Mouse Autosomal Homolog of DAZ, a Candidate Male Sterility Gene in Humans, Is Expressed in Male Germ Cells before and after Puberty

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INTRODUCTION

One-sixth of human couples are infertile but otherwise healthy (Hull et al., 1985). In most cases of human infertility, the underlying cause cannot be identified with certainty. In theory, some cases could be due to genetic defects that disrupt germ cell development while sparing the soma. Such “pure sterile” genes have been identified in Drosophila and Caenorhabditis elegans, for example, and their characterization is now leading to an understanding of molecular processes unique to germ cells in invertebrates (Castrillon et al., 1993; Ellis and Kimble, 1994). In mammals, by contrast, no pure sterile factor has been unequivocally, biochemically defined, though in some cases the responsible genetic loci have been mapped.

Perhaps the best characterized pure sterile locus in humans is the Azoospermia Factor (AZF), on the long arm of the Y chromosome. A role for the human Y chromosome in spermatogenesis was first suggested in 1976 by Tiepolo and Zuffardi, who reported the occurrence of grossly deleted Y chromosomes in six men with azoospermia (no sperm in semen). Based on these findings, they hypothesized that Yq harbors an Azoospermia Factor gene or gene complex required for spermatogenesis. In recent years, strong evidence in support of Tiepolo and Zuffardi’s model has been obtained, and AZF has been precisely localized. We and our colleagues have demonstrated that a particular portion of Yq—the “AZF region”—was deleted in 13% of azoospermic men (Reijo et al., 1995). The fathers of these azoospermic men had intact Y chromosomes, confirming that the AZF deletions in their sons were the cause of the sons’ infertility.

Testis biopsies from azoospermic men with AZF deletions revealed a surprisingly wide range of histologic findings. Germ cells were found to be completely absent in some individuals (“Sertoli cell only syndrome”), while early spermatogenic cells were present in others (“testicular maturation arrest”). In two of the latter cases, spermatogenesis sometimes progressed beyond meiosis, to the stage of condensed spermatids. As previously suggested, this wide array of testicular phenotypes could be explained by a defect (with variable expressivity) in spermatogonia, the stem cells responsible for constantly replenishing the spermatogenic lineages.
in the testes, or in the differentiation of primordial germ cells to spermatogonia (Reijo et al., 1995).

The molecular identity of AZF has not been established definitively, but a strong candidate is DAZ (Deleted in Azospermia), the only transcription unit so far identified in the AZF region (Reijo et al., 1995). (YRRM1 and YRRM2, corresponding to the cDNAs MK5 and MK29, respectively, had also been proposed as AZF candidates (Ma et al., 1993) but subsequent studies revealed that these transcription units mapped outside the AZF region (Reijo et al., 1995)). DAZ encodes a putative RNA-binding protein and, as judged by Northern blotting of human tissue RNAs, is expressed predominantly in testes (Reijo et al., 1995).

Given the strong possibility that DAZ might be AZF, a pure male sterile factor, we wished to learn more about its pattern of expression. Is DAZ expressed in germ cells or in somatic cells of the testis? Is DAZ expressed early or late in the pathway of spermatogenesis? Would the pattern of DAZ expression readily account for the testicular pathology observed in AZF-deleted men? These questions may be difficult to pursue in humans given the limited availability of suitable tissue specimens. To pursue these questions in an experimentally tractable species and to explore the degree to which the DAZ gene and its product have been conserved during mammalian evolution, we characterized the mouse homolog of human DAZ.

MATERIALS AND METHODS

Mice. Wildtype (BALB/c) mice were from the laboratory of R. J. Aienisch; mice with W or S1 mutations (in the White spotted and Sted genes, respectively) were obtained from Jackson Laboratories (Bar Harbor, ME).

Isolation of DNA and RNA. Preparation of mouse DNA was as previously described (Simpson and Page, 1991). RNA was prepared using Trizol reagent (Gibco BRL, Grand Island, NY). Essentially, cells or tissue were resuspended in approximately 10 vol Trizol, and 0.2 vol of chloroform was added to each sample. After centrifugation to remove cell debris, RNA in the supernatant was precipitated with isopropanol, rinsed with ethanol, and resuspended in deionized water.

Hybridizations and sequencing. Northern blotting, Southern blotting, and library screening techniques were as previously described (Simpson and Page, 1991; Page et al., 1987) with hybridizations as follows: 20 h at the appropriate temperature (42°C for all Southern and Northern and 37°C for screening the cDNA library) in 50% formamide, 5× SSC, 1× Denhardt's, 20 mM Na phosphate, pH 6.6, 0.005% denatured salmon sperm DNA, 1% sodium dodecyl sulfate, 10% dextran sulfate. The blots were washed three times for 15 min each at 65°C in 0.1× SSC, 0.1% sodium dodecyl sulfate. The gene encoding the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (G3PDH) was used as a reference probe to control for the loading of different quantities of RNA (Tso et al., 1985). Nucleotide sequencing of DAZ cDNA clones was performed as previously described (Fisher et al., 1990).

The mouse Dazh gene was mapped by genetic linkage analysis using an interspecific backcross: (C57BL/6 × Mus spretus) F1 × M. spretus (J ackson Laboratories). Genomic DNAs from backcross progeny were digested with TaqI, Southern blotted (2.5 μg per lane), and hybridized with Dazh cDNA pDP1580, which was found to detect M. spretus TaqI fragments of 4.5, 2.3, and 0.5 kb as distinct from C57BL/6/j fragments of 4.5, 2.5, 2.3, 0.6, and 0.5 kb. Ninety-four backcross progeny were genotyped for this TaqI polymorphism, and comparison with genotype data from other markers (J ackson Laboratory collaborative data) allowed Dazh to be positioned.

Fluorescence in situ hybridization. Dazh genomic clone pDP1642 was isolated from a mouse library (strain 129; Wu et al., 1994) by hybridization of plaque lifts with Dazh cDNA pDP1580 at 42°C. Sequencing of the ends of three EcoRV fragments subcloned into the Bluescript KS(+) vector (Stratagene, Inc., La Jolla, CA) revealed that pDP1642 contained sequence colinear with Dazh cDNA. The 10-kb genomic insert was labeled with biotin-11-dATP by nick-translation (Gibco BRL, Gaithersburg, MD). Metaphase chromosomes were prepared from male C57BL/6 and M. spretus lymphocytes using 0.075 M KCl as hypotonic buffer and methanol/acetic acid (3:1, v/v) as fixative. The hybridization was carried out as previously described (Edelhoff et al., 1993). After incubation with goat anti-biotin antibody, slides were rinsed in 2× SSC, 0.1% Tween 20, 0.15% bovine serum albumin. A second incubation with fluorescein-labeled anti-goat IgG and a rinse in modified 2× SSC followed. The chromosomes were banded using Hoechst 33258-actinomycin D staining and propidium iodide counterstaining. Chromosomes and hybridization signals were visualized by fluorescence microscopy using a dual-band pass filter (Omega, Brattleboro, VT) and a Vector detection system (Vector, Burlington, CA).

RESULTS

DAZ Homologs Are Autosomal in Mice and Other Mammals

We previously demonstrated that, in apes, homologs of the human DAZ gene are located on the Y chromosome, where they may exist in multiple copies (Reijo et al., 1995). To identify homologs in more distantly related mammals, we hybridized a human DAZ exon (325.7; Reijo et al., 1995) to a “Noah’s ark” Southern blot of genomic DNAs from male and female mice, rabbits, dogs, and cattle (Fig. 1). In each of the four species,
FIG. 2. Mapping of Dazh to mouse chromosome 17. (A, Top) Fluorescence in situ hybridization of mouse Dazh genomic clone pDP1642 to lymphocyte metaphase chromosomes from a male C57BL/6J mouse. (Bottom) Chromosomes stained with Hoechst 33258-actinomycin D and counterstained with propidium iodide. (B) Genetic map location determined by following segregation of a Dazh TaqI restriction fragment length polymorphism in a (C57BL/6 × M. spretus)F1 × M. spretus backcross.

hybridization to a single fragment was observed, and in each species the single fragment was present in both males and females. Thus, these more distantly related mammalian species all possess a DAZ homolog, but the homologs are apparently not located on the Y chromosome.

To resolve whether these non-Y homologs were autosomal or X-linked, we mapped the mouse homolog, which we will refer to as Dazh (DAZ homolog), by two independent methods. First, Dazh was mapped to mouse chromosome 17 by fluorescence in situ hybridization (FISH) to metaphase chromosomes. Similar results were obtained using metaphase spreads prepared from either C57BL/6J, an inbred lab strain, or the species M. spretus (Figs. 2A and 2B). Of 51 C57BL/6J cells examined, 15 (29%) showed signals on both chromatids of chromosome 17 at regions C–D. Of 73 M. spretus cells examined, 28 (38%) showed signals on both chromatids of chromosome 17 at regions C–D. This chromosomal assignment was independently confirmed and refined by genetic linkage mapping. Following the segregation of a TaqI restriction fragment length polymorphism in a C57BL/6 × M. spretus backcross (Jackson Laboratory Panel BSS), we observed that Dazh did not recombine with the marker D17Mit9 and mapped between the markers Tpx2 and D17Bir8. Although we have not rigorously mapped the rabbit, dog, and cow homologs, normalization of the Southern autoradiogram shown in Fig. 1 (by hybridizing known autosomal probes to the same blot; not shown) suggests that the homologs in these species are also autosomal rather than X-linked.

Mouse Dazh Encodes a Putative RNA-Binding Protein

To determine whether the mouse homolog is transcribed, we hybridized human DAZ cDNA clone pDP1576 to a Northern blot of RNAs prepared from various adult mouse tissues. We observed a 3.5-kb transcript in the testis, much as we had previously seen (Reijo et al., 1995) in human tissues using the same hybridization probe (results not shown; see similar experiment using mouse cDNA probe below).

To characterize mouse Dazh transcription further, we screened an adult testis cDNA library (Mardon and Page, 1989) by hybridization using as probe human DAZ exon 325.7. The inserts of three cDNA clones identified by this screen were sequenced in their entirety.
MOUSE AUTOSOMAL HOMOLOG OF DAZ

FIG. 3. (A) Nucleotide sequence of mouse Dazh cDNA clone pDP1580 and, immediately beneath, predicted amino acid sequence of the encoded protein. The RNP/RRM domain is boxed. A 72-nucleotide unit tandemly repeated seven times in human DAZ (5) but present only once here in mouse Dazh is underlined. The GenBank accession No. is U46694. (B) Comparison of mouse Dazh and human DAZ (5) proteins. Dots represent identity to mouse Dazh. Note that human DAZ contains seven tandem repeats of a 24-amino-acid unit present only once in mouse Dazh. The predicted mouse Dazh protein contains only one copy of this 24-residue unit. The mouse Dazh protein contains an RNP/RRM motif of the sort found in many proteins that bind RNA and after the Pubertal Onset of Spermatogenesis (or in some cases single-stranded DNA) (Fig. 3B). In this 85-amino-acid domain, the human and mouse proteins differ at only 9 residues. Outside the putative RNA-binding domain, the human and mouse proteins exhibit less conservation, though both are rich in proline, glutamine, and tyrosine residues, as are many RNP/RRM proteins (Burd and Dreyfuss, 1994; Kenan et al., 1991).

Mouse Dazh Is Expressed in Male Germ Cells before and after the Pubertal Onset of Spermatogenesis

As described above, Northern analysis employing a human DAZ probe suggested that mouse Dazh is expressed predominantly in the testis. We confirmed and extended these findings using mouse Dazh cDNA clone pDP1580 as probe. By Northern analysis, we searched for Dazh transcripts in the following adult tissues from both female and male mice: brain, gonad, heart, kidney, liver, lung, and spleen. As before, we detected expression predominant in the testis. We confirmed and extended these findings using mouse Dazh cDNA clone pDP1580 as probe. By Northern analysis, we searched for Dazh transcripts in the following adult tissues from both female and male mice: brain, gonad, heart, kidney, liver, lung, and spleen. As before, we detected expression predominant in the testis.
While it is formally possible that Dazh might be expressed in Sertoli or other somatic testicular cells—and be induced only in the presence of germ cells—no gene has, to our knowledge, been shown to exhibit such behavior in the testis. It is far more likely, we concluded, that Dazh is expressed in the germ cells of the testis.

We next set out to determine the stage or stages of male germ cell development in which Dazh is expressed. During the first postnatal week, spermatogonia stem cells are formed from prospermatogonia present at birth (McCary, 1993). During the next several weeks of life, the first cohort of fully differentiating germ cells proceed through spermatogenesis in a relatively synchronous manner. As a result, simple Northern blotting of RNAs from testes of various ages can provide much information as to the developmental stages and cell types in which a germ-cell-specific gene is expressed. As shown in Fig. 4B, a 3.5-kb Dazh transcript (the same size as seen in the adult testis) was faintly detectable in the testes of 1- and 3-day-old mice, when the only germ cells present are prospermatogonia. The abundance of the Dazh transcript increased dramatically by Day 6, when the first spermatogonia appear; reached a plateau at about Day 10, when the first wave of spermatogenic cells entered meiosis; and remained relatively constant thereafter. Thus, the temporal and quantitative pattern of Dazh expression correlates with the presence of prospermatogonia and with the appearance and expansion of the spermatogonial population to which the prospermatogonia give rise.

**DISCUSSION**

**Autosomal Homologs of DAZ in Mice and Other Mammals**

We had previously shown that, in apes, the Y chromosome carries one or more homologs of human DAZ (Reijo et al., 1995). By contrast, we found no evidence of a Y-linked DAZ homolog in any of the four nonprimate mammals (mouse, rabbit, dog, and cattle) studied here. As judged by Southern blotting of male and female DNAs—and more definitive mapping studies in the mouse—these mammals appear to carry a single, autosomal homolog of DAZ (Figs. 1 and 2). These observations substantiate the emerging view that the genetic content of the Y chromosome has been in rapid flux during mammalian evolution (e.g., Graves, 1995). This conclusion is all the more telling when one appreciates that the Y's meiotic partner, the X, has been the most stable chromosome during placental mammalian evolution (Rugarli et al., 1995; Palmer et al., 1995).

Nucleotide similarity between Y-linked human DAZ and autosomal mouse Dazh (85% identity in the coding region) suggests that they derived from a common ancestral gene during mammalian evolution. It is not obvious whether this ancestral gene was autosomal or Y-
linked, but a male–female common band observed on Southern blots of human and ape DNAs (Fig. 1; Fig. 5. in Reijo et al., 1995) may be evidence of an autosomal homolog in these species as well.

In the mouse, Dazh maps to 17, a chromosome whose t complex is well known to students of mammalian spermatogenesis because of its male transmission distortion and recessive male-sterile loci (Silver, 1993). As Dazh maps about 9 cM distal to the t complex, it cannot be responsible for t complex phenomena as conventionally defined. However, chromosome 17 has also been repeatedly implicated in hybrid sterility, in which the male progeny of certain interspecies crosses suffer spermatogenic failure (Pilder et al., 1993; Forejt and Ivanyi, 1974). Indeed, four of six published mammalian hybrid sterility loci map to mouse chromosome 17 (Pilder et al., 1993; a fifth, unpublished locus also maps to this chromosome; S. Pilder, pers. comm. February 1996, Philadelphia, PA). The loci studied to date map within the t complex, and the possible existence of hybrid sterility loci elsewhere on chromosome 17 has not yet been explored. Given the expression pattern of mouse Dazh and that its human Y-linked homolog may be a male-sterile factor, this potential link to hybrid sterility warrants experimental study.

Dazh Expression in Prospermatogonia and Spermatogonia

Having found that mouse Dazh is transcribed in the adult testis, we explored the developmental timing of its expression and whether this takes place in the soma or in germ cells. We addressed these issues by Northern analysis, taking advantage of (1) the availability of mutants (W, S1) that lack germ cells but retain the somatic components of the testis and (2) the relatively synchronized progression of male germ cells during the first month after birth, when spermatogonial stem cells are formed and the first wave of fully differentiating descendants proceed through spermatogenesis. Our studies revealed that mouse Dazh is expressed (1) primarily if not exclusively in the testis, (2) in germ cells but not in somatic cells, and (3) at all postnatal timepoints, including those before and after the pubertal onset of spermatogenesis. The level of Dazh expression increased steadily until about 10 days after birth, long before the first wave of spermatogenesis was completed, and remained relatively constant thereafter.

Taken together, these results strongly suggest that Dazh is expressed in spermatogonia (the only germ cells present in newborn males) and in the expanding population of spermatogonial stem cells to which they give rise during the first postnatal week. Dazh continues to be expressed after puberty, most likely in the long-lived spermatogonial populations that constantly replenish the spermatogenic lineage. (At present we cannot exclude that Dazh might also be expressed in early spermatogonial derivatives (spermatocytes), a possibility to be explored by cell fractionation or in situ hybridization.) While a few other germ-cell-specific genes have been found to be expressed in spermatogonia or spermatogonia (Starborg et al., 1992; Tanaka et al., 1994), the temporal expanse of Dazh's expression is unprecedented (Hecht, 1993; Meistrich and van Beek, 1993) and suggests that Dazh participates in differentiation, proliferation, or maintenance of germ cell founder populations well before, during, and after the pubertal onset of spermatogenesis.

Dazh is also transcribed, though at a much lower level, in the adult ovary. It will be interesting to determine whether this reflects Dazh activity in oocytes or in the somatic components of the ovary.

Similarity of Mouse Dazh and Human DAZ

Two observations suggest that, in vivo, the human DAZ and mouse Dazh proteins perform similar functions on identical or near-identical RNA (or possibly single-stranded DNA) substrates. First, the amino acid sequences of the human and mouse proteins are similar throughout, and especially in the RNP/RRM domain, that is their most striking feature (Fig. 3). Second, the human and mouse genes are both expressed predominantly in the testis. Indeed, in situ hybridization studies have revealed that, in adult human testes, DAZ is transcribed in spermatogonia (D. Menke, G. Mutter, and D.C.P., unpublished results), suggesting that the human and mouse genes may be expressed in male germ cells at similar or identical developmental stages. Taken together, these data suggest that the human and other mammalian DAZ genes may function in spermatogonia (and their immediate precursors, prospermatogonia) throughout postnatal life.

Is DAZ the Azoospermia Factor in Humans?

If human DAZ plays a critical role in spermatogonia or their precursors, then this is consistent with our previous predictions for AZF. Men with deletions of the AZF region of the Y chromosome exhibit spermatogenic defects of varying severity. Severe cases are characterized by the complete absence of germ cells, including spermatogonial stem cells (Sertoli cell only syndrome; Reijo et al., 1995), while in mild cases spermatogenesis proceeds to completion at reduced output, resulting in severe oligospermia (Reijo et al., 1996). As we have previously suggested (Reijo et al., 1995), this range of phenotypes could be readily explained by a variably expressed ("leaky") defect in spermatogonia or spermatogonia. Although there is no definitive evidence equating DAZ with AZF, our findings provide an important circumstantial link: both DAZ and AZF may function in spermatogonia or their precursors.

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Note added in proof. Dazha is identical to the Dazla gene as described by Cooke et al. (Hum. Mol. Genet. 5: 513–516, 1996).