Hominoid-specific SPANXA/D genes demonstrate differential expression in individuals and protein localization to a distinct nuclear envelope domain during spermatid morphogenesis

V.A. Westbrook1,2, P.D. Schoppee1, G.R. Vanage1,3, K.L. Klotz1, A.B. Diekman1,4, C.J. Flickinger1, M.A. Coppola1 and J.C. Herr1,5

1Department of Cell Biology, Center for Research in Contraceptive and Reproductive Health, University of Virginia, Charlottesville, VA, USA
2Present address: Department of Biochemistry and Molecular Genetics, University of Virginia, Box 800733, Charlottesville, VA 22908, USA
3Present address: Institute for Research in Reproduction, Mumbai, India
4Present address: Department of Biochemistry and Molecular Biology, University of Arkansas for Medical Sciences, 4301 West Markham Street, Slot 516, Little Rock, AR 72205, USA
5To whom correspondence should be addressed at: Department of Cell Biology, Center for Research in Contraceptive and Reproductive Health, University of Virginia, Box 800732, 1300 Jefferson Park Avenue, Charlottesville, VA 22908, USA. E-mail: jch7k@virginia.edu

Human sperm protein associated with the nucleus on the X chromosome consists of a five-member gene family (SPANXAI, SPANXAX, SPANXB, SPANXC and SPANXD) clustered at Xq27.1. Evolved from an ancestral SPANX-N gene family (at Xq27 and Xp11) present in all primates as well as in rats and mice, the SPANXA/D family is present only in humans, bonobos, chimpanzees and gorillas. Among hominoid-specific genes, the SPANXA/D gene family is considered to be undergoing rapid positive selection in its coding region. In this study, RT–PCR of human testis mRNA from individuals showed that, although all SPANX/ A/D genes are expressed in humans, differences are evident. In particular, SPANX C is expressed only in a subset of men. The SPANXA/d protein localized to the nuclear envelope of round, condensing and elongating spermatids, specifically to regions that do not underlie the developing acrosome. During spermiogenesis, the SPANXA/d-positive domain migrated into the base of the head as the redundant nuclear envelope that protrudes into the residual cytoplasm. Post-testicular modification of the SPANXA/d proteins was noted, as were PEST (proline, glutamic acid, serine, and threonine rich regions) domains. It is concluded that the duplication of the SPANX-N gene family that occurred 6–11 MYA resulted in a new gene family, SPANXA/D, that plays a role during spermiogenesis. The SPANXA/d gene products are among the few examples of X-linked nuclear proteins expressed following meiosis. Their localization to non-acrosomal domains of the nuclear envelope adjacent to regions of euchromatin and their redistribution to the redundant nuclear envelope during spermiogenesis provide a biomarker for the redundant nuclear envelope of spermatids and spermatozoa.

Key words: cancer/hominoids/spermatozoa/spermiogenesis/testis

Introduction

As early as the first drafts of the human genome, comparative genomic analyses suggested that a small subset of hominoid lineage-specific genes might encode traits conferring important adaptive advantages and offer clues to human evolution (Lander et al., 2001). Absent in lower primates including orangutan, rhesus macaque and tamarin, the five-member SPANXA/D family of genes at Xq27.1 was recently identified only in Homo sapiens, Pan paniscus, Pan troglo dytes and Gorilla gorilla. The ancestral gene family, SPANX-N, was identified in humans as five genes mapped to Xq27.1 and Xp11, with orthologous SPANX-N genes being present in lower primates and rodents (Kouprina et al., 2004). The SPANXA/D coding regions showed a 2-fold acceleration in the rate of synonymous and non-synonymous substitutions compared with non-coding regions, suggesting that the SPANXA/D coding regions were evolving much faster than intronic and 5′ untranslated regions under positive selective pressure (Kouprina et al., 2004). The five SPANXA/D genes each contained two exons and a small intron of 648 bp and were tightly clustered on the X chromosome (Westbrook et al., 2004). SPANXA, SPANXC and SPANXD encoded proteins of 97 amino acids, whereas SPANXB contained an 18-nucleotide insertion and encoded a 103-amino acid protein that is absent in Gorilla and Pan (Westbrook et al., 2000; Kouprina et al., 2004).

Bioinformatics and northern blot analyses in humans reveal the expression of the SPANXA/D genes among normal tissues only in testes (Westbrook et al., 2000). The characterization of the cellular localization and subcellular compartmentalization of SPANX proteins in the testis is a focus of this study. SPANXA/D also showed dysregulated...
expression in various human tumours (Zendman et al., 1999, 2003b; Goydos et al., 2001; Wang et al., 2003; Westbrook et al., 2004), including melanomas, breast carcinomas, glioblastomas and ovarian carcinomas, placing them in the category of cancer-testis antigens. The SPANXa/d protein has been localized to the nucleus or cytoplasm in over 50% of metastatic melanomas, with the incidence of SPANXa/d protein increasing in the more metastatic specimens (Westbrook et al., 2004). Abnormal expression of the SPANXa/d protein has also been noted in haematological cancers, with patients showing serum immune responses to SPANXa/d proteins (Wang et al., 2003). The connection of the SPANXa/D genes to cancer has been further underscored by fine mapping the SPANXa/D gene cluster within two cancer susceptibility loci: TGCT1, encoding a testicular germ cell tumour susceptibility gene (Rapley et al., 2000, 2003; Holzki et al., 2004), and HPCX, encoding a susceptibility gene for familial prostate cancer (Lange et al., 1999; Schleutker et al., 2000; Lander et al., 2001; Matikainen et al., 2001; Bochum et al., 2002; Stephan et al., 2002; Kibel et al., 2003).

The present study was undertaken to understand the patterns of SPANXa/D expression in individuals and to define the subcellular localization of the SPANXa/d protein during spermiogenesis. Among the most important findings are the following: (i) the SPANXc gene is expressed in only a subset of individuals; (ii) the SPANXa/d proteins appear post-meiotically; and (iii) the SPANX proteins undergo unique morphogenetic movements during spermatid nuclear condensation, being restricted to non-acrosomal regions of the spermatid nuclear envelope. This study leads to the conclusion that the replication of the SPANXc gene(s) that was initiated 6–11 MYA when primates were becoming more human-like resulted in a gene family SPANXa/D that may play an interesting role in post-meiotic spermatid nuclear differentiation.

Materials and methods
The University of Virginia Human Investigation Committee approved all studies involving human semen donors. Informed consent was obtained from each participant in the semen donor programme after an explanation of the nature and possible consequences of the studies. All procedures involving the use of animals were conducted in accordance with the Guide for Care and Use of Laboratory Animals (National Academy Press, Washington, DC, USA).

Preparation of spermatozoa
Ejaculated chimpanzee (P. troglodytes) spermatozoa were generously provided by Dr Rick Lee at the M.D. Anderson Cancer Center (Bastrop, TX, USA). Human semen, donated by healthy volunteers following 3 days of sexual abstinence, was centrifuged following liquefaction at room temperature, and the washed spermatozooe were either extracted or frozen for future use. Primate spermatozoa from freshly collected ejaculates were washed following liquefaction at room temperature and frozen. Frozen ejaculates were thawed at 37°C, and spermatozoa were pelleted by centrifugation at 400 × g and washed in Ham’s F10 medium (Irvine Scientific, Santa Ana, CA, USA). For immunofluorescent microscopy, washed spermatozoa were fixed at 4°C with 2% formaldehyde (Electron Microscopy Sciences, Fort Washington, PA, USA) in phosphate-buffered saline (PBS) for 20 min and washed thrice by centrifugation. Cells were air-dried onto slides and washed three times with PBS. For western blot analysis, spermatozoa were prepared as described below.

Preparation of testis sections
Testes were obtained from patients undergoing elective orchietomies. For fresh specimens, tissues were fixed overnight with 10% neutral buffered formalin (Sigma Chemical, St Louis, MO, USA). The samples were dehydrated through an ethanol series, embedded in paraffin, sectioned and mounted on slides. Formalin-fixed, paraffin-embedded tissue sections of chimpanzee testes were generously provided by the Coulston Foundation (Alamogordo, NM, USA). Before use, sections were dewaxed, rehydrated and treated with 0.25% hydrogen peroxide to block endogenous peroxidase activity. Slides were treated as described below for immunofluorescent microscopy.

Preparation of dissociated spermatogenic cells
Dissociated spermatogenic cells were prepared as previously described (Romrell et al., 1976; Westbrook-Case et al., 1995). Samples of human testis were obtained from surgical specimens. The tissue was decapsulated and minced in Ham’s F10 medium (Irvine Scientific). The tissue was incubated in Ham’s F10 containing 0.5 mg/ml of collagenase (Type XI, Sigma Chemical) for 20 min at 33°C with constant agitation of 60–80 cycles per minute. The dissociated tubules were allowed to settle, and the supernatant was decanted. The isolated tubules were washed twice with Ham’s F10 medium in this manner and incubated in Ham’s F10 containing 2.5 mg/ml of trypsin (Sigma Chemical) and 0.1 U/ml of micrococcal nuclease (Sigma Chemical) for an additional 20 min at 33°C with constant agitation of 60–80 cycles per minute. The resulting cell suspension was pipetted gently and washed thrice by centrifugation at 250 × g. Dissociated testicular cells were fixed at 4°C with 2% formaldehyde in PBS for 20 min and washed thrice by centrifugation. Cells were air-dried on slides and washed three times with PBS before immunofluorescence staining.

Immunofluorescent microscopy
Immunofluorescent staining was performed on the slides of chimpanzee spermatozoa, human-dissociated testicular cells and human and chimpanzee testis sections. To permeabilize the cells, we incubated the slides in methanol and washed them with PBS. Non-specific protein-binding sites were blocked by incubating the slides in PBS with 10% normal donkey serum (NDS). SPANXa/d antisera was produced by immunizing guinea pigs with recombinant SPANXa protein, as previously described (Westbrook et al., 2000). This immunoreagent recognizes 19 SPANXa/d isoforms by two-dimensional (2D) gel electrophoresis.

Slides were incubated with preimmune guinea-pig serum (1:250), immune guinea-pig serum (1:250) or mouse monoclonal antibodies (MHS-10 to the intra-acrosomal protein SP-10 (Herr et al., 1990a and 1990b) diluted in PBS with 1% NDS (PBS–NDS). The slides were washed and incubated with fluorescein isothiocyanate (FITC)– or tetramethylrhodamine B isothiocyanate (TRITC)-conjugated F( Ab)2 fragments of donkey anti-mouse immunoglobulin G (IgG)/IgM (1:200) or donkey anti-guinea-pig IgG (1:200) preabsorbed against guinea pigs and mice, respectively (Jackson ImmunoResearch, West Grove, PA, USA), in PBS–NDS. To control for cross-reactivity in this dual-labelling experiment, we incubated some control slides with guinea-pig primary antibodies and anti-mouse secondary antibodies or with mouse primary antibodies and anti-guinea-pig secondary antibodies. Preimmune serum or secondary antibodies alone were also used as negative controls for immunostaining. Slides were washed with PBS, fixed in 2% formaldehyde, washed and mounted with Slow-Fade Light (Molecular Probes, Eugene, OR, USA) containing 4′,6-diamidino-2-phenylindole (DAPI) II counterstain (Vysis, Downers Grove, IL, USA). Cells were observed by differential interference contrast (DIC) and epifluorescence microscopy using a Zeiss axiophot microscope. Digital images were obtained using Openlab cell imaging software (Improvision, Lexington, MA, USA).

Immunoelectron microscopy
For post-embedding immunolabelling, washed testis segments were fixed on ice with 4% formaldehyde, 0.5% glutaraldehyde and 1% tannic acid in 0.1 M sodium phosphate buffer, pH 7.4, rinsed in buffer, dehydrated through an ethanol series and embedded in Lowicryl K4M resin (Electron Microscopy Sciences). Thin sections were mounted on nickel grids and immunostained. Primary mouse antibodies were used at a concentration of 1:200, and 5 nm gold-conjugated secondary goat anti-mouse antibodies (Goldmark, Phillipsburg, NJ, USA) were employed at a concentration of 1:50. Grids were rinsed with PBS and water, stained with uranyl acetate and carbon coated. Specimens were viewed and photographed with a JEOL 100CX electron microscope.

Polyacrylamide gel electrophoresis and western blot analysis
Human spermatozoa and testes were obtained as described earlier. For sodium dodecyl sulphate (SDS)–polyacrylamide gel electrophoresis (PAGE), cell and tissue samples were extracted in 1% (w/v) SDS and centrifuged at 12 000 × g to remove insoluble debris. Protein concentrations of sperm extracts were determined by bicinchoninic acid (BCA) protein assay (Fierce Chemical, Rockford, IL, USA) using a bovine serum albumin (BSA) standard. Polypeptides were separated by one-dimensional (1D) SDS–PAGE using 15% polyacrylamide.
gels (Laemmli, 1970) with equal amounts of total protein loaded per lane. Benchmark prestained protein ladder (Gibco-BRL, Grand Island, NY, USA) standards were used to estimate the apparent molecular weight of analysed proteins. The proteins were transferred to nitrocellulose for immunostaining following standard immunoblot protocols (Towbin et al., 1979). SPANXa/d antiserum was produced by immunizing mice with recombinant SPANXa protein, as previously described (Westbrook et al., 2000). Sperm extracts were included on the gel as a positive control for the SPANXa/d protein. Immunoblots were washed with PBS (150 mM NaCl and 20 mM sodium phosphate, pH 7.5), incubated with blocking buffer (PBS, 0.05% Tween-20 and 5.0% non-fat dry milk), washed with PBS-Tween and incubated with anti-SPANXa/d antibodies diluted in blocking buffer. Preimmune serum were utilized as a negative control for immunostaining with mouse antiserum. After washing, blots were incubated with horse-radish peroxidase-conjugated goat anti-mouse IgG/IgM F(ab)2 fragments (Jackson ImmunoResearch) in blocking buffer and washed. Immunoreactive proteins were visualized by staining with tetramethylbenzidine (TMB)/peroxide substrate (AlerCHEK, Portland, ME, USA). RT–PCR and restriction enzyme analyses of testicular RNA

Testicular mRNA was obtained from the tissue specificity core of the Center for Research in Contraceptive and Reproductive Health at the University of Virginia. Pooled testicular mRNA was obtained from Ambion (Austin, TX, USA). To amplify SPANXa/d transcripts, we performed RT of mRNA followed by PCR amplification of the SPANXa/d cDNA. Briefly, 2 μm of poly(A)+ RNA from each tissue was reverse transcribed in the following manner. Poly(A)+ RNA, 0.5 μg (0.5 mg/ml) oligo(dT)12–18 primer and 4 μl of 5x buffer [250 mM Tris–HCl (pH 8.3), 375 mM KCl and 15 mM MgCl₂] were mixed in a 14-μl reaction volume, heated to 65°C and cooled slowly to 37°C. To this reaction, 1 μl of placental ribonuclease inhibitor, 2 μl of 100 mM dithiothreitol, 1 μl of 10 mM dNTPs and 2 μl (400 U) Moloney murine leukemia virus (MMLV) RT were added and mixed. The reaction components were incubated at 37°C for 60 min for the RT reaction to occur. To control for genomic DNA contamination, some reactions contained no RT enzyme.

SPANXa/d was amplified in a 50-μl PCR containing 5 μl of first-strand cDNA, 25 μl of IQ™ SYBR® Green Supermix (1x contains 50 mM KCl, 20 mM Tris–HCl, pH 8.4, 0.2 mM each dNTP, 25 U/ml of iTag DNA polymerase, 3 mM MgCl₂, SYBR Green 1 and 10 μm fluorescein; Bio-Rad Laboratories, Hercules, CA, USA) and 0.2 μg of the degenerate forward (5’-CCTRCYRWGACATYG-AAGAACC-3’) and reverse (5’-GCCSAAGKGTGAGRTGATGAC-3’) primers designed to amplify all four SPANXa/d gene products (R: A/G; Y: C/T; W: A/T; S: C/G and K: G/T). These primers produce a 355-bp fragment of SPANXa, SPANXc and SPANXf and 373 bp fragment of SPANXb. The positive controls included the amplification of each SPANXa/d cDNA from a plasmid and the amplification of pooled testis cDNA purchased from Ambion. The reaction mixtures were first heated to 95°C for 5 min, and then amplification was performed as follows: denaturing at 94°C for 30 s, annealing at 60°C for 30 s and extension at 72°C for 30 s. A final extension step at 72°C for 2 min was included following 35 cycles. A portion of the reaction (5 μl) was subjected to electrophoresis in a 2% agarose gel to be certain the amplification was successful. The PCR product was then purified over a column using the QAquick PCR purification kit (Qiagen, Valencia, CA, USA) according to the manufacturer’s protocol. Water was used to elute the cDNA.

The DraI, XhoI, SfiI, BsmBI and HaeIII restriction enzymes were purchased from New England Biolabs (Beverly, MA, USA). Each reaction (20 μl) consisted of 15 μl of PCR product in water, 2 U of enzyme, 20 μg of BSA and the recommended buffer. The restriction digestion was performed for 1 h at the recommended temperature after which the reactions were heat inactivated (when applicable), according to the manufacturer’s protocol. The resulting DNA fragments were separated in a 4% agarose gel (NuSieve 3:1; BioWhittaker Molecular Application, Rockland, ME, USA) made with TAE buffer (40 mM Tris-acetate and 2 mM EDTA, pH 8.5), stained for 10 min with SYBR Green (diluted 1:10 000 in TAE buffer; Molecular Probes) and visualized using a FluorImager 595 (Molecular Dynamic, Sunnyvale, CA, USA).

Results

Genomic organization of human SPANX and other cancer-testis antigens

The genomic region where the SPANX genes map has a high occurrence of cancer-testis antigens. The SPANX genes were clustered on the X chromosome in region q27.1, with the exception of the SPANX5 gene that was mapped to Xp11 (Figure 1). The SPANXa/d genes, which are more centromeric, were closely linked to the SPANX-N1/N4 genes within a 5-Mb region on the X chromosome. All SPANX genes were closely linked to genes encoding cancer-testis antigens of the MAGE and GAGE families. To date, the expression of

Figure 1. Map of the ‘recently evolved’ SPANXa/d and ‘ancestral’ SPANX-N gene families on human chromosome X. Five SPANXa/d genes (blue) are present in differing orientations (arrows) on the DNA strands, including identical duplications of SPANXa/d, designated SPANXa1 and SPANXa2. Four SPANX-N genes (green), N1–N4, are closely mapped to the SPANXa/d genes. The SPANX-N5 gene is mapped to Xp11. Other genes or putative genes that have been mapped to this region are indicated, including several cancer-testis antigens of the MAGE and GAGE gene families (red).
all SPANX-A/D genes, as well as SPANX-N3 and SPANX-N4, has been demonstrated by either cDNA or expressed sequence tag (EST) cloning. However, although numerous transcripts for the SPANX-A/D genes have been described, only one cDNA for SPANX-N4 and one cDNA and one EST for SPANX-N3 have been reported (Table I). The expression of SPANX-N1, SPANX-N2 and SPANX-N5 genes has not been demonstrated to date.

**SPANX protein sequence analyses and identification of PEST regions**

Previous studies have shown that the SPANXa/d proteins were highly charged proteins with isoelectric points (pls) ranging from 5.0 to 5.5 based on 2D western blots (Westbrook et al., 2001). In this study, we examined the polypeptide sequences encoded by each of the SPANX genes using bioinformatics approaches. Utilizing the ProtParam tool (http://us.expasy.org/tools/protparam.html), the human SPANXa/d proteins had pls ranging from 5.0 to 5.9, which was similar to pls demonstrated utilizing 2D western blot analysis (Westbrook et al., 2001). These polypeptides had >30% charged amino acids and were glutamic acid rich (Table I).

The human SPANX-N1/N5-deduced amino acid sequences were also glutamic acid and serine rich, with the number of charged amino acids >30%. However, the pls for these proteins ranged from 3.9 to 9.2. As described earlier, only human SPANX-N3 and SPANX-N4 have shown any evidence of expression utilizing bioinformatics approaches.

The polypeptide sequences for SPANX proteins of *P. troglodytes, G. gorilla, Pongo pygmaeus, Macaca mulatta* and *Mus musculus* also showed high glutamic acid content, >50% charged amino acids and pls ranging from 4.1 to 9.5 (mouse). The sequences for *P. paniscus* were not examined because an open reading frame could not be obtained from the available genomic sequences. Interestingly, like human SPANXa/D, but not human SPANX-N, mouse SPANX-N showed strong evidence of mRNA expression based on cDNA and EST cloning. All cDNAs and ESTs from the mouse were sequenced from the testis or germ cells.

Figure 2 shows a multiple sequence alignment of the SPANX proteins from various species including conserved PEST sequences (yellow) and nuclear localization signals (blue). The PESTfind algorithm (http://emb1.bcc.univie.ac.at/embnet/tools/bio/PESTfind/) defines PEST sequences as hydrophilic stretches between 12 and 60 residues in length rich in proline (P), glutamic acid (E) or aspartic acid (D) and serine (S) or threonine (T). They are flanked by positively charged lysine (K), arginine (R) or histidine (H) residues, but positively charged residues within the PEST sequence itself are disallowed (Rogers et al., 1986; Rechsteiner, 1990; Chevaillier, 1993; Rechsteiner and Rogers, 1996). PESTfind produces a score ranging from −50 to +50, with scores >45 considered significant. Using this algorithm, PEST sequences were predicted in all human, chimpanzee and gorilla SPANXa/d protein sequences as well as in SPANX-N sequences, with the exception of human SPANX-N5, chimpanzee SPANX-N1 and gorilla SPANX-N1 polypeptides. Significant PESTfind scores ranged from +5.72 to +18.12 (Figure 2, Table II). The human SPANXb, human SPANX-N3, human SPANX-N4 and orangutan SPANX-N3 proteins each contained two predicted PEST regions.

**Not all SPANX-A/D genes are expressed in individual testes**

To determine which SPANX-A/D genes were expressed in different individuals, we performed RT–PCR on testicular mRNA from nine men using primers that recognize all SPANXa/d sequences. These RT–PCR products were then digested with restriction enzymes specific for each of the SPANXa/d sequences and analysed by gel electrophoresis. SPANXa, SPANXb, SPANXc and SPANXd plasmids were used as positive controls for the specificity of the primers and the restriction enzymes. A pool of testicular mRNA (Ambion) served as a positive control for the expression of all SPANX-A/D genes.

The expected size of the uncut RT–PCR products is 355 bp for SPANXa, SPANXc and SPANXd and 373 bp for SPANXb (Figure 3) [Note: SPANXd and SPANXe GenBank designations represent polymorphisms of the SPANXd gene, whereas SPANXb and SPANXf represent polymorphisms of the SPANXb gene (Westbrook et al., 2004)]. Restriction enzymes Dral, XhoI, BsmBI and SfiI were chosen for these experiments to differentiate among the four SPANXa/d cDNAs. The predicted digestion patterns and fragment sizes are shown in a stick diagram above each panel in Figure 3. Table III describes the four SPANXa/d cDNAs cut by these individual enzymes, including the expected fragment sizes, and summarizes the incidence at which the SPANXa/D genes were detected in individual testes.

XhoI, which cuts only SPANXd and SPANXe, generating 237 and 118 bp products, showed digested fragments of the expected sizes in the testis pool, the SPANXd-positive control and all individual testes except for sample 3. As expected, XhoI did not cut the PCR products from the SPANXa-, SPANXb- or SPANXc-positive controls (Figure 3B). This indicated that eight of nine testes expressed SPANXD.

SPANXa does not contain a unique restriction enzyme site. Therefore, the strategy to confirm that SPANXa was present involved digesting the PCR products with two enzymes and comparing the resulting band patterns against the plasmid controls. The stick diagram (Figure 3A) demonstrates that DraI cuts SPANXa to yield 210 and 145 bp products and cuts SPANXd to yield 145, 118 and 92 bp products. DraI does not cut SPANXc. The PCR product from testis 3 yielded only 210 and 145 bp fragments that matched the SPANXa control, indicating that testis 3 expressed SPANXa but not SPANXD.

When both SPANXa- and SPANXd-positive controls were present and the sample was digested with both DraI and XhoI, bands of 237, 210, 145, 118 and 92 bp were identified (Figure 3A). Digestion of the SPANXd-positive control with DraI and XhoI resulted in only four bands, the absence of the 210-bp band being indicative of the absence of SPANXa. In Figure 3A, DraI/XhoI digestion of the SPANXa- and SPANXd-positive controls, the pooled testes and all of the other individual testis samples, except testis 8, resulted in five restriction fragments of the expected sizes. This result indicated that with the exception of testis 8, all testis samples contained both SPANXa and SPANXd. Digestion of PCR product from testis 8 resulted in only four bands; the 210-bp band was absent. This pattern demonstrated the presence of SPANXd and the absence of SPANXa in testis sample 8.

BsmBI, which cuts SPANXc generating 257 and 98 bp products, digested the testis pool, the SPANXc-positive control and testes 3, 6 and 9 (Figure 3C). However, BsmBI did not cut SPANXa-, SPANXb- and SPANXd-positive controls, the pooled testes and all of the other individual testis samples, except testis 8, resulted in five restriction fragments of the expected sizes. This result indicated that with the exception of testis 8, all testis samples contained both SPANXa and SPANXc. Digestion of PCR product from testis 8 resulted in only four bands; the 210-bp band was absent. This pattern demonstrated the presence of SPANXd and the absence of SPANXa in testis sample 8.

SfiI, which cuts SPANXb/generating 263, 101 and 9 bp products, showed digested products of the appropriate sizes in the testis pool, SPANXb-positive control and all the testis samples (Figure 3D). This indicated that SPANXB expression occurred in all testes examined.

**Localization of SPANXa/d protein in human and chimpanzee spermatids and spermatocytes**

Immunolocalization was performed to determine the subcellular localization of the SPANXa/d proteins during different stages of spermiogenesis using several anti-SPANX immunoreagents. Previous
Table 1. Protein analyses of the SPANXa/d and SPANX-N polypeptide sequences from species known to date

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<th>Gene</th>
<th>Species</th>
<th>Protein accession number</th>
<th>Locus</th>
<th>Expression data</th>
<th>Number of amino acids in polypeptide sequence</th>
<th>Number of negatively charged amino acids</th>
<th>Number of positively charged amino acids</th>
<th>Total number of positively and negatively charged amino acids</th>
<th>Percentage of charged amino acids in the polypeptide sequence</th>
<th>Isoelectric point (pI)</th>
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Data show the high percentage of charged amino acids in these proteins, the deduced pI and amino acids that are rich in these polypeptide sequences.
immunofluorescent studies of ejaculated human spermatozoa (Westbrook et al., 2000, 2001) utilized anti-recombinant SPANx/d antibodies from four individual mice, four individual guinea pigs and Olmsted affinity-purified mouse antibodies. Each immunoreagent localized the SPANx/d protein to the nuclear envelope within the cytoplasmic droplet and within nuclear craters. This pattern of localization was consistent with the localization of PEST sequences within the polypeptide sequence. The presence of PEST sequences was determined using the PESTfind program, which analyzed genomic sequences for potential PEST motifs. Table II summarizes the identification of PEST sequences in SPANx proteins, including the localization of PEST sequences within the polypeptide sequence and PESTfind scores. Figure 2 provides a comparison of SPANx/d and SPANx-N proteins, some derived from genomic sequences, highlighting the highly conserved nuclear localization signals (blue) and PEST motifs (yellow).
was confirmed by immunoelectron microscopy (Westbrook et al., 2001). Preadsorption of mouse and guinea-pig anti-SPANXa/d antibodies with recombinant SPANX protein abolished staining seen on human spermatozoa. Anti-SPANX antibodies raised against recombinant SPANXa recognize SPANXa, the most variable protein of this family, and SPANXc proteins on 2D gels as determined by mass spectrometry (Westbrook et al., 2001). SPANXa/d protein sequence similarity ranges from 79 to 97%.

To examine the localization of SPANXa/d during spermiogenesis, we performed indirect immunofluorescent labelling using anti-SPANXa/d antibodies on paraffin-embedded testis sections from humans and chimpanzees (Figures 4 and 5, respectively), enzyme-disassociated human testicular cells (Figure 6) and chimpanzee-ejaculated spermatozoa (Figure 7). The MHS-10 monoclonal antibody, which recognizes the acrosomal matrix protein SP-10 (Herr et al., 1990a,b), was used to stage spermatids in the testes based on the degree of acrosomal development.

In both human and chimpanzee testes, SPANXa/d staining was restricted to post-meiotic spermatids (Figures 4 and 5), which were identified by their characteristic nuclei and the presence of the acrosomal protein SP-10. Spermatogonia, spermatocytes and somatic cells did not demonstrate SPANXa/d or SP-10 immunofluorescent staining. In both species, some spermatids stained with the acrosomal marker SP-10 but did not stain with anti-SPANX antibodies, suggesting that SPANXa/d is present in some cohorts of spermatids but not in others. Furthermore, not all spermatozoa within the chimpanzee ejaculate demonstrated SPANXa/d protein staining (Figure 7). This localization of SPANXa/d to a subpopulation of spermatids and spermatozoa is consistent with the localization of SPANXa/d to 50% of human ejaculated spermatozoa that has been previously reported (Westbrook et al., 2001). Although cohorts of SPANX-negative spermatids were observed in the testes, the exact percentage of SPANX-positive spermatids in an individual testis has not been determined nor has the incidence of SPANX-positive spermatids been compared in different individuals. All negative controls described in Materials and methods showed only minimal background staining (data not shown).

In Golgi-phase spermatids (Figure 6A), the SP-10 protein was observed within the nascent acrosomal vesicle, and the SPANXa/d protein was localized to the nuclear periphery (Figure 6A). As the acrosomal vesicle expanded and flattened onto the anterior aspect of

Figure 3. Restriction enzyme digest analyses of testicular SPANXa/d RT–PCR products. The stick diagram above each gel represents the expected sizes of the restriction enzyme fragments for that enzyme. Individual gels show digestion products for DraI and XhoI, XhoI, BsmBI and SfiI. Each lane contains the resulting uncut and cut fragments of these specific restriction enzyme digests following RT–PCR of testicular mRNA. SPANXa, SPANXb, SPANXc and SPANXd are positive control plasmids for each of the four individual mRNAs. The expected size of the uncut RT–PCR products of SPANXa, SPANXc and SPANXd is 355 bp and of SPANXb is 373 bp. Ambion Testis Pool is mRNA obtained from a pool of donors. Testes 1–9 are mRNA samples from individual testes. Table III describes the SPANXa/d mRNAs cut by different restriction enzymes, including the expected fragment sizes, and summarizes the incidence of SPANX-A/D genes expressed in each testis.
the nucleus during the cap phase, SPANXa/d staining remained on the nuclear periphery but was absent in the region of the nuclear envelope underlying the developing acrosome (Figure 6B). Intranuclear regions known in the andrology literature as nuclear craters also demonstrated SPANXa/d staining (Figure 6C). As in mature spermatozoa (Westbrook et al., 2001), the craters observed in spermatids by immunofluorescence corresponded to SPANX-positive membrane whorls within nuclear vacuoles of spermatozoa as observed by immunoelectron microscopy (Figure 8A). These nuclear vacuoles frequently underlay the acrosome (arrows, Figure 8A).

In late cap-phase spermatids, as the acrosome spreads across the anterior nucleus, the spermatid cytoplasm begins to redistribute posteriorly forming the cytoplasmic lobe (Figure 6D). In successive stages of acrosomal development, SPANXa/d protein remained associated with the nuclear envelope posterior to the acrosome (Figure 6D–F), whereas the envelope underlying the acrosome remained unstained. Electron microscopy studies of spermatids at these stages revealed SPANXa/d immunogold staining of nuclear envelope posterior to the acrosome (Figure 8B). The nuclear chromatim adjacent to SPANX-positive regions remained euchromatic, whereas the remainder of the nuclear contents underwent condensation.

During the acrosome and maturation stages of human and chimpanzee spermiogenesis, the SPANXa/d protein migrated to the base of the spermatid nucleus and in some sections was observed to protrude into the cytoplasmic droplet and residual cytoplasm opposite the developing acrosome (Figures 4C, green; 5, green and 6G and H). Here, the protein associated with whorls of redundant nuclear envelope. This immunofluorescent localization within the cytoplasmic droplet is consistent with the localization of SPANXa/d to the redundant nuclear envelope observed in mature ejaculated human spermatozoa (Westbrook et al., 2001). Ejaculated chimpanzee spermatozoa also showed staining of nuclear vacuoles and the cytoplasmic droplet, as previously described for human spermatozoa (Figure 7). Meanwhile, as early as cap-phase spermatids, the SPANXa/d-negative domain underlying the acrosome began to extend beyond the margin of the acrosome (see non-stained interval between SP-10 domain and SPANX domain in the nucleus during the cap phase).
Although during evolution, the vast majority of duplicated genes are deleted or degraded into pseudogenes (Li et al., 2001; Glazko and Nei, 2003; Glazko et al., 2005), the present study confirms that SPANXA/D duplications are preserved as functional messages and suggests that these genes may contribute a selective advantage during spermatid nuclear morphogenesis.

Mechanisms underlying individual differences of expression

Although all the SPANXA/D genes are transcribed in human testes, individual differences in expression occur. SPANXb/f transcripts were identified in all nine individuals, and SPANXA and SPANXd transcripts were found in eight of nine men, but SPANXc transcripts were identified in only three of the nine individuals. Genomic DNA was not available from the individuals who provided the testicular RNA exam ined in this study; therefore, it is not yet certain whether SPANXA/D gene expression is differentially regulated and, if so, by what mechanism(s) or whether certain individuals lack a SPANX gene or contain interrupted or mutated coding regions. The ethnic origin of these testis donors is unknown. Therefore, it has not been determined whether differential expression may be due to ethnic or geographic origins.

In all nine individual testes examined, at least three of the four SPANX transcripts were expressed, suggesting if some individual genomes lack a SPANXA/D gene, it is likely a single gene. Because the SPANXc gene is absent in African great apes (Kouprina et al., 2004), it is likely a single gene. Because the SPANXc gene is absent in African great apes (Kouprina et al., 2004), it is likely a single gene. Because the SPANXc gene is absent in African great apes (Kouprina et al., 2004), it is likely a single gene. Because the SPANXc gene is absent in African great apes (Kouprina et al., 2004), it is likely a single gene. Because the SPANXc gene is absent in African great apes (Kouprina et al., 2004), it is likely a single gene.
Figure 7. Immunofluorescent/differential interference contrast (DIC) micrographs of ejaculated chimpanzee spermatozoa stained with 4′,6-diamidino-2-phenylindole (DAPI) II for DNA (blue) and tetramethylrhodamine B isothiocyanate (TRITC) for the SPANXa/d protein (red). SPANXa/d staining is observed in the posterior head associated with the cytoplasmic droplet and in the nuclear vacuoles (inset) of chimpanzee spermatozoa. This is consistent with SPANXa/d staining of human spermatozoa × 1000.

2004) and only three of nine humans show transcription of SPANXc mRNA in the testis, SPANXC in particular may be undergoing rapid evolution. An alternative explanation for the apparent lack of SPANX-C or other SPANX transcripts in some individuals might be SPANX-A/D gene conversion, the transfer of DNA sequences between two homologous genes within this family. Recent genomic studies indicate that the SPANXC locus is replaced by SPANXD or SPANX in some individuals (Kouprina et al., under review).

X-Linked gene expression and spermiogenesis

SPANX/A/D genes are remarkable in having undergone duplication on the X chromosome while exhibiting, among normal tissues, testis-specific expression and mRNA/protein localization exclusively in post-meiotic spermatids (Westbrook et al., 2000; Zendman et al., 2003b). Testis-specific expression of human SPANX/A/D is consistent with the available data, indicating the expression of the orthologous mouse SPANX-N in the testis. Only a few X-linked genes (Hendriksen et al., 1995; Moss et al., 1997; Turner et al., 1998) appear to follow a pattern of post-meiotic expression in the testes. This appears to be due to post-meiotic repression of X and Y gene expression caused by pachytene asynapsis and inactivation of the sex chromosomes (Turner et al., 2006). However, several recent articles demonstrate post-meiotic expression of a subset of X-linked genes (Ellis et al., 2005). The cysteine-rich perinuclear theca (CRYPT) family shares some similarities to SPANX gene family. The CYP family contains 15 genes, seven of which are X linked. Of these seven, six contain two exons and one is intronless (Hansen et al., 2006). These X-linked genes are expressed in post-meiotic spermatids and encode highly charged proteins localized to the post-acrosomal perinuclear theca (Kitamura et al., 2004; Hansen et al., 2006).

The hominoid SPANXa/d proteins are first detected in the nuclear envelope of early round spermatids in the Golgi phase of acrosomal biogenesis and reach their greatest deployment within the nucleus in cap-phase round spermatids before nuclear condensation. As nuclear condensation and elongation proceed, SPANXa/d proteins migrate as a distinct post-acrosomal domain of the nuclear envelope towards the base of the nucleus. In mature spermatids, the SPANXa/d proteins then associate with the redundant nuclear envelope within the residual cytoplasm. The SPANXa/d domain of the nuclear envelope is thus causal to the acrosome and reorganizes as acrosome biogenesis progresses, ultimately constricting into the redundant nuclear envelope. To date, five testes from individual men have been immunostained with the SPANX antibody using immunoperoxidase (not shown) and immunofluorescent staining of paraffin-embedded testis sections. All testes showed the results described. In addition, enzyme-dissociated cells from two men showed the same results. However, the immunofluorescent staining of testicular paraffin sections and enzyme-dissociated spermatids used in our study precluded precise quantification of the percentage of spermatids staining positively for SPANX protein. Although it is clear from these studies that only a subset of spermatids stained for the SPANX protein, further morphometric analyses will be needed to define the exact proportion of SPANX-positive spermatids.

The localization of SPANXa/d protein in chimpanzee spermatids and ejaculated spermatozoa to nuclear vacuoles and to the redundant nuclear envelope was similar to that observed in human spermatozoa (Westbrook et al., 2001). As the SPANX/A/D genes appear to be restricted to hominoids (Kouprina et al., 2004), the identical localization in human and chimpanzee sperm supports similar roles for SPANXa/d proteins in these species.

These results differ significantly from reports on the localization of SPANX published by Salemi et al. (2004, 2006). Using an anti-peptide mouse polyclonal antiserum on glutaraldehyde-fixed seminal specimens, these authors (Salemi et al., 2004) showed staining on 96% of spermatozoa including head (39.2%), midpiece (22.8%) or both sites (34.4%). In addition, staining of spermatagonia, spermatocytes and spermatids was also found with this antiserum in the testis. The findings of Salemi contrast significantly with our previous findings (Westbrook et al., 2000) and with those of Zendman et al. (2003a,b) that demonstrate post-meiotic expression of SPANX and differ as well from the precise localizations of the present study in the spermatid nuclear craters and post-acrosomal nuclear envelope as staged in relationship to acrosome biogenesis. The protein localization, described in this study, correlates well with in situ hybridization of SPANX mRNAs that were found only in post-meiotic spermatids (Westbrook et al., 2000). Taken together, past literature and the current study demonstrate SPANX mRNA and protein localization exclusively in spermatids.

Unlike the localization of SPANX to 96.4% of spermatids and spermatozoa by Salemi et al. (2004), our previous studies (Westbrook et al., 2001) demonstrated that 50% of ejaculated spermatozoa showed staining of the nuclear craters and cytoplasmic droplet, corresponding to the redundant nuclear envelope. This staining of the nuclear envelope in spermatozoa and spermatids has been demonstrated with many reagents: four individual mouse polyclonals made against SPANXa recombinant protein, immunoaffinity-purified mouse polyclonals, four individual guinea-pig polyclonal antisera raised against SPANXa recombinant protein and three rabbit polyclonal antibodies raised against three different SPANX peptides (Kouprina et al., under review). Furthermore, staining was abolished when these anti-peptide antibodies were preadsorbed with their specific peptides, but not different peptides. Staining was also abolished if guinea-pig polyclonal antisera were preadsorbed against SPANXa recombinant protein.

Furthermore, two rabbit polyclonal antisera, which we raised against specific SPANX peptides, showed staining over the entire cell in virtually all spermatozoa. However, this staining remained following preadsorption of these two anti-peptide antibodies with their specific peptides, indicating background, artefactual and non-specific staining was being observed. These results with rabbit anti-peptide antisera may
Figure 8. Electron micrographs of human spermatids following post-embedding labelling with anti-SPANXa/d antibodies. In this cap-phase spermatid (A), staining was not observed associated with the nuclear envelope (ne) underlying the developing acrosome (a). However, gold particles (5 nm) were present on membrane whorls lying within a nuclear vacuole (NV) that opened into the subacrosomal space. In this elongated spermatid (B), gold particles were associated with both the nucleoplasmic and the cytoplasmic faces of the nuclear envelope (ne) posterior to the acrosome (a) and adjacent to the manchette microtubules (M, white arrows). Staining of the nuclear envelope was not observed underlying the developing acrosome (a, bars mark posterior aspect of acrosome) or in the region immediately posterior to the acrosomal bulbs where the nuclear envelope is closely apposed to the nucleus. The inset shows a fold of the nuclear envelope at the posterior region of the spermatid nucleus which exhibited a concentration of gold particles indicative of SPANXa/d staining. M, manchette; N, nucleus. A × 20,000; B × 16,000.
between the posterior edge of the acrosomal bulbs and the anterior boundary of SPANXa/d-positive domain (Figure 6D and E). This correlation further supports the interpretation that the distinctive patterns of SPANXa/d immunofluorescence result from the exclusion of SPANXa/d from the thickened subacrosomal regions of the nuclear envelope beneath and posterior to the acrosome.

Ultrastructural observations have also revealed scalloping (waves) in the nuclear envelope at the caudal end of the nucleus in condensing spermatids (Holstein and Roosen-Runge, 1981). Our SPANXa/d immunofluorescent observations of similar stages (Figure 6F) also showed a wavy appearance (Figure 6D and E). As noted earlier, in elongating spermatids, the area immediately posterior to the acrosome, described as the post-acrosomal dense lamina, is known to lack nuclear pores (Holstein and Roosen-Runge, 1981). However, it is noteworthy that posterior to this region, the sac-like expansions of redundant nuclear envelope retain nuclear pores (Holstein and Roosen-Runge, 1981) and that the pattern of SPANXa/d localization correlates with the nuclear pore-containing regions.

**SPANX localization and chromatin**

The localization of SPANXa/d protein in spermatids and sperm must also be considered in the light of chromatin dynamics during spermiogenesis. DNA condensation by transition proteins and protamines is thought to protect the male genome from physical, chemical and mutagenic injury during epididymal storage and transport through the male and female reproductive tracts. Most of the highly condensed chromatin remains transcriptionally inert. Interestingly, the relationship of the nuclear envelope to regions of euchromatin and heterochromatin demonstrates ultrastructural differences proximal and distal to the posterior ring, a biochemical and biophysical barrier between the anterior and posterior aspects of the sperm head. Anterior to the posterior ring where the inner and outer lamellae of the nuclear envelope are closely apposed, largely obliterating the lumen of the perinuclear cisterna, the nuclear envelope is tightly associated with the condensed nuclear chromatin and lacks nuclear pores (Fawcett, 1970). However, distal to the posterior ring and embedded within the cytoplasmic droplet, where the nuclear envelope evaginates forming the redundant nuclear envelope, a nuclear space devoid of condensed chromatin is found. This heterogeneity in both the sperm nuclear envelope and the adjacent chromatid may be significant and relate to growing evidence that sperm retain poly(A)⁺ mRNAs (Ostermeier et al., 2002; Dadoune et al., 2004). While gene transcription in ejaculated spermatozoa remains controversial, it is striking that SPANXa/d localizes to nuclear craters, intranuclear structures in spermatids lacking condensed chromatin, and to the redundant nuclear envelope that also lacks condensed chromatin. Thus, from the light and electron microscopic observations, the hypothesis may be advanced that SPANXa/d proteins play a role in maintaining domains of euchromatin in condensing spermatids.

**Analyses of the SPANX protein sequences**

More than 19 tightly clustered, argyrophobic spots corresponding to SPANXa/d proteins have previously been identified on 2D western blots of ejaculated human sperm extracts, a heterogeneity that cannot be accounted for simply by four gene products (Westbrook et al., 2001). The western blot analysis presented in this study confirms that at least some post-translational modifications of SPANX-a/d occur from the time spermatozoa leave the testis to the time of ejaculation. The exact nature of these modifications remains to be elucidated.

The highly charged nature of the SPANXa/d proteins has been demonstrated previously by 2D western blots and bioinformatics approaches. This study shows that all SPANX (a/d and N) proteins

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### Figure 9

Western blot analysis of human sperm and testicular proteins immunostained for SPANXa/d. Polyclonal antiserum (I) raised against recombinant SPANXa/d recognized polypeptide bands in sperm and testis extracts of 15–20 and 15–16 kDa, respectively. Only light background staining was observed with preimmune serum (pi). A, amido black stain.

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**Integration of SPANXa/d localization with fine structural literature**

Electron micrographs have shown that approximately half of the surface of the acrosomal vesicle abuts the indent nuclear envelope (Holstein and Roosen-Runge, 1981) in early round spermatids. As the acrosome spreads and flattens, the underlying nuclear envelope has been characterized as lacking both nuclear pores and perinuclear cisterna (Holstein and Roosen-Runge, 1981). Fine structural studies also show that, as chromatin condensation and nuclear elongation proceed, the nuclear envelope underlying the acrosome is thickened by a dense layer that obliterates the perinuclear cisterna and extends slightly beyond the free edge of the acrosomal cap (Holstein and Roosen-Runge, 1981). This small gap was apparent as a SPANXa/d-negative domain

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**Offer one explanation for the discrepancy between the results of our previous and present studies and the results reported by Salemi et al.**
described to date have a high percentage of charged amino acids (~30%). Nevertheless, the pIs range from 3.9 to 9.5 with most of the proteins of either an acidic or a basic nature (i.e. few demonstrate neutral pIs). This suggests that a general charged nature, as opposed to specific positive or negative residues, may be important in the functional biochemistry of the SPANX proteins in the testes or spermatozoa.

The localization of SPANXa/d to a subpopulation of spermatids and spermatozoa raises questions as to the mechanism(s) of this phenomenon. PEST regions serve as signals for proteolytic degradation (Rechsteiner, 1990; Rechsteiner and Rogers, 1996; Klonowski, 2001). They are present in metabolic enzymes, transcription factors, protein kinases, protein phosphatases, cyclins and immunogenic peptides presented on major histocompatibility complex (MHC) I molecules (Schimke, 1973; Chevailler, 1993; Gomes and Barnes, 1997; Niedermann et al., 1997; Sekhar and Freeman, 1998). PEST-containing proteins appear to be targeted for ubiquitin-26S proteasome-mediated degradation, although calpain cleavage has also been implicated in the degradation of PEST-containing proteins (Eto et al., 1995; Rechsteiner and Rogers, 1996). The deletion of PEST regions in numerous proteins has been shown to considerably increase protein stability, and the transfer of PEST regions to stable proteins leads to rapid degradation of the fusion protein. The presence of predicted PEST sequences in all SPANX/A-D genes, as well as in most SPANX-N genes, suggests that selective pressure is operating to conserve this motif and regulate the precise temporal and spatial distribution of the SPANXa/d protein in post-meiotic spermatid nuclei.

**Implication of SPANXa/d testicular localization for cancer immunotherapy**

Interest is growing for anti-tumour vaccines employing SPANXa/d because (i) SPANXa/d mRNA and protein expression has been described in >50% of melanomas (Westbrook et al., 2004), (ii) SPANXb expression has been found in multiple myelomas and other haematological malignancies (Wang et al., 2003) and (iii) SPANXb immunogenicity has been demonstrated in humans with haematological tumours (Wang et al., 2003). The present findings that SPANXa/d proteins are restricted in humans to post-meiotic spermatids on the adluminal side of the blood testis barrier suggest that the treatment of men and women with SPANXa/d vaccines would be tumour specific without affecting other organs or tissues and that the treatment of men would probably spare spermatogonia and spermatocytes and thus preserve fertility.

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**References**


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