Identification of the promoter sequences involved in the cell specific expression of the rat somatostatin gene

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ABSTRACT

DNA sequences containing the 5' flanking region of the rat somatostatin gene were linked to the coding sequence of the bacterial chloramphenicol acetyl transferase gene. This recombinant plasmid is active in expressing CAT activity in the neuronally derived, somatostatin producing CA-77 cell line. Deletion analyses of the somatostatin promoter show that the sequences proximal to position -60, relative to the cap site are required for expression of this promoter. A 4 base pair deletion of residues -46 through -43 within the somatostatin promoter results in a down mutation in vivo suggesting the existence of an element critical for the expression of the promoter in CA-77 cells. In addition, the somatostatin recombinant and its 5' deletion constructs preferentially express CAT activity in CA-77 cells, whereas only basal level of expression is observed in HeLa, BSC40, and RIN-5F cell lines, pointing to the cell specific nature of this promoter.

INTRODUCTION

The expression of some eukaryotic genes is remarkably cell specific. A single gene can be actively transcribed in one cell type and be almost totally silent in another. The reasons for this cell type specificity are only now beginning to be understood (1, 2). Accumulating evidence suggests that for many genes the critical regulatory step in gene expression is the rate of transcription initiation (3). Studies with a number of eukaryotic and viral promoters have elucidated some of the sequence elements that contribute to transcriptional initiation events. The "TATA" and "CAAT" box sequences present in numerous genes at approximately positions -30 and -80, respectively, appear to be important but nonsufficient elements, determining the precise start site and, to some degree, the frequency of initiation of transcription (4, 5). Studies on the SV40 early (6) and HSV TK promoters (7, 8) have identified regions between -50 and -100 which are essential for full promoter activity. These regions contain several copies of the sequence GGGGCC, known as the GC-rich box (9, 10). It has been shown that a cellular protein, Spl (11), specifically interacts with the GC box (12). GC-rich

sequences have been found in the promoter sequences of a number of cellular
genes (13-18).

Another class of transcriptional control elements known as enhancer
sequences have been identified in a number of viral as well as cellular
promoters (19). Enhancer sequences act in cis to potentiate transcription of
adjacent promoters. Enhancers can also contribute cell-specific expression as
is evident from the polyoma enhancer which functions in differentiated but not
undifferentiated embryonal carcinoma (EC) cells (20). In addition, the
immunoglobulin heavy chain enhancer stimulates transcription of the adjacent
promoter only in cells of B lineage (21-23), and the insulin, chymotrypsin and
elastase enhancers are functional only in the cell types which normally are
active in the expression of the respective promoter (24, 24).

We have focused our attention on genes which are selectively expressed
in neuronal and endocrine cells (26-31). The gene encoding the 14 residue
peptide somatostatin is selectively expressed in brain (32), as well as
endocrine cells of the pancreas (33) and gastrointestinal tract (34, 35). A
chimeric plasmid containing the 5' flanking region of the somatostatin gene
linked to a "reporter" gene, chloramphenicol acetyltransferase (CAT) (36) was
introduced into neuronally derived and non-neuronally derived cell lines and
expression of CAT activity was measured. The manuscript describes experiments
which determine the elements required for tissue specific expression of the
rat somatostatin gene.

MATERIALS AND METHODS

Cell lines: HeLa cells, BSC40, and RIN-5F (37) cells were all maintained
in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf
serum. The BSC40 cell line was derived from monkey kidney cells. RIN-5F is a
rat pancreatic insulinoma cell line which expresses the endogeneous insulin
but not the somatostatin gene (37). The CA-77 cell line originated from a rat
medullary thyroid carcinoma and expresses the endogenous somatostatin gene
(26). It is maintained in defined medium, composed of 1:1 DMEM:F-10,
supplemented with 5 µg/ml transferrin, 10 µg/ml insulin and 3 X 10^-8 M
selenous acid. During passage of the CA-77 cells, the plating medium is
composed of 1:1 DMEM:F-10 and is supplemented with 5% horse serum and 2.5%
fetal calf serum.

Plasmid constructions.

Plasmid pBXA-750 was constructed by cloning an 800 b.p. fragment derived
from the somatostatin gene 5' upstream sequences, corresponding to bases -750
to +50, in front of the bacterial chloramphenicol acetyl transferase gene.

The CAT gene was subcloned from plasmid pSV2CAT into PBR322 in a way that two unique sites, HindIII and BamHI exist upstream from the CAT coding sequence, resulting in plasmid PCATB'. PCATB' is a promoterless CAT plasmid, containing the poly(A) splice site and poly(A) addition site of SV40 at the 3' end of the CAT gene sequence. The 800 b.p. somatostatin fragment (-750 to +50) was obtained from a lambda phage subclone, pRAHE5 described in ref. (29). A unique XbaI site located at +50 in pRAHE5 was converted to a BamHI site. The fragment of interest (-750 to +50) was obtained as a HindIII-BamHI fragment and cloned between the unique HindIII and BamHI sites of the plasmid harboring the CAT gene.

Deletion mutants were constructed by two methods. The plasmids pBXA-250 and pBXA-70 were made by utilizing the restriction sites SalI (-250) and BglII (-70), respectively. Deletion mutants were also obtained by Bal31 nuclease treatment of plasmid PBXA-750, linearized at the HindIII site. After Bal31 nuclease treatment, a HindIII linker was added at the endpoint of the deletion followed by digestion with BamHI, a site that exists at +50. The HindIII-BamHI fragment was isolated from a 1% agarose gel and cloned into the unique HindIII-BamHI sites of the CAT gene containing plasmid, PCATB'. This method ensures that the same PBR322 sequences are flanking the endpoint of each deletion. The endpoint of key deletions was identified by sequencing, using the dideoxy method (38). The name of each plasmid is indicative of the number of nucleotides present, relative to the transcriptional cap site.

Plasmid PBXSSTA4, containing a 4bp deletion of residues -46 to -43 of the somatostatin promoter was constructed in a two step cloning, utilizing plasmid PBXSST-t. This plasmid was constructed by cloning the HindIII-PvuII fragment of PBXA-750, containing the 5' flanking region of the rat somatostatin gene linked to the 5' portion of the CAT gene (PvuII site), into the HindIII-DRAI sites of PBR322. PBXSST-t has lost the ampicillin resistance gene and contains a unique Aat2 restriction enzyme site at position -47 of the rat somatostatin promoter. Plasmid PBXSST-t was digested with Aat2, treated with S1 nuclease and religated. Removal of four residues, -46 to -43, results in the formation of a PstI site. The HindIII-BamHI fragment of the deletion construct was cloned in front of the CAT gene as described earlier, resulting in plasmid PBXSSTA4.

Eukaryotic Cell Transfections and CAT Assays.

Plasmid DNA was transfected into CA-77 and HeLa cells by a modification of the method of Potter et al. (39). Cells (4 x 10^6 cells/ml) were placed in
a sterile 1 ml disposable cuvette in 1 X HBS buffer, containing 140 mM NaCl, 3 mM KCl, 0.7 mM Na$_2$HPO$_4$, 20 mM Hepes pH 6.0, 0.2% dextrose and 20 µg/ml of plasmid DNA and incubated on ice for about 5-10 min. Platinum electrodes were inserted into the cuvette and joined to a set of capacitors which could deliver 300 Volts with a decay time of 45 msecs. After discharge, the cells were kept on ice for 10 min. and then plated directly, in media containing serum. The cells were harvested 48 hrs later and assayed for CAT activity.

HeLa, BSC40 and RIN-5F cells were transfected with 20 µg of CsCl purified plasmid DNA using the CaPO$_4$ coprecipitation technique (40). The cells were transfected at 20-40% confluency in 100 mm dishes. Four hours after addition of the DNA the cells were subjected to 20% glycerol treatment for 2 min and then placed in medium containing serum. Cells were harvested 48 hrs. after addition of the DNA and extracts were assayed for CAT activity according to the method of Gorman et al. (36). A modification of the Gorman protocol involved treatment of the extract with 5mM EDTA and a heat inactivation step at 60°C for 10 min (41). The heat inactivation treatment of the cellular extract utilized for the measurement of CAT activity, improved the amount of conversion of $^{14}$C-acetylated chloramphenicol, especially for CA cell extracts.

**Primer Extension Analysis.**

Messenger RNA transcription initiation site analysis was performed with total RNA isolated from CA-77 cells 48 hours after electroporation of plasmid PBXΔ-750. Cytoplasmic RNA was prepared by lysing the cells in 0.5% NP-40 buffer followed by phenol-chloroform extractions (42). A single stranded oligonucleotide of sequence 5' CAACG GTGGT ATATC CAGTG 3', complementary to nucleotides 15-34 of the coding sequence of the CAT gene (43) was end labeled using [$\gamma$-$^{32}$P]ATP (7,000 Ci mmol$^{-1}$ 1CN) and T$_4$ polynucleotide kinase (NEN). Approximately 2ng of labeled primer (specific activity of $10^8$cpm/µg) were mixed with 80µg of RNA from transfected cells in 30µl of 10mM Tris pH 8.0, 100mM NaCl, 1mM EDTA. The reaction mixture was heated to 70°C, slowly cooled to 55°C and incubated at 55°C for 1hr. The annealed primer and RNA were precipitated with one-tenth volume of 3M sodium acetate and one volume isopropanol, then resuspended in 50mM Tris pH 8.3, 0.14M KCl, 10mM MgCl$_2$, 4mM dithiothreitol containing deoxynucleotides at a final concentration of 1mM each in a final volume of 40µl. 20 units of reverse transcriptase were added and the reaction was allowed to proceed for 1hr at 42°C. After phenol-chloroform extraction, the extension products were analysed on 8% sequencing gels. A parallel sequencing reaction was performed utilizing plasmid PBXΔ-750 and the CAT primer described above, by the dideoxy methods (38).
Figure 1

A. Construction of recombinant PBXΔ-750. Plasmid PBXΔ-750 was constructed from PRSAHE5 a lambda phage subclone, containing 750bp of 5' upstream sequences and the first somatostatin gene exon. The unique XbaI site at +50 in PRSAHE5 was converted to a BamHI and the 800bp HindIII-BamHI fragment was cloned into the unique BamHI, HindIII sites of a PBR322 promoterless CAT plasmid, resulting in plasmid PBXΔ-750.

B. Analysis by primer extension of CAT transcripts in electroporated CA-77 cells. The autoradiogram shown presents data from primer extension using total cytoplasmic RNA prepared from CA-77 cells after electroporation with plasmid PBXΔ-750, performed as described under "Materials and Methods". Lanes indicated A, G, C, T are sequencing ladders of plasmid PBXΔ-750 utilizing the CAT primer. The arrow indicates the start site of transcription.
Nucleic Acids Research

Autoradiography of the primer extended product was for 10 days at -80°C with intensifying screen.

RESULTS

Plasmid Constructions.

In order to determine the sequences necessary for transcriptional activity, the upstream region of the somatostatin gene was cloned in front of the bacterial chloramphenicol acetyl transferase gene as shown in Figure 1A. The resulting plasmid, PBxΔ-750, contains 750 b.p. of upstream sequences, oriented 5' to 3' in front of the CAT structural gene. The transcriptional activity of the somatostatin promoter fragment in construct PBxΔ-750 was examined by introducing this plasmid in CA-77 cells. This cell line, developed by Roos and his colleagues (44), originated from a rat medullary thyroid carcinoma and was previously utilized to isolate somatostatin cDNA

<table>
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<th>Deletions</th>
<th>% Activity in CA-77 cells</th>
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<tr>
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<td>PBxΔ-500</td>
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</tr>
<tr>
<td>PBxΔ-315</td>
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<tr>
<td>PBxΔ-250</td>
<td>94</td>
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<tr>
<td>PBxΔ-101</td>
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<td>PBxΔ-70</td>
<td>94</td>
</tr>
<tr>
<td>PBxΔ-60</td>
<td>100</td>
</tr>
<tr>
<td>PBxΔ-43</td>
<td>4.8</td>
</tr>
</tbody>
</table>

The name of each deletion plasmid is indicative of the number of nucleotides present relative to the cap site. CAT activity was determined according to the method of Gorman et al. (36), following electroporation of the plasmids in CA-77 cells. The relative activities are expressed as a percentage of the activity of the largest fragment tested.
clones (26). The CA-77 cell line provides a good system to study the somatostatin promoter, since it expresses the endogeneous somatostatin gene and it therefore synthesizes factors which might be involved in the expression of this promoter. As shown in Table I, plasmid PBxA-750, when introduced into Ca-77 by electroporation, is active in directing the synthesis of CAT enzyme. Primer extension analysis utilizing a primer to the CAT gene (Figure 1B) shows that the RNA synthesized by plasmid PBxA-750 initiates at the correct position.

**Sequences Required for Expression.**

In order to determine which sequences are necessary for the transcriptional activity of the somatostatin promoter fragment, deletions were constructed in plasmid PBxA-750. Various upstream sequences of the promoter were deleted, maintaining the same vector flanking sequences at the endpoints of the deletions. Plasmid DNA from each deletion was introduced into CA-77 cells and the CAT activity was measured. Table I shows the activity of each deletion plasmid. The results are expressed as a percentage of the activity of the entire promoter fragment. Removal of progressively larger portions of 5' sequences from the somatostatin promoter in constructs PBxA-500, PBxA-420, PBxA-315, PBxA-250 and PBxA-101 did not greatly affect the activity of the remaining promoter fragment. The following deletions are of particular note; PBxA-70, PBxA-60, and PBxA-43. Even though only 70 b.p. of 5' upstream sequences remain in PBxA-70, the transcriptional activity of the fragment is 100%. A GC rich box-like sequence is located at position -65, Figure 2.

Removal of the GC rich box, which is the recognition site for factor Spl, in

![Diagram of the 5' flanking region of the rat somatostatin gene. The stipple boxes indicate the position of the TATAA and CATT homologies. The solid black boxes (A-F) are alternating purine-pyrimidine stretches (31). The lower part shows the nucleotide sequence between position -70 and +1. Numbers indicate position with respect to the initiation site and direction of transcription.](image)
deletion PBXA-60 does not effect the transcriptional activity of the remaining fragment. Transfection with plasmid PBXA-43 resulted in marked decrease in CAT activity, to a level of approximately 4.8%. These studies show that only sequences proximal to -60 are required for transcription of the somatostatin promoter. To confirm these results, we took advantage of a unique Aat2 restriction enzyme site localized at position -47 in the rat somatostatin promoter and obtained a somatostatin deletion construct, PBXSSTΔ4 (Materials and Methods and Fig. 3A). PBXSSTΔ4 is identical in all other respects to PBXA-750, with the exception of the 4bp deletion of residues -46 through -43. Plasmid PBXSSTΔ4 was introduced into CA-77 cells by electroporation. The level of CAT expression is shown in Figure 3B. The 4bp deletion reduced the level of expression to 9% of the wild type promoter. Therefore, within the sequences (-60 to +1) required for expression of the rat somatostatin promoter resides an element critical for expression of this promoter in CA-77 cells.

Cell Specific Expression.

The expression of construct PBXΔ-750 was also examined in non-neuronal cell lines in order to determine whether the activity of the somatostatin

Figure 3

A. Method of construction of a 4bp deletion within the -60 + 1 region of the rat somatostatin promoter (also see Materials and Methods).
B. CAT assays of plasmid PBXΔ-750 (wild type promoter), PBXSSTΔ4 (a 4bp deletion of residues -46 through -43) and PBXΔ-43 after electroporation in CA-77 cells. The percent of chloramphenicol conversion to the acetylated form and percent expression is shown below each lane.
promoter in a transient expression system follows the tissue specific
eexpression observed in vivo. To compensate for the variability of tissue
culture cell lines to take up exogenous DNA we have used the plasmid RSVCAT as
a standard to monitor transfection efficiency. Plasmid RSVCAT contains the
Rous sarcoma virus LTR promoter directing the synthesis of the CAT gene (45).
Since most neuronally derived cell lines such as CA-77 cells are refractory to
the CaPO4 coprecipitation method of transfection (40), we utilized the method
of electroporation (39) as a way of introducing plasmid DNA into CA-77 cells.
In addition, Crabb and Dixon (41) have recently demonstrated that extracts
from a variety of cells are capable of deacetylating acetylchloramphenicol.
The deacetylating activity varies dramatically from cell to cell. The
deacetylating activity can be completely inactivated by heating the cell
extracts to 60°C for 10 minutes in the presence of EDTA. This allows one to
reliably and accurately measure CAT activities in different cell lines. In
order to determine the activity of plasmid PBXA-750 in non-neuronal cell lines
we initially utilized HeLa cells to examine the expression of the somatostatin
promoter. Plasmids RSVCAT and PBXA-750 were introduced into HeLa cells by
electroporation as well as by the CaPO4 coprecipitation methods. The results
shown in Figure 4 were obtained. Plasmid RSVCAT is expressed in both CA-77

Figure 4. Expression of plasmid PBXA-750 in the neuronally derived CA-77 cell
line and HeLa cells. Plasmid PBXA-750 and the control plasmid RSVCAT
were introduced into CA-77 by electroporation. HeLa cells were
transfected by the methods of electroporation (39) and by CaPO4 (40).
Table 2: Comparison of activity of the somatostatin deletion constructs in cell lines of neuronal and non-neuronal origin.

<table>
<thead>
<tr>
<th></th>
<th>PBxΔ-750</th>
<th>PBxΔ-250</th>
<th>PBxΔ-70</th>
<th>PBxΔ-43</th>
<th>RSVCAT</th>
</tr>
</thead>
<tbody>
<tr>
<td>CA-77</td>
<td>15.7</td>
<td>14.3</td>
<td>14.3</td>
<td>0.7</td>
<td>100</td>
</tr>
<tr>
<td>HeLa</td>
<td>0.85</td>
<td>0.21</td>
<td>0.14</td>
<td>0.14</td>
<td>100</td>
</tr>
<tr>
<td>BSC40</td>
<td>1.0</td>
<td>0.6</td>
<td>1.0</td>
<td>0.45</td>
<td>100</td>
</tr>
<tr>
<td>RIN5F</td>
<td>1.1</td>
<td>1.1</td>
<td>1.1</td>
<td>1.1</td>
<td>100</td>
</tr>
</tbody>
</table>

CAT activity of somatostatin promoter deletion constructs and control plasmid RSVCAT, transfected in parallel in various cell types was calculated by counting regions of the TLC in a liquid scintillation counter. Shown above are normalized values of percent conversion of acetylated 14C-chloramphenicol, by setting the expression of control plasmid RSVCAT as 100%.

and HeLa cells. In contrast, the 5' flanking region of the rat somatostatin gene results in significant CAT enzyme expression only in CA-77 cells. The method of introduction of plasmid RSVCAT and PBXΔ-750 into HeLa cells, namely electroporation versus CaPO4 coprecipitation did not affect the expression profile of these two plasmids in HeLa cells. Therefore, the possibility that this difference in the expression of the somatostatin promoter between HeLa and CA-77 cell lines is due to the method of introduction of the plasmid DNA is ruled out.

In order to gain more information on the sequences that mediate specificity, we tested some representative deletions, namely PBXΔ-750, PBXΔ-250, PBXΔ-70, and PBXΔ-43 in four different cell lines, CA-77, HeLa, BSC40, and RIN-5F. A parallel experiment was performed with each cell line, using RSVCAT as a control. Table 2 shows the normalized values of the CAT assays by setting the expression of the RSVCAT control plasmid as 100%. Plasmids PBXΔ-750, PBXΔ-250, and PBXΔ-70 exhibit the highest level of expression in CA-77 cells, while the expression of construct PBXΔ-43 decreases dramatically. In contrast, the level of CAT activity of each somatostatin construct tested in HeLa, BSC40 or RIN-5F cells is within the range of expression of deletion PBXΔ-43. Because all assays are performed under identical condition, the amount of chloramphenicol acetylated gives a direct measure of the strength of the somatostatin promoter fragment tested. This
observation suggests that the 5' flanking sequences of the rat somatostatin promoter (-70 to +1) contains all the cis-acting elements necessary for specific transcription in CA-77 cells. The preferential expression of the somatostatin promoter in CA-77 cells is probably due to a difference in trans-acting factors existing in CA-77 cells but not in HeLa, BSC40 or RIN-5F cells.

DISCUSSION

In the present study we performed deletion analyses of the somatostatin promoter region, in order to define the sequences which are involved in the expression of the somatostatin gene. These promoter deletions constructs were introduced into cell lines of both neuronal and non-neuronal origin. We show that sequences between -750 and -70 of the promoter can be deleted without any appreciable loss of transcriptional activity of the remaining fragments in CA-77 cells. Within the -70 region at position -65 (Figure 2), there exists a GC rich sequence homologous to the GC-rich box. Deletion of this GC-rich sequence, in construct PBXA-60, did not diminish the level of expression, indicating that the GC-rich homology is either nonfunctional or nonessential for the expression of the somatostatin promoter. In contrast, deletion of the sequences between -60 to -43 decreased the activity of the promoter to 4% level of expression. Thus, the upstream border of sequences required for transcription of the somatostatin promoter is located at nucleotide position -60, relative to the transcriptional cap site. In addition, elimination of 4 base pairs between -46 to -43 results in a strong down mutation in vivo. This 4bp deletion disrupts the cAMP consensus sequence 5'-TGACGTCA-3', which is highly conserved in genes that appear to be regulated via the cAMP-pathway.

Montminy, et al., (46) has recently shown that the somatostatin gene contains a cAMP responsive element. In mapping the location of this sequence, they demonstrated that it resides between -70 and -30. The importance of this region in both expression and regulation underscores the importance of defining the details of the specific interactions which must occur within this region.

Although we have determined by deletion analyses that at least some of the sequences necessary for transcription reside between -60 and -40 in the somatostatin promoter, there may be additional sequences within the 5' upstream region which also play important roles in regulating expression. For example, McKnight and Kingsbury showed that most of the nucleotides within the CAAT-like sequence of the TK promoter could be substituted without complete loss of promoter function (7). However, a detailed study demonstrated that
the CAAAT-like sequences of the TK promoter are involved in specific interaction with the CTF factor (47). The deletion analyses of the somatostatin promoter show that the CAAT-box like and GC-rich sequences are located outside of the transcriptionally important region of the promoter. The explanation for this observation is either that the CAAT-like and GC-rich box-like sequences of the somatostatin promoter are not functional homologues of the CAAT-box and GC-rich box or that they are nonessential for transcription of the somatostatin promoter.

A similar rationale might also explain the absence of an effect of the sequences in the somatostatin promoter which have the potential for forming extensive Z DNA structures (31). These sequences located at -670 to -300 are evolutionarily conserved with respect to both position and length. Removal of these sequences has no affect on the transcriptional activity of the remaining somatostatin promoter fragments.

Transient expression of the somatostatin deletion constructs in non-neuronal cell lines such as HeLa, BSC40 and RIN-5F resulted only in basal level of expression. The mode of introducing the DNA into HeLa cells, either by electroporation or CaPO4 coprecipitation did not alter the expression profile of the plasmids tested. Thus, the level of expression of the somatostatin promoter construct is at least 15 fold higher in CA-77 cells than in HeLa, BSC40 or RIN-5F cells, suggesting that expression of this promoter is cell type specific. Since only basal level of expression is observed with all deletions tested in the non-neuronal cell types, the element rendering the promoter cell type specific must be localized within the -70 to -43 region.

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