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The LIM family transcription factor Isl-1 requires cAMP response element binding protein to promote somatostatin expression in pancreatic islet cells

(cAMP response element/cell specificity/phosphorylation independence)

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ABSTRACT Many eukaryotic genes are regulated by cAMP through a conserved cAMP response element (CRE). Here we show that, in the pancreatic islet cell line Tu6, a well-characterized CRE in the somatostatin gene does not provide cAMP responsiveness but functions as an essential element for its basal activity. DNA-binding and functional analyses indicate that the cAMP-responsive factor CREB regulates somatostatin expression in these cells without requirement for phosphorylation at the protein kinase A-regulated Ser-133 phosphorylation site. In addition to the CRE site, cell-specific expression of the somatostatin gene requires a second promoter element, which binds the recently characterized LIM family protein Isl-1. Thus, Isl-1 and CREB appear to synergize on the somatostatin promoter to stimulate high-level expression in Tu6 cells. The ability of CREB to function in a phosphorylation-independent manner suggests a mechanism by which this protein can regulate gene transcription.

The tissue-specific expression of numerous genes appears to require specialized trans-activators, which often act autonomously to stimulate transcription. The GHF-1/pit-1 (1) protein, for example, stimulates high-level expression of the growth hormone gene in pituitary somatotrophs without apparent requirement for additional factors. Other tissue-specific activators, however, must apparently associate with additional factors in order to regulate expression. The T-cell-specific factor TCF-1α (2), for example, requires adjacent cAMP response element (CRE) and ETS-1 binding sites (3) in order to stimulate T-cell receptor α gene expression.

In the present study, we have examined the mechanism underlying the cell-specific expression of the somatostatin gene in a pancreatic tumor cell line Tu6 (4). Promoter deletion studies indicate that somatostatin expression in these cells requires two promoter elements. The first site recognizes the islet cell factor Isl-1 (5) and the second is a consensus CRE (6) that has previously been shown to bind the CRE binding protein CREB. The Isl-1 protein would thus appear to fall into that category of proteins requiring additional factors to stimulate transcription.

MATERIALS AND METHODS

Cell Culture and Transient Assays. MSL-G2-Tu6 cells (referred to here as Tu6; a gift of O. Madsen, Hagedorn Research Laboratory, Gentofte, Denmark) and RIN-5AH cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (HyClone). PC12 and F9 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 5% horse serum. Transfections were done as described with a cotransfected Rous sarcoma virus–β-galactosidase (RSV–β-Gal) plasmid as internal control (7).

The somatostatin reporter vectors were constructed by standard cloning techniques. The effector plasmids Gal4CREB and Gal4CREB-M1 were made by inserting CREB and CREB-M1 cDNAs into the simian virus 40–Gal4 expression vector pSG424 (8). CREB-M1 differs from CREB in a missense mutation that converts Ser-133 to Ala-133. Gal4-ATF2 was a gift from M. R. Green (Boston). The effector plasmid RSV–Isl-1 was made by inserting a *Hin*dIII/*Bgl* II fragment containing the Isl-1 cDNA into the expression vector RSV-SG (9).

RNA Analysis. Total RNA was prepared from Tu6, PC12, or RIN-5AH cells by a standard guanidinium/phenol chloroform method. Northern blot and RNA protection assays were performed as described (9, 10).

Two-Dimensional Tryptic Mapping. Two-dimensional tryptic mapping experiments were carried out as described (7).

***In Vitro* Transcription Assays.** Tu6 nuclear extracts were prepared according to the method of Dignam *et al.* (11). Immunodepletion of endogenous CREB protein was accomplished by incubating the extract with a CREB immunoaffinity resin (12) for 2 hr at 4°C. Recombinant CREB and CREB-M1 proteins were prepared by expression in bacteria using pET vectors and were purified as described (12).

For *in vitro* transcription assays, 150 ng of 4X/99 plasmid template was incubated with 0.5 μg of recombinant CREB protein for 15 min at 4°C. Then 150 μg of immunodepleted Tu6 extract was added to the reaction mixture. Transcription and primer extension were done as described (13).

DNA Binding Analyses. Southwestern blots were performed as described using a multimerized somatostatin CRE probe (12). Preparation and immunoaffinity treatment of nuclear extracts were done as described above.

DNase I protection assays were performed as described (14) using an end-labeled probe that contained somatostatin sequences from −141 to +58. Recombinant CREB protein was prepared as described above. Recombinant Isl-1 protein was prepared from bacteria as a TrpE fusion protein as described (5).

Western Blot Analysis. The CREB antiserum 244, raised against a peptide corresponding to amino acids 128–160 of the CREB protein, has been characterized (12). The Isl-1 antiserum 5254 was isolated from rabbits immunized with a synthetic peptide corresponding to amino acids 112–126 of the reported sequence for rat Isl-1. Blocked Isl-1 antiserum was prepared by incubating 1 mg of the Isl-1 peptide with 1 ml of antiserum at 4°C for 16 hr. Partially purified recombi-

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Abbreviations: CRE, cAMP response element; CREB, CRE binding protein; CAT, chloramphenicol acetyltransferase; PKA, protein kinase A.

nant Isl-1 was prepared by inserting the entire coding sequence of Isl-1 into the T7 polymerase expression vector pET-8c and fractionating bacterial lysates from transformed cells by DNA-cellulose chromatography. For Western blot analysis, 25 μg each of Tu6 and PC12 nuclear extracts and 10 ng of partially purified recombinant Isl-1 protein were fractionated on SDS/polyacrylamide gel, blotted onto nitrocellulose filters, and incubated with either Isl-1 (1:200), preadsorbed Isl-1 (1:200), or CREB (1:500) antiserum and then developed with an alkaline phosphatase-conjugated anti-rabbit secondary antibody (Bio-Rad).

RESULTS

The somatostatin gene contains a conserved CRE motif that mediates hormonal induction in a number of cell lines. Tu6 cells express high levels of somatostatin (Fig. 1*A Left*) but are unresponsive to cAMP stimulation (Fig. 1*A Right*). PC12 pheochromocytoma cells do not express somatostatin endogenously (Fig. 1*A Left*) but can express a transfected somatostatin gene in stable lines selected with a cotransfected neomycin-resistance marker (6). In contrast to Tu6 cells, the transfected PC12 cell line KS-2 stimulates somatostatin transcription 5- to 10-fold in response to cAMP (ref. 6; Fig. 1*A Right*).

The disparate response to cAMP in Tu6 and PC12 lines prompted us initially to examine the relative importance of the CRE site in directing somatostatin expression in these cells. Using the chloramphenicol acetyltransferase (CAT) reporter construct −1.4CAT, which contains 1.4 kilobases (kb) of somatostatin 5′ flanking sequence, we performed transient CAT assays and normalized transfection efficiency between cell types to β-galactosidase activity derived from a cotransfected RSV–β-Gal plasmid (16) (Fig. 1*B*). Tu6 cells expressed the −1.4CAT somatostatin fusion gene vector at levels 20- to 50-fold higher than in PC12 cells (Fig. 1*B*), suggesting that this region of the somatostatin promoter did indeed contain elements that mediated cell-specific expression. Remarkably, deletion of the CRE site from the −1.4CAT plasmid severely abrogated somatostatin promoter activity. Thus, although the CRE site did not function to promote cAMP responsiveness in Tu6 cells, it was nevertheless critical in mediating high-level expression of the somatostatin gene.

The ability of the CRE site to promote high-level expression of somatostatin without directing a transcriptional response to cAMP suggested that another CRE binding protein besides CREB might be responsible for this activity. Indeed, a number of CRE binding proteins have been characterized to date, although their functions remain largely uncharacterized. To determine which of these proteins was predominantly expressed in Tu6 cells, we performed Southwestern blotting analysis (Fig. 1*C*). Using a double-stranded somatostatin CRE oligonucleotide as probe, we detected a major CRE binding protein of 43 kDa in both Tu6 and PC12 extracts. Immunoaffinity chromatography of these extracts with a polyclonal CREB antiserum removed a majority of the CRE binding activity in both cell lines, and Western blotting experiments further demonstrated that the levels of CREB protein were comparable between Tu6 and PC12 cells (see Fig. 4*D*, lanes 8 and 9).

Southwestern analysis showing that CREB accounts for a majority of the CRE binding activity in Tu6 cells suggested that this protein may indeed participate in directing cell-specific expression of the somatostatin gene. The absence of any cell-specific differences in CREB expression, however, prompted us to ask whether the phosphorylation of CREB at the protein kinase A (PKA)-regulated Ser-133 phosphoacceptor site might differ between PC12 and Tu6 cells. Two-dimensional tryptic maps (Fig. 1*D*) of immunoprecipitates

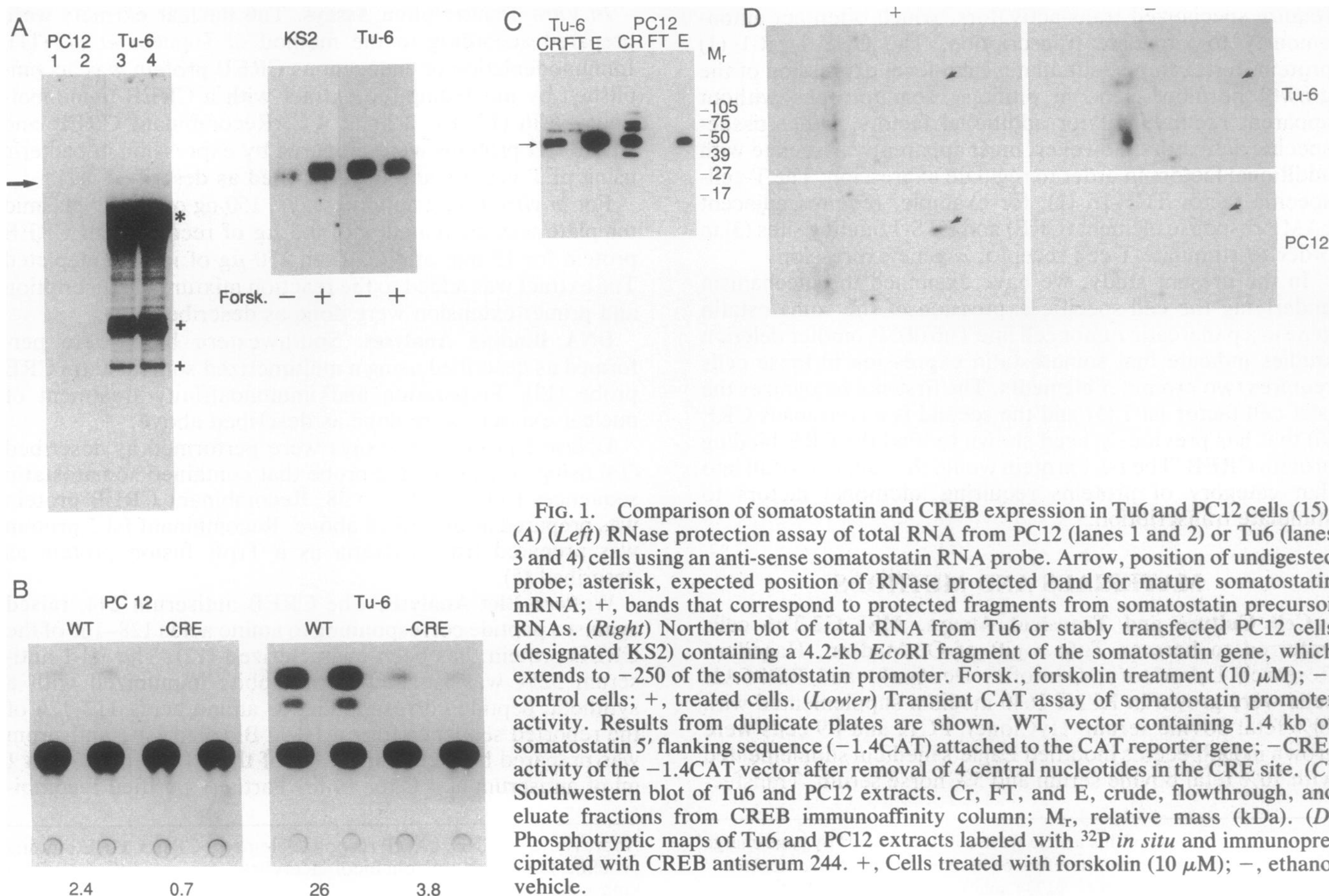

FIG. 1. Comparison of somatostatin and CREB expression in Tu6 and PC12 cells (15). (*A*) (*Left*) RNase protection assay of total RNA from PC12 (lanes 1 and 2) or Tu6 (lanes 3 and 4) cells using an anti-sense somatostatin RNA probe. Arrow, position of undigested probe; asterisk, expected position of RNase protected band for mature somatostatin mRNA; +, bands that correspond to protected fragments from somatostatin precursor RNAs. (*Right*) Northern blot of total RNA from Tu6 or stably transfected PC12 cells (designated KS2) containing a 4.2-kb *Eco*RI fragment of the somatostatin gene, which extends to −250 of the somatostatin promoter. Forsk., forskolin treatment (10 μM); −, control cells; +, treated cells. (*Lower*) Transient CAT assay of somatostatin promoter activity. Results from duplicate plates are shown. WT, vector containing 1.4 kb of somatostatin 5′ flanking sequence (−1.4CAT) attached to the CAT reporter gene; −CRE, activity of the −1.4CAT vector after removal of 4 central nucleotides in the CRE site. (*C*) Southwestern blot of Tu6 and PC12 extracts. Cr, FT, and E, crude, flowthrough, and eluate fractions from CREB immunoaffinity column; M_r, relative mass (kDa). (*D*) Phosphotryptic maps of Tu6 and PC12 extracts labeled with ^{32}P *in situ* and immunoprecipitated with CREB antiserum 244. +, Cells treated with forskolin (10 μM); −, ethanol vehicle.

from cells labeled with $^{32}P_i$ showed no difference in basal phosphorylation of the Ser-133 phosphoacceptor in PC12 versus Tu6 cells. Moreover, forskolin treatment induced phosphorylation of CREB at this site in PC12 cells, but no such stimulation was observed in treated Tu6 cells. Additional CREB phosphopeptides were observed to various degrees in Tu6 and PC12 cells, which did not seem to be significantly regulated by forskolin. In fact, these correspond to casein kinase II phosphorylation sites (M.M., unpublished data), which when mutagenized do not affect PKA-dependent CREB activity. Thus, the inability of these cells to stimulate somatostatin expression in response to cAMP would appear to be explained by an inability to induce CREB phosphorylation at Ser-133.

To test whether CREB could direct high-level expression of the somatostatin gene in Tu6 cells, we constructed an expression vector encoding a Gal4–CREB fusion protein. This fusion protein contains the yeast Gal4 DNA binding domain attached to the N terminus of CREB. Correspondingly, we replaced the CRE site in the 1.4-kb somatostatin promoter with two Gal4 recognition sequences. The absence of other proteins with the same DNA binding specificity as Gal4 in these cells thus permitted us to directly assess CREB function. When assayed in Tu6 cells, the −1.4(Gal4)CAT reporter plasmid had no detectable activity in Tu6 cells, further illustrating the importance of the CRE site in directing somatostatin expression. Cotransfection of the Gal4 CREB effector plasmid induced the activity of the −1.4(Gal4)CAT reporter 10- to 15-fold (Fig. 2*A*), demonstrating that Gal4-CREB could indeed restore activity to the somatostatin promoter. No such induction of the −1.4(Gal4) reporter was observed in F9 cells (data not shown), demonstrating that such induction was cell specific. As predicted from the two-dimensional tryptic mapping experiments, the mutant Gal4-CREB-M1, which contains a Ser-133 to Ala-133 substitution at the PKA phosphoacceptor site, was as active as wild-type CREB in Tu6 cells. These results indicate that, although phosphorylation at Ser-133 is critical for cAMP responsiveness, CREB directs high-level expression of the somatostatin gene in Tu6 cells through a different mechanism.

To determine whether other CRE binding proteins could also stimulate somatostatin expression in Tu6 cells, we obtained an expression vector encoding the CRE binding protein ATF2/CRE-BP1 fused to the Gal4 DNA binding domain. This Gal4-ATF2 (17) expression plasmid can induce the activity of a cotransfected Gal4CAT reporter gene 5- to 10-fold in several cell lines (J.L., unpublished data; ref. 17). When compared to Gal4-CREB (Fig. 2*A*, lanes 11 and 12), however, the Gal4-ATF2 plasmid was far less able to stimulate expression of the somatostatin −1.4(Gal4) reporter. The results thus support the contention that CREB is specifically required for somatostatin promoter activity in Tu6 cells.

To characterize further the effect of CREB on somatostatin expression, we performed *in vitro* transcription assays with Tu6 nuclear extracts (Fig. 2*B*). Immunodepletion of CREB (lanes 3 and 4) caused a dramatic reduction in somatostatin promoter activity relative to undepleted extract (lanes 1 and 2). Addition of recombinant CREB restored this transcriptional activity (lanes 5 and 6); and recombinant CREB-M1 protein was equipotent in reconstituting expression (lanes 7 and 8). As immunodepletion of Tu6 extract specifically removes CREB without affecting the levels of other CRE binding proteins like ATF2 (Fig. 1*C*), these results further emphasized the importance of CREB in regulating somatostatin expression. Thus other CRE binding proteins do not appear to be capable of supporting somatostatin expression in these cells.

The absence of cell-specific differences in CREB expression, basal phosphorylation, or activity on the minimal CRE-CAT reporter Δ2.5 (Fig. 3 *Lower Left*) prompted us to ask whether other functional elements cooperate with the CRE site to stimulate somatostatin expression in Tu6 cells (Fig. 3). Deletion analysis suggested that, in addition to the CRE site from −56 to −32, sequences from −350 to −71 in the somatostatin promoter were also important for high-level expression. Conversely, insertion of a somatostatin promoter fragment extending from −304 to −71 restored high-level expression to the minimal somatostatin promoter construct Δ2.5 lacking any cell-specific expression in Tu6 cells (Fig. 3 *Lower Left*). Other regions of the somatostatin promoter were unable to reconstitute activity. Hence, they either do

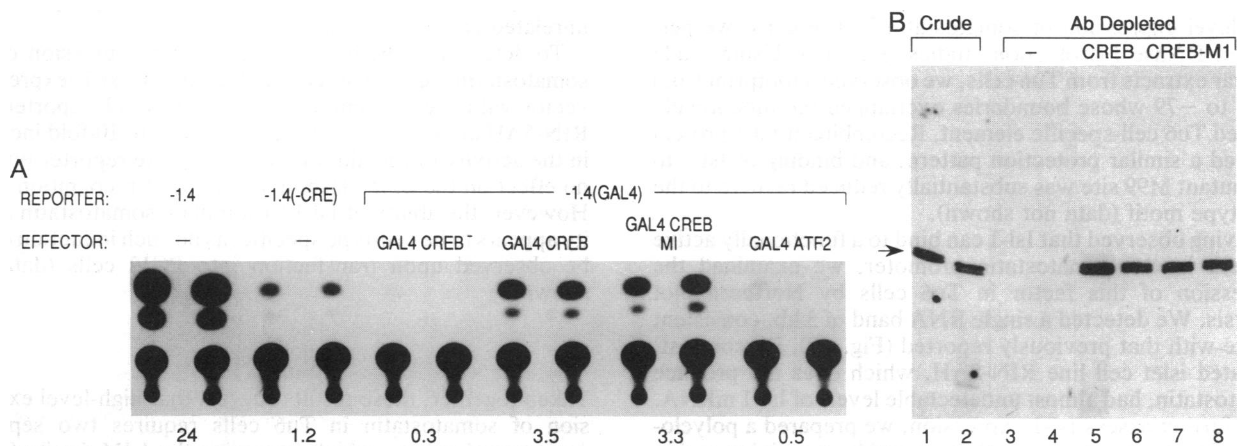

FIG. 2. (*A*) Transient assay of CREB effector and somatostatin reporter vectors in Tu6 cells. Reporter, CAT reporter construct used in each assay; −1.4, wild-type somatostatin reporter vector containing 1.4 kb of promoter sequence; −1.4(−CRE), somatostatin reporter vector with CRE site deleted (see Fig. 1); −1.4(GAL4), 1.4-kb CAT reporter with two copies of the GAL4 recognition site inserted in place of CRE at −48; Effector, eukaryotic expression vectors; −, no effector plasmid; GAL4 CREB⁻, control simian virus 40 expression plasmid encoding GAL4 DNA binding domain from amino acids 1–147 attached to CREB in the anti-sense orientation; GAL4 CREB, expression plasmid with CREB in the sense orientation; GAL4 CREB-M1, CREB with Ser-133 to Ala-133 missense mutation; GAL4 ATF2, GAL4 DNA binding domain attached to CRE binding protein ATF2/CRE-BP1. Numbers below chromatogram indicate percentage conversion of [^{14}C]chloramphenicol. (*B*) Primer-extension analysis of somatostatin transcription in Tu6 nuclear extracts. Crude, crude Tu6 nuclear extract; Ab depleted, transcriptions performed with immunodepleted extract (lanes 3–8); CREB and CREB M1, recombinant proteins (0.5 μg) added to immunodepleted extract. Arrow points to expected size (55 bases) of primer-extended product.

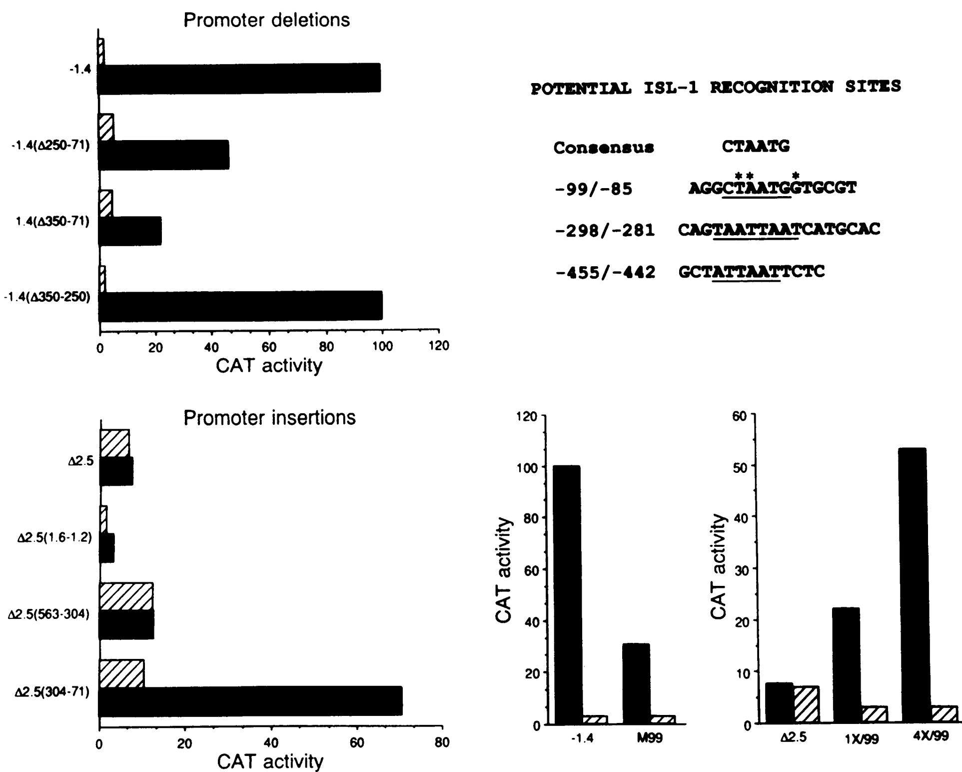

FIG. 3. Analysis of somatostatin promoter activity in PC12 and Tu6 cells. (*Upper Left*) Deletional analysis of somatostatin promoter. Bar graph shows CAT activity of somatostatin promoter constructs in PC12 (hatched bars) and Tu6 cells (solid bars). CAT activity for each construct is expressed as percentage wild-type −1.4CAT construct (100%). Numbers following Δ indicate promoter sequences deleted from −1.4CAT reporter. (*Lower Left*) Insertional analysis of somatostatin promoter activity. Promoter sequences inserted into minimal Δ2.5 reporter vector are indicated in parentheses. (*Upper Right*) Potential Isl-1 recognition sites in the somatostatin promoter. Consensus recognition site from insulin promoter is shown in boldface. Numbers indicate 5′ to 3′ position of sequences. Sequences containing Isl-1 motif are underlined. (*Lower Right*) Activity of −99 Isl-1 motif. M99, −1.4CAT vector with Isl-1 site at −99 mutated at residues indicated by asterisk (above). 1X/99, minimal Δ2.5 reporter with single Isl-1 site inserted; 4X/99, four Isl-1 sites inserted. Activities are expressed as percentage −1.4CAT reporter.

not mediate cell-specific expression or are functionally dependent on additional elements not contained within those fragments.

Analysis of promoter sequences spanning the −304 to −71 segment revealed two consensus recognition sites (CTAATG) for the islet-specific factor Isl-1 (5) (Fig. 3 *Upper Right*). To assess the importance of these motifs, we mutagenized the proximal −99 Isl-1 site and observed a 70% reduction in cell-specific activity (Fig. 3 *Lower Right*). Insertion of a single Isl-1 motif into the Δ2.5 construct enhanced promoter activity 5- to 10-fold in Tu6 cells, and multimerizing the Isl-1 site further increased activity to nearly 30-fold in Tu6 over PC12 cells.

To determine whether Isl-1 might indeed mediate the high-level expression of somatostatin in Tu6 cells, we performed DNase I protection studies (Fig. 4*A*). Using crude nuclear extracts from Tu6 cells, we observed a footprint from −105 to −79 whose boundaries overlapped the functionally defined Tu6 cell-specific element. Recombinant Isl-1 protein showed a similar protection pattern, and binding of Isl-1 to the mutant M99 site was substantially reduced relative to the wild-type motif (data not shown).

Having observed that Isl-1 can bind to a functionally active element in the somatostatin promoter, we examined the expression of this factor in Tu6 cells by Northern blot analysis. We detected a single RNA band of 3 kb, consistent in size with that previously reported (Fig. 4*B*). By contrast, a related islet cell line RIN-5AH, which does not produce somatostatin, had almost undetectable levels of Isl-1 mRNA.

To further assess Isl-1 expression, we prepared a polyclonal antiserum against a synthetic peptide containing amino acids 112–126 of the predicted Isl-1 protein sequence (Fig. 4*D*). This antiserum recognized a 47-kDa protein in bacterial extracts expressing recombinant Isl-1 protein from a pET-Isl expression vector. Antibody recognition was completely blocked by preadsorbing the antiserum with synthetic Isl-1 peptide (Fig. 4, lanes 5–7). Western blot analysis of Tu6 nuclear extracts with the Isl-1 antiserum revealed two immunoreactive bands of similar molecular mass (47 kDa). The smaller of these appears to comigrate with the bacterial Isl-1 band, suggesting that the larger species may represent a modified form of the protein.

Surprisingly, PC12 extracts also expressed an Isl-like species, which was ≈5 kDa smaller than either the recombinant or the Tu6 proteins. To determine whether the difference in migration might arise from proteolytic degradation or other artifacts, we compared CREB expression in the same extracts by Western blot analysis. Indeed, both Tu6 and PC12 extracts contained comparable amounts of CREB, which migrated at the same molecular mass (Fig. 4*D*, lanes 8 and 9). Moreover, differences in Isl-1-like species have been observed with several different extracts, suggesting that PC12 and Tu6 cells express different forms of the protein. Alternatively, the species detected in PC12 cells may represent an unrelated cross-reacting protein.

To determine whether Isl-1 can direct expression of the somatostatin gene, we cotransfected an Isl-1 expression vector with the multimerized 4X/(−99) CAT reporter into RIN-5AH cells (Fig. 4*C*). Isl-1 caused a 5- to 10-fold increase in the activity of the multimerized Isl-1 site reporter but had no effect on the Δ2.5 plasmid lacking Isl-1 recognition sites. However, the ability of Isl-1 to stimulate somatostatin activity appears to be cell-type specific, as no such induction could be observed upon transfection into PC12 cells (data not shown).

DISCUSSION

Taken together, these results suggest that high-level expression of somatostatin in Tu6 cells requires two separate promoter elements, which recognize the LIM family factor Isl-1 and CREB, respectively. LIM family members have been characterized by the presence of a cysteine-rich motif near their N terminus (18). At least three of these members—lin-11, mec-3, and Isl-1—also contain a homeodomain (5, 18) and hence constitute a distinct class of presumptive transcriptional regulators. While Isl-1 was identified by virtue of its ability to bind to specific sequences on the insulin promoter, our data provide evidence that Isl-1 functions as a transcriptional activator. Furthermore, the inability of an

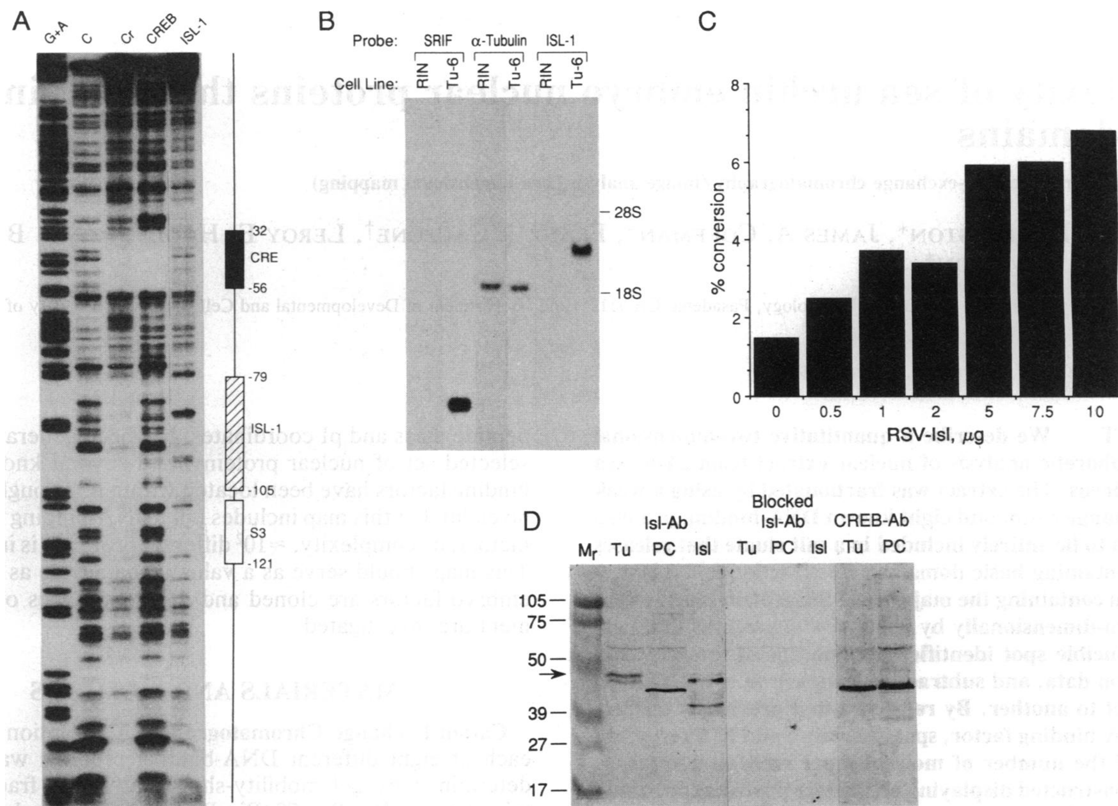

FIG. 4. Analysis of Isl-1 expression and activity on somatostatin promoter. (*A*) DNase I protection assay of somatostatin promoter fragment. Protein incubated with end-labeled DNA fragment is indicated above each lane. G+A, size marker; C, no extract control; Cr, crude nuclear extract from Tu6 cells; CREB, recombinant CREB protein; ISL-1, recombinant Isl-1 protein. Schematic shown alongside indicates regions protected on somatostatin promoter. Isl-1, Isl-1 recognition site; S3 additional footprinting activity in Tu6 extracts not corresponding to functional activity by CAT assay. (*B*) Comparison of somatostatin (SRIF) and Isl-1 mRNA levels in RIN-5AH and Tu6 cells. Probe and RNA source are indicated above each lane. α-Tubulin probe was used as internal standard. Positions of 18S and 28S rRNAs are shown on right. (*C*) Bar graph showing activity (percentage conversion) of 4X/99 CAT reporter in RIN-5AH cells when cotransfected with increasing amounts of RSV-Isl-1 effector plasmid. Amount of effector plasmid used is indicated in μg. (*D*) Western blot analysis of Isl-1 and CREB expression in Tu6 and PC12 cells. Lanes: 1, molecular size marker with relative mass indicated in kDa; 2–4, PC12, Tu6, and recombinant Isl-1 protein, respectively, incubated with Isl-1 antiserum; 5–7, extracts incubated with "blocked" Isl-1 antiserum preadsorbed with synthetic Isl-1 peptide used as immunogen; 8 and 9, Tu6 and PC12 extracts incubated with CREB antiserum 244. Arrow points to 47-kDa immunoreactive band in Tu6 and recombinant bacterial extracts.

Isl-1 expression vector to regulate reporter activity in PC12 cells suggests that additional islet cell factors may be required for somatostatin expression.

Although Isl-1 activity would appear to be critical for somatostatin expression in Tu6 cells, it does not appear to be sufficient. Deletion of the CRE, for example, severely reduces promoter activity. Our data suggest that CREB also functions importantly in this process without requirement for phosphorylation at the regulated PKA site. The mechanism underlying somatostatin expression in Tu6 cells may thus be most readily explained by synergistic interactions between CREB and Isl-1 proteins. Moreover, the inability of ATF2 to direct somatostatin expression relative to CREB in these cells suggests that CREB may provide some specific activity that is essential for Isl-1 function.

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