (PCE), is recognizably conserved in the nearby 5′-flanking regions (from −100 to −200) of all 12 murine pancreatic acinar cell-specific genes so far sequenced (2, 32, 33). This conserved element is contained within the minimal transcriptional regulatory regions sufficient for acinar cell specificity in cell transfection experiments (2, 5, 10, 13) and transgenic mice (6, 8, 22, 23) for all the acinar cell-specific genes tested. Selective mutation of the PCE abolishes expression in transfected pancreatic acinar cell lines (2, 5, 10, 13). As a homomultimer, the chymotrypsin gene PCE activated expression in transfected acinar cells (18).

The PCEs of the acinar cell-specific genes, including the A element of the EI enhancer, bind a transcription factor complex (PTF1) found selectively in pancreatic tissue and in acinar tumor cell culture lines (5, 10, 26). Mutations that inactivate the PCE in transfected cells also abolish PTF1 binding (10). PTF1 comprises two DNA-binding proteins that recognize a pair of conserved sequence motifs within the PCE (27). PTF1 is first detectable at the onset of acinar cell-specific gene expression during embryonic pancreatic development (25). These observations indicate that PTF1, through binding to the PCE, is the principal determinant for the transcription of acinar cell-specific genes, although other transcriptional elements (e.g., the B and C elements of the EI enhancer) in the regulatory regions of the acinar genes are required for activity in both cultured cells (13) and animals (34). Weinrich et al. (36) have characterized a similar PCE-binding activity (XPF1), although its relationship with PTF1 and its role in pancreatic gene activation are not yet clear.

(ii) The C element. As a homomultimer, the C element is inactive in transgenic mice (Fig. 2) and transfected acinar, insulinoma, or fibroblast cells in culture (reference 14 and unpublished results). Nonetheless, the mutational inactivation of the C element demonstrated that it plays a crucial role in the

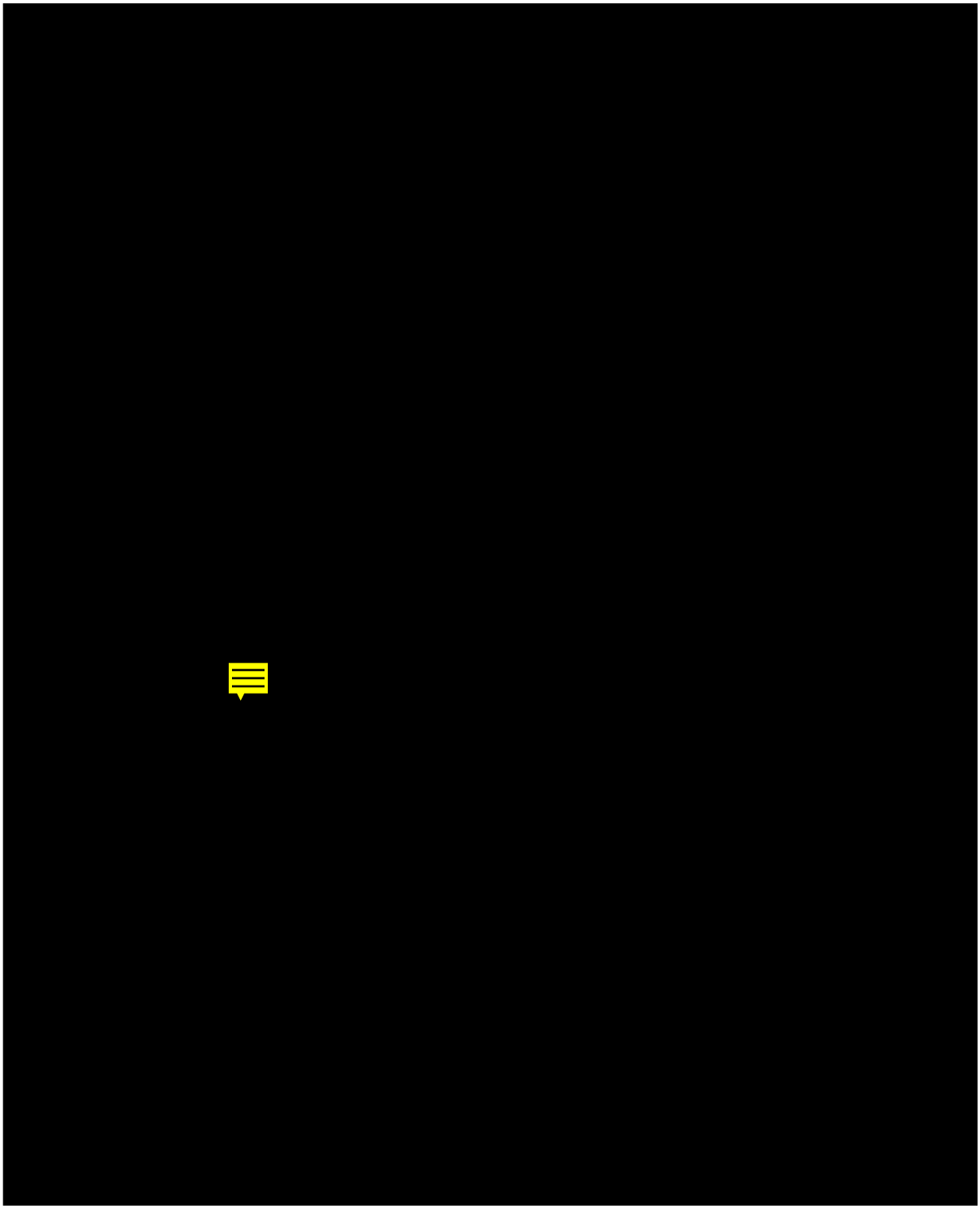

FIG. 6. Genetic test of the role of each EI enhancer element in the regulation of the transcription of the rat EI gene. The diagrams on the left predict the consequences of silencing each enhancer element for expression in acinar and islet cells. The proposed roles for the transcription factors (solid symbols) in acinar and islet cells are described in Discussion. Photomicrographs of the hGH immunolocalization in pancreatic samples for either the wild-type enhancer-hGH (top panel) or mutant enhancer-hGH transgenes (bottom panels) are at the right. Arrowheads point to the edge of the islet(s) in each panel. Bars, 20 μm. As detailed in the text, the experimental results verify each prediction. Transgene copy numbers were 3 for mouse 610-4-20-14 (wild-type enhancer), 5 for mouse 834-6 (A mutant), 27 for mouse 785-1 (B mutant), and 6 for mouse 759-5 (C mutant).

TABLE 4. Expression of mutant EI enhancer-hGH fusion transgenes in exocrine and endocrine compartments of the pancreas

Transgene	No. of mice examined[a]	No. of mice in which hGH was detected by immunocytochemistry in:		
		Acinar cells	Islet cells	Neither cell type[b]
−205EIp.hGH[c]	9	9	6	0
A mutant	8	1	4	4
B mutant	8	4	0	4
C mutant	5	4	0	1

[a] Number of independently derived founder or progeny from independent lines examined for hGH by immunocytochemistry.
[b] Number of mice in which hGH was undetectable in either acinar or islet cells by immunocytochemistry.
[c] Results for the complete three-element enhancer summarized from reference 14.

enhancer in transfected acinar cells (13) and an important though redundant role in mice (34). In animals, the C element is required in the enhancer for islet expression specified by the B element (Fig. 6). Moreover, either the B or the C element is necessary for acinar cell expression in the normal enhancer (Fig. 6) (34). Finally, the C element enhances the activity of synthetic A and B elements (Tables 1 and 2). Alone or in combination with other enhancer elements, it displays no cell type specificity but, rather, increases the activity in cell types specified by the other elements.

(iii) The B element. The B element plays different roles in acinar and islet cells. It was initially identified as a mutation-sensitive region absolutely required for activity of the EI enhancer in pancreatic acinar tumor cells in culture (13). However, as a homomultimer, the B element is inactive in pancreatic acinar cells (14). In combination with the A element, it increases the fraction of transgenic animals that express in acinar cells (the expression penetrance) and increases the level of transgenic mRNA (Tables 1 and 2). Consequently, in acinar cells its role is to augment the activity of the A element, which specifies acinar cell activity, by increasing both the likelihood of gene activation and the level of transcription. Its role in acinar cells appears to be redundant with that of the C element, because mutational inactivation of either the B or C element alone does not detectably affect acinar cell expression in mice (Fig. 6), whereas inactivation of both abolishes expression (34).

Pancreatic acinar cells contain a B-element-binding activity which appears to mediate this augmenting role (35). The binding activity is a heteromeric factor complex which binds the 12-bp B element. Scanning mutations in the region of the B element affect the binding of this complex and transcriptional activity in acinar cells congruently, indicating that the binding complex mediates the acinar activity.

Although as a homomultimer the B element cannot activate transcription in acinar cells, it can activate transcription in islets. This activity is highly selective: expression is not detectable in any of the 19 other major organs examined. The B element retains this specificity within its normal regulatory context as well, because the normal enhancer directs islet expression along with the appropriate acinar cell expression. Indirect evidence for the inherent islet activity of the B element is the presence of a repressor domain, immediately upstream of the enhancer, which suppresses the islet activity but not the acinar activity of the enhancer (14). The repressor is the final regulatory activity that imposes the appropriate acinar cell specificity characteristic of the endogenous EI gene. Definitive proof of the islet activity comes from the effects of inactivating the B element in its natural position in the enhancer, which abolishes islet but not acinar cell expression (Fig. 6).

The region of the B element required for islet activity and the binding factor which appears to mediate the activity are distinct from the acinar cell factor and its binding site (14). The β-cell activity of the B element, assayed by transfection in an insulinoma cell line, resides in an 8-bp central subregion within the 12-bp core required for the acinar cell activity. Therefore, binding sites for two distinct factors with compatible nucleotide sequences are nested within the B element. The B element binds a nuclear protein (originally designated βTF-1) from insulinoma cells that is not found in cell lines derived from other tissues (14). Scanning mutations across the B element affect its transcriptional activity in transfected insulinoma cells and its ability to bind βTF-1 congruently, indicating that βTF-1 mediates the islet-specific transcriptional activity.

This islet activity of the B element represents a regulatory link between the endocrine and exocrine pancreas. Recent evidence (32a) indicates that βTF-1 is the islet homeodomain protein variously called STF-1, IPF1, IDX-1, IUF1, and XIHbox8 (15, 19, 20, 29, 38), and, more recently, PDX, for pancreas and duodenum homeobox factor. In addition to the observation that nucleotide sequences of the known binding sites for this islet factor are similar to the 8-bp βTF-1 site, the known functional IPF1-, STF-1-, and IDX-1-binding sites are effective competitors for the formation of the βTF-1 complex on the EI B element. Moreover, independent antibody preparations against STF-1 and XIHbox8 supershift the βTF-1 complex.

PDX/βTF-1 is present in the nuclei of incipient pancreatic cells at the onset of pancreatic rudiment evagination from the primitive gut (20). As pancreatic development progresses, PDX/βTF-1 is associated principally with insulin-containing endocrine cells, although sizable fractions of somatostatin-, glucagon-, and pancreatic polypeptide-containing cells also contain PDX/βTF-1 (7). Shortly thereafter in pancreatic development, PDX/βTF-1 is also associated with emerging amylase-containing acinar cell precursors (7) but becomes progressively restricted during development, so that in adult rats and mice its pancreatic expression is limited to the islets (7, 19, 20, 24). Thus, PDX/βTF-1 is transiently present in the embryonic pancreatic cells that have begun to activate the program for acinar cell differentiation. These observations indicate that PDX/βTF-1 may be available for binding the B element of the EI enhancer at the time of EI gene activation and suggests that PDX/βTF-1 may play a role in the activation of acinar cell gene transcription in early pancreatic development. Consistent with such a regulatory role in acinar cell differentiation, mice with both PDX/βTF-1 alleles inactivated are born missing both endocrine and exocrine pancreas (11).

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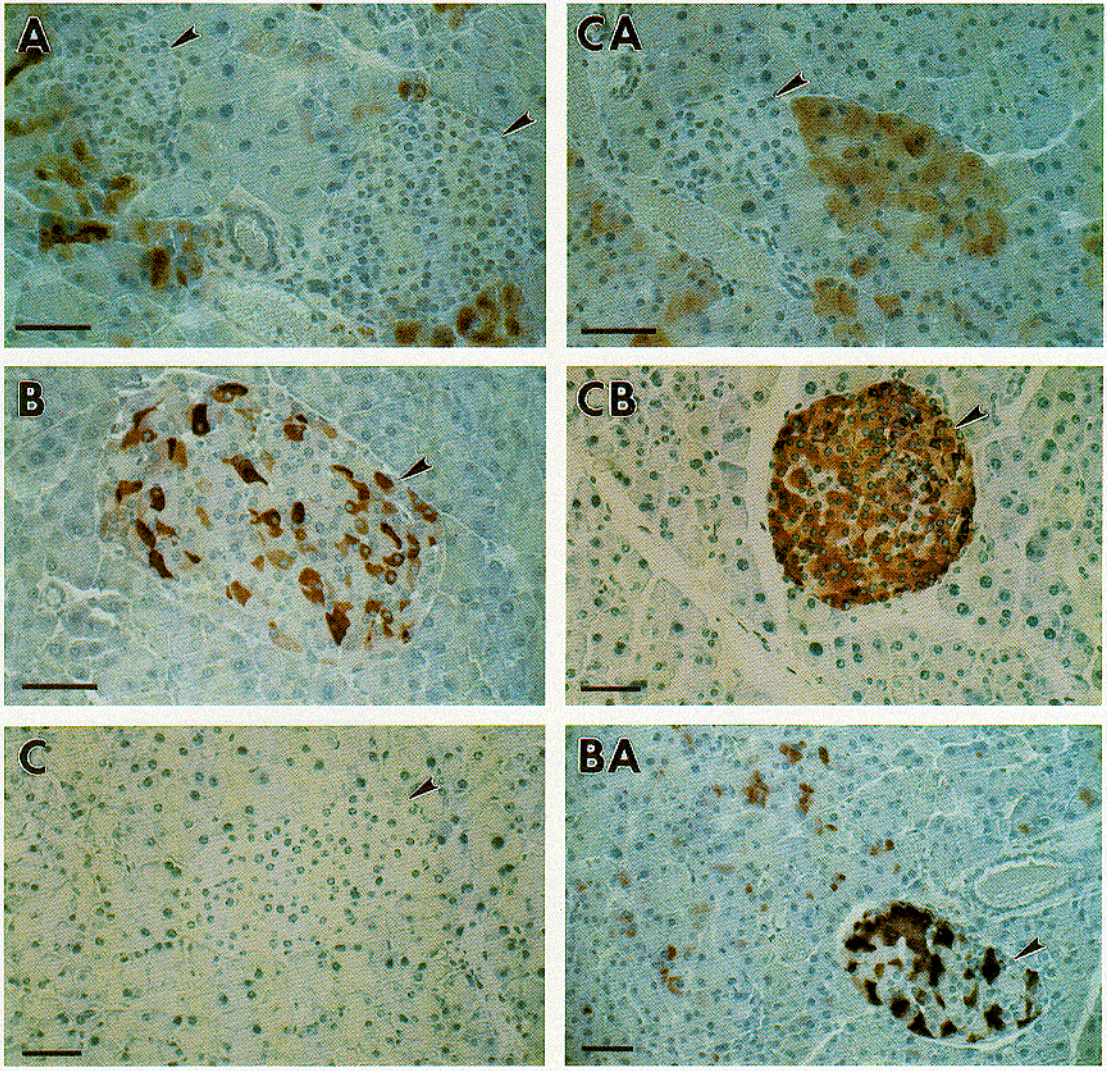
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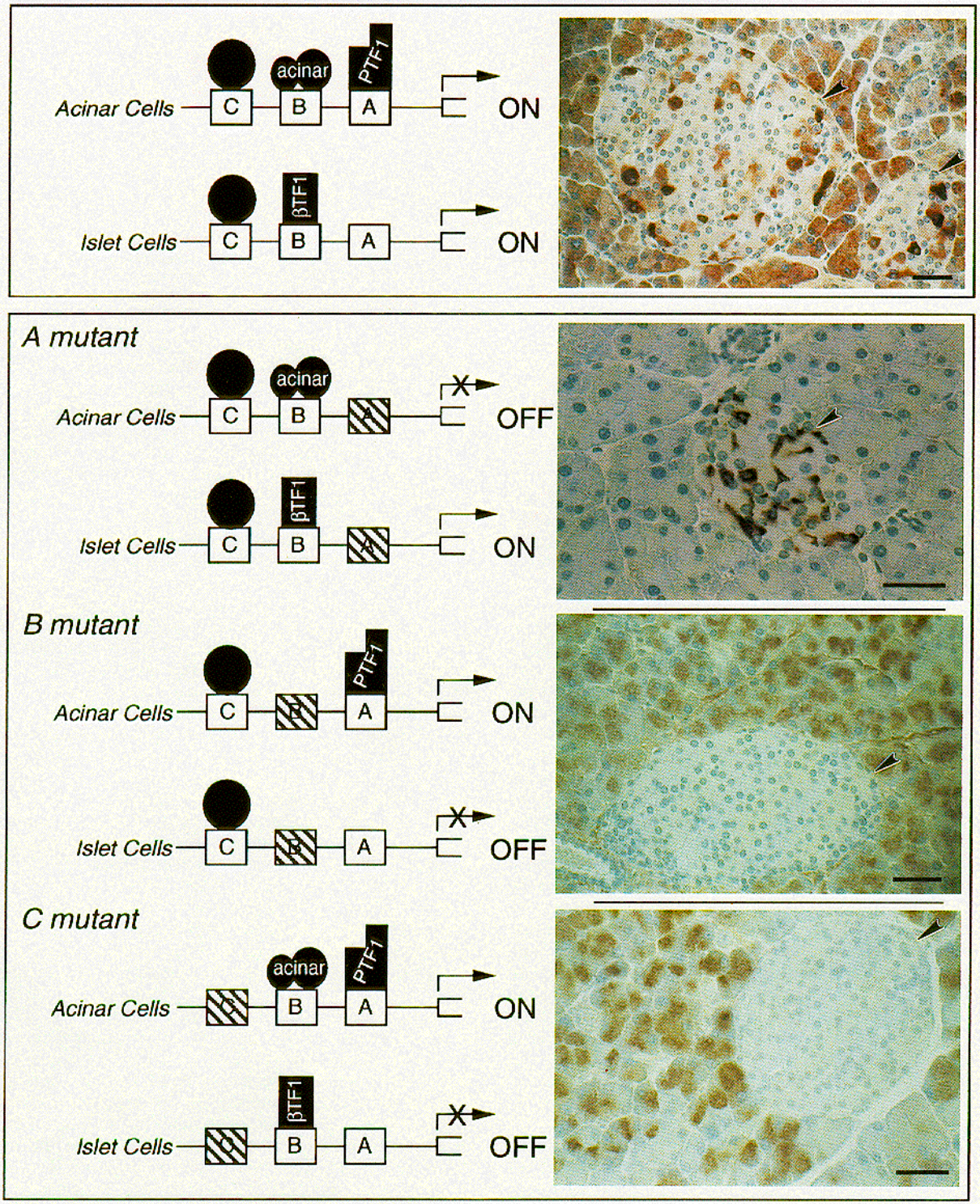
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