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Identification of a Transcriptional Enhancer Important for Enteroendocrine and Pancreatic Islet Cell-Specific Expression of the Secretin Gene

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It is well established that the gene encoding the hormone secretin is expressed in a specific enteroendocrine cell, the S cell. We now show that the secretin gene is transiently expressed in insulin-producing B cells of the developing pancreatic islets in addition to the intestine. Furthermore, secretin is produced by most established islet cell lines. In order to identify and characterize the regulatory elements within the secretin gene that control tissue-specific expression, we have introduced secretin reporter gene constructions into the secretin-producing HIT and STC-1 cell lines as well as the nonexpressing INR1-G9 glucagonoma line. Analysis of deletion mutants revealed that sequences between 174 and 53 bp upstream from the transcriptional start site are required for maximal expression in secretin-producing cells. This positive element functioned independently of position and orientation. Further deletions into the enhancer resulted in a stepwise loss of transcriptional activity, suggesting the presence of several discrete control elements. The sequence CAGCTG within the secretin enhancer closely resembles that of the core of the B-cell-specific enhancer in the insulin gene. Point mutations introduced into this putative element led to greater than 85% reduction in transcriptional activity. Gel mobility shift assays suggested that a factor in B cells closely related or identical to proteins that bind to the insulin enhancer interacts with the CAGCTG motif in the secretin gene.

The hormone secretin is produced by a highly specialized enteroendocrine cell type, the S cell, in the small intestine. The distribution of secretin cells is restricted along both the vertical and horizontal axes of the intestine. In most species, secretin cells are present in highest abundance in the proximal small intestine and decrease distally in the jejunum, ileum, and colon. Secretin cells are found in villi and zones of transition between villi and crypts of the intestinal epithelium (25). In the rat, Northern (RNA blot) analysis confirmed that the small intestine was the primary site of secretin gene expression (23). In contrast to most other species, there does not appear to be a proximal-to-distal decreasing gradient in secretin production in the small intestine of the rat.

Enteroendocrine cells are developmentally related to the endocrine cells of the pancreas, both arising from the embryonic gut endoderm (26). Several hormones, including glucagon and somatostatin, are synthesized by both enteroendocrine and islet cell types. Cell-specific control of insulin (9) and glucagon (36) gene expression in B and A cells of the islets, respectively, occurs at the level of transcription. Enhancer elements localized in the 5′-flanking region of the insulin and glucagon genes appear to restrict transcription to the appropriate insulin- and glucagon-producing islet cell lines.

In addition to the established intestinal sites of secretin biosynthesis, secretin-immunopositive cells in cultures of neonatal rat pancreas (38) and more recently in two tumor cell lines derived from transgenic mice expressing viral oncogenes under control of the insulin gene (37) have been described. One cell type, STC-1, was developed from a secretin-producing intestinal endocrine tumor expressing simian virus 40 large T antigen and polyomavirus small T antigen directed by the insulin gene 5′-flanking region. The possibility that the secretin cell is related to pancreatic B cells was further supported by the demonstration of secretin production by a second cell type, BTC-3, derived from a pancreatic B-cell tumor-expressing large T antigen.

The presence of immunoreactive secretin in the STC-1 and BTC-3 cell lines prompted us to examine other islet cell lines for secretin gene expression. We now show that secretin is produced by a number of established islet cell lines. In addition, the secretin gene is transiently expressed in normal B cells in developing pancreatic islets. Consistent with these observations, we have also developed an islet cell model to study transcriptional regulation of the secretin gene, utilizing both secretin-producing and nonproducing cell lines. Secretin reporter gene constructions were preferentially expressed in secretin-producing cells and not in cell lines that did not express the endogenous secretin gene. Analysis of deletion mutants revealed that as little as 174 bp of 5′-flanking sequence could support maximal transcriptional activity. Further characterization of this region suggested that the secretin gene is transcriptionally regulated by a multielement, cell-specific enhancer located between 53 and 174 bp upstream from the transcriptional initiation site. Examination of the secretin *cis*-active regulatory domain reveals regions which do not resemble known enhancers for

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other peptide hormone genes as well as sequences similar to elements of the insulin gene enhancer involved in cell type-specific transcriptional regulation.

MATERIALS AND METHODS

Northern blot hybridization assays. Total RNA from the small intestine of Sprague-Dawley rats (Taconic Farms) and cell lines was extracted with guanidine thiocyanate and then with acid-phenol (5). Following homogenization of fresh pancreas in guanidine thiocyanate, RNA was precipitated with lithium chloride (2). For Northern blot analyses, 25 μg of RNA was separated on a 1.3% formaldehyde–agarose denaturing gel, transferred to Nytran membranes (Schleicher & Schuell) by capillary blotting, and hybridized under conditions described previously (23) except as noted in the text. To obtain sufficient pancreatic RNA samples for analysis, pancreas were pooled from six to eight fetuses and three to four postnatal animals. To account for variations in sample loading in each lane, the filters were washed and rehybridized to a β-actin cDNA probe. Blots were also probed with a rat insulin I cDNA probe to compare secretin expression with that of another islet hormone.

Immunocytochemical analysis. Rat fetuses (n = 5) at a gestational age of 18 days were fixed in Bouin's solution, dissected along the midline, and embedded in paraffin at 60°C. Serial 5-μm-thick sections were cut and mounted on chrome- and alum-coated glass slides. The sections were initially used to screen for the presence of secretin-immunoreactive endocrine cells within the developing pancreas and intestine. Secretin antiserum (kind gift from J. M. Polak, Hammersmith Hospital, London) was diluted to 1:500 in phosphate-buffered saline (PBS) containing 10% horse serum. In order to detect the secretin-positive cells in the pancreas, a higher concentration (1:500) of antiserum was required than is routinely used for detection of the intestinal endocrine cells (1:2,000). At this concentration, the antiserum demonstrated some cross-reactivity with the related peptide glucagon. The sections were incubated in the antiserum for 24 h at 4°C in a humidified atmosphere. After the sections were washed in PBS, the bound antibodies were localized by incubation of the sections in fluorescein-conjugated goat anti-rabbit immunoglobulin G (IgG; Jackson Laboratories, BioCan Scientific, Toronto, Canada), diluted 1:2,000 in PBS containing 10% horse serum for 2 h at room temperature.

The absorption studies used the secretin antiserum absorbed with secretin at 0.1 and 1 μmol/ml, glucagon at 1 and 10 μmol/ml, vasoactive intestinal peptide at 10 μmol/ml, and gastric inhibitory peptide at 10 μmol/ml. The antiserum was absorbed overnight and then used as the primary layer in the staining sequence outlined above. The double staining experiments used rabbit antisecretin at a final dilution of 1:500 codiluted with one of the following: guinea pig anti-insulin antiserum (MRC Regulatory Peptide Group; final dilution, 1:2,000), murine antiglucagon tissue culture medium (kind gift from M. Gregor, Klinikum Steglitz, Berlin, Germany; final dilution, 1:100), or murine antisomatostatin (MRC Regulatory Peptide Group; final dilution, 1:1,000) in PBS with 10% horse serum. The incubation times for the double staining mixtures were identical to those for the single staining procedure. After being washed in PBS, the sections were incubated with fluorescein-conjugated goat anti-rabbit IgG (Jackson Laboratories; final dilution, 1:2,000) codiluted with either rhodamine-conjugated porcine anti-guinea pig IgG (Jackson Laboratories; final dilution, 1:1,000) or rhodamine-conjugated equine anti-mouse IgG (Jackson Laboratories; final dilution, 1:2,000) in PBS with 10% horse serum as the secondary antiserum.

Plasmid constructions. To generate a series of deletion mutants for transient-transfection assays, an *Apa*I-*Nco*I restriction fragment containing 1,600 bp of the secretin gene 5′-flanking region plus 32 bp of exon 1 was isolated from a 4.5-kb *Eco*RI fragment cloned into pIBI31 (22) and subcloned into pGEM5. The 1,600-bp insert was excised with *Bam*HI and *Sal*I, subcloned into pUC19, reexcised with *Sma*I and *Hin*dIII, and subcloned into the *Sma*I and *Hin*dIII sites of pA3-LUC, a promoterless firefly luciferase reporter gene (27). Deletion mutants were generated by limited digestion with *Bal*31 endonuclease from the *Sma*I site at the 5′ boundary of −1600pA3-LUC. Linkers with *Kpn*I ends were added to the *Bal*31-deleted fragments, and the secretin gene sequences were reintroduced into the *Kpn*I and *Hin*dIII sites of pA3-LUC. To generate reporter gene constructions containing additional 5′-flanking region, a rat genomic library in EMBL-3A (Clontech Laboratories, Inc.) was screened with a probe complementary to the 5′ end of the existing clone. An *Eco*RV-*Nco*I fragment containing sequences from −4800 to +32 was inserted into the *Sma*I and *Nco*I sites in −1600pA3-LUC, replacing the original bp −1600 to +32 insert.

To characterize the 3′ boundary of the secretin gene regulatory region, a series of 3′-directed deletion mutants were generated and placed upstream of either the Rous sarcoma virus (RSV) or enhancerless herpes simplex thymidine kinase (TK) promoter. Fragments containing deletions at the 3′ end were excised and subcloned to introduce polylinker sequences that facilitated cloning into the *Kpn*I and *Sal*I sites of pRSV180A3-LUC (27) or the *Kpn*I and *Sac*I sites of pT81LUC (34). Several deletion mutants were produced by using existing restriction sites (*Nar*I, −168; *Pvu*II, −127; *Cla*I, −97). The −510 to −53 and −174 to −53 constructions were created by first subcloning a *Kpn*I-*Cla*I fragment into the same sites of pBluescript and then introducing two complementary synthetic oligonucleotides corresponding to sequences between −97 and −53 into the *Cla*I and *Sac*I sites of the vector. The inserts were excised with *Kpn*I and *Sac*I and subcloned into pT81LUC. The inserts were excised from pT81LUC with *Kpn*I and *Xho*I and subcloned into the *Kpn*I and *Sal*I sites of pRSV180A3-LUC. Additional 3′ deletion constructions were generated by DNA amplification with *Taq* polymerase primed with 3′-directed oligonucleotides extending upstream from −71, −85, −105, and −140 of the secretin gene and +824 to +846 (5′-CTTATCATGTCTGGATCCCCGGG-3′) of the pA3-LUC vector. The primers were designed to generate a *Sac*I restriction site to facilitate cloning of the amplified fragment into the *Kpn*I and *Sac*I sites of pT81LUC. Amplified promoter regions were sequenced to confirm the absence of mutations introduced by polymerase chain reaction.

Site-directed mutagenesis. Point mutations were introduced into the potential E box (−124 to −130) sequence CAGCTG by using *Taq* polymerase primed with an oligonucleotide containing the sequence TAGTTG substituted for the wild-type sequence and an oligonucleotide corresponding to sequences (+824 to +846) in the pA3-LUC vector. The amplified fragments were digested with *Kpn*I and *Cla*I and inserted into the same sites of p-174/-53T81LUC after excision of the wild-type sequences. All mutant reporter gene constructions were sequenced to confirm the introduction of the desired mutation by the chain termination method with modified T7 DNA polymerase (39).

Cell culture. The HIT T-15 M2.2 (hereafter called HIT) (9), RIN 1046-38A, RIN 1056C, RIN B6 (1, 4), and BTC-3 (10) cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM; 4.5 mg of glucose per liter) supplemented with 10% fetal calf serum. STC-1 cells (37) were cultured in DMEM supplemented with 2.5% fetal calf serum and 12.5% horse serum. INR1-G9 (40) and GH4 cells were cultured in RPMI 1040 and Ham's F10 media, respectively, supplemented with 10% fetal calf serum. All media were supplemented with 100 U of penicillin, 100 μg of streptomycin, and 292 μg of L-glutamine per ml. Cells were cultured at 37°C in a humidified atmosphere of 5% CO_2–95% air.

Transient-expression assays. One day prior to transfection, cells were plated at a density of 2.5×10^6 per 100-mm dish. A total of 30 μg of DNA, consisting of 2.5 to 5.0 μg of secretin reporter gene DNA, 1 μg of pRSVCAT, and sheared salmon testis carrier DNA, was introduced into cells by standard calcium phosphate coprecipitation, followed by a 2-min treatment with 15% glycerol. Cell lysates were prepared 24 to 48 h after transfection by extraction with 1% Triton X-100–100 mM potassium phosphate (pH 7.8)–1 mM dithiothreitol. Enzymatic assays measuring luciferase activity were performed in a Monolight 2010 luminometer (Analytical Luminescence Laboratory) by published methods (7). To account for variations in cell transfection efficiency, luciferase activity in cell lysates was normalized to the amount of chloramphenicol acetyltransferase (CAT) protein as determined by an enzyme-linked immunosorbent assay (5 Prime-3 Prime, Inc.).

Primer extension analysis. Total cellular RNA was extracted from HIT cells 48 h after transfection with 10 μg of reporter gene DNA and 20 μg of carrier DNA. The ^{32}P-end-labeled primer (5′-CCAGGAACCAGGGCGTATCTCTT CATAGCCTT-3′, 10^7 cpm/pmol), corresponding to sequences between +133 and +164 of the luciferase gene, was annealed to 30 to 50 μg of RNA at 62°C for 2 h (7). The primer was extended for 1 h at 37°C with Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories). The size of the extension products was determined by electrophoresis on denaturing 6% polyacrylamide gels, using the −174pA3-LUC sequence as a ladder.

Gel mobility shift assays. Gel mobility shift assays were performed as described by Fried and Crothers (12) with crude nuclear extracts prepared from HIT cells by the method of Dignam et al. (8). Complementary oligonucleotides were annealed, and the double-stranded species was isolated by electrophoresis on a native polyacrylamide gel and used as a probe or competitor fragments. Labeled probe was generated by filling in 5′ protruding ends with [α-^{32}P]dCTP and the large fragment of DNA polymerase I to a specific activity of greater than 10^7 cpm/pmol. Approximately 7.4 fmol of probe was incubated with 0.5 μg of nuclear extract protein for 15 min at 23°C in a total volume of 20 μl, containing 12 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7.9), 4 mM Tris-Cl, 1 mM EDTA (pH 8.0), 1 mM dithiothreitol, 12% glycerol, 0.3 mg of bovine serum albumin per ml, and 50 μg of poly(dI-dC) per ml. For competition experiments, competitor DNA was incubated with the protein for 15 min prior to addition of the probe at 23°C. The DNA-protein complexes were fractionated on a nondenaturing 4% polyacrylamide gel (acrylamide-bisacrylamide, 29:1) containing 2.5% glycerol and buffered with 0.5× TBE (45 mM Tris base, 45 mM boric acid, 1 mM EDTA). Radioactivity was detected by autoradiography of the dried gel. The probe consisted of sequences between −143 and −111 plus several additional bases at each end

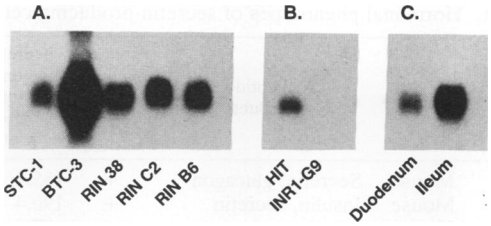

FIG. 1. Identification of secretin-producing cell lines by Northern blot analysis. Total cellular RNA (25 μg) from each cell line (A and B) and from rat small intestine (C) was loaded onto each lane, electrophoresed, transferred to nylon membranes, and probed as described under Materials and Methods. To facilitate detection of hamster secretin transcripts, the blot was first washed at somewhat reduced stringency (panel B, 0.2× SSC at 70°C for 30 min) and then washed further at higher stringency (panels A and C, 0.05× SSC at 85°C for 30 min). Autoradiographs represent exposures with two intensifying screens at −85°C for 48 h.

(5′-tcgaGGAGGCCGGACGACAGCTGGGGGGGCGGCC CTG-3′; additional bases in lowercase letters). The following double-stranded sequences were used in DNA competition studies:

Wild type (probe):
5′-tcgaGGAGGCCGGACGACAGCTGGGGGGGCGGCCCTG-3′
E box:
5′-tcgaGACGACAGCTGGG-3′
Mutant E box:
5′-tcgaGACGATAGTTGGG-3′
NIR element (rat insulin 1 gene)
5′-gatcCCCTCTCGCCATCTGCCTACCTACCCT-3′
FAR element (rat insulin 1 gene)
5′-gatcTCATCAGGCCATCTGGCCCCTTGTTAAT-3′
AP-1:
5′-GCTAGTGATGAGTCAGCCGGATC-3′

RESULTS

Identification of secretin-producing cell lines. The presence of immunoreactive secretin in the intestinal cell line STC-1, derived from transgenic mice expressing viral oncogenes under the control of the insulin promoter (37), prompted us to screen several islet-derived cell lines for secretin gene expression. Nine established islet cell lines were examined for secretin transcripts by Northern blot analysis with a rat secretin antisense RNA probe (23). In the rat (RIN 1046-38A, RIN B6, and RIN 1056C2), mouse (BTC-3), and hamster (HIT) islet cell lines as well as in STC-1 cells, the probe hybridized to a single mRNA species (Fig. 1) equal in size to the 750-nucleotide secretin transcripts in the rat duodenum and ileum. Secretinlike immunoreactivity, when determined, was readily detectable in each of the cell lines expressing secretin transcripts (Table 1). In addition, one glucagon-producing subclone (clone 6) of the αTC1 cell line expressed secretin transcripts, whereas another glucagon-producing subclone (clone 9) did not (Table 1). In one islet cell line, INR1-G9, a hamster glucagonoma (Fig. 1), neither secretin mRNA nor the peptide could be detected. Therefore, expression of the endogenous secretin gene in islet cell lines did not appear to correlate with the expression of any other islet hormones (Table 1). In particular, not all cell lines that produced secretin produced insulin. Furthermore, some glucagon-producing cell lines were capable of supporting secretin gene expression.

Developmental regulation of secretin gene expression in the pancreas. Expression of a hormone in transformed cell lines

TABLE 1. Hormonal phenotypes of secretin-producing cell lines[a]

Cell line	Species	Peptide products[b]	Secretin mRNA present	Secretinlike immunoreactivity (pg/mg of cell protein)
STC-1	Mouse	Secretin, glucagon	+	155.1 ± 27.0
BTC-3	Mouse	Insulin, secretin	+	190.4 ± 26.0
HIT	Hamster	Insulin	+	87.5 ± 3.6
RIN 38A	Rat	Insulin, gastrin, CCK, angiotensinogen	+	19.4 ± 7.3
RIN B6	Rat	Gastrin	+	12.3 ± 4.5
RIN 1056C2	Rat	CCK, angiotensinogen	+	Not done
αTC1 C16	Mouse	Glucagon	+	Not done
INR1-G9	Hamster	Glucagon	−	Undetectable
αTC1 C19	Mouse	Glucagon	−	Not done

[a] Cell lines were examined for the presence of secretin transcripts. Secretinlike immunoreactivity was determined by radioimmunoassay for each cell line, and the results are expressed as the means of at least three independent experiments ± standard error of the mean.

[b] The peptides detected in the cell lines, as reported previously (16, 35, 37, 40), are listed. CCK, cholecystokinin.

but not in corresponding normal tissues may indicate the existence of a multipotential progenitor cell present in early development. In view of the present data demonstrating expression of the secretin gene in several islet-derived cell lines, we examined the pancreas of developing and adult rats for the expression of secretin mRNA by Northern blot analysis. In the pancreas of fetal and neonatal rats, a single mRNA species comparable in size to the 750-nucleotide secretin transcripts in duodenum hybridized to the probe (Fig. 2). Pancreatic secretin mRNA reached its highest abundance at day 19 of gestation. Levels fell significantly after birth and were below the limits of detection in adult animals. The ontogeny of secretin gene expression in the pancreas is distinctly different from that of the insulin gene. In agreement with others, we show (Fig. 2) that insulin is expressed at low levels in the fetus, rising to high levels after birth (1, 17). The observed abundance of secretin transcripts during pancreatic development did not result from unequal loading of different lanes, as seen from the pattern of β-actin abundance.

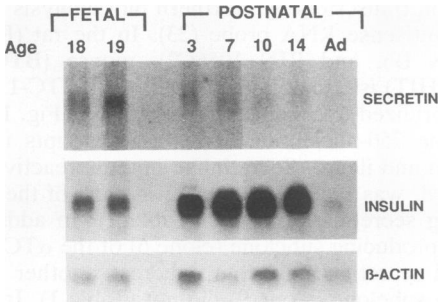

FIG. 2. Analysis of secretin mRNA from the pancreas at different stages of development by Northern blot hybridization. Total pancreatic RNA (25 μg) from developing animals was loaded in each lane. Ages are indicated in days. Ad, adult rat pancreas. The blot was washed in 0.05× SSC at 85°C for 30 min and exposed for 3 days at −85°C with two intensifying screens. After the autoradiographic signal was allowed to decay, the blot was rehybridized to a rat insulin I cDNA probe, washed in 0.2× SSC at 65°C for 30 min, rehybridized to a mouse β-actin cDNA probe, and washed in 0.2× SSC at 55°C for 30 min. The membrane was exposed to film for 14 h at −85°C with two intensifying screens.

Anatomic localization of secretin in the developing rat pancreas. Secretin-immunoreactive endocrine cells were readily observed in the fetal pancreas at day 18 of gestation, when secretin transcripts are relatively abundant. At this stage of development, the endocrine cells were not found in discrete islets but in clusters. Secretin-positive cells were decreased in number by day 21, observed occasionally at postnatal day 14, and could no longer be identified in adult animals (not shown). To determine whether secretin-immunoreactive cells in the pancreas belonged to one of the established islet lineages, sections of pancreas from day 18 of gestation were double stained for secretin plus insulin, glucagon, or somatostatin. Most secretin-immunoreactive cells (>80%) were identified as insulin-producing beta cells by double immunofluorescence. All secretin-positive cells remaining after absorption of the antiserum with glucagon were positive for insulin and represented a subset of beta cells (Fig. 3). The majority of fetal beta cells did not stain for secretin immunoreactivity (Fig. 3). Glucagon preadsorption of the secretin antiserum eliminated the staining of glucagon-containing alpha cells (not shown). No colocalization of somatostatin and secretin was observed (not shown). Preabsorption studies demonstrated that endocrine cell staining in the pancreas was not affected by the addition of either vasoactive intestinal peptide or gastric inhibitory peptide.

Cell-specific regulation of secretin gene expression in cell lines. To localize sequences in the 5′-flanking region that control cell-specific transcription of the secretin gene, a series of secretin reporter genes were constructed, containing sequential deletions of 5′-flanking DNA placed upstream of the structural gene encoding firefly luciferase. Reporter gene constructions containing between 4,800 and 39 bp of the secretin 5′-flanking region were introduced into HIT cells, which produce secretin, and INR1-G9 cells, an islet cell line which does not express the secretin gene. As little as 174 bp of flanking sequence was sufficient for maximal reporter gene expression in HIT cells (Fig. 4). Removal of an additional 15 bases, however, reduced transcriptional activity by nearly 80%. Deletion to −126 and subsequently to −97 further reduced transcriptional activity. Constructions shorter than −97 had basal transcriptional activity which was minimally higher than that of the promoterless pA3-LUC reporter. In contrast, none of the constructions were efficiently expressed in the INR1-G9 cells (Fig. 4). The dramatic reduction in luciferase activity in HIT cells with deletions below −174 and the failure to unmask expression in INR1-G9 cells suggest that sequence localized within −174 bp of the transcriptional initiation site functions as a positive *cis*-active domain in secretin-producing cells. The gradual loss of transcriptional activity observed in the HIT cells with deletions below −174 suggests that there may be multiple components to the positive element.

Enhancerlike properties of the positive *cis*-active element. To define the 3′ boundary of the transcriptionally active region in the secretin gene, a series of 3′-directed deletions of the secretin 5′-flanking region were constructed and placed upstream of either the enhancerless RSV promoter (Fig. 5A) or the herpes simplex virus TK (data not shown) promoter fused to the luciferase gene. A construction extending from −510 to −53 increased the expression of the enhancerless pRSV180A3-LUC construct approximately 70-fold (Fig. 5A). Deletion of an additional 18 bp resulted in a 60% reduction in activity, and an additional deletion to −97 eliminated enhancement entirely. The pattern of activity

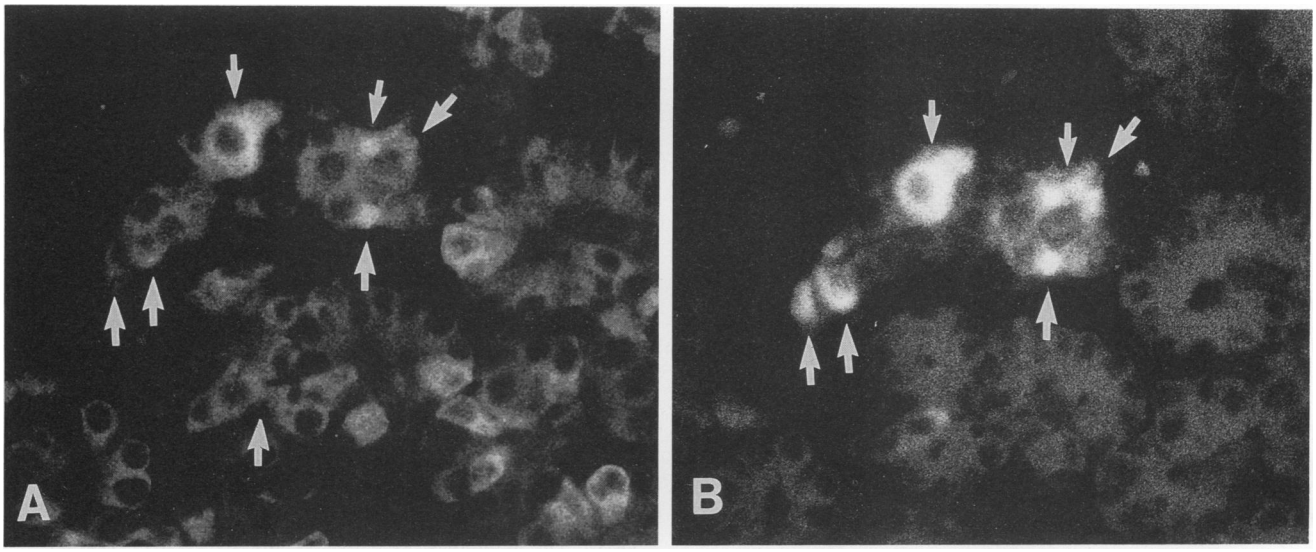

FIG. 3. Anatomic localization of secretin-producing cells in the fetal rat pancreas by immunofluorescent staining. (A) A 5-μm section of fetal rat pancreas (day 18) immunostained with insulin antiserum and localized with rhodamine. (B) The same section showing secretin-immunoreactive cells localized with fluorescein. All secretin-positive cells appear to stain for insulin and are indicated by arrows. Magnification, ×1,200.

observed with the TK promoter paralleled that of the enhancerless RSV, indicating that the region determined to be transcriptionally active could utilize functionally heterologous promoters. The minimal element −174 to −53, as defined by deletional analysis, also functioned efficiently in either orientation in HIT cells and retained partial activity (20-fold enhancement of the RSV promoter) independent of position, suggesting that these sequences function as enhancers (Fig. 5A). Transient-expression assays carried out in the INR1-G9 cells revealed that sequences between −53 and −85 in the secretin enhancer weakly stimulated transcription from the heterologous RSV promoter (Fig. 5A) but not from the secretin promoter (Fig. 4).

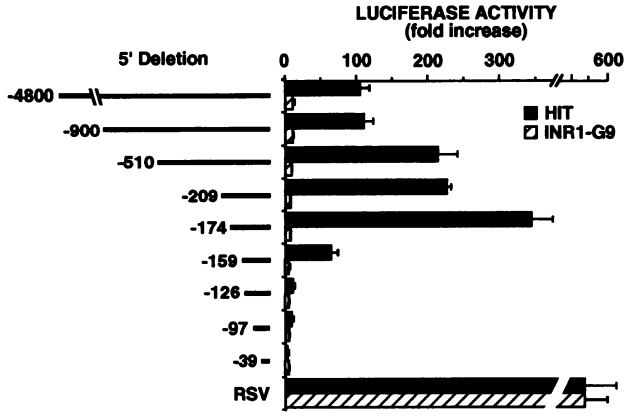

FIG. 4. Deletional analysis of *cis*-active DNA elements in the rat secretin gene 5′-flanking region. A series of constructions containing 5′-directed deletions of the secretin 5′-flanking region plus 32 bp of exon 1 were fused to the structural gene for firefly luciferase. The reporter gene constructions were introduced into secretin-producing HIT and nonproducing INR1-G9 cells for transient-expression assays. After 48 h, cell extracts were assayed for luciferase activity. Activities were normalized for transfection efficiency as described in Materials and Methods and are expressed as fold increase over the activity generated from the promoterless construction pA3-LUC. The plasmid pRSV400-A3LUC (RSV) served as a positive control. Activities represent the mean ± standard error of the mean of at least three independent transfection experiments.

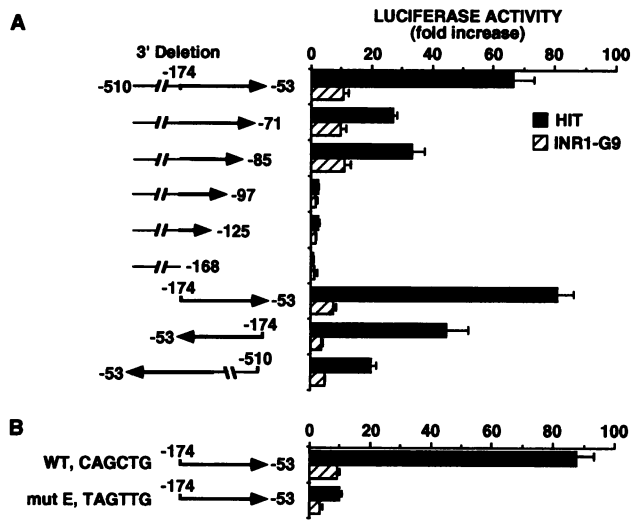

FIG. 5. Characterization of the secretin enhancer element. (A) A series of 3′-directed deletions, with the boundaries illustrated, were placed upstream of the enhancerless RSV promoter fused to the luciferase gene. The reporter gene constructions were transiently transfected into HIT and INR1-G9 cells. Arrows denote the orientation of the secretin gene fragment relative to that of the promoter. Luciferase activities are expressed as fold increase over the luciferase activity generated from the enhancerless RSV promoter. Other details are described in the legend to Fig. 4. (B) Sequences from −174 to −53 were placed upstream of an enhancerless herpes simplex virus TK promoter fused to the luciferase gene. WT, wild-type CAGCTG sequence at −130. The CAGCTG sequence has been replaced by TAGTTG in the mutant E box (mut E) construction, as described in the text. Luciferase activities are expressed as fold increase over the activity generated from the TK promoter alone.

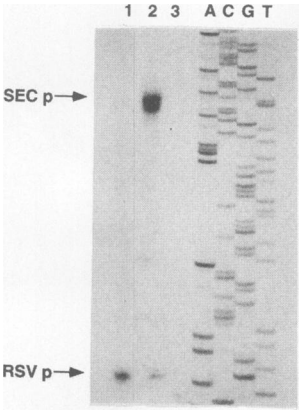

FIG. 6. Determination of the initiation site of transiently expressed luciferase transcripts in HIT cells by primer extension analysis. A ^{32}P-end-labeled probe specific for the luciferase sequence from +133 to +164 was annealed to total RNA from HIT cells transfected with either pRSV400-A3-LUC (lane 1), −174pA3-LUC (lane 2), or −174/−53pRSV180LUC (lane 3) and then extended with reverse transcriptase. The sizes of extension products were determined following electrophoresis on a denaturing 6% polyacrylamide gel alongside a sequencing ladder of −174pA3-LUC primed with the same oligonucleotide. SECp and RSVp denote the positions of the primer extension products (231 and 179 nucleotides) predicted if transcription initiated from the start sites known to be utilized by the secretin and RSV promoters, respectively.

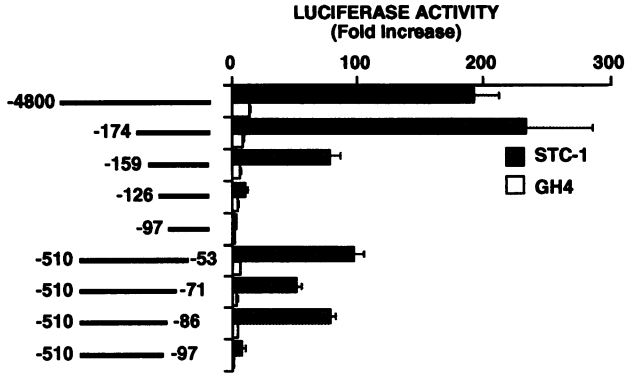

FIG. 7. Activity of the secretin enhancer in STC-1 and GH4 cells. The reporter gene constructions shown in Fig. 4 and 5 were introduced into STC-1 and GH4 cells for transient-expression assays as described in the text and those figure legends. The luciferase activities of 5′ deletion mutants are expressed as fold increase over the activity generated from the promoterless construction pA3-LUC. The luciferase activities of the 3′ deletion mutants are expressed relative to that of the enhancerless construction pRSV180A3LUC. Activities represent the mean ± standard error of the mean of at least three independent transfection experiments.

To verify that the luciferase activity arose from reporter genes transcribed from the appropriate promoter, total RNA isolated from transiently transfected HIT cells was examined by primer extension analysis (Fig. 6). Examination of the shortest fully active construction, −174pA3-LUC, revealed the presence of a single luciferase transcript which initiated at a position identical to that of the secretin gene in rat intestine. Similar analysis of the minimal enhancer element, from −174 to −53, placed 5′ to the enhancerless RSV promoter demonstrated a single luciferase transcript which initiated at the transcriptional start site established for the RSV promoter.

The ability of secretin-luciferase reporter gene constructions to activate transcription in other secretin-expressing and nonexpressing cell lines was also examined (Fig. 7). Secretin-reporter gene constructions transiently transfected into the secretin-producing intestinal STC-1 cell line were expressed with efficiencies comparable to those of HIT cells. As was the case for the HIT cells, as little as 174 bp of 5′-flanking sequence supported maximal luciferase expression. Removal of sequences between −53 and −85 did not result in as large a loss of transcriptional activity in STC-1 cells as in HIT cells. Constructions containing as little as 97 bp of 5′-flanking sequence retained minimal residual enhancer activity in STC-1 cells. The ability of the secretin regulatory domain to weakly but significantly enhance transcription in secretin-nonproducing GH4 cells may indicate the presence of sequences that function as general activators of transcription.

Role of insulin gene enhancerlike sequences in secretin gene expression. The sequence CAGCTG is similar to the core of the enhancer elements found in the rat insulin I gene (21, 31), the gastrin gene (42), exocrine pancreatic genes (29), and the immunoglobulin heavy-chain genes (11). To determine whether the E box-like sequence was transcriptionally active within the secretin gene, the CAGCTG sequence was replaced by TAGTTG. This mutation was designed on the basis of previously described point mutations which decreased the transcriptional activity of the immunoglobulin μ E2 protein and interfered with binding to the E12 protein (19, 28, 29, 31). Transcriptional activity was reduced by approximately 85% when TAGTTG replaced the wild-type sequence, suggesting that the CAGCTG sequence is necessary for efficient transcription of the secretin gene in HIT cells (Fig. 5B).

Interaction of nuclear proteins with the secretin gene CAGCTG motif. Gel mobility shift assays demonstrate that 0.5 μg of HIT nuclear proteins bind to a probe containing the sequence between −143 and −111, producing two major retarded bands migrating as a doublet (Fig. 8, lane 1) which are completely competed by the unlabeled probe sequences (lanes 2 and 3). The inability of a nonspecific unrelated AP-1 sequence to displace binding suggested that these complexes resulted from sequence-specific binding (lanes 12 and 13). An excess of unlabeled oligonucleotide containing only the core CAGCTG sequence competed effectively for islet nuclear proteins and resulted in the loss of both DNA-protein complexes, suggesting that the proteins bound to this core motif (lanes 4 and 5). An excess of an oligonucleotide with transcriptionally less active TAGTTG sequence substituted for CAGCTG was also preincubated with the HIT nuclear extracts for competition studies (lanes 6 and 7). Even a 500-fold molar excess of the mutant sequence was unable to displace binding of the proteins to the wild-type sequence, suggesting that the proteins complexed to the CAGCTG sequence may be important for transcriptional activity. To determine whether the CAGCTG sequence in the secretin gene and the related NIR and FAR elements in the rat insulin I gene interacted with similar proteins, the NIR and FAR sequences were used for DNA competition studies. Binding of the HIT nuclear proteins to the CAGCTG sequence in the secretin gene was also displaced by an excess of the rat insulin I NIR (8, 9) element sequence. The FAR (10, 11) element bound with apparent lower affinity, resulting in partial competition of the complexes at a 500-fold excess. These results indicate that the HIT nuclear proteins which

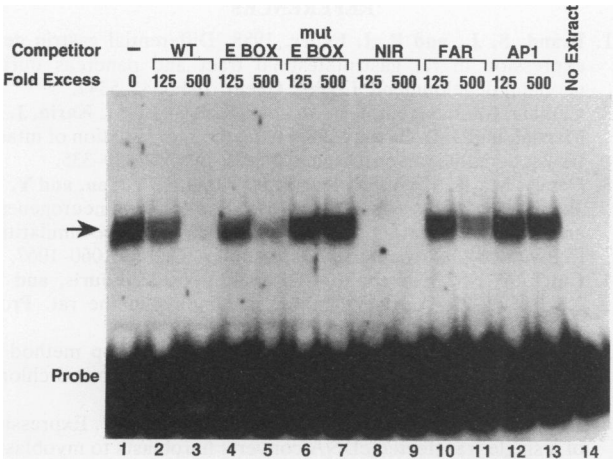

FIG. 8. Gel mobility shift analysis of proteins binding to the secretin gene CAGCTG sequence. Binding reactions were performed with labeled 41-bp double-stranded oligonucleotides containing sequences spanning −143 to −111 of the secretin gene and 0.5 μg of HIT cell nuclear protein. The nomenclature of each competing fragment and corresponding sequences is described in Materials and Methods. WT denotes competition with the unlabeled probe sequences (wild type). The molar ratio of competitor to probe is shown above each lane. Lane 14 contains probe alone with no nuclear extract. The position of the retarded doublet complex is denoted by an arrow.

bind to the secretin gene E box also bind to the enhancer elements in the insulin gene. The proteins in the retarded complexes appeared to interact with DNA sequences in the following order of decreasing affinity: NIR element, secretin enhancer, and FAR element.

DISCUSSION

The gene encoding secretin is selectively expressed in a specific enteroendocrine cell type, the S cell, localized primarily in the small intestine. The existence of extraintestinal secretin has not been well established, in part because of limitations in the specificity and sensitivity of different secretin antisera used in radioimmunoassays. Isolation of a cDNA encoding secretin and its subsequent use as a hybridization probe revealed that secretin transcripts are expressed throughout the intestinal tract. Although secretin transcripts and peptide were undetectable in the adult rat pancreas, secretin immunoreactivity in primary cultures of neonatal pancreatic islets has been described (38). The potential for islet cells to produce secretin is further supported by the existence of two secretin-producing cell lines, STC-1 and BTC-3, derived from transgenic mice expressing viral oncogenes under control of the rat insulin promoter (37). The BTC-3 cell line originated from a pancreatic β-cell tumor in transgenic mice. The STC cell line was derived from an intestinal endocrine tumor in transgenic mice expressing both simian virus 40 large T antigen and polyomavirus small T antigen under the control of the insulin promoter (37). We have subsequently examined several islet cell lines for the presence of secretin transcripts. Expression of the endogenous secretin gene in all but two of the islet-related cell lines examined did not appear to correlate with a specific hormonal phenotype and may therefore reflect the multipotentiality of these cell lines (35).

Examination of the rat pancreas by RNA blot hybridization and immunocytochemical methods revealed that the secretin gene was transiently expressed in B cells of developing animals. The ontogeny of secretin gene expression in the pancreas parallels the pancreatic expression of several other regulatory peptides. Gastrin (1), thyrotropin-releasing hormone (15), and peptide YY (24) are expressed at their highest levels before birth in nascent islets and are subsequently repressed in adult animals. In the present study, secretin gene expression in the pancreas appears to be limited to B cells during development. Both the proportion of B cells that coexpress secretin and the intensity of secretin immunostaining appear to fall after birth, arguing against the existence of a distinct secretin-producing cell lineage in the islets. These findings suggest that developing B cells in fetal and neonatal rats can transiently support secretin gene expression, which is subsequently repressed in mature B cells. The cross-reactivity of most secretin antisera with the related peptide glucagon makes it difficult to rule out low-level expression of secretin in glucagon-producing A cells. The transient expression of secretin in developing pancreatic endocrine cells and in several multipotential islet cell lines suggests that the intestinal S cell may be developmentally related to the endocrine cells of the pancreas. The belief that enteroendocrine and pancreatic endocrine cells arise from the embryonic gut endoderm is consistent with this hypothesis (26).

The islet cell lines described here provide an attractive model for characterization of cell-specific transcriptional regulation of the secretin gene. The availability of cell lines that express their endogenous secretin gene, as well as an additional islet line that does not, provides a model system by which to identify transcriptional control elements specific for secretin-producing cells. Transient-expression assays with a series of reporter gene constructions containing progressive deletions indicate that transcription of the secretin gene in secretin-producing cells is mediated by a positive, *cis*-active regulatory element spanning 120 bp of 5′-flanking region. Sequential removal of sequences from the 5′ and 3′ ends of this sequence resulted in a stepwise reduction in enhancer activity, suggesting that the enhancer consists of multiple elements. In addition to the positive *cis*-active element, the deletional analysis suggests the existence of weak negative elements between −900 and −174.

The sequence from −174 to −131 of the *cis*-regulatory domain in the secretin gene, when compared with a relational data base of transcription factor-binding sites (14), does not appear to contain any known consensus factor-binding motifs. However, this region appears to be important for the transcriptional activity of the secretin gene in secretin-producing cells. Examination of the enhancer from −130 to −53, however, reveals sequences that resemble the consensus binding sites for known transcription factors. We sought to examine the role of the CAGCTG motif in secretin gene expression because of the importance of closely related elements in the islet-specific transcription of the rat insulin I (21) and gastrin (42) genes. The sequence CAGCTG at −130 includes the core binding motif for members of the helix-loop-helix family of transcription factors, CANNTG. The introduction of point mutations into the secretin E box motif, corresponding to mutations which inactivate the insulin enhancer, similarly reduced the transcriptional activity of the secretin gene. These observations suggested that transcription of the secretin gene may depend on interactions with factors similar or identical to those which activate insulin gene expression.

Helix-loop-helix DNA-binding proteins have been impli-

cated in cell-specific and developmental regulation of transcriptional activity for a wide variety of genes in many non-islet tissues and in numerous species. Examples include the immunoglobulin enhancer-binding proteins E12 and E47 (32), the closely related pancreatic enhancers Pan 1 and 2 (33), transcription factor AP-4 (18), the muscle-specific regulatory proteins MyoD (6) and myogenin (43), and the *Drosophila* regulatory protein products of the daughterless and achaete-schute genes (3). The NIR and FAR CANNTG elements of the insulin gene appear to bind to the widely distributed E12 protein (13). Gel mobility shift assays indicate that islet nuclear proteins interact with the CAGCTG sequence in the secretin gene and that the same protein(s) recognizes the CANNTG motifs in the rat insulin gene. Transcriptional regulation by a common protein factor with the insulin gene may explain, in part, selective secretin gene expression in B cells.

Two GC-rich sequences resembling Sp1 consensus-binding sites (20) were identified between −122 and −113 (GGGGCGGCCC) and between −68 and −59 (GGGGCGG TGC) in the secretin gene. Deletion of the downstream GC sequence resulted in an approximately 60% loss of transcriptional activity of secretin reporter genes in HIT cells. Owing to the low activity of deletion mutants which span the upstream GC sequence, we cannot comment on its role in secretin gene expression. Whether the GC-rich sequences interact with Sp1 is not clear. DNA-binding proteins other than Sp1 have been shown to recognize consensus Sp1-binding sites. GC-rich elements in the pro-opiomelanocortin (41) and gastrin (30) promoters do not appear to interact with Sp1 itself, indicating a potential role for sequences that flank the core sequence in determining factor binding.

The molecular mechanisms responsible for the disappearance of secretin from the islets after birth is unknown. Gastrin gene expression is similarly repressed after birth in islets, presumably through an islet-specific interferon beta-like negative element (1, 42). An analogous negative element could not be identified in the secretin gene by the DNA transfection studies in transformed cell lines reported here. However, the presence of a pancreas-specific secretin gene repressor activated in the postnatal pancreas may explain the disappearance of secretin from this tissue.

The results of the present study suggest that cell-specific transcriptional regulation of the secretin gene is mediated by a *cis*-active regulatory domain containing multiple positive elements. The CANNTG element as well as the GC-rich elements resemble several transcription factor recognition sequences. The analysis of deletion mutants indicates that additional sequences not clearly related to those of known DNA-binding proteins are also important for transcriptional regulation of the secretin gene. Efficient cell-specific transcription of the secretin gene may therefore require *cis*-active elements that bind to factors found specifically in secretin cells as well as elements that interact with DNA-binding proteins which are present in many cell types.

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