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Absence of *DAZ* gene mutations in cases of non-obstructed azoospermia

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Sequence-tagged site (STS) analysis of the Y chromosome long arm (Yq) of azoospermic males has identified a minimum common deleted region of several hundred kilobases in ~13% of cases. A candidate azoospermia gene, *DAZ* (deleted in azoospermia), has been isolated from this region. *DAZ* has also been shown to be absent in severely oligozoospermic males albeit at a much lower frequency. These data, although highly suggestive, do not constitute formal proof that *DAZ* actually plays a role in azoospermia, as no small intragenic deletions, rearrangements or point mutations in the gene have been found. In this study we report the screening of DNA from 168 azoospermic/oligospermic males for the presence of the *DAZ* gene. Deletions involving *DAZ* were detected in five out of 43 (11.6%) azoospermic males whereas none were found in the remaining 125 oligospermic patients. We present the genomic structure of the 5′ end of the *DAZ* gene together with its sequence analysis in 30 non-obstructed azoospermic males. No mutations in *DAZ* were found in any of the patients sequenced. These data provide no formal proof that *DAZ* is AZF. Thus the possibility is still valid that another gene(s) mapping to the minimum deletion interval may be responsible for, or contribute to, the observed phenotypes. Alternatively, if *DAZ* is AZF, they suggest that the most frequent cause of gene inactivation is via large deletions possibly mobilized by Y chromosome repetitive sequences.

Key words: DAZ/genetics/male infertility/sequence analysis/Y chromosome

Introduction

Evidence for a direct involvement of the human Y chromosome in male infertility was first provided by Tiepolo and Zuffardi (1976). These authors described six azoospermic males with cytogenetically visible terminal deletions of Yq at Yq11. The paternal Y chromosome of four of the males was shown to be normal indicating that the deletions had arisen *de novo*, leading to the hypothesis that DNA in this region must encode genetic factor(s) controlling spermatogenesis. Since then, the genetic complexity at this azoospermia locus (AZF) has been addressed by several authors using molecular genetic techniques to detect submicroscopic Yq microdeletions in DNA extracted from sterile males (Ma *et al.*, 1992; Vogt *et al.*, 1992; Nagafuchi *et al.*, 1993). Two candidate *AZF* genes have emerged from these studies. Ma *et al.* (1993) first isolated a family of Y chromosome genes encoding proteins with RNA-binding motifs termed *RBM* (RNA binding motif). This family is expressed exclusively in the testis in spermatogonia and primary spermatocytes and is conserved on the Y chromosome in most mammals studied. Due to its complex organization (there are at least 20 *RBM* loci on Yp and Yq most of which are probably pseudogenes) its exact role in spermatogenesis remains controversial (Chandley and Cooke, 1994). A new candidate has been isolated recently by Reijo *et al.* (1995). These authors reported screening 89 azoospermic males with more than 84 sequence-tagged site (STS) markers on Yp and Yq. They detected overlapping Yq deletions in 12 of the individuals and used exon trapping strategies to isolate an apparently single copy gene termed *DAZ*, (deleted in azoospermia) from the critical region. Like *RBM*, *DAZ* is well conserved, transcribed in adult germ cells and encodes an RNA binding protein motif. Interestingly, although *RBM* did not appear to be deleted in any of the azoospermic subjects studied by Reijo *et al.* (1995), not all the deleted azoospermic males studied by Ma *et al.* (1992, 1993) were deleted for *DAZ* (e.g. patient Jolar). This raises the question as to the exact relationship between these two genes and their role in the process of spermatogenesis. In this respect, the recent report of Reijo *et al.* (1996a) showing that two out of 35 severely oligospermic males were deleted for *DAZ* is intriguing. It shows that the absence of *DAZ*, although severely compromising spermatogenesis, does not completely preclude the production of mature spermatozoa. Additional evidence for the role of *DAZ* in azoospermia is provided by the finding that its fruit fly homologue, *boule*, is essential in the meiotic stages of *Drosophila* spermatogenesis (Eberhart *et al.*, 1996).

In the most extensive study of sterile males to date, Vogt *et al.* (1996) examined 370 males with idiopathic azoospermia or severe oligospermia for the presence of 76 Yq11 loci. They present evidence for a more complex system involving three distinct AZF loci in Yq11 termed AZFa (proximal), AZFb (central) and AZFc (distal, corresponding to the deleted *DAZ* region). A meticulous genotype/phenotype

correlation indicated that expression of gene(s) within AZFa and AZFb are needed before proliferation or at meiosis respectively, and that AZFc is active later during meiosis or in spermatids.

The aim of the present study was two-fold. Firstly, to assess the frequency of *DAZ* deletions in our patient population of azoospermic and oligospermic males. Secondly, to analyse the sequence of the *DAZ* gene in non-deleted azoospermics for small mutations, thereby providing formal proof of the involvement of *DAZ* in male sterility.

Materials and methods

DNA extraction and polymerase chain reaction

DNA was extracted from 10 ml of peripheral blood using standard procedures. It was screened for the presence of the *DAZ* and *SRY* genes by polymerase chain reaction (PCR) under the following conditions: DNA 100 ng, 10 mM Tris–HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 100 μM each dNTP, 100 pmol each specific primer, and 1 IU *Taq* polymerase in a 50 μl reaction volume. After an initial denaturation step at 94°C for 4 min, cycle parameters were 94°C for 30 s, 60°C for 30 s, and 72°C for 60 s for 30 cycles. Primer sequences are given below.

Isolation of the cDNA DAZ *clone p*DAZ*bcm*

Primers flanking the open reading frame of the *DAZ* cDNA were designed according to the published sequence (Reijo *et al.*, 1995). They were then used to isolate a reverse transcription (RT)-PCR product from human testis RNA using a Marathon-Ready 1st strand cDNA (Clontech). A 1.0 kb product was obtained, cloned into pBluescriptII (Statagene) and sequence analysis of a single clone confirmed that the *DAZ* cDNA product had been correctly obtained.

Genomic structure of DAZ

A cosmid library, highly enriched for the Y chromosome by flow sorting, was kindly provided by Dr Y.C.Lau (San Francisco). This library, consisting of ~3000 arrayed cosmids, was screened using the p*DAZ*bcm cDNA labelled with [^{32}P]-dCTP by random priming (Prime-it kit; Stratagene) according to the manufacturer's instructions. Cosmid filters were hybridized for 16 h at 65°C in 7% sodium dodecyl sulphate (SDS), 0.5 M $NaPO_4$ containing 2×10^6 c.p.m./ml of probe, followed by washing 3×30 min in 0.1× sodium chloride/sodium citrate (SSC) at 65°C. DNA obtained by positively hybridizing individual clones was restricted with EcoRI, separated on a 0.8% agarose gel and transferred by Southern blot to Zetaprobe GT nylon membrane. The filter was probed with several [^{32}P]-labelled exon specific oligonucleotides and two EcoRI fragments of 2 and 2.8 kb were identified which spanned the 5′ RNA binding domain of the gene. These fragments were excised from the gel, cloned and partially sequenced using the dideoxy chain termination method and sequenase2 T7 polymerase (USB). Their sequence was then compared with that of the *DAZ* cDNA and in this way the intron/exon structure was deduced. Probing the cosmid blot with oligonucleotides from the 3′ non-coding region of *DAZ* did not reveal any hybridizing fragments.

Sequencing DAZ *from azoospermic males*

Two intron primers, DAZ51 and DAZ16, were designed to specifically amplify a 2.8 kb genomic fragment encompassing the 5′ end of the *DAZ* gene as shown in Figure 1. To aid in subsequent sequencing, the DAZ51 primer was 5′ biotinylated. PCR was conducted with the Expand, long template PCR system (Boehringer Mannheim) using buffer 1, 500 ng DNA, 200 μM each dNTP, 100 μM each specific primer, and 1 IU Expand polymerase in a 100 μl reaction volume. Cycle parameters were: initial denaturation 94°C for 2 min followed by 92°C for 10 s, 60°C for 30 s, 68°C for 3 min for 10 cycles; followed by 92°C for 10 s, 60°C 30 s, 68°C for 3 min + 20 s increment/cycle for 25 cycles ending with a 7 min extension at 68°C. After confirming amplification on a 0.8% agarose gel, excess primers and nucleotides were removed by centrifugation through a Micron 100 micro-concentrator (Amicon). The PCR product was then incubated with paramagnetic streptavidin Dynabeads M-280 (Dynal) for 30 min, denatured by adding 0.1N NaOH and the biotinylated strand captured and washed using a strong magnet. Direct solid phase sequencing was performed on aliquots of the product using the dideoxy chain termination method as above. Five intron primers, (DAZ16, DAZ15, DAZ14, DAZ13, DAZ12) located close to the intron/exon junctions were used to sequence the exons as shown in Figure 1. The sequence of primers used was:

254F	GGGTGTTACCAGAAGGCAAA
254R	GAACCGTATCTACCAAAGCAGC
SRYF	GAATATTCCCGCTCTCCGGA
SRYR	GCTGGTGCTCCATTCTTGAG
DAZ51	CACTATGCTATATTGTTTCTCCAACG (biotinylated 5′ end)
DAZ12	AGTTTGTAACAGGGCCCACAT
DAZ13	TTGTCTGATACTTATAGAA
DAZ14	TTTTACACAAGTCCGTGTG
DAZ15	GGCTGCACATGACGAGCA
DAZ16	CCACAGAGGGAAGGATGACTA

Results

In all, 168 male idiopathic infertile patients presenting at the Baylor College of Medicine, Department of Urology Male Infertility Clinic, Texas, USA, together with 55 normal males, were screened for the presence of the *DAZ* and *SRY* genes. The results of the screening and the clinical findings can be seen in Table I. Of the 168 patients examined, 43 could be classified as non-obstructed azoospermic (no spermatozoa in the ejaculate), 28 as severely oligospermic ($<1\times10^6$ spermatozoa/ml), 87 as oligospermic ($1–20\times10^6$ spermatozoa/ml), nine were found to have congenital bilateral absence of the vas deferens (CBAVD) due to mutations in the cystic fibrosis gene (CFTR) and one patient was found to be an XX male. None of the patients was karyotyped except for the XX male. *SRY* could be detected in all of these patients showing that the extracted DNA could be amplified normally. The *DAZ* gene was found to be deleted in five of the azoospermic males, representing 11.6% of the total. *DAZ* was not deleted in any other clinical category of infertile males except in the XX male where it is most probable that only a small fragment of the short arm of the Y is present in the otherwise female XX genome. Further analysis of the five patients deleted for *DAZ* with more STS markers showed that three of them harboured large terminal deletions involving approximately half of Yq whereas the other two had interstitial deletions of ~1–5 Mb which did not extend into the heterochromatic portion of Yq (data not shown).

Screening of the flow-sorted Y cosmid library with the *DAZ* cDNA identified 12 cosmids containing the *DAZ* gene. Five of the cosmids were identical as judged by restriction analysis, and the other seven overlapped extensively. All cosmids

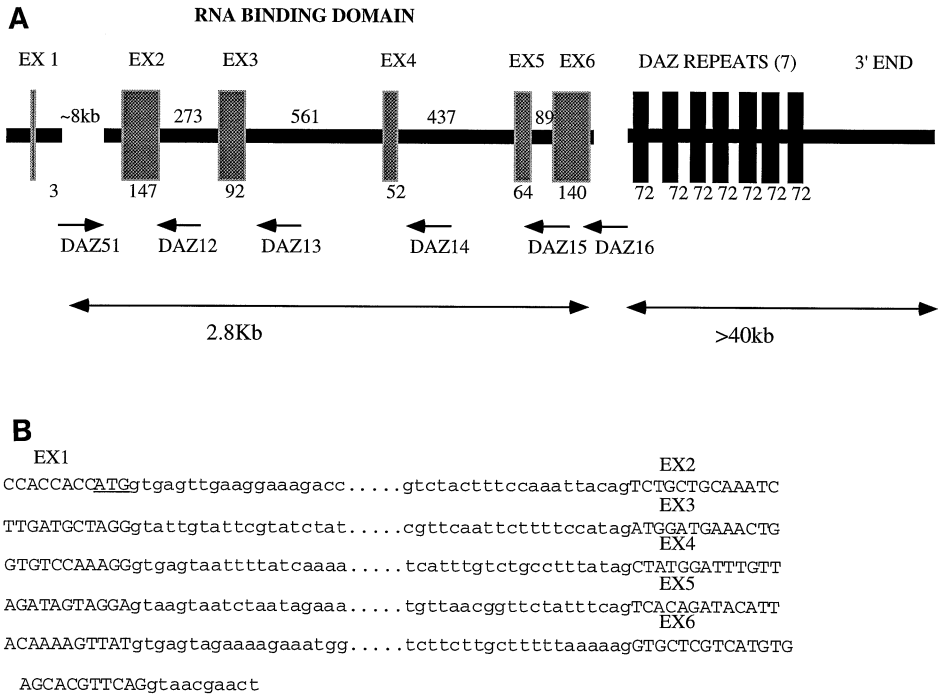

Figure 1. (**A**) Genomic stucture of the 5′ end of the *DAZ* gene. Sizes of the introns and exons are given in base pairs (bp). Position of the primers used for amplification and sequencing are shown. (**B**) Sequence of the exon/intron boundaries of the 5′ end of the *DAZ* gene.

Table I. Results of screening 168 males, presenting at the Baylor College of Medicine, Department of Urology Male Infertility Clinic for idiopathic male infertility, together with 55 normal male controls, for deletions of the Y-linked *DAZ* gene

Patients	*n*	ΔDAZ	Percentage
Azoospermic	43	5	11.6
Severe oligozoospermic	28	0	–
Oligozoospermic	87	0	–
CBAVD	9	0	–
XX male	1	1	100
Normal controls	55	0	–

Azoospermic = no spermatozoa present in ejaculate; severe oligozoospermic = $<1\times10^6$ spermatozoa/ml; oligozoospermic = $1–20\times10^6$ spermatozoa/ml; normal = $>20\times10^6$ spermatozoa/ml.

contained the 5′ end of the gene including at least one copy of the 72 bp repeat. None of the cosmids contained any coding sequences 3′ to the repeated blocks suggesting that the repeats and 3′ end extend over a minimum of 40 kb of genomic DNA.

The partial structure of the 5′ end of the *DAZ* gene is presented in Figure 1A. The first coding exon includes only the initiator ATG followed by an intron of ~8 kb. The remainder of the 5′ coding sequence up to the *DAZ* repeats is contained in five exons spanning 1.9 kb. The sequence at the intron/exon boundaries is shown in Figure 1B. Each of the 72 bp *DAZ* repeats represents a single exon and, together with the remaining 3′ end of the gene, extend over a minimum of 40 kb.

The 5′ end of the *DAZ* gene encompassing the RNA binding domain, was directly sequenced from 30 non-deleted azoospermic males. The sequences were then compared with themselves and with that of the published *DAZ* cDNA. No mutations of any kind were found in any of the five exons examined. In addition, no sequence polymorphisms could be seen in the intervening intronic DNA sequenced.

Discussion

The results of screening our infertile male population for the presence of *DAZ* showed that 11.6% were deleted for this gene. None of the deletions were intragenic and involved relatively large amounts of Y chromosome DNA. Although the fathers were not available for testing, it is assumed that the deletions were *de novo* in origin as *DAZ* deletions have not been found in normal, fully fertile males. Testis biopsies of the five deleted males showed a variable phenotype. Most tubules contained only Sertoli cells but in all patients rare tubules containing a few germ cells in various stages of arrest could be detected. These data are in keeping with those of previous reports which show that approximately the same percentage of azoospermic males have variable sized micro-deletions in Yq11, completely removing the *DAZ* gene and giving a similar heterogeneous histological pattern (Kobayashi *et al.*, 1994; Reijo *et al.*, 1995; Najmabadi *et al.*, 1996; Vogt *et al.*, 1996).

In light of the report by Reijo *et al.* (1996a) that *DAZ* deletions can be associated with severe oligozoospermia, we screened 28 severely oligozoospermic ($<1\times10^6$ spermatozoa/ml) and 87 oligozoospermic males ($1–20\times10^6$ spermatozoa/ml) for the presence of *DAZ*. In contrast, in our oligospermic population no evidence for deletions of this gene was seen. This suggests that *DAZ* deletions in oligospermics are rarer and a larger number of patients will need to be screened to obtain an accurate estimate of deletion frequency.

It should be emphasized that, although *DAZ* is a compelling candidate azoospermia gene, formal proof of its role is lacking,

as all deletions identified to date are relatively large. Indeed, in the present study, three of the five deletions found in the azoospermic males were large terminal deletions leaving only two clear cases of microdeletion representing 4.7% of cases. The finding of small intragenic deletions, rearrangements or point mutations would provide direct proof for the role of *DAZ*. In addition, the possibility exits that the degree of infertility may well be related to the type of mutation found. We therefore derived the intron/exon structure of the 5′ end of the *DAZ* gene and used it to design a direct, solid phase sequencing strategy as detailed in Figure 1. The 5′ end of the gene was targeted as it contains the potential RNA binding domain which is most likely to be of functional importance. However, no evidence for mutation was found in any of the five terminal *DAZ* exons amplified and sequenced from 30 non-obstructed azoospermic males.

The sequencing strategy also provided partial data on the intervening introns. A consistent T+C was found instead of a single base in intron 3 near the exon 4 boundary indicating that we had amplified two distinct copies of the *DAZ* gene (data not shown). As the change occurred within an MboI site (i.e. GATC or GACC) we were able to confirm this finding by amplifying the intron and restricting the product with MboI. Three bands were seen of the predicted size consistent with the amplification of a minimum of two copies of the *DAZ* gene. A careful re-examination of the sequencing data confirms that neither copy of the *DAZ* gene shows any exonic changes. In all probability this second copy of *DAZ* represents the homologous *SPGY* gene reported by Vogt *et al.* (1996) and Maiwald *et al.* (1996). Indeed, it has recently been shown that the DAZ locus is in fact more complex than previously thought having arisen by translocation, duplication and deletion of an ancestral *DAZLA* gene located on human 3p (Saxena *et al.*, 1996; Yen *et al.*, 1996; McElreavy *et al.*, personal communication).

Taken together, these data suggest that either *DAZ* is not AZF or alternatively that DNA deletion is the primary cause of inactivation of the locus. As the indirect evidence linking deletions of *DAZ* with infertility is strong we favour the latter hypothesis. This implies that the infertility seen in the 87% of patients not deleted for *DAZ* must have another aetiology. Undoubtedly a percentage of these will be due to mutations/deletions in other Y located genes mapping to AZFa or AZFb. In this respect, *RBM* remains a strong contender. We suggest, however, that mutations in autosomal genes must play a major role. Indeed, a re-examination of the medical records of several infertility clinics including our own suggests a surprisingly high familial component (Lilford *et al.*, 1994; our unpublished observations; McElreavy *et al.*, Institute Pasteur). In fact *DAZ* is a very recent addition to the Y chromosome being Y-located only in New World monkeys and humans. In all other mammals tested the *DAZ* homologues (*Dazla)* are autosomal and there is no Y copy (Cooke *et al.*, 1996; Maiwald *et al.*, 1996; Reijo *et al.*, 1996b). In humans a highly homologous functional autosomal *DAZLA* gene also exists on chromosome 3p (Saxena *et al.*, 1996; Yen *et al.*, 1996). What role, if any, the *DAZLA* gene plays in fertility and its functional relationship to its Y-linked homologue is currently under investigation.

Clear candidates for other autosomal loci playing a role in spermatogenesis are genes involved in cell–cell signalling pathways, mismatch repair, control of meiosis/mitosis and chromatin remodelling. Recent reports of targeting such genes in mice (Baker *et al.*, 1995, 1996; Bitgood *et al.*, 1996; Blendy *et al.*, 1996; Nantel *et al.*, 1996; Roest *et al.*, 1996) has led to the production of sterile males whose testicular histology resembles that seen in some types of human male sterility. We are currently trying to assess their importance in our infertile male population.

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