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The Cell-Specific Elastase I Enhancer Comprises Two Domains

FRED KRUSE, CYNTHIA T. KOMRO, CAROLYN H. MICHNOFF, AND RAYMOND J. MACDONALD*
The Department of Biochemistry, The University of Texas Health Science Center at Dallas, Dallas, Texas 75235

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Two separate domains within the 134-base-pair rat elastase I enhancer and a third domain at the enhancer-promoter boundary are required for selective expression in pancreatic acinar cells. The domains were detected by a series of 10-base-pair substitution mutations across the elastase I gene regulatory region from positions −200 to −61. The effect of each mutant on the pancreas-specific expression of a linked chloramphenicol acetyltransferase gene was assayed by transfection into pancreatic 266-6 acinar cells and control NIH/3T3 cells. The two enhancer domains are nonredundant, because mutations in either eliminated (>100-fold reduction) expression in 266-6 cells. DNase I protection studies of the elastase I enhancer-promoter region with partially purified nuclear extracts from pancreatic tissue and 266-6 cells revealed nine discrete protected regions (footprints) on both DNA strands. One of three footprints that lie within the two functional domains of the enhancer contained a sequence, conserved among several pancreas-specific genes, which when mutated decreased linked chloramphenicol acetyltransferase expression up to 170-fold in 266-6 cells. This footprint may represent a binding site for one or more pancreas-specific regulatory proteins.

The novel biochemical functions of differentiated cells arise through the differential expression of genes contained within all cells. A common underlying mechanism of the cell-specific expression of genes that determine the phenotype of differentiated cells is transcriptional regulation (4, 5). An understanding of the genetic and molecular mechanisms that activate cell-specific gene transcription requires an understanding of the nature of DNA control sequences, the transcription factors that bind those sequences, and the productive consequence of the binding that activates transcription. To investigate these control mechanisms, we have characterized the cell-specific expression of the rat pancreatic elastase I (EI) gene (29) as an example of a transcriptionally regulated gene that contributes to the characteristic phenotype of a differentiated cell type.

EI is a representative member of the subfamily of serine proteases expressed selectively and to high levels in the acinar cells of the exocrine pancreas (16). The pancreatic serine protease subfamily comprises at least nine members, including three isozymes of trypsin, three isozymes of chymotrypsin, and two isozymes of elastase (elastases I and II) as well as glandular kallikrein. These serine proteases are part of the complement of hydrolytic enzymes that are synthesized, stored, and secreted in massive amounts for intestinal digestion and dominate the differentiative phenotype of the exocrine pancreas. EI mRNA accumulates to 10,000 molecules per average adult pancreatic acinar cell, whereas in other tissues EI mRNA levels are at least 1,000-fold lower and are often below detection (30). The acinar cell-specific expression of EI is determined transcriptionally (17). The level of EI mRNA is not modulated by hormones (26) or diet (27). Thus, the control of EI gene expression is simple but rigorous: it is on at a high level in pancreatic acinar cells and essentially off in all other cells.

The gene control sequences necessary and sufficient for rigorous, acinar cell-specific transcription have been defined in transgenic animals. Transgenic mice bearing the entire rat EI gene and extensive flanking sequences express elastase selectively in the pancreas in a manner indistinguishable from the expression of the normal rat and mouse genes (30). The 5′-flanking region of the rat EI gene confers pancreatic transcriptional specificity to a human growth hormone (hGH) reporter gene in animals (20, 21). Moreover, a 134-base-pair (bp) element within the proximal upstream sequences (nucleotides −72 to −205) is sufficient to direct pancreas-specific expression of the hGH gene by using either the hGH or EI promoter (13). When tested in transgenic animals, this regulatory element behaves as a cell-specific enhancer that directs high levels of hGH reporter gene expression in pancreatic acinar cells regardless of its orientation or position relative to the hGH gene (13). At novel positions upstream or within the hGH reporter gene, or when linked to the hGH promoter, the EI enhancer generates DNase I-hypersensitive sites in pancreatic chromatin coincident with the position of the enhancer (13). Therefore, the nucleotide sequence of the enhancer causes the formation of chromatin hypersensitive sites, presumably through the binding of transcription factors to form a pancreas-specific transcription complex. The EI enhancer also directs the correct timing of hGH reporter gene activation during pancreatic development (13). Thus, the minimal regulatory element of the rat EI gene is a cell-specific enhancer that confers appropriate developmental timing and the correct site and level of transcription.

Through the analysis of the effects of 10-bp scanning mutations on expression after transfection into a mouse pancreatic acinar tumor cell line, we demonstrated that the EI enhancer contains two distinct domains; both are required for acinar cell-specific transcription. The domains include binding sites for sequence-specific DNA binding proteins, indicating that the functional sequence elements of the EI enhancer bind factors necessary for pancreatic expression.

MATERIALS AND METHODS

Substitution mutagenesis. A 213-bp *Sal*I-to-*Bam*HI fragment (from nucleotides −205 to +8) containing the enhancer, promoter, and mRNA start site of the cloned EI gene (29) was subcloned from pE0.2hGH (20) into bacteriophage M13mp9. Fourteen individual 10-bp substitution mutants scanning nucleotides −200 to −61 of the EI fragment

* Corresponding author.

were prepared by oligonucleotide-directed mutagenesis (33) of the subcloned EI gene fragment with synthetic oligonucleotides that paired with 13 nucleotides of the wild-type sequence on either side of a 10-nucleotide target sequence. The base sequence for each substitution (see Fig. 2) was identical and consisted of the overlapping restriction site recognition sequences for *Eco*RV and *Xba*I. The mutations were verified by Sanger et al. dideoxy sequencing (24) and restriction endonuclease site analysis with *Eco*RV and *Xba*I enzymes.

Plasmid constructions. The 213-bp EI fragment and each of the 213-bp EI substitution mutant fragments were isolated by digestion with *Sal*I and *Bam*HI and fused to the *Escherichia coli* chloramphenicol acetyltransferase (CAT) (see Fig. 1) gene in the following manner. A 1,630-bp *Hin*dIII-*Bam*HI fragment containing the CAT gene was isolated from pSV2-CAT (10) and subcloned into M13mp8. A new *Bam*HI restriction site was created 12 bp upstream of the CAT translational start site and 13 bp downstream of the *Hin*dIII site by oligonucleotide-directed mutagenesis. The 12-bp sequence upstream of the CAT translational start site was altered to match the EI mRNA sequence between nucleotides +11 and +22, which lies immediately upstream of the EI translational start site. The modified CAT-containing fragment was removed from M13mp8 and inserted into the *Bam*HI site of pUC19. This promoterless modified CAT plasmid was designated mCAT. CAT plasmids containing the EI control region were constructed by placing either the 213-bp EI wild-type or mutant fragments between the *Sal*I and *Bam*HI sites of the mCAT plasmid immediately upstream of the mCAT gene. The construct containing the unaltered EI control region was designated EI-mCAT; constructs containing a 10-bp substitution are designated according to the position of the substitutions (e.g., −91/−100).

RSV-CAT and RSV-gpt, plasmids in which the long terminal repeat of Rous sarcoma virus (RSV) is fused to either CAT (9) or the *E. coli* gene coding for the enzyme xanthine-guanine phosphoribosyltransferase (gpt) (11), were the generous gifts of Cornelia Gorman.

Cell culture, DNA transfection, and measurement of CAT and gpt activity. Mouse pancreatic 266-6 cells (21) and mouse NIH/3T3 cells were grown in Dulbecco modified Eagle medium (DMEM) containing penicillin, streptomycin, and 10% fetal calf serum. Subconfluent cultures of both cell types on 100-mm plates were cotransfected with 10 μg of test plasmid (i.e., EI-mCAT or the EI-mCAT substitution mutants) and 10 μg of RSV-gpt DNA after coprecipitation with calcium phosphate (12). After 18 h the precipitate was washed off the cells, once with Tris-saline (20 mM Tris chloride [pH 7.4], 150 mM NaCl) containing 3 mM EGTA {[ethylene bis(oxyethylenenitrilo)]tetraacetic acid} and then once with Tris-saline. The cells were then incubated in fresh growth medium for an additional 30 h. The cells were harvested by washing three times with phosphate-buffered saline, covered with 1 ml of Tris-EDTA-saline (40 mM Tris chloride [pH 7.4], 1 mM EDTA, 150 mM NaCl), scraped from the plates, and collected by centrifugation in 1.5-ml microfuge tubes.

Preparation of cell extracts and CAT enzyme assays were essentially as described by Gorman (8). Protein was determined by the method of Bradford (2). CAT enzyme units were defined as nanomoles of [^{14}C]chloramphenicol converted to acetylated [^{14}C]chloramphenicol per microgram of cell extract per hour. The gpt enzyme units were defined as micromoles of [^{14}C]xanthine converted to [^{14}C]xanthine monophosphate per microgram of cell extract per hour. CAT enzyme activity was normalized for transfection efficiency by measuring the level of gpt enzyme activity derived from cotransfecting RSV-gpt. The gpt enzyme was assayed by the method of Chu and Berg (3). Normalized CAT activity was then defined as CAT units divided by gpt units.

Preparation of nuclear extracts from rat liver and pancreas. Nuclei were isolated from rat livers and pancreases by modifications of the methods of Osborne et al. (22) and Shapiro et al. (D. J. Shapiro, P. A. Sharp, W. W. Wahli, and M. J. Keller, submitted for publication). All steps were performed at 0 to 4°C. Briefly, livers and trimmed pancreases from male Sprague-Dawley rats were washed in ice-cold phosphate-buffered saline containing 1 mM EDTA, cut into small pieces, then homogenized for eight strokes in a motor-driven Potter-Elvejhem homogenizer in 4 ml of buffer A (10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid] [pH 7.9], 880 mM sucrose, 1 mM EDTA, 1 mM dithiolthreitol [DTT], 0.1 mM leupeptin, 0.6 mM phenylmethylsulfonyl fluoride [PMSF]) per g of initial tissue weight. For liver preparations buffer A was supplemented either with 2 mM magnesium acetate and 1.5 mM $CaCl_2$ or with 0.1 mM EGTA–0.75 mM spermidine–0.15 mM spermine; footprinting results were similar with extracts prepared with either set of cations (see Results). For pancreas preparations buffer A was supplemented with 0.1 mM EGTA–0.75 mM spermidine–0.15 mM spermine. After the homogenate was filtered through cotton gauze, buffer A was added for a final dilution of 6 ml/g of initial tissue weight, then layered over buffer B (buffer A containing 20% glycerol) in a ratio of 3 to 5 ml of homogenate per ml of buffer B. Crude nucleus pellets were collected by centrifugation of the homogenate for 10 min at 800 × *g* in a swinging bucket rotor. The nucleus pellets were suspended in 0.6 ml of buffer A per g of initial tissue weight and then mixed with 0.9 volume of buffer C (10 mM HEPES [pH 7.9], 2.2 M sucrose, 1 mM DTT, 0.1 mM leupeptin, 0.6 mM PMSF). For liver preparations buffer C was supplemented with 1 mM EDTA–5 mM magnesium acetate, and for pancreas preparations buffer C was supplemented with 0.1 mM EDTA–0.75 mM spermidine–0.15 mM spermine. The nucleus suspensions (26 ml) were dispensed into tubes for an SW28 ultracentrifuge rotor (Beckman Instruments, Inc.), underlayered with 10 ml of buffer C, and centrifuged at 62,000 × *g* for 1 h. The nucleus pellet was washed twice by suspension and centrifugation at 2,600 × *g* for 10 min in 10 ml of buffer D (20 mM HEPES [pH 7.9], 0.75 mM spermidine, 0.15 mM spermine, 0.2 mM EDTA, 2 mM EGTA, 25% glycerol, 2 mM DTT, 0.1 mM leupeptin, 0.6 mM PMSF).

The nucleus pellets were suspended in 0.1 to 0.2 ml of buffer D per g of initial tissue weight and mixed with 5 M NaCl for a final concentration of 0.42 M. Proteins were extracted by mixing for 1 h on a Clay-Adams Nutator. Insoluble material was removed by centrifugation in a Beckman SW60 rotor at 175,000 × *g* for 75 min. The extracted proteins were precipitated with the addition of solid $(NH_4)_2SO_4$ to a final 70% saturation. The precipitated protein was dissolved in a minimal volume of buffer E (20 mM HEPES [pH 7.9], 20% glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.2 mM EGTA, 2 mM DTT, 0.6 mM PMSF, 50 μM leupeptin) and dialyzed. After insoluble material was removed by centrifugation at 16,000 × *g* for 1 h, the supernatant was collected and stored at −70°C.

Preparation of 266-6 pancreatic cell extracts. Protein extracts were prepared from 266-6 cells by the method of Shapiro et al. (submitted). Briefly, 266-6 cells were grown at 37°C in DMEM as described above to a density of approxi-

mately 10^6 cells per cm^2. The cells were washed once in ice-cold phosphate-buffered saline containing 1 mM EDTA. Cells were scraped and rinsed off the bottom of the tissue culture flasks in 10 ml of ice-cold phosphate-buffered saline–EDTA per flask. All subsequent steps were carried out at 0 to 4°C. The cells were collected by centrifugation at 170 × *g* for 10 min, suspended in 5 ml of buffer F (10 mM HEPES [pH 7.9], 0.75 mM spermidine, 0.15 mM spermine, 0.1 mM EDTA, 10 mM KCl, 1 mM DTT, 0.6 mM PMSF, 0.1 mM leupeptin) per ml of initial packed cell volume and incubated for 10 min on ice. These cells were collected by centrifugation at 300 × *g* for 10 min, suspended in 2 ml of buffer F per ml of initial packed cell volume, and homogenized with three strokes of the tight-fitting pestle of a Dounce homogenizer. Then 0.1 ml of buffer G (5 mM HEPES [pH 7.9], 75 μM spermidine, 15 μM spermine, 1 mM KCl, 20 μM EDTA, 0.1 mM DTT, 60 μM PMSF, 10 μM leupeptin, 67.5% sucrose) was added per ml of initial packed cell volume and homogenized with three strokes of the loose-fitting pestle. Nuclei were collected by centrifugation at 16,300 × *g* for 20 s in a Sorvall HB-4 rotor. The crude nucleus pellet was suspended in approximately 2 ml of buffer H (20 mM HEPES [pH 7.9], 0.75 mM spermidine, 0.15 mM spermine, 0.2 mM EDTA, 2 mM EGTA, 25% glycerol, 2 mM DTT, 0.6 mM PMSF, 0.1 mM leupeptin) per ml of initial packed cell volume. The nuclear protein was extracted in 0.42 M NaCl, concentrated, and dialyzed as described above for liver and pancreatic cell nuclei.

Column chromatography of 266-6 and tissue extracts. Liver, pancreas, and 266-6 extracts were chromatographed on DE-52 cellulose (Whatman Ltd.) at 2 to 3 mg of protein per ml of resin equilibrated in buffer I (20 mM HEPES [pH 7.9], 1 mM EDTA, 6 mM $MgCl_2$, 100 mM KCl, 20% glycerol, 1 mM DTT, 0.1 mM leupeptin, 0.6 mM PMSF). The flow-through fractions containing footprinting activity were pooled and stored at −70°C.

The 266-6 DE-52 flowthrough fraction was fractionated further by heparin agarose column chromatography in buffer I at 10 mg of protein per ml of resin. The resin was washed with buffer I until the absorbance at 280 nm returned to baseline. Protein was eluted with a linear gradient of 0.1 to 0.5 M KCl in buffer I. Individual fractions were dialyzed overnight against buffer I before use in footprint assays.

DNase I footprinting. DNase I protection assays were performed essentially as described by Osborne et al. (21). Nuclear extracts (5 to 37 μg) of 266-6 cells, rat pancreas, or liver were mixed with 2 to 5 fmol (12,000 cpm) of a ^{32}P-labeled DNA fragment in 50 μl of a buffer containing 25 mM Tris chloride (pH 7.9), 6.25 mM $MgCl_2$, 50 mM KCl, 0.5 mM EDTA, 0.5 mM DTT, 0.1 mM PMSF, 10% glycerol, 2% polyvinyl alcohol, and 0.5 μg of poly(dI-dC) (Pharmacia Fine Chemicals). After incubation on ice for 15 min, 50 μl of 5 mM $CaCl_2$–10 mM $MgCl_2$ was added, followed by incubation at room temperature for 1 min. The samples were then digested with 5 to 160 ng (in 1 to 12 μl) of DNase I (DPFF; Worthington Diagnostics) for 1 min at room temperature. The reactions were terminated by the addition of 150 μl of 1% sodium dodecyl sulfate–20 mM EDTA–200 mM NaCl–67 μg of wheat germ tRNA per ml. The nucleic acids were recovered by phenol-chloroform extraction and ethanol precipitation, suspended in 3 μl of 95% formamide–1 mM EDTA–0.05% bromophenol blue–0.05% xylene cyanol green–25 mM NaOH, denatured at 90°C for 5 min, and resolved by electrophoresis in 8% polyacrylamide sequencing gels (19).

A DNA fragment containing the enhancer and promoter regions from the rat EI gene (nucleotides −205 to +8) was used in the DNase I protection assays. This fragment was generated from pE0.2hGH (20) by digestion with *Hin*dIII to label upstream from the enhancer or *Bam*HI to label downstream of the promoter. The 5′ ends were labeled with [γ-^{32}P]ATP and polynucleotide kinase; the 3′ ends were labeled with [α-^{32}P]dCTP and the Klenow fragment of DNA polymerase I. After a second digestion with the appropriate enzyme (*Hin*dIII or *Bam*HI), the labeled fragment was gel purified and electroeluted (32). In addition to rat EI sequences (nucleotides −205 to +8), the DNA probes contained 15 bp from a polylinker located 5′ to nucleotide −205.

RESULTS

EI-directed expression in transfected pancreatic tumor cells. Experiments with transgenic mice defined a pancreatic acinar cell-specific enhancer between nucleotides −205 and −72 and a functional promoter between nucleotides −71 and +8 of the EI gene 5′-flanking region (13). To more precisely define the limits and nature of the EI transcription control elements, we utilized a mouse pancreatic acinar cell line, 266-6. Mouse 266-6 cells were derived from a pancreatic acinar cell carcinoma excised from a transgenic mouse (20) that expressed simian virus 40 T antigen selectively in the exocrine pancreas. The transgene responsible for production of the tumor consisted of 7.2 kilobases of rat EI 5′-flanking sequence fused to the simian virus 40 T-antigen structural gene. The 266-6 cells in culture accumulate approximately 2 to 5% of the level of EI mRNA as normal mouse pancreas tissue (21; unpublished observations). Thus, these tumor cells continue to express EI, albeit at a much-reduced level.

We tested whether the pancreatic cell line would selectively express CAT fusion gene constructs driven by the EI gene enhancer-promoter region by cotransfecting both 266-6 cells and NIH/3T3 cells with EI-mCAT and RSV-gpt. EI-mCAT contained the rat EI enhancer-promoter element between nucleotides −205 and +8 linked to the CAT reporter gene (Fig. 1); RSV-gpt contained the RSV long terminal repeat fused to the *E. coli* gpt reporter gene (11). The expression of the RSV-gpt construct in the transfected cells served as an internal control for differences in transfection efficiency.

Whereas an enhancer-promoter-less CAT plasmid (mCAT) was essentially inactive in both cell types, EI-mCAT was selectively expressed in the pancreatic acinar cell line (Table 1). EI-mCAT was as inactive as mCAT in NIH/3T3 cells, but 50- to 100-fold more active in the pancreatic acinar cells. In contrast RSV-mCAT, chosen because the RSV enhancer-promoter is a powerful transcriptional activator in a variety of transfected cell lines with little or no cell specificity, was nearly equally expressed in both pancreatic acinar cells and NIH/3T3 cells. These results demonstrated the ability of the EI gene region between nucleotides −205 and +8 to direct selective pancreatic acinar cell expression.

Substitution mutagenesis of the EI control region defines two enhancer domains. To identify domains within the EI regulatory region responsible for pancreas-specific expression, we scanned the 213-bp EI fragment by substitution mutagenesis. Ten base segments starting at nucleotide −200 and ending at −61 were altered, resulting in 14 independent substitution mutants (Fig. 2). Each substitution mutant was fused to mCAT and cotransfected with RSV-gpt into 266-6 and NIH/3T3 cells to test for effects on acinar cell-specific expression (Fig. 3). Scanning down the EI fragment from nucleotide −200 to −61, a number of the substitution muta-

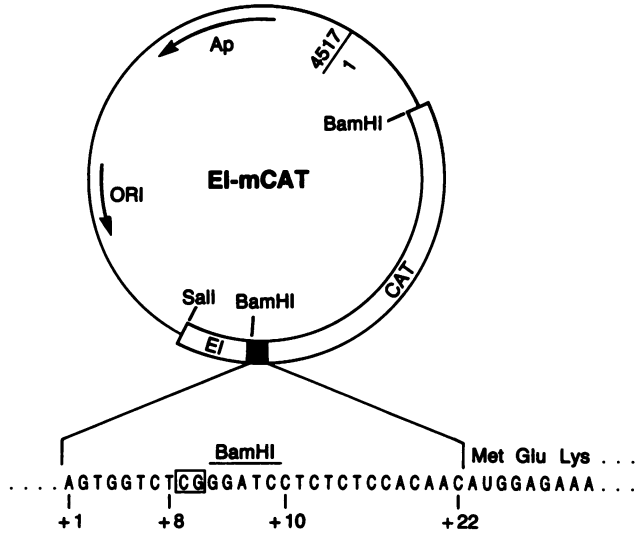

FIG. 1. EI-mCAT construction. The rat EI fragment from nucleotides −205 to +8 was linked to an mCAT reporter gene as described in Materials and Methods. The filled-in box identifies the region where EI was fused to mCAT and consists of the untranslated sequence of EI message from nucleotides +1 to +22 (minus +9) interrupted by the *Bam*HI site at nucleotide +8 (the boxed-in CG dinucleotide is part of a *Bam*HI linker sequence added to nucleotide +8 of EI) (20).

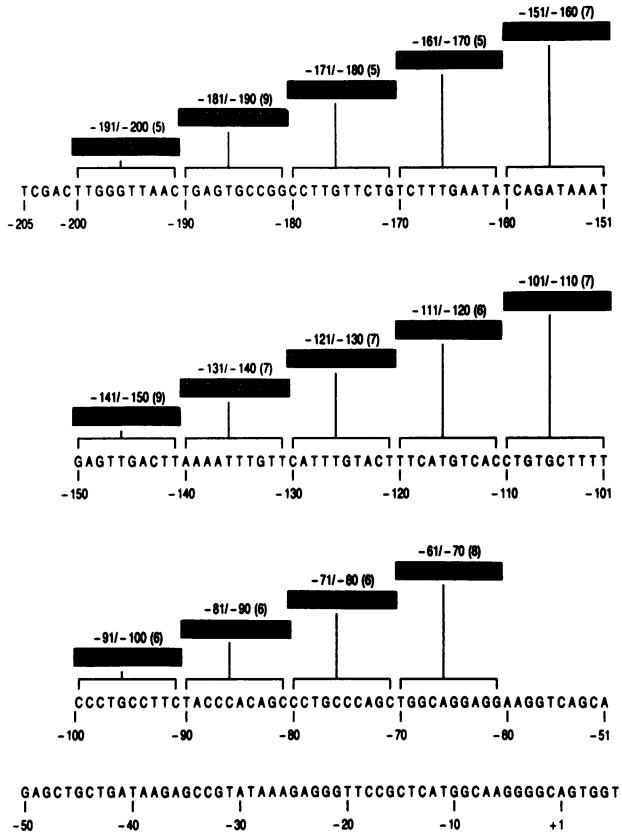

FIG. 2. Substitution mutants of the EI 5′-flanking region. Fourteen individual 10-bp substitution mutants were prepared between nucleotides −200 and −61 of the EI fragment (nucleotides −205 to +8) by oligonucleotide-directed mutagenesis (see Materials and Methods). The base sequence at each substitution (shown in the boxes) is identical and consists of the overlapping restriction endonuclease sites for *Eco*RV and *Xba*I. The numbers in parentheses are the number of EI base changes for a given substitution. The lowercase letters correspond to EI bases that remain unchanged.

tions significantly disrupted the production of CAT activity in 266-6 cells. The results from several experiments in which 266-6 and NIH/3T3 cells were transfected with EI-mCAT and various EI-mCAT mutants are summarized in Table 2.

The results of the scanning mutations delineate two and at least part of a third domain within the EI sequence from nucleotides −200 to −61 that are required for EI-mCAT transcription in 266-6 cells. Two EI enhancer domains, EIeB from nucleotides −140 to −190 and EIeA from nucleotides −90 to −120, lie within the EI gene fragment (nucleotides −205 to −72) previously shown to possess all the properties of a cell-specific enhancer when tested in transgenic mice (13). Inactivation of either domain by mutagenesis abolishes expression in 266-6 cells. The EIeB domain may contain two sudomains from nucleotides −140 to −160 and −170 to −190 since the −161/−170 mutation decreased CAT activity only fivefold compared with EI-mCAT. The third mutation-sensitive domain from nucleotides −61 to −80 covers the boundary between enhancer and promoter elements. It is unclear whether the mutations in this domain affect promoter or enhancer function or possibly both.

The presence of a negative regulatory element that inhibits expression in nonpancreatic cells would be revealed by substitution mutations that increased expression in NIH/3T3 cells. None of the substitution mutants produced significant CAT activity in NIH/3T3 cells (Table 2). By this analysis there is no evidence for a strong negative regulatory element within the EI control region.

TABLE 1. Selective expression by the EI enhancer in transfected cells

Gene construct	Normalized CAT activity[a] in cells		Activity ratio (266-6 to NIH/3T3)
	Pancreatic 266-6	NIH/3T3	
mCAT	0.02 ± 0.01	0.06 ± 0.03	0.33
RSV-mCAT	590 ± 200	740 ± 260	0.80
E1-mCAT	11 ± 6.8	0.12 ± 0.02	92

[a] Data are expressed as mean values ± standard errors from four independent transfections. Normalized CAT activity is defined in Materials and Methods as CAT units divided by gpt units.

EI enhancer and promoter binding proteins. DNase I protection assays were performed to identify nuclear proteins in pancreatic 266-6 cells that specifically bind to the EI enhancer and promoter elements. Nuclear extracts were prepared from 266-6 cells by the rapid and efficient procedure of Shapiro et al. (submitted) that uses spermine and spermidine instead of divalent metal cations to stabilize nuclei while minimizing nuclease and protease activation. To enrich for nuclear DNA-binding proteins, the 266-6 cell extracts were chromatographed on DE–52 cellulose. Flowthrough fractions contained protein which produced the regions of DNase I protection (Fig. 4). All detectable footprinting activity in the crude nuclear extracts was recovered in the DE-52 flowthrough fraction, which was used in all subsequent assays.

A DNA fragment containing the enhancer, promoter, and transcription initiation site of EI gene (nucleotides −205 to +8) was used as the probe in the DNase I protection assays (Fig. 4). Nine discrete regions (I through IX) in the 5′-

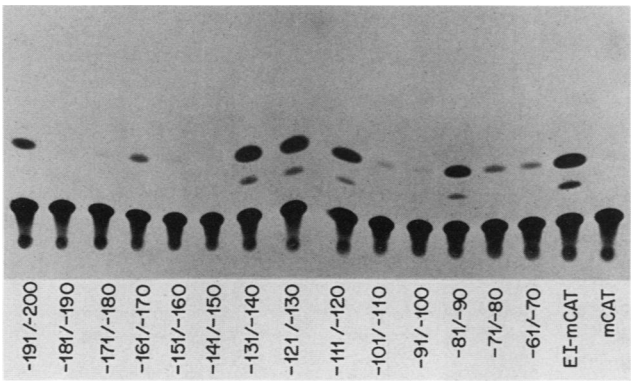

FIG. 3. CAT enzyme activity from extracts of 266-6 cells transfected with mCAT, EI-mCAT, and each of the substitution mutants. Extract protein (60 μg) for each transfected construction was assayed for CAT activity as described in Materials and Methods. The autoradiograph shows the CAT assays performed in experiment 2 of Table 2. The CAT enzyme activity for the −111/−120 mutant appears to be greater than the normalized value listed in Table 2. However, as determined by the cotransfected gpt activity in cells with the −111/−120 construct, this was due to an increased transfection efficiency. The values in Table 2 are normalized for differences in transfection efficiency.

flanking region of the EI gene were protected from DNase I digestion by 266-6 extracts. Most of the footprints showed protected regions on both the coding and noncoding strands of the DNA. Several footprints were bordered by DNase I-hypersensitive sites. Treatment of the DE-52 extract with proteinase K or heat (99°C for 15 min) before the footprint assays abolished all footprints (data not shown). Thus, all protected regions were the result of protein binding to the DNA.

In the enhancer domain EIeB, the most distinctive footprint was observed between nucleotides −141 and −159 (VII), with less protection adjacent to this region (nucleotides −164 to −173, VIII) (coding strand, Fig. 4). In domain EIeA, footprint VI was present on the coding strand (nucleotides −108 to −114) but only marginally detected on the noncoding strand. Three consecutive footprint regions (III through V) were centered around a sequence with dyad symmetry at the enhancer-promoter boundary at nucleotide −72. Two other regions were protected in the promoter domain of the EI gene at the following locations: one immediately upstream from the start of transcription (footprint I) and one near a TATA box sequence (footprint II). Notably, the mutation-insensitive region between the two domains of the enhancer (nucleotides −120 to −140) was devoid of footprints. However, this region contained several DNase I-hypersensitive sites. The significance of DNase I-hypersensitive sites without apparent footprints nearby is unclear. One possibility is that proteins are present at too low a concentration to cause detectable footprints but can still create DNase I-hypersensitive sites proximal to the DNA-binding sites.

Because 266-6 cells have significantly lower levels of EI gene expression than pancreas tissue, we sought to verify that 266-6 cells have the same complement of EI DNA-binding proteins as pancreatic tissue. Because nuclei prepared from tissue require additional purification before the preparation of footprinting extracts, we modified the method of Osborne et al. (22); to control nuclease and protease activities we used spermine and spermidine in place of divalent metal cations. Nonetheless, DNase I protection by 266-6 and pancreatic extracts prepared by the two procedures were very similar (Fig. 5), indicating that 266-6 cells and pancreas may have several DNA-binding proteins in common. The main difference between the two DNase I protection patterns was a narrower and less well protected region VII for pancreatic tissue.

To investigate whether cell-specific DNA-binding proteins could be demonstrated by this analysis, the DNase I protection patterns for pancreatic and liver nuclear extracts prepared similarly were compared (Fig. 5). Both strands of the EI gene region at nucleotides −205 to +8 were assayed; the results for the coding strand are shown. The footprints with liver extracts were similar to those with 266-6 and pancreatic extracts in the promoter region and at the enhancer-promoter boundary of the EI gene (footprints I through V). A few key differences were noted in the enhancer region. A

TABLE 2. Effects of scanning mutations on acinar cell-specific expression

Gene construct	Relative CAT enzyme activity[a]									
	Expt 1		Expt 2		Expt 3		Expt 4		Avg	
	266-6	NIH/3T3	266-6	NIH/3T3	266-6	NIH/3T3	266-6	NIH/3T3	266-6	NIH/3T3
EI-mCAT	(100)	1.2	(100)	0.60	(100)	4.2	(100)	2.4	(100)	2.1 ± 0.90
−61/−70					7.3	0[b]	17	1.8	12 ± 6.8	0.90 ± 1.2
−71/−80					7.3	0.30	13	1.0	10 ± 4.0	0.60 ± 0.49
−81/−90					55	0	103	1.0	79 ± 34	0.50 ± 0.70
−91/−100	1.2	3.0	0	0					0.60 ± 0.84	1.5 ± 2.1
−101/−110	0.06	2.1	1.4	0.91					0.73 ± 0.95	1.5 ± 0.84
−111/−120	5.6	4.6	12.6	3.4					9.1 ± 4.9	4.0 ± 0.85
−121/−130	36	0.80	62	0			95	0.50	64 ± 20	0.43 ± 0.29
−131/−140	120	2.4	68	0.10			171	0.20	120 ± 36	0.90 ± 0.92
−141/−150	0	2.9	0	0.60			0.16	1.8	0.05 ± 0.06	1.8 ± 0.81
−151/−160	0.35	4.6	0	1.8					0.18 ± 0.25	3.2 ± 2.0
−161/−170	26	5.1	21	0.20			13	3.4	20 ± 4.6	2.9 ± 1.7
−171/−180	0	2.4	2.9	0					1.4 ± 2.0	1.2 ± 1.7
−181/−190					0.20	0	0.05	0.80	0.12 ± 0.11	0.40 ± 0.56
−191/−200					19	1.5	26	0	22 ± 4.9	0.75 ± 1.1

[a] Expressed as the percentage of normalized CAT activity (defined in Materials and Methods) derived from 266-6 cells transfected with EI-mCAT in each experiment. Averaged values are expressed as mean values ± standard errors.

[b] CAT activity below the level of detection: equal to or less than the background CAT activity value obtained by transfection of 266-6 cells with salmon sperm DNA.

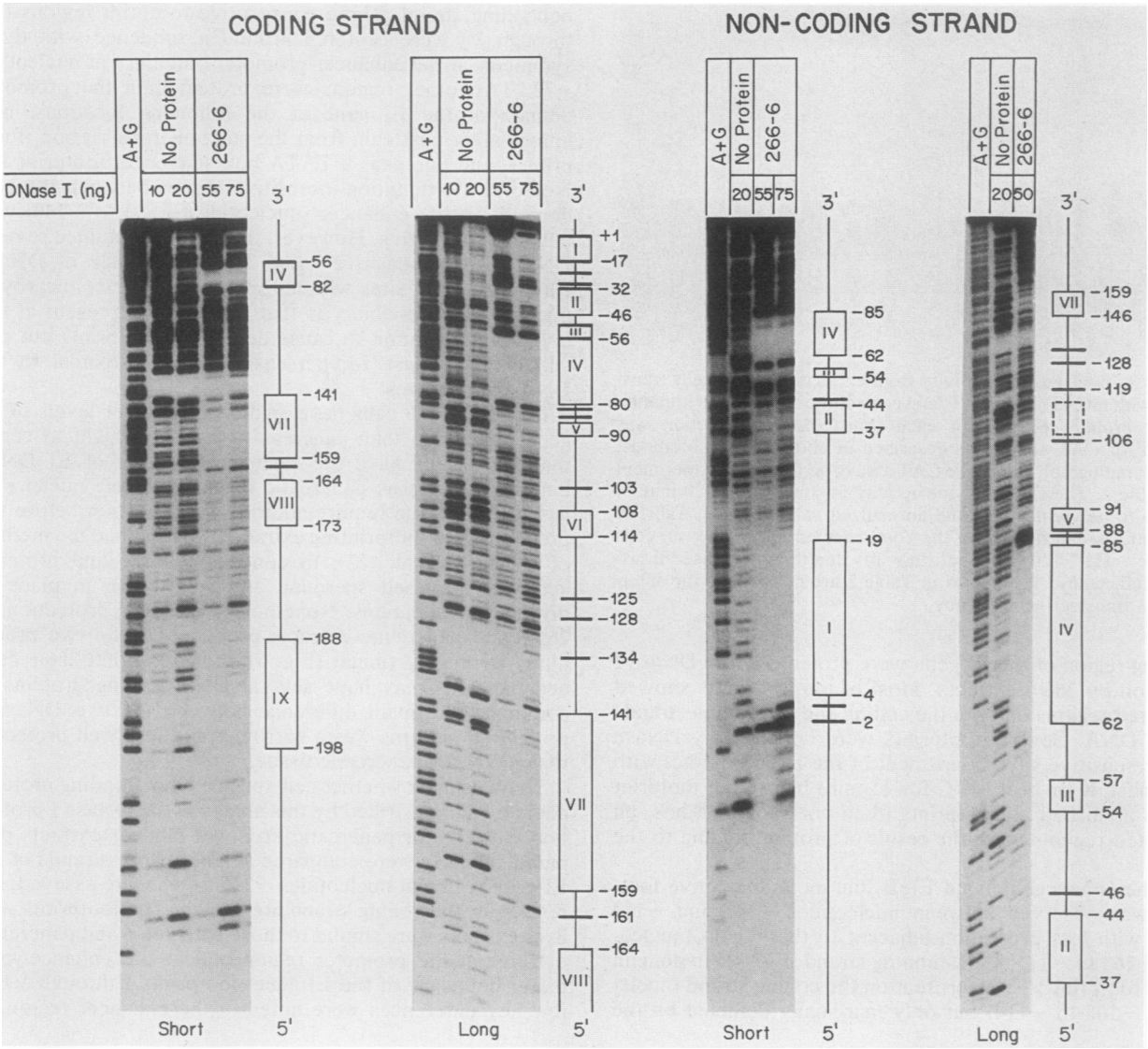

FIG. 4. DNase I footprinting analysis of the enhancer and promoter regions of the rat EI gene. The coding strand of the EI control region from nucleotides −205 to +8 was 5′ end-labeled 15 nucleotides upstream of nucleotide −205 at a *Hin*dIII site in a polylinker. The noncoding strand was labeled at the 3′ end of the same *Hin*dIII site. DNase I protection assays were performed as described in Materials and Methods with 37 μg of DE-52 flowthrough protein from 266-6 cells and 5 fmol of the DNA probes. The amount of DNase I included in each reaction is shown above each lane. Long and short runs are included to adequately show regions of protection at both ends of the DNA fragment. DNase I-protected regions I through IX are shown to the right of each autoradiograph; hypersensitive sites are denoted by thick horizontal lines along each drawing. Nucleotide positions at footprint boundaries are noted. Maxam and Gilbert A+G sequencing reactions of the same DNA fragment are included to provide size markers.

novel footprint between nucleotides −124 and −131 was produced with liver extracts. This footprint maps between the two functional domains of the enhancer in the region that is largely insensitive to mutation. Furthermore, the weak footprint in region VI on the coding strand appears to be absent with liver extracts; however, the DNase I pattern on the noncoding strand is similar in this region for pancreatic and liver extracts. Further investigation is needed to determine whether the differences in footprints between pancreatic cells and liver cells are due to the binding of proteins that specify pancreas-specific expression.

The 266-6 nuclear extracts fractionated further by heparin agarose chromatography gave the most definitive footprints and confirmed less well-defined protected regions (Fig. 6). There was no detectable loss of footprinting activity between protein applied to and protein eluted from the heparin agarose column. Partial resolution of footprinting activity for regions I and III through VI from regions VII and VIII (and possibly from region II) suggest that these sets of footprints are due to the binding of different proteins. Protein that produced region VII and VIII footprints (nucleotides −146 to −172) was eluted first in fractions 38 through 42. The region VIII footprint was stronger with heparin agarose fractions than with the protein applied to the column. Footprint activity for regions I and III through V were present in fractions 40 through 46. Similarly, the region VI footprint was produced with the coding strand and fractions 40 through 46 (data not shown). The region IV footprint

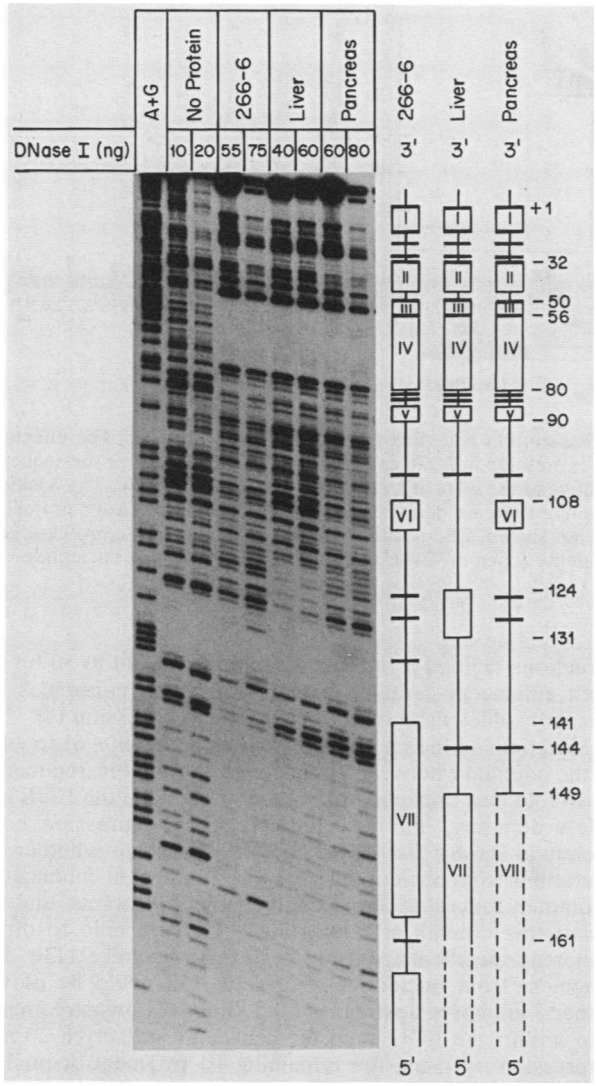

FIG. 5. Comparison of rat EI DNA binding proteins from 266-6 cells, rat liver, and pancreas. DNase I footprinting assays were performed as described in Materials and Methods. The rat EI enhancer-promoter fragment was labeled on the coding strand 15 nucleotides upstream of nucleotide −205. Reactions contained no protein or DE-52 flowthrough protein from 266-6 cells (37 μg), rat liver (13 μg), or rat pancreas (23 μg). The amount of DNase I in each reaction is noted above each lane. DNase I protected regions (boxes) and hypersensitive sites (horizontal lines) are shown to the right of the autoradiograph. Maxam and Gilbert A+G sequencing reactions serve as size markers.

appears to consist of two distinct binding domains between nucleotides −63 to −78 and nucleotides −78 to −85. Use of the 5'-labeled noncoding strand revealed strong DNase I protection at nucleotides −63 to −78 with heparin agarose fractions 40 through 48. However, nucleotides −78 to −85 showed strong DNase I protection with heparin agarose fractions 42 through 44 and significantly weaker DNase I protection with fractions 40 and 46 (data not shown).

DISCUSSION

Functional analysis of the EI regulator. The EI gene fragment from nucleotides −205 to +8 bearing enhancer and promoter elements directed the pancreas-specific expression of a passive reporter gene (the gene for human growth hormone) in transgenic mice (20). In transfection studies with cells in culture, the same EI gene fragment fused to the CAT reporter gene directs CAT expression in pancreatic 266-6 cells 50- to 100-fold greater than in nonpancreatic NIH/3T3 cells. In contrast, the CAT gene driven by the nonselective enhancer and promoter of the RSV long terminal repeat was nearly equally expressed in both cell lines. The ratio of specific (EI-mCAT) to nonspecific (RSV-mCAT) activity in 266-6 cells compared with NIH/3T3 cells was 115 (92:0.8).

A mutational analysis of the sequence from nucleotides −200 to −61 of the EI regulator revealed two domains, EIeA and EIeB, within the EI enhancer and part of a third domain (from nucleotides −61 to −80) at the enhancer-promoter boundary (Fig. 7). Each domain was required for cell-specific CAT activity. The EIeA and EIeB domains appear to be nonredundant enhancer elements, because mutations in either eliminate (>100-fold reduction) CAT expression in

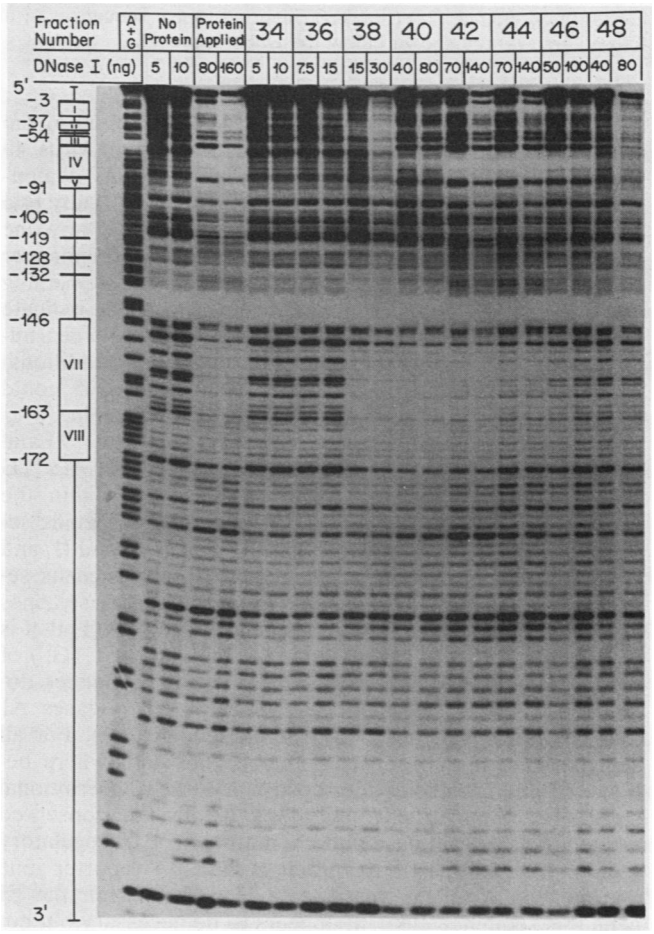

FIG. 6. Heparin agarose elution profile of proteins that bind EI enhancer and promoter DNA. The *Hin*dIII-to-*Bam*HI fragment (nucleotides −205 to +8) was labeled at the 3' *Hin*dIII end, and footprint reactions were performed as described in Materials and Methods. This fragment (5 fmol) was incubated with 42 μg of protein applied to or 4 to 17 μg protein eluted from the heparin agarose column. The amount of DNase I in each reaction is noted above each lane. The figure left of the autoradiogram illustrates a composite of the footprint regions (open boxes) and hypersensitive regions (horizontal lines) observed with the heparin agarose fractions 34 through 48. Maxam and Gilbert A+G sequencing reactions serve as size markers.

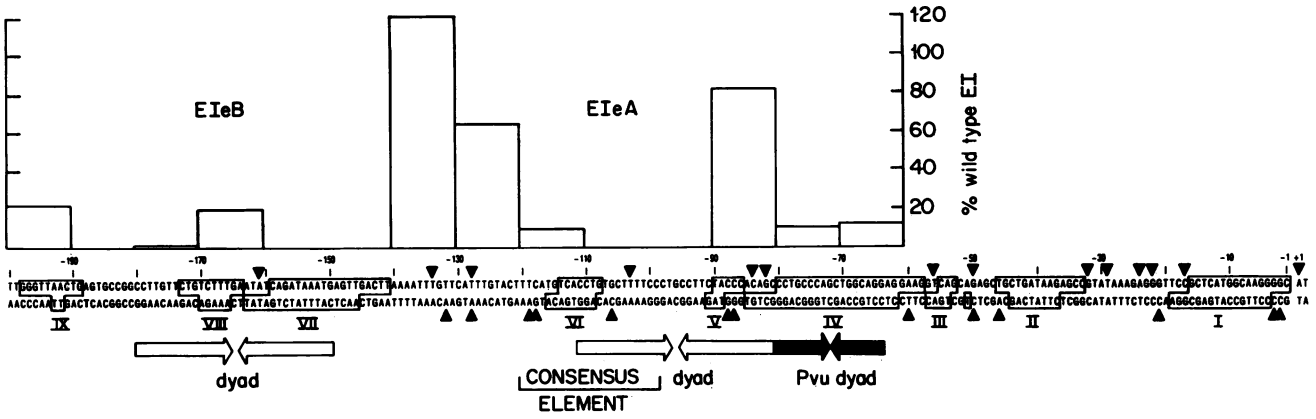

FIG. 7. Coincidence of EI gene sequences required for cell-specific transcription and sites for DNA binding proteins. The effects of the10-bp substitution mutations on the level of CAT activity after transient expression in 266-6 cells are shown lined up above the sequence of the EI regulatory region. The data are taken from Table 2 and plotted relative to the level of CAT expression for EI-mCAT. The positions of footprints are shown as the boxed-in regions for each strand; hypersensitive sites are denoted by arrowheads. The DNase I protection results are a compilation of data from Fig. 4, 5, and 6 and additional data not shown. The position of the pancreatic serine protease gene consensus sequence (28) (bracket), inverted repeat sequences of modest fidelity (open arrows), and the inverted repeat at nucleotide −72 (closed arrows) are shown below the DNA sequence.

266-6 cells. Multiple domains have also been found in the immunoglobulin heavy-chain (15) and the simian virus 40 (14) enhancers. However, the individual domains demonstrate considerably functional redundancy. Loss of any two of the four E motifs in the heavy-chain enhancer or any one of the A, B, or C elements in the simian virus 40 enhancer can be at least partially compensated by the remaining domains. Although experiments in transgenic mice indicate that complete EI enhancer activity is present between nucleotides −205 and −72 (13), it is possible that an additional EI enhancer domain upstream of nucleotide −205 could compensate for an inactivated EIeA or EIeB domain.

The EIeA domain located between nucleotides −90 and −120 contains a 20-bp sequence (−118 CATGTCACCTG TGCTTTTCCC −99) recognizably conserved within the proximal 5′-flanking sequences of five pancreas-specific serine protease genes (elastases I and II, trypsins I and II, and chymotrypsin B) (29). Six bases within the consensus sequence are completely conserved in the five protease genes and lie within a septamer core sequence (CACCTGT) that is closely related to the core sequence motif (CACCTG$^{G}_{C}$) of the immunoglobulin kappa and heavy-chain enhancer domains (7). Pancreatic amylase and carboxypeptidases A1 also have a similar 20-bp conserved sequence (1). For all seven genes this consensus sequence lies somewhere between 55 and 240 nucleotides upstream of the transcriptional start site. As with EI, mutations within the conserved sequence of the amylase and chymotrypsin B 5′-regulatory regions reduced the transcription of the CAT reporter gene in transfected rat pancreatic AR4-2J cells (1). For the EI gene this sequence element appears to be necessary but not sufficient for pancreatic transcription.

The EIeB domain (nucleotides −140 to −190) has recently been shown to be necessary for correct expression in transgenic mice (13). In transgenic mice, an EI gene fragment at nucleotides −205 to −72 could direct the pancreas-specific expression of a hGH gene with its promoter intact, whereas a fragment from nucleotides −151 to −72 (missing the majority of the EIeB region) could not (13). The amylase and chymotrypsin B pancreas-specific enhancers do not appear to possess a second separate regulatory domain similar to EIeB, since deletion analysis from both the 5′ and 3′ directions defined a relatively small region (40 to 50 bp for both enhancers centered around the 20-bp consensus sequence) sufficient to direct cell-specific expression (1).

Mutations in the EI domain from nucleotides −61 to −80, at the boundary between enhancer and promoter, reduced 8- to 10-fold but, unlike many of the mutations in the EIeB and EIeA domains, did not eliminate CAT expression completely in 266-6 cells. At present, it is not clear whether the mutations from nucleotides −61 to −80 affected enhancer or promoter function. Surprisingly, gene constructs that divided this domain at nucleotide −72 were able to direct pancreas-specific expression in transgenic mice (13). The fragment from nucleotides −205 to −72 could be moved either 3 kilobases upstream or 0.3 kilobase downstream to a site within the hGH reporter gene and still drive correct expression by using the remaining EI fragment at nucleotides −71 to +8 as a promoter. These and other transgenic experiments demonstrated that a functional enhancer resides within nucleotides −205 to −72 and a functional promoter resides within nucleotides −71 to +8. This apparent discrepancy in the importance of this domain may be due to fundamental differences in the transgenic animal and cell transfection assays employed. Alternatively, cutting at nucleotide −72 divides this symmetrical domain precisely at its center, and both halves may function when separated, whereas a 10-bp mutation which destroys the function of either half may inhibit the action of the intact domain.

Correlation of functional elements and protein-binding sites. The two enhancer domains defined functionally by the mutagenesis experiments include four sites for sequence-specific binding proteins (Fig. 7). The coincidence of sequences which bind a factor with those required for transcription supports the functional significance of factor binding to these sites. The distal, central, and proximal portions of EIeB contain binding sites IX, VIII, and VII, respectively. The distal portion of EIeA contains site VI, which spans the central septamer core (CACCTGT) within the pancreatic consensus sequence (see above). Binding sites III through V are located at the enhancer-promoter boundary, and two additional binding sites, I and II, are located in the proximal promoter region. The diversity of the protected sites strongly suggests that different proteins bind

within the EI control region. This was confirmed in part by heparin agarose chromatography, which partially resolved three classes of protein responsible for three different sets of footprints.

Not all mutation-sensitive regions (i.e., nucleotides −181 to −188 and −91 to −100) have binding sites, however. Clearly, these sequences have a critical functional role; the absence of detectable factor binding may be due to low levels or low affinities of binding factors or their inactivation in extracts.

The nucleotide sequence of the EI footprint region IV has a nearly perfect twofold symmetry spanning 17 bases (Fig. 7), and the pattern of flanking protected and hypersensitive sites suggests symmetrical protein binding within this extended region. In addition, enhancer domains EIeA and EIeB are associated with two sequence elements with weak dyad symmetry (Fig. 7). Both dyads are homologous to a half-dyad consensus CCTGTNN$^{T}_{C}$TTNCAN$_{0-3}$G (13) that is also contained within the chymotrypsin B region sufficient for cell-specific expression (1) and is found in the proximal 5′-flanking sequences of elastase II and trypsin I as well. A number of eucaryotic and procaryotic DNA-binding proteins recognize DNA sequences with twofold symmetry (6, 23, 25, 28, 31). Future work will determine what role, if any, the dyad sequences play in protein binding and regulation of the EI gene.

A key question is the identity of regulatory factors that dictate the tissue specificity of EI gene transcription. The DNase I protection patterns of the EI enhancer-promoter region with pancreatic 266-6 and liver nuclear extracts were identical within the first 90 bp upstream from the start of transcription, suggesting the presence of common DNA-binding proteins in both tissue types for regions I through V. Although factors capable of binding to this region in vitro are present in liver as well as pancreas, the hypersensitive site characteristic of the active gene in vivo is present in the chromatin of pancreas but not of liver (13). This discrepancy may be due to a lack of access of factors to this region in liver chromatin or to the absence of a tissue-specific factor crucial to the formation of an active transcription complex.

Further upstream in the enhancer region, the sets of footprints generated by pancreatic and liver extracts differed. A novel protected region at nucleotide −130 was detected with liver extracts. The factor(s) responsible is unlikely to be a repressor that prevents EI gene transcription in liver, because mutations in this region do not elevate expression of EI-mCAT in nonpancreatic cells. A second difference between 266-6 and liver cells was the pattern of footprints at nucleotide −153 (footprint VII). The proteins which bind to this region may be different in liver and pancreatic cell extracts. A third potential difference was the presence of footprint VI on the coding strand with 266-6 and pancreatic extracts but not liver extracts. This footprint covered the septamer core within the sequence conserved among the EI and other pancreatic gene enhancers. Mutations within the EI septamer core decreased expression of CAT constructs up to 170-fold in pancreatic 266-6 cells (Fig. 7). Similarly, mutations within the conserved sequence of chymotrypsin B decreased expression 50-fold (1). Therefore, the conserved pancreatic sequences containing the septamer core and the associated DNA-binding protein(s) are the most likely candidates for the control elements that determine pancreas-specific transcription of EI. Recent evidence indicates that the formation of cell-specific transcription complexes requires a collection of cell-specific and general transcription factors bound to gene regulatory regions (for a review, see reference 18). The apparently large number of EI enhancer-promoter binding proteins and the presence of most of them in pancreas and liver suggest that the action of the EI enhancer may involve a complex collection of cell-specific and general transcription factors as well.

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