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A Single Element of the Elastase I Enhancer Is Sufficient To Direct Transcription Selectively to the Pancreas and Gut

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The elastase I (EI) gene is expressed at high levels in the exocrine pancreas and at lower levels in other regions of the gut. The transcriptional enhancer of the EI gene, from nucleotides −205 to −72, recapitulates the expression of the endogenous gene in transgenic mice; it directs not only pancreatic acinar cell expression of a human growth hormone (hGH) transgene but also expression to the stomach, duodenum, and colon. This pattern of selective expression limited to the gastroenteropancreatic organ system is specified by the A element, one of three functional elements in the EI enhancer. When multimerized, the A element directed expression of a hGH reporter gene selectively to the pancreas, stomach, and intestine in transgenic mice. Immunofluorescent localization of hGH indicated that the A element multimer transgenes were expressed in the acinar cells of the pancreas as well as in Brunner's gland cells of the duodenum. The A element binds a pancreatic acinar cell-specific factor, PTF1. Our results show that while the A element is responsible for directing tissue and cell type specificity, other elements of the enhancer must be involved in the regulation of the level of gene expression.

Cell-specific transcriptional enhancers are complex assemblies of multiple DNA elements and their bound transcription factors. To understand their mechanism of action, it is necessary to understand the role of the individual functional elements and their bound factors in controlling the cell type and strength of activation of the basal transcriptional machinery at the promoter. The tissue-specific transcriptional enhancer of the pancreatic elastase I (EI) gene confers high-level expression in transgenic mice independent of its position relative to the promoter and with a heterologous promoter (15). The enhancer forms a tissue-specific chromatin DNase I-hypersensitive site indicative of a nucleoprotein transcription complex which follows the enhancer when it is moved in transgene constructs either near the promoter, far upstream, or within the reporter gene.

The minimal region that retains these properties spans 134 bp (from −72 to +205) and is composed of three functional elements (A, B, and C). All three elements are necessary for expression in cultured pancreatic acinar cells (20) and important in pancreatic expression in transgenic mice (38). The A element comprises the pancreatic consensus element (PCE), a 17-bp sequence found in the 5′ flanking region of other pancreatic acinar cell digestive enzyme genes as well (2, 8, 37). Multiple copies of the chymotrypsin PCE were sufficient to selectively stimulate the transcriptional activity of a luciferase reporter gene in a pancreatic acinar cell line 30- to 50-fold without affecting expression in insulinoma or fibroblast cell lines (26). The PCE contains a bipartite binding site, with A and B box binding motifs separated by one or two DNA turns (6). The B box motif (CACCTG) coincides with the consensus for E box binding sites for the helix-loop-helix DNA-binding proteins (19, 27, 28). The A box (TTTCCC) has no similarity to other known DNA consensus sequences. The PCE from the amylase gene was used to partially purify a binding activity from pancreatic nuclear extracts (6). This binding activity, designated PTF1, is specific for DNA sequences containing a PCE (6, 16). PTF1 contains two DNA-binding proteins, a 48-kDa subunit which binds to the A box and a 64-kDa subunit which binds to the B box (34). The appearance of PTF1 during the development of the mouse pancreas coincides with the onset of expression of the digestive enzyme genes (32). These observations suggest that PTF1 mediates the action of the PCE in pancreatic acinar cells.

Recent observations indicate that expression of the genes for these enzymes is not limited to the pancreas, but also occurs in stomach and other gut tissues. mRNA for mouse trypsin, elastase, and amylase has been detected in the glandular stomach (4, 8), and amylase has been immunolocalized to the Chief cells of the gastric glands (8). Mouse EI gene expression has been detected in duodenum and colon as well (21). We have defined the role of individual enhancer elements in the transcriptional activation of the pancreatic EI gene in a subset of organs derived from the embryonic gut.

Here we report that the A element of the EI enhancer directs transcription of reporter genes specifically to the pancreas, stomach, and intestine of transgenic mice. This gastroenteropancreatic specificity recapitulates that of the complete EI enhancer and is independent of the other enhancer elements and the EI promoter. In vitro binding studies indicate that the EI A element is specifically bound by the pancreatic transcription factor PTF1.

MATERIALS AND METHODS

Transgene construction and analysis of transgenic mice. Transgenes were constructed by using routine recombinant DNA procedures (35). The construction of the −205EI.hGH transgene was described previously (31); it contains the rat EI enhancer and promoter sequences from nucleotides −205 to +8 fused to the human growth hormone (hGH) reporter gene at +3. The plasmids $6A_{26}$EIp.hGH and $6A_{26/32}$EIp.hGH were

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made by fusing six tandem repeats of the A_{26} or $A_{26/32}$ oligonucleotides (see Fig. 2) to the EI promoter at −92. The plasmid $6A_{26}$tkp.hGH was constructed by inserting an A_{26} hexamer into the −105 *Bam*HI site of −105tkp.hGH (21). To facilitate the removal of the $6A_{26}$tkp.hGH transgene from the plasmid vector sequence, the *Eco*RI site at the 3′ end of the hGH gene was destroyed and a 10-bp oligonucleotide containing a *Xho*I recognition site (GCCTCGAGGC) was inserted. The transgenes were separated from plasmid vector sequences by restriction endonuclease digestion and isolated by preparative agarose gel electrophoresis.

Transgenic mice were created by microinjection of fertilized mouse eggs; mice harboring the transgene were identified, and transgene copy numbers were quantified by dot-blot hybridization of tail DNA as described elsewhere (3) with an hGH cDNA probe (36). Each animal analyzed in this study was either an independently generated founder or a single progeny of another independent founder mouse.

Northern (RNA) blot analysis. Tissue RNA was isolated from adult mice either by guanidine thiocyanate extraction (24) or RNA STAT-60 (Tel-Test "B", Inc., Friendswood, Tex.) (5, 18). Segments of the gastrointestinal tract used for RNA analyses included the entire stomach, 5 to 6 cm of duodenum beginning immediately adjacent to the stomach, the proximal jejunum, the distal ileum, and the distal colon. Pancreatic tissue was microdissected away from the serosal lining of the duodenum and jejunum to eliminate pancreatic contamination of duodenal RNA. Total RNA from each tissue was separated by methyl mercury agarose gel electrophoresis (1) and transferred to a Zetaprobe membrane. Endogenous EI mRNA was detected with a cDNA probe for mouse EI (7), and amylase mRNA (14) was detected with an oligonucleotide 23 bases in length specific for pancreatic amylase (+372 to +394) mRNA. Expression of the transgenes was monitored by hybridization with an hGH cDNA probe (36).

Electrophoretic mobility shift analysis. Nuclear extracts from tissue culture cells were prepared by a modification of the method of Dignam et al. (9), as reported previously (21). Nuclear extracts from tissues were prepared by a modification of the procedure by Gorski et al. (12). Each buffer contained a mixture of protease inhibitors: leupeptin (5 mg/liter), antipain (0.5 mg/liter), chymostatin (1 mg/liter), α1 antitrypsin (1 mg/liter), soybean trypsin inhibitor (0.5 mg/liter), *N*-α-*p*-tosyl-L-lysine chloromethyl ketone (0.5 mg/liter), *N*-tosyl-L-phenylalanine chloromethyl ketone (1 mg/liter), phenylmethylsulfonyl fluoride (50 mg/liter), and 1,10-phenanthroline (6 mg/liter). Minced tissue was resuspended in 3 volumes (per gram of tissue) of homogenization buffer and homogenized with a motor-powered Teflon-glass homogenizer. The homogenate was further diluted with the addition of 5.5 volumes of homogenization buffer, 27 ml of the homogenate was layered over 10 ml of homogenization buffer, and the nuclei were collected by centrifugation in an SW28 rotor at 24,000 rpm for 40 min. The nuclei were resuspended in 4 ml of nuclear resuspension buffer per SW28 tube and lysed by the dropwise addition of NaCl to a final concentration of 0.42 M. The insoluble material was removed by centrifugation in an SW60 rotor at 40,000 rpm for 90 min. The proteins were precipitated by the addition of ammonium sulfate to a final saturation level of 75% and collected by centrifugation in an SS34 rotor at 8,000 rpm for 30 min. The protein pellet was resuspended in dialysis buffer {0.5 to 1.0 ml/5 to 10 g of tissue, 20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 7.9], 20% glycerol, 0.2 mM EDTA, 0.2 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid], and 2 mM dithiothreitol} and dialyzed twice for 2 h against 500 ml of dialysis buffer. The extract was quick frozen in powdered dry ice and stored at −70°C.

The mobility shift binding and electrophoresis conditions used were as described previously (21) with the exception that 0.5 to 2.9 μg of *Escherichia coli* DNA per binding reaction were substituted for poly(dI-dC) · poly(dI-dC).

Methylation interference analysis. Double-stranded A_{26} oligonucleotides were partially methylated by treatment with 0.5% dimethyl sulfate in 200 μl of DMS buffer (50 mM sodium cacodylate [pH 8.0], 10 mM $MgCl_2$, and 1 mM EDTA) at 37°C for 30 min. The reaction was stopped by the addition of 50 μl of DMS stop buffer (1.5 M sodium acetate [pH 7.0], 1.0 M 2-mercaptoethanol, and 100 μg of tRNA per ml). The DNA was purified by two rounds of precipitation with ethanol. Standard binding reactions (21) were carried out, and the nucleoprotein complexes were resolved by native gel electrophoresis. The complexes were visualized by autoradiography, and the oligonucleotides were isolated from the complexes by electroelution. The DNA was cleaved by incubation with 125 μl of 10% piperidine at 90°C for 30 min. The samples were then lyophilized four times, and the DNA fragments were resolved on a 15% polyacrylamide sequencing gel.

Immunofluorescence analysis. Pancreatic and duodenal tissues were immersed in Carnoy's fixative and embedded in paraffin. To ensure that the duodenum was examined thoroughly for hGH-expressing cells, extended regions of the proximal duodenum including the pyloric junction were sectioned longitudinally and immunostained. Standard indirect immunofluorescence techniques were used to detect hGH protein in 2- to 5-μm-thick pancreatic or duodenal sections by overnight incubation with affinity-purified goat anti-hGH antibody (Scantibodies Laboratory, Inc.) diluted to 1:500 (1:8,000 for pancreatic sections containing −500EI.hGH) followed by 1 h of incubation with Texas red-conjugated donkey anti-goat immunoglobulin G (Jackson ImmunoResearch Laboratories; ML grade) diluted to 1:100.

RESULTS

The 134-bp EI enhancer region accurately reproduces the pattern of expression of the endogenous EI gene. The mouse EI gene is expressed at high levels in the acinar cells of the pancreas, but low-level expression also occurs in the stomach, duodenum, and colon (Fig. 1, top panel). These observations expand the expression repertoire of the EI gene beyond the previously believed specificity for the pancreas (37). In addition, a shorter RNA of insufficient size to encode a complete preproelastase I protein was detected in kidney and prostate.

The enhancer of the rat EI gene responsible for pancreatic expression has been shown to reside within 134 bp located in the 5′ flanking region from −205 to −72 (15). To determine whether the enhancer also directs transcription appropriately in the gut tissues, RNA from 19 tissues of a transgenic mouse carrying the −205 to +8 EI enhancer/promoter fragment fused to the hGH reporter gene (−205EI.hGH) was examined for the presence of hGH mRNA (Fig. 1, middle panel). High levels of hGH mRNA were detected by Northern blot analysis in the pancreas, and lower levels were detected in the duodenum and colon. hGH mRNA in the stomach was also detected upon longer exposure of the Northern blot (data not shown).

Another pancreatic acinar gene, mouse amylase 2, is known to be expressed in the stomach (4); its presence is limited to the Chief cells of the glandular region (8). Expression in portions of the intestine has not been examined. Probing a Northern blot of tissue RNAs with an oligonucleotide specific for the pancreatic amylase mRNA revealed the mRNA in the pan-

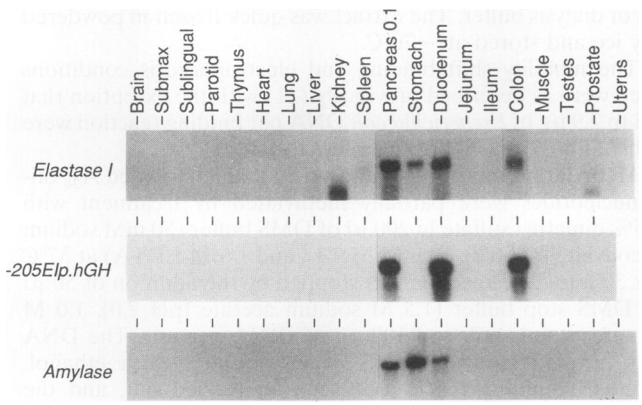

FIG. 1. Northern blot analysis of the expression of endogenous mouse EI, amylase, and the −205EI.hGH transgene. All lanes contained 10 μg of tissue RNA except the pancreas lane, which contained 0.1 μg. (Top) Endogenous EI mRNA levels in tissues from nontransgenic mice. (Middle) hGH RNA levels from a male transgenic mouse carrying three copies of the −205EI.hGH transgene. (Bottom) Endogenous amylase mRNA levels in nontransgenic mice.

creas, stomach, and duodenum, but not in the colon (Fig. 1, bottom panel). These results show that expression of the pancreatic acinar cell digestive enzymes is not limited to the pancreas and stomach but extends to other gut tissues as well and may be a property of the entire complement of the pancreatic hydrolytic enzymes.

The A element of the EI enhancer directs tissue-specific expression in transgenic mice. Previous analysis of the EI enhancer revealed the presence of three functional elements, A, B, and C (20, 38). Using overlapping mutations, we have further refined the boundaries of the A, B, and C elements (Fig. 2A). The A element extends from nucleotides −96 to −115 and contains the PCE. The B element spans nucleotides −146 to −160 and binds general transcription factors as well as an islet β-cell-specific factor (21). The C element is located between nucleotides −166 and −195 and augments but does not direct pancreas-specific expression (21).

This report focuses on the role of the A element in the EI enhancer. To determine whether regulatory information within the A element was sufficient to determine tissue-specific gene expression, we tested a gene construct ($6A_{26}$EIp.hGH) containing six tandem copies of the A element (A_{26}) linked to the EI promoter (EIp) from nucleotides −92 to +8 and driving the hGH reporter gene (Fig. 2B). The multimerized A element constructs were inactive in transfection studies with pancreatic exocrine and endocrine tumor cell lines as well as nonpancreatic cell lines (data not shown). Because mice were more permissive for the expression of a partial EI enhancer than transfected acinar tumor cells (38), we asked whether the A element multimer was active in transgenic mice.

Mice expressing the transgenes were identified by Northern blot analysis of pancreatic RNA obtained by partial pancreatectomies. Out of 12 mice examined, 2 expressed the $6A_{26}$EIp.hGH transgene in the pancreas (data not shown). Transgenes containing the EI promoter but not containing additional regulatory elements are inactive in transgenic mice (15). Therefore, expression of $6A_{26}$EIp.hGH may be attributed to the multimerized A element.

Extended Northern blot tissue surveys were performed on the two mice that expressed the $6A_{26}$EIp.hGH transgene to determine the specificity of expression. Mouse 658-6, which had a reduced (approximately 10-fold) pancreatic hGH mRNA level relative to that of the complete enhancer (e.g., construct −205EI.hGH [Fig. 1]), also had hGH mRNA in the stomach and duodenum (Fig. 3, panel 1). There was no detectable expression in the colon. These results suggested that the A element has all the sequence information necessary to direct transgene expression in the pancreas, stomach, and duodenum. For mouse 651-3, transgene expression was detected at a low, uniform level in all tissues examined (data not shown). This result likely represents a case of the transgene integrated in a chromosomal region that is constitutively active in all tissues, perhaps near a housekeeping gene.

To test the possibility that an artificial sequence element created at the junctions of the homomultimer was affecting expression, as well as to test whether changing the phasing between elements by half a helix turn might increase the level of expression, the sequence of the A element repeat was altered (see Fig. 2B, $A_{26/32}$) by adding three nucleotides on either side of the A_{26} oligonucleotide (the additional nucleotides are transversions of the EI gene sequence at those positions). Five of eleven founder mice bearing this transgene construct ($6A_{26/32}$EIp.hGH) expressed the transgene in the pancreas, but at levels about 100-fold lower than constructs containing the complete enhancer. Extensive Northern blot tissue surveys for two of the mice (739-6-4 and 740-2) (Fig. 3, second and third panels) showed transgene expression in the pancreas, duodenum, and colon similar to that from the endogenous EI gene, from transgenes containing the EI enhancer and promoter from −205 to +8 (−205EI.hGH), and from the shorter A element ($6A_{26}$EIp.hGH). However, this modification of the A element may have decreased pancreatic and gastric expression relative to the intestine. Additional sites of expression in the gut (jejunum and ileum) and the kidney were also observed. The tissue specificities of the $A_{26/32}$ transgene were identical for the two mice, indicating that the homomultimer and not the chromosomal integration site determined which tissues expressed the transgene.

To eliminate the possibility that the EI promoter was required for the tissue specificity of the homomultimer transgenes, it was replaced with the minimal promoter from the herpes simplex virus thymidine kinase gene (construct $6A_{26}$tkp.hGH, Fig. 2B). The fraction of transgenic mice expressing the transgene in the pancreas was low (1 of 11), similar to what was observed with the $6A_{26}$EIp.hGH mice. The $6A_{26}$tkp.hGH transgene was expressed at low levels in the duodenum, jejunum, and ileum, as well as the pancreas (Fig. 3, bottom panel). hGH mRNA was not detected in the stomach or colon.

The expression of the $6A_{26/32}$EIp.hGH and $6A_{26}$tkp.hGH transgenes in the jejunum and ileum was not seen for either the endogenous EI gene or the −205EI.hGH transgene containing the minimal EI enhancer. This inappropriate expression cannot be a consequence of combining the A multimer with a specific promoter, because it occurred with both the cognate EI promoter and the heterologous thymidine kinase promoter. Furthermore, the $A_{26/32}$ and A_{26} elements have different end sequences, so this result cannot be due to the artificial creation of a recognition site for an intestine-specific transcription factor at the junctions of the repeated elements. Additionally, ileum and jejunum expression as a consequence of the transgene site of chromosomal integration is unlikely, because three independent transgenic mice gave similar results. Therefore, the A element must have the information to direct expression in the pancreas and a variety of gut tissues, but within the context of the intact enhancer transcriptional activity in the jejunum and ileum is suppressed.

In summary, we extensively examined the A element mul-

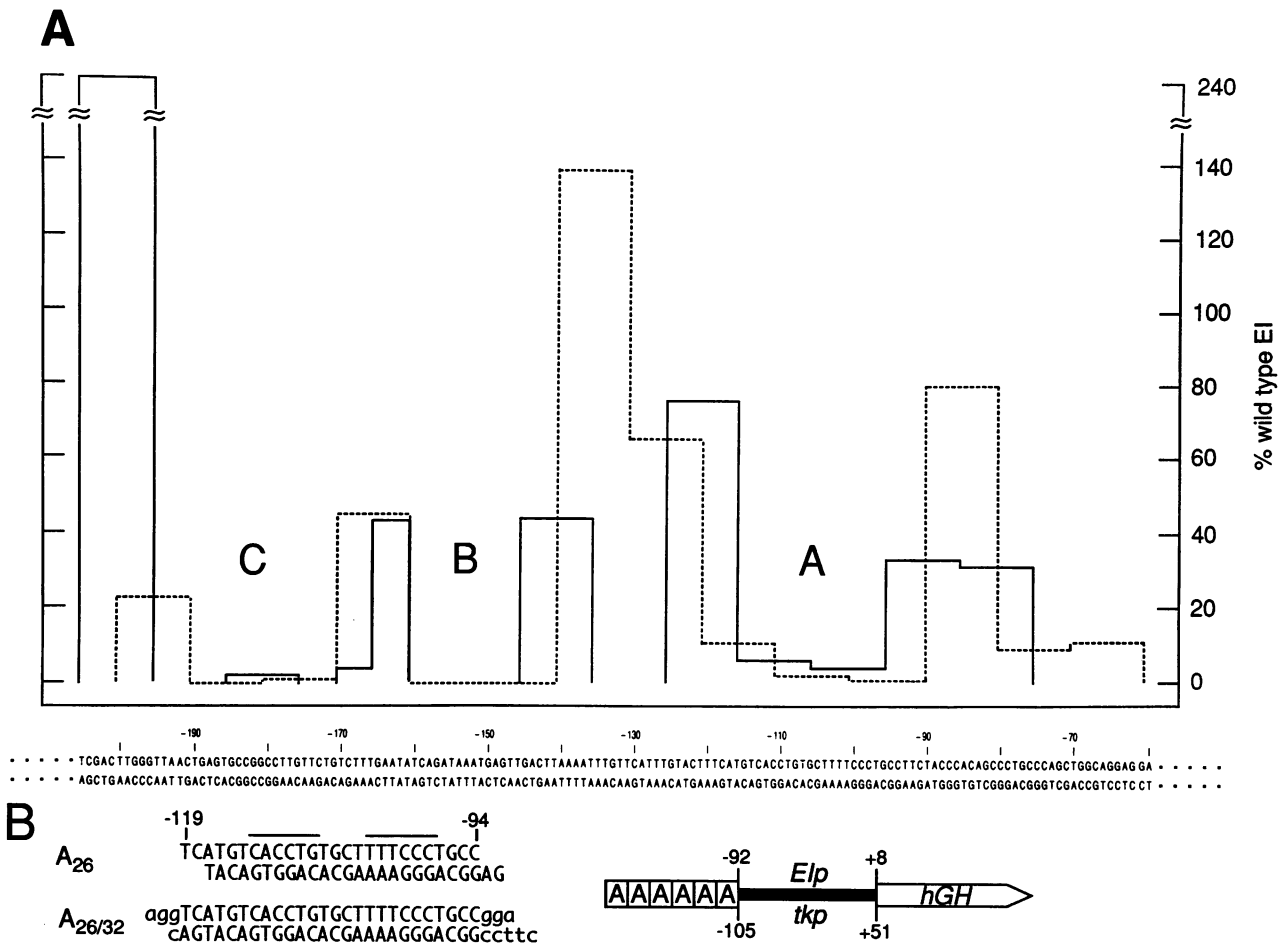

FIG. 2. Overlapping 10-bp substitutions define three functional elements in the EI enhancer. (A) The effects of mutations on the activity of the EI enhancer in transient transfections of a mouse pancreatic acinar cell line (266-6) are indicated above the nucleotide sequence of the EI enhancer. Ten bases of EI enhancer sequence were replaced with the sequence TCTAGATATC starting at nucleotide −205. The dashed lines indicate previously reported results of a set of nonoverlapping 10-bp substitutions (20), and the solid lines indicate new results from an additional set of 10-bp substitutions, offset by 5 bp from the original substitutions, that further define the enhancer elements. Also included as solid lines are the results from two 5-bp transversions from −166 to −170 and −161 to −165. The three mutationally sensitive regions are designated A, B and C. (B) Nucleotide sequences of the two A element oligonucleotides used in the construction of the transgenes. The $A_{26/32}$ oligonucleotide contains the same 26 bases as the A_{26} oligonucleotide (−119 to −94) but has 3 bases (lowercase letters) flanking each end that are transversions of the wild-type enhancer sequence. Both double-stranded oligonucleotides had a 2-bp 5′ overhang for head-to-tail multimerization. A schematic of the two transgenes used in this study is shown to the right. The $6A_{26}$EIp.hGH and $6A_{26/32}$EIp.hGH constructs contained six tandem repeats of the oligonucleotide linked to the EI promoter at −92 to +8 fused to the human growth hormone reporter gene at +3. The $6A_{26}$tkp.hGH construct contained six repeats of the A_{26} oligonucleotide linked to the herpes simplex virus thymidine kinase promoter (−105 to +51) fused to the hGH gene at +3.

timer transgene expression profile of five mice carrying three different A multimer transgene constructs (summarized in Table 1). Four of the five mice showed highly selective expression in the pancreas and gut. Pancreatic levels of transgenic mRNA, however, were 1 to 2 orders of magnitude below that reproducibly obtained with the intact EI enhancer. All nonpancreatic sites of expression of the endogenous EI were represented in one or more of the four transgenic lines (the duodenum was represented in all four). These results indicate that the A element contains the sequence information necessary to direct pancreas- and gut-specific expression independent of its cognate promoter.

The A element directs acinar cell-specific expression in transgenic mice. To identify the pancreatic cell type(s) expressing the various A_{26} multimer transgenes, sites of hGH expression were examined by immunofluorescent localization. Pancreatic expression of transgenes containing the −500 to +8 EI 5′ flanking region fused to the hGH reporter (−500EI.hGH) is limited to the acinar cells (Fig. 4B and reference 21), consistent with the site of expression of the endogenous EI gene. For transgenes $6A_{26}$EIp.hGH (Fig. 4A) and $6A_{26}$tkp.hGH (data not shown), hGH protein was detected in acinar cells and not other pancreatic cell types. There was no detectable pancreatic acinar cell immunofluorescence from three different $6A_{26/32}$EIp.hGH lines even though transgene expression was detected at low levels in the Northern blot analyses. However, in one $6A_{26/32}$EIp.hGH animal (739-6-4) we observed immunofluorescence in a few scattered cells in the islet of Langerhans (data not shown).

The duodenum was also examined to determine which cell

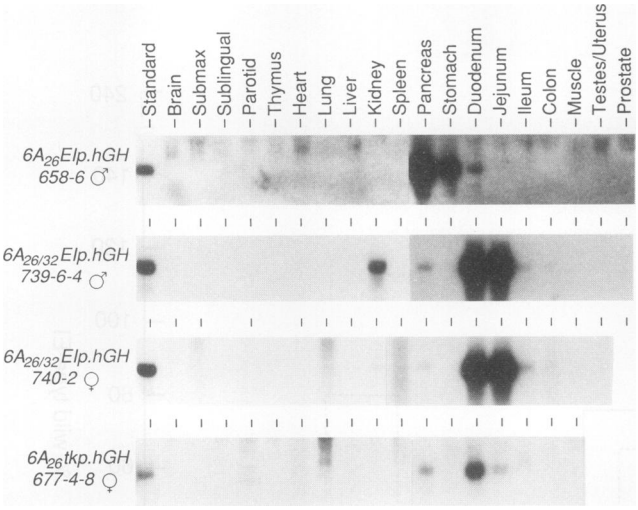

FIG. 3. Northern blot analyses of hGH mRNA in tissues of transgenic mice bearing the A element multimer transgenes. All lanes contained 10 μg of RNA. The standard lane had 0.1 μg of transgenic pancreatic RNA added to 10 μg of rat liver RNA, resulting in a signal equivalent to 100 hGH mRNAs per cell, or 1% of the average level of expression directed by a complete EI enhancer (15). Transgene copy numbers were 20 for mouse 658-6, 13 for 739-6-4, 29 for 740-2, and 6 for 677-4-8.

type expressed the $6A_{26/32}$EIp.hGH transgene. hGH immunofluorescence was limited to scattered Brunner's gland cells in the upper reaches of the duodenum (Fig. 4C). However, fluorescence in individual cells was very intense and may account for the mRNA level in total duodenal RNA (Fig. 3). Similar hGH immunofluorescence was seen in Brunner's glands in the duodena of mice containing the −205EI.hGH transgene (Fig. 4D). Therefore, Brunner's glands are a consistent site of expression, whether the A element is present in the normal context of the EI enhancer or as a homomultimer independent of other enhancer regions.

The A element binds a pancreatic acinar cell-specific complex. To determine whether a common DNA binding activity may mediate A element activity in the pancreas and gut tissues, we examined electrophoretic mobility shift complexes for nuclear extracts from several expressing and nonexpressing tissues (Fig. 5A). Three major complexes were detected with the pancreatic extract. Two of these (labeled a and b) were common to all the tissues examined, whereas the slowest migrating complex was unique. We have been unable to detect this unique binding activity in nonpancreatic tissues including the stomach and duodenum even after increasing the amount of nuclear extract protein in the assay severalfold or by attempting to enrich for A element binding activity by affinity chromatography (data not shown). The inability to detect this binding complex in nuclear extracts from the stomach and duodenum may be due to its presence in these gut tissues at low levels in a small fraction of cells, consistent with the very low level of expression of the endogenous EI gene in these tissues relative to that in the pancreas.

To characterize further the cell specificity of the A element binding activities, we examined pancreatic and nonpancreatic cell lines for the presence of A element binding activity similar to that seen with pancreatic tissue. Nuclear extracts from the cell lines AR4-2J (17) and 266-6 (20), pancreatic acinar cell lines that express EI (38); ARI-P, a line derived from the same pancreatic acinar cell tumor as AR4-2J, but which does not express the acinar cell-specific digestive enzymes (17); RIN 1046-38, a pancreatic endocrine cell line that expresses insulin (33); and Rat2, a fibroblast cell line (39) were analyzed. With the EI A element, a complex of the same mobility as that derived from pancreatic tissue was formed with extracts from the two acinar cell lines tested, but not from the three nonacinar cell lines tested. The same complex also formed with the homologous elements containing the PCE from the chymotrypsin B (Fig. 5B) and amylase 2.1 (data not shown) gene enhancers. Moreover, the formation of the pancreas-specific mobility complex with the EI A element is effectively inhibited by both the chymotrypsin and amylase elements but not by an unrelated oligonucleotide (Fig. 5C). The chymotrypsin element differs from the EI A element at 7 of the 14 nucleotides outside the A and B boxes of the PCE, and the amylase gene PCE region has no discernible similarities other than the A and B boxes. Therefore, the unique binding activity is restricted to pancreatic exocrine cell lines that retain differentiated properties of acinar cells, and it is specific for the PCE.

The pancreas-specific complex is PTF1. The cell-type distribution and the electrophoretic mobility of the pancreatic acinar cell-specific binding complex are consistent with its being the previously characterized PTF1, which binds the PCE of several acinar cell genes including EI (6). To confirm its

TABLE 1. Summary of gene expression in normal and transgenic mice

Gene or transgene and mouse no.	No. of pancreatic expressors/total no. of animals[a]	Presence (+) or absence (−) of expression in indicated site						No. of instances of expression in other tissues/no. of tissues examined
		Pancreas	Stomach	Duodenum	Ileum	Jejunum	Colon	
Endogenous EI	4/4	+	+	+	−	−	+	1/14[b]
−205EI.hGH	26/29	+	+	+	−	−	+	0/13[c]
Endogenous amylase	1/1	+	+	+	−	−	−	0/14
$6A_{26}$EIp.HGH	2/12							
658-6		+	+	+	−	−	−	0/13
$6A_{26/32}$EIp.hGH	4/11							
739-6-4		+	−	+	+	+	+	1/13[d]
740-2		+	−	+	+	+	+	1/10[d]
$6A_{26}$tkp.hGH	1/11							
677-4-8		+	−	+	+	+	−	0/11

[a] Each animal used in this study was an independent transgenic founder or a progeny mouse of an independent founder.
[b] Bladder.
[c] A complete 19-tissue survey of a single mouse was done.
[d] Kidney.

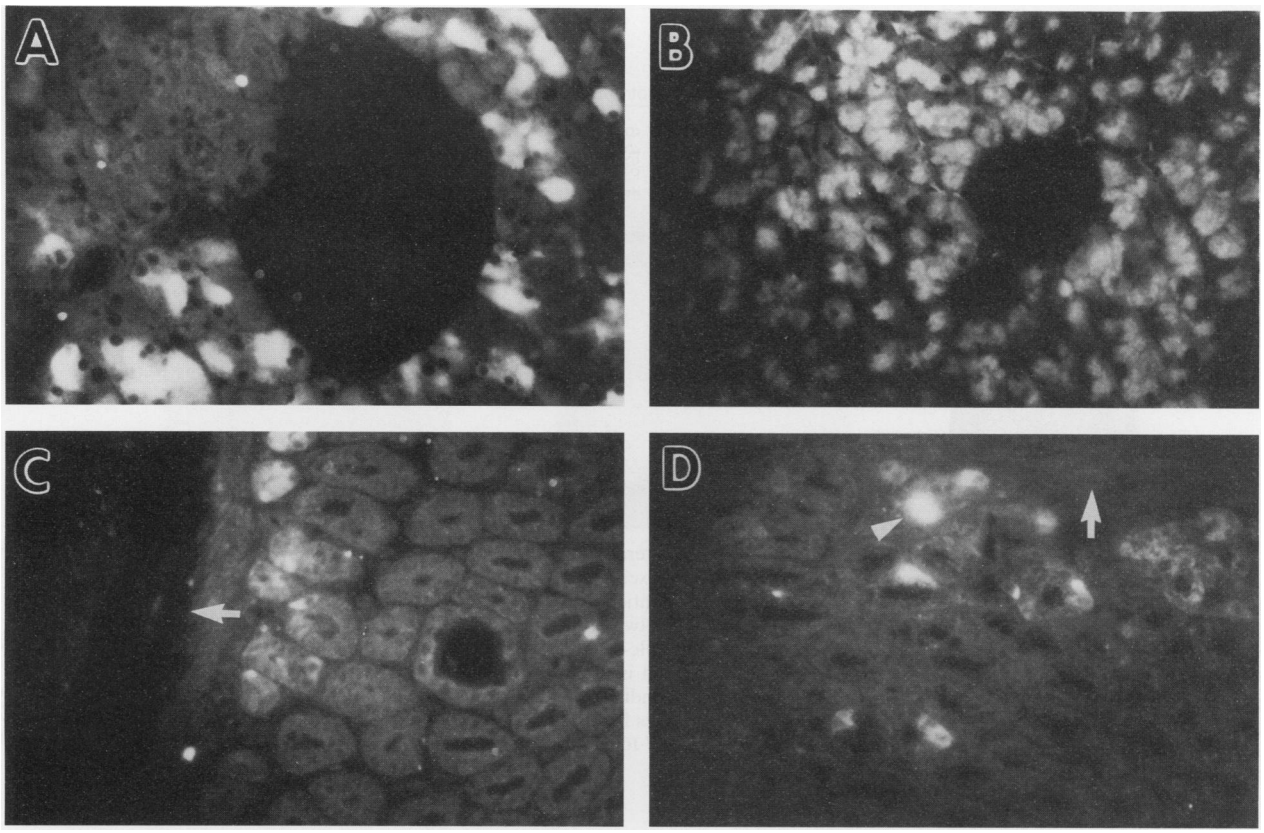

FIG. 4. Immunofluorescence detection of hGH in pancreatic acinar cells and duodenal Brunner's glands. Immunofluorescence staining of hGH in pancreatic acinar cells from a mouse of line 658-6 bearing 20 copies of the $6A_{26}$EIp.hGH transgene (A) and from mouse 778-5 with 9 copies of the −500EI.hGH transgene (B); immunofluorescence localization of hGH in Brunner's gland cells of the upper duodenum from mouse 739-6-4 with 13 copies of the $6A_{26/32}$EIp.hGH transgene (C) and from mouse 610-4-20 with 3 copies of the −205EI.hGH transgene (D). Arrows in panels C and D indicate the smooth muscle layer of the duodenal wall. The arrowhead in panel D indicates an intensely fluorescing cell of a Brunner's gland slightly out of the focal plane. Magnification, ×520.

identity, we compared the G nucleotide contact points of the pancreas-specific complex (designated PTF1 in Fig. 6A) and the nonspecific a and b complexes with those known for PTF1. Formation of the pancreas-specific complex was sensitive to methylation of the two G residues (nucleotides −108 and −115) on the coding strand of the B box of the PCE (Fig. 6B) and to methylation of several G residues in the A and B boxes on the noncoding strand (at nucleotides −98, −99, −100, −110, and −111) (Fig. 6C). In contrast, binding was not affected by methylation of a guanine −113 in the B box and the two guanines between the A and B boxes. Methylation of the pair of guanines at −94 and −95 also partially interferes with binding. These results (summarized in Fig. 6D) largely agree with the published observations for PTF1 (6).

The interference pattern of the a complex was limited to the B box for both coding and noncoding strands (Fig. 6B and C) and was identical to the PTF1 complex over this site. Because the a complex is not pancreas-specific and binds only the B box of the PCE, it is unlikely that this complex by itself plays a role in the positive action of the A element in pancreatic transcription. The B box is nearly identical to the μE box motif that in vitro binds homodimers of the ubiquitous E2A family members E12 and E47 and heterodimers of E12 and E47 with other bHLH factors (29). The a complex can be inhibited with oligonucleotides from the MCK enhancer (23) containing the E box motif, suggesting that ubiquitous E box binding proteins form the a complex (data not shown). The b complex displayed no detectable sites of interference (Fig. 6B and C). Therefore, the pancreas-specific complex appears to be PTF1, whereas the other two binding activities are clearly distinct.

DISCUSSION

The A element is responsible for the spectrum of EI gene specificity in vivo. Through analysis in transgenic mice, we have identified a 26-bp element (the A element) that largely recapitulates the appropriate tissue-specific activity of the EI gene transcriptional enhancer. Hexamer repeats of the A element are capable of directing expression of a naive hGH reporter gene to the pancreas and selective regions of the gut. Pancreatic expression is limited to the acinar cells, the cell type that expresses the genes for EI and the other pancreatic digestive enzymes. These results demonstrate strikingly that a single key element of a complex enhancer can reproduce the tissue and cell specificity of the enhancer even within the context of a complex animal. The PCE motif within the A element binds the pancreatic acinar cell-specific transcription factor PTF1. We propose that bound PTF1 mediates the cell-specific activity of the A element in transgenic mice.

By examining the range of tissue- and cell-specific expression in transgenic mice, we uncovered expression in the gut that would not have been possible to examine by cell transfection

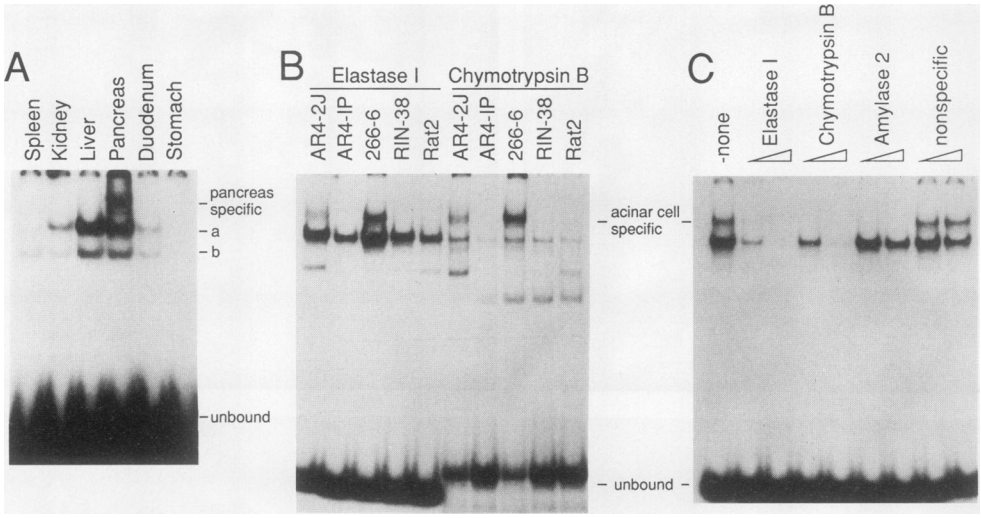

FIG. 5. Electrophoretic mobility gel shift analysis of protein-DNA interactions between the EI A element and nuclear extracts from pancreatic and nonpancreatic cell types. (A) Detection of pancreas-specific complexes using the A_{26} double-stranded oligonucleotide. Nuclear extracts from six different mouse tissues were examined for the binding activities. Identical amounts of protein (8 μg) were used in each binding reaction. The complex unique to the pancreas is labeled pancreas specific; the other two complexes are labeled a and b. (B) Detection of acinar cell-specific complexes with either the double-stranded A_{26} oligonucleotide or a double-stranded chymotrypsin oligonucleotide (−213 to −186) containing the pancreatic consensus binding site (2, 26). The acinar cell-specific band is indicated. (C) DNA sequences containing the pancreatic consensus element compete for the binding of acinar cell-specific complexes. Binding reactions were carried out in the presence of 50- (left lanes) and 500-fold (right lanes) molar excesses of double-stranded oligonucleotides from the EI (−119 to −94) A element (A_{26}), the chymotrypsin (−213 to −186) enhancer core element (26), the amylase 2-IV domain (−157 to −121) (6), and a random sequence 21-mer that does not contain the pancreatic consensus element.

studies. All mice that expressed the A multimer transgenes ($6A_{26}$EIp.hGH, $6A_{26}$tkp.hGH, and $6A_{26/32}$EIp.hGH) in the pancreas also expressed them in the duodenum. Stomach and colon expression was detected sporadically. This activity of the A element in selective parts of the gastrointestinal tract is consistent with normal EI gene expression. The endogenous mouse EI gene is expressed to high levels in the pancreas and to lower levels in the stomach, duodenum, and colon. This same pattern of tissue specificity is seen in transgenic mice carrying a −205EI.hGH transgene. Therefore, the first 205 bp of 5′ EI gene flanking sequence is responsible not only for the pancreatic transcription but for the stomach, duodenum, and colon transcription as well. Similarly, the endogenous rat pancreatic trypsin gene is also expressed in the stomach (8), and a pancreatic amylase gene is expressed in the stomach (4, 8) and duodenum (Fig. 1). Moreover, the nearby 5′ flanking regions of the rat pancreatic amylase and trypsin genes direct transgene expression to the stomach as well as the pancreas (4, 8); expression in other potential sites of the gut has not been examined. These results indicate that low-level expression in several tissues of the gastrointestinal tract may be a common attribute of the pancreatic hydrolytic enzymes. The PCE, present in the EI A element and regulatory regions of the other pancreatic genes, contains regulatory information for this nonpancreatic expression as well.

The organs of the gastroenteropancreatic system share a number of attributes. The stomach, intestine, and pancreas all develop from the primitive gut, contain similar sets of neuroendocrine cells that synthesize polypeptide hormones, and have cells with the morphology of acinar cells. Consequently, it is not surprising that the pancreatic hydrolytic enzymes are shared among these organs as well. The multiple tissue specificity of the EI A element coincides with the domain of this organ system.

Duodenal expression directed by the A element or the minimal EI enhancer is limited to a small fraction of the Brunner's gland cells in the submucosal region of the proximal duodenum. In most mammals, Brunner's glands contain mucus-secreting cells with ill-defined secretory granules, smooth endoplasmic reticulum, and a small oval-shaped nucleus (11, 13). However, in some species, such as mice, rabbits, and horses, a second cell type that has the hallmarks of protein-secreting serous cells very similar to pancreatic acinar and stomach Chief cells (11, 22) is present. The serous cells contain small dense secretory granules located apically, rough endoplasmic reticulum, and a rounded nucleus. In rabbits, these cells are few in number, stain positive for lipase (also found in the pancreas), and are interspersed with the mucous cells (25). Localization of transgenic hGH in the duodenum to serous cells of the Brunner's glands would explain why hGH-positive cells were so few.

Pancreatic acinar cell-specific proteins bind to the EI A element. The pancreatic acinar cell-specific complex that forms on the EI A element appears to be PTF1. Affinity purification using the amylase 2 gene PCE has yielded a protein complex, PTF1, which includes two DNA-binding polypeptides that bind the A and B boxes of the PCE (34). The affinity-purified PTF1 binds the EI A element in vitro, indicating that the PTF1 factors isolated are not specific for the amylase gene PCE (6). Our methylation interference results closely match those seen with affinity-purified PTF1 (6) and indicate that the interactions we see most likely result from PTF1 binding in the pancreatic nuclear extracts.

Affinity purification using the chymotrypsin PCE yielded a 60-kDa protein, XPF-1, that binds to both the A and B boxes (41). The DNase I footprint of XPF-1 covers the A and B boxes not only of chymotrypsin B but also of EI and amylase 2.1. Because methylation interference studies for XPF-1 have

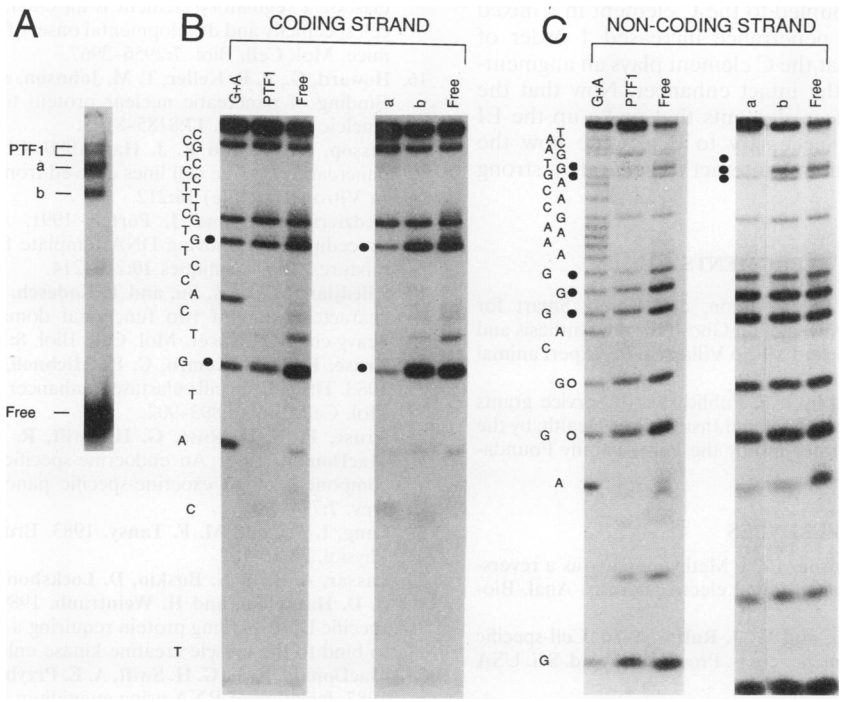

FIG. 6. Methylation interference analysis of DNA-protein complexes formed with the EI A_{26} oligonucleotide. (A) Gel shift of complexes formed between the A_{26} oligonucleotide and pancreatic nuclear extract. The three complexes are labeled PTF1, a, and b. (B and C) Methylation interference analysis on the three complexes from a pancreatic nuclear extract. Results are shown for both the coding and noncoding strands. Lane 1, G+A sequencing reaction; lane 2, PTF1; lanes 3 and 6, the unbound oligonucleotide. Lane 4, the a complex; lane 5, the b complex. The closed and open circles indicate sites of strong and weak interference, respectively. (D) Summary of the results from the methylation interference studies.

not been published, we cannot rule out the possibility that XPF-1, not PTF1, is what we have detected in our gel shift analysis. It seems unlikely, however, that a single DNA-binding protein would give the same methylation interference pattern as PTF1. In neither case has there been definitive proof that these factors are the ones that are acting in vivo to give pancreas-specific gene expression. The proof awaits the cloning and analysis of the genes for these factors.

The results from transgenic mice indicate that there must be tissue-specific factors interacting with the A element in the pancreas, stomach, and duodenum. We failed to detect A element binding activities in nuclear extracts from stomach or duodenum that were not also seen in extracts from nonexpressing tissues. The immunofluorescent staining results indicate that the cells expressing the A element transgene in the duodenum are few. The Chief cells which have been shown to express amylase in the stomach are also infrequent. Consequently, PTF1 may be present in total tissue nuclear extracts commensurate with the very low levels of endogenous EI gene and transgene expression, but below the level detectable by conventional mobility shift assays.

Regulation of the EI gene in the acinar cells of the pancreas. One approach to assessing the contribution of individual elements within complex transcriptional enhancers is to link multiple copies of an element to a passive reporter gene and test that DNA construct in either cell transfections or transgenic mice (10, 26, 30, 40). However, even with multiple copies of the element, the activity may still be far less than that of the intact enhancer (10, 30). This is also the case for the EI A_{26} element. Although A element multimers direct pancreatic and gut-specific expression, the expression is low: only 7 of the 34 independent transgenic mice (20%) expressed the multiple A element transgenes in the pancreas; in contrast, 26 of 29 independent transgenic mice (90%) expressed hGH transgenes with a single-copy 134-bp minimal enhancer containing all three elements (21). Moreover, levels of the hGH reporter mRNA were approximately 1 to 2 orders of magnitude below those for mice with transgenes containing the 134-bp EI minimal enhancer. Consistent with its low activity in vivo, the multimerized A element is ineffective in cultured acinar cells; by transfection assays, the $6A_{26}$EIp.hGH construct is inactive in two independent pancreatic acinar tumor cell lines. The poor expression penetrance (the fraction of transgenic mice that express the transgene) and the low level of transgenic hGH mRNA indicate that A multimers reconstitute a weak regulatory region compared with the 134-bp enhancer.

A similar observation was made for the activity of the multimerized B element of the EI enhancer in mice (21). The B element multimer by itself can direct pancreatic islet β-cell expression, but the penetrance and level of expression of the

transgene are low. When coupled to the C element in a mixed multimer, both levels and penetrance increased 1 order of magnitude (21), implying that the C element plays an augmenting role in the context of the intact enhancer. Now that the actions of individual enhancer elements that make up the EI enhancer are known, it is necessary to determine how the elements and their bound factors interact to generate a strong tissue-restricted enhancer.

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