A human *DAZ* transgene confers partial rescue of the mouse *Dazl* null phenotype

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ABSTRACT In a subset of infertile men, a spectrum of spermatogenic defects ranging from a complete absence of germ cells (sertoli cell only) to oligozoospermia is associated with microdeletions of the *DAZ* (deleted in azoospermia*)* gene cluster on human distal Yq. *DAZ* encodes a testis-specific protein with RNA-binding potential recently derived from a single-copy gene *DAZL1* (DAZ-like) on chromosome 3. Y chromosomal *DAZ* homologues are confined to humans and higher primates. It remains unclear which function unique to higher primate spermatogenesis *DAZ* may serve, and the functional status of the gene recently has been questioned. To assess the extent of functional conservation we have tested the capacity of a human *DAZ* gene contained in a 225-kb yeast artificial chromosome to complement the sterile phenotype of the *Dazl* null mouse (*Dazl*$^{-/-}$), which is characterized by severe germ-cell depletion and meiotic failure. Although *Dazl*$^{-/-}$ mice remained infertile when the *DAZ* transgene was introduced, histological examination revealed a partial and variable rescue of the mutant phenotype, manifest as a pronounced increase in the germ cell population of the seminiferous tubules and survival to the pachytene stage of meiosis. As well as constituting definitive proof of the spermatogenic role of the *DAZ* gene product, these findings confirm the high degree of functional conservation between the *DAZ* and *DAZL1* genes, suggesting they may constitute a single target for contraceptive intervention and raising the possibility of therapeutic up-regulation of the *DAZL1* gene in infertile men.

The existence of a Y borne fertility gene or "azoospermia factor" (AZF) was suggested first by the observation of microscopically visible deletions of distal Yq in a proportion of infertile or subfertile men. In the four examples tested, the deletions were shown to have arisen *de novo*, in the paternal germ line, supporting the likelihood of a causal link (1). Molecular techniques employing Y chromosome-specific markers subsequently revealed that 5–10% of severely oligozoospermic (abnormally low sperm count) or azoospermic (zero sperm count) men have cytogenetically invisible Yq microdeletions (2–7). These fall into three classes mapping to discrete subintervals of the Yq region, termed AZFa, b, and c (8), and positional cloning strategies have led to the isolation of candidate genes from each subinterval. The predicted product of the AZFa candidate resembles a ubiquitin C-terminal hydrolase (9) whereas both the AZFb and AZFc candidates encode distinct putative RNA-binding proteins, designated *RBM* (RNA-binding motif) and *DAZ* (deleted in azoospermia), respectively, which are expressed exclusively in germ cells (3, 10). Both gene products contain a single RNA-recognition motif (RRM), are present in multiple copies

on the Y chromosome (3), and were derived from ancestral single-copy autosomal genes. Phylogenetic studies have revealed that in the case of *RBM* the transposition and amplification events preceded the radiation of mammals (10–13) whereas *DAZ* was copied to the Y chromosome much more recently, in the old-world primate lineage (14, 15). This difference in evolutionary history of the two genes is reflected in their patterns of functional conservation. The proposed role of *RBM* in spermatogenesis must reflect a degree of functional specialization subsequent to transposition because the progenitor, *hnRNPG*, is a ubiquitously expressed gene with a function in pre-mRNA metabolism and is not implicated in spermatogenesis (16). Because no single-copy *RBM* homolog is known in an organism amenable to genetic manipulation, such as *Drosophila* or mouse, experimental proof of its role in spermatogenesis is difficult to obtain.

In contrast to *RBM*, the proposed role of *DAZ* in gametogenesis is shared with its autosomal ancestor and is very ancient. The ancestral form of *DAZ* is identifiable by a high degree of sequence homology in phylogenetically diverse species and is implicated in the execution of both oogenesis and spermatogenesis (14, 15, 17–24). Targeted mutagenesis of the mouse *DAZ* homolog, *Dazl*, on chromosome 17 produced a null phenotype in the male of sterility, characterized by failure of germ–cell maintenance and maturation, which resembles defects seen in AZFc patients (25). *Dazl* is expressed in the germ cells of both sexes, and the null female exhibits complete failure of folliculogenesis and sterility. The human autosomal ancestor, *DAZL1* (DAZ-like), which is 90% identical to *DAZ*, also is expressed in the germ cells of males and females (18), and the possibility of its implication in some cases of male and/or female infertility merits investigation. Intriguingly, *Drosophila melanogaster* and *Caenorhabditis elegans* exhibit reciprocal patterns of sex specificity in their requirements for the *DAZ* progenitor. Thus, whereas C. *elegans daz-1* is required in the hermaphrodite for oogenesis and dispensable in the male (23), the *Drosophila* homolog, *boule* is required for spermatogenesis but not oogenesis (17). Although these differences between species in the effects of loss of *DAZL* function could signify some fundamental divergence in the gametogenesis mechanism, it seems more likely that another gene(s) can substitute for DAZL function in some contexts.

Because a single-copy gene suffices to support spermatogenesis in all other species, the requirement for an additional Y chromosomal DAZ gene cluster in man and old-world primates requires explanation. In comparison with their autosomal counterpart, the DAZ genes have undergone a number of structural rearrangements (18). These include a tandem

Abbreviations: *DAZ*, deleted in azoospermia; *Dazl*, mouse autosomal DAZ-like; *DAZL1*, human autosomal DAZ-like; AZF, azoospermia factor; *RBM*, RNA-binding motif; STS, sequence-tagged site; YAC, yeast artificial chromosome; RT-PCR, reverse transcription–PCR; HSV-TK, herpes simplex virus thymidine kinase.
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amplification of exon 7, giving rise to a 24-aa repeat with a copy number of between 7 and 14 ("DAZ repeat"). However, the functional significance of this feature is unknown, and throughout most of the coding region including the RRM, the sequence has been highly conserved, suggesting the retention of similar RNA targets. Although it is possible that the Y chromosomal genes have acquired some novel function, not shared by the ancestral form and required only in higher primates, the similar phenotypic consequences of loss of mouse *Dazl* and human *DAZ* also argue against such a functional distinction. Alternatively, the significance of the DAZ gene cluster may be only quantitative, serving to increase overall gene dosage and product levels, thereby enhancing spermatogenic efficiency. Gene amplification after transposition to the nonrecombining portion of the Y chromosome may have been favored further by the need to maintain gene function in the face of an accumulation of deleterious mutations through the operation of Müllers ratchet (26). In man, the variable penetrance of AZFc deletions that remove the entire *DAZ* gene cluster is consistent with a degree of genetic redundancy. The phenotypic spectrum ranges from a total lack of germ cells (sertoli cell only) to oligozoospermia, and, in one case, a male lacking the *DAZ* gene cluster is known to have fathered a son who was infertile (3, 8). An extreme view is that the Y chromosomal *DAZ* cluster is a nonfunctional relic. The infertility associated with *DAZ* deletions could be accounted for by the deletion of other, as yet uncharacterized genes within the interval with testis-specific expression patterns (27, 28). In support of this possibility there are no reported instances of *DAZ* point mutations in infertile men, and a recent study of nucleotide substitution rates within the *DAZ* locus revealed a pattern consistent with an absence of selective pressure (29).

Our approach to assessing the degree of functional overlap between *DAZ* and its autosomal counterparts was to test the capacity of a *DAZ* transgene to rescue the sterility of the *Dazl* knockout mouse. This report describes the introduction of a 225-kb yeast artificial chromosome (YAC) clone from the AZFc region containing a *DAZ* gene into mice lacking a functional copy of *Dazl*. The *DAZ* transgene displayed an appropriate male germ cell-specific pattern of expression in the mouse and conferred a partial rescue of the mutant phenotype manifest as a pronounced increase both in the survival of spermatogonia and the frequency of maturation to the pachytene stage of meiotic prophase. The implications of the present findings for the functional relationship between *DAZ*/*Dazl* are discussed.

MATERIALS AND METHODS

Introduction of YAC S12 into the $Dazl^{Tm1Hgu}$/$Dazl^{Tm1Hgu}$ Genetic Background. YAC S12 (yS12) contains a 225-kb human Y chromosome insert. The S12 YAC was transferred to the yeast host CGY2570 (30) and retrofitted with modified vector arms based on pCS990 (centromeric arm) (30) and pJS89 (acentric arm) (31). pCGS990 contains a conditional yeast centromere whose activity is abolished under selective growth conditions (30). Under these conditions, S12 YAC copy number increased 10- to 20-fold, allowing sufficient DNA to be prepared for injection (not shown). YAC DNA was prepared as described (32). S12 YAC DNA was injected into pronuclei at a concentration of 1 ng/μl. The G_0 mice were F_2 hybrids from a C57BL × CBA/Ca cross. The single G_0 male transgenic mouse was backcrossed to a CB57BL × CBA/Ca F_1 female to establish the S12 transgenic line, TgN (hDAZy1)1Hgu, which will be referred to as TgS12. TgS12 positive males were crossed to $Dazl^{Tm1Hgu}$/+ females (25). $Dazl^{Tm1Hgu}$/+;TgS12 individuals were mated to test for phenotypic rescue in $Dazl^{Tm1Hgu}$/$Dazl^{Tm1Hgu}$;TgS12 progeny. *Dazl* genotype was determined by PCR as described (25), and the presence of yS12 was detected by PCR with *TRP1* primers f488

(5′-gctctcttgccttccaacc-3′) and f492 (5′-caacctaaggaggatgttt-tgg-3′).

Nucleic Acid Preparation. Genomic DNA was prepared from mouse tail tips or kidney by using standard techniques. Total RNA was prepared by using RNAZol B reagent (AMS Biotechnology, Oxford, U.K.) according to the manufacturer's instructions. Poly(A)$^+$ RNA for 5′ rapid amplification of cDNA ends PCR was prepared by using oligo(dT)-coupled magnetic beads (Dynal, Great Neck, NY).

YAC PCR Analysis. Y chromosome-specific sequence tagged site (STSs) used to map the extent of yS12 have been described (33). Primers dazint1 (5′-gtcgacaacaaagcagga-3′) and dazint2 (5′-gtcgacatcataattacg-3′) confirmed the retention of sequence immediately upstream of *DAZ* exon 2 in the S12 YAC. The absence of exon 1 in TgS12 transgenic mice was confirmed with primers daz1 (5′-ggttttccttacaccttagc-3′) and daz2 (5′-aggctcaaggaggaacagag-3′).

Reverse Transcription—PCR (RT-PCR). First-strand cDNA synthesis reactions and RT-PCR were performed by using a preamplification system kit (GIBCO) with 1 μg of total RNA and oligo(dT) primer. RT-PCRs contained 1 μl cDNA and *DAZ* forward and reverse primers at 1 μM (forward primer, daz3 (5′-ccgaagcatacaaatggtgg-3′; reverse primer, daz4 (5′-tgttcacaggtacttcttgg-3′). Control reactions were performed by using S16 ribosomal protein primers (14).

5′ Rapid Amplification of cDNA Ends PCR. Reactions were performed by using the Marathon cDNA Amplification kit (CLONTECH). First-round PCR used *DAZ* gene-specific primer 1 (5′-gaaccgtatctaccaaagcagcttcc-3′). Nested PCR used *DAZ* gene-specific primer 2 (5′-gccaagccgtggaatggtagcaatg-3′). PCR products were gel-purified (Qiagen gel-extraction kit), cloned into a plasmid vector by using the pGEM-T kit (Promega), and sequenced by using a BigDye Terminator sequencing kit and automated sequencing apparatus (Applied Biosystems).

Filter-Hybridization Analysis. Northern and Southern hybridization was carried out according to standard methods by using nylon filters and hybridizing in 7% SDS/0.5 M sodium phosphate, pH 7, at 65°C. Southern hybridization probes were either restriction fragments isolated from cloned genomic *Eco*RI fragments in plasmid pUC19 (p7C46A/pG5D provided by P. Yen, Harbor–UCLA Medical Center, Los Angeles, CA) or gel-purified PCR products (p8A/*DAZ*3TER). The 1.5-kb probe p8A (*DAZ* 5′ flanking) was generated by using forward primer l411P4 (5′-gaccggaggctatgaacagg-3′) and reverse primer l411P5 (5′-caattggcccttgaggagcc-3′) on a human genomic DNA template. p8A is located 10 kb upstream of *DAZ* exon 1 in cosmid l411 (D. Page, available at http://www.genome.wi.mit.edu). *DAZ*3TER (*DAZ* 3′ untranslated region) was generated by using forward primer daz3 and the T3 vector primer (5′-aattaaccctcactaaaggg-3′) on a *DAZ* cDNA template cloned in phagemid vector pBK-CMV (provided by P. Yen). The Northern hybridization probes were *DAZ*3TER or mouse β-actin (mouse actin PCR product cloned into pGEM1) (34).

Immunohistochemistry and *in Situ* Hybridization. Mouse testes were fixed in Bouins for 6–8 h and stored in 70% ethanol processed into paraffin wax, and 5-μm sections were cut. Immunohistochemistry for proliferating cell nuclear antigen was performed by using a mAb at 1:100 (Dako); positive nuclei were visualized by using standard methods (35). Radiolabeled sense and antisense riboprobes were synthesized from *DAZ*3TER subcloned in pCRIITOPO (Invitrogen) and used to perform *in situ* hybridization according to standard methods (35); sections were exposed to emulsion for 33 days.

Western Blotting. Methods for protein extraction, Western blotting, and antibody detection are described in ref. 36. *DAZ* antibody 133 is specific for human DAZ (R. Reijo, personal communication).
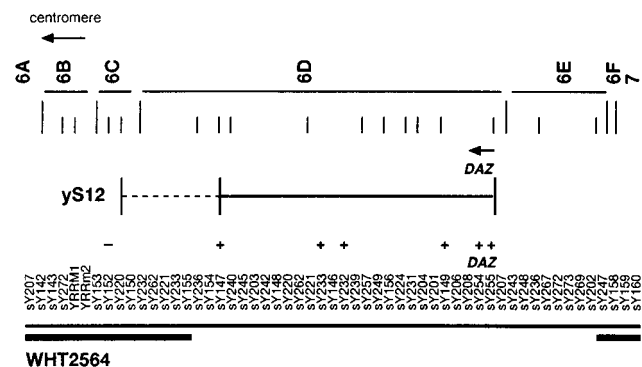
RESULTS

Introduction of a YAC from the Human *DAZ* Genomic Region Gene into Mice. YAC clone S12 (yS12) contains a 225-kb insert originally reported to span the AZFc deletion in an azoospermic patient KLARD (10). Further analysis of the STS content of yS12 revealed that the YAC extends over only a portion of the minimal AZFc region and tests positive for sY254 and sY255 located within the *DAZ* gene (3) plus additional 3′ flanking STS markers (Fig. 1) and is negative for STSs representing CDY, PRY, TTY2, and BPY2 (27).

yS12 was introduced into a C57BL × CBA/Ca F_1 mouse background by pronuclear injection. A single transgenic G_0 male (M1) was obtained, and Southern hybridization to M1 genomic DNA with probes specific to each YAC vector arm indicated the presence of a complete YAC (not shown). M1 was backcrossed to C57BL × CBA/Ca F_1 females to generate the transgenic mouse line TgN(hDAZy1)1Hgu, referred to hereafter as TgS12. Fluorescence *in situ* hybridization (FISH) with a YAC vector arm probe revealed an integration site close to the centromere of chromosome 7 (not shown). A crude estimate of YAC copy number was obtained from the FISH signal in interphase nuclei. A maximum of two hybridization signals were seen per nucleus indicating a probable copy number of 1 or 2 (not shown).

yS12 Contains a Single Copy of *DAZ*. To assess the integrity of the *DAZ* sequence within the TgS12 mouse, Southern blots of TgS12 mouse and human genomic DNA were probed with sequences derived from the indicated locations within the *DAZ* genomic region (Fig. 2*A*). Probe pG5D, a genomic *Eco*RI fragment representing exons 2–6, and *DAZ*3TER, a *DAZ*-specific cDNA-derived PCR product representing the 3′ untranslated region, gave similar patterns of hybridization in human and mouse, but p7C46AP, the genomic *Eco*RI/*Pst*I fragment including *DAZ* exon 1, failed to produce a hybridization signal in the TgS12 mouse (Fig. 2*B*). The presence of a breakpoint in yS12 between exons 1 and 2 was confirmed by PCR (*Materials and Methods*). The absence of sequences further upstream of exon 1 was confirmed by hybridization with an additional probe p8A (not shown). Coupled with the STS content, this structural information is consistent with the interpretation that TgS12 contains a single, incomplete copy of the *DAZ* gene, excluding exon 1 and promoter sequences, and probably derived from the centromere proximal end of the *DAZ* gene cluster (Fig. 1).

Tissue Specificity of Transgene Expression. In the TgS12 transgenic mouse *DAZ* transcripts were detectable by RT-PCR

Fig. 2. (*A*) Restriction map of *DAZ* gene indicating the positions of probe fragments and *DAZ* exons 1–11. Scale in bp is shown. (*B*) Southern analysis of genomic DNA from wild-type mouse (*a*), human male (*b*), and TgS12 transgenic mouse (*c*) with probes derived from the *DAZ* genomic region. Probes (p7C46AP, pG5D, and *DAZ*3TER) used are indicated at the bottom.

in testis RNA but not in ovary RNA or in the range of nongonadal tissues that were examined, indicating an appropriate tissue distribution of *DAZ* transgene expression (Fig. 3*A*). Northern hybridization with *DAZ* 3TER confirmed the presence of *DAZ* transcript in the transgenic testis, although comparison with the signal in human testis RNA indicated a much reduced level of transcript in the TgS12 mouse (Fig. 3*B*). At least three bands are seen in the human testis RNA, presumably representing transcripts containing different numbers of *DAZ* repeats (37). A single size class of transcript, within the range seen in human, is apparent in the TgS12 mouse testis. RNA *in situ* hybridization to sectioned adult testes of TgS12 transgenic mice with a *DAZ*-specific probe revealed a pattern of cell type specificity that mirrored *DAZ* expression in human testis. Transcripts were confined to the germ-cell component of the testis lumen and appeared most

Fig. 1. STS map of the AZFc region, including the positions of the *DAZ* gene cluster and *RBM* (*YRRM*) genes adapted from Reijo *et al.* (3). At the top are 22 intervals defined by patient deletions and YAC endpoints. Below that are minimum and maximum possible extents of yS12 revealed by testing for the presence (+) or absence (−) of indicated STS markers. The broken bar indicates the minimal AZFc region as defined by the deletion in azoospermic patient WHT2564 (3). All STSs have been described (3, 41).

Fig. 3. (*A*) Transcription profile of *DAZ* transgene in a range of tissues (lane 1, ovary; lane 2, testes; lane 3, female brain; lane 4, male brain; lane 5, female kidney; and lane 6, male kidney) from TgS12 transgenic mice assayed by RT-PCR. *DAZ* and S16 ribosomal protein primers described in *Materials and Methods*. (*B*) Northern hybridization to adult testis RNA of wild-type mouse (lane 1), TgS12 transgenic mouse (lane 2), and human (lane 3) with probe *DAZ*3TER (*Upper*). The diffuse signal in human RNA represents a range of *DAZ* transcripts containing different numbers of the 24-aa *DAZ* repeat. There is no apparent cross-hybridization to mouse *Dazl*. Signal obtained with a mouse actin probe is shown (*Lower*).

abundant in spermatocyte stages (Fig. 4). Thus, despite its structural rearrangement, the *DAZ* transgene displayed a highly conserved pattern of spatial regulation.

Structure and Expression of the DAZ Transgene. Because Southern blotting indicated that exon 1 and the promoter region are absent from the *DAZ* transgene, 5′ rapid amplification of cDNA ends (RACE) analysis was performed to determine the structure of the *DAZ* transcript in the TgS12 mouse. Sequence analysis of RACE products confirmed the absence of *DAZ* exon 1 from the cDNA sequence and revealed the attachment of at least 150 bp of herpes simplex virus thymidine kinase (HSV-TK) sequence to the second exon of the *DAZ* transcript at nucleotide position 258 (Fig. 5*B*). This arrangement most probably is the result of an interstitial deletion of the yS12 construct fusing *DAZ* exon 2 with the *HSV-TK* gene located upstream in inverted orientation within the YAC left arm (Fig. 5*A*). *DAZ* exon 1 encodes only the initial methionine. The predicted product of the fusion transcript therefore would consist of the remaining amino acids of DAZ plus an N-terminal sequence of 14 foreign aa (Fig. 5*C*). Western analysis with a DAZ-specific antibody confirmed the presence of an immunoreactive transgene product and revealed variegation of transgene expression between individual males (Fig. 6). The retarded migration of the transgene product relative to DAZ protein from the testis of an individual human male may be attributable to a difference in *DAZ* repeat number in addition to the N-terminal extension of the fusion protein. The lower abundance of the transgene protein product may reflect the observed difference in transcript level seen by Northern hybridization (Fig. 3*B*) and/or reduced translational efficiency.

yS12 Confers Partial Rescue of the *Dazl* Phenotype. To test its ability to complement the mouse *Dazl* mutation, yS12 was introduced into a $Dazl^{Tm1Hgu}/Dazl^{Tm1Hgu}$ knockout background (hereafter referred to as $Dazl^{-/-}$) by genetic crossing (see *Materials and Methods*). Progeny homozygous null for *Dazl* and carrying the yS12 *DAZ* transgene ($Dazl^{-/-}$;TgS12) were assessed immunohistochemically for phenotypic rescue by using sectioned adult testes stained with an anti-proliferating
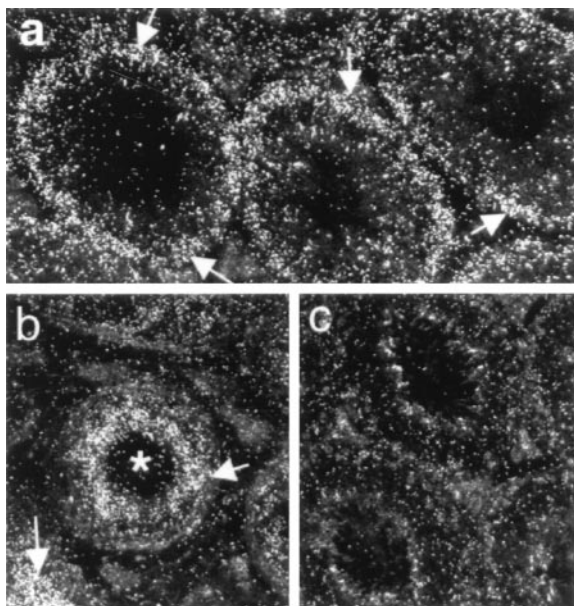

FIG. 5. (*A*) Structural rearrangement of yS12 resulting in fusion of *HSV-TK* and *DAZ* sequences. Positions and orientations of the *DAZ* gene and the *LYS2, TRP1,* and *HSV-TK* genes within the modified YAC are indicated. (*B*) Interstitial deletion has resulted in a fusion of *HSV-TK* sequence to the *DAZ* gene with elimination of *DAZ* exon 1 (including initiation codon) and intron 1. The location of a potential initiation codon is indicated by an asterisk. (*C*) Predicted amino acid sequence of the fusion transcript. *DAZ* nucleotide and amino acid sequences (in *B*) and amino acids introduced by fusion (in *C*) are underlined.

cell nuclear antigen antibody to detect dividing cells (Fig. 7 *A* and *B*). In comparison with testis sections from $Dazl^{-/-}$ controls lacking the *DAZ* transgene (Fig. 7 *C* and *D*), these mice exhibited clear evidence of phenotypic rescue. Although fertility was not restored and there was no evidence of complete spermatogenesis, a high proportion of seminiferous tubules in the transgenic males contained substantial germ-cell numbers and a lumen populated with numerous prophase spermatocytes. In contrast, tubules of control mice often were devoid of germ cells or contained only a few, peripherally located spermatogonial cells, which rarely progressed into meiosis. The scatter diagram in Fig. 8 illustrates data from 19 adult males: 11 genotype $Dazl^{-/-}$;TgS12, five $Dazl^{-/-}$, and 3 wild type. Individuals were scored for the dual criteria of percentage of prophase-positive tubules and number of prophase nuclei per positive tubule. Although individual males varied considerably in the degree of phenotypic rescue, as the distribution in Fig. 8 shows, $Dazl^{-/-}$;TgS12 animals exhibited consistent and, in some cases, dramatic enhancement compared with $Dazl^{-/-}$ controls. In the five $Dazl^{-/-}$ individuals shown, the percentage of prophase-positive tubules ranges from 3% to 38% (mean, 18%). Corresponding values in $Dazl^{-/-}$;TgS12 individuals range from 38% to 90% (mean, 60%). On the criterion of number of prophase nuclei per positive tubule, $Dazl^{-/-}$;TgS12 mice scored from 6 to 20 compared with a range of 3 to 7 in $Dazl^{-/-}$ controls (means of 10 and 3, respectively). No correlation between the degree of rescue and the homozygous or hemizygous status of the YAC transgene (as determined by fluorescence *in situ* hybridization

FIG. 4. Detection of *DAZ* mRNA transcripts in human testis and in a $Dazl^{-/+}$;TgS12 mouse by *in situ* hybridization. Abundant silver grains were present within the seminiferous epithelium (∗) of human (*b*, positive control) and the $Dazl^{-/+}$;TgS12 transgenic mouse (*a*). Most mRNA appeared to be localized in pachytene spermatocytes (arrows). As expected, no *DAZ* mRNA was detected in wild-type mice (*c*).
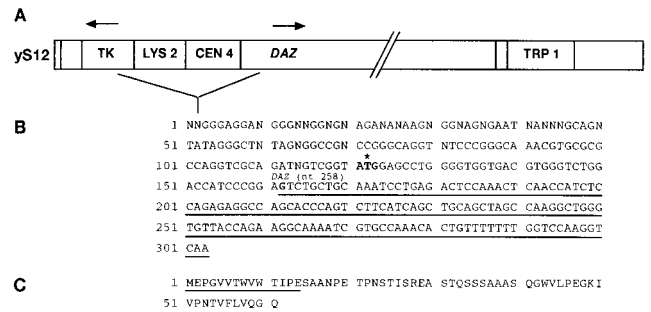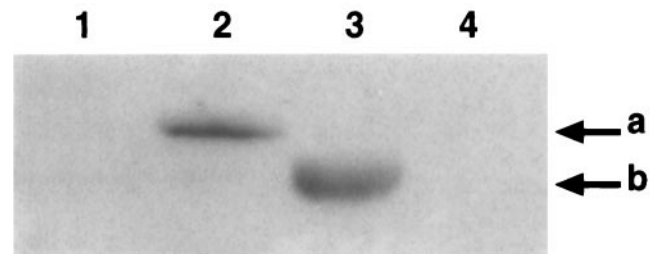

FIG. 6. Western blot of adult human and mouse testis proteins probed with DAZ-specific antibody 133. Lanes: 1, TgS12 transgenic mouse number 1; 2, TgS12 transgenic mouse number 2; 3, human; 4, wild-type mouse. Signals corresponding to the 50-kDa immunoreactive protein in the TgS12 transgenic mouse number 2 (a) and the 45-kDa DAZ protein (b) are indicated. TgS12 mice are Dazl$^{+/+}$.
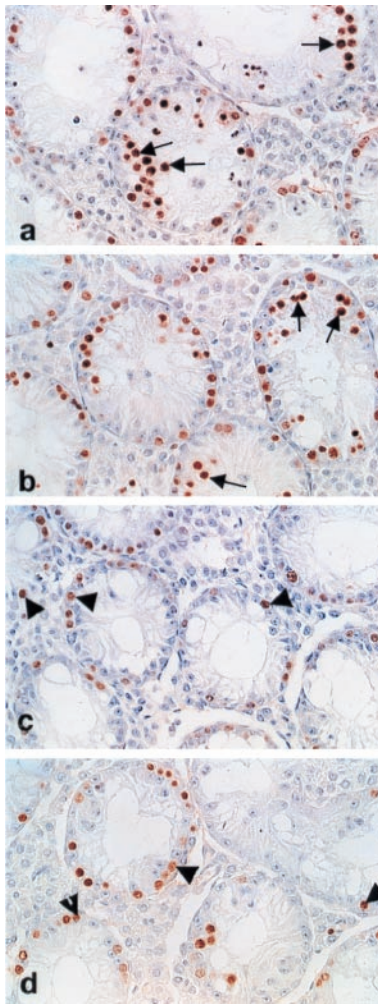
FIG. 7. Immunohistochemical staining of sectioned adult mouse testes with anti-proliferating cell nuclear antigen antibody to detect proliferating cells. In the $Dazl^{-/-}$ mouse a proportion of tubules appears devoid of proliferating germ cells, and the remainder contain only peripherally located, premeiotic spermatogonial stages (*c* and *d*, arrowheads). The $Dazl^{-/-}$;TgS12 transgenic mouse exhibits a pronounced increase in the spermatogonial population and the presence of numerous prophase spermatocytes (arrows) in the tubular lumen, indicating partial restoration of meiosis (*a* and *b*). The $Dazl^{-/-}$;TgS12 and control $Dazl^{-/-}$ mice were derived from the same cross of $Dazl^{Tm1Hgu}/+$;TgS12 × $Dazl^{Tm1Hgu}/+$;TgS12 animals. The individual animals are identified as a and b ($Dazl^{-/-}$;TgS12) and c and d ($Dazl^{-/-}$) in Fig. 8.

analysis on metaphase chromosome spreads; not shown) was detected in the subset of seven $Dazl^{-/-}$;TgS12 animals tested.

DISCUSSION

We have shown that a YAC clone containing a truncated copy of a human *DAZ* gene is capable of partially rescuing the infertility of the male *Dazl* knockout mouse. Consistent with the data from deletion analysis of infertile men, the present findings constitute persuasive evidence that the product of human *DAZ* is active in spermatogenesis and has retained functional homology with its murine autosomal ancestor. The probability of a causal link between the elimination of *DAZ* sequences and spermatogenic impairment in men with AZFc deletions thereby is strengthened.

Although the failure of the S12 YAC to restore fertility could reflect incomplete functional homology between the *DAZ* and *Dazl* gene products, it equally could result from a quantitative insufficiency or structural impairment of the *DAZ*
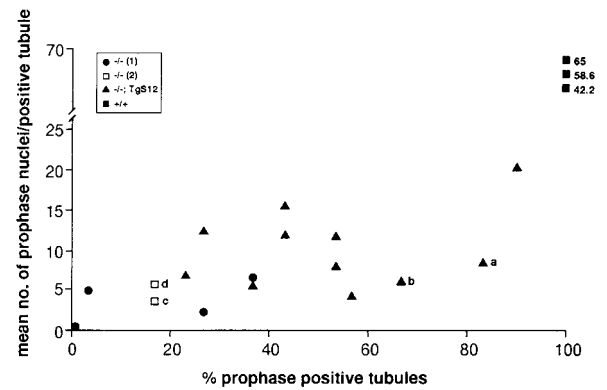

FIG. 8. Scatter diagram indicating the range of phenotypic rescue conferred by yS12. Testis sections from adult mice of genotype +/+, $Dazl^{-/-}$;TgS12 (−/−;S12), and $Dazl^{-/-}$ (−/−) were probed with anti-PCNA antibody and scored for the percentage of prophase-positive tubules and mean number of prophase nuclei per positive tubule. Each point represents data from at least 10 (+/+) or 30 tubules ($Dazl^{-/-}$;TgS12 and $Dazl^{-/-}$) of an individual male. $Dazl^{-/-}$ individuals were generated in one of two genetic crosses: $Dazl^{Tm1Hgu}/+$ × $Dazl^{Tm1Hgu}/+$ (**1**) or $Dazl^{Tm1Hgu}/+$;TgS12 × $Dazl^{Tm1Hgu}/+$;TgS12 (**2**). Points corresponding to the $Dazl^{-/-}$;TgS12 individuals (a and b) and $Dazl^{-/-}$ individuals (c and d) represented in Fig. 7 are indicated.

transgene. Both Northern and Western analyses indicated a low level of *DAZ* expression in the transgenic mouse testis relative to human testis. This reduced expression level simply may reflect a difference in *DAZ* gene copy number because the Y chromosomal *DAZ* cluster is thought to consist of up to seven gene copies (38). Additional possibilities are incomplete conservation of cis- or trans-acting regulatory elements, a negative chromosomal position effect on transgene expression, or both. From our structural analysis of the *DAZ* transgene, which revealed the absence of exon 1 and 5′ flanking sequences, it seems likely that an enhancer element could be lacking, although the observed testis-specific expression pattern does suggest that at least partial regulatory information resides internally or 3′ of the transcribed region. The variability of both transgene expression as revealed by Western blotting and phenotypic rescue would be consistent with a degree of transgene positional silencing, possibly in consequence of the centromeric-insertion site. Furthermore, as a result of structural rearrangement, *DAZ* exons 2–11 are fused with the upstream *HSV-TK* YAC gene, leading to the addition of at least 14 foreign, N-terminal amino acids and the elimination of the native initial methionine. It is possible that these alterations have compromised the function of the transgene and the substitution for the native ATG of a relatively inefficient translation initiation site may have contributed to the reduction in transgene protein product.

Several lines of evidence point to there being a quantitative requirement in gametogenesis for *DAZ* gene family products. In humans, the penetrance of the phenotype in men with Y chromosomal *DAZ* deletions is highly variable, ranging from a complete lack of germ cells (Sertoli Cell Only syndrome) to meiotic arrest at various stages. Even within an individual testis, variegation is apparent between seminiferous tubules (3, 8). In both mouse and *Drosophila*, heterozygosity for the *DAZ* autosomal homologue reveals a degree of haploinsufficiency, and only partial rescue of the null phenotype was provided by a *boule* transgene (17, 25). Mouse *Dazl* is known to be expressed throughout much of the spermatogenic program, from the primordial germ-cell stage up to sperm maturation (25, 39, 40). Therefore, it is likely that the gene operates on different RNA targets and exercises different functions at different stages. The *Dazl* phenotype encompasses at least three classes of defect. The earliest apparent aberration is a

reduction in germ-cell numbers, which is followed at puberty by a meiotic failure manifest as an absence of spermatogenic stages beyond pachytene. The heterozygous phenotype reveals a later function in spermiogenesis. These distinct functions of Dazl protein may be differentially dosage-sensitive. Specifically, our results suggest that the level of product provided by the *DAZ* transgene is sufficient to increase germ-cell survival and permit the initiation of meiosis in the absence of Dazl but below the threshold necessary to support completion of the spermatogenic program. To test the possibility that a higher dosage of DAZ protein could provide full phenotypic rescue, we currently are generating further transgenic lines carrying a range of *DAZ* constructs to obtain increased levels of gene expression.

The quantitative nature of the requirement for *DAZ* protein may explain the presence of the *DAZ* multigene family on the human Y chromosome, in addition to its autosomal progenitor *DAZL1*, if this results in a selective advantage of increased spermatogenic efficiency. Alternatively, the structural changes after transposition to the Y chromosome, including acquisition of the *DAZ* repeat, may have engendered subtle functional divergence such that *DAZ* provides a function complementary to *DAZL1*. The present study points to the feasibility of probing the structure/function relationships of the human *DAZ* and *DAZL1* genes in a mouse model system. From the cross-species conservation demonstrated by the partial rescue of the *Dazl* phenotype it follows also that *DAZ* has at least partial identity of function with its human autosomal counterpart, *DAZL1.* In the clinical field, a therapeutic possibility arising from partial redundancy of *DAZ* and *DAZL1* in the human male would be the mitigation of spermatogenic defects in *DAZ*-deleted men by up-regulation of the function of their autosomal *DAZL1* genes.

1. Tiepolo, L. & Zuffardi, O. (1976) *Hum. Genet.* **34,** 119–124.
2. Kobayashi, K., Mizuno, K., Hida, A., Komaki, R., Tomita, K., Matsushita, I., Namiki, M., Iwamoto, T., Tamura, S., Minowada, S., *et al.* (1994) *Hum. Mol. Genet.* **3,** 1965–1967.
3. Reijo, R., Lee, T. Y., Salo, P., Alagappan, R., Brown, L. G., Rosenberg, M., Rozen, S., Jaffe, T., Straus, D., Hovatta, O., *et al.* (1995) *Nat. Genet.* **10,** 383–393.
4. Najmabadi, H., Huang, V., Yen, P., Subbarao, M. N., Bhasin, D., Banaag, L., Naseeruddin, S., Dekretser, D. M., Baker, H. W. G., McLachlan, R. I., *et al.* (1996) *J. Clin. Endocrinol. Metab.* **81,** 1347–1352.
5. Qureshi, S. J., Ross, A. R., Ma, K., Cooke, H. J., Intyre, M. A., Chandley, A. C. & Hargreave, T. B. (1996) *Mol. Human Reprod.* **2,** 775–779.
6. Stuppia, L., Mastroprimiano, G., Calabrese, G., Peila, R., Tenaglia, R. & Palka, G. (1996) *Cytogenet. Cell Genet.* **72,** 155–158.
7. Pryor, J. L., Kent-First, M., Muallem, A., Van, B. A., Nolten, W. E., Meisner, L. & Roberts, K. P. (1997) *N. Engl. J. Med.* **336,** 534–539.
8. Vogt, P. H., Edelmann, A., Kirsch, S., Henegariu, O., Hirschmann, P., Kiesewetter, F., Kohn, F. M., Schill, W. B., Farah, S., Ramos, C., *et al.* (1996) *Hum. Mol. Genet.* **5,** 933–943.
9. Brown, G. M., Furlong, R. A., Sargent, C. A., Erickson, R. P., Longepied, G., Mitchell, M., Jones, M. H., Hargreave, T. B.,

10. Cooke, H. J., Attara, N. A., *et al.* (1998) *Hum. Mol. Genet.* **7,** 97–107.
10. Ma, K., Inglis, J. D., Sharkey, A., Bickmore, W. A., Hill, R. E., Prosser, E. J., Speed, R. M., Thomson, E. J., Jobling, M., Taylor, K., *et al.* (1993) *Cell* **75,** 1287–1295.
11. Laval, S. H., Glenister, P. H., Rasberry, C., Thornton, C. E., Mahadevaiah, S. K., Cooke, H. J., Burgoyne, P. S. & Cattanach, B. M. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 10403–10407.
12. Elliott, D. J., Ma, K., Kerr, S. M., Thakrar, R., Speed, R., Chandley, A. C. & Cooke, H. (1996) *Hum. Mol. Genet.* **5,** 869–874.
13. Delbridge, M. L., Harry, J. L., Toder, R., Oneill, R. J. W., Ma, K., Chandley, A. C. & Graves, J. A. M. (1997) *Nat. Genet.* **15,** 131–136.
14. Cooke, H. J., Lee, M., Kerr, S. & Ruggiu, M. (1996) *Hum. Mol. Genet.* **5,** 513–516.
15. Reijo, R., Seligman, J., Dinulos, M. B., Jaffe, T., Brown, L. G., Disteche, C. M. & Page, D. C. (1996) *Genomics* **35,** 346–352.
16. Soulard, M., Dellavalle, V., Siomi, M. C., PinolRoma, S., Codogno, P., Bauvy, C., Bellini, M., Lacroix, J. C., Monod, G., Dreyfuss, C., *et al.* (1993) *Nucleic Acids Res.* **21,** 4210–4217.
17. Eberhart, C. G., Maines, J. Z. & Wasserman, S. A. (1996) *Nature (London)* **381,** 783–785.
18. Saxena, R., Brown, L. G., Hawkins, T., Alagappan, R. K., Skaletsky, H., Reeve, M. P., Reijo, R., Rozen, S., Dinulos, M., Disteche, C. M., *et al.* (1996) *Nat. Genet.* **14,** 292–298.
19. Shan, Z., Hirschmann, P., Seebacher, T., Edelmann, A., Jauch, A., Morell, J., Urbitsch, P. & Vogt, P. H. (1996) *Hum. Mol. Genet.* **5,** 2005–2011.
20. Yen, P. H., Chai, N. N. & Salido, E. C. (1996) *Hum. Mol. Genet.* **5,** 2013–2017.
21. Seboun, E., Barbaux, S., Bourgeron, T., Nishi, S., Algonik, A., Egashira, M., Nikkawa, N., Bishop, C., Fellous, M., McElreavey, K., *et al.* (1997) *Genomics* **41,** 227–235.
22. Carani, C., Gromoll, J., Brinkworth, M. H., Simoni, M., Weinbauer, G. F. & Nieschlag, E. (1997) *Mol. Hum. Reprod.* **3,** 479–483.
23. Karashima, T., Sugimoto, A. & Yamamoto, M. A. (1997) *Worm Breeders Gazette* **15,** 65–66.
24. Houston, D. W., Zhang, J., Maines, J. Z., Wasserman, S. A. & King, M. L. (1998) *Development (Cambridge, U.K.)* **125,** 171–180.
25. Ruggiu, M., Speed, R., Taggart, M., Mckay, S. J., Kilanowski, F., Saunders, P., Dorin, J. & Cooke, H. J. (1997) *Nature (London)* **389,** 73–77.
26. Rice, W. R. (1994) *Science* **263,** 230–232.
27. Lahn, B. T. & Page, D. C. (1997) *Science* **278,** 675–680.
28. Yen, P. H. (1998) *Genomics* **54,** 5–12.
29. Agulnik, A. I., Zharkikh, A., Boettger-Tong, H., Bourgeron, T., McElreavey, K. & Bishop, C. E. (1998) *Hum. Mol. Genet.* **7,** 1371–1377.
30. Smith, D. R., Smyth, A. P., Strauss, W. M. & Moir, D. T. (1993) *Mamm. Genome* **4,** 141–147.
31. Shero, J. H., McCormick, M. K., Antonarakis, S. E. & Hieter, P. (1991) *Genomics* **10,** 505–508.
32. Schedl, A., Grimes, B. & Montoliu, L. (1996) *Methods Mol. Biol.* **54,** 293–306.
33. Vollrath, D., Foote, S., Hilton, A., Brown, L. G., Beerromero, P., Bogan, J. S. & Page, D. C. (1992) *Science* **258,** 52–59.
34. Tokunaga, K., Taniguchi, H., Yoda, K., Shimizu, M. & Sakiyama, S. (1986) *Nucleic Acids Res.* **14,** 2829.
35. Majdic, G., Sharpe, R. M., O'Shaughnessy, P. J. & Saunders, P. T. (1996) *Endocrinology* **137,** 1063–1070.
36. Harlow, E. & Lane, D. (1988) *Antibodies: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY).
37. Yen, P. H., Chai, N. N. & Salido, E. C. (1997) *Mamm. Gen.* **8,** 756–759.
38. Glaser, B., Yen, P. H. & Schempp, W. (1998) *Chromosome Res.* **6,** 481–486.
39. Seligman, J. & Page, D. C. (1998) *Biochem. Biophys. Res. Commun.* **245,** 878–882.
40. Habermann, B., Mi, H. F., Edelmann, A., Bohring, C., Backert, I. T., Kiesewetter, F., Aumuller, G. & Vogt, P. H. (1998) *Hum. Reprod.* **13,** 363–369.
41. Foote, S., Vollrath, D., Hilton, A. & Page, D. C. (1992) *Science* **258,** 60–66.