The AZFa gene DBY (DDX3Y) is widely transcribed but the protein is limited to the male germ cells by translation control

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Received June 24, 2004; Revised and Accepted July 21, 2004

We explored the function of the human DEAD-box Y RNA helicase DBY (DDX3Y) gene located in the (AZFa) region on the human Y chromosome (Yq11.21). Deletion of this Y interval is known to be a major cause for the occurrence of a severe testicular pathology, the Sertoli-cell-only (SCO) syndrome. DBY has a structural homologue on the short arm of the X chromosome DBX (DDX3X) (Xp11.4). We found widespread transcription of both genes in each tissue analyzed, although predominantly in testis tissue. However, translation of DBY was detected only in the male germ line, whereas DBX protein was expressed in all tissues analyzed. In testis tissue sections, DBY protein was found predominantly in spermatogonia, whereas DBX protein was expressed after meiosis in spermatids. We conclude that although both RNA helicases are structurally very similar, they have diverged functionally to fulfill different roles in the RNA metabolism of human spermatogenesis, and that deletion of the DBY gene is the most likely cause of the severe testicular pathology observed in men with AZFa deletions.

INTRODUCTION

The azoospermia factor a (AZFa) deletion interval has been mapped to proximal Yq11 (Yq11.21) (1). It is 792 kb in size and contains two protein-coding Y genes, USP9Y (ubiquitin specific protease 9 Y) and DBY (DEAD-box RNA helicase Y; DDX3Y) (2–4). Since AZFa deletions are found at high frequency in men with a complete germ cell loss [the Sertoli-cell-only [SCO] syndrome] (5,6), it has been suggested that the AZFa genes are functional during an early phase of human spermatogenesis (7,8). Both AZFa genes have a homologue on the short arm of the X chromosome (Xp11.4), USP9X [DFFRX (9)] and DBX (DDX3X) (10); [DDX3 (11); DDX14 (12); DDX3X (13)]. Their high sequence similarity to the Y copies (>94%) throughout the complete coding regions suggests that both genes should have a similar cellular function. Functional replacement of the yeast DEX1 RNA helicase with the human DBX gene or its mouse homologue DIPas1/PL10 (14,15) suggests that DBX proteins, and thus probably also DBY proteins, are required for the initiation of translation. However, a nuclear function is indicated as well, because the lack of DBX proteins in hamster ET24 cells resulted in an arrest of the cellular division cycle at the G1 phase (16).

Although northern blot analysis has shown expression of both AZFa genes in multiple tissues (10), USP9Y and DBY gene mutations were found to be associated only with distinct testicular pathologies; no somatic pathologies were reported. A point mutation at a USP9Y splicing site truncating the protein by ~90% was found in one man with spermatid arrest (17), and complete USP9Y deletion resulted in a similar testicular pathology (18, patient SAYER). DBY deletions were found in men with different testicular pathologies including the SCO syndrome (19). Therefore, it has been proposed that the AZFa genes are functional only in the male germ line and that the complete SCO syndrome observed in the testis tissue of men with a complete AZFa deletion is caused...
after deletion of both AZFa genes, USP9Y and DBY, or after deletion of only the DBY gene (17, 19, 20).

The highly homologous X copies, USP9X and DBX, are expected to function during spermatogenesis as well. It is therefore surprising that these are not able to compensate for the loss of USP9Y and DBY function in men with an AZFa deletion. One explanation might be that both AZFa genes form complexes with their X homologues in the male germ line, whereas another might be that the Y copy has a different role in spermatogenesis not provided by the X copy.

We addressed this challenging query first with respect to the DBY and DBX gene pair because DBY deletions were found in men with the most severe testicular pathology (19), and because some homologues of DBY in mouse (DIPas1/PL10) and frogs (Xan3) were found to be expressed in the male and female germ lines, respectively (21–23). Their conserved peptide boxes and overall similarity are distinct from that of the EIF1A (24), P68 (25) and VASA (26) homologues in these species and most comparable with that of the human DBY and DBX proteins. They, therefore, can be designated as the DBY protein subfamily of the highly conserved DEAD-box RNA helicases (27) with comparable molecular weights and a high sequence similarity (~90%) throughout the whole coding sequence, supporting the possibility of a similar germ line function for these RNA helicases in humans as well.

We studied the functions of the DBY and DBX genes in the human male germ line, separately. Their transcriptional profiles were distinguished by gene copy-specific RT–PCR analyses, their translational profiles by polyclonal antisera which mark specifically only the DBY- and/or DBX-encoded proteins in western blots and in testicular tissue sections. Our results suggest a translational control for DBY transcripts such that protein is synthesized only in the male germ line. The predominant expression of DBY protein in spermatogenesis reveals that deletion of the DBY gene in men with AZFa deletions is the most likely cause of their severe testicular pathology.

RESULTS

DBY and DBX are transcribed predominantly in testis tissue

The DBY and DBX genes were first identified from two different cDNA clones each (DBY1, DBY2 and DBX1, DBX2; GenBank accession nos AF000984, AF000985 and AF000982, AF000983, respectively) extracted from a testis cDNA library (10). In this early study, the authors suggested ubiquitous transcription of the DBY gene at similar levels in all the tissues analyzed, although testis-specific shorter DBY transcripts (3 kb) were indicated by their northern blot as well. The DBY hybridization probe used for this northern blot, however, also hybridized strongly with an RNA sample from human ovary, suggesting significant cross-reaction with the homologous DBX transcript (Fig. 2, in 10). The real level of DBY and DBX transcripts in each tissue analyzed could therefore not be determined by this experiment. For that reason, we first repeated the RNA blot experiment using a more specific DBY hybridization probe. With this probe, we detected predominant expression of DBY in testis tissue, where there were at least five different RNA species with lengths of 5.2, 3.9, 3.2, 2.9 and 2.7 kb (Fig. 1A). The 5.2 kb RNA was also found in all other tissues analyzed although at a lower and rather variable level. This DBY RNA blot pattern is thus significantly different from that of the earlier study, demonstrating variable quantities of DBY transcripts in the human tissues analyzed and distinguishing not one but five distinct RNA species in testis tissue by their different transcript lengths.

However, this DBY probe also shows weak cross-reaction with DBX transcripts, as indicated by its hybridization to the 5.2 kb RNA population in the ovary RNA sample (Fig. 1A). Thus, we cannot exclude the possibility that some of the other transcripts detected by the DBY probe are derived from the DBX gene.

We therefore used an alternative method to distinguish the transcripts of the DBY and DBX genes: semi-quantitative RT–PCR amplification using primer pairs specific for either the DBY or DBX transcript and their comparative analysis on a single agarose gel (Fig. 1B). We found DBY transcripts in all male tissues analyzed and in male leukocytes, but not in female leukocytes, which confirmed the specificity of the DBY primer pairs. DBX transcripts were also found in all male tissues and in male and female leukocytes, but always in a significantly lower quantity than DBY transcripts. The highest level of DBX transcripts was detected in testis tissue. Evaluation of the semi-quantitative expression patterns in the linear range of the fluorescent intensities (Fig. 1C) shows that there are tissue-specific differences in DBY and DBX transcript levels. In each male tissue, more DBY than DBX products were identified by PCR in the same RNA sample.

The antisera DBY-10/DBX-10 detect the individual DBY and DBX proteins specifically

The specificity of our polyclonal DBX and DBY antisera (DBY-10 and DBX-10) for the detection of only DBY or only DBX proteins were shown to be very high by specific peptide blot tests and peptide specific affinity chromatography (see Materials and Methods). Most crucial would be, however, an analysis of their cross-reaction on western blots containing protein extracts from Escherichia coli cells expressing full-length recombinant DBY and DBX proteins (for details see Materials and Methods). The DBY-specific antiserum only detects the recombinant DBY protein, whereas the DBX-specific antiserum only detects the recombinant DBX protein (Fig. 2). In the same kind of experiment, the DBXY-20 antiserum prepared with a specific peptide of DBY and DBX (see Materials and Methods) was able to detect both DBX and DBY proteins (data not shown). The three polyclonal antisera, DBY-10, DBX-10 and DBXY-20, should therefore also be able to distinguish DBY and DBX proteins on western blots of protein extracts from human tissues.
DBY proteins are only detectable in human testis tissue

We prepared cellular protein extracts from clinical biopsies of human male brain, kidney and testis tissue, and from female ovary tissue, for the analysis of DBY and DBX proteins. After fractionation on polyacrylamide gels they were blotted onto nylon filters and incubated with DBY- and DBX-specific antisera (Fig. 3A). No cross-reaction with the DBY-10
antiserum was observed with the ovary protein sample, as expected, but surprisingly the male brain and kidney protein extracts did not display any cross-reaction either. A distinct cross-reaction with the DBY-10 antiserum is indeed the DBY protein and that this protein is expressed only in testis tissue. When we incubated the same western blot—after stripping the filters with 2% SDS and 100 mM 8-mercaptoethanol and DBX protein expression was also found with other testicular specimens with complete spermatogenesis. Our results therefore strongly suggest that the DBY and DBX RNA helicases, despite their high sequence homology, have a different function in the developmental sequence of the human male germ line. Whereas DBY protein function is contributing mainly to the premeiotic spermatogonia phase, DBX proteins are functioning after meiosis in spermatids.

DISCUSSION
RNA helicases of the DEAD (Asp-Glu-Ala-Asp) box family have an ancient origin. They are found in almost all organisms and are associated with many processes in the life of an RNA molecule, ranging from synthesis to degradation (27). They rearrange inter- or intra-molecular RNA structures or RNA–protein complexes and all contain the same structurally conserved core element characterized by seven peptide domains conserved from yeast to human (27). This core element is flanked by divergent amino- and carboxy-terminal sequences determining the specific function of each RNA helicase and allowing the designation of distinct subfamilies.

In this paper, we present strong experimental evidence that the human DBX and DBY DEAD-box RNA helicases, although structurally very similar (>95%) along the complete length, are functionally expressed at two different phases of the male germ line (Fig. 3B). Moreover, although both genes are transcribed in all tissues analyzed and in leukocytes, the DBY protein was observed only in testis tissue, whereas the DBX protein was found in male brain and kidney as well as in female ovary tissue. These results support the
Figure 3. (A) Expression of DBY and DBX proteins in different human tissues. The western blot prepared from a 12% polyacrylamide gel containing similar quantities of protein extracts from human male brain, kidney, testis and female ovary tissues (a) was successively incubated with the DBY-10 (b) and DBX-10 (c) specific antisera as described in Materials and Methods. With the DBY-10 antiserum a single protein band of 73 kDa was detected only in the testis protein extract (b); with the DBX-10-specific antiserum a protein band of 73 kDa was detected in all tissues analyzed (c). No additional protein bands were detectable in blot b even after extending the luminescent film exposure time or using the higher sensitivity of the FEMTO kit for detection analyses. (B) Immunohistochemical analyses of the location of the DBY and DBX proteins in tissue sections of human testis optimized with two staining methods (ACE and DAB) and the ABC detection system as described in Materials and Methods section. The testicular tissue sections were first incubated with the DBY-10 and DBX-10 antisera (a, b and c). Using ACE staining after DBY-10 incubation, we localized DBY proteins predominantly in spermatogonia (see arrows with ‘spg’ in a and b picture); with DAB staining after DBX-10 incubation we localized DBX proteins predominantly in spermatids (see arrows with ‘spd’ in c picture). Occasionally, DBY-10 also stains some pre-leptotene/leptotene spermatocytes (see arrow with ‘lep-spc’ in picture a). When we incubated testicular tissue sections with the DBXY-20 antiserum, a combined staining of spermatogonia (spg) plus spermatids (spd) was consistently observed (picture d), confirming our western blot results that this antiserum is able to detect both the DBY and DBX proteins.
suggestion of Skaletsky et al. (28) who grouped the DBY gene with the X-degenerate Y gene class with a likely widespread function of the ancient X gene copy but a male-specific function of the Y gene copy.

Structural homologues of the human DBY and DBX genes in mouse [Dby/Dbx] (29); PL10/D1Pas1 (21,22), in frog [Xan3 (23)] and in zebrafish [ZFP110a (30)] are also functionally involved in the male and female germ lines. The DBY DEAD-box subfamily is therefore reminiscent to the Vasa DEAD-box subfamily with a similar conserved germ line function (31). However, whereas the location of VASA proteins in fetal and adult germ cells seems to be conserved from Drosophila to human (31) the location of proteins encoded from the DBY family in the male germ line seems to be distinct in mouse and human. Thus there is yet no evidence for a requirement of the mouse Dby gene at any stage of the mouse male germ line (32), whereas in human, DBY deletions cause a severe testicular pathology (19) and accordingly DBY proteins were found predominantly in spermatogonia (Fig. 3B). The mouse homologues, Dby and Dbx, are located in the Sxra interval of the short Y arm (Dby) and X arm (Dbx), respectively, and their transcription products were found in multiple tissues (29), whereas an autosomal retrogene of Dbx (PL10/D1Pas1 on chromosome 1; 101.5 cM) is expressed only in testis tissue (21,22).

The creation of intron-less retrogenes from their progenitor genes by retroposition of their transcripts seems to be a common mechanism for male germ line genes when they are located on the X chromosome and have a function at meiosis (33). Indeed, D1Pas1 protein is expressed predominantly in the nuclei of germ cells undergoing meiosis (22), where transcription of the mouse Dby and Dbx genes is repressed because of the presence of sex chromosome inactivation (MSC) and formation of the sex vesicle (34). A similar autosomal functional retrogene for the human DBY gene was not found (35). One may therefore assume that in human this back-up is not necessary because the DBY protein expressed until leptotene (see Fig. 3B) is stable enough to last also through the long and metabolically very active stage of the meiotic pachytene. Another possibility might be that in humans a functionally similar DEAD-box RNA helicase is replacing or complementing the DBY helicase function in the pachytene germ cells. Putative candidates are the VASA protein most abundantly expressed in spermatocytes (31) and the EIF2γA protein encoded from the autosomal retrogene EIF2γA (on 12p13.2–12.3) of the X chromosomal EIF2γX locus located in Xp22.1–22.2 (36). In contrast to its parental X gene copy, EIF2γA is expressed only in testis tissue. It has retained his ORF and is 97.5% homologous to the EIF2γX gene sequence. If this retrogene encodes a functional EIF2γA protein it is most likely required for the testis specific initiation of translation processes similar to we could show for the DBY DEAD RNA helicase in this paper (Fig. 3). Unfortunately, the location of the EIF2γ protein in the human male germ line is not yet known, but it might be interesting to recall in this context the germ line function of the EIF2γ mouse homologue, Eif2-γ3y, which is required for normal spermatogonial proliferation and progression of the mouse male germ cells through the meiotic prophase (32).

From the viewpoint of evolution, the Sxra interval on the short arm of the mouse Y chromosome has been considered to be functionally equivalent to the human AZFa interval, because after Sxrb deletion spermatogonia proliferation is disrupted in the mouse male germ line as was found after AZFa deletion in human. The associated functional mouse gene locus was therefore called spermatogonia proliferation y (Spy) locus (37). Indeed, the gene content of both spermatogenesis loci, Spy and AZFa, seemed to be comparable: both contain the syntenic triplets, Usp9y, Dby and Uty (homologues to UTY in proximal Yq11 adjacent to the AZFa deletion interval), which are structurally also conserved on the Y chromosome of cats and pigs (38,39). However, other Y genes which were also mapped to the mouse Spy locus, Eif2–s3y3, Smcy, Ube1y, Zfy, were not found in the human AZFa locus, suggesting that the functional conservation of both spermatogenesis loci might be limited. Accordingly, expression of the Eif2–s3y gene might then perhaps be considered as the functional equivalent of the human DBY gene and probably also vice versa, as discussed already in the last paragraph.

The presence of the DBY protein only in human testis tissue and its predominant location in spermatogonia suggests that it contributes to the function of human spermatogonia and that this function is essential because its deletion in men with AZFa or DBY deletions results in a severe testicular pathology including the SCO syndrome (1,19). An intriguing speculation in this context might be the assumption of some functional interactions between DBY and DBX transcripts (or proteins) in the male germ line constituting a haplo-insufficient genotype and genetic dominance of the DBY locus similar as proposed by Wilkie (40). This would also help to explain the variability of the testicular pathologies observed in some men with DBY deletions (19). Indeed, there is a higher level of DBY transcripts than DBX transcripts in all tissues analyzed and especially in testis tissue where only DBY and not DBX transcripts are prominent (Fig. 1). Accordingly, we would expect a different transcription profile for the DBX gene in men with DBY deletions. It is also possible, however, that the variability of the testicular pathologies in men with DBY deletions could be due to a primarily postmeiotic spermatogonial disruption after DBY deletion, and that the SCO syndrome is due to secondary age-dependent effects, like those described for patients with AZFc deletions (7,41). The SCO syndrome as a secondary age-dependent effect was also described in two mice strains (X0Sry/Eif2s3y and Xsxra0/0/Eif2s3y) which both lack Dby, the DBY mouse homologue (32).

MATERIALS AND METHODS
RNA analysis
Two human multiple tissue northern blots MTN-I and II (Clontech, Heidelberg, Germany) were hybridized with a 32P-labelled
PCR product generated from the 3' end of DBY cDNA (forward primer: 5'-GCTGATAGATTGCTGGATGAG-3'; reverse primer: 5'-TGCTTTCCGCGACTGACCC-3'). Sequences and PCR-conditions are deposited in the GenBank STS database (accession no. BV208342). Pre-hybridization and hybridization were carried out in 5 ml of ExpressHybTM solution according to the manufacturer's instruction. A 32P-labelled PCR product of the β-actin gene (GenBank accession no. NM_001101; forward primer: 5'-ATCCTGCTGCTGAACTTC-3'; reverse primer: 5'-GCTGATCACCACGTCTGCCT-3') expressed in each tissue was used as an internal control probe.

A semi-quantitative RT–PCR method (42) was performed to measure the levels of DBY and DBX mRNA separately with DBY- and DBX-specific primer pairs. The DBY primers are described above, the DBX specific forward primer (5'-GAAGCTCTAGAGTTTCTAC-3') and reverse primer (5'-TCTCAACATCAGTGAACCTTC-3') amplify a cDNA sequence of 422 nt between exon 3 and exon 7 only of DBX (GenBank accession no. BV208341). All cDNA–PCR reactions were carried out in a T96-Gradient cycler (Biometra, Goettingen, Germany). For the RNA extractions from the tissues indicated we used the RNeasy Mini Kit (Qiagen, Hilden, Germany) and followed the manufacturer's protocol. RT–PCR reactions were performed with ~40 ng oligo-dT primed cDNA, according to the following conditions: denaturation at 94°C for 45 s, annealing at 60°C (for DBX) and 62°C (for DBY) for 45 s and extension at 72°C for 45 s. After the chosen number PCR cycles (10–40), the corresponding PCR reaction was extended at 72°C for 5 min, cooled in ice-water and stopped with 0.5 M EDTA buffer. An amount of 4 µl of each reaction mix was run on a 1% agarose gel and quantitatively evaluated after staining with ethidium bromide and visualization on an UV transilluminator. Image analysis was performed with the Gel Doc™ 2000 IMAGER system and the Quantity One 1-D software (BIO-RAD, Munich, Germany).

**PROTEIN ANALYSIS**

**Preparation of polyclonal antisera**

The DBY and DBX proteins belong to the highly conserved DEAD box family of RNA helicases which has multiple members also in the human genome (13). Screening the GenBank/SWISSPROT/TREMBL database for human DEAD-box RNA helicases with a significant similarity to the DBY and DBX protein sequences only the proteins derived from the DX3x (GenBank accession no. AF061337) and DDX14 (GenBank accession no. U50553) genes were identified. Both genes seem to be identical to DBX because they had the same sequences, encode the same number of amino acids (662 aa) and mapped on the same site on the X chromosome (Xpl1.4). For the preparation of specific DBY and DBX antisera in rabbits, we therefore selected synthetic peptides from the N-terminal region of the DBY (DBY-10: VKKNPDELDQ) and DBX (DBX-10: AVENALGDLQ) protein. These were synthesized by Peptide Speciality Laboratories GmbH (Heidelberg, Germany). We also prepared a polyclonal antisera from a peptide sequence which is only found in proteins of the DBY subfamily (DBXY-20: ASTASKGRYIPPHELKNEAS).

Purified peptides were conjugated to a carrier protein (maleimide-activated keyhole limpet hemocyanin; KLH; 1 mg peptide/1 mg KLH) via their C-terminal cysteine residue using the Inject Activated Immunogen Conjugation Kit (Pierce, Bonn, Germany) and subcutaneously injected into New Zealand White rabbits following a standard booster protocol. Specificity and titre of the antisera were controlled by hybridization to ovalbumin coupled parental peptides spotted at different concentrations onto nitrocellulose membrane stripes (Sartorius, Goettingen, Germany). The DBY-10 antiserum detected only the parental DBY peptide and the DBX-10 antiserum only the DBX peptide. Positively reacting antisera were enriched from the crude blood serum by ammonium sulfate precipitation and purified by affinity chromatography (SulfoLink Coupling Gel Support resin; Pierce). All fractions containing a positive cross-reaction to only their parental peptide were brought to neutral pH conditions with 1 M Tris buffer (pH 8) and stored in siliconized tubes with 50% glycerol at −80°C.

**Preparation of DBY and DBX His-tagged recombinants expressed in M15 [pREP4] cells**

His-tagged fused DBX and DBY full-length recombinants were constructed by cloning in-frame the full-length cDNA products of both genes in the BamHI and SalI cloning sites of the pQE80L expression vector (The QIAexpressionistTM, Qiagen). Expression of the full-length His-tagged-DBY and DBX recombinants in M15 [pREP4] cells was induced with 1 mM IPTG (0, 30 and 60 min) and increasing quantities detected on western blots after incubation with Tetra-His anti-serum (Qiagen). Two clones with the highest expression titre (DBYfl-K13 and DBXfl-K68) were preserved with 15% glycerol at −80°C. For the competition assays large quantities of the DBY and DBX recombinants were prepared in 200 ml LB medium after growing of the cells to OD600 ~0.7–0.9 and 30 min induction with 1 mM IPTG. The recombinant proteins were purified after sonication (5 s bursts with microtip) by His-Tag affinity chromatography using a imidazol gradient between 0.1 and 0.5 M. Fractions of the pure protein were collected and stored at −80°C.

**Western blot**

The protein extracts from frozen tissue samples were homogenized on ice using standard protocols. Aliquots of 30 µg were loaded onto a 12% SDS–polyacrylamide gel for their size fractionation. Sample blocks (brain, kidney, ovary and testes) were stained with 0.2% Serva Blue R250 (Serva, Heidelberg, Germany), or electrophoretically transferred onto an Immobilon-P membrane (Millipore, Eschborn, Germany). After pre-incubation of the membranes in blocking solution [5% skimmed milk powder in 1X TNT (50 mM Tris, 150 mM NaCl, 5 mM EDTA and 0.05% Tween 20; pH 7.6)] for 30 min at room temperature, they were incubated with the affinity-purified antisera (1:100 v/v) for 1.5 h. For the competition experiments, both antisera were pre-incubated with different concentrations of the parental peptides, DBY-10 or DBX-10, or the purified recombinant DBY and DBX proteins, respectively. Visualization of the
hybridization pattern was achieved by incubation with an anti-rabbit IgG-peroxidase conjugate (1:30,000 v/v) as the secondary antibody (Dianova, Hamburg, Germany) in the same blocking solution for 30 min and the Western Lightning Chemiluminescence Reagent Plus kit (Perkin Elmer, Langen, Germany) according to the manufacturer’s instructions.

Immunohistochemistry
Paraffin blocks of embedded testicular tissue fixed in buffered formaldehyde. Bouin or Stieve solutions were derived from surgical biopsies of men with obstructive azoospermia selected for a normal pattern of germ cells in the testis tubules (kindly provided by M. Bergmann, Giessen). Tissue blocks were sectioned at 5 μm and mounted on SuperFrost Plus glass slides (Neolab, Heidelberg, Germany). After overnight storage at 37°C, sections were de-paraffinized with xylene (3 x 10 min) and re-hydrated with ethanol–water series. After washing for 3 min in permeabilization buffer (0.1 M Tris, 0.1 M NaCl, 0.1% Triton X-100; pH 7.4), the slides were incubated with 0.2 M boric acid, pH 7, at 60°C overnight. Standard avidin–biotin–complex (ABC) immunoperoxidase protocols (LINARIS, Wertheim, Germany, or Zymed, San Francisco, CA, USA) were used for staining the slides after incubation with the DBX-10 (1:100 v/v) and DBXY-20 (1:2000 v/v) antisera. Unspecific cross-reactions were blocked with 3% hydrogen peroxide in methanol, gentle shaking for 30 min at room temperature (RT). After pre-incubation of all slides in 3% goat serum (60 min at RT) incubation with the polyclonal antisera was performed overnight at 4°C in 1% goat serum. Slides (ABC treated) were stained with ACE (acetyl cabazole; Dako Cytomation, Glostrup, Denmark) or DAB (3,3’-diaminobenzidine tetrachloride:hydrogen peroxide; WAK Chemie, Steinbach, Germany) and subsequently counterstained with Gill’s hematoxylin II (VWR International, Bruchsal, Germany) before mounting in Immuno-Mount (Shandon, Pittsburgh, PA, USA).

ACKNOWLEDGEMENTS
We are indebted to Professor Martin Bergmann (University Giessen) for his generous and continuous support during the experimental phase of optimizing our immunohistochemical staining protocols and also training personally one of us (J.Z.). Hans Richard Rackwitz is thanked for his numerous experimental suggestions during preparation of the specific DBY and DBX peptides and antisera. The students Isabelle Dürk, Cornelia Kröger and Nina Rind are thanked for their experimental contributions to the comprehensive purification work of the crude DBY and DBX antisera and recombinant DBY and DBX proteins, respectively. Mrs Marie Luise Diarra and Prof. Peter Sinn (Section Gynecol. Pathology, University Women Hospital, Heidelberg, Germany) are thanked for their continuous support in preparing the testicular tissue sections and in the microscopic evaluation and imaging of the different immunohistochemical DBY and DBX staining patterns. This work was supported by a grant from the Deutsche Forschungsgemeinschaft to P.H.V. (DFG: Vo403/10-5).

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