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Testis-specific protein, Y-encoded (TSPY) expression in testicular tissues

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TSPY, the 'testis-specific protein, Y-encoded', is the product of a tandem gene cluster on human proximal Yp. In order to gain insight into the function of this locus, we have analysed (i) the diversity of RNAs transcribed from the cluster, (ii) the sequence homology of the deduced TSPY to other proteins, and (iii) its protein properties both in tissue extracts and in tissue sections, using a TSPY-specific antiserum. We have identified a set of distinct *TSPY* transcripts with diverse exon compositions. We show that TSPY has homology with other human and non-human proteins, including SET and NAP, factors that are suggested to play a role in DNA replication. Protein analysis revealed TSPY to occur mainly in a modified, putatively phosphorylated form. By immunostaining it was detected in distinct subsets of spermatogonia. TSPY was also strongly immunostained in early testicular carcinoma *in situ* (CIS), while seminomatous tumour cells stained less intensely. The spermatogonial cells of two XY-TFM- females gave a strong immune response. The data presented here point to a phosphorylation-dependent TSPY-function in early spermatogenesis, immediately prior to the spermatogonia-to-spermatocyte transition, and in early testicular tumorigenesis.

INTRODUCTION

TSPY is a gene family located on the proximal part of human Yp with each of its 20–40 copies embedded in a single unit of the *DYZ5* tandem repeat array (1–3). This array maps to the region immediately adjacent to the centromere on human Yp, thus rendering it exempt from pseudoautosomal recombination. *TSPY*-homologous gene families have also been found on the Y-chromosome of the great apes (4,5), cattle and other large mammals [(6) and own unpublished data], whereas *TSPY*-homologous sequences have not so far been isolated from rodents. By RNA analyses, expression of *TSPY* sequences was found exclusively in the testis both in prenatal and adult stages (2,4,6,7).

The differentiation of germ cells and the production of mature spermatozoa is an intricately regulated process, which, if deregulated, ends in tumour formation and/or infertility. The understanding of how this complex machinery is genetically controlled is only at its very beginning (8), as reviewed in (1). A link between spermatogenic processes and testicular tumour development is suggested by the finding that germ cell-derived tumours, especially gonadoblastomas, often develop on the basis of gonadal dysgenesis (9,10). There is evidence to invoke one or more Y-chromosomal causative genes, because gonadoblastomas have almost exclusively been encountered in cases where Y-chromosomal material was detected (9).

The organisation of *TSPY* as a tandem repeat gene family renders its functional analysis more difficult than it would be for a single-copy gene. It is, for instance, necessary to determine to what extent sequence diversity at the genomic level is reflected in transcript complexity and which of the transcripts encode functional products. Human *TSPY* sequences are characterised by sequence divergence of up to 10% (2,7). So far, two *TSPY* transcripts, JA923 (7) and Y-231 (4), differing in exon composition have been published, the latter of which may be functional. In this study, the question of transcript functionality was readdressed. Properties of the protein, such as biochemical parameters, tissue specificity, and the cellular site of expression in the testis were studied using a TSPY-specific polyclonal antiserum generated against a recombinant polypeptide derived from the JA923 open reading frame, which encompasses the Y-231-type exon-1-encoded amino acid sequence. We also discuss the results of database searches for proteins with homologies to TSPY.

RESULTS

Sequence diversity of TSPY mRNAs

Transcript diversity was assessed by RT–PCR and direct sequencing of cDNAs prepared from testicular tissues of seven individuals. RT–PCR was performed using cDNAs primed with *TSPY*-specific as well as poly-dT primers to avoid a possible

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product bias due to one or the other of the methods. Three major products amplified with the primers Mu and TD3 (indicated in Fig. 1B) of 569, 257, and 170 bp in length, respectively, were isolated and sequenced. The RT–PCR products obtained by specific cDNA priming and, as a control, the product amplified with the same primers from genomic DNA, are shown in Figure 1A. Products were of equal numbers and sizes in all seven cDNA samples analysed (not shown). Specific and oligo-dT-priming revealed no differences in product amounts and sizes, thus all variants appeared to be equally polyadenylated. The most abundant and longest product (11) was found to be nearly identical with Y-231, except for a major difference at the exon 4–5 border (Figure 1B,C). The exon 5 of this product is 11 bp shorter than its Y-231 equivalent, most likely due to the use of a different splice acceptor site 11 bases downstream. Thus, the open reading frame is shifted and the deduced amino acid sequence is 14 amino acids longer. In none of the seven individuals tested was a transcript with the Y-231-type exon 4–5 border found. We assume that, in our samples, Y-231 is a very rare variant. Two further single bp differences between the Y-231 and TSPY cDNAs analysed here were detected in the cDNAs 5′ region, which further support the view that the products analysed here are transcribed from genomic TSPY copies that are distinct from the Y-231-type copy. On the basis of these data and in view of the sequence homologies to related proteins shown below, we suggest that the major transcript seen here is the most promising candidate for a functional TSPY (referred to as TSPY^{major}). The TSPY variants A and B, schematically drawn in Figure 1B, show altered splice patterns with respect to exon 1. Since no genomic products having a corresponding size were found, genomic deletions were excluded. Instead, the deletion breakpoints almost perfectly match the consensus sequences of donor and acceptor splice sites (12). This sequence context strongly suggests that both transcript variants have formed by the use of a cryptic splice donor site (TSPY-A and TSPY-B) and a cryptic splice acceptor site (TSPY-A) within exon 1 (Fig. 1B). Whether these products are generated by alternative splicing of one and the same transcript or from different distinct transcripts with individual splice donor and acceptor sites is as yet unclear.

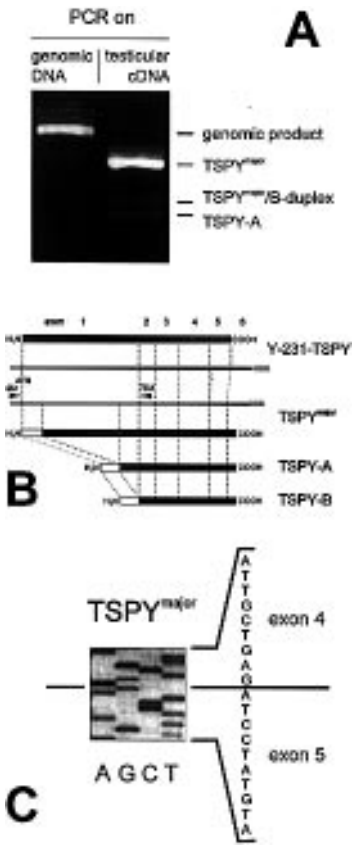

Figure 1. RT–PCR analysis of *TSPY* transcripts. (**A**) EtBr-image of the RT–PCR products amplified termed TSPY^{major} (569 bp), TSPY-A (lowest band of 170 bp), and TSPY-B (contained in the heteroduplex band between TSPY^{major} and TSPY-A). (**B**) Positions of primers used in this RT–PCR analysis and organisation of the *TSPY*-mRNA sequences of the TSPY^{major} and the Y-231 type. The TSPY^{major} protein product is aligned with TSPY-A and TSPY-B products (as deduced from the RT–PCR products) in order to illustrate coding regions and the exon compositions found. (**C**) autoradiograph and nucleotide sequence of the exon-4-to-exon-5 border of the TSPY^{major} transcript.

TSPY is related to the SET and NAP 1 proteins

Detailed database and sequence analyses revealed that TSPY is a member of a protein super family including the proto-oncogene SET, the nucleosome-assembly factor NAP-1, and a not further characterised protein from *plasmodium falciparum.* The alignment of human TSPY^{major} and bovine TSPY (unpublished data) with SET (Fig. 2) reveals stretches of conserved amino acids distributed over the entire length of the protein sequences. The major difference between TSPY and SET is the C-terminal stretch of acidic moieties found in SET, but not in TSPY. This acidic domain was shown *in vitro* to promote DNA replication, presumably by interacting with nucleosomes (13–15). The most carboxy-terminal group of homologous amino acids shared by all three proteins in this alignment is only present in the TSPY^{major} reading frame but not in the Y-231 frame, further supporting the idea that TSPY^{major} is functional.

Table 1 illustrates amino acid identity, similarity, and the number of gaps present in a selection of pairwise alignments between TSPY, SET, and NAP sequences from different species. The homology values drop and the numbers of gaps rise with increasing evolutionary distances between TSPY, SET, and NAP, as expected for sequences diverging from a common ancestor.

Biochemical analysis using the TSPY antiserum 837/3

For the biochemical analysis of the TSPY gene product, we used a rabbit antiserum (837/3) raised against a fusion protein corresponding to the JA923-deduced amino acid sequence (JA923-TSPY, shown in Fig. 3A), almost identical with the first 149 amino acids of the TSPY^{major} gene product (shown in Fig. 3A). In order to test for the specificity of the antiserum, competition experiments were done with the carrier of the fusion protein, TrpE, on its own, preimmune serum and with sequence-specific peptides using the full-length immunisation peptide or a deleted (shortened) peptide, which covers the region of identity between both types of transcripts, the JA923-TSPY-type and the TSPY^{major}-type (shown in Fig. 3A). Both the fusion protein corresponding to the immunisation peptide and the shortened peptide were able to block the antiserum from binding on western blots and in immuno-histochemical stainings on tissue sections.

On western blots of testicular tissue extracts derived from adult males (Fig. 3B), four bands corresponding to proteins of ~60, 38,

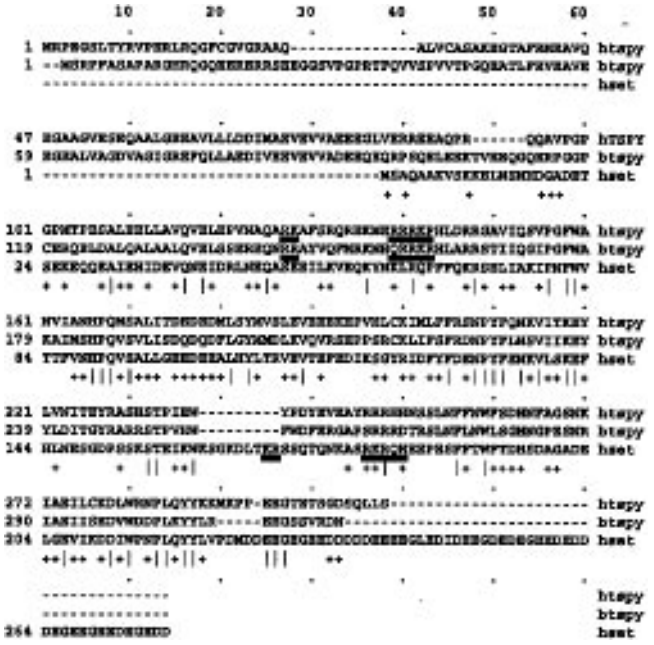

Figure 2. Alignment of TSPY, and SET amino acid sequences. |: identical residues, +: columns where SET is identical with one or similar to one or both TSPY amino acids, referring to the following grouping (LIVAM) (AGP) (STC) (RKH) (DE) (QN) (FWY). **h**: human, **b**: bovine. Underlined amino acids are potential nuclear localisation signals (NLS).

Table 1. Sequence homologies between TSPY, SET and NAP amino acid sequences of different species

	hTSPY	bTSPY	hSET	hNAP	mNAP
	#gaps / %identity / %similarity at gap weight: 3.0; gap length weight: 1.0				
hSET	1 / 31.6 / 56.3	1 / 26.2 / 52.3			
rSET	1 / 30.5 / 52.7	1 / 24.0 / 48.7	0 / 98.3 / 99.1		
dSET	2 / 30.3 / 56.3	2 / 25.6 / 51.8	3 / 56.5 / 77.4		
hNAP	5 / 14.3 / 35.5	3 / 13.5 / 34.8	4 / 22.4 / 44.4		
mNAP	5 / 14.3 / 35.8	3 / 13.9 / 34.9	4 / 21.7 / 44.8	0 / 98.2 / 99.7	
yNAP	6 / 15.3 / 39.2	9 / 18.1 / 42.1	5 / 24.5 / 45.9	5 / 31.3 / 57.3	5 / 22.1 / 49.0
p. falciparum	4 / 21.2 / 48.6	2 / 17.2 / 45.6	4 / 20.7 / 58.6	6 / 24.5 / 47.0	6 / 24.1 / 47.0

h: human, b: bovine, m: murine, r: rat, d: *drosophila*, y: yeast, *p. falciparum*: *plasmodium falciparum*. The analysis was perfomed using GAP contained in the software package HUSAR, EMBL, Heidelberg.

33 and 29 kDa—referred to as p60, p38, p33, and p29—were detected. TrpE-protein-blocked antiserum and unblocked antiserum gave the same band pattern, preimmune serum detected no protein and control polypeptide-blocked antiserum prevented the reaction with p38, p33, and diminished the p29 signal. In size, p33 corresponds to the TSPY predicted for both Y-231 (4) and TSPY^{major}. p38 may be a phosphorylated modification of p33, as suggested by a western analysis of testis extracts treated with alkaline phosphatase prior to the immunoblotting. Phosphorylation appears to cause a mobility shift of the protein, which assumes an apparent molecular weight of 38 kDa, a biophysical phenomenon that is reversed by dephosphorylation (Fig. 3C). p33 and p38 thus appear to be alternative forms of one and the same polypeptide. p29 may represent carbonic anhydrase, as suggested by cross-reaction with

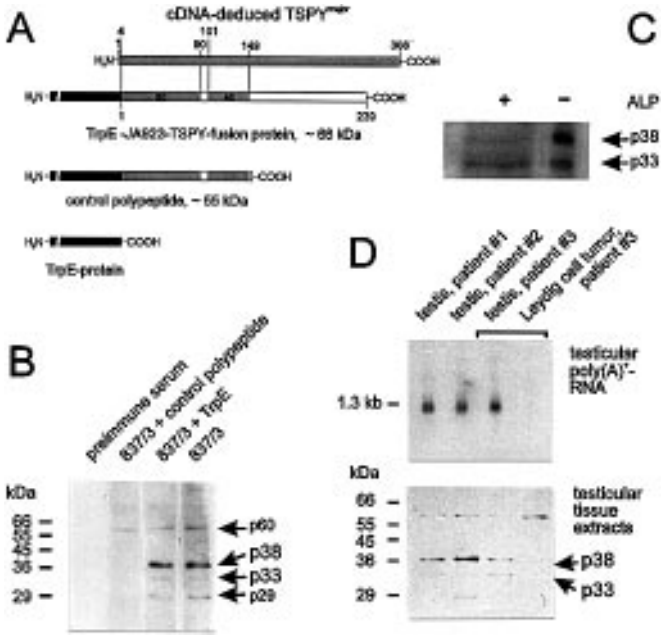

Figure 3. Analyses with the anti-TSPY-antibody 837/3. (**A**) Schematic alignment of the TSPY^{major} amino acid sequence with the TrpE-fused recombinant immunisation polypeptide (66 kDa), the TrpE-fused control polypeptide (55 kDa) and the TrpE-fusion protein. (**B**) Specificity of the antiserum 837/3 by western blotting. PI: preimmune serum, 837/3 preabsorbed with the control-polypeptide, the TrpE-fusion protein alone and without preabsorbing. (**C**) Western analysis of testicular protein extracts treated with and without 10 U alkaline phosphatase. (**D**) Northern and western analysis of testicular tissues from three different patients along with a Leydig cell tumour from the third patient. The northern blot was probed using radiolabeled TSPY cDNA clone JA923, the western blot was performed using the anti-TSPY antiserum 837/3.

erythrocyte carbonic anhydrase present in the molecular weight marker mix (not shown) and by the reaction with blood cells in the *in situ* immunostaining (see below).

Western analysis with testicular protein extracts from three different patients is shown in Figure 3D. All three patients had received bilateral orchidectomy. Patients 1 and 2 had suffered from prostate cancer and patient 3 from a Leydig cell tumour in one testicle. The western analyses were accompanied by northern analyses of poly(A)$^+$ RNAs prepared from the same tissues. Except for the Leydig cell tumour, all tissues, including the unaffected testis of patient 3, revealed the major 1.3 kb northern signal (2,4) and the characteristic band-quartet in the western analysis. p29 was also found in the *TSPY*-transcript-negative tissue of the Leydig cell tumour, a result which supports the idea that p29 does not originate from *TSPY*-transcripts. The western analysis of a variety of human tissues also revealed a few signals in other tissues, but none of these was sensitive to control-peptide-blocked antiserum (data not shown). In summary, it is strongly suggested that p33 (p38) is TSPY.

In situ analysis of the TSPY-expressing cell type in the testis

In order to elucidate the topology of TSPY expression in the testis, sections of testicular tissue with normal spermatogenesis were immunostained *in situ* using the antiserum 837/3. As in the western analysis, TSPY-specific immunostaining was assessed

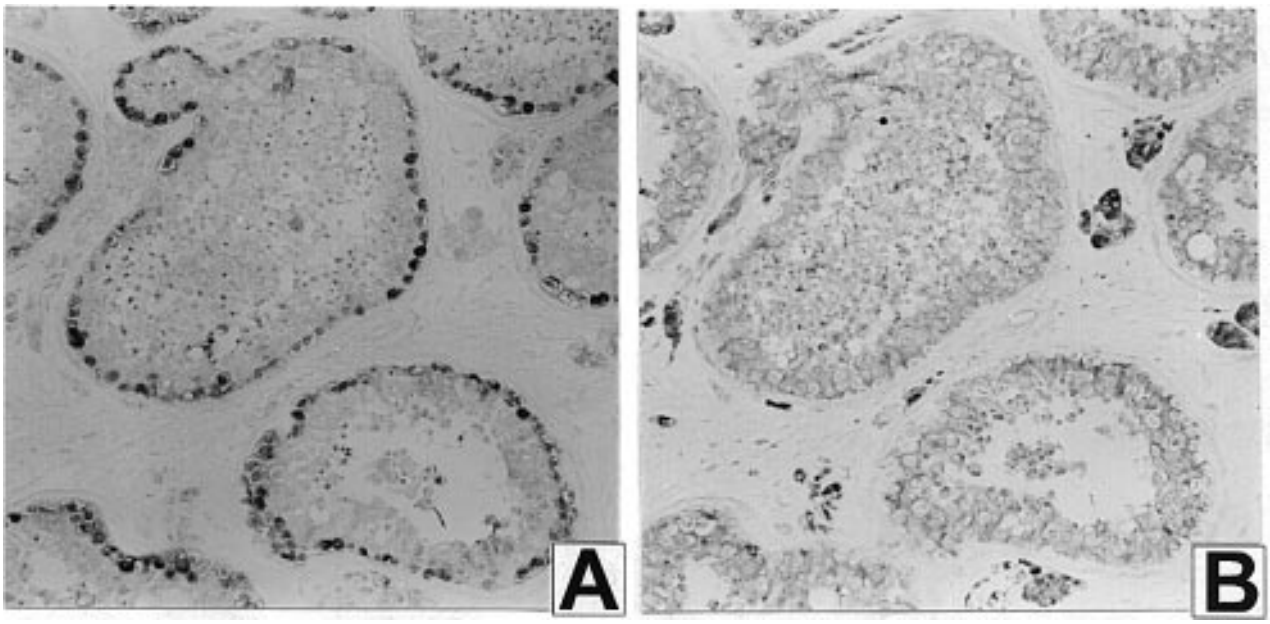

Figure 4. Cellular localisation of TSPY in normal testicular tissue. Immunostaining of normal testis with antiserum 837/3, preabsorbed with (**B**) and without (**A**) control-polypeptide. TSPY is localised predominantly in the cytoplasm of groups of spermatogonia (100-fold magnification). The reaction with the intertubular Leydig cells in (**B**) is an artifact presumably caused by the binding of antibody control peptide complexes to Leydig cell antigens. Such reactions are occasionally observed when polyclonal antibodies are used.

using antiserum blocked with the control polypeptide. The antiserum strongly reacts with groups of spermatogonia (Fig. 4A,B). Immunostaining occurs predominantly in pairs of spermatogonia, with neighbouring cells showing decreased dye intensities. A small number of primary spermatocytes appeared weakly stained (not shown). Staining is mainly concentrated in the cytoplasm of the cells, but some spermatogonia also appear to carry grains of the dye on their nucleoplasm. It should be pointed out that an amino acid motif which could serve as a nuclear localisation signal (NLS) is present in the amino acid sequence of $TSPY^{major}$ (see Fig. 2). Carbonic anhydrase (p29, see above) is only expressed in Sertoli cells of the adult testis and was thus excluded to cause the signal on spermatogonia found (16). The antiserum with and without control polypeptide was also applied to sections of human liver, lung, stomach, pancreas, intestine, colon, brain, thyroid gland, carotic arteria, lymph node and skin, but none of the tissues reacted specifically, in accordance with the testis-specificity of TSPY expression seen at the mRNA level.

In situ analysis of TSPY in testicular tumours and testicular tissues of XY-TFM females

Germ cell tumours are believed to arise from the carcinoma *in situ* (CIS) stage of the testis, where tumour cells are located at the basal membrane of the tubulus (17). These cells share a number of morphological features and cell surface markers with embryonic germ cells (18,19) supporting the hypothesis that they are persisting primordial germ cells (19). Up to a point where a CIS begins to develop into a rapidly growing germ cell tumour, proliferation of CIS cells is thought to proceed slowly (17). We have stained sections of early and later stages of testicular tumours employing the antiserum 837/3. As can be seen in Figure 5A,B, CIS cells show strong immunostaining. The concentration of the dye mostly on and around the cell nucleus is probably artificial, because CIS cells contain large cytoplasmic stocks of glycogen that are washed out during the tissue fixation process, causing the cytoplasm to stack around the nucleus and at the plasma membrane. Figure 5B shows a CIS stage together with both tumour cells on their way out of the seminiferous tubule and cells forming an interstitially located solid seminoma. Also, the migrating cells and groups of cells forming a solid tumour are specifically immunostained, the latter to a much lesser extent than CIS cells.

TSPY-immunostaining was also performed with testicular tissues of two XY-female siblings with testicular feminisation (TFM) due to a point mutation in the androgen receptor gene (T. Dörk *et al.*, in preparation). Histologically, dysgenetic testes were found showing spermatogonia-like and Sertoli-like cells. Though present in all tubules, spermatogonia-like cells stained positively for TSPY in restricted areas only (Fig. 6). These cells did not react with an antiserum against the placenta-like alkaline phosphatase (not shown), an embryonic antigen expressed on CIS and embryonic testicular cells (20). Thus, the 837/3-positive cells are thought to represent residual spermatogonia rather than early tumour cells.

DISCUSSION

We have shown that at least three major transcripts, differing in size, abundance, and splice pattern, are expressed from the human *TSPY* locus, a tandem repeat gene family with some 20–40 elements on proximal Yp. The transcript heterogeneity could, in principle, be caused either by the transcription of structurally different genes within the cluster, or by one or several structurally identical copies permitting alternative splice patterns. In contrast

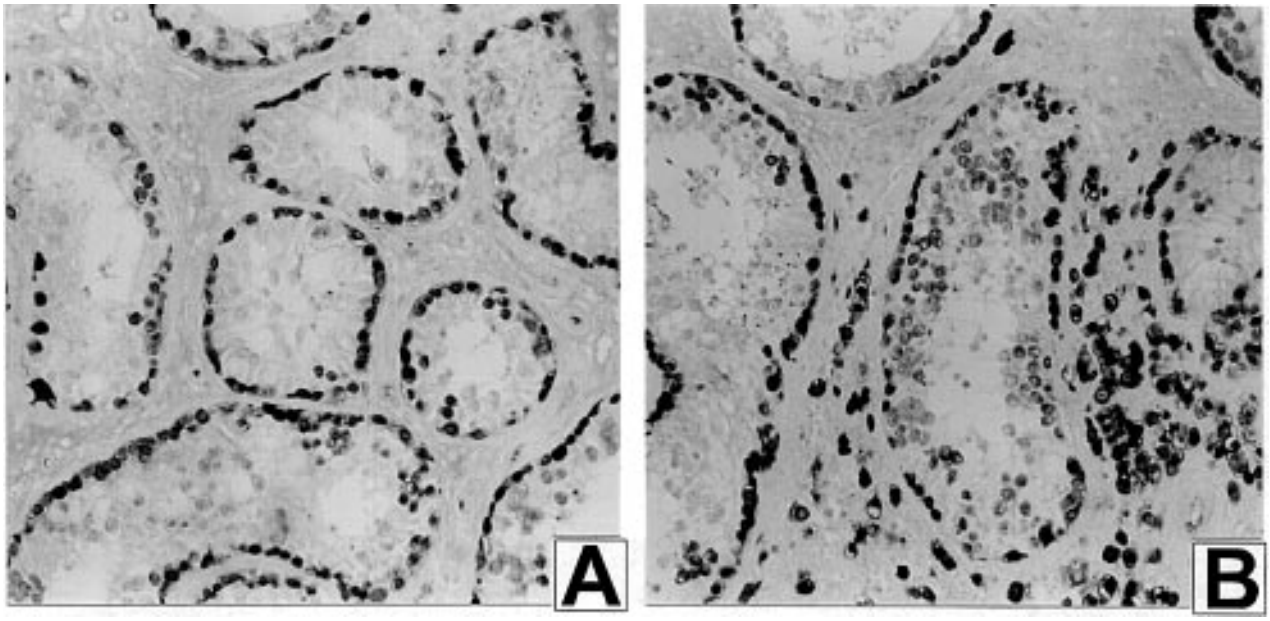

Figure 5. TSPY expression in early testicular tumorigenesis. (**A**) Testicular carcinoma *in situ* (CIS). (**B**) CIS and interstitial tumour cells are stained by the TSPY antiserum 837/3.

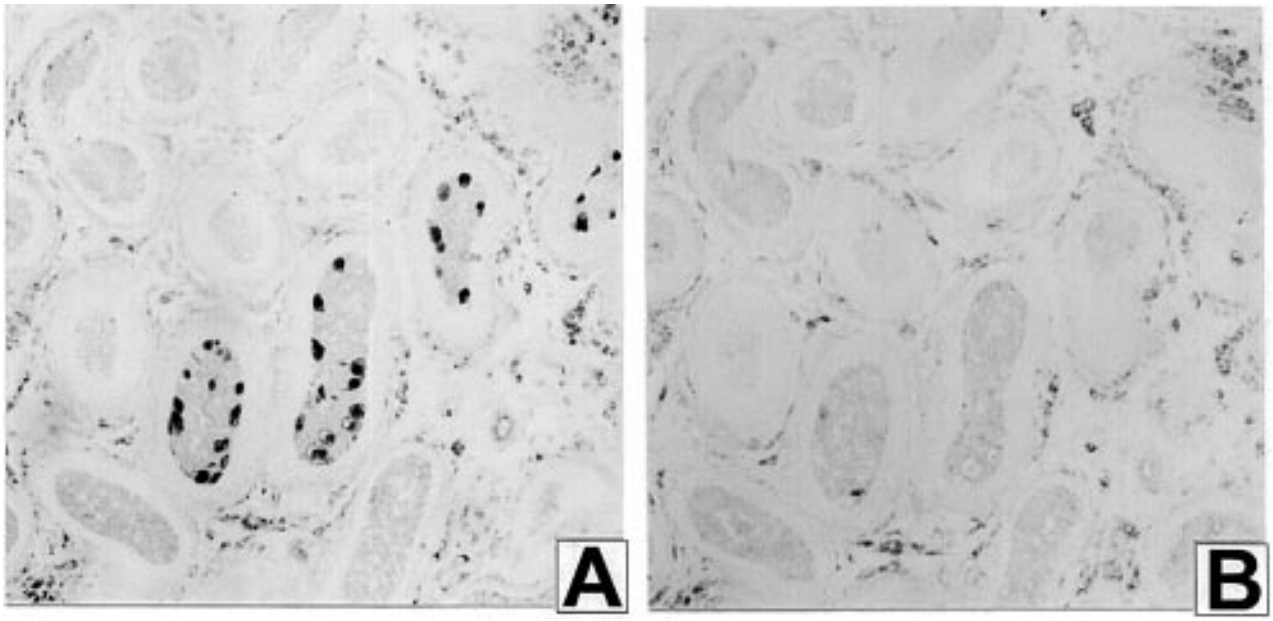

Figure 6. TSPY expression in germ cells in a testis of a 46,XY female due to a mutation in the androgen receptor gene, leading to testicular feminisation. Cells thought to represent residual spermatogonia are stained by the TSPY-antiserum 837/3. Immunostaining using the antiserum 837/3 preabsorbed without (**A**) and with the control polypeptide (**B**) in a 100-fold magnification.

to observations made at the genomic level (1,2,4), no sequence heterogeneity due to single base-pair exchanges or small deletions/insertions was seen among these transcripts. It is not possible, however, to assess the number of expressed *TSPY* copies from these data.

On the basis of structural comparisons with the SET/NAP protein, the product of the largest cDNA, termed above as $TSPY^{major}$, is also likely to represent the functional transcript. In comparison with the cY-231 reading frame, its region of homology with SET has significantly extended at the C-terminus. The evolutionary conservation of amino acid sequences of TSPY proteins in man and cattle as well as between TSPY and SET is suggested to reflect some important, though still unknown physiological function. SET and the more distantly related NAP have been described as activating factors of the replication process (13–15) and as binding-partners of cyclin B with a

cyclin/CDK complex (15,22). More recently, SET has also been identified as a potent inhibitor of protein phosphatase 2A, a major mammalian protein serine threonine phosphatase that regulates diverse cellular processes (23). Although it is presently unclear how these observations fit together in a cellular network of proliferation and differentiation processes, further advances in the characterisation of SET function may provide some important clues also to the biological role of the *TSPY* gene family.

Our data indicate that TSPY occurs in two alternative forms of apparent relative molecular masses of 33 and 38 kDa. Alkaline phosphatase treatment experiments suggest p38 to be the phosphorylated p33. SET with its predicted molecular weight of 33 kDa is likewise predominantly found in a phosphorylated form of apparent 38 kDa and was shown by phosphopeptide mapping to be phosphorylated at two serine residues (25). This suggests that functional regulation by phosphorylation may be a common feature of the SET/TSPY protein family.

A TSPY function specific for the proliferation of germ cells is suggested by the immunohistochemical data of TSPY expression. The marked variation in immunostaining of spermatogonia with pairs of spermatogonia showing equivalent staining indicates that TSPY may be involved in spermatogonial proliferation. If the rare staining in spermatocytes labels TSPY originating from mother spermatogonia, then TSPY protein expression may mark the branch point between mitotic proliferation and meiotic differentiation.

That TSPY expression is somehow tied to the mitotic division of early germ cells is also indicated by immunostaining in CIS and early seminoma cells. CIS cells are thought to originate from embryonic germ cells persisting in the testis, as suggested by the presence of embryonic antigens detected with specific antibodies (18,19). Although the anti-TSPY-antiserum stained both the CIS cells and normal spermatogonia, together with the evaluation of morphological parameters (for review see 17), TSPY immunostaining may add to the recognition of early germ cell tumorigenesis. In this context, it is of interest to note that in the dysgenetic testicular tissue of the two XY-TFM patients, TSPY is expressed in patches of spermatogonia-like cells while the majority of tubules remain negative. It is tempting to speculate that the TSPY-negative cells are resting, while the TSPY-positive cells are actively proliferating, with a potential to transform to a malignant state. Interestingly, Tsuchiya *et al.* (27) have recently shown that *TSPY* maps into the candidate region for the Y-linked gonadoblastoma locus.

In summary, *TSPY* gives rise to a pool of heterogeneously composed transcripts, at least one of which yields a protein that is a member of a superprotein family together with SET and NAP. Immunohistologically, TSPY was detected to be concentrated in the cytoplasm of spermatogonia in normal as well as pathological tissue. TSPY was found in restricted areas of the dysgenetic testes of two XY-female siblings with TFM. TSPY was also found in early testicular tumour cells at the CIS-stage and in the early forms of seminomatous testicular tumours. On the basis of the data presented here, its structural relationship to SET and NAP, TSPY is suggested to serve a function related to spermatogonial proliferation.

MATERIALS AND METHODS

Production of antisera

For the preparation of rabbit antisera, an *Eco*RI/*Hin*dIII fragment of cDNA clone pJA923, spanning the entire open reading frame (7), was subcloned into the pATH3 expression vector to yield the TrpE/JA923 fusion construct pJA10EX. After indoleacrylic acid-induced expression in RRI-cells (28) the fusion protein was separated from a whole cell lysate by SDS–PAGE and purified from the gel by band excision and electroelution. Rabbits were immunized according to standard methods (29). The resulting sera were preabsorbed with Sepharose-bound proteins extracted from TrpE-expressing RRI cells.

Western analysis

For western analysis, 50 µg protein (per lane) of total tissue (for preparation see below) were separated on 12% gels in SDS-PAGE (30), and transferred onto nitrocellulose membrane (Hybond C extra) by semi-dry blotting (Biometra). Signals were immunodetected with antiserum 837/3 diluted 1/2000, using the ECL-chemiluminescence system (Amersham), and autoradiography on Kodak Biomax films.

RNA preparation

Poly(A)$^+$ RNAs were isolated using the Fast Track RNA Isolation kit (Invitrogen) following the manufacturer's protocols. Total cellular RNA was extracted using the method reported in (31).

RT–PCR and DNA sequencing

RT–PCR analyses were carried out with cDNAs reversely transcribed from total cellular RNA using the First Strand Synthesis kit (Pharmacia) following the manufacturer's instructions. cDNA synthesis was primed either with a sequence-specific primer or with the poly-(dT) primer supplied with the kit. RT–PCR was carried out with the TSPY primers Mu (5′-GGCCCTTCGCGCGCAGTCCC-TTAG-3′, forward, position –45 to –22 of the TSPY^{major}-cDNA) and TD3 (5′-GTCAGTGATCAGGGCTGACATCTG-3′, reverse, position 521 to 545 of the TSPY^{major} cDNA), located in exons 1 and 2 of the Y-231-type TSPY cDNA, respectively.

Tissues and preparation of cellular extracts

Testicular tissues used in the western analyses and dephosphorylation assays were from patients with orchidectomy due to prostate cancer (patients 1 and 2 in Fig. 3D) or a Leydig cell tumour (patient 3, Fig. 3D), respectively. Approximately 100 mg of tissue were homogenized in 0.25 M sucrose, 20 mM Hepes, 1 mM EDTA, 1 mM DTT, pH 7.4, in a Dounce homogenizer, cell debris removed by short centrifugation at 4°C and 12 000 *g*. Supernatants of this tissue extract were subjected to protein analysis using a Lowry assay (DC Protein Assay, Biorad) and stored at –70°C prior to western blotting.

Dephosphorylation assays

Total testis protein (16 µg) was incubated in a 20 µl volume either with or without the addition of 10 U calf intestine alkaline phosphatase (Boehringer Mannheim) for 2 h at 30°C. The reaction was stopped by mixing the components with 6.6 µl 4 × Laemmli sample buffer and boiling the sample after reaction prior to western analysis.

Tissue sections and immunohistochemistry

Tissue sections were prepared after fixation of the tissues in Bouin's fixative and embedding in paraffin. Sections of 5–6 µm

were used for immunohistochemical analyses using a modified peroxidase ABC detection protocol (32).

Computer sequence analyses

Multialignment of the sequences given in Figure 2 was performed combining data of the program MULTALIGN with the data from pairwise alignments of the indicated sequences with the program GAP. MULTALIGN and GAP are included in the HUSAR software provided by the EMBL.

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