Alternative promoter usage and tissue specific expression of the mouse somatostatin receptor 2 gene

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Abstract We have cloned the 5′ upstream regulatory region of the mouse somatostatin receptor 2 gene. Its genomic organization is novel among all somatostatin receptor genes. It contains two previously unrecognized exons, separated by introns larger than 25 kb, and three tissue and cell specific alternative promoters. The first promoter in front of exon 1 is active only in AtT-20 tumor cells. The second promoter, located 5′ to exon 2, is used in brain, pituitary, adrenals, pancreas, NG 108-15 and AtT-20 cells. Furthermore, it contains putative DNA elements for regulation by glucocorticoids, estradiol and cAMP. A third promoter, located in exon 3, is additionally used in lung, kidney and spleen.

Key words: Somatostatin receptor 2; Alternative promoter usage; Tissue specific gene expression

1. Introduction

Somatostatin 14 and its N-terminally extended form somatostatin 28 are widely distributed throughout the central nervous system and peripheral tissues. They act as neurotransmitters or neuromodulators, and in particular as inhibitors of neurotransmitter release and endocrine and exocrine secretion (for a review see [1]). The multiple physiological effects of somatostatin are known to be mediated via five distinct types of G protein coupled somatostatin receptors (sst1–sst5) [2–10]. A further degree of genetic diversification of ssts has been reported for the subtype 2, which is alternatively spliced to generate the two isoforms sst2A and sst2B with different C-termini [11,12]. Sst2 is involved in inhibition of growth hormone release from the pituitary [13], pancreatic glucagon secretion [14] and gastric acid secretion from stomach [15]. At the cellular level, sst2 mediates somatostatin evoked inhibition of cAMP accumulation by coupling to adenylyl cyclase via G_i proteins [10,16]. As shown more recently, sst2 also couples to a tyrosine phosphatase, which has been proposed to be involved in inhibition of tumor cell growth observed after clinical administration of somatostatin analogs such as octreotide [17,18]. Little is known about the genomic structure of sst2 and sst genes in general and the molecular mechanisms controlling transcription in the various tissues. In fact, only genomic sequences immediately adjacent to the coding regions of sst genes have been the subject of investigation to date [19–23]. Here, we report the cloning of the 5′ upstream regulatory region (5′ URR) of the mouse sst2 gene. We provide evidence for the existence of two previously unrecognized exons and introns and three individual, alternative promoters located within 50 kb upstream of the protein coding region. In RT-PCR experiments the usage of the three promoters was examined in various tissues and cell types.

2. Materials and methods

2.1. Cloning and mapping of genomic fragments of sst2

The exonic sequences of sst2 are contained on two independent bacteriophage P1 clones of a mouse embryonic stem cell library (Genome Systems, St. Louis, MO, USA). From P1 clone #24 exon 1 was obtained, while P1 clone #88 contained exons 2–4. For subcloning and mapping P1 DNA was first characterized by restriction enzyme analysis and Southern blot analysis. Then, appropriate fragments were subcloned in pBluesript SK^- (Stratagene, Heidelberg, Germany) according to standard methods [24]. As hybridization probes served randomly ^{32}P-dATP (Hartmann, Braunschweig) labeled fragments of a sst2B cDNA [11]. To determine the nucleotide sequence overlapping inserts of subclones were sequenced on both strands by the dideoxy chain termination method using the Sequenase II kit (USB, Braunschweig, Germany). The size of the first intron could not be determined, since exons 1 and 2 are contained on different, non-overlapping P1 genomic library clones. As revealed by hybridization, exon 1 is located at a distance of at least 15 kb from the 3′ end of the P1 clone #24 insert, and exon 2 at least 10 kb from the 5′ end of the P1 clone #88 insert. The first intron thus must be larger than 25 kb. The size of intron B was determined by hybridization of DNA isolated from P1 clone #88 with probes for the adjacent exons 2 and 3. A *Not*I fragment of about 40 kb contained both exons, whereas various fragments up to 25 kb in size obtained by digestion of P1 DNA with other restriction enzymes only contained either exon 2 or exon 3. The size of intron B is thus between 25 and 40 kb.

2.2. Primer extension experiments

Primer extension reactions were preformed with 1 μg poly(A)$^+$ RNA (RNeasy and Oligotex mRNA, Qiagen) and 750 nM oligonucleotide primer (sequences see Fig. 2). The antisense DNA was extended with 5 units Tth polymerase (Boehringer, Mannheim, Germany) under conditions suggested by the manufacturer with additionally 10 μCi α-^{32}P-ATP. Cycling conditions were 30 s at 95°C, 30 s at 60°C and 1 min at 75°C for 30 cycles. The products were analyzed on a 8% denaturing polyacrylamide gel.

2.3. RT-PCR experiments

Total RNA was extracted from various mouse tissues using RNeasy columns (Qiagen). cDNA was transcribed with random hexamer and oligo dT primers and Superscript II RT (Gibco BRL). Nucleotide sequences of primers were as follows: P1 upstream primer: 5′-GCTGGAGGTAGTCATTGAGCTT-3′, P2 upstream primer: 5′-CCCGGGCAAGCTCTCTCA-3′, P3 upstream primer: 5′-AGGAAGATCTCTAGGCAGCTTG-3′, exon 3 downstream primer: 5′-TGATGGTCTTCATCTTGGCGT-3′. PCR was performed under the following conditions: 30 cycles of 1 min at 96°C, 1 min at 55°C and 2 min at 72°C. To check the integrity of the RNA/cDNA a 605 bp sequence of the glycerine aldehyde phosphate dehydrogenase gene (GAPDH) was amplified from the same preparations for 25 cycles of 1 min at 96°C, 1 min at 60°C and 3 min at 72°C. Primers were: upstream 5′-TGGCAGCTTTCTCCAGGCGGC-3′ and downstream 5′-CCCACGGCAAGTTCAACGGCA-3′. PCR products were electrophoresed on 1% agarose gels, blotted and hybridized under stringent conditions (5×SSC, 20% formamide; o/n at 63°C) with a randomly labeled radioactive probe. The washed blots (0.2×SSC, 1%

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                                                                  YY1
   1 ATGAACCTCC AACCAATCGT CTCATTCTTG GTTTGGACTG AAAGCCCATG GTAATTTAGA AAAGATACTA TAACCTGAGG
                                                                                    ERE
  81 ACAAATCAAG ACCAGAAAAT GAAAACATTT AGAGTACATG AGCAGATTTT TTTTTTGTCT TAATATATTT CCAATACAGT
                                                                                YY1
 161 CATTCAGCAA ACAGAAACAA GTAACAACAA CAGAAATCAG ACAAAGCAAC TGGAACTGCC AGAAAACACT GACCTTAAAA
 241 GTGCTAGTGC GTTCTCTCAA AGGACTTAGC ACTCTCGGTT TGCACAGGAA GTTCTAATAC CTTATTACTT TGTCTAATAA
 321 TCAGGTAATA AAAAAGGTCT GAGACACTCT TGGGACCCTA GACACACAGA AATGAGAGCA GGAAAAGACG GCAAGGTCAG
 401 CCAAGTCGGC AAGCTCCAGC TCTCTGCTCG AAGGCGTCGG AACCTTAGAA GAGGCAAGGA GACTGGCCTG TCTGCCTACA
                                                                                YY1
 481 GGAGGCATTT AACTGCTTCT GCCAAGCAGG GTCTTATAAA AATGACAGGA AGCCAAGAAC AAGTCCATAC CTGACAAATA
 561 ACGACAATCG AGATGGTGAT GTCAGGCCCA ATAAAGCATT TTGGCAAGTT AAGAATGTGC ACCTGTGAGA ACTGAGGACG
 641 ACTTGACAGA CTCCTGTTTC CCTGGCTCTT TCTCGGCAAA CTAATCGTGG TGACTGAAGA GTCTACCCTG AGCTTTACAG
 721 ATGCACGGAT CCAACAACTG CTGCTCTCCT GGGCATCAGG AAGACAAAAC GACAGCGGTG CTTACATGCA GAAGTATTCT
 801 GCGCACGATG GAACAATGAG CCAGAGAGGA TTTAGATGGG GGAGAGGGGT GCTCTGCAGA TATACTGGTG AGACCGGCCA
                                  GRE
 881 GGAAAGGAAA GGACACCCAA CACACAGACA CGTGTGTGCT TACTCATCCT CAGAGCCTCA GAGGCCTCTG GCCACACTGC
                                  GRE
 961 CATCGAGCAT GCTTTCCCCC AGAGCAGACT GTACCTTCGC AGCTATAGCT GTACACTGCC ACGGCTGACC TGTCCACCAG
                                  YY1
1041 GTTCTCATCG TGATGCCAGC TCACCGCCAT CTTCCCCATG CCGAAATAGG GCTCCTCTTT CAAGTAGGGC ATCTTCTGAG
1121 GATCCATGAA GTTTAGCAAA GTCACGTTGT AGGCTGCTCT GCTCTTAAGG TCCACTTCAT CATCACAGGA CGGCCCCACG
1201 CCGGCCCTGG GGAACTCTGC CATGCACAGT GGCACAGCAT CTTCATTGGC CTTCTCTCTG ACAGCCAGTT CTTCCAAGGC
     •
1281 TGGATGGTCT CCAGCTGGAG GTAGTCATTG AGCTTTAGGA AGGTCTGACA TGCAGCGGCG ATCTCAGCCT CTGTGTACTT
1361 GACAGTGCAC CCTTCACGGG CCAGGGCACC GTGAGAGTCT GGTGTTCAAG TACTTGTAGG TGCAGCCTGG GTCCCCGATG
1441 AGGATGCGAG ATACTGGGGT GAGCACATCT TTGCCTTGGA TCCTCACCAC GTCCCGAAAC AAGCAGCCAT GCTTATCGAG
1521 TGTGAGAAAG GCCTCGGGCA CCTCCTTATG CAGCTCCTCT GGTATGCTGC CGGCCTCTCG GAAAACCAGT TTAGGGTATT
1601 TCAGCTGCCA CTAAAATAAA ACCAATGAGG GGGAGACAGg tacacttggc tatcg....Intron A (>25 kb).....
1656 aactgggagc tcaattaacc ctcactaaag ggagtcgact cgatcaaaaa caggatggct gcatcaggac ccacagagaa
1736 agctaatcgg ggggtttgtt ttctgatttt tcatgttcta gaaaccacca gggtgcactc actgtctctg tctgtctgtc
1816 tgtctgtctg tctctgcccc tctctctctc tctctctctc ccctctctct ctcctctctc tcctctctct ctctctctct
1896 ctctctctgg gggagtgtta agaggtgaag gaagggaggc tacaggattg attatgactt attaaactat tggaacatcg
1976 gctgaatcat cacagtcaaa aaataaatag ctgcaacttg atgcgacaca tctcccactt cttgcgagtc cctccttgct
2056 cccgcagagc gccacagcgc gcgtaggcgg aacggttggg ctggggctgg gtcagaccag caagcagtga ctgacgggct
2136 aaattaaagc tagaagagac tgcatggccc ggggcctcca gttgggagag cggtggagcc cgcggagcct gctggctgca
2216 ggaactgaag ccaggtgccc cgcgcatgca cacctgcagg cgcctgcacg ggagacacgc ttgctgctgt cacccctttt
                                  GRE
2296 tcctctgccc ttagcctgac tctgcaacct gtgccctgtt ccccggactg gaccgtggta ggcacggctt tgcaaacttg
2376 agaacacgtc ttaaaggtcc cggagctagt ctaggtcacc caagtcgccg caccagctgc ctggaatttg ttgggtaaaa
2456 ccagaccgag tcactgaaat cggaaagaaa gccacgcgcg ccctcatagg gaggagtcgt ggagacactg acatcgcttc
2536 accatccaac agcatcaagg tctgctgtct tatcctggcc acccgcaggt tttctccact ctgctcaccg gtccaacagg
                                  CRE
2616 acccggcagg cggagccaag ctgccgtgac gtcacgggcg ggggcgtggg gaagtgtgcc caggggctgc cacccgcccg
                                                                                    ERE
2696 ggccactgct agcggcgcag ccaccggcgc gctcgcgagg ccaccggcgc ctggagcacc agtccgcggc tgggcgtcaa
                                  •
2776 tgatctacag gccaagctag ctctctcggg gcccgggcaa gctctctcag acgccagaag ggccagcacg agccagCCAA
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2856 **GGAAGCTGGG AGCCCCGGGA GCCACTGCTG AGCGCCAAAC ACCCGCTAAG CCTGCTGTGC TCCTGGACGC CCAGCTGGGT**

2936 **AAAAGAACAG CTTCTGGGTG CTAGAGAACA CAGGGAAGCC AAGTGCTCGA GGAAAACCAA G**gtgatgggg tcca.....

3010*Intron B (25-40 kb)...* gagctccagg ctagctcagc tcaaggaaga aggaggaggg gaaagggagg agggagagag

3070 gggaggagga ggaggaagaa gagaggagga agaagaagga agagagggag tgatgtggga gggaaagaaa agaaggaagg

3150 agggaggaag agggggagat ggctccgtca ataaagcacc tgacttgcaa gcaagaagac ctgagttcaa cctccagaac

3230 cacataaaac tgccggtata tttgtataca tatctgtaat cacagccgtg ggggaggcag acacaggtgg atctctgcag

3310 ctcactgaac agctagccca agctacatgg tgaaacatca tgactcaaaa aagggtgagg ctatcaaacc tatgtaaaca

3390 cacacacaca aacacacaca tacaccatgc agcagcacac atgaatacat gaaaaaaaaa gaggaattgg gggggcatgg

3470 aacgtgaaac taagaagatg aacaagaaac agagggagcc taagtagcct gggactagac ggctactttt tctgcttctt

3550 ttcccag**ATG TCACAGTAGA CTCTTGGCCC CCAGAGCCCT GTGAGGCGAG AGGAAGATCT CTAGGCAGCT TGGTTCTAGA**

3630 **CGGAGTGGAA AGCAGCCATG GAGATGAGCT CTGAGCAGTT**

M E M S S E Q ...

Fig. 1. (*Continued*). Nucleotide sequences of the 5′ region of the mouse sst2 gene. The sequences of exons are shown in bold, intronic sequences are shown in lower case letters. Putative binding sites for several transcription factors are also shown in bold. The dots above indicate transcription initiation sites. Both 5′ splice sites follow the AG/GT rule. The 3′ splice site of intron A has the classical NYAG/ but lacks the pyrimidine-rich stretch in front. The 3′ splice site of intron B matches perfectly to the consensus sequence (Y)nNYAG/ ('/' is the junction point, N is A, C, G or T, and Y is C or T).

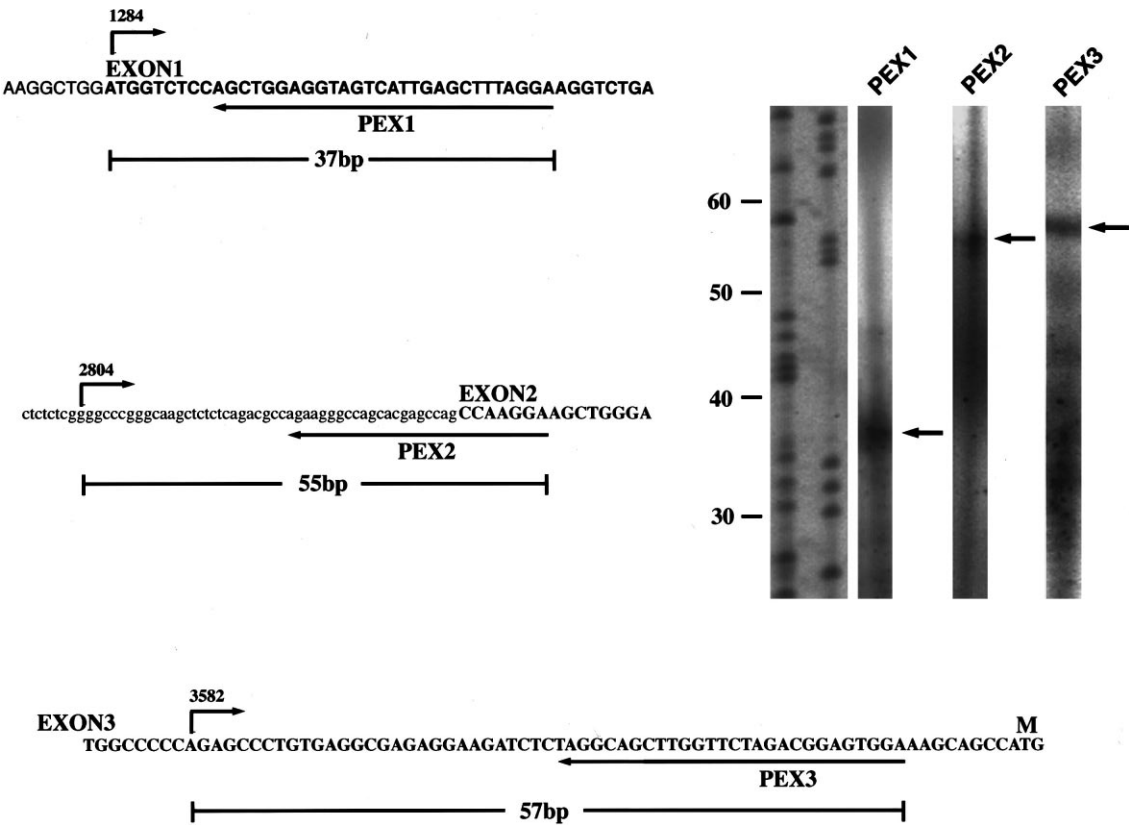

Fig. 2. Determination of transcription initiation sites. Primer extention experiments using poly(A)$^+$ RNA isolated from AtT-20 cells revealed three initiation sites which are depicted schematically along with sequences and primers used (PEX1, PEX2, PEX3). Short arrows indicate the respective extended fragments in the gel aligned to a co-electrophoresed sequencing reaction for size determination (C and G reaction of pBluescript SK$^-$ with an end-labeled M13 reverse primer).

SDS; 2 times 15 min at 68°C) were exposed to Kodak X-OMAT films.

3. Results

3.1. The 5′ upstream region of the mouse sst2 gene is divided by two large introns

We previously reported the cloning of a cDNA encoding the mouse sst2B isoform, which has a long 5′ untranslated region, containing 590 bp upstream of the first ATG codon [11]. Using fragments of this cDNA as probes genomic sequences of the upstream region were subcloned from two P1 library clones prepared from mouse 129/SvJ embryonic stem cells (Genome Systems, St. Louis, MO, USA). The nucleotide sequences (EMBL Nucleotide Sequence Database, accession numbers AJ005518, AJ005519, AJ005520) are shown in Fig. 1, Fig. 3A shows the upstream region of the gene schematically. The 5′ URR of the sst2 gene comprises three exons, separated by two large introns: the first exon consists of 356 bp (nt 1284–1639). It is separated from exon 2 by at least 25 kb intronic sequences (intron A). Exon 2, which consists of 144 bp (nt 2852–2996) follows intron B, which is 25–40 kb in size. The sequences encoding the receptor protein start in exon 3 (1083 bp, nt 3557 onwards). The exon-intron junctions are similar to consensus splice sites [25]. Sequence analysis of the 5′ flanking regions of the first three exons revealed several motifs similar to binding sites for transcription factors which typically occur in promoters (see Fig. 1): a classical cAMP response element (CRE) [26], several putative responsive elements for glucocorticoids and estrogen (GRE [27], ERE [28], respectively) and putative binding sites for Yin Yang 1 (YY1)[29]. However, no classical core promoter elements such as TATA or CAAT boxes are present in the sst2 gene sequences.

3.2. The sst2 gene has three distinct transcription initiation sites

To determine the transcription initiation site(s) of the mouse STR2 gene primer extension experiments were performed, which are shown in Fig. 2. The start of exon 1 is defined by an initiation site located at nt 1284. Additionally, a second initiation site is located 48 bp 5′ to exon 2 (nt 2804) and a third transcription start site is located at the 26th bp of exon 3 (nt 3582). These multiple initiation sites suggest the existence of three individual promoters in the sst2 gene.

3.3. The three sst2 promoters are used cell and tissue specifically

Next, the transcriptional activity of these promoters was tested. For the RT-PCR experiments cDNAs were prepared from various mouse tissues and two cell lines. A fragment of

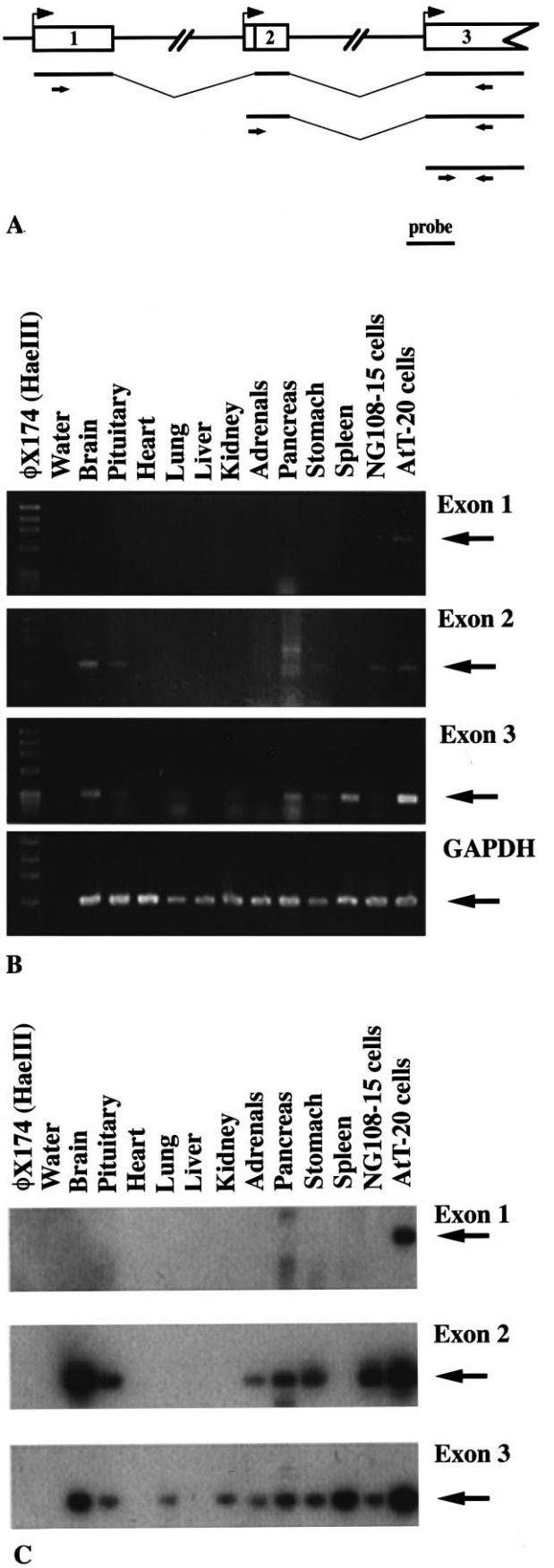

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Fig. 3. Tissue and cell dependent usage of the sst2 gene promoters. A: Rationale of the experiments. One common primer located in exon 3 was used as the 3′ downstream primer in combination with three different 5′ upstream primers. Note that the specific upstream primer for promoter 2 transcripts is located within the intron, so that only transcript starting here and not spliced mRNA species starting in exon 1 are recognized. The upstream primer for the promoter 3 transcripts is located within exon 3. B: RT-PCR with cDNAs of various tissues and cells. Products of the three primer pairs specific for the sst2 promoters, P1 (810 bp), P2 (500 bp) and Pc (279 bp), and GAPDH control (605 bp) are indicated by arrows. C: Hybridization of the gels with a sst2 probe indicated in A.

the ubiquitous GAPDH gene cDNA was amplified as a control. The results of the experiments are depicted in Fig. 3. Fig. 3A shows the rationale of the experiments schematically with the location of the different primers used. The result of a typical experiment is shown in Fig. 3B. The specificity of the PCR products was demonstrated by hybridization of the bands with a radioactively labeled probe specific for exon 3 sequences (Fig. 3C). The experiments show that the three promoters are used in a tissue and cell specific manner: a PCR product of the first promoter was only found in cDNA generated from mouse pituitary AtT-20 cells. Specific PCR products from the second promoter located in front of exon 2 were detected in tissues of brain, pituitary, adrenals, pancreas and stomach, in NG 108-15 neuroblastoma×glioma hybrid cells and AtT-20 cells. In addition to the cells and tissues which contain P2 promoter transcripts, cDNA species starting exclusively from the third promoter were found in lung, kidney and spleen. No sst2 transcripts were detected in heart and liver. To demonstrate that these products were not amplified merely from incomplete spliced transcripts we performed additional RT-PCR reactions with primers located 5′ of the three determined transcription initiation sites (nt 1226, 2756 and 3508). As expected, use of these upstream primers together with the common 3′ primer did not result in PCR products (data not shown).

4. Discussion

Our results provide a more complete picture of the structure of the sst2 gene. The gene covers a range of at least 50 kb on the genome. The 5′ URR comprises two exons and two long introns. The N-terminal part of the receptor protein including all of the transmembrane domains is coded by exon 3. A fourth short exon is critical for the generation of the two receptor variants sst2A and sst2B as reported earlier [11]. Interestingly, the gene contains three transcription initiation sites, arranged in such a way that transcripts may start at the 5′ border of each of the first three exons. Consistent with the assumption that the gene then contains three promoters, a number of typical promoter elements are present within the sequences preceding the initiation sites: three putative binding sites for factor YY1 are present within the sequences in front of exon 1. This transcription factor is currently recognized as an important regulator protein whose name reflects its ambivalent function to serve in many cases as a repressor of the transcriptional machinery but also as a transcriptional activator under certain conditions [29]. The presence of such binding sites may explain why the first promoter is silent in most tissues. As shown earlier sst2 mRNA levels are also regulated by glucocorticoids and estradiol [30,31]. Potential response elements for such regulation are present on the first and second promoters. The second promoter is active in tissues in which somatostatin has essential physiological importance such as brain, pituitary and pancreas. This promoter also contains a classical CRE. It was observed earlier that sst2 mRNA is upregulated by agents that elevate the intracellular cAMP level [32], which may be accomplished via this element. Together, these observations suggest an important physiological role of the second promoter. Currently, the activities of all promoters in transfected cells and the putative transcription factor interactions are being investigated in our laboratory. Recently, a promoter was identified immediately upstream of the sst2 protein coding region in the human gene. It contains an initiator element that binds a novel transcription factor and a TC-rich sequence, which was discussed as a possible transcription factor binding site [23]. The human sequences harboring these elements are highly homologous to the mouse gene. Using RNase protection the authors localized a transcriptional start site in the human gene which corresponds almost exactly to the intron B/exon 3 junction of the mouse gene. The TC-rich sequence in the human gene corresponds in the mouse gene to the pyrimidine-rich stretch typically occurring at 3′ splice sites. The high homology to the murine splice junction includes the possibility that the human gene may also contain additional upstream exons, which is currently being tested by us. In the mouse gene transcripts start 26 bp within exon 3. Transcripts originating exclusively from this third promoter were found in lung, kidney and spleen (Fig. 3). Due to the start site of transcription of the third promoter within the exon the upstream primer P3 will always also recognize spliced mRNA species derived from exons 1 and 2. Thus, the question whether the third promoter is additionally active in those cells and tissues in which one of the other promoters is used or only in lung, kidney and spleen cannot be answered by RT-PCR and requires further investigation, e.g. the use of promoter-transgenic animals. Another novel feature is the observation of sst2 expression in lung. Previous studies performed with rat tissue showed only expression of sst4 in lung [19]. On the other hand, sst2 has been repeatedly found to be expressed in human lung carcinoma cells [33,34].

In the last few years the use of multiple promoters has been shown to be a frequently used mechanism creating diversity and flexibility in gene expression (for a review see [35]). Alternative transcripts of one gene can be generated that either are differentially regulated in different tissues [36] or developmental stages [37], or respond in different ways to various stimuli [38]. We have shown for the first time that a somatostatin receptor gene belongs to this group of genes featuring a complex 5′ untranslated region and tissue dependent alternative promoter usage. Further investigations will reveal whether the other sst types have similar genomic structures.

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