Characterization, expression pattern and chromosomal localization of the spermatogenesis associated 6 gene (Spata6)

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We report the cloning and characterization of the spermatogenesis associated 6 gene (Spata6) encoding a predicted protein of 488 amino acids. It exhibits similarity with the motor domain of kinesin related proteins and with the Caenorhabditis elegans neural calcium sensor protein (NCS-2). The gene encodes three mRNAs of ~2.6, ~1.8 and ~1.2 kb. The expression of the 2.6 kb mRNA is detected at low levels in testis, ovary, thymus and placenta, while the 1.8 and 1.2 kb transcripts are exclusively expressed in testis. The 1.8 and 1.2 kb transcripts are specified in haploid germ cells. Data from in situ hybridization experiments suggested that mRNA expression of Spata6 in spermatids is higher than in spermatocytes and spermatogonia. RT–PCR analysis and whole mount in situ hybridization demonstrate that the Spata6 transcript is expressed during embryonic development and is localized in neural tube, somites and limb buds of mouse embryo. The Spata6 gene consists of 15 exons ranging in size between 40 and 596 bp. The 2.6 and 1.8 kb transcripts have different 5’ untranslated sequences but have the same translational initiation site and therefore may encode the same protein with a predicted molecular weight of 49.7 kDa. The 1.2 kb transcript is derived from a proximal promoter between exons 7 and 8, and contains a translation initiation codon AUG, which is in frame with initiator AUG codon of the 2.6 and 1.8 kb transcripts. Therefore, the 1.2 kb transcript may code for a truncated protein of 32 kDa. Western blot analysis with the antiserum raised against a synthetic peptide from the C-terminal of the deduced Spata6 protein detects only a single protein of 53 kDa in all tissues studied. The Spata6 gene was localized to chromosome 5, region q34-35 in the rat and to chromosome 1, region p32-35 in the human. In an effort to determine the function of Spata6, we inactivated the mouse gene in embryonic stem cells through homologous recombination. Although the heterozygous mutant cells were able to generate low coat colour chimeric mice, all chimeras did not transmit the targeted allele to their progeny suggesting that a high contribution of Spata6\(^{+/−}\) cells lead to the lethality of the chimeric embryos.

Key words: cDNA/expression/gene/mutation/neural tube/Spata6/spermatogenesis

Introduction

Spermatogenesis, the development of spermatogonial stem cells into spermatozoa, is a highly ordered process. This process occurs within the seminiferous tubules and is divided into three stages. The first consists of mitotic proliferation and differentiation of the spermatogonial stem cells into diploid primary spermatocytes. The second stage consists of meiotic division of the primary spermatocytes to produce haploid spermatids. During the final step, spermiogenesis, spermatids are morphologically transformed into highly differentiated spermatozoa. Many proteins are involved in this process of differentiation. Some of these proteins represent testis-specific isoforms of genes that are expressed in other tissues and are generated by post-transcriptional processing/alternative splicing or polyadenylation (Venables, 2002). Others are exclusively expressed in testis and are anticipated to act as critical factors for initiation and progression of spermatogenesis (Eddy and O’Brien, 1998).

In an effort to examine the molecular basis of spermatogenesis, we have used a differential cDNA screening method to isolate clones representing genes expressed exclusively in testis. In this report, we report the isolation, characterization, expression pattern and chromosomal localization of the spermatogenesis associated 6 gene (Spata6), which is expressed in three mRNA forms. Expression of two Spata6 transcripts is restricted to the haploid spermatids. To determine the physiological role of the Spata6, we have mutated the gene in embryonic stem (ES) cells through homologous recombination, generated chimeric mice deriving from four homologous recombinant ES lines and studied the contribution of the recombinant cells in different tissues and spermatozoa of some chimeric mice.

Materials and methods

Animals, embryo preparation and tissue culture

All mice used in these experiments were kept in air-conditioned and light-controlled rooms. Mice mutants W/W, Tjfn/Y, ol/tol and qk/qk (Lyon and Searle, 1989) were purchased from the Jackson Laboratory (Bar Harbor, USA). Female mice were mated in the afternoon and on the following morning the
vaginal plug was determined as 0.5 day post-coitum (d.p.c.). Embryos between 8.5 and 14.5 d.p.c. were decapitated and used immediately for RNA isolation or fixed in 50 mM PBS containing 4% paraformaldehyde. All animal experiments were carried out according to the best practices approved by the Medical Faculty of the University of Göttingen and Research Advisory Committee.

The determination of the mouse and rat Spata6 cDNA sequence

A rat testis cDNA library was screened by the –/+ screening method (Sargent, 1987). The [32P]-labelled cDNA to be used as the probe was synthesized from poly(A)-rich RNA prepared from rat testis using MML reverse transcriptase (Gibco BRL) and poly T primer. Several cDNA clones that gave strong hybridization signals with the testis cDNA probe were used to characterize the gene expression pattern by Northern blot analysis. A 800 bp cDNA fragment (rS 0.8) that hybridized with three testicular transcripts of 2.6, 1.8 and 1.2 kb was used to identify the full-length cDNA. Mouse and rat testis Agt11 cDNA libraries (Clontech, Palo Alto, CA, USA) were screened on nitrocellulose filters using a [32P]-labelled 800 bp rat cDNA (rS 0.8) probe. The hybridization was performed under standard conditions (Sambrook et al., 1989). Three mouse clones containing 1.1 (mS 1.1), 1.9 (mS 1.9) and 2.1 (mS 2.1) kb cDNA fragments, and four rat clones with 1.4 (rS 1.4), 1.6 (rS 1.6), 1.8 (rS 1.8) and 2.1 (rS 2.1) kb cDNA fragments were isolated, subcloned into pBluescript SK (Stratagene, La Jolla, CA, USA) and sequenced using an ABI 373 DNA sequencer (Applied Biosystems, Weiterstadt, Germany). To identify the full-length mouse Spata6 cDNA and the 5’ end of the three mouse Spata6 mRNAs, we performed 5’ RACE analysis. Total RNA and poly(A)+-enriched RNA isolated from mouse testis using Trizol reagent (Gibco BRL) and the oligotest-spin column method (Qiagen, Hilden, Germany) respectively. To determine the 5’ sequence of the 2.8, 1.8 and 1.2 kb transcripts, a Marathon-ACE (Clontech) was performed on mouse testis cDNA using Spata6-specific antisense primers: Ho9 5’-GAG GAA ATC GTA GGC CTA ACG-3’; Ho4 5’-GCT GGA TTA CAT TAA AGC ACTG-3’ and Ho6 5’-GAT GGA AGG ACT TTA TAC CAA-3’ (base pairs 1529–2329). The cDNA probe was labelled using digoxigenin-11-UTP and whole mount in situ hybridization was performed according to standard procedures as described previously (Xu and Wilkinson, 1998). The digoxigenin-labelled probes were detected with NBT/BCIP (Boehringer). The stained embryos were embedded in 7% gelantine/15% suroce and 50 μm sections were cut. Experiments were performed twice with eight embryos each.

Polyclonal antibodies and Western blot analysis

The polyclonal antibodies to Spata6 were raised by immunization of rabbits with peptide NH2-SHQAHARHLCDERDPEREDE-COOH (Figure 4). The coupling of the peptide to bovine serum albumin (BSA) was performed using glutaraldehyde and affinity purification of antibody was performed as described previously (Harlow and Lane, 1988). The tissues were homogenized in 10× vol of SEM buffer [0.32 mol/l sucrose, 1 mmol/l EDTA and 0.1% (v/v) mercaptoethanol] and adjusted to a final protein concentration of 10 μg/ml. Twenty micrograms of each homogenate were loaded into a pre-cast 4–12% vol sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) Bis-Tris gel (Invitrogen, Groningen, Netherlands). After electrophoresis, the proteins were transferred to PVDF membranes (Amersham) as described by Harlow and Lane (1988) and thereafter the membranes were incubated with the Spata6-specific antibodies overnight at 4°C. After washing, the bound antibodies were detected using the avidin-biotin-peroxidase complex system (DAKO, Denmark).

Chromosomal localization

Fluorescent in situ hybridization (FISH) was performed as described previously (Pinkel et al., 1988; Stephanova et al., 1996). The probe used for FISH was clone 3E, containing a 7 kb EcoRI fragment from the rat Spata6 gene and the human Spata6 P1 clone (ICRFp700p/98116q6).

Generation of Spata6 chimeric mice

A λ phage clone carrying a mouse genomic fragment of Spata6 was isolated from a 129/Sv genomic mouse library (Stratagene) by using the cDNA fragment containing sequences of exons 3 and 4. For the determination of the restriction map of the Spata6 locus and localization of the exonic sequences, the 12.5 kb isolated genomic fragment was subcloned into the pZERO-TM-2 vector (Invitrogen) and examined by Southern blot analysis. The Spata6-targeting vector was constructed using plasmid pPNT (Tybulewicz et al., 1991), which contained a neomycin resistance gene driven by a PGK promoter (pgk-neo) and a herpes simplex virus thymidine kinase gene (tk). The 5.7 kb Srf fragment containing a sequence of intron 2 was isolated and ligated with Xhol-digested pPNT vector after treating with Klenow enzyme (clone Spata6/1). The 2.7 kb Spel/Nor fragment (Nor site from polylinker of phage clone) containing a sequence of intron 4 was used as the 3’ homologous arm and inserted into 42 R (5’-CAA ATG AGA AGA GCC AGC CGG CCG-3’), and reverse transcriptase superscript II (Gibco BRL) at 42°C for 1 h. The PCR amplification was carried out using sense primer HA 25F (5’-GTG TTC ACC TCC AAA CTG TGA TGA-3’) and antisense primer HA42R, and Taq polymerase for 35 cycles at 94°C for 30 s, 58°C for 2 min, and 72°C for 3 min. The control PCR was performed with hypoxanthine phosphoribosyltransferase (Hpirt) sense (5’-GTC AAG GCC ATA TCA AAC AAC AAC-3’) and antisense (5’-CCT GGA TTA CAT TAA AGG ACT-3’) primers.

In situ hybridization

The mouse testes were fixed in 4% paraformaldehyde in PBS at 4°C overnight and embedded in paraffin. Five micrometer sections were cut, dewaxed in xylene, and then processed essentially as described by Dressler and Gruss (Dressler and Gruss, 1989). The [35S]UTP-labelled riboprobes were synthesized using T7 or SP6 RNA polymerases (Boehringer, Mannheim, Germany) from linearized plasmid templates containing the Spata6 cDNA (rS 0.8). The hybridization was carried out for 16 h at 55°C and washed to final stringency at 60°C in 2× SSC/50% formamide. The tissue sections were exposed to X-ray film (Amersham) for 20 h and to NTB2 emulsion (Amersham) for 2–5 days.

Whole mount in situ hybridization

The antisense riboprobes for whole mount in situ hybridization were prepared from pBluescript KS (Stratagene) plasmid containing a rS 0.8 cDNA fragment (base pairs 1529–2329). The cRNA probe was labelled using digoxigenin-11-UTP and whole mount in situ hybridization was performed according to standard procedures as described previously (Xu and Wilkinson, 1998). The digoxigenin-labelled probes were detected with NBT/BCIP (Boehringer). The stained embryos were embedded in 7% gelante/15% suroce and 50 μm sections were cut. Experiments were performed twice with eight embryos each.
Characterization and expression of Spata6

**Results and discussion**

**Expression pattern of the Spata6 isoforms**

We have used a differential cDNA screening method to isolate clones representing genes expressed exclusively in testis. A rat testis cDNA library was screened using radiolabelled first-strand cDNA probes prepared from poly(A)-rich RNA of testis and liver. One cDNA clone was used (rS 0.8) containing an insert of 800 bp sequence hybridized in Northern blot analysis with three mRNAs of ~2.6, ~1.8 and ~1.2 kb. The 2.6 kb transcript was relatively low in testis, ovary, thymus and placenta, while the 1.8 and 1.2 kb transcripts were exclusively expressed in the testis (Figure 1A). The tissue distribution and presence of the three Spata6 mRNA isoforms in mouse testis is identical to that in rat (Figure 1A and data not shown). To evaluate the expression pattern of different transcripts during testis development and to determine the testicular cell types expressing the different Spata6 isoforms, we performed Northern blot analyses. As shown in Figure 1B, a weak expression of the 2.6 kb isoform was detected throughout testicular development. The 1.8 and 1.2 kb transcripts could not be detected in mouse testis till post-natal day 15. The expression of the two testis-specific isoforms could first be detected (only after X-ray film overexposure) at day 20. Thereafter, we observed an increasing level of expression of the two testis-specific isoforms. During mouse spermatogenesis, a first wave of spermatogonia is differentiated by mitotic division to give rise to diploid spermatocytes ~10 days after birth. The primary spermatocytes undergo two meiotic divisions at ~17 days of age. The correspondence of these events with the appearance of the 1.8 and 1.2 kb transcripts suggests that both isoforms are specifically expressed in haploid germ
cells and may play an important role in spermatid maturation or sperm function.

We also examined the expression of the three Spata6 transcripts in different testicular cell types and in testes of mouse mutants, in which spermatogenesis is arrested at different stages (Figure 1C). As expected, only the 2.6 kb transcript was expressed in MA10-derived Leydig cells, 15P-1-derived Sertoli cells, testis of W/Wv mutant mice (which lack germ cells) and in testes of Tfm/Y mice (in which spermatogenesis is arrested at the stage of primary spermatocytes). The expression of the three transcripts in testes of olt/olt and qk/qk mutant mice, in which spermatogenesis is arrested in spermatid stage, clearly demonstrates the haploid-specific expression of the 1.8 and 1.2 kb isoforms. We wish to note that the level of the 2.6 kb mRNA is higher in testes of Tfm/Y than that of W/Wv mutant mice indicating the increased expression of the 2.6 kb transcript in early spermatocytes.

To confirm the Northern blot analysis data, which suggest that all testicular cell types contain messages for the Spata6 gene, in situ hybridization on testis sections was performed (Figure 2). The 800 bp cDNA (rS 0.8) that detects the three Spata6 isoforms in Northern blot analyses was used to generate the [35S]UTP-labelled sense and antisense probe. As shown in Figure 2, the Spata6 mRNA was detected in all germ cell stages, Leydig cells and Sertoli cells, although at different levels of expression. A strong accumulation of silver grains was restricted to the cell layers close to the lumen. This area corresponds to histological localization of the post-meiotic cells (round and elongated spermatids). However, the expression of Spata6 in pachytene spermatocytes is higher than in earlier spermatocytes, spermatogonia, Sertoli cells and Leydig cells. No specific signal was observed in mouse testis tissue sections that were hybridized with a sense riboprobe (data not shown).

Sequence analysis in the EST databank revealed that a number of EST clones from mouse embryos are identical to Spata6 cDNA indicating the embryonic expression of Spata6. To examine the expression of Spata6 during embryonic development, RT-PCR was performed (Figure 3A). The Spata6 expression was detected in blastocysts and in whole embryos from E8.5 to E12.5. After E13.5, the level of expression is decreased. Production of the control Hprt products was observed throughout, demonstrating the presence of intact loaded RNA. (B) Whole mount in situ hybridization. At E9.5 (a, left) and E10.5 (a, right), the Spata6 gene is expressed in neural tube, somites and limb bud. (b) Dorsal view of E9.5 embryo shows the restricted expression of Spata6 gene in neural tube and somites. The restricted expression pattern of the Spata6 gene was confirmed by analysis of transverse sections (c, d, e). Spata6 expression is specifically restricted in dorsal region of neural tube (c, d, e) and in the distal part of developing limb bud (c).
E10.5 (a, right) is found in the neural tube, in somites and limb buds (a and b). A transverse section of embryo revealed that Spata6 expression is restricted to the dorsal region of neural tube (Figure 3B, d and e) and to the distal part of developing limb buds (Figure 3B, c).

Sequence analysis of Spata6 cDNA

To facilitate further molecular analysis of Spata6 cDNA and to determine the sequence of the three Spata6 isoforms, 800 bp Spata6 cDNA (rS 0.8) was used to screen mouse and rat testis cDNA libraries. Three mouse clones containing 1.1 (mS 1.1), 1.9 (mS 1.9) and 2.2 (mS 2.2) kb cDNA fragments and four rat clones with 1.4 (rS 1.4), 1.6 (rS 1.6), 1.8 (rS 1.8) and 2.1 (rS 2.1) kb cDNA fragments were isolated, subcloned into pBluescript KS(+) and sequenced. Sequence analysis of the cDNA clones revealed that each cDNA fragment contained identical sequence and had the same polyadenylation site at the 3¢ UTR, and the differences in length are due to an extended 5¢ end. To determine the complete length of the mouse cDNA, Marathon-RACE (Figure 4). (A) The nucleotide and deduced amino acid sequence of the mouse Spata6 cDNA. The amino acid sequence of mouse Spata6 is shown in the single-letter code below the nucleotide sequence. The predicted two translation start sites ATG located at the same reading frame are in bold letters. The first ATG in exon 2 may represent the translation start site for the Spata6 2.6 and 1.8 kb isoforms, while the second ATG codon in exon 8b represents that of the Spata6 1.2 kb isoform. Arrows indicate the intron±exon boundaries in the mouse Spata6 gene. The peptide sequence spanning residues 389±408 (underlined) was used to generate the rabbit polyclonal antiserum. The sequences extending from the amino acids 60±143 and from 178±432, which exhibit similarity with the motor domain of kinesin-related proteins and C.elegans NSC-2 respectively, are indicated in parentheses. (B) Nucleotide sequence of exon 8a, which is located in the 5¢ untranslated region of the Spata6 1.2 kb isoform.
was performed with two antisense primers from the 5′ end of the mH 2.2 clone. The longest PCR fragment of 360 bp (mS 0.36) was subcloned and sequenced. The complete sequence of mouse Spata6 cDNA was assembled from cDNA sequences obtained from the five isolated mouse cDNA sequences and was deposited in GenBank (accession no. AF291465). The complete nucleotide sequence and the predicted amino acid sequence of the mouse Spata6 cDNA are shown in Figure 4. The sequence includes three ATG initiation codons, which are located at positions 792, 967 and 1468 of the sequence. The first ATG is in frame with the termination codon TGA at position 2259, predicting a translation product of 488 amino acids with a molecular weight of 49.7 kDa. The determined nucleotide sequence of the rat Spata6 (accession no. 291466) cDNA showed 95% identity to mouse cDNA sequence. The human EST (accession no. AK000869) have a similarity of 89% to the mouse Spata6 cDNA sequence. Yamano et al. (2001) have cloned a partial cDNA sequence of rat Spata6, which is identical to our cDNA sequence. Using the isolated cDNA in Northern blot analysis, they could only identify one testis-specific transcript.

Computer searches of the mouse Spata6 with protein sequence databases using the PSI-BLAST program (Altschul et al., 1997) revealed that the Spata6 sequence, as a whole, showed no significant homology to known polypeptides, suggesting that the Spata6 is a protein of novel class. However, it should be noted that the sequence extending from the amino acid 60 to 143 (Figure 4A) has 45% similarity with the highly conserved motor domain of different members of the kinesin-related protein (KRP) family, which are involved in translocation of vesicles and organelles along microtubules and in spindle function during both mitosis and meiosis (Goodson et al., 1994; Vale and Fletterick, 1997). Furthermore, the sequence extending from the amino acid 178–432 exhibits 46% similarity to the Caenorhabditis elegans neuronal calcium sensor protein (NCS-2).

**Genomic structure of the Spata6 gene**

To understand the gene structure underlying the three Spata6 splice variants observed by Northern blot analysis, the 129/Sv mouse genomic phage and cosmid libraries were screened with six cDNA fragments spanning the full length of the mouse cDNA sequence. Despite several screenings of the libraries with the different Spata6 cDNA fragments, we were not able to isolate genomic fragments containing all exonic sequences of the mouse Spata6. Therefore, we isolated the missing exons from the rat genomic libraries. Nine different positive clones were identified. The DNA fragments containing exonic sequences were subcloned and the exon/intron boundaries of the Spata6 gene were determined by DNA sequencing. Figure 5A illustrates a schematic representation of the rodent Spata6 gene. The Spata6 gene consists of 15 exons ranging in size between 40 and 596 bp (Figure 5A).

**Molecular origin of the Spata6 transcripts**

To determine whether the three transcripts are derived from alternative splicing in a tissue specific manner or are derived from different promoters, Northern blot analyses using different Spata6 cDNA probes containing the nucleotide sequence of different exons were performed. Northern blot hybridizations with probes containing the cDNA sequence of exon 1 (from 1 to 596 bp of the cDNA) revealed...
that the sequence of exon 1 is only included in the 2.6 kb transcript and
the sequences of exons 2–7 are present in the 2.6 and 1.8 kb Spata6
mRNAs (Figure 5B). Furthermore, Northern blot hybridization with
cDNA probe (E8) containing the sequence of exon 8 and cDNA probe
(E11/12) containing the sequences of exons 11 and 12 were found to
hybridize with all three transcripts (Figure 5B). Altogether, these
results suggest that exons 8–15 are present in all three transcripts
and the 1.8 kb transcript starts with exon 2 (Figure 5B and C).

Marathon PCR on a mouse testis cDNA library with a primer
located in the sequence of exon 9 results in amplification of a 220 bp
fragment. Sequence analysis of the subcloned product showed that the
220 bp fragment possesses a novel 51 bp at the 5′ terminus. To identify
the genomic fragment containing this novel 51 bp cDNA sequence,
DNA of the cosmid clone (Hcos-D), which contains exons 7 and 8,
was digested with different restriction enzymes, blotted and
hybridized with the 51 bp oligonucleotide probe. Southern blot
analysis and sequencing of the hybridizing genomic fragment revealed
that the 51 bp sequence is located ~1.5 kb upstream of exon 8.
Therefore, we designated this identified exon as exon 8a (Figure 5A
and C).

Northern blot analysis revealed that the 51 bp sequence could only
be detected in the 1.2 kb Spata6 mRNA (Figure 5B). Sequencing of
the genomic fragments containing this novel 51 bp showed that exon
8a contains the novel 51 bp sequence and is represented as the 5′
untranslated region of the 1.2 kb Spata6 mRNA (Figure 4B).
Furthermore, the results suggest that the smallest Spata6 transcript
may be derived from a proximal promoter between exons 7 and 8a,
which might be testis-specific.

Taken together, the results of the cDNA sequence and RNA
analyses suggest that the 2.6 and 1.8 kb transcripts are different in the 5′
untranslated sequence, but can be translated into the same protein
with a molecular weight of 49.7 kDa. The 1.2 kb transcript possesses
translational initiation codon at position 1468. The predicted
translation codon of the 1.2 kb transcript is in frame with protein
encoded by the 2.6 and 1.8 kb transcripts and therefore would result in
a truncated protein of predicted molecular weight of 32 kDa. To
address the question, if more than one protein product corresponding
to the Spata6 mRNA isoforms are present, we raised antisera
(pAH1) against the oligopeptide containing the amino acid sequence
from 389 to 408 of the deduced Spata6 protein (Figure 4). Western
blot analysis showed that the antisera (pAH1) only detects a 53 kDa
protein in extracts from testis, thymus, brain and ovary (Figure 6).
This result suggests that the 53 kDa protein is the product of the 2.6
and 1.8 kb Spata6 transcript. Further Western blot analysis using
testicular extract from 5-, 10-, 14-, 20- and 25-day-old mice and from
adult mice as well as from olf/olf and gk/gk mutant mice, in which
spermatogenesis is arrested in spermatid stage, was performed.
Neither in testicular extracts of developmental stages or in testicular
extracts of the mutants, could a 32 kDa protein be found, only the
53 kDa protein was detectable (data not shown). It is possible that the
failure to detect the putative protein coded by the 1.2 kb transcript is
due to reduced expression of the 1.2 kb mRNA relative to the 2.6 kb
transcript (Figure 1A–C). Therefore, protein preparation from an
enriched spermatid fraction could prove if a translated product of the
1.2 kb mRNA is present at all.

Chromosomal localization of the rat and human SPATA6
gene

The Spata6 gene was initially localized using 11 mouse×rat cell
hybrids (Szpirer et al., 1984). The 800 bp cDNA probe of rS 0.8
detected a 14 kb EcoRI restriction fragment in rat DNA and an 8 kb
fragment in mouse DNA. Of all the panels, there were positive for
the presence of the 14 kb rat Spata6 genomic fragment. Perfect

concordance was observed between the Spata6 hybridizing fragment
and rat chromosome 5 (data not shown), whereas all other chromo-
somes showed two or more discrepancies. Regional chromosome
localization of the Spata6 was obtained by FISH. The 800 bp cDNA
probe generated bright double chromatid signals on chromosome 5
only. The position of double chromatid signals along chromosome 5
was measured on 15 chromosomes and found to be comprised of
between 71 and 77% of the chromosome length (starting from the
centromere), allowing the assignment of Spata6 to 5q34-q35
(Figure 7A).

Chromosomal in situ hybridization was also performed on human
metaphase chromosomes using a human P1 clone (ICRFP700
P18116Q5) that was obtained after screening a human genomic
library. Of 60 metaphase cells, 48 exhibited specific labelling of
chromosome 1, in the region p32-35 (Figure 7B) and form part of a
syntenic group that is conserved and whose homologues map on rat
chromosome 5 and mouse chromosome 4 (Truett et al., 1995). A
homologous human EST clone (accession no. AT790763) was
localized on mouse chromosome 4. In this region of human
chromosome, loci for several disorders and syndromes such as
Walker–Warburg syndrome (OMIM 236670), Van der Woude
syndrome 2 (OMIM 606713) and ptosis, hereditary congenital 1
(OMIM 178300) that may be related to SPATA6, have been mapped.
No known human syndromes or disorders, which are correlated with
male infertility, seem to be associated with this locus as determined by
an OMIM search. The curly tail (ct), a semidominant mutation in
mouse that causes exencephaly (~3% of embryos), spina bifida (10%)
and curled tail (50%), was mapped to distal mouse chromosome 4
(Van Staaten and Copp, 2001). Based on the chromosomal localiza-
tion and the expression of the Spata6 gene in the neural tube, we
hypothesize that the curly tail might be the result of mutations in
the mouse Spata6 gene. However, amplification and direct sequencing of
exons containing coding sequences and exon–intron boundaries of
Spata6 did not detect any differences between DNA derived from ct/ct
and wild-type mice (data not shown). Thus, most likely, Spata6 and ct mutations are not allelic.

Targeted disruption of Spata6 and characterization of chimeric mice

To elucidate the function of Spata6, we have disrupted the gene in ES cells through homologous recombination. The Spata6 targeting vector was achieved by substituting exons 3 and 4 of the gene with the neomycin phosphotransferase II (neo) in reverse orientation relative to the Spata6 transcription unit (Figure 8A). Thus, transcripts made from the targeted allele and spliced from exon 2 to exon 5 would contain an in-frame stop codon and encode a protein of 87 amino acids. The targeting vector was linearized and used to transfect RI ES cells. Targeted integration into the Spata6 was verified by Southern blot analysis using a hybridization probe located 5′ to the targeted construct (5′ probe in Figure 8A). As expected for a homologous recombination event, the wild-type locus showed a 17 kb KpnI fragment and the targeted locus a 14 kb KpnI fragment (Figure 8B). Of the clones screened, three of 74 clones had undergone homologous recombination. To confirm that these three recombinant ES clones had undergone correct homologous recombination at the 3′ junction and contained no additional integrations of the targeted vector, Southern blot with KpnI-digested DNA prepared from these three recombinant ES clones were hybridized with the 3′ internal probe (Figure 8A). As expected for correct homologous recombination, the probe only detected the 13-KpnI fragment (Figure 8C). Injection of blastocysts with cells from the ES clones nos 31 and 71 resulted in 13 male and five female chimeras, in which the chimerism ranged from 5–50% according to coat colour. All these chimeric males and females did not transmit the targeted allele to their offspring and the average litter size was not significantly different from that obtained from breeding 129/Sv males with C57BL/6J females. Failure of chimeras derived
from two ES clones to sire agouti offspring lead us to retransfect ES cells with the targeted construct and we isolated two homologous recombinant ES clones. All eight chimeric males generated by injection of blastocysts with cells of both ES clones produced only black progeny. In contrast, four chimeric males, which were generated by injection of blastocysts with non-homologous recombinant ES clones, transmitted the transgenic vector to their offspring. Analysis of 10 metaphase cells derived from each of three homologous recombinant ES clones displayed a diploid karyotype (data not shown). These results indicate that the failure of Spata6 chimeras derived from the homologous recombinant ES cells to sire agouti offspring was not due to ES defects or technical procedures.

The failure of the chimeras to sire agouti progeny is due either to the fact that the Spata6+/- cells do not contribute efficiently to the development of different tissues, especially the germ cells or the disruption of one allele of Spata6 interferes with the Spata6+/- derived spermatozoa during reproduction. To address this question, we have performed a PCR assay based on microsatellite polymorphisms to evaluate the contribution of the 129/Sv and C57BL/6j cells in different tissues and in cauda epididymal sperm isolated from five chimeras. As shown in Figure 8D, the ratio of the Spata6+/- as judged by the level of the amplified 129/Sv-specific fragment, was very low or undetectable in different tissues. However, the contribution of the Spata6+/- cells was relatively higher in kidney and in the skeletal muscle of two chimeric mice. In testis and sperm, the 129/Sv-specific fragment was undetectable (Figure 8D and E). These results and the expression of Spata6 during early embryonic development led us to suggest that a high contribution of Spata6+/- cells causes the lethality of the chimeric embryos. Similar phenomena have been observed by targeted disruption of the neural cell adhesion molecule (Ncam) gene. All live-born chimeras derived from Ncam+/- have a low level of ES cell contribution, whereas those with high levels of contribution have severe growth retardation, defects in somites and neural tube formation, and die between embryonic day 8.5 and 10.5 (Rabinowitz et al., 1996). It has been shown that the failure of the protamine 1 (Prm1) and 2 (Prm2) chimeras to sire agouti offspring was not due to absence of 129/Sv sperm, but is caused by a haploinsufficiency of one allele of Prm1 or Prm2, which prevents genetic transmission of both mutant and wild-type alleles (Cho et al., 2001).

Taken together, the expression pattern of Spata6 in the neural tube and failure to produce live-born chimeras with a high contribution of Spata6+/- cells suggests that the dosage effect of Spata6 is necessary

Figure 8. Targeting disruption of the Spata6. (A) Restriction map of the wild-type allele, targeting vector and targeting allele are shown together with the relevant restriction sites. A 3 kb SstI/SpeI fragment containing exon 3 (E3) and exon 4 (E4) was replaced by a pgk-neo selection cassette, which is in reverse orientation relative to the Spata6 transcription unit. TK, thymidine kinase cassette; K, KpnI; S, SstI; Sp, SpeI; N*, NotI site from polylinker of phage clone. The positions of the 5' external and 3' internal probe used in Southern blot analyses are shown. (B) Southern blot analysis of transfected ES clones. Genomic DNA extracted from ES clones was digested with KpnI and probed with the 5' external probe indicated in (A). The wild-type Spata6 allele yields a 17 kb KpnI fragment, whereas the targeted allele yields a 14 kb fragment, as indicated in (A). (C) DNA from three homologous recombinant ES clones 31, 67 and 71 was digested by KpnI and probed with a 3' internal probe. A probe detected only a 13 kb KpnI fragment. (D) PCR assay using microsatellite markers was performed to determine the degree of chimerism in different tissues of chimeric male mice. The 129- and C57-specific fragment was amplified using a genomic DNA of the 129/Sv and the C57BL/6j mouse strain. (E) Ratio of 129/Sv and C57BL/6j sperm in cauda epididymis of the chimeric male (Ch1), which transmitted the transgenic targeting vector to their offspring, and six chimeric mice (Ch2-Ch7), which do not transmit the targeted allele to their progeny, was determined using the PCR-based microsatellite polymorphisms.
for normal development of the neural tube and/or neural tube closure. Neural tube defects (NTD) are among the most common and severe congenital malformations in human. They occur worldwide with an incidence of between one and nine per 1000 births (Emery and Rimoin, 1990). Perhaps mutations in SPATA6 might also cause some of the human NTDs. However, this is our speculation, which has to be confirmed experimentally. Currently, we are screening for mutation(s) in the SPATA6 gene in NTD patients.

The specific expression of the 1.2 and 1.8 kb transcripts in the post-meiotic germ cells may be necessary for spermatogenesis or for sperm function during fertilization. Therefore, production of conditional knockout mice may be the most promising method for the direct assessment of the function of Spata6 during embryogenesis and spermatogenesis.

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