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The Rat Elastase I Regulatory Element Is an Enhancer That Directs Correct Cell Specificity and Developmental Onset of Expression in Transgenic Mice

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A total of 134 base pairs of the 5′ flanking sequence of the elastase I gene is sufficient and necessary to direct expression of the passive human growth hormone gene (hGH) to the exocrine pancreas. We demonstrate that this elastase I regulatory region contains a transcriptional enhancer which directs acinar cell-specific expression in transgenic animals. The elastase I enhancer specifies correct expression of the linked hGH gene in an orientation- and position-independent manner and can activate a heterologous promoter. The enhancer also directs the appropriate temporal activation of the hGH gene in the developing pancreas. Transcription is initiated correctly for the elastase I or hGH promoter, and the transcripts are correctly processed regardless of the enhancer position within or outside the fusion gene. The elastase I enhancer generates coincident DNase I-hypersensitive sites in pancreatic chromatin when moved 3 kilobases upstream or within the first intron of the hGH gene and when associated with the hGH promoter.

The transcriptional activation of genes during cellular differentiation requires the interaction of *trans*-acting regulatory proteins with *cis*-acting DNA control sequences. Many DNA control elements that direct cell-specific transcription resemble viral enhancers, because they can activate heterologous promoters as well as their natural promoter and they can act independent of orientation, at a distance, and at positions downstream from a transcriptional start site (36). Enhancers of cellular genes have been identified by linking potential enhancer elements to reporter genes and introducing the test gene construct into cells via transfection (2, 16, 45). Cell-specific expression is then measured by comparison of the expression of the construct in an appropriate differentiated cell line and in an inappropriate cell line. It is as yet unknown whether the functional definition of enhancers (distance, promoter, and orientation independence) can be extended to their action on chromosomally located genes in animals. Introduction of fusion genes bearing regulatory elements into transgenic mice permits the analysis of regulated expression in a wide array of cell types, all with the identical introduced gene at the same chromosomal position (31). This permits a test of the effects of altered position, orientation, or sequence of an enhancer on its ability to direct correct cell-specific expression with appropriate developmental timing in animals.

Although transgenic mice have not, thus far, been used to precisely map regulatory elements, a large number of transgenes have been expressed in the appropriate cell types despite being integrated in a foreign genome (31). Cell-specific expression has been obtained with transgenes coding for differentiative products of several cell lineages, including lymphoid (5), erythroid (7, 44), exocrine (43), endocrine (19), and myoid (40) lineages. Moreover, the timing of expression of developmentally regulated transgenes has been shown to occur correctly (17, 21, 25, 30). Thus, the information necessary for correct cell-specific and developmental regulation is present within the transgenic sequences and is able to function in novel locations in the chromosomes of an animal.

The rigorous regulation of the pancreatic elastase I (EI) gene provides a simple paradigm of cell-specific transcription. The EI gene is a representative member of the serine protease multigene subfamily selectively expressed in the acinar cells of the exocrine pancreas (24). The enzymes encoded by this pancreatic family are synthesized, stored, and secreted at high levels for intestinal digestion. These genes essentially are not expressed in other cell types of the body; similar enzymatic activities found in other tissue represent products of related but different genes. For example, levels of EI mRNA in nonpancreatic tissues are often 100,000-fold (or more) lower than levels in pancreatic tissues (43). The EI gene is activated at day 13 to 14 of gestation of the rat (18) and is expressed throughout the life of an animal at a level largely unaffected by hormones (37) or diet (38). Thus EI gene expression is simple and rigorous: it is on at a high level in pancreatic acinar cells and off in other cell types. Because the expression of EI appears to be largely, if not exclusively, determined by transcriptional means (22), this gene provides a useful model of the molecular and genetic mechanisms of cell-specific control of transcription in animals.

When the rat EI gene with extensive 5′ and 3′ flanking sequences is introduced into mice, it is expressed in a manner that mimics its normal pancreas-specific expression: transgenic mice have high levels of rat EI mRNA in the pancreas but low to undetectable levels in nonpancreatic tissues (43). The pancreas-specific expression of the rat

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transgene as well as the normal endogenous mouse EI gene is determined transcriptionally (22, 29). Moreover, the 5′ flanking region of the rat EI gene can direct the expression of other structural genes to the pancreas of transgenic mice. Fusion transgenes containing only 5′ flanking sequences of the EI gene linked to the structural gene for human growth hormone (hGH) are expressed selectively and to high levels in the acinar cells of the pancreas (28). The EI regulatory sequences confer pancreas-specific transcription of the fusion gene at rates comparable to that of the endogenous mouse EI gene (29). The presence of the rat EI regulatory sequence generates a DNase I-hypersensitive region in close proximity to the promoter in pancreatic chromatin of transgenic mice, but not in chromatin from a nonpancreatic tissue such as liver (29). The presence of hGH mRNA of correct size indicates correct transcription of the fusion gene and processing of the primary transcript. The structural genes for the simian virus 40 T antigen, the *Escherichia coli* neomycin phosphoribosyl transferase, and the EJ *ras*, linked to the EI regulatory region, are also selectively expressed in the exocrine pancreas (29, 33). Clearly, EI regulatory sequences can direct pancreas-specific expression of heterologous genes in transgenic mice.

In transgenic mice bearing EI-hGH (E-GH) transgenes, hGH protein was synthesized only in the appropriate pancreatic cell type, the acinar cells that normally synthesize elastase and the other digestive enzymes (28). hGH synthesized by the exocrine pancreas does not exert a physiological response because it is secreted into the pancreatic duct system, channeled to the intestine, and degraded. Indeed, transgenic mice with E-GH fusion genes do not grow larger than normal littermates. Normal growth confirmed the lack of expression of E-GH transgenes in nonpancreatic tissues, because even low-level hGH expression in tissues that secrete into the circulation causes increased growth (32). Moreover, EI-directed expression of the simian virus 40 T-antigen gene, a dominant marker of gene expression, causes pancreatic acinar cell carcinomas in transgenic mice and no tumors of other cell types (29). Therefore EI-directed transcription of heterologous genes is not only tissue specific but also cell specific.

In the work described in this paper we demonstrated that the regulatory domain of the EI gene is a cell-specific enhancer of only 134 base pairs (bp) or less and can function independently of other EI gene sequences to direct pancreas-specific expression. The EI enhancer is also sufficient to activate expression of a passive reporter gene at the appropriate time during embryonic development. These results show that the functional definition of an enhancer can be extended to chromosomally located genes in animals. Strict transcriptional regulation by this enhancer is maintained in a foreign genome, at many chromosomal locations, and during the complex development of the mouse.

MATERIALS AND METHODS

Construction of E-GH fusion genes. Fusion gene constructs were assembled by cloning into pUC13. Rat EI gene regions were derived from the genomic lambda clone λE1b (42). Fusion genes that contained the EI promoter and transcription start site had the *Acc*I site at +8 of the EI gene converted to a *Bam*HI site by the addition of a synthetic linker then ligated to the *Bam*HI site at +3 of the hGH gene (28). Fusion genes that contained the hGH promoter and start site had EI 5′ flanking sequences ligated to a synthetic *Sal*I site attached to −83 of the hGH gene (39); in some instances it was necessary to add a synthetic *Sal*I linker to the EI fragment. The fusion gene E-GH 11 had the EI regulatory region on a *Pvu*II fragment inserted into the *Bal*I site within the first intron of the hGH gene; E-GH 10 had the EI *Pvu*II fragment inserted into the *Stu*I site at −3 kilobases (kb) of the EI flanking region. Recombinant plasmid DNAs bearing the fusion genes were purified by CsCl buoyant density centrifugation and digested with appropriate restriction endonucleases to release the intact fusion gene from plasmid sequences. The fusion gene DNA fragment was separated by agarose gel electrophoresis, eluted from the agarose by perchlorate extraction (8), precipitated in 70% ethanol, and dissolved in 5 mM Tris hydrochloride (pH 7.5)–0.2 mM EDTA for microinjection.

Production and identification of transgenic mice. A few hundred molecules of the plasmid-free fusion gene DNA was microinjected into the male pronucleus of F_2 hybrid mouse eggs (obtained by mating C57Bl/6×SJL hybrid adults) as described by Brinster et al. (4). The eggs were reimplanted into pseudopregnant mice and allowed to develop to term. Quantitative dot blot assays of transgene copy number were performed as described by Brinster et al. (4) by using 5 μg of tail DNA and hybridizing with a ^{32}P-labeled hGH probe comprising 2 kb of the hGH gene.

Hybridization analysis of hGH RNA. Tissue RNA was isolated by the guanidine thiocyanate procedure (9, 23). Poly(A)$^+$ RNA was enriched by binding to oligo(dT)-cellulose (1). hGH RNA levels were quantified by solution hybridization with synthetic oligonucleotide probes. The hybridizations were set up as described previously (11, 28), with either a 21-nucleotide oligomer complementary to a region in hGH exon 4 or a 24-nucleotide oligomer complementary to a region in hGH exon 2. The oligonucleotides were end labeled with polynucleotide kinase and [γ-^{32}P]ATP to a specific activity of about 3,000 Ci/mmol and purified either by cetylpyridinium bromide precipitation (15) or gel electrophoresis. The number of hGH RNA molecules was calculated on a per-cell basis from the hybridization results by using the previously reported (43) values of total RNA per cell for various tissues.

Northern blot (RNA blot) analysis of RNAs was performed essentially as described previously (43), except for modifications necessary for oligonucleotide probes. Hybridizations were performed with 10^6 cpm of the ^{32}P-labeled 24-nucleotide hGH exon 2 oligomer per ml in 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.05 M sodium phosphate buffer (pH 7.0)–0.2% bovine serum albumin–0.2% polyvinylpyrrolidone–0.2% Ficoll 400 (Pharmacia)–250 μg of herring sperm DNA (Sigma Chemical Co., St. Louis, Mo.) per ml–0.1% sodium dodecyl sulfate–0.1% sodium PP_i for 4 to 12 h at 42°C. Filters were washed in 6× SSC–0.05% sodium PP_i–0.1% sodium dodecyl sulfate for 5 min at room temperature and then in the same solution without sodium dodecyl sulfate for 5 min at room temperature and for 15 min at 37°C.

Primer extension analyses. The location of transcriptional initiation of various E-GH gene constructs was detected by primer extension. cDNA synthesis from poly(A)$^+$ pancreatic RNA from transgenic mice by using the hGH exon 2 oligonucleotide as primer was performed as follows. ^{32}P-labeled oligonucleotide (1 pmol; 3 μCi/pmol), 0.2 μg of poly(A)$^+$ RNA, and 5 U of avian myeloblastosis virus reverse transcriptase (Molecular Genetics Resources, Tampa, Fla.) were incubated for 1 h at 42°C in a 10-μl reaction mix containing 30 mM NaCl, 20 mM Tris hydrochloride (pH 8.3), 10 mM $MgCl_2$, 20 mM dithiothreitol, and the four

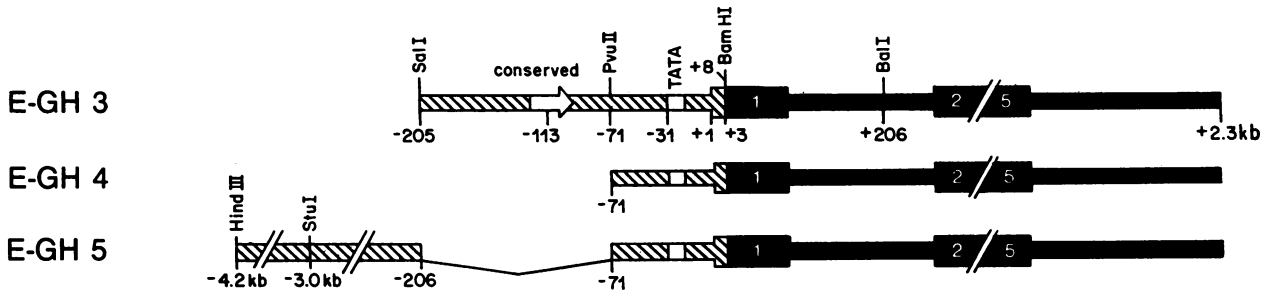

FIG. 1. Fusion gene constructs for localizing the pancreas-specific regulatory region of the EI gene between −205 and −72. Fragments of the EI 5′ flanking region (▨) including the transcription start site and the first 8 nucleotides of the transcript were fused to the hGH gene (▬) at +3. The hGH gene contained all five exons, four introns, and 0.5 kbp of 3′ flanking sequences. The positions of the elastase TATA box at −31 and the sequence centered at −113 (arrow) conserved among the pancreatic serine protease genes are indicated.

deoxynucleoside triphosphates at 2 mM each. Approximately 0.5% of the reaction mixture was analyzed by electrophoresis in a DNA sequencing gel (26). To determine the G+A sequence of the extended primer, the extension reaction was scaled up fivefold and the products were precipitated in 70% ethanol and electrophoresed in a DNA sequencing gel. The extended primer band was cut out, electroeluted (49), and cleaved at G and A residues by formic acid and piperidine treatment by the method of Maxam and Gilbert (26).

DNase-I hypersensitive site assay. Nuclei were isolated from about 0.1 g of pancreas or liver essentially as described by Mulvihill and Palmiter (27), with the exception that buffers NA, NB, and NC contained 1 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA). Nuclei were stored in buffer NC at −70°C and thawed on ice before use. Nuclei equivalent to approximately 160 μg of DNA were digested at 37°C with 0.2 μg of pancreatic DNase I (Worthington Diagnostics, Freehold, N.J.) in buffer NC containing 5 mM $MgCl_2$ and 1 mM $CaCl_2$. Samples (20 μg) were removed prior to and at 0.5, 1, 2, 3, 5, 10, and 15 min after DNase I addition, digested with 100 μg of proteinase K per ml in 1% sodium dodecyl sulfate for 1 h at 37°C, and then extracted once with phenol-chloroform and once with chloroform. Total nucleic acids were precipitated in 70% ethanol–0.1 M NaCl. The nucleic acids were dissolved in 10 mM Tris hydrochloride–1 mM EDTA (pH 7.5), treated with RNase A, and digested to completion with *Bgl*II. The genomic DNA was analyzed by Southern blotting (41) with a ^{32}P-labeled, nick-translated 0.9-kb *Pvu*II-*Bgl*II fragment of the hGH gene.

Immunofluorescent localization of hGH. Time-mated transgenic mice were sacrificed at various times during gestation. Transgenic fetuses were identified by dot blot hybridization of head DNA. Tissues were placed in Carnoy fixative for a minimum of 48 h and then embedded in paraffin. Indirect immunofluorescent staining was performed on deparaffinized 5-μm sections. Antiserum to hGH (rabbit), obtained from the National Institute of Arthritis, Metabolism and Digestive Diseases, was diluted 1:10 with 10% normal rabbit serum. The secondary antibody, fluorescein isothiocyanate-labeled goat anti-rabbit immunoglobulin (Cooper Biomedical-Cappel), was diluted 1:20.

RESULTS

Location of the EI regulatory element. E-GH fusion genes were constructed by linking the 5′ EI gene region including the first 8 nucleotides of the mRNA sequence to +3 of the hGH structural gene, which includes four introns, five exons, and 0.5 kbp of 3′ flanking sequences (Fig. 1). The elastase sequences provide the transcription start site, the TATA box and promoter, and additional upstream regulatory elements. The hybrid mRNA produced by these fusion genes contains only the first 8 untranslated nucleotides of EI mRNA linked to the nearly complete hGH mRNA with the hGH amino acid coding domain intact. Tissue levels of hGH mRNA were measured by quantitative solution hybridization with a synthetic oligonucleotide probe (see Materials and Methods).

We have shown previously that transgenic mice bearing fusion genes with 4.2 kbp (construct E-GH 1), 0.5 kbp (E-GH 2), and 0.2 kbp (E-GH 3) of EI 5′ flanking sequences linked to the hGH gene expressed hGH mRNA only in cells of the exocrine pancreas (28). Animals bearing fusion genes with as few as 205 bp of 5′ flanking nucleotides (fusion gene construct E-GH 3; Table 1 [28]) had levels of hGH mRNA in the pancreas that were equal to or greater than the mRNA levels produced by the normal, endogenous EI gene (10,000 mRNAs per cell). In contrast, hGH RNA was not present in any of the seven nonpancreatic tissues examined. Therefore this fusion gene containing EI gene sequences between −205

TABLE 1. Expression of E-GH 3, E-GH 4, and E-GH 5 in transgenic mice[a]

Construct	Mouse	No. of genes/cell	No. of hGH mRNAs/cell in[b]:		
			Pancreas	Kidney	Liver
E-GH 3	47-4[c]	3.3	<10	<10	<10
	48-4[c]	39	<10	<10	<10
	49-3[c]	2.1	3,340	<10	<10
	136-2	3.4	16,100	<10	<10
	132-5	3.4	33,100	<10	<10
	129-3	3.4	51,000	<10	<10
E-GH 4	9 total	2–26	<10	<10	<10 (130)[d]
E-GH 5	12 total	8–107	<10	<10	<10

[a] Samples were taken from seven nonpancreatic tissues (intestine, kidney, liver, parotid gland, spleen, submaxillary, and testes or ovary) and analyzed for each mouse; results for kidney and liver are given. Results for all other tissue samples were similarly negative (<10 hGH mRNAs per cell).

[b] The designation <10 indicates that no hGH mRNA was detectable above background hybridization. Generally this is less than 10 mRNA molecules per cell but depends upon the individual assay.

[c] These mice had transgenic constructs with 3.5 kbp of the pBR322 cloning vector shown to inhibit expression in animals. All other mice in this report had constructs devoid of plasmid DNA.

[d] A single mouse of the nine tested had a detectable hGH mRNA level, and only in the liver (level indicated.)

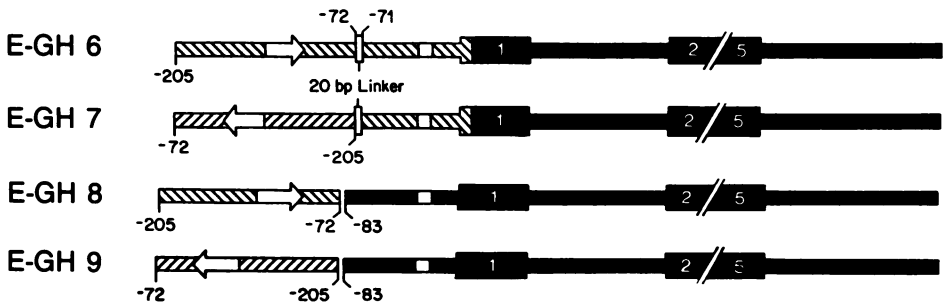

FIG. 2. Fusion gene constructs used to test whether the EI regulatory region directs pancreas-specific expression independently of orientation and identity of the promoter. The EI ( ) regulatory region was linked in either orientation to the hGH gene ( ) with its own promoter or with the EI promoter. Constructs E-GH 6 and E-GH 7 have a 20-bp linker (derived from the cloning cassette of pUC13 between *Sal*I and *Sma*I) to facilitate flipping the elastase regulatory region.

and +8 has sufficient regulatory information to direct pancreas-specific expression.

Deletion of EI flanking sequences to −71 (E-GH 4) abolished expression of the transgene (Table 1). Nine transgenic mice bearing 2 to 26 copies of this fusion gene had no detectable hGH mRNA in the pancreas or the seven nonpancreatic tissues examined. These gene-trimming experiments demonstrate that regulatory information for pancreas-specific gene expression is present within the 134-bp upstream region of the EI gene between the *Sal*I site at −205 and the *Pvu*II site at −72.

Whereas E-GH fusion genes with 4.2 kbp of EI 5′ sequences linked to the hGH gene are expressed in an appropriate pancreas-specific manner (28), deletion of just the suspected regulatory region between −205 and −72 (E-GH 5) abolishes expression (Table 1). Twelve independently derived transgenic mice bearing 8 to 107 copies of the E-GH 5 fusion gene had no detectable hGH mRNA in the pancreas or the seven nonpancreatic tissues examined. Therefore the presence of the region from −205 to −72 is necessary for pancreas-specific expression; other sequences within 4.2 kb of the 5′ end of the EI gene cannot sustain pancreatic expression in the absence of this region.

Properties of the EI regulatory region. We next set out to determine whether the EI gene regulator (nucleotides −205 to −72) had the properties of a cell-specific enhancer when tested in transgenic mice. Since the molecular mechanism is not understood, enhancers are currently defined experimentally as *cis*-acting regulatory sequences that activate transcription from the correct start site of a linked gene (i) independent of orientation, (ii) with heterologous promoters, and (iii) at a distance far upstream or even from within or downstream of a gene. These experiments tested the ability of a short (134-bp) DNA control element removed from the normal context of its gene and surrounding sequences to direct transcription precisely to a single cell type in animals.

To facilitate testing of the effect of reversing the orientation of the EI regulator, a 20-bp polylinker sequence derived from pUC13 was inserted into the *Pvu*II site at −72 of E-GH 3. This fusion gene construct (E-GH 6), with the regulatory region in its normal orientation adjacent to the EI promoter, was still expressed selectively in the pancreas of transgenic mice (Table 2). Reversing the orientation of the region from −205 to −72 (construct E-GH 7) had no discernible effect on expression. Seven of eight transgenic mice had high pancreatic levels (an average of 28,000 mRNAs per cell) with little or no expression in kidney or liver.

To test whether the EI regulator could direct pancreas-specific transcription from a heterologous promoter, the *Sal*I-*Pvu*II fragment of EI from −205 to −72 was linked to the hGH gene with its cognate promoter. Two of three transgenic mice bearing fusion genes with the EI fragment in a normal orientation (E-GH 8) had high pancreatic mRNA levels (an average of 72,000 hGH mRNAs per cell) and no detectable hGH mRNA in the kidney or liver (Table 2). Reversing the orientation of the EI regulatory fragment linked to the hGH promoter (E-GH 9) had no discernible effect on expression. Thus the EI regulator directs transcription from a heterologous promoter independent of orientation. Moreover, because the regulatory fragment from −205 to −72 was the only EI gene sequence contained within E-GH 8 and E-GH 9 gene constructs, these results also demonstrate that this region is sufficient to direct pancreas-specific expression with high fidelity.

Approximately 30% of transgenic mice produced from

TABLE 2. Expression of E-GH 6, E-GH 7, and E-GH 9 in transgenic mice

Construct	Mouse	No. of genes/cell	No. of hGH mRNAs/cell in:		
			Pancreas	Kidney	Liver
Orientation independent					
E-GH 6	220-1	0.9	3,200	<10	<10
E-GH 7	229-1	0.6	<10	<10	<10
	230-3	1.2	1,300	<10	<10
	312-5	6.1	4,500	<20	<50
	310-1	2.6	6,400	<20	<30
	312-6	3.4	7,700	31	33
	229-3	3.3	28,000	<10	<10
	309-3	7.5	49,000	<20	<10
	310-2	21	100,000	<10	120
Heterologous promoter					
E-GH 8	123-1	1.4	<10	<10	<10
	169-5	4.2	68,000	<20	<10
	249-3	7.1	84,000	<10	<10
E-GH 9	292-3[a]	0.8	1,500	<10	<10
	295-6[a]	1.9	1,700	<10	200
	258-3	18	40,000	<10	<20
	259-5[a]	5.0	470	<20	<10
	259-5-9	19	264,000	<20	<10

[a] These G_0 mice were mosaic for the transgene on the basis of a low transmission frequency coupled with a higher transgene copy number in transgenic progeny.

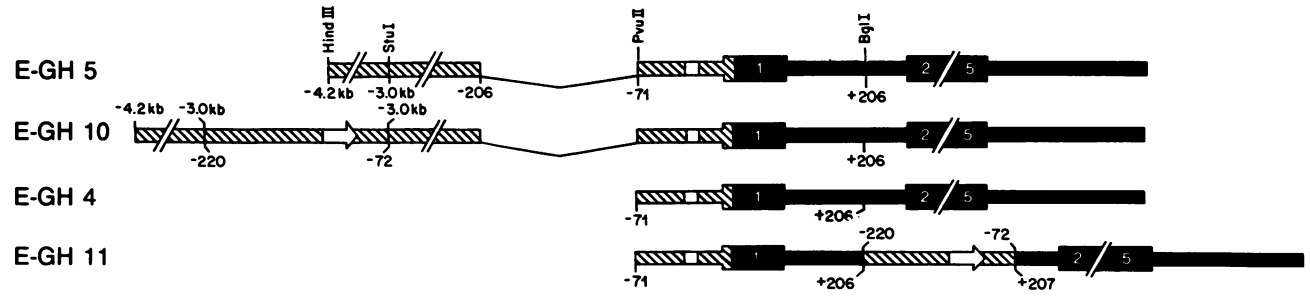

FIG. 3. Fusion gene constructs used to test whether the EI (▨) regulatory region directs pancreas-specific expression when moved to a position far upstream or within the hGH (■) gene. E-GH 10 has the EI regulatory region reinserted into the E-GH 5 construct at the *Stu*I site at −3 kb within the normal rat EI flanking sequences. E-GH 11 has the EI regulatory region inserted into the inactive E-GH 4 construct at the +206 *Bal*I site within intron 1 of the hGH gene.

microinjection of DNA into early embryos are both germ cell and somatic cell mosaics (47). Several transgenic mice with low pancreatic expression of a functioning transgene were bred and shown to be mosaic on the basis of a low transmission rate of the transgene coupled with a higher gene copy number in transgenic offspring. When levels of the transgenic hGH RNA were examined in tissue samples from both founder mice and offspring, expression in nonpancreatic tissues remained low, and in several cases the pancreatic levels increased. It appears that only a fraction of the population of pancreatic acinar cells of some mosaic mice acquires and expresses the transgene. For example, when mouse 259-5 and its offspring 259-5-9 were compared, levels of hGH RNA in kidney and liver tissue remained undetectable, while the level in pancreas tissue increased 560-fold (Table 2). Thus, the level of expression in pancreas tissue of G_0 mice often may be an underestimate owing to mosaicism, since few G_0 mice are tested for transgene transmission and expression in progeny.

We next tested the effects of distance on the cell-specific function of the EI regulator. The regulatory region was inserted into the inactive E-GH 5 fusion gene, which has the region from −205 to −72 deleted, at a site 3 kbp upstream of the EI promoter (E-GH 10; Fig. 3). This use of E-GH 5 maintains EI 5′ sequences between the reinserted regulator and the EI promoter. The reinserted EI regulatory region restored pancreas-specific expression in all six transgenic mice obtained (Table 3). Pancreatic levels of hGH mRNA (average 16,000 mRNAs per cell) were not significantly below the levels produced by constructs with the EI regulator adjacent to the promoter. The E-GH 10 fusion gene consistently gave significant expression in liver tissue (an average of 200 RNAs per cell), although the level of hGH mRNA in the liver was at least 60-fold lower than the level in the pancreas of the same mouse. Therefore one effect of placing the EI regulator at a distance was the release of constraints on expression of hGH RNA sequences in at least one nonpancreatic tissue. This occurs without noticeably diminishing the level of pancreatic expression. The nonpancreatic expression did not cause the mice to grow noticeably larger than normal, as would be expected if the transcripts produced a biologically functional hGH.

We then tested whether the EI regulator directed pancreas-specific expression in mice when placed downstream of a transcription start site. The EI regulatory fragment was inserted into the first hGH intron of the inactive E-GH 4 fusion gene, which retains only the first 71 proximal flanking nucleotides of EI (Fig. 3). Of 10 transgenic mice bearing this E-GH 11 construct, 8 had moderate (150 to 500 mRNAs per cell) or high (1,500 to 14,000 mRNAs per cell) levels of hGH mRNA in the pancreas but not in other tissues (Table 3). Animals with low pancreatic levels had only a fragment of the transgene (e.g., mouse 187-1-1) or were confirmed or suspected of being mosaic with the transgene probably absent from most cells of the pancreas. This construct was highly pancreas specific: none of the 10 transgenic mice bearing E-GH 11 had hGH RNA in the kidneys; only 2 had detectable hGH RNA in the liver (Table 3). The combined analyses of the EI regulator demonstrate that it acts as a transcriptional enhancer specific for pancreatic acinar cells.

TABLE 3. Expression of E-GH 5, E-GH 10, and E-GH 11 in transgenic mice

Construct	Mouse	No. of genes/cell	No. of hGH mRNAs/cell in:		
			Pancreas	Kidney	Liver
Far upstream					
E-GH 5	12 total	8–107	<10	<10	<10
E-GH 10	300-1	3.8	7,800	<10	<10
	301-5	4.6	10,000	<20	70
	305-1	3.2	13,000	<10	<10
	300-2	8.4	16,000	<20	54
	300-8	8.6	18,000	<10	280
	305-4	5.7	23,000	<10	80
	306-4	13	31,000	40	450
Within the hGH gene					
E-GH 4	9 total	2–26	<10	<10	<10
E-GH 11	211-2	5.8	<10	<10	<10
	188-2[a]	0.3	60	<10	ND[b]
	188-2-10	1.4	<60	<30	36
	187-1[a]	0.2	<30	<10	<10
	187-1-1	0.6	150	<10	<10
	194-1[a]	0.5	210	<20	<10
	194-1-17	1.1	96	<10	ND
	192-5	1.0	470	<10	<10
	192-3	1.2	500	<10	<10
	193-3	3.4	1,500	<10	<10
	193-3-1	11	4,100	<15	50
	212-1	22	4,200	<10	<10
	211-5	3.9	7,400	<10	<10
	211-3	11	14,000	<20	<10

[a] These G_0 mice were mosaic for the transgene on the basis of a low transmission frequency coupled with a higher transgene copy number in transgenic progeny.

[b] ND, Not determined.

FIG. 4. Structure of the E-GH 12 fusion gene. The EI flanking region from −150 to −72 (▨) was linked to the hGH promoter and structural gene (▬).

The inactive E-GH 4 recipient gene described in the previous experiment contains only 79 nucleotides of EI gene sequence (nucleotides +8 to −71). The pancreas-specific reactivation of this gene by the insertion of the EI regulatory region at a distant site (E-GH 11) demonstrates that the elastase region between −71 and +8 comprises a functional promoter (Fig. 3, Table 3). This promoter can direct transcription from the correct start site (see below) when activated by an appropriate regulatory element such as the EI enhancer.

Deletion to −150 inactivates the EI enhancer. The regulatory sequences within the EI region from −205 to −72, linked to the hGH promoter and structural gene (E-GH 8 and E-GH 9; Fig. 2), direct appropriate pancreas-specific expression (Table 2). Deletion of the sequences between −205 and −150 (E-GH 12), however, reduces hGH RNA levels in the pancreas 2 to 3 orders of magnitude (Fig. 4; Table 4). In addition, most of the tissue specificity was lost, since hGH RNA levels in the kidneys and liver increased to levels nearly as high as in the pancreas. The apparent low expression of E-GH 12 in mice might have been due to extreme mosaicism, in which few somatic cells contained the transgene and expressed it at a uniformly high rate across most tissues. To determine whether mosaicism could account for the low transgene expression, the levels of hGH RNA in pancreas, kidney, and liver tissue were measured for transgenic progeny of mice 319-1 and 322-6 bearing 15 and 25 copies per cell of the E-GH 12 construct, respectively. The progeny had hGH RNA levels in these three tissues comparable to the parental levels (Table 4). Therefore mosaicism cannot account for the low-level expression. The deletion of regulatory sequences between −205 and −150 caused both decreased pancreatic expression and the loss of tissue specificity.

Despite significant hGH RNA levels in the three tissues examined, none of the transgenic animals bearing the E-GH 12 construction had enhanced growth. Previously, transgenic mice with very low levels of hGH mRNA in the liver were shown to have enhanced growth (32). Poly(A)$^+$ RNA from pancreas, kidney, or liver tissue of several transgenic mice bearing E-GH 12 were examined by Northern blot (RNA blot) analysis. No hGH RNA band at 950 nucleotides corresponding to correctly transcribed and processed hGH mRNA was detected, although the amount of RNA was at least 10-fold more than necessary to detect a discrete band (data not shown). Both the lack of enhanced growth and the absence of hGH mRNA of authentic size indicate that the E-GH 12 fusion gene, with its deletion down to −150, produces only aberrant transcripts.

The EI enhancer directs transcription from correct start sites. To verify that the hGH mRNA in pancreatic RNA preparations from transgenic mice bearing E-GH fusion genes was correctly transcribed and processed, the size of the pancreatic hGH RNA was determined by Northern blotting (Fig. 5). Pancreatic RNA from the fusion transgenes used to define the enhancerlike nature of the EI regulator had a single hGH-hybridizing RNA of 950 nucleotides. The presence of a single hGH RNA band of the appropriate size for hGH mRNA suggests that the site of transcription initiation, the choice of the polyadenylation site, polyadenylation, and splicing were correct for each fusion transgene.

To confirm the site of transcription initiation for the

TABLE 4. Expression of E-GH 12 in transgenic mice

Mouse	No. of genes/cell	No. of hGH mRNAs/cell in[a]:		
		Pancreas	Kidney	Liver
319-5	33	<10	64	13
319-3	2.6	30	33	62
323-3	3.9	100	26	46
319-1[b]	3.7	100	30	110
319-1-4	15	110	32	52
322-6	25	180	26	81
322-6-1	25	87	120	46
317-5	4.4	180	66	140
322-8	8.9	300	32	69

[a] hGH mRNA levels are the result of two to four determinations for each tissue.
[b] This G_0 mouse was mosaic.

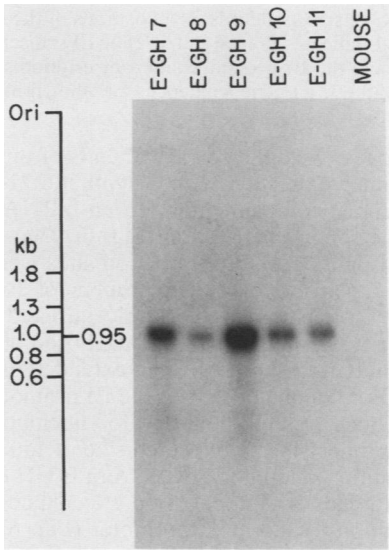

FIG. 5. Northern blot analysis of the hGH mRNAs produced by E-GH fusion genes. Approximately equal amounts of pancreatic poly(A)$^+$ RNA preparations were resolved by agarose gel electrophoresis in the presence of methylmercury hydroxide, transferred to diazophenylthioether paper, and hybridized with an hGH-specific oligonucleotide probe (see Materials and Methods). The fusion gene constructions are designated above each lane: E-GH 7, the EI enhancer region in its reverse orientation linked to the hGH gene driven by the elastase promoter (mouse 310-2, Table 2); E-GH 8, the enhancer region in a normal orientation linked to the hGH gene and promoter (mouse 249-3, Table 2); E-GH 9, the enhancer region in reverse orientation linked to the hGH gene and promoter (mouse 259-5-9, Table 2); E-GH 10, the enhancer region inserted 3 kb upstream from its normal position near the promoter (mouse 306-4, Table 3); E-GH 11, the enhancer region inserted into the first intron of the hGH gene driven by the elastase promoter (mouse 211-3, Table 3); MOUSE, no transgene (control).

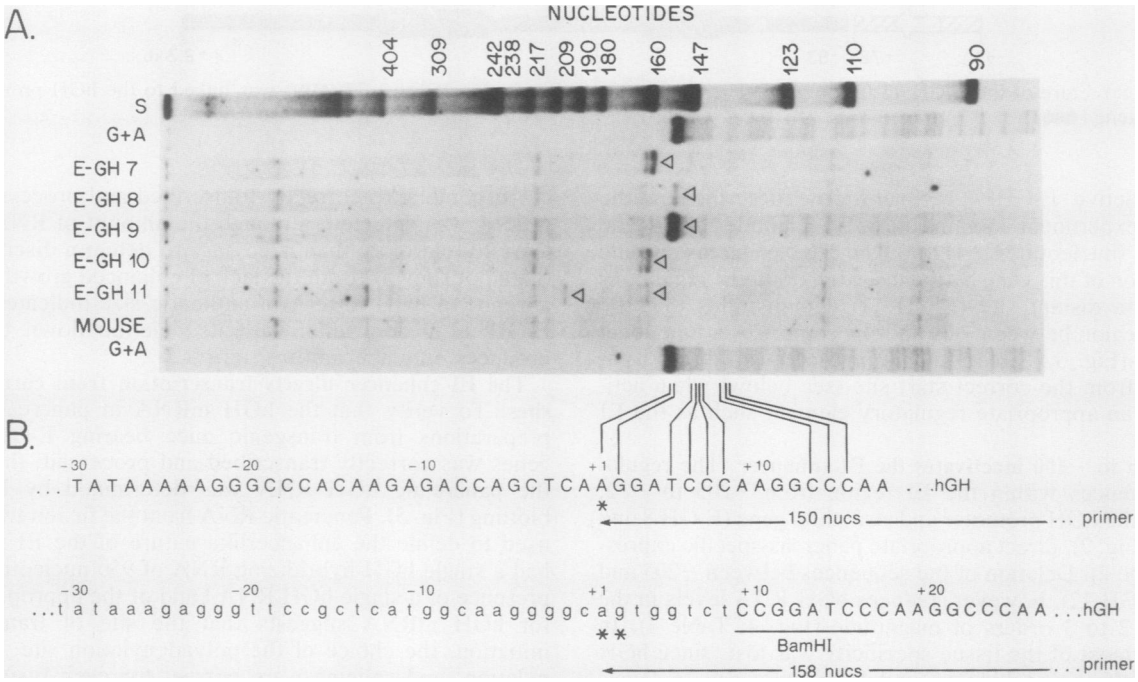

FIG. 6. (A) Primer extension analysis of the 5′ ends of the mRNAs produced by E-GH fusion genes. A ^{32}P-labeled oligonucleotide derived from exon 2 of the hGH gene was used to prime cDNA synthesis with reverse transcriptase by using pancreatic poly(A)$^+$ RNA from transgenic mice (same as in Fig. 5) bearing the E-GH fusion genes designated for each lane. Lane G+A contains the purine chemical cleavage products of the primer extension cDNA of lane E-GH 9 to align the mRNA start with the known gene sequence. Lane S contains ^{32}P-labeled *Hpa*II-cut pBR322 size standards. (B) Positions of the mRNA start sites within the known sequences of the E-GH fusion genes are indicated by asterisks. +1 indicates the normal hGH or EI transcript start site. Top sequence: the hGH promoter region, start site, and 5′ mRNA region. Bottom sequence: the EI promoter and transcription start site plus the first 8 EI transcription unit nucleotides joined by a *Bam*HI linker to the hGH gene at +3. EI gene sequences are shown in lowercase; hGH gene sequences are in uppercase.

introduced genes, we analyzed the 5′ ends of the pancreatic RNAs by primer extension with a synthetic 21-base oligonucleotide primer complementary to an mRNA region derived from the second hGH exon (Fig. 6). Depending upon whether hGH or EI promoter-initiation sites were used in a fusion gene construct, extended primers of two different lengths were anticipated for correctly initiated transcripts (Fig. 6B). For fusion genes with the hGH promoter and start site, correct initiation should give an extended primer of 150 nucleotides. For fusion genes with the EI promoter and start site, the extended primer should be 158 nucleotides.

Extended primers of the expected size of 150 nucleotides were obtained for pancreatic RNAs from E-GH 8 and E-GH 9, the fusion genes with the 134-bp EI enhancer linked in either orientation to the hGH promoter (Fig. 6). To verify that the extended primer also had the correct nucleotide sequence corresponding to the 5′ end of hGH mRNA, we isolated the extended primer for the E-GH 9 transcript, cleaved it at purine residues with formic acid-piperdine treatment, and displayed the cleavage products in the same gel as the extended primers. The G-A sequence results confirm the correct position of the mRNA cap site (lane G+A, Fig. 6).

Primer extension analysis of transcripts from E-GH 7, with the EI enhancer adjacent to the EI promoter, and E-GH 10, with the enhancer moved 3 kbp upstream from the EI promoter, showed that the transcripts were initiated at the normal start site of the EI gene (Fig. 6). Transcripts from E-GH 11, with the regulatory region within the first hGH intron, produced two extended primers of different lengths with about equal intensity. One, at about 158 nucleotides, is indicative of initiation at the normal EI site; the other, of 197 nucleotides, is indicative of an aberrant initiation site. Except for the partly aberrant initiation of the E-GH 11 construct, the primer extension analyses demonstrated that the pancreas-specific enhancer of EI correctly initiates transcription from its normal promoter or the heterologous hGH promoter in transgenic mice.

The EI enhancer generates a DNase I-hypersensitive site. A pancreas-specific DNase I-hypersensitive site characteristic of a regulatory sequence with protein bound to it was generated over the EI enhancer and promoter when 0.5 kb of the EI 5′ flanking sequences are linked to the hGH structural gene (29). We asked whether the enhancer fragment was the sole EI gene sequence necessary to generate a hypersensitive site when linked to a heterologous (hGH) promoter and whether the hypersensitive site would follow when the enhancer was moved far upstream or to a position within the hGH gene (Fig. 7).

Transgenic mouse 306-4-5 had a tandem array of 13 copies of the E-GH 10 construct, which has the EI enhancer fragment repositioned 3 kb upstream. Digestion of pancreatic nuclear DNA with *Bgl*II generates an intense 7.0-kb band from the 13 tandem repeats and minor bands of 8.8, 5.7, and 5.0 kb derived either from junction fragments with mouse DNA or from rearrangements within one or two gene copies within the array. Treatment of pancreatic nuclei from this mouse with DNase I prior to *Bgl*II digestion yields a prominent 4.4-kb band. The DNase I-generated endpoint of this fragment maps to the position of the EI enhancer 3 kb upstream from the transcription start site (Fig. 7A). Minor hypersensitive sites map to positions 2.7, 1.6, and 1.0 kb

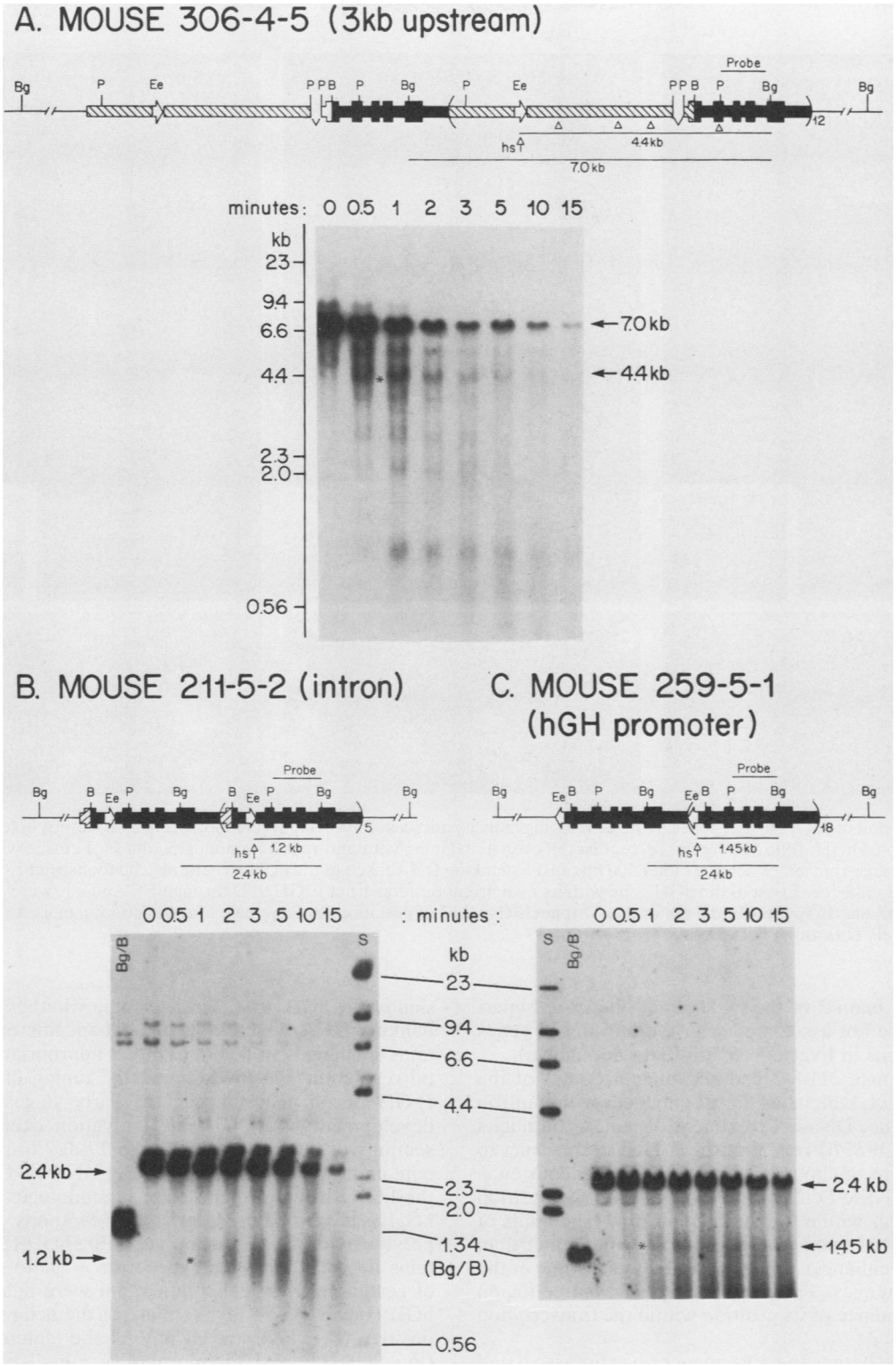

FIG. 7. DNase-I hypersensitive sites in pancreatic chromatin follow the EI regulatory region. The maps for three different E-GH fusion gene constructs show the presumed tandem head-to-tail organization of the integrated copies. ▨, EI gene sequences; Ee, position of the EI enhancer; ▬, hGH gene sequences; ——, genomic DNA regions. The position and extent of the *Pvu*II-to-*Bgl*II probe used for the indirect end-labeling procedure are shown above each map. The *Bgl*II fragment derived from digestion of the tandemly repeated genes and the DNA fragment generated by DNase I digestion of chromatin are shown below each map. The arrows designated hs indicate the positions of the hypersensitive sites. Lanes 0 through 15 contain nuclear DNA after digestion with DNase I for the indicated times between 0 and 15 min. Asterisks indicate the DNase I-generated hypersensitive bands. Lanes S contain *Hin*dIII-digested lambda DNA size standards. (A) Results for transgenic mouse 306-4-5 bearing 13 copies of construct E-GH 10. Arrowheads indicate additional hypersensitive sites. (B) Results for transgenic mouse 211-5-2 bearing six copies of construct E-GH 11. The autoradiogram includes a *Bgl*II-*Bam*HI digest of nuclear DNA not treated with DNase I to give a known 1.34-kb hybridizing fragment from the hGH genes (lane Bg/B) for more accurate sizing of the DNase I-generated fragment. (C) Results for transgenic mouse 259-5-1 bearing 19 copies of construct E-GH 9.

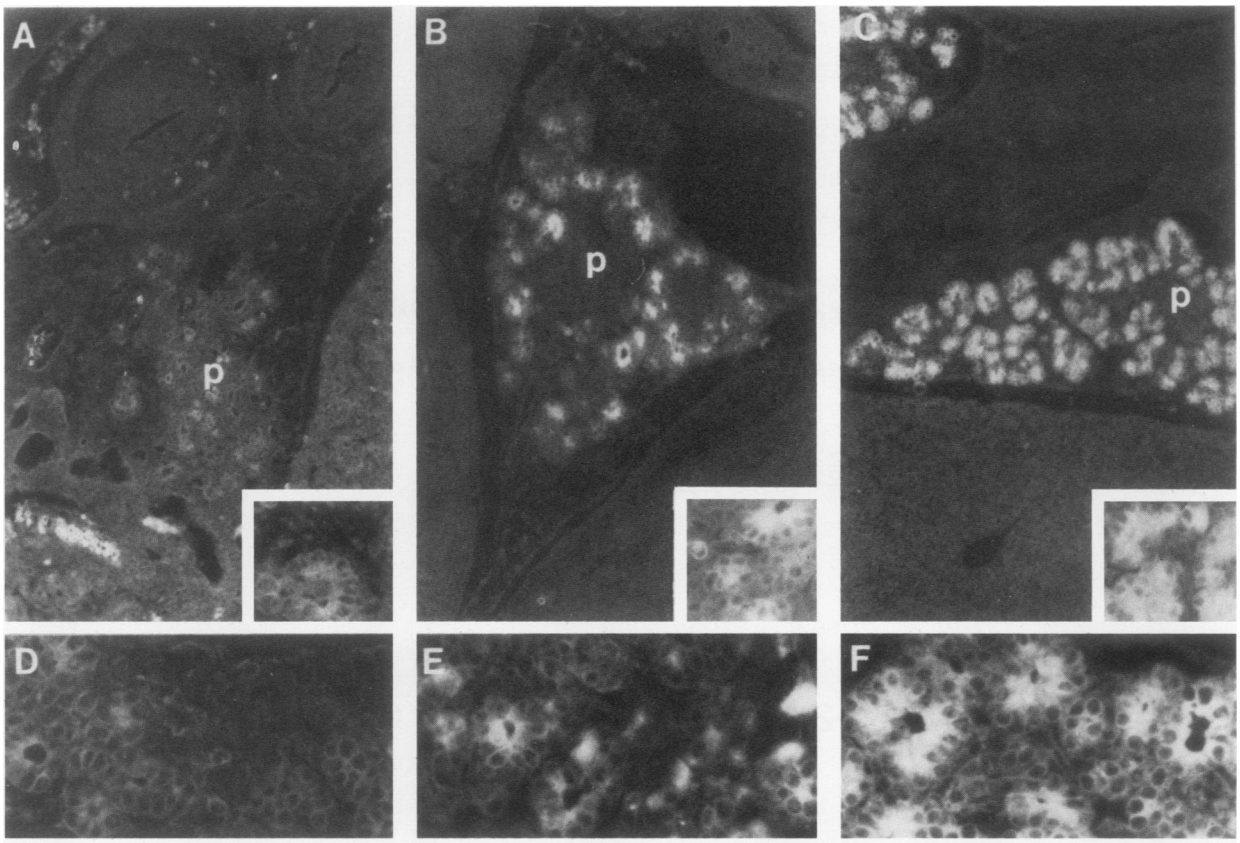

FIG. 8. Expression of E-GH fusion genes in the developing mouse pancreas. Indirect immunofluorescence on Carnoy-fixed tissue sections by using antiserum to hGH, followed by fluorescein isothiocyanate staining with anti-rabbit immunoglobulin G. Fetuses were derived from females mated to transgenic males bearing either 500 bp of EI 5′ flanking DNA fused to the hGH structural gene (construct E-GH 2; A through C) or the 134-bp EI enhancer fused to the hGH gene with its own promoter (construct E-GH 9; D through F). Animals were sacrificed on day 14 (panels A and D), day 16 (panels B and E), or day 17 (panels C and F) of gestation. P, Pancreas. Magnification: panels A through C, ×100; inserts, ×260; panels D through F, ×375.

upstream and to exon 2 of the hGH gene. Nuclease-hypersensitive sites are not associated with the introduced genes at similar positions in liver chromatin (data not shown).

Transgenic mouse 211-5-2 had six tandem copies of the E-GH 11 construct, which has the EI enhancer within intron 1 of the hGH gene. DNase I treatment of pancreatic nuclei cleaved the 2.4-kb *Bgl*II repeat of the E-GH 11 construct to a smaller fragment of about 1.2 kb with heterogeneous ends (Fig. 7B). The DNase I cleavage region maps to the position of the EI enhancer within the first hGH intron. The limits of this hypersensitive region extend about 250 bp, greater than the length of the enhancer fragment, and include some of the hGH intron sequences. The extended hypersensitive region may be a consequence of its position within the transcription unit.

To determine whether the EI enhancer has an associated nuclease hypersensitive site when linked to and driving a heterologous promoter, we analyzed the DNase I sensitivity of the E-GH 9 construct in pancreatic chromatin of mouse 259-5-1 (Fig. 7C). DNase I cleaves the *Bgl*II repeat fragment at the position of the EI enhancer adjacent to the hGH promoter to give a fragment of 1.45 kb. The low intensity of this DNase I-generated fragment may indicate that only a few of the 19 copies of the fusion gene were active and had transcription factors bound to the enhancer region.

The EI enhancer directs appropriate developmental expression of the hGH gene. To determine whether the elastase 5′ flanking DNA which controls cell-specific expression contains sequences sufficient to direct appropriate developmental regulation, we investigated the timing of expression of E-GH fusion genes during the early stages of pancreatic development when EI gene activation occurs. Histologic sections of pancreas from 14- to 17-day transgenic fetuses containing 500 bp of EI 5′ flanking DNA (E-GH 2) linked to the hGH structural gene were immunologically stained for hGH as described in Materials and Methods. (E-GH 2 is the previously described fusion gene E0.5hGH [28], which contains EI 5′ flanking sequences from −500 to +8.) At day 14 of gestation a few pancreatic acini were lightly stained for hGH (Fig. 8A). At day 16 many of the acini were distinctly positive (Fig. 8B), and at day 17 the staining was intense (Fig. 8C). By day 17 all morphologically distinct acinar cells contained hGH. The accumulation of hGH coincides with the timing for the activation of the rat EI gene in rats (18) and the rat EI transgene in mice (data not shown).

To show whether the EI enhancer alone was sufficient to direct correct temporal expression during pancreogenesis, we assayed the appearance of hGH in transgenic fetuses bearing the E-GH 9 fusion gene, which has the EI enhancer fragment linked in reverse orientation to the hGH promoter and gene. The temporal pattern and intensity of staining for hGH in acinar cells from the pancreas of E-GH 9 fetuses

were indistinguishable from that observed in E-GH 2 fetuses. Acinar cell-specific staining was evident at day 14 (Fig. 8D); at day 16 both the number of acini and intensity of staining had increased dramatically (Fig. 8E), and they increased further at day 17 (Fig. 8F). These results demonstrate that the DNA control sequences contained within the EI enhancer not only confer acinar cell-specific expression to a heterologous gene and promoter, but also direct appropriate temporal activation in the developing pancreas.

DISCUSSION

The developmental regulation of a gene is defined by three parameters: the level of expression, the site (tissue specificity) of expression, and the timing of expression. For many genes, including pancreatic EI, that are expressed in a cell-specific manner, these parameters are determined transcriptionally. The cell-specific control of expression mediated by the EI regulator is largely, if not exclusively, transcriptional (22, 29). We have identified and characterized a short (134-bp) regulatory element within the proximal upstream region (between −205 and −72) of the rat EI gene that correctly specifies all three developmental regulatory parameters when tested in animals. This *cis*-acting regulator directs appropriately high pancreas-specific expression and activates expression at the correct stage of pancreatic development when tested in animals. The regulatory region (−205 to −72) appears necessary for pancreas-specific expression, because deletion of this small region from a construction with 4.2 kb of 5′ flanking sequences eliminates expression. This region also is sufficient for pancreas-specific expression; other EI gene regions, including the promoter, are not required.

The EI regulator behaves as a cell-specific enhancer when tested in germ line transformed mice. Linked to an inactive hGH gene, it activates pancreas-specific expression when present in either orientation and at novel positions far upstream or within the gene. In contrast to the situation with most other enhancers tested by transfection into cultured cells (3, 46), expression was not appreciably diminished by moving the EI enhancer as far as 3 kb upstream of its normal position adjacent to the promoter. The EI enhancer directs expression from its cognate promoter or from the heterologous hGH promoter. Levels of expression with the hGH promoter were similar to those with its homologous promoter. Regardless of the position of the EI enhancer, transcription of the hGH gene in the pancreas is initiated correctly. These results also demonstrate that the flexibility of enhancer function is not limited to cell transfection assays but in fact is manifested with chromosomally located genes in animals.

The deletion of the 55 distal nucleotides from the −205 to −72 active enhancer dramatically reduces its ability to direct pancreas-specific expression. The overall effect can be resolved into two distinguishable components that suggest the removal of two kinds of regulatory elements. First, high levels of pancreatic expression fall 100- to 1,000-fold, as if an important positively acting element required for pancreatic transcription was deleted. Second, nonpancreatic expression appears at levels similar to the pancreatic level, as if a negatively acting regulatory element that suppresses nonpancreatic transcription was deleted. Both of these effects are stable, heritable properties of the shortened enhancer element. Thus deletion of the sequences between −205 and −150 removes regulatory signals necessary both for high pancreatic and suppressed nonpancreatic expression. Unexpectedly, the low level of expressed RNA represents aberrant transcription. What causes the shortened enhancer to abandon the adjacent hGH promoter and transcription start site and apparently activate nonspecific start sites is not clear.

Sequence motifs within the regulatory region may correlate with function. The EI enhancer region contains a 20-bp sequence (centered around −110) that is the only recognizably conserved sequence within the proximal 0.5-kb 5′ flanking sequences of five pancreas-specific serine protease genes (EI and EII, chymotrypsin B, and trypsins I and II) examined (43). A similar sequence is present, although less well conserved, for pancreatic RNase, amylase, and carboxypeptidase A and B genes as well (3). In all instances the conserved sequence is present between 90 and 240 nucleotides upstream from the transcriptional start site. Mutations within the conserved sequence associated with the chymotrypsin B and amylase genes diminish expression when their effect is tested by transfection into the AR4-2J pancreatic acinar tumor cell line (3). A total of 55 bp of the chymotrypsin B flanking region containing the conserved sequence was sufficient to enhance expression of a reporter gene in the AR4-2J cells. However, the EI enhancer fragment −150 to −72, which also contains this sequence, is not sufficient to direct pancreas-specific expression in mice (E-GH 12; Fig. 4). Therefore, although the conserved sequence may be involved in pancreas-specific transcription, it is not sufficient.

We note that the consensus sequences of EI, chymotrypsin B, and trypsin I genes are also part of a region of imperfect dyad symmetry with half-site consensus sequence CCTGTNNC/TTTNCAN$_{0-3}$G. The conserved CCTGT at each end of the dyad occurs approximately three turns apart on the DNA helix and is a potential recognition site for a protein dimer in head-to-head orientation. The EI gene has a second copy of this dyad centered around −161, which is deleted in the shortened enhancer fragment of E-GH 12. The EI enhancer may require both copies of this dyad for correct expression in animals.

Gene regulatory regions are associated with chromatin sites that are highly sensitive to digestion by DNase I and other endonucleases (10, 12). Cell-specific regulatory elements have associated chromatin hypersensitive sites only in appropriate cell types in which they are active or capable of being activated (6, 14). β-Globin transgenes in novel locations acquire characteristic erythroid-cell-specific DNase I-hypersensitive sites (34). The association of hypersensitive sites with promoter elements, viral or cellular enhancers, and hormone responsive control elements implies that the binding of regulatory proteins alters chromatin structure (13). Jongstra et al. (20) demonstrated that moving the simian virus 40 enhancer to other positions within the simian virus 40 genome causes the concomitant movement to the same position of a nucleosome-free DNase I-hypersensitive site. Thus it is the nucleotide sequence of the viral enhancer that directs factor binding, and this binding occurs irrespective of the location of the enhancer within the viral genome.

We have demonstrated that the 134-bp EI enhancer, when fused to the hGH gene and introduced into the germ line of mice, generates a nuclease-hypersensitive site in pancreatic chromatin which follows the enhancer irrespective of its position. This demonstrates that a chromosomal DNase I-hypersensitive site generated by a cellular enhancer is transposed by the movement of the enhancer. These results prove directly that the nucleotide sequences within the

enhancer itself cause the formation of a chromatin hypersensitive site.

Between days 10 and 12 of rat fetal development, the pancreas develops from a foregut diverticulum to an epithelial structure resembling a single pancreatic acinus. This period is characterized by marked histological differentiation without any significant cytodifferentiation (35). Between days 13 and 15, acinar cell-specific mRNAs, secretory enzymes, and secretory granules appear and begin to accumulate. The accumulation of serine protease mRNAs and the zymogens they encode increases dramatically during the interval between day 15 and birth at day 20 (18, 48). The first indication of EI gene activation is the accumulation of elastase I mRNA at days 13 to 14 (18).

The appearance of hGH protein detected by immunofluorescence in 14-day embryonic pancreatic rudiments bearing E-GH fusion genes coincides with the onset of accumulation of normal pancreatic digestive enzymes. Thus the minimum enhancer region that correctly specifies extent and cell-specific control also correctly specifies timing. Moreover, because the E-GH 9 construct has the enhancer linked to the hGH promoter in a reverse orientation, correct timing also appears independent of orientation and nature of the promoter, two important enhancer properties. The DNA sequence information for all three developmental functions are probably coincident. Therefore correct timing, extent, and site of expression may all be mediated by the binding of the same regulatory factor(s). In this instance, the developmental timing of gene activation would be determined by the appearance during pancreatic acinar cell differentiation of one or more enhancer-binding factors.

The EI enhancer domain bounded by nucleotides −205 and −72 contains all the regulatory information to activate all appropriate gene transcription processes in a pancreas-specific manner. The formation of pancreas-specific DNase I-hypersensitive sites that reside over the EI enhancer in pancreatic chromatin indicates that pancreas-specific factors bind to the enhancer. All necessary sequence information for this tissue-specific binding resides within the short 134-bp fragment. If localized or extended alterations in chromatin structure are necessary to activate the gene, information for these processes must reside within the same enhancer fragment. Furthermore, if there are important constraints on nuclear architecture, such as positioning the active gene at or near the nuclear membrane or in association with the nuclear matrix in a pancreas-specific manner, then this fragment must contain information for these processes as well.

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