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Transcriptional control of human steroid sulfatase

Alessia Nardi[a], Elena Pomari[a], Doriano Zambon[b], Paola Belvedere[a], Lorenzo Colombo[a], Luisa Dalla Valle[a,*]

[a] Comparative Endocrinology Laboratory, Department of Biology, University of Padova, Via U. Bassi 58/B, 35131 Padova, Italy
[b] Orthopaedic Clinic, University of Padova, Via Giustiniani 3, 35128 Padova, Italy

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ABSTRACT

Steroid sulfatase (STS) is a membrane-bound microsomal enzyme that hydrolyzes various alkyl and aryl steroid sulfates, leading to the *in situ* formation of biologically active hormones. The entire human STS gene spans over approximately 200 kbp of which the first 100 kbp include the regulatory region, while the STS-coding region is located downstream. Previous studies indicated that STS expression, in different human tissues, could be regulated by at least six different promoters associated with alternative first exons. Here, we describe two new splicing patterns: the first, found in the prostatic cell line PC3, is based upon a partially coding new first exon (0d) that is spliced to a new second exon (1e). The second variant was found in the ovary and it is characterized by the novel splicing of the untranslated exon 0b to exon 0c, which is then spliced to the common exon 1b. We also report the results of a multiplex ligation-dependent probe amplification (RT-MLPA) analysis for the simultaneous detection, in qualitative and/or semi-quantitative terms, of the transcription patterns of *STS* in different tissues.

1. Introduction

Steroid sulfatase (STS) is a membrane-bound microsomal enzyme that hydrolyzes biologically inactive steroid sulfates, such as dehydroepiandrosterone sulfate (DHEA-S), estrone sulfate (E1-S) and cholesterol sulfate, to active steroids. This enzyme is a member of a superfamily comprising 12 different mammalian sulfatases [1]. The human STS gene is located on the distal short arm of the X chromosome, maps in Xp22.3-Xpter and has been initially cloned, sequenced and characterized from placental RNA [2]. STS is believed to be virtually ubiquitous in small quantities; its presence has been well established by immunohistochemistry, biochemical analysis or RT-PCR of its mRNAs in placenta and breast, but also in skin, liver, lung, ovary, adrenal gland and brain [3].

In the mouse, STS has been demonstrated to be important in many developmental pathways, such as embryogenesis and differentiation of skeletal system, as well as in neurosteroid synthesis and immune response [4].

Tissue STS levels in humans can vary under different physiological conditions, but also between normal and cancerous samples. In malignant breast tissue, STS mRNA expression was found to be significantly higher than in normal tissue [5], consistently with the higher STS activity detected in pathological samples [6]. This finding suggested that STS could play a significant role in supporting the growth of hormone-dependent tumors and there is now abundant evidence that the hydrolysis of E1-S to estrone (E1) is the main source of estrogens in tumors [6–8]. Actually, expression of STS has been shown to be a strong prognostic factor for disease-free survival in breast cancer [9] and in ovarian cell adenocarcinoma [10]. Similarly, in human prostate cancer, STS is considered significant not only for *in situ* androgen production by freeing DHEA from DHEA-S [11,12], but also for intra-tumoral estrogen production.

Information about the molecular regulation of STS transcription is still limited. Recently, by means of 5′-RACE analysis, six different promoters were found to drive *STS* expression, giving rise to transcripts with unique first exons [13,14]. These exons are distributed along 117 kbp of the 5′-flanking region of the gene and all of them, except exon 1d that was found only in one tissue, vary in length due to the occurrence of multiple transcription start sites (TSSs). Only exons 1a, associated with the promoter that drives expression in placenta, and 1b, that presents the broadest tissue distribution, are partially coding with putative ATGs in frame with another ATG in exon 2. Four alternatively spliced STS transcripts that differ at their first exons were also identified in MCF-7 mammary epithelial cells by Zaichuk et al. [15]. These transcripts, that correspond to those previously identified [13,14], were shown to have distinct expression patterns in human tissues and in different categories of breast tumors, suggesting cell-type-specific co-regulation.

In this work, we report two additional alternatively spliced transcripts in a human tissue and cell line. Moreover, we set up a

* Corresponding author. Tel.: +39 049 8276188; fax: +39 049 8276199.
E-mail address: luisa.dallavalle@unipd.it (L. Dalla Valle).

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Table 1
Primer sequences used in the RT-PCR, 5′-RACE and RT-MLPA analyses.

Target	Primer		Sequences	Position	GenBank no.
RT-PCR expression analyses					
STS coding region	STS-1	Sense	5′-CTGACTTCTGTCACCACCCT-3′	637 → 656	J04964
STS coding region	STS-2	Antisense	5′-GTCCATGTTGCTAGTGGGCT-3′	1409 → 1390	J04964
β-ACTINA	Q-Beta-1	Sense	5′-CACCAACTGGGACGACATGGAG-3′	303 → 324	BC013380
β-ACTINA	Q-Beta-2	Antisense	5′-GGCCTGGATGGCCACGTACAT-3′	409 → 469	BC013380
5′-RACE analysis					
STS coding region	STS-4	Antisense	5′-AGGGTCAGGATTAGGGCTGCT-3′	889 → 869	J04964
STS coding region	STS-6	Antisense	5′-AGGTGCTGAGTGAGTTTCACT-3′	409 → 389	J04964
STS coding region	STS-10	Antisense	5′-CAGTAGGAGGAAAGGGATCT-3′	242 → 223	J04964
RT-MLPA					
STS coding region	STS-14	Antisense	5′-AGGGTGGTGACAGAAGTCAGTC-3′	656 → 635	J04964
STS coding region	STS-16	Antisense	5′-CTTGATGCTGCGTGGCTCTC-3′	280 → 261	J04964
Exon 1b	STS-1bR	Antisense	5′-GTGCAGGTTCAGCAACTGAG-3′	60 → 40	AM282552
B2M	β-2-microgl.	Antisense	5′-TGACTTTCCATTCTCTGCTTG-3′	120 → 102	AY187687

multiplex ligation-dependent amplification procedure (RT-MLPA) to obtain a fast and overall picture of the STS transcriptional patterns in different tissues with at least semi-quantitative indications of the most expressed STS variants.

2. Materials and methods

2.1. Collection of human tissues and RNA extraction

Anonymous human biopsy materials (listed in Table 5 with results of RT-MLPA) were taken from surgically removed organs following national and institutional ethical guidelines and with full patient consent and review board approval. When available, normal tissue samples were excised as far distant as possible from the tumor or pathological area. Human prostate cancer adherent cell lines LNCaP, DU145 and PC3, were cultured in RPMI 1640 medium, supplemented with 5% fetal calf serum and 2 mM L-glutamine. Cells were incubated at 37 °C under 5% CO_2. Total RNA was extracted with Trizol reagent (Invitrogen, Milan, Italy) and kept at −80 °C until use.

2.2. Expression analyses

Reverse transcription (RT) of 2 μg total RNA was performed in 20 μl final volume with random hexamers and the ThermoScriptTM RT-PCR System Kit (Invitrogen). After incubation for 10 min at 25 °C, RT was carried out for 30 min at 55 °C, followed by RT termination for 5 min at 85 °C. Total single-stranded cDNA (sscDNA) was amplified by PCR in a 25 μl reaction volume containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5–2 mM $MgCl_2$, 0.2 mM of each dNTP, 0.2 μM of the respective primers, and 1.25 U of Biotherm Taq DNA polymerase (Società Italiana Chimici, Rome, Italy). Oligonucleotide primers are listed in Table 1 and amplification conditions in Table 2. The extension phase of the last cycle was prolonged by 10 min. As RNA quality control, RT-PCR of β-actin mRNA was performed for every sample with specific primers. For each PCR, negative controls were prepared by replacing the cDNA solution with sterile water.

2.3. Determination of the transcription start sites of the STS gene expressed in various tissues

The TSSs of the STS gene were determined with the method of RNA ligase-mediated rapid amplification of cDNA 5′-ends (RLM-5′-RACE) using the FirstChoice RLM-RACE Kit (Ambion, Celbio, Milan, Italy), as previously described [14]. STS sscDNAs were PCR-amplified using the 5′-RACE outer primer (5′-GCT GAT GGC GAT GAA TGA ACA CTG-3′) and a specific 3′-reverse primer, either STS4, designed on exon 5, or STS6, designed on exon 3 of the STS gene [16]. The amplification procedure is listed in Table 2. The diluted products were subjected to second and third rounds of amplification using the 5′-RACE inner primer (5′-CGC GGA TCC GAA CAC TGC GTT TGC TGG CTT TGA TG-3′) and two specific 3′-reverse primers, STS6 and STS10, selected on exons 3 and 2, respectively. The template cDNAs of the second and third rounds of PCR were diluted between 1:50 and 1:500, according to the intensity of the previous amplification. In some cases, only a second round of PCR with STS10 was sufficient to obtain specific transcripts. The cDNA from the last PCR was purified from the sliced gel band in a preparative gel and either directly sequenced or, when more than a single band was present, ligated into a pGEM-T vector using the pGEM-T Vector System I (Promega, Milan, Italy). Plasmids from positive colonies were purified and sequenced.

2.4. Nucleotide sequencing

Sequencing was performed on dsDNA directly from PCR products or after cloning with the ABI PRISM Dye Terminator Cycle Sequencing Core Kit (Applied Biosystems). Electrophoresis of sequencing reactions was completed on an ABI PRISM 3100 DNA Sequencer. Homology searches were carried out using the Basic Blast program, Version 2.0, at http://www.ncbi.nlm.nih.gov/BLAST/, and alignments were performed with the ClustalW program at http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_clustalwan.html. Promoter analysis of the 5′-flanking regions of the exon 0d-associated promoter was conducted with the Cister program (Cis-element Cluster Finder,

Table 2
Amplification procedure of the PCR reactions.

Gene target	Primers	[Mg^{++}]	Annealing (°C)	Extension (s)
STS	STS1–STS2	2 mM	56	40
STS	5′-RACE outer–STS4	2 mM	Touch down 58-52	60
STS	5′-RACE outer–STS6	2 mM	Touch down 58-52	60
STS	5′-RACE inner–STS6	2 mM	58	30
STS	5′-RACE inner–STS10	2 mM	58	30
β-Actin	Q-Beta1–Q-Beta2	1.5 mM	60	15

Table 3
Hemi-probes sequences used in the RT-MLPA analyses. Sequences in bold and italic correspond to the PCR primers.

Hemi-probe	Sequence
RPO-ex2	5′P-TGAAGATCCCTTTCCTCCTACTGT***TCTCTAGATTGGATCTTGCTGGCAC***-3′
RPO-ex5	5′P-GAAATGGCACCTTGGGATGAGCTG***TCTCTAGATTGGATCTTGCTGGCAC***-3′
RPO-ex1b	5′P-AGCCCGATGCCCTTGGTTTGACTCTACTAAGAGC***TCTAGATTGGATCTTGCTGGCAC***-3′
RPO-B2M	5′P-CTCCAAAGATTCAGGTTTACTCACGTCATCCAGCAG***TCTAGATTGGATCTTGCTGGCAC***-3′
LPO-ex0a	5′-***GGGTTCCCTAAGGGTTGGA***CATCAAAGTGCGCCCACAGCAGCAACCTTGGAGCAGGCAGGAAG-3′
LPO-ex0b	5′-***GGGTTCCCTAAGGGTTGGA***GCACACTACCCACCCAGAAGAAGTCCGTCCATGTCAAAGATGAGGAAG-3′
LPO-ex0c	5′-***GGGTTCCCTAAGGGTTGGA***CTTGAGTCTTCCAGTAAAGAGCCCTCTCTCTGAAAGCCTTGTAAGAACTGGAAG-3′
LPO-ex1a	5′-***GGGTTCCCTAAGGGTTGGA***CACAAGCTGGAGATGCCTTTAAGGAAGA-3′
LPO-ex1b	5′-***GGGTTCCCTAAGGGTTGGA***GCAAGATCGTCTTCAGCTGTTCATAGCGAAGA-3′
LPO-ex1c	5′-***GGGTTCCCTAAGGGTTGGA***GGTGGGAAGACTAGAAGGACATCGATATTGAGAAGA-3′
LPO-ex1d	5′-***GGGTTCCCTAAGGGTTGGA***CCTGTTATTCTTACAAGGAATGAAATGCAGTGAGGAAGA-3′
LPO-ex1e	5′-***GGGTTCCCTAAGGGTTGGA***CTGTATTTTGGACACCTTGGGCTGAGTCATCTTCATGTCTTAGGAAGA-3′
LPO-ex4	5′-***GGGTTCCCTAAGGGTTGGA***GCTAAGCTTCTGAAGGATCAAGGTTATTCAACAGCACTGATAGG-3′
LPO-B2M	5′-***GGGTTGCCTAAGGGTTGGA***CCTTAGCTGTGCTGCGCTACTCTCTCTTTCTGGCCTGGAGGCTATCCAGCGTA-3′

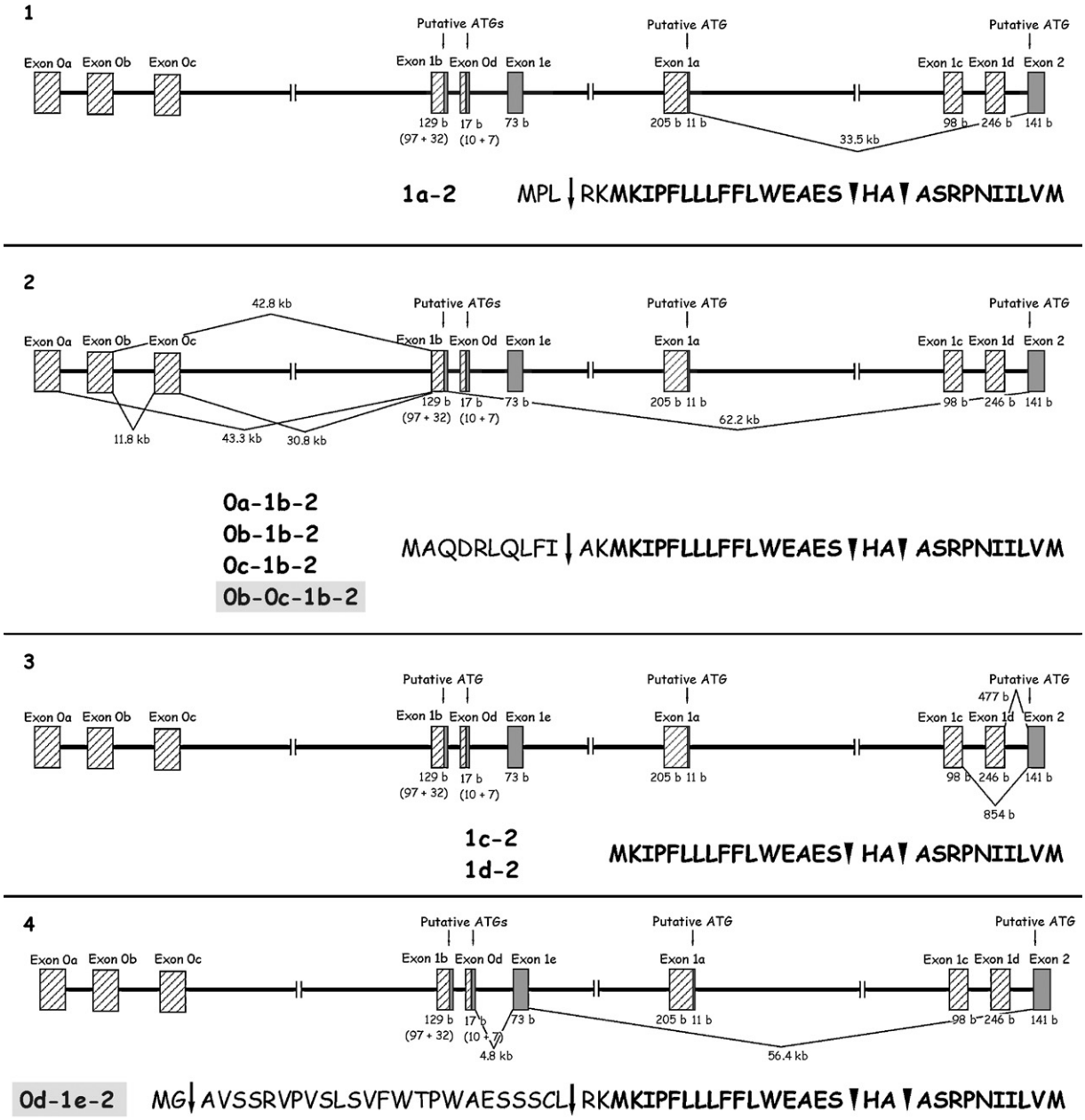

Fig. 1. Intron/exon structure of the alternative transcripts of the STS gene. Exons are shown as boxes and introns as lines. Filled and striped boxes indicate the translated and untranslated parts of the exons, respectively. Numbers indicate sizes of exons and introns. Vertical arrows indicate putative translation start codon positions. The angled lines connect potential splice donors and acceptors. The new transcripts are boxed in grey. In each panel is reported the N-terminal region of the STS proteins encoded using different putative translation start codons located in exons 1a, 1b, 0d and 2. Arrows show the intron position between exons 1a and 2, between exons 1b and 2 and between exon 0d and 1e and 1e and 2. Triangles indicate the potential cleavage sites for signal peptidase.

Fig. 2. Sequences of the different first exons and exon 2 of the STS gene and determined transcription start site positions. In italics: first 12 nt of introns; in bold: 5′- and 3′-splice signals. Putative translation start codons are in bold and boxed. The cDNA sequences of the new STS transcripts have been submitted to the EMBL Nucleotide Sequence Database under the accession numbers FM209048 for exon 0d (sequenced from PC3 cell line) and FM209047 for exon 0b–0c (sequenced from ovary).

http://zlab.bu.edu/~mfrith/cister.shtml), with a cut-off score of 90%.

2.5. RT-MLPA analysis

The MLPA method, as described by Schouten [17] and adapted for RNA-derived cDNA [18], was used to analyse alternative STS gene transcriptions. The hemi-probe sets for STS alternative transcripts were designed following recommendations by MRC-Holland (Designing synthetic MLPA probes, http://www.mlpa.com/pages/support_desing_synthetic_probespag.html). They are targeted against different STS first exons and are listed in Table 3. In order to avoid detection of contaminating DNA fragments, all target sequences have an exon boundary close to the probe ligation site. For each probe target sequence, a specific RT primer was designed that is complementary to the RNA sequence immediately downstream of the probe target sequence (Table 1). As house-keeping gene for normalization, we used the β-2-microglobulin (B2 M) gene.

Total RNA samples (2 μg) were mixed with 1 μl of each probe-specific RT primer (1 pmol) in a final volume of 15 μl and heated for 5 min at 70 °C to melt the secondary structure of the RNA. After this step, 10 μl of a mix composed by M-MLV reaction buffer, dNTPs (1.25 pmol), 25 U of Recombinant RNasin Ribonuclease Inibitor, 200 U of M-MLV-Reverse Transcriptase (Promega) was added. RT was carried out for 1 h at 42 °C. Inactivation of MMLV-RT enzyme was carried out for 2 min at 98 °C.

Samples of 2.5 μl of cDNA were then mixed with 1.5 μl SALSA-MLPA buffer (1.5 M KCl, 300 mM Tris–HCl (pH 8.5) and 1 mM EDTA) (MCR-Holland, Resnova, Roma, Italy), 1.5 μl of probe mix (1–4 fmol of each synthetic hemi-probe, except that for B2 M that was used 1/400 less concentrated) and 1.5 μl of water, to a final volume of 8 μl. Samples were heated for 1 min at 95 °C and incubated for 16 h at 60 °C to achieve optimal hybridisation. Ligation of annealed oligonucleotides was performed at 54 °C for 15 min by diluting samples to 40 μl with dilution buffer (2.6 mM $MgCl_2$, 5 mM Tris–HCl (pH 8.5), 0.0013% non-ionic detergents, and 0.2 mM NAD) containing 1 U Ligase-65 enzyme (MCR-Holland). The ligase enzyme was inactivated by heating at 98 °C for 5 min.

To amplify ligation products by PCR, 10 μl of the ligation reaction was added to 30 μl PCR buffer at room temperature. While at 60 °C, 10 μl of a buffered solution containing PCR primers (10 pmol), dNTPs (2.5 nmol) and 2.5 U SALSA polymerase (MRC Holland) were added. PCR was performed for 35 cycles (30 s at 95 °C, 30 s at 60 °C, and 1 min at 72 °C). The extension phase of the last cycle was prolonged by 20 min.

Samples were amplified with one reverse primer (GTG CCA GCA AGA TCC AAT CTA GA) and one forward FAM-labelled primer (GGG TTC CCT AAG GGT TGG A), and then analysed on the ABI PRISM 3100 after the addition of ROX 500, a fluorescence marker. Data were evaluated using Peak Scanner software V. 1.0 (Applied Biosystems). Expression levels of individual genes are characterized by different peaks, where the peak areas represent the amounts of fluorescently labelled amplification products of unique length. For relative quantification of gene expression, the peak areas of target genes are divided by the peak area of the house-keeping gene in order to normalise values for sample comparisons.

3. Results and discussion

In our previous works [13,14], we have demonstrated, by means of 5′-RACE analysis, that the expression of the human STS-encoding gene is driven by at least six alternative first exons associated with different promoters (results summarized in Fig. 1, together with the new results). To advance our investigation, we initially studied by 5′-RACE other available tissues (signed by * in Table 5) expressing consistent levels of STS, as demonstrated by RT-PCR analysis

Table 4
Results of the 5′-RACE analyses.

	First exon	Length (nt)
Du-145	0a	263
	0b	140
Lung-N	1a	52
Ovary-1C	1c	153
	0b–0c	42–156
Ovary-2N	0a	328
Ovary-2C	0b	140
PC3	0c	136
	0d–1e	17–73
Uterus-1C	0b	143

with the set of primers STS1 and STS2 that anneals inside the coding region. Besides, we tried to set up an RT-MLPA analysis for the simultaneous detection, at least in qualitative terms, of alternative *STS* transcription patterns in different tissues. In this case, we used all the new tissues available as well as some samples analysed in a previous work (signed by ♦ in Table 5) [14]. Tissues, that were either barely positive for STS expression or producing very low quantities of total RNA and thus not suitable for 5′-RACE, were also included in RT-MLPA analysis (signed by # in Table 5).

3.1. 5′-RACE analysis

5′-RACE analysis was performed with two or three rounds of PCR. Single specific 5′-RACE products were generated in normal and cancerous ovary and lung (ovary-2-N and 2-C; lung-N and C) and in a sample of uterine adenocarcinoma (uterus-1-C), whereas multiple bands were found with the other tissues. After sequencing of the amplified fragments, we observed the utilization, in the new samples, of the same alternative first exons previously identified [14], but also of two additional transcript variants (see below). The results of this analysis are summarized in Table 4 and the transcription patterns are shown in Fig. 1. The new transcript variants are pointed out in panels 2 and 4 of Fig. 1 by a grey box. The sequences of the two new variants, found in ovary-1C and in the prostatic cell line PC3, are reported in Fig. 2 and were submitted to GenBank with the accession numbers FM209047 and FM209048, respectively.

The transcript variant found in ovary-1P is characterized by an alternative pattern of splicing, never described before. It is composed of the untranslated exon 0b, spliced to exon 0c with an intervening intron of 11.82 kb, which displays splice signals consistent with the GT/AG rule. Exons 0b and 0c are 42 nt- and 156 nt-long, respectively; exon 0c is then spliced to the common exon 1b.

The new splicing pattern found in the prostatic cell line PC3 contains a novel first exon, called exon 0d, spliced to another new exon 1e after an intron of 4.8 kb. Exon 0d is 17 nt-long, whereas 1e contains 73 nt. Exons 0d is downstream and very close to exon 1b (Fig. 1, panel 4), being separated by only 875 nt. Exon 1e is spliced to exon 2 after an intron of 56.4 kb. Both new introns display canonical splice signals. Exon 0d is partially coding, as it contains an ATG in frame with the coding sequences of exon 1e and exon 2. This putative initiation codon only partially matches the Kozak consensus sequence.

Due to the presence of different putative ATGs in exons 1a, 1b, and 2, as previously demonstrated [14], and now also in exon 0d, the N-terminal region of the encoded proteins may be different, with the longest protein translated from exon 0d. In Fig. 1, we show the four different deduced N-terminal regions with the common amino acid sequence deriving from exon 2 in bold. As previously reported [14], these differences in the N-terminal region should not affect the catalytic activity of STS, because they bear upon the signal peptide in all proteins, as demonstrated by sequence analysis with the "SignalP" program (http://www.cbs.dtu.dk/services/SignalP/). This was established also for the protein deriving from the last transcription pattern (exons 0d-1e-2) that presents a signal peptide with a probability of 0.998.

Sequence analysis using the Cister program revealed that no TATA box upstream of the new first exon 0d of human *STS*. In addition, a preliminary analysis of the proximal promoter showed six putative sites that bind Sp1, a transcription factor that is a mediator of the indirect effects of estrogen receptors on transcription interactions [19] and six putative sites that bind the E26 transformation-specific transcription factor (Ets), recognized as a key regulator of differentiation, hormone responses and tumorigenesis in endocrine organs and target tissues [20].

3.2. RT-MLPA analysis

MLPA and RT-MLPA [18] are relatively new techniques that permit the simultaneous PCR amplification and quantification of

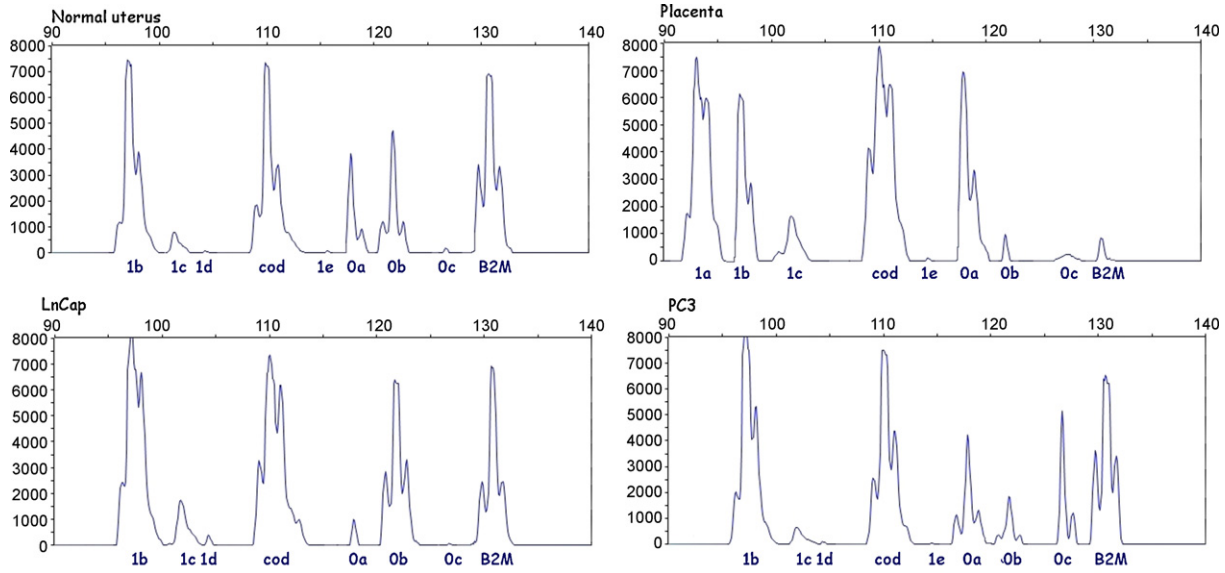

Fig. 3. Examples of RT-MLPA with STS probe panel. Expression levels of individual genes are shown by different peaks. For relative quantification of gene expression, the peak areas of target genes were divided by the peak area of a house-keeping gene.

Table 5
Results of the RT-MPLA analyses.

Tissues		SEX	Exon 1a	Exon 1b	Exon 1c	Exon 1d	Exon 1e	Cod. reg.	Exon 0a	Exon 0b	Exon 0c
Adrenal gland ♦	C	F	N/D	1.99	0.04	N/D	N/D	1.37	0.08	0.69	0.01
Colon-1 #	C	F	0.02	2.12	0.06	0.01	0.01	1.48	0.30	0.55	0.01
Colon-2 #	C	M	0.03	2.68	0.08	0.02	N/D	1.97	0.35	0.57	0.01
Dermal papilla-1 ♦	N	F	0.01	3.06	0.06	0.04	N/D	2.37	0.32	0.49	0.36
Dermal papilla-2 #	N	M	0.02	2.27	0.03	0.02	N/D	1.65	0.31	0.43	0.21
DU-145 *	M	N/D	1.29	0.16	0.01	0.01	1.27	0.08	0.95	0.01	
Fermoral head Cartilage #	N	F	0.03	0.54	0.02	0.08	N/D	0.45	0.06	0.24	N/D
	A	F	N/D	2.16	N/D	N/D	0.02	2.56	0.41	0.84	N/D
LnCap #	M	N/D	1.61	0.30	0.03	N/D	1.50	0.07	0.99	N/D	
Lung * (N) # (C)	N	M	0.42	1.10	0.19	N/D	N/D	1.48	0.09	0.65	0.01
	C	M	0.14	1.44	0.11	0.01	0.01	1.43	0.20	0.82	N/D
Mammary gland ♦	C	F	N/D	2.56	0.03	0.03	N/D	1.74	0.02	0.81	0.01
Ovary-1 *	C	F	N/D	1.18	0.07	N/D	N/D	1.14	0.01	0.64	N/D
Ovary-2 *	N	F	N/D	2.37	0.10	0.02	0.01	1.67	0.74	0.52	0.01
	C	F	N/D	1.20	0.24	0.09	N/D	1.48	0.04	1.13	N/D
Ovary-3 ♦	C	F	0.01	0.99	0.03	N/D	N/D	0.85	0.01	0.58	N/D
Ovary-4 ♦	C	F	0.04	0.83	0.05	0.02	N/D	0.83	0.01	0.44	N/D
Pancreas ♦	C	M	N/D	1.39	0.02	N/D	N/D	1.07	0.02	0.56	0.01
Placenta ♦	N	F	11.82	5.91	0.68	N/D	N/D	14.17	5.97	0.25	N/D
PC3 *	M	N/D	1.66	0.09	0.01	0.01	1.15	0.38	0.18	0.33	
Skin-1 #	N	F	N/D	2.14	0.09	N/D	N/D	1.49	0.03	0.72	0.25
Skin-2 #	N	F	N/D	1.45	0.07	0.02	N/D	1.22	0.12	0.44	0.20
Thymus #	N	F	N/D	1.00	0.06	0.01	N/D	0.75	0.06	0.47	N/D
	C	F	N/D	1.69	N/D	0.01	N/D	0.54	0.06	1.41	N/D
Uterus-1 # (N) * (C)	N	F	N/D	1.39	0.10	N/D	N/D	0.99	0.34	0.45	0.01
	C	F	N/D	1.27	0.08	0.01	N/D	1.38	0.24	0.56	0.01
Uterus-2 ♦	C	F	N/D	1.08	0.01	N/D	N/D	0.63	0.01	0.37	N/D
Uterus-3 ♦	C	F	N/D	1.58	0.02	N/D	N/D	0.97	0.01	0.49	N/D

Tissues or cell lines used in this work: (*) Samples on which both 5′-RACE and MLPA analyses were performed. (#) Samples on which only RT-MLPA analysis was performed due to low STS expression or low quantities of total RNA. (♦) Samples analysed in a previous work (Dalla Valle et al. [15]) and used for RT-MLPA analysis. N, normal tissue; C, cancer tissue; A, arthrosic cartilage of the femoral head; N/D, not detected.

different sequences. In this technique, hemi-probes and DNA (or cDNA) are denatured and then hybridised together. After hybridisation to their target sequences the probes are enzymatically ligated but only when they are both hybridised to adjacent sites. The ligated product is then PCR amplified *via* constant sequences on the probes and amplified products are analysed using capillary electrophoresis. Each ligated probe gives rise to an amplification product of unique length. In RT-MLPA hemi-probes were designed to span exon boundaries, precluding the detection of potentially contaminating genomic DNA. We chose this analysis instead of real time RT-PCR as it is less time-consuming, less expensive, requires low quantities of RNA (down to 10 ng of total RNA) and, with only one reaction, it is possible to investigate all the STS transcription patterns up to now identified. The B2 M gene was used as a control probe, as recommended by MCR-Holland (Fig. 3). Finding a good house-keeping gene is a difficult goal when using different tissues, as in this work. However, the aim of our study was not to achieve an absolute quantification of transcripts, but rather to inspect the overall *STS* transcription pattern specific of each tissue. Normalised STS transcript values were obtained by dividing the peak area of each transcript by the peak area of the control transcript in the same run. These normalised values were then used to compare results from different samples (Fig. 3). As *B2M* is a highly expressed gene, its abundant probe would engage primers for amplification at the expense of the other probes thus lowering their absolute signals To solve this problem, we reduced, the hemi-probe concentration of B2M to 1/400 in the probe mix.

MLPA data are reported in Table 5. As shown, exon 1a is expressed at very high levels in placenta. Nevertheless, this transcript has been found also in normal and cancerous lung, thus confirming the results obtained by 5′-RACE analysis. Low levels of expression were found also in other tissues. Exon 1c, previously found in thyroid and peripheral mononuclear leukocytes, is expressed in all tissues analysed, except arthrosic cartilage of the femoral head, but always at very low levels. These results are in agreement with those of previous works [14,15]. Exon 1d and particularly exon 1e were expressed in a restricted number of tissues and at low expression levels. Exon 1b was instead expressed in all samples analysed with levels ranging from 9 to 52% of exon 1b placental expression and from 4.6 to 26% of exon 1a placental expression.

Although exons 0a, 0b and 0c are always linked to exon 1b, we analysed also their specific patterns of expression, as they are presumably driven by different promoters. The highest levels of expression were found for exon 0b and the lowest for exon 0c. In placenta, transcripts containing exon 1b are associated mainly with exon 0a, and not 0b, as found in many other tissues.

From a quantitative point of view, two problems emerge from the examination of the MLPA results. First, the sum of transcripts with exons 0a, 0b and 0c is always lower (except for placenta in which it is similar) than transcripts with exon 1b. This could be explained by either a better annealing efficiency of the hemi-probes for this transcript or the presence of other, still unknown, first exons linked to exon 1b.

Similarly, data of the coding region are generally lower with respect to the sum of data from exons 1a, 1b, 1c, 1d, and 1e; in four samples they are similar and higher in just two. These results suggest that the detection efficiency of the hemi-probe set for the coding region is lower with respect to the other hemi-probe sets. In the samples in which data are similar or higher, a possible explanation could be the expression of completely unknown transcripts that were not revealed. It is important to underline that the presence of an STS pseudogene in the Y chromosome limited our selection choice of the hemi-probe sets.

4. Conclusions

Steroid sulfatase is an important enzyme involved in steroid hormone metabolism. In recent years, STS has emerged as a key player in the maintenance of high intra-tumoral estrogen and androgen levels through intracrine biosynthesis from sul-

fated precursors, and, hence, in the growth stimulation of steroid hormone-dependent tumors.

The studies on the transcriptional control of *STS* have demonstrated the possibility of a tissue-specific regulation, as previously found for other steroidogenic enzymes, such as the aromatase gene, in which alternative splicing is considered to play important roles not only in tissue-specific expression, but also in the alteration of aromatase expression through developmental or neoplastic process within the same tissue.

In particular, eight different transcriptional patterns have been characterized so far, indicating that the expression of the STS enzyme is tightly regulated, in line with its importance in cellular functions. In some tissues, no STS transcripts containing the first exon 1a (the placental one) could be found, thus strengthening the hypothesis of a tissue-specific control of transcription. Recently, the study by Zaichuk et al. showed that, in breast carcinoma, steroid sulfatase transcription is under the control of estrogen and is up-regulated by 17β-estradiol *via* direct binding of the estrogen receptor to estrogen response elements (EREs) located in the promoter regions driving 1a and 1b transcripts [15]. According to the authors, the diversity in the distribution of STS variants among human breast carcinomata presumably reflects specific regulation and function of each of the isoform during tumor progression.

Apart from this work [15], no information is available regarding other transcription factors involved in STS promoter regulation. This kind of knowledge is instead very important to understand how STS is differentially expressed in various tissues and under different physiological or pathological conditions in the same tissue.

In order to acquire a rapid and not expensive STS transcriptome, we have set up an RT-MLPA protocol that allows the qualitative and semi-quantitative analyses of all the known STS transcripts. Since very low quantities of RNA are needed, this protocol can be applied to small samples of tissues obtained by biopsy or even by laser capture microdissection.

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