Characterization of the 5′-flanking promoter region of the rat somatostatin receptor subtype 3 gene

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Abstract We investigated the 5′-flanking promoter region of the rat somatostatin receptor subtype 3 (rSSTR3). Using a cDNA probe, genomic clones containing the 5′-flanking promoter region of the rSSTR3 gene were isolated. A sequence of 5.4 kb directly upstream from the start codon was analyzed and two introns were found in the 5′ untranslated region (UTR) of the cDNA sequence. The transcriptional initiation site was determined by 5′ rapid amplification of cDNA ends (RACE), primer extension and RNase protection analysis with cerebellar RNA. Two major transcriptional initiation sites were found at position −1040 (tsp1) and −856 (tsp2) relative to the translational initiation site. Like a number of other promoters of G-protein-coupled receptors, the rSSTR3 gene lacks TATA and CAAT motifs and includes G+C-rich regions. Functional analysis of the promoter region by transfecting rSSTR3 luciferase-reporter gene constructs into rat pituitary GH_3 cells and HEK 293 cells indicated that a 107-bp region upstream of tsp2 was sufficient to drive transcription. Furthermore a 562-bp region at position −1304 to −1865 upstream of the ATG start codon exerted a negative regulatory effect on transcriptional activity.

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Key words: Somatostatin; Promoter; 5′ Untranslated region; 5′ Rapid amplification of cDNA ends

1. Introduction

Many neurotransmitter receptors occur in numerous different subtypes or isoforms, which frequently exhibit only marginal differences in terms of receptor function. This is particularly true for the five somatostatin receptors (SSTR1–5), which show very similar patterns in coupling to effector molecules like adenylyl cyclase or G-protein gated inwardly rectifying potassium channels [1,2]. One of the main differences between receptor subtypes appears to be the spatial and temporal pattern of expression, which may enable different cell types to express SSTRs at the appropriate time during development.

SSTR subtypes 1–5 are expressed in the mammalian central nervous system, and it has been shown that the spatial and temporal expression pattern of SSTRs in the CNS is highly subtype specific [3]. Thus, in the cerebellum SSTR1 is mostly observed in fetal and neonatal rats, whereas SSTR3 is the major subtype detected in the cerebellum of adult animals [4]. SSTRs are also highly expressed in various tumors derived from neuroendocrine tissues; hormone secretion from these tumors can be inhibited by treatment with several stable peptide analogues of SST, and this constitutes a major clinical use of somatostatin and its analogues. The regulation of SSTR expression is therefore of clinical importance, as the effect of SST analogue treatment on the expression levels of individual SSTR subtypes in these tumors may determine the efficiency of the long-term SST treatment.

Limited information is available on the regulation of SSTR expression; the expression of SSTR1 in the pituitary GH_3 cell line has been shown to be regulated by the transcription factor Pit1 (Baumeister, Wegner, Richter and Meyerhof, manuscript submitted). The mouse SSTR2 gene is subject to alternative splicing in its 5′-untranslated region, giving rise to three different mRNA species that are regulated by three different promoters [5]. A common feature among the SSTR subtypes that have been analyzed so far (rSSTR1, mSSTR4, hSSTR5) is the lack of TATA and CAAT sequences and the presence of G+G-rich regions in the 5′-flanking promoter region [6–8].

Here we report a structural and functional analysis of the 5′-flanking region of the gene for the rat SSTR3. The highest mRNA levels for this receptor have been detected in the hippocampus and the cerebellum of adult animals, whereas the mRNA is absent from fetal tissues [4,9]. Conflicting results have been obtained with respect to the presence of SSTR3 in the pituitary, one of the main targets of somatostatin released from the hypothalamus. We have shown previously that SSTR3 mRNA is present in the GH_3 pituitary tumor cell line, though the main SSTR subtype expressed in these cells is SSTR1 [6]. In order to characterize the SSTR3 gene, we have determined its transcriptional start site using mRNA isolated from rat cerebellum and from the GH_3 cell line. A functional analysis of the putative promoter region was performed by transfection of luciferase reporter constructs in GH_3 cells.

2. Materials and methods

2.1. Isolation of genomic clones

A rat genomic library in lambda DASH II (Stratagene) was screened by standard protocols [10] using a [α-^{32}P]-labelled fragment consisting of the first 450 bases of the published rSSTR3 cDNA sequence [4]. One positive clone, λ-19, was analyzed by restriction mapping and Southern blot hybridization. *Eco*RI fragments were subcloned into pBluescript II SK$^+$ (Stratagene) and sequenced.

2.2. RNA preparation

Total RNA was isolated from adult rat cerebelli and GH_3 cells using the guanidine isothiocyanate/cesium chloride extraction method [10]. Poly(A)$^+$ RNA was selected using oligo(dT)-latex particles (Oligotex mRNA Kit, Qiagen, Hilden, Germany) according to the manufacturer's instructions.

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Abbreviations: HEK, human embryonic kidney; NRSE, neuronal restrictive silencing element; RACE, rapid amplification of cDNA ends; SST, somatostatin; SSTR, somatostatin receptor; UTR, untranslated region

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2.3. Rapid amplification of cDNA ends (5′-RACE)

cDNA was transcribed using murine Moloney leukemia virus-reverse transcriptase (USB, Cleveland, OH, USA) and the specific oligonucleotide 5′-TCTTCAGATGGCACCCA-3′ which is complementary to nucleotides −472 to −456 relative to the translational initiation site of the SSTR3 mRNA. Twenty μg of total rat cerebellar RNA was used in the synthesis reaction. Further tailing carried out using terminal deoxynucleotide transferase (Pharmacia, Freiburg, Germany) as described [11], followed by a first amplification step in the presence of Taq polymerase, the non-specific oligonucleotide Ri-Ro(dT) and the specific oligonucleotide 5′-CAGAAGCTTCTTC-CAAAGATGACAGAAATG-3′ (Ra1), complementary to nucleotides −484 to −513 of the SSTR3 cDNA. In the second amplification step, the two specific oligonucleotides 5′-AGAGGATC-CGAAAAAGGCAGGGACAGAGGG-3′ (Rax), complementary to −718 to −747 and 5′-TGGGGATCCTGACAGAGAAGGATTGA-GCCA-5′ (Ra4), complementary to −777 to −806 were used as specific primers together with the non-specific primer Ri [11]. Products were analyzed by Southern blotting with ^{32}P-end-labelled specific oligonucleotides after electrophoresis in 1% agarose gels and subcloned into pBluescript II Sk$^+$ for sequence analysis.

2.4. Primer extension

Primer extension was performed essentially as described [10] using up to 10 μg poly(A)$^+$ RNA isolated from either rat cerebelli or GH_3 cells. Yeast tRNA was used as a negative control. ^{32}P-end-labelled antisense oligonucleotides complementary to nucleotides −718 to −747 (Rax), −777 to −806 (Ra4), −884 to −903 5′-GACAGCAG-GTGCCAGACTGA-3′ (PE2), −915 to −944 5′-TTAGGATCCGA-GGCCAGACCGGGAGAGGGA-3′ (Ra5), relative to the translational initiation site of the coding strand were used. Primers and RNA were hybridized at 42°C overnight and extended with MMLV reverse transcriptase (USB). The reaction products were analyzed on a 6% polyacrylamide/50% urea gel.

2.5. RNase protection

RNase protection assays were performed as described [12]. Several genomic regions (−1294 to −1044, −1125 to −777, −1125 to −625) were amplified by PCR and subcloned in pBluescript II Sk$^+$. Labelled antisense RNA was generated using T7 RNA polymerase and [α-^{32}P]CTP following linearization of the construct with *Eco*RI, yielding transcripts that contained complementary sequences to the corresponding regions of the genomic DNA. The RNase protection assays were performed using 25 μg total RNA from cerebellum or 25 μg yeast tRNA. RNAs were hybridized overnight at 45°C. For RNase digest, various concentrations of RNase A and RNase T1 were used. The resulting protected fragments were analyzed on a 6% polyacrylamide gel containing 50% urea. A dideoxy DNA sequencing reaction was run as a size marker, and the difference in migration properties between DNA and RNA was accounted for by calculation using a factor derived from the migration behavior of the unprotected band.

2.6. Cell culture and transfection

Rat pituitary GH_3 tumor cells were cultured in Ham's F10 medium containing 15% horse serum, 2.5% fetal calf serum, 1 mM L-glutamine. Human embryonic kidney (HEK) 293 cells were cultured in DMEM containing 10% fetal calf serum, 2 mM L-glutamine and 0.1 mg/ml penicillin/streptomycin. rSSTR3 luciferase reporter plasmids were generated by inserting the appropriate fragments of the genomic into the multiple cloning site of pGL3Basic (Promega, Madison, WI, USA).

400 μl of cell suspension at a density of 5×10^6–1×10^7 cells/ml were transfected with 10 μg pGL3-Basic or equivalent amounts of rSSTR3 promoter/pGL3-Basic constructs by electroporation (BTX Electroporation System, BTX, San Diego, CA, USA). To determine the transfection efficiency, each plate was cotransfected with 5 μg of CMVlacZ vector. The chosen parameters for electroporation were cuvettes with 4-mm gap, 260 V, 800 mF, 720 W.

Cells were harvested 24 h after transfection by scraping and subsequent centrifugation. The cell pellet was washed once with phosphate buffered saline and resuspended in 300 μl lysis buffer (100 mM Tris-acetate, pH 7.8; 10 mM Mg-acetate; 1 mM EDTA; 1% Triton X-100). After sonication, the cell debris was separated from cytoplasmic proteins by centrifugation at 14 000 rpm for 10 min. For luciferase assays, 10 μl cell extract was mixed with 50 μl luciferase assay reagent (Promega). Measurements were performed in a luminometer (MicroLumat LB 96 P/EG&G Berthold). For β-galactosidase assays, 20 μl cell extract was used with *o*-nitrophenyl-β-D-galactopyranoside (Sigma; 0.8 mg/ml final concentration in 0.1 M sodium phosphate, pH 7.5) as substrate. Enzyme activity was detected by measuring light absorbance at 420 nm.

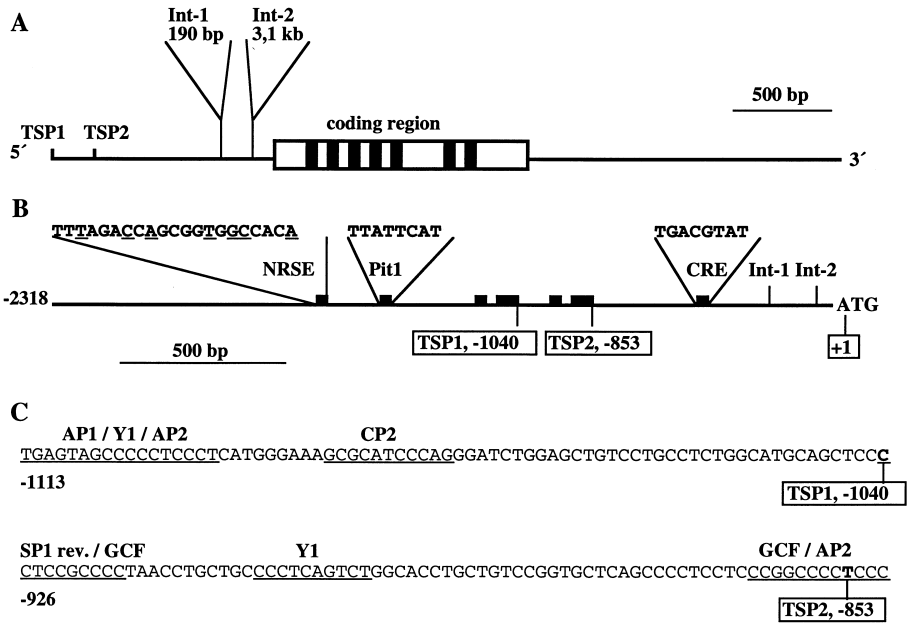

Fig. 1. Structural organization of the rSSTR3 gene. A: Scheme of the rSSTR3 gene showing the location of the two introns (Int-1, Int-2) and the two transcriptional start sites TSP-1 and TSP-2. B: Organization of the 5′ region of the rSSTR3 gene upstream of the translational start codon. The position of consensus sites for several transcription factors is indicated by thick bars. In the case of the neuronal restrictive silencing element (NRSE), deviations detected in the SSTR3 gene are underlined. C: Partial sequence upstream of the two transcriptional start sites showing the location of consensus sequences for the transcription factors AP1, Y1, AP2, CP2, Sp1 and GCF.

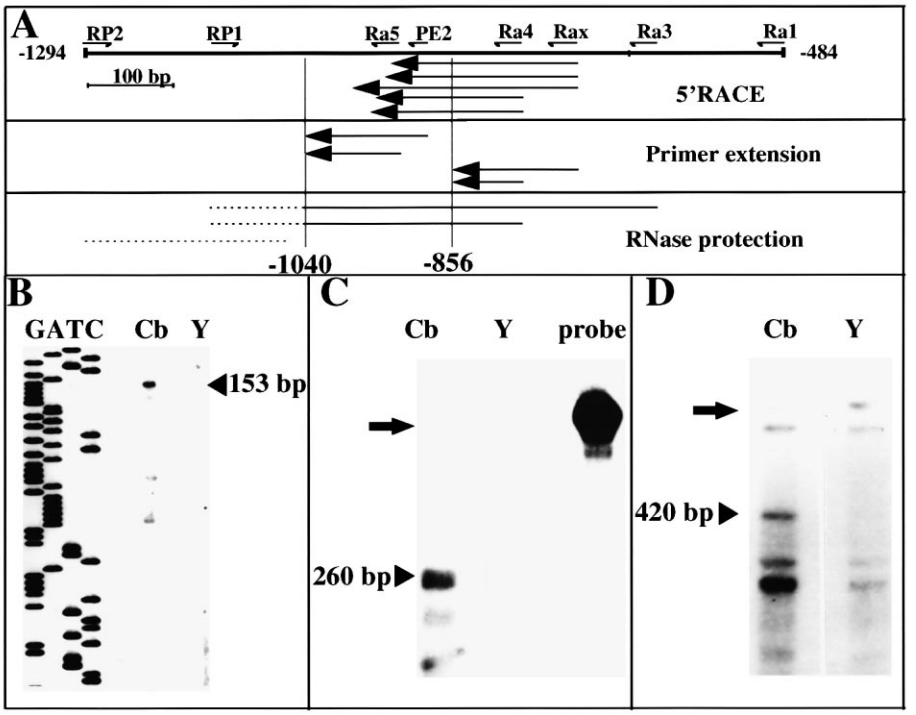

Fig. 2. Analysis of the transcriptional start site of the SSTR3 gene. A: Summary of the results obtained by 5′-RACE, primer extension and RNase protection. 5′-RACE and primer extension products are indicated by arrows; in the lower panel the probes used for RNase protection experiments are indicated; the non-protected parts are indicated by dotted lines, the protected parts by solid lines. The oligonucleotides used for primer extension, 5′-RACE and the generation of probes for RNase protection are shown on top. The two major transcriptional start sites at −856 and −1040 are indicated by vertical lines. B: Primer extension using the primer PE2 performed on poly(A)$^+$ RNA isolated from rat cerebellum (Cb) and yeast tRNA (Y). The size of the transcript was determined by a sequencing reaction performed on a plasmid containing the genomic clone using the same primer PE2. The major transcript of 153 bp is indicated by an arrow. C: RNase protection was performed using cerebellar total RNA (Cb) or yeast tRNA (Y); as probe a fragment of the genomic clone corresponding to position −1125 to −777 was used. The concentration of RNase A was 40 mg/ml, the concentration of RNaseT1 was 300 units/ml. In lane P the undigested probe was loaded. The arrowhead indicates the position of the specifically protected band, the arrow marks the undigested probe. D: A fragment corresponding to position −1125 to −625 was used. The concentration of RNase T1 was 200 units/ml. Experimental conditions and figure legends are otherwise identical to C.

3. Results

3.1. Structure of the rSSTR3 gene

By screening a rate genomic library with a fragment corresponding to the 5′-end of the known cDNA for rSSTR3, 4 positive clones were identified. Two *Eco*RI fragments (3.4 kb and 6.1 kb) of one of these clones contained the 5′-flanking sequence and the coding region of the rSSTR3 gene. The structure of the 5′-flanking sequence of the gene is shown in Fig. 1. By comparison of the published cDNA [4] and the genomic sequence reported here, two introns were identified in the 5′-UTR. The size of the first intron is 187 bp, the size of the second intron is about 3.1 kb, 2.1 kb of which were sequenced.

Similar to the promoter regions of the other SSTRs [6–8], the sequence upstream of the translational start site of the SSTR3 gene contains neither a canonical TATA box nor a CAAT box.

3.2. Determination of the transcriptional start site

The known cDNA sequence of SSTR3 has a length of 3.9 kb, whereas the SSTR3 mRNA is detected at about 4.4 kb in Northern blot analysis [4]. As the difference of 500 bp cannot be accounted for by the poly(A) tail alone, we attempted to determine the transcriptional start site of the SSTR3 gene. First we employed the 5′-RACE technique to approach the 5′-end of the mRNA derived from adult rat cerebellum. Upon sequence analysis several RACE products were identified (see Fig. 2) which were colinear with the genomic sequence, adding roughly 300 bp to the known mRNA sequence. Based on this sequence information, several primers were built to be used in primer extension analysis. Primer PE2 yielded a major specific band corresponding to a transcriptional start site at position −1040 (Fig. 2A; the numbering used from now on is relative to the ATG start codon and does not count the two introns). This start site was also found when the primer Ra5 was used. A second putative transcriptional start site was detected with primers Rax and Ra4 at a distance of 856 bases from the start codon. Both start sites were also detected when the primer extension experiment was performed on poly(A)$^+$ RNA isolated from GH_3 cells (not shown).

To investigate if further introns were present in this region of the genomic clone, we performed RNAse protection analysis with cerebellar total RNA using ^{32}P-labelled RNA probes transcribed in vitro from fragments of the genomic clones cloned into pBluescript. A probe encompassing the range from position −1125 to −777 yielded a specifically protected band of approx. 260 bp (Fig. 2C). This is in agreement with a transcriptional start site at position −1040. A second probe, ranging from position −1125 to −625 on the genomic sequence, protected a fragment of 420 bp (Fig. 2D). This result again fits to a start site at position −1040. No specifically

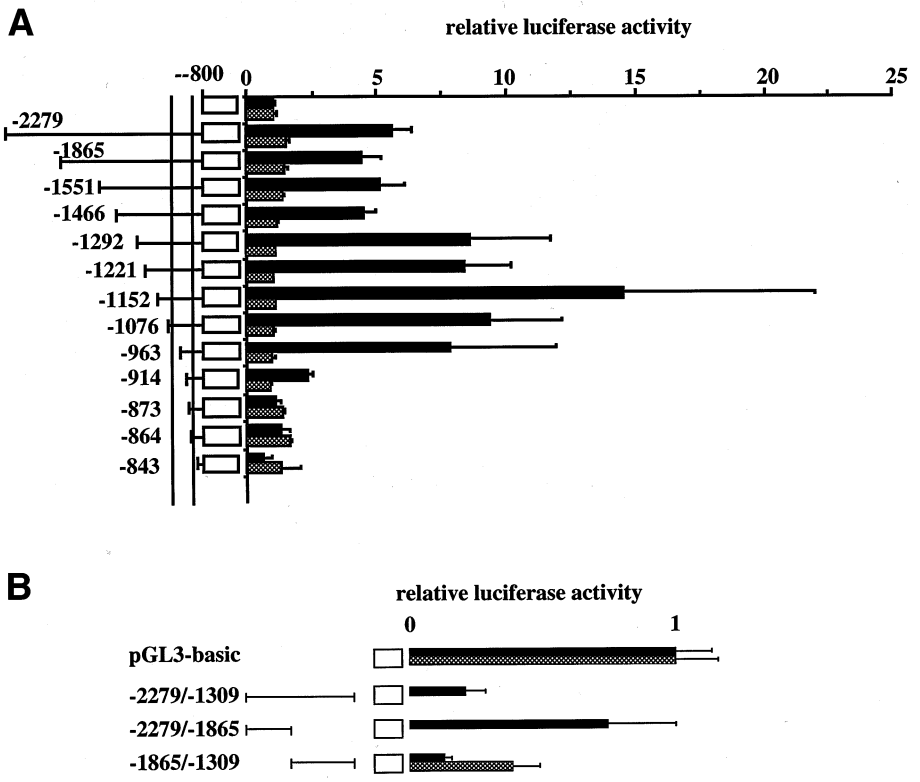

Fig. 3. Functional analysis of the SSTR3 promoter. A: Stepwise truncation from the 5′-end of the promoter sequence. Promoter constructs in the luciferase reporter gene vector pGL3-basic were transfected into GH_3 (filled bars) or HEK cells (open bars) together with a CMV-lacZ construct. Luciferase activity was normalized to the β-galactosidase activity and is shown as the relative increase when compared to results obtained with the control vector pGL3-basic. The numbers indicate the 5′-ends of the constructs; the 3′-end is at position −800 in all cases. The two vertical lines indicate the positions of the two transcriptional start sites at positions −1040 and −856. B: Identification of a repressing activity in the 5′-end of the promoter. Experimental conditions were identical to A. The numbers indicate the 5′- and 3′-ends of the constructs used. Experiments using the constructs −2279/−1309 and −2279/−1865 were performed on GH_3 cells only.

protected bands were observed with a fragment containing sequence from position −1294 to −1044 upstream to the first starting point at position −1040, confirming that sequence upstream to this point is not included in the messenger RNA (data not shown). Adding the 385 bp of the 5′ sequence identified here to the 3881 bp of the published rat SSTR3 mRNA sequence and considering a poly(A) tail of about 200 residues, the resulting total length of the mRNA closely matches the length of 4400 bp that has been observed in Northern blot analysis [4].

3.3. Functional analysis of the rat SSTR3 promoter

A functional analysis of the SSTR3 promoter region was performed by transient transfection of luciferase reporter gene constructs into GH_3 cells. This pituitary tumor cell line does express endogenous SSTR3, albeit at a low level. The dominant somatostatin receptor subtypes in this cell line are SSTR1 and SSTR2 [6,13]. Nevertheless we could detect the SSTR3 transcriptional start sites using poly(A)$^+$ RNA isolated from GH_3 cells (see above).

Transfection of the luciferase gene under the control of a 1.2-kb fragment which included the putative promoter region upstream of the transcriptional start sites into GH_3 cells led to a 5-fold increase in luciferase activity in cell extracts, when compared to cells transfected with the luciferase vector without an insert. This increase was specific to GH_3 cells as it was not observed upon transfection into HEK 293 cells (Fig. 3A) which do not express endogenous SSTR3. Further truncation of this construct from the 5′-end had no effect on promoter activity until a position at −1292 was reached. With this construct a further increase in luciferase activity was observed, which was again not paralleled in HEK293 cells. The highest promoter activity was observed with a fragment ranging from position −1152 to −800. A significant reduction in activity was then observed when the 5′-flanking region was shortened from position −963 to −914. Thus a 107-bp fragment (−963 to −856) immediately upstream of the second transcriptional start site at position −856 is sufficient to drive transcription of the rSSTR3 gene in GH_3 cells.

A second set of experiments was performed with constructs in which the 5′-UTR was not truncated at position −800 but at position −200. In these experiments rSSTR3 promoter activity was also GH3-cell specific, but it became apparent that additional regulatory elements might be present in the 5′-UTR of the gene between −856 and −200, as the promoter activity did not decrease to control levels when these constructs were truncated beyond the second transcriptional start site at position −856 (data not shown).

From our data it appears that the SSTR3 promoter activity is restricted by elements in the 5′ part of the investigated sequence. To analyze this further, several constructs were generated which contained only parts of this region linked to a promoterless luciferase gene. In cell extracts of GH3 cells transfected with these constructs a reduced luciferase activity

was observed when compared to control cells transfected with the empty vector, consistent with a negative regulatory effect of the 5′ region of the promoter (Fig. 3B). The strongest inhibitory effect, a reduction to 10% of the basal level determined in control cells, was observed with the region from −1865 to −1309; this part of the sequence was also effective in HEK cells, leading to a reduction of the promoter activity to 40% of basal values (Fig. 3B).

4. Discussion

The data presented here provide the first analysis of the 5′-regulatory region of the SSTR3 gene; two features become apparaent from this analysis that distinguish rSSTR3 from the other SSTRs: first, the 5′-UTR is by far the longest among those SSTR genes characterized so far [6,14,15]. Secondly, SSTR3 contains two introns upstream of its translational start codon which were identified by comparing the cDNA sequence with that of the genomic clone. A similar observation was made only with the mouse SSTR2 cDNA, which contains two small exons at its 5′-end which are separated by large introns of about 25 kb [5]. Whereas these give rise to differentially spliced isoforms, no signs of alternative splicing were detected during sequence analysis of the SSTR3 cDNA [4].

Our functional analysis of the SSTR3 promoter region clearly shows that the sequence upstream of the second transcriptional start site at position −856 confers cell type specific expression of the receptor. Thus, while the promoter is functional in GH_3 cells, which express endogenous SSTR3 at moderate levels, no promoter activity was observed in HEK cells which are devoid of endogenous SSTRs. In addition, a factor from GH_3 (and not HEK) nuclear extracts specifically binds to the promoter region upstream of the second transcriptional start site which is sufficient to initiate transcription in GH_3 cells (data not shown). So far the identity of this tissue-specific transcription factor for SSTR3 has not been resolved. Although consensus sequences for the transcription factors Sp1, GCF and Y1 have been identified, these factors are rather ubiquitously expressed and are therefore not likely to be involved in the tissue-specific regulation of SSTR3.

We detected one consensus sequence for the transcription factor Pit-1 in the rSSTR3 promoter (−1394 to −1387). Pit-1 is responsible for the GH_3-cell specific expression of the rSSTR1 gene (Baumeister, Wegner, Richter and Meyerhof, manuscript submitted), but it is obviously not required for expression of rSSTR3 in the same cells as it can be deleted without any effect on promoter activity. This further illustrates the differential regulation of SSTR gene expression in one particular cell type.

The 5′ region of the investigated genomic clones appears to suppress SSTR3 promoter activity. This inhibitory effect became evident upon linkage of this region to the pGL3 basic vector and transfection into GH_3 cells. One possible explanation is the occurrence of a sequence (−1607 to −1587) which bears similarity to a consensus sequence for a neuronal restrictive silencer element [16]. Binding of a nuclear protein (which is not expressed in neurons) to this sequence restricts gene expression in non-neuronal tissues. This mechanism might be responsible for the rather low expression of rSSTR3 in GH3 cells.

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