

The *AZF*a 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 (*AZF*a) 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 *AZF*a deletions.

INTRODUCTION

The azoospermia factor a (*AZF*a) 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 *AZF*a 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 *AZF*a genes are functional during an early phase of human spermatogenesis (7,8). Both *AZF*a genes have a homologue on the short arm of the X chromosome (Xp11.4), *USP9X* [*DFFRX* (9)] and *DBX* (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 *DED1* 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 *AZF*a 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 *AZF*a 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 *AZF*a deletion is caused

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after deletion of both *AZF*a 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 *AZF*a deletion. One explanation might be that both *AZF*a 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 spermatogonia reveals that deletion of the *DBY* gene in men with *AZF*a 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.

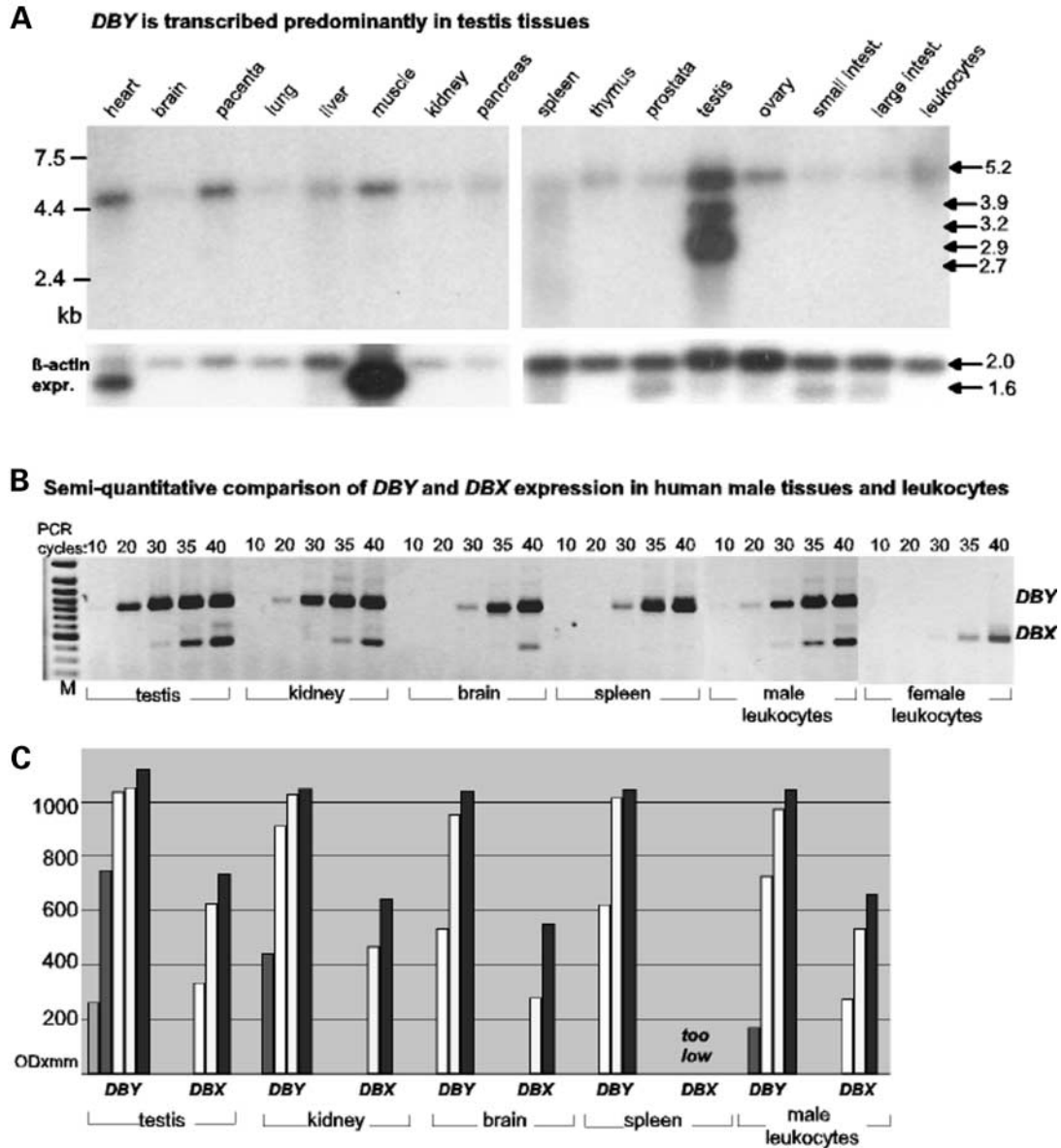


Figure 1. (A) Predominant transcription of the *DBY* gene in human testis tissue. The northern blots (MTN I and II from Clontech) contained the same quantity of polyadenylated RNA samples in each lane extracted from the tissues indicated. Only placenta and ovary tissues were of female origin. As a control for the loading of each lane, we subsequently hybridized the same blots with a β -actin probe demonstrating a high expression of its splicing variant (1.6 kb length) especially in muscle tissue. The *DBY* probe used identified at least five different RNA species in testis tissue with their length indicated at the right. (B) Semi-quantitative comparison of *DBY* and *DBX* expression by RT-PCR experiments in the male tissues indicated and in male and female leukocytes after different numbers of PCR cycles (10, 20, 30 and 40). The similar expression profile of the β -actin gene in all RT-PCR experiments was used as an internal control (data not shown). Further internal standardization was reached by comparing the fluorescent intensities of the different PCR products in the same gel using the same experimental conditions for the *DBY* and *DBX* RT-PCR experiments. For this purpose we chose different lengths for the RT-PCR products of *DBY* and *DBX* transcripts (*DBY* 814 bp; *DBX* 422 bp). More *DBY* than *DBX* transcripts are produced in each tissue analyzed, with the highest expression level of both genes in testis tissue. No *DBY* transcripts were detected in female leukocytes confirming the specificity of the used *DBY* primer pairs. The 'M' lane at the left contains an aliquot of the 100 bp DNA ladder (MBI Fermentas, St Leon-Rot, Germany) as a length marker. To mark the large difference of the fluorescent staining intensities between the *DBY* and *DBX* PCR products in the same agarose gel we performed their digital image analysis using the BIO-RAD imager software (C). Although the shorter *DBX* PCR products are expected to give a lower fluorescent staining intensity per molecule than the longer *DBY* PCR products, the large quantitative differences found are much greater than this small effect. In spleen, *DBX* expression is too low to be detected in the 4 μ l PCR aliquots used, but could be seen after loading a 10 μ l aliquot (data not shown).

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

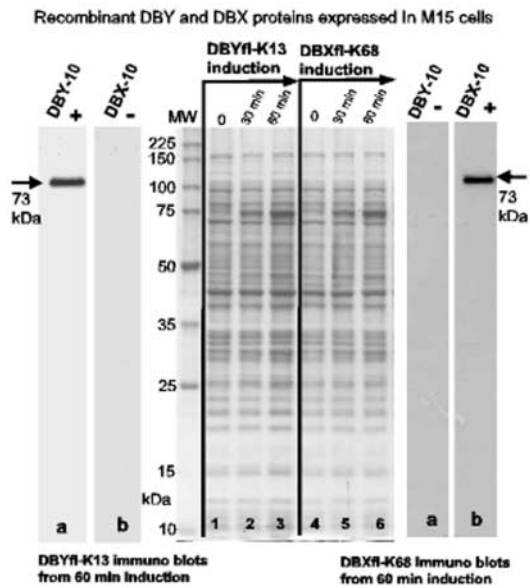


Figure 2. The polyclonal DBY-10 and DBX-10 antisera only detect the corresponding parental DBY and DBX proteins from the recombinants DBYfl-K13 (expressing DBY protein) and DBXfl-K68 (expressing DBX protein) in *E. coli* M15 cells after IPTG induction for 0, 30 and 60 min, respectively. The three lanes, 1–3, at the left show the time-points of DBYfl-K13 expression; lanes 4–6 show the time-points of DBXfl-K68 expression. Gels and blots were calibrated for a similar sample loading. Two western blot strips (one of DBYfl-K13 expression and one of DBXfl-K68) were prepared from the same protein extracts after the 60 min induction period and first hybridized with the DBY-10 antiserum (a), then with the DBX-10 antiserum (b) after stripping the filters with 2% SDS and 100 mM β -mercaptoethanol in a 62.5 mM Tris pH 6.8 buffer at 50–55°C. DBY-10 only cross-reacted with the recombinant DBY protein (a), DBX-10 only with the recombinant DBX protein. For protein gels we used the ‘broad-range protein’ molecular weight marker (10–225 kDa; Promega: cat# V8491) indicated at the left (MW). For further details see Materials and Methods.

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 to a single protein band was only found in the protein extract from the testis tissue (Fig. 3A). Its molecular weight of 73 kDa corresponds to the expected size of the DBY protein. We did not find cross-reaction to the other male tissues even when we extended the detection time or used the most sensitive kit available (SuperSignal® West Femto; Pierce, Bonn, Germany). When we pre-incubated the DBY-10 antiserum with its parental DBY-10 peptide or the recombinant DBY protein, cross-reaction to the testis protein was abolished as well. We therefore conclude that the testis protein detected by 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—with the DBX-10 antiserum, a protein with a similar size (73 kDa) was detected in each tissue analyzed (Fig. 3A). We found no cross-reaction on this western blot with the pre-immune serum of the rabbits used in the DBY-10 and DBX-10 immunization protocol. When we incubated the DBXY-20 antiserum with this western blot, the same staining pattern as seen with the DBX-10 antiserum was found (data not

shown). The protein of 73 kDa marked with the DBX-10 antiserum should therefore represent the DBX protein expressed in human male brain, kidney and testis tissue, and in the female ovary.

DBY and DBX proteins are expressed in germ cells of different spermatogenic phases

On the basis of the specificity of the DBY-10 and DBX-10 antisera (Fig. 2), we could now explore the expression of DBY and DBX separately in tissue sections from the human testis. The DBY-specific antiserum, DBY-10, stained predominantly the cytoplasm of spermatogonia suggesting a cytoplasmic function for DBY proteins in these germ cells (Fig. 3B). Occasionally, a prominent staining of the cytoplasm of some pre-leptotene/leptotene spermatocytes was observed as well. The DBX-specific antiserum, DBX-10, also stained germ cells but of a different spermatogenic phase, after meiosis: namely spermatids. To exclude the possibility that these pictures are non-specific cross-reactions, we confirmed our basic result by incubating some sections of the same testicular tissue with (1) the DBXY-20 antiserum which is expected to mark both proteins and (2) the pre-immune sera of the rabbits used for preparation of both antisera. No cross-reaction was obtained with the pre-immune sera, but the DBXY-20 staining picture seemed to combine the location of the DBY and DBX proteins staining the cytoplasm of spermatogonia and spermatids (Fig. 3B). The same pattern of DBY 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

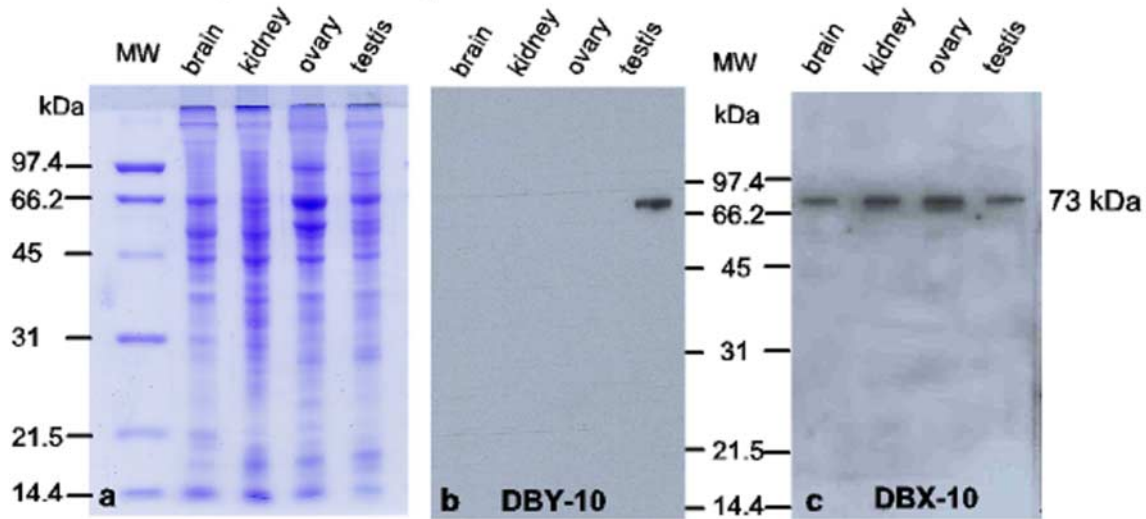
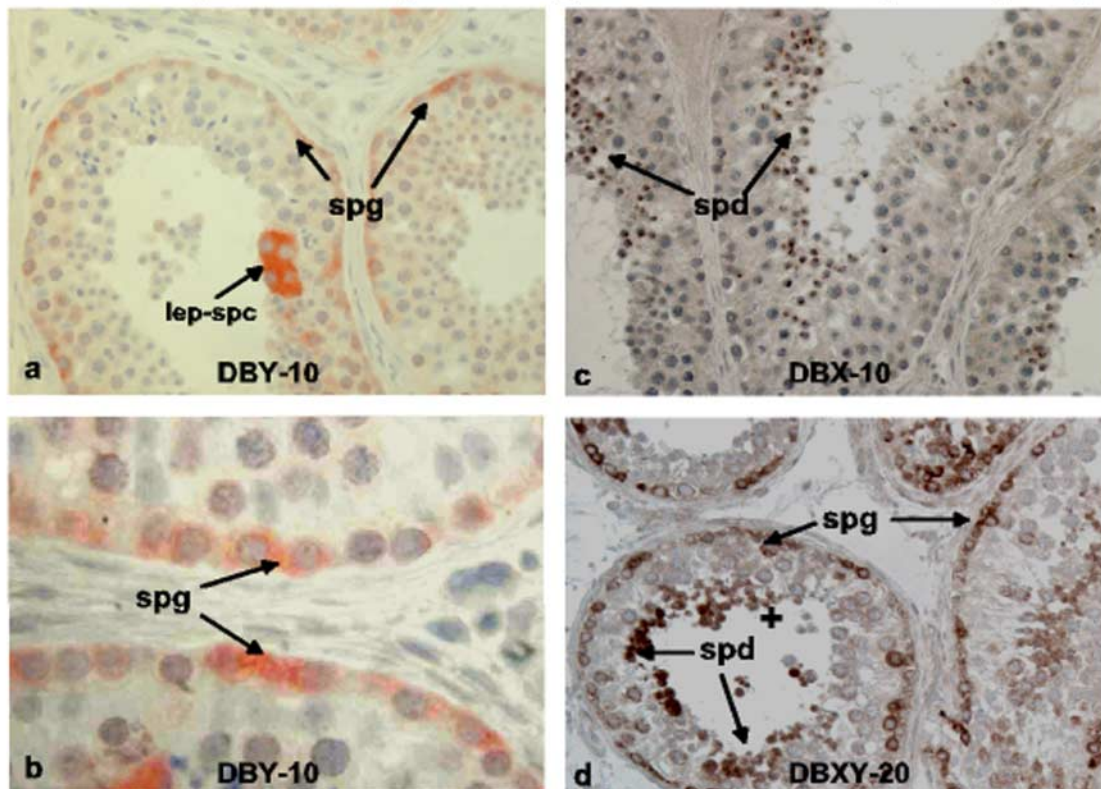
A DBY and DBX proteins are expressed in human testis tissue**B DBY and DBX proteins are expressed in germ cells of different spermatogenic phases**

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/DIPas1* (21,22)], in frog [*Xan3* (23)] and in zebrafish [*ZjPl10a* (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 *Sxr^b* 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/DIPas1* 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, *DIPas1* 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 meiotic sex chromosome inactivation (MSCI) 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 EIF2 γ A protein encoded from the autosomal retrogene *EIF2 γ A* (on 12p13.2–12.3) of the X chromosomal *EIF2 γ X* locus located in Xp22.1–22.2 (36). In contrast to its parental X gene copy, *EIF2 γ A* is expressed only in testis tissue. It has retained his ORF and is 97.5% homologous to the *EIF2 γ X* gene sequence. If this retrogene encodes a functional EIF2 γ A 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 EIF2 γ 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 *EIF2 γ* mouse homologue, *Eif2-s3y*, 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 *Sxr^b* interval on the short arm of the mouse Y chromosome has been considered to be functionally equivalent to the human *AZFa* interval,

because after *Sxr^b* 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-s3y*, *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* transgene and not that of the *Dby* transgene was able to restore normal spermatogonial proliferation in mouse strains with *Spy* deletion (32). The evolutionary pressure on Y genes functional for spermatogenesis in mouse and humans seems therefore to be different on the scale of a single gene and more comparable if setting the focus on the spermatogenic functions of the complete Y gene content of the *Spy* and *AZFa* loci, both involved in the same genetic network controlling the proliferation of spermatogonia. If this holds true, the mouse *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 spermatogenic 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 *XSxr^b0/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'-GCTGATAGGATGCTGGATATGG-3'; reverse primer: 5'-TGCTTCCGCGACTAGCACC-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 ExpressHyb™ 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'-ATCCTGCGTCTGGACCTG-3'; reverse primer: 5'-GCTGATCCACATCTGCTG-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'-GAAGCTACTAGAGGTTTCTAC-3') and reverse primer (5'-TCTCAACATCACTGAAACTTTC-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 cyler (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 μ l 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 *DDX3* (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 (Xp11.4). 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: VVKNDPELDQ) and *DBX* (*DBX*-10: AVENALGLDQ) protein. These were synthesized by Peptide Speciality Laboratories GmbH (Heidelberg, Germany). We also prepared a polyclonal antiserum from a peptide sequence which is only found in proteins of the *DBY* subfamily (*DBXY*-20: ASTASKGRYIPPHLRNKEAS).

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 Imject 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 sulphate 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 *Bam*HI and *Sal*I cloning sites of the pQE80L expression vector (The QIAexpressionist™; 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 antiserum (Qiagen). Two clones with the highest expression titre (*DBY*fl-K13 and *DBX*fl-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 blots

The protein extracts from frozen tissue samples were homogenized on ice using standard protocols. Aliquots of 30 μ g 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 1 \times 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 × 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 Immu-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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