

# Male Infertility Caused by a *de Novo* Partial Deletion of the *DAZ* Cluster on the Y Chromosome\*

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## ABSTRACT

Deletions in distal Yq interval 6 represent the cause of 10–15% of idiopathic severe male infertility and map to a region defined *AZFc* (azoospermia factor c). The testis-specific gene *DAZ* is considered a major *AZFc* candidate, and its deletion has been associated with a severe disruption in spermatogenesis. However, *DAZ* is actually a multicopy gene family consisting of seven clustered copies spanning about 1 megabase. Only deletions removing the entire *DAZ* gene cluster together with other genes have been reported in infertile males. Because no case of spermatogenic failure has been traced to

intragenic deletions, point mutations, or even deletions not involving all the *DAZ* copies, the definitive proof for a requirement of *DAZ* for spermatogenesis is still debatable. Here we report the first case of a partial deletion of the *DAZ* cluster removing all but one of the copies. This deletion is present in a patient affected with severe oligozoospermia who had a testicular phenotype characterized by a great quantitative reduction of germ cells (severe hypospermatogenesis). The absence of this deletion in the fertile brother of the patient suggests that this *de novo* mutation indeed caused the spermatogenic failure. (*J Clin Endocrinol Metab* 85: 4069–4073, 2000)

DELETIONS IN THE *AZFc* (azoospermia factor c) region on distal Yq interval 6 represent the cause of 10–15% of idiopathic azoospermia and severe oligozoospermia and most frequently involve the *DAZ* (deleted in azoospermia) gene family (1–6). Although *DAZ* is not the only gene present in this region (7–9), it is a major *AZFc* candidate. This possibility is supported by the high homology of *DAZ* with a *Drosophila* male infertility gene, *boule* (10), which mutation causes spermatogenic arrest. Furthermore, more recent proofs of the spermatogenic role of the *DAZ* gene product arose from the observation that a human *DAZ* transgene is capable of partially rescuing the sterile phenotype of mouse knockout for the homologous gene *Dazl* (11). The critical role for *DAZ* in human male germ cell development is also supported by the evidence that this gene is testis-specific, with transcription limited to germ cells (12–14), even if its function remains unclear, and the postulated RNA binding property of this gene has not yet been demonstrated (1, 15, 16).

Most difficulties in understanding the biological function of *DAZ* and the actual genotype-phenotype relationship probably arise from the multicopy nature of this gene, present as a cluster of seven copies spanning about one megabase (8, 15, 17). Because deletions of *DAZ* in infertile patients are generally assessed by PCR on genomic DNA

extracted from peripheral leukocytes, only deletions removing the whole *DAZ* gene cluster can be detected. Therefore, intragenic deletions, point mutations, or even deletions not involving all the *DAZ* copies, have not yet been found, and it remains still unknown whether each *DAZ* copy is effectively expressed and active in the testis. The definitive proof for a requirement of *DAZ* for spermatogenesis is therefore still debatable.

Here we report the first evidence of a *de novo* partial deletion of the *DAZ* cluster, removing all but one of the copies, in a patient affected by severe oligozoospermia and a testicular phenotype of severe hypospermatogenesis. These data further support and elucidate the role of this gene in human spermatogenesis.

## Materials and Methods

### Clinical analysis

The patient (PD51) was part of a previously selected group of patients in whom we performed a PCR-based screening of Yq microdeletions (18). The study was approved by the Hospital Ethical Committee, and informed consent was obtained from the patient. The patient was 30 yr old and suffered infertility for 3 yr. Semen samples were obtained on three different occasions, separated by a 3-week interval, following a 3-day period of sexual abstinence; and complete semen analyses were performed according to WHO guidelines (19). Plasma concentrations of FSH (Ares-Serono, Milan, Italy), LH (Ares-Serono), and testosterone (Radim, Rome, Italy) were determined by RIA. A comprehensive history and general investigation excluded any possible causes of testicular damage, such as cryptorchidism, varicocele, seminal tract infections, drug use, endocrinopathies, postmumps orchitis, testicular trauma, or torsion (idiopathic infertility). Details of Yq sequence tagged site (STS)-PCR analysis have been given previously (18), as well as details of testicular fine-needle aspiration cytology (FNAC) technique and analysis (20–22). Briefly, testicular FNAC was performed using 23-gauge (0.6-mm) butterfly needles and aspirating with a 20-mL syringe. The cellular material was placed on microscope slides, air-dried for 24 h,

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stained with May-Grünwald and Giemsa (Merck KgaA, Darmstadt, Germany), and examined under a light microscope at  $\times 125$ ,  $\times 400$ , and  $\times 1250$  magnifications. At least 200 spermatogenic cells were counted per smear, and the following forms were identified and expressed as relative percentages: spermatogonia, primary and secondary spermatocytes, early and late spermatids, and spermatozoa. The interposed Sertoli cells were expressed as the Sertoli index (the number of Sertoli cells per 100 spermatogenic cells), which as been found to be a reliable index of the tubular germ potential (20–22). As described in previous studies (20–22), cytological analysis allowed us to identify the following: 1) complete absence of germ cells, defined as Sertoli cell-only syndrome; 2) quantitative reduction of the germ line, with respect to Sertoli cells, indicating different degrees of hypospermatogenesis; 3) spermatogonia or spermatocytes arrest; 4) spermatids arrest; and 5) normal germ line with increased percentage of mature spermatozoa, indicating an obstruction of the efferent ducts.

#### DNA probes and Southern blot

Probes from various regions of the *DAZ* gene were used to define the deletion in patient PD51 (Fig. 1A). Probe A (98.1-ex 2) is a 1.0-kb PCR fragment containing intron 1 and exon 2 sequences. It was amplified from male genomic DNA using primers corresponding to nt 446–470 and nt 1471–1490 of a *DAZ* cosmid clone 63C9 (GenBank AC000021). Probe B (7A69F/Pst B) is a 1.7-kb *Pst*I genomic fragment containing the entire intron 6, one copy of exon 7, and small segments of exon 6 and intron 7. Probe C (DAZe4-P8/T7) is a 1.0-kb PCR fragment amplified from a *DAZ* complementary DNA clone e4 and contains the 3' end of the *DAZ* gene (16). Probe D, used for fiber-fluorescence *in situ* hybridization (fiber-FISH) analysis (7C46A), is a 6-kb *Eco*RI genomic fragment containing the 5' end of the gene, including exon 1 and 4 kb of the 5' flanking region. Southern blot experiments were performed using standard methods (23), with  $^{32}$ P-labeled DNA probes prepared by the random primed method (Boehringer Mannheim, Milan, Italy). Blots were exposed at  $-70^\circ\text{C}$  for several days.

#### FISH

Metaphase chromosomes and interphase nuclei were prepared from peripheral blood lymphocytes, using standard methods. Plasmid probes 7C46A for the *DAZ* gene (probe D, Fig. 1A) and pHu14 for the *SRY* gene were labeled with biotinylated deoxy-ATP or digoxigenated deoxyuridine 5-triphosphate (Roche, Molecular Biochemicals, Mannheim, Germany) by nick translation (Life Technologies, Inc.) and hybridized to chromosomes, nuclei, and DNA fibers, as previously described (24). Fiber-FISH analysis was performed using sodium-hydroxide-treated slides (25).

#### Results

While screening infertile men for Y-chromosome deletions, by STS-PCR (18), we identified one severely oligozoospermic patient, PD51, whose DNA gave unclear PCR results. PD51 was first assayed with 38 STSs located within deletion intervals 5 and 6 of the Y chromosome (18). The PCR products of all STSs tested, including those flanking the *DAZ* region, were of the expected size and intensity, with the exception of five STSs within the *AZF*c region. In fact, although PD51's DNA was able to direct the synthesis of sY277, sY254, sY279, sY283, and sY255 (1, 6), the yields were much lower than the normal controls, suggesting partial deletion of the *DAZ* cluster (data not shown). To further investigate the possible deletion in PD51, Southern hybridization was performed using probes from various regions of the *DAZ* gene (Fig. 1A). Probe A contained sequences from intron 1 and exon 2. It detected an intense 1.7-kb *Eco*RI fragment derived from the Y-linked *DAZ* genes and a 2.2-kb fragment

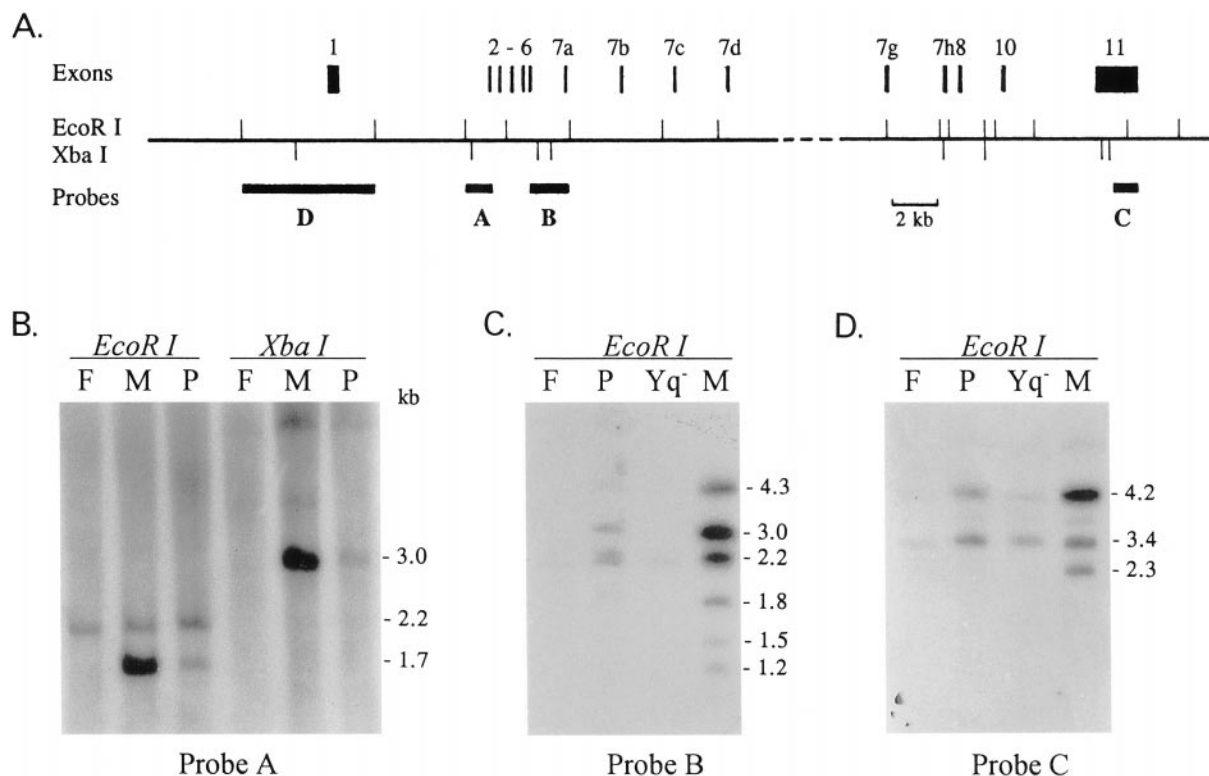


FIG. 1. Genomic structure, probes, and Southern analysis of the *DAZ* gene cluster in patient PD51. A, The genomic structure of a *DAZ* gene. The locations of exons, restriction sites for *Eco*RI and *Xba*I, and the probes used for Southern blot (probes A, B, and C) and fiber-FISH (probe D) are indicated. B, C, and D, Southern blots with probe A, B, and C, respectively (indicated at the bottom), with the restriction enzymes indicated at the top. The samples are: F, normal female; M, normal male; P, patient PD51; Yq-, a patient with Yq deletion (no. 9, Ref. 18).

derived from the autosomal *DAZL1* gene (26) in a normal male, and only the 2.2-kb fragment in a normal female (Fig. 1B). Probe A also detected a major 3.0-kb fragment of *DAZ*, in addition to other minor fragments, in the normal male when the genomic DNA was digested with *Xba*I. The Y-linked *Eco*RI and *Xba*I fragments, but not the autosomal fragments, in PD51 were of significantly reduced intensity compared with those in the normal male, indicating that PD51 had a deletion of most of the *DAZ* genes. Similar reductions in signal intensities of Y-linked fragments in PD51 were also observed using additional probes from either the middle (probe B) or the 3' end (probe C) of the *DAZ* gene (Fig. 1A). It is noted that there are multiple copies of exon 7, which encodes one *DAZ* repeat unit, in a given *DAZ* gene (15, 16). Therefore, probe B, which contained exon 7 and its flanking sequences, detected multiple Y-linked *Eco*RI fragments of variable intensities in a normal male (Fig. 1C). It also detected an autosomal 2.2-kb fragment, which was present in a normal female and in a patient with complete Yq deletion (patient no. 9, with a deletion breakpoint in interval 5A) (18). PD51 contained only some of the Y-linked fragments detected by probe B, indicating the retaining of the middle portion of some *DAZ* genes. Probe C contained the 3' end of the gene and detected an intense 4.2-kb band and two weaker bands of 3.4 kb and 2.3 kb in the normal male and two fragments from the autosomal *DAZL1* gene that are of similar size as some of the Y-linked fragments but of weaker intensity (Fig. 1D). From the relative intensities of the fragments detected by probe C, it is concluded that PD51 retained some 3' end sequences of the *DAZ* gene.

Taken together, these results suggested that PD51 lost most of the *DAZ* copies and that this deletion caused the spermatogenic failure. To test this hypothesis, we performed the same Southern blot experiments in the patient's brother, who was normally fertile. The brother's DNA showed the normal presence of all bands; and also, the relative intensity of the fragments was identical to that of the control male (data not shown). Therefore, the deletion was not present in

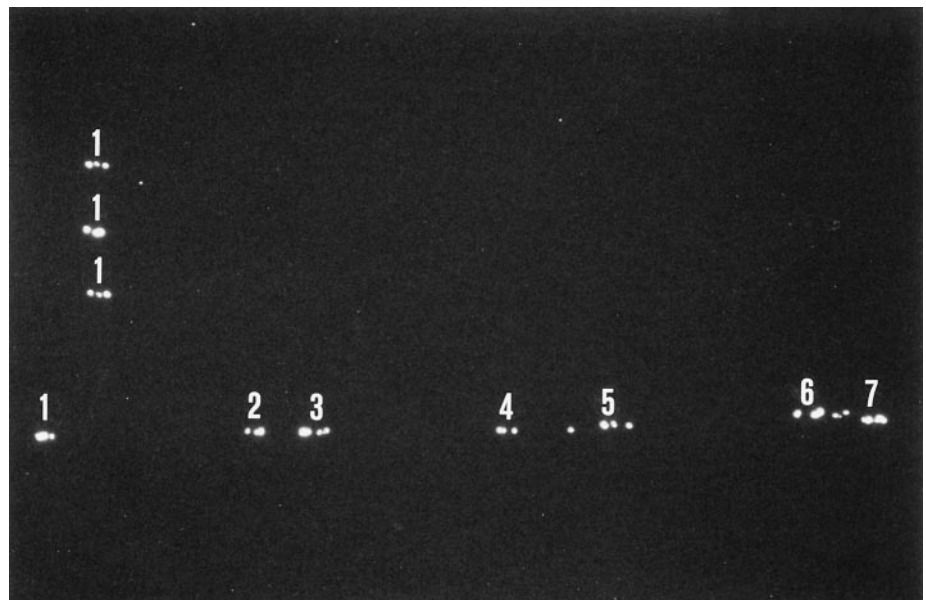
the patient's brother, allowing us to consider the mutation a *de novo* event, and therefore the cause of the testiculopathy.

To further characterize the exact copy number of the *DAZ* genes retained in PD51, we compared signal intensities of the *Eco*RI fragments detected by probe A (Fig. 1B), because, in this case, there was no overlapping between the Y-linked and autosomal fragments. Using the autosomal 2.2-kb *Eco*RI fragment as an internal standard, it was determined, from the relative intensities of the 1.7-kb *Eco*RI fragments in PD51 and in the normal male, that PD51 retained a single *DAZ* gene, assuming that the normal male had seven *DAZ* genes (17).

Our initial attempts to detect the presence of the 5' end of the *DAZ* gene in PD51, by Southern hybridization, were unsuccessful because of weak signals and high backgrounds. To this aim and to directly analyze the copy number of *DAZ* genes present in PD51, we performed FISH experiments using probe D from the 5' end of the *DAZ* gene (Fig. 1A). FISH analysis on metaphases and nuclei from PD51 showed the presence of very tiny signals, compared with those on slides from his brother and healthy controls (data not shown). Therefore, we performed fiber-FISH experiments with this probe, and we found a single short array on 20 relaxed chromatin fibers from PD51; whereas, in his brother, a cluster of about 7 arrays was found (Fig. 2), as expected in a normal fertile man (17). These results not only showed the presence of the 5' end of *DAZ* but also confirmed the single copy of *DAZ* in PD51. Our findings therefore show that PD51 retained only 1 complete copy of *DAZ* gene.

Semen analysis of patient PD51 repeatedly revealed severe oligozoospermia (mean sperm concentration,  $0.25 \pm 0.17 \times 10^6/\text{mL}$ ), with very poor morphology, viability, and motility. His testicular volumes were 17 and 15 mL (normal range, 16–25). The basal FSH plasma concentrations were slightly higher than normal ( $10.5 \pm 2.1 \text{ IU/L}$ ; normal range, 2–8 IU/L), whereas basal LH and testosterone levels were in the normal ranges. Testicular FNAC showed, in both testes, a strong quantitative reduction in the absolute number of germ cells, with respect to Sertoli cells (Sertoli index > 300), a

FIG. 2. Fiber-FISH analysis using probe 7C46A (probe D) for the *DAZ* gene. From the top: three different fibers from patient PD51 show a single short array (number 1 indicates one *DAZ* copy); whereas, on a fiber from the fertile brother of patient PD51, about seven arrays may be seen (numbers 1–7 indicate seven *DAZ* copies).





condition defined as severe hypospermatogenesis (20–22). In this form, each cell type is observed in relatively normal proportions (*i.e.* no maturation arrest was present). A representative picture of the testicular cytology observed in patient PD51 is shown in Fig. 3. Taken together, these data indicate a severe primary testiculopathy, involving only the spermatogenic system.

### Discussion

The *DAZ* gene family constitutes a major candidate for the *AZFc* phenotype, but the definitive proof for its role in human spermatogenesis is still lacking, mainly because no detrimental mutations within the gene have been detected in affected patients. In fact, only deletions removing the entire gene cluster (seven copies, spanning about one megabase) have been found to date (1, 2, 6, 18, 23).

To support the involvement of *DAZ* deletions in determining male infertility, we have performed Southern blot and fiber-FISH experiments, in a severely oligozoospermic patient, in which unclear results were obtained by standard PCR technique (18). By using probes from various regions of

the *DAZ* gene, we were able to demonstrate that the patient retained only one complete copy of the *DAZ* genes, by Southern analysis, and confirmed this finding by fiber-FISH analysis. Furthermore, to clearly support the hypothesis that such deletion was the actual cause of the spermatogenic disruption, we analyzed the fertile brother of the patient, and we found that he carried all the seven *DAZ* copies. These results showed that the partial deletion of the *DAZ* cluster was a *de novo* event arisen in the germ cells of the father and is likely the etiological factor of the testiculopathy in patient PD51. However, we cannot conclude with certainty that this partial deletion actually has determined the spermatogenic. Nevertheless, no other possible causes of testicular damage was evident, and the patient was classified as idiopathic, severely oligozoospermic. Furthermore, the finding that the fertile brother of the patient did not show any alteration in the *DAZ* genes strongly supports the pathogenic role of the partial deletion.

Oligozoospermia may be related to various spermatogenic alterations, including reduction of germ cells (hypospermatogenesis), maturation arrest at different levels (spermatogonia, spermatocytes, spermatids), or partial obstruction of the seminal pathways (20). Therefore, to look for a phenotype-genotype relationship, we analyzed the testicular structure of patient PD51, other than the seminal parameters. We found that the partial deletion of the *DAZ* cluster produced important effects on spermatogenesis and caused a severe primary testiculopathy. Semen analysis revealed severe oligozoospermia. The testes were quite small and the basal FSH plasma concentrations were slightly higher than normal, whereas plasma levels of LH and testosterone were in the normal range. Testicular cytology revealed, in both testes, a strong quantitative reduction in the absolute number of germ cells, with the presence of both premeiotic and postmeiotic spermatogenic cells, defining a histologic diagnosis of severe hypospermatogenesis without spermatogenic arrest.

A clear phenotype-genotype relationship in patients with deletion of the entirety of the *DAZ* cluster has not yet been demonstrated, and *AZFc* deletions may be found both in azoospermic and oligozoospermic men (1, 2, 4–6, 18, 23). Furthermore, different testicular histologies may be found in these patients. The spermatogenic failure observed in patient PD51 seems to suggest that the loss of most copies of *DAZ* produces a depopulation of germ cells, rather than their complete absence, and that *DAZ* probably acts during the first phases of the spermatogenic process. Therefore, it could be speculated that, in patients with deletion of the entirety of the *DAZ* cluster, the loss of other possible gene(s) in the *AZFc* region may exacerbate the spermatogenic disruption, leading to more severe phenotypes, as observed in patients with, for example, Sertoli cell-only syndrome. The recent findings of other genes, such as *BPY2* (7) and *CDY* (7, 8, 27), as well as new exons (9) in the vicinity or within the *DAZ* cluster, seem to support this idea.

Another area of interest is the number of *DAZ* genes that are transcribed and active in the testis. Unfortunately, we could not determine whether *DAZ* was expressed at some level in the spermatogenic cells of PD51, because *DAZ* messenger RNAs have not been detected in ejaculated sperm but

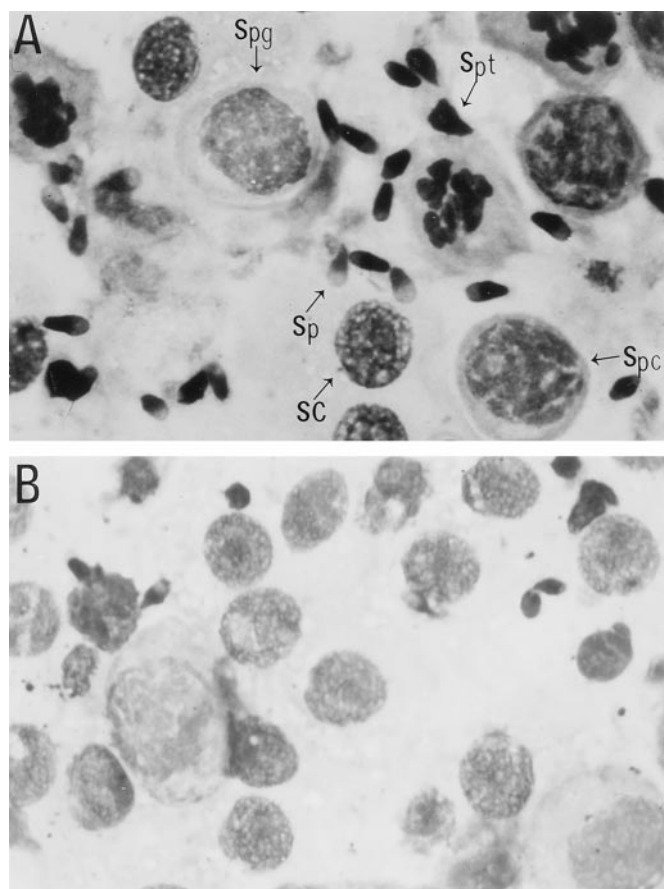


FIG. 3. Representative picture of the testicular cytology associated with partial deletion of the *DAZ* cluster in patient PD51. A, Photomicrograph of normal spermatogenesis from a fertile man. Each spermatogenic cell type is present; B, Severe hypospermatogenesis in patient PD51, characterized by a great quantitative reduction of germ cells. Spg, Spermatogonia; Spc, spermatocytes; Spt, spermatids; Sp, mature spermatozoa; SC, Sertoli cells (May Grünwald-Giemsa staining,  $\times 1250$  magnifications). Bar; 5  $\mu$ m.

only in more immature spermatogenic cells (spermatogonia and spermatocytes) (12, 14). Furthermore, we had no testicular material for expression analysis, because FNA was performed during the diagnostic workup of the patient before molecular experiments, and the patient denied any further analysis.

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