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Polymorphic expression of DAZ proteins in the human testis

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BACKGROUND: *DAZ* is a male infertility gene located at the *AZFc* region of the Y chromosome. There are four copies of the *DAZ* gene that share a strong homology but are not identical to one another. In the present study, we carried out cDNA cloning and immunoblot analyses to determine whether all of the *DAZ* genes are actively expressed in the human testis.

METHODS: *AZFc* deletion was detected by sequence-tagged site polymerase chain reaction (PCR) of genomic DNA isolated from blood samples. *DAZ* cDNAs were cloned with RT–PCR followed by sequence analysis. The expression of DAZ proteins in human testis was determined by immunoblot and compared with *DAZ* cDNA expression.

RESULTS: Immunoblot analysis revealed four DAZ protein bands in testis samples that showed no deletions in the *AZFc* region. No specific bands were observed in samples from *AZFc* deletion patients. Testis samples from individuals with the partial *AZFc* deletion, gr/gr, showed two DAZ-specific bands. Interestingly, the sizes of DAZ-specific bands varied among individuals. Analysis of *DAZ* transcripts in testis samples revealed that the DAZ proteins were translated from the largest of the multiple transcripts originating from each single *DAZ* gene.

CONCLUSIONS: All four *DAZ* genes are expressed in the human testis, and their products are highly polymorphic among men.

Key words: DAZ / Y chromosome / male infertility / testis

Introduction

A genetic component of male infertility was introduced when microdeletions in the Y chromosome were observed in infertility patients (Tiepolo and Zuffardi, 1976). Frequently deleted loci in the Y chromosome were defined as *Azoospermia Factor* (*AZF*), and related genes were considered candidate genes for male infertility (Reijo *et al*., 1995; Ferlin *et al*., 1999, 2007). *Deleted in Azoospermia* (*DAZ*) in the *AZFc* region has attracted particular interest because its involvement in germ cell development is evolutionally conserved. In *Caenorhabditis elegans*, *DAZ-1* mutants are sterile through blockage of oogenesis at the pachytene stage of meiotic prophase I (Karashima *et al*., 2000). Mutations of *boule*, the *Drosophila* homolog of *DAZ*, result in a primary defect of meiotic cell division in male germ cells (Eberhart *et al*., 1996). *Dazl* knockout mice show defects in gamete production (Ruggiu *et al*., 1997), and the leptotene–zygotene stage of meiotic prophase I is the final point reached in male germ cell development (Saunders *et al*., 2003).

DAZ family proteins have one or more RNA recognition motif (RRM) domains and multiple DAZ repeats whose function remains to be characterized. It was proposed that DAZ family proteins are involved in mRNA metabolism during spermatogenesis (reviewed in Yen, 2004; Reynolds and Cooke, 2005). Dazl proteins co-fractionate with the polysome pool, suggesting that Dazl-bound mRNAs are translationally active (Tsui *et al*., 2000; Maegawa *et al*., 2002). In fact, DAZ family proteins specifically interact with a group of mRNAs to augment translation *in vivo* (Collier *et al*., 2005; Reynolds *et al*., 2005, 2007). However, an interaction of DAZ with PUM2, a potential repressor of translation, has also been reported (Moore *et al*., 2003). It was recently proposed that the mouse Dazl protein functions as an adaptor to facilitate transport of a set of mRNAs to specific sites within male germ cells (Lee *et al*., 2006).

In humans, there are three genes in the DAZ family: *DAZ*, *DAZL* and *BOULE*. It has been proposed that the ancestral *BOULE* gave rise to *DAZL* by duplication and transposition in the early vertebrate lineage and that *DAZ* arose during primate evolution by transposition and

amplification of *DAZL* (Saxena *et al*., 1996; Xu *et al*., 2001). *DAZ* was amplified to four copies in the Y chromosome, possibly through tandem duplication and inversion events during evolution (Saxena *et al*., 2000; Kuroda-Kawaguchi *et al*., 2001). The four *DAZ* genes share more than 99% homology but are distinct in terms of the number of RRM domains and DAZ repeat sequences (Saxena *et al*., 2000; Kuroda-Kawaguchi *et al*., 2001; Fernandes *et al*., 2002; Vogt and Fernandes, 2003). It has been well documented that individuals with complete deletion of *AZFc* show spermatogenic defects with variable penetration rates (Reijo *et al*., 1995; Ferlin *et al*., 1999, 2007; Kuroda-Kawaguchi *et al*., 2001). It has also been reported that partial deletions of *AZFc* such as gr/gr are often transmitted from father to son and represent a significant risk factor for spermatogenic failure (Repping *et al*., 2003). The b2/b3 deletion, another type of partial deletion of *AZFc*, poses no risk on male fertility (Repping *et al*., 2004). This suggests that an intact or a partial set of *DAZ* genes may be required for the completion of spermatogenesis.

Despite their implication in male fertility, it is still elusive whether all four *DAZ* genes are actively expressed or not. Previous attempts have revealed a single DAZ band in immunoblot analyses (Habermann *et al*., 1998; Reijo *et al*., 2000). More recently, Huang *et al*. (2008) provided evidence that all four *DAZ* genes are transcribed, but failed to detect DAZ proteins by immunoblot analysis. In this study, we determined whether four *DAZ* genes are actively expressed in the human testis. The results show that all *DAZ* genes are active and polymorphic.

Materials and Methods

Human samples and deletion screening in the AZFc region

Human blood and testis samples from 13 patients were obtained with informed consent and IRB approval of the Seoul National University Hospital. The samples were stored at −80°C until further experiments.

Deletions in the *AZFc* region were screened using genomic DNA as templates for sequence-tagged site polymerase chain reaction (STS-PCR) with the following STSs: sY255 (GenBank accession number: G65827), sY1191 (GenBank accession number: G73809) and sY1291 (GenBank accession number: G72340). PCR primers for *ZFY* and *SRY* were also used as controls (Simoni *et al*., 2004). PCR was performed using the following conditions: a preheating at 95°C for 15 min, 35 cycles of 94°C for 30 s; 55°C for 30 s; 72°C for 30 s; and a final extension at 72°C for 4 min. The PCR products were analyzed on 2% agarose gels.

Deletion types were predicted and designated as a 'gr/gr deletion' when only sY1291 was negative (Fernandes *et al*., 2002; Repping *et al*., 2003), an '*AZFc* deletion' when all of three STSs were negative (Kuroda-Kawaguchi *et al*., 2001) and a 'no *AZFc* deletion' when all of three STSs were positive. When the only sY1191 was negative, it was termed 'sY1191-negative deletion', being previously regarded as a 'b2/b3 deletion' or a 'g1/g3 deletion' (Fernandes *et al*., 2004; Repping *et al*., 2004). From deletions in the *AZFc* region, the *DAZ* copy number in each sample was deduced.

Plasmids and antibodies

The cDNA clones of human *DAZ2* (GenBank accession number: BC113006), *DAZL* (GenBank accession number: BC027595) and *BOULE* (GenBank accession number: BC033674) were purchased from RZPD (Berlin, Germany). The coding regions of the cDNAs were subcloned into *pGEM-T easy* vector (Promega), and subsequently into *pGEX-4T* vector (GE Healthcare) for antibody generation or into *pcDNA3.1-HA* vector (Invitrogen) for expression in mammalian cells.

Two rabbit polyclonal antibodies were raised against bacterially expressed GST-DAZ2 and GST-DAZL fusion proteins, resulting in anti-DAZ and anti-DAZL antibodies. The specific antibodies were further affinity-purified using an immunoblot method (Harlow and Lane, 1999). In brief, the antisera were incubated with a strip of nitrocellulose membrane blotted with GST-DAZ2 or GST-DAZL fusion proteins, and eluted with 100 mM glycine (pH 2.5). Monoclonal antibody against HA epitopes was purchased from Sigma (St Louis, MO, USA). Peroxidase-conjugated anti-rabbit or anti-mouse IgG (Sigma-Aldrich) was used as a secondary antibody.

Cell culture, transfection and immunoblot analysis

293T cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, and maintained in 5% CO_2 at 37°C. Plasmids were transfected using polyethylenimine (Sigma-Aldrich) according to the manufacturer's instruction. Twenty-four hours after transfection, the cells were lysed and subjected to immunoblot analysis.

Human testis samples or cultured cells were lysed in 1× SDS sample buffer (50 mM Tris–HCl, pH 6.8, 100 mM dithiothreitol, 2% SDS, 0.1% Bromophenol Blue and 10% glycerol). About 20 μg of protein extract was loaded in each lane of 10% polyacrylamide gels and then transferred into nitrocellulose membranes. The membranes were blocked in 5% skim milk in TBST (20 mM Tris, 150 mM NaCl and 0.3% Triton X-100) for 30 min, and then incubated with anti-DAZ (1:100 dilution), anti-DAZL (1:100 dilution), preimmune serum (1:1000 dilution) or anti-HA (1:10 000 dilution) overnight at 4°C. After being washed three times with TBST for 5 min, the membranes were incubated with peroxidase-conjugated secondary antibodies at a dilution of 1:15 000 for 30 min. After the membranes were washed three times with TBST for 5 min, the peroxidase activity was detected using ECL reagent.

Immunohistochemistry

Human testis samples were fixed in Bouin's solution (Sigma-Aldrich), and paraffin-embedded tissues were sectioned at 6 μm. The sections were deparaffinized, hydrated and boiled for 15 min in 10 mM citrate (pH 6.0). After cooling at room temperature, the sections were blocked in 1% goat serum for 30 min and then incubated with anti-DAZ (1: 10 dilution) or preimmune serum (1:100 dilution) for 1 h. After being washed with PBST (phosphate-buffered saline with 0.1% Triton X-100), the sections were incubated with either biotinylated or FITC-conjugated secondary antibody for 30 min. The sections were washed with PBST and then the biotinylated antibody was visualized with avidin:biotinylated peroxidase complex (Vector). The sections stained by immunoperoxidase methods were further washed, developed with 3,3′-diaminobenzidin tetrachloride (Sigma-Aldrich) and counterstained with hematoxylin (Sigma-Aldrich).

Cloning and expression of DAZ transcripts

Testicular RNA was extracted from human testis samples using NucleoSpin® RNA XS (Macherey-Nagel) according to the manufacturer's instruction, and then reverse-transcribed using random hexamers. The resultant cDNAs were amplified by PCR using the following primers: *DAZ* coding region (forward: tttccttacaccttagcctttgg, reverse: tcccaatcaaattggacataca), *DAZ* 3′UTR (forward: ggagcaaaggagaaatctgtgg, reverse: tcccaatcaaattggacataca), *DAZL* (forward: cacagcctctgctcctcct, reverse: aaaccagcaacttcccatgt), *GAPDH* (forward: tggcgtcttcaccaccat, reverse: caccaccctgttgctgta). PCR was performed using the following conditions: a preheating at 94°C for 5 min, 35 cycles of 94°C for 30 s; 55.5°C for 30 s; 72°C for 3 min; and a final

extension at 72°C for 7 min. The PCR products were then analyzed on 0.9–2% agarose gels.

Notably, the primers for *DAZ* coding region were designed to attach to the untranslated regions common to all of the already-known seven *DAZ* transcripts: *DAZ1* (GenBank accession number: NM 004081), *DAZ2* transcript variant 1 (GenBank accession number: NM 020363), *DAZ2* transcript variant 2 (GenBank accession number: NM 001005785), *DAZ2* transcript variant 3 (GenBank accession number: NM 001005786), *DAZ3* (GenBank accession number: NM 020364), *DAZ4* transcript variant 1 (GenBank accession number: NM 001005375) and *DAZ4* transcript variant 2 (GenBank accession number: NM 020420). This enabled us to clone all of the *DAZ* transcripts in human testis.

To clone *DAZ* transcripts, the amplified PCR products were phenol/chloroform extracted and then ligated into *pGEM-T easy* vector (Promega). After color screening to select the bacterial colonies containing proper inserts, the plasmids were further confirmed by sequence analyses. For sequencing, an additional primer (reverse: agggcactgcggtggcatct) that attaches to the end of DAZ repeat was used. The screening procedure was repeated three times, resulting in more than 40 *DAZ* cDNA clones for each testis sample.

For mammalian expression of the *DAZ* cDNAs, the *pGEM-T easy* vector containing an entire protein coding region of *DAZ* was digested by EcoRI, and the resulting fragment was subcloned into *pcDNA3.1*(+) vector (Invitrogen). Subsequent cell culture, transfection and immunoblot analysis were performed as described above.

Results

Detection of DAZ proteins in human testis

We generated and affinity-purified polyclonal antibodies against GST-DAZ2 and GST-DAZL fusion proteins. In order to test the specificity of the antibodies, we carried out immunoblot analysis with ectopically expressed DAZ family proteins and found the DAZ antibody detected both HA-DAZ and HA-DAZL proteins (Fig. 1A). The DAZL antibody detected HA-DAZL strongly and HA-DAZ weakly (Fig. 1A). Neither DAZ nor DAZL antibody detected HA-BOULE. In human testis lysates (#001), the DAZ antibody detected three specific bands with similar intensities, whereas no band was observed

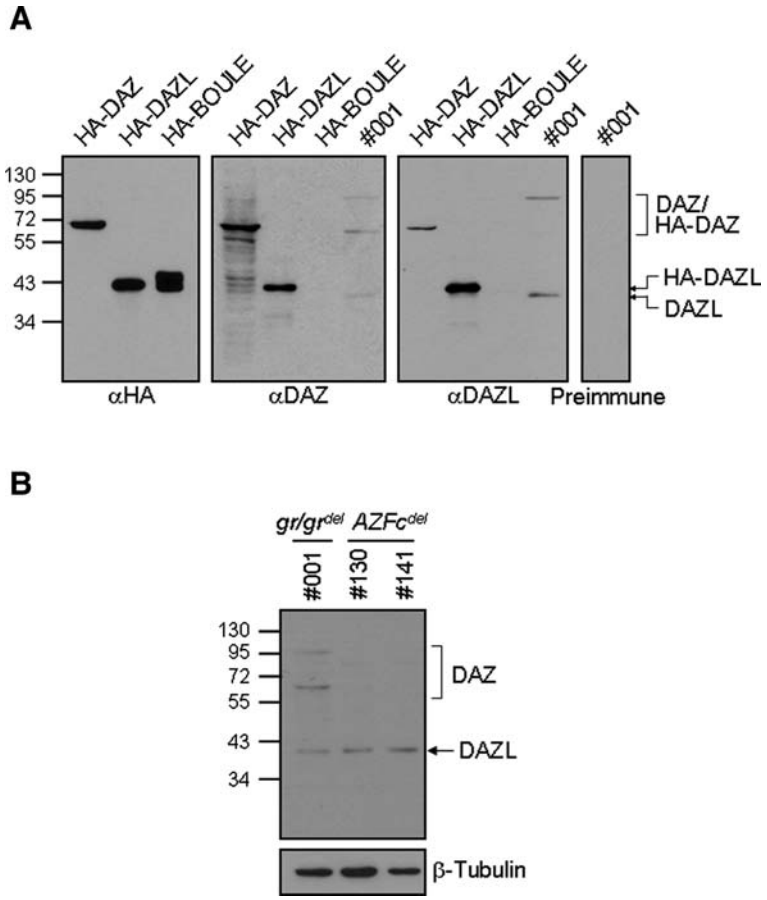

Figure 1 Specificity of the DAZ antibody.
(**A**) Immunoblot analyses were carried out using antibodies against DAZ, DAZL and the HA epitope, or preimmune serum. HA-DAZ, HA-DAZL and HA-BOULE were prepared by transient transfection into 293T cells. A human testicular lysate (#001) was also included. Multiple DAZ bands and a discrete HA-DAZ band are indicated with brackets. The HA-DAZL and endogenous DAZL bands are indicated with arrows. Note that both DAZ and DAZL proteins cross-reacted with DAZ or DAZL antibodies. (**B**) Immunoblot analysis of DAZ in the human testis. Protein extracts (20 μg) were loaded on the gel. Genotypes of human samples included a *gr/gr* deletion (#001) and two *AZFc* deletions (#130 and #141). The DAZ and DAZL bands are indicated with a bracket and an arrow, respectively. The same membrane was re-probed with a β-tubulin antibody.

with preimmune serum (Fig. 1A). The DAZL antibody detected an identical set of bands in the same human testis lysates (Fig. 1A). By estimating the sizes of the detected bands, we predicted that the 40 kDa band corresponded to the endogenous DAZL protein, whereas the 95 and 65 kDa bands might correspond to endogenous DAZ proteins.

To confirm detection of the endogenous DAZ protein with our DAZ antibody, we carried out immunoblot analysis with human testicular lysates from individuals lacking the *DAZ* gene (Fig. 1B). Genomic deletions in the *AZFc* locus where *DAZ* copies are located were detected using STS-PCR analysis (Reijo *et al.*, 1995; Repping *et al.*, 2003; Table I). Individual #001 had a gr/gr deletion with two of four *DAZ* gene copies, whereas individuals #130 and #141 had total deletions of the *AZFc* locus. Individual #001 showed three specific bands of 95, 65 and 40 kDa, as seen previously (Fig. 1B). Individuals #130 and #141, however, showed only a 40 kDa DAZL band (Fig. 1B). These results indicate that the DAZ antibody detected both endogenous DAZ and DAZL proteins specifically. In addition, these results suggest that multiple types of DAZ proteins are expressed in the human testis.

Immunohistochemical analysis was carried out to determine localization of DAZ protein in the human testis. The DAZ antibody immunostained the cytoplasm of spermatogonia and primary spermatocytes specifically (Fig. 2). Since our DAZ antibody cross-reacts with DAZL, the specific signals should represent both DAZ and DAZL proteins. Nonetheless, the result indicates cytoplasmic localization of DAZ protein in the premeiotic germ cells.

Table I ***AZFc*** **deletions and predicted** ***DAZ*** **copy number of the human samples used in this study**

Individual	Deletion in *AZFc* region			Deletion type	*DAZ* copy number
	sY255	sY1191	sY1291		
#077	−	−	−	$AZFc^{del}$	0
#112	−	−	−	$AZFc^{del}$	0
#130	−	−	−	$AZFc^{del}$	0
#141	−	−	−	$AZFc^{del}$	0
#001	+	+	−	gr/gr^{del}	2
#105	+	+	−	gr/gr^{del}	2
#128	+	+	−	gr/gr^{del}	2
#052	+	−	+	1191^{del}	2
#155	+	−	+	1191^{del}	2
#114	+	+	+	No	4
#119	+	+	+	No	4
#146	+	+	+	No	4
#151	+	+	+	No	4

+, positive; −, negative. $AZFc^{del}$, whole *AZFc* deletion; gr/gr^{del}, gr/gr deletion; 1191^{del}, sY1191-negative deletion; No, no deletion in *AZFc* region.

Polymorphic DAZ proteins in human testis

The human testis samples were obtained from 13 individuals, whose genotypes are summarized in Table I. The sampled genotypes included intact *AZFc* with four *DAZ* genes, gr/gr deletions with two *DAZ* genes remaining and *AZFc* deletions without any *DAZ* genes. We also obtained testicular samples from two individuals without the sY1191-specific band, who had previously been reported to have two *DAZ* genes (Fernandes *et al.*, 2004; Repping *et al.*, 2004). Immunoblotting revealed multiple DAZ-specific bands with diverse molecular weights in addition to the DAZL-specific band of 40 kDa (Fig. 3). These results suggest that *DAZ* genes produce polymorphic DAZ proteins ranging from 50 to 95 kDa in size. Interestingly, the number of the DAZ bands matched the predicted copy number of *DAZ* genes; individuals with intact *AZFc* produced four DAZ-specific bands, whereas those with the gr/gr deletion produced two DAZ-specific bands (Fig. 3). No DAZ band was detected in individuals with *AZFc* deletion (Fig. 3). Individual #114 appeared to have three DAZ bands, but the second largest band was actually an overlap of two specific bands of DAZ2 and DAZ4, as shown in Supplementary

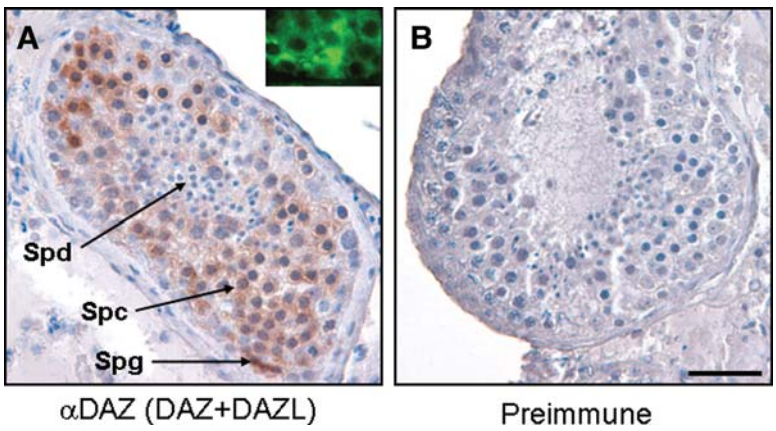

Figure 2 Immunohistochemical analysis of the human testis with the DAZ antibody.
Paraffin sections of the normal human testis (sample #155) were immunostained with the DAZ antibody (**A**) or preimmune serum (**B**). The inset is an indirect immunofluorescence staining of the same human testis sample, revealing positive signals at the cytoplasm of premeiotic germ cells. Spg, spermatogonium; Spc, spermatocyte; Spd, spermatid. Scale bar, 50 μm.

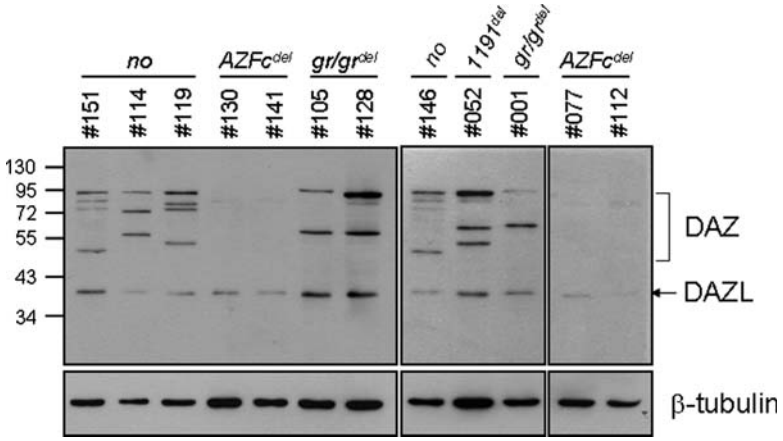

Figure 3 Polymorphic expression of DAZ proteins in the human testes.
Immunoblots were carried out using the DAZ antibody. Testis lysates were prepared from 12 individuals whose *AZFc* genotypes are indicated. The DAZ- and DAZL-specific bands are indicated with a bracket and an arrow, respectively. β-Tubulin was detected as a loading control.

Fig. S1B. The sY1191-negative individual produced three DAZ-specific bands (Fig. 3), suggesting a possible rearrangement, such as a deletion followed by a duplication, at the *AZFc* region of the Y chromosome (Repping *et al*., 2004).

Cloning of DAZ transcripts expressed in human testes

To further investigate the generation of DAZ protein bands of diverse sizes, we decided to examine the transcription of different-sized coding sequences. In order to identify the *DAZ* transcripts in the testicular samples, we conducted RT–PCR with primer sets common to all *DAZ* genes (Fig. 4A). When we used primers targeting the 3′UTR of *DAZ*, we were able to detect a discrete 816 bp PCR fragment in all testicular samples except those with *AZFc* deletions (Fig. 4B). With primers targeting the full-length coding region of *DAZ*, we detected multiple PCR fragments ranging from 2 to 4 kb (Fig. 4B). As a control, we detected the *DAZL*-specific PCR fragment in all testicular samples irrespective of *AZFc* deletions (Fig. 4B). These results suggest that the coding regions of the *DAZ* transcripts are diverse in size.

Next, we cloned the *DAZ* transcripts translated in the testis samples used in this study. The PCR fragments of the full-length *DAZ* coding region were cloned and sequenced. We then determined the origins of the transcripts based on the genomic sequences of *DAZ* (Saxena *et al*., 2000; Fernandes *et al*., 2002; reviewed in Vogt and Fernandes, 2003). Transcript origins were distinguished by the number of RRM domains and the type of DAZ repeat sequences (Fig. 4A). *DAZ1* contains three RRM domains and a single Y-type DAZ repeat, whereas *DAZ2* has one RRM and an A-type DAZ repeat. *DAZ3* contains one RRM and a Y-type DAZ repeat. Finally, *DAZ4* is similar to *DAZ1* but contains two RRM and a Y-type DAZ repeat.

The sequencing data revealed that human testis expresses *DAZ* transcripts from diverse coding sequences. For example, we cloned 46 colonies from individual #146 and identified nine different *DAZ* cDNAs (Fig. 5A). The cDNAs of three individuals with intact *AZFc* loci originated from all four *DAZ* genes (Fig. 5A; Supplementary Fig. S1A and B). Interestingly, gr/gr deletion samples contained transcripts from only two different *DAZ* gene copies (Fig. 5B; Supplementary Fig. S1C and D). Furthermore, the *DAZ* transcripts in the gr/gr deletion samples were paired according to the expected intra-chromosomal recombinations (Repping *et al*., 2003): samples from individuals #001 and #105 contained *DAZ3* and *DAZ4*, whereas individual #128 expressed *DAZ1* and *DAZ2* (Supplementary Fig. S1C and D).

To identify transcripts encoding DAZ proteins in the testis, *DAZ* cDNA clones were ectopically expressed in 293T cells, immunoblotted with the DAZ antibody and compared with the testis sample from which the clone originated. Most of the *DAZ* cDNAs were translated into proteins with the expected molecular weights (Fig. 5; Supplementary Fig. S1). All DAZ-specific bands in the testis samples matched cDNA clones expressed in cultured cells. Individuals with intact *AZFc* expressed four different types of DAZ proteins (Fig. 5A; Supplementary Fig. S1A and B). Interestingly, the expressed DAZ proteins originated from the largest transcripts from each *DAZ* gene. For example, of the three *DAZ3* clones isolated from individual #146, only the protein band of *DAZ3* clone #146-6 matched that of the real human testis sample (Fig. 5A). The gr/gr-deleted individuals expressed two DAZ proteins, either DAZ1 and DAZ2 or DAZ3 and DAZ4 (Fig. 5B; Supplementary Fig. S1C and D). Again, the longest transcripts from each *DAZ* gene were expressed. These results collectively indicate that all *DAZ* genes actively produce polymorphic DAZ proteins.

Discussion

In the present study, we used immunoblotting to detect DAZ protein in human testis and identified cDNA clones that correspond to the expressed DAZ proteins. We found that all four *DAZ* genes actively produce DAZ proteins. First, the number of DAZ-specific bands matched the number of *DAZ* genes in the genome, as individuals with intact an *AZFc* region or a gr/gr-deletion had four or two DAZ-specific bands, respectively. No DAZ band was detected in *AZFc*-deleted individuals. Second, the DAZ-specific bands originated from each *DAZ* gene existing in the genome of each individual. Four DAZ-specific bands in the individuals with intact *AZFc* matched the products

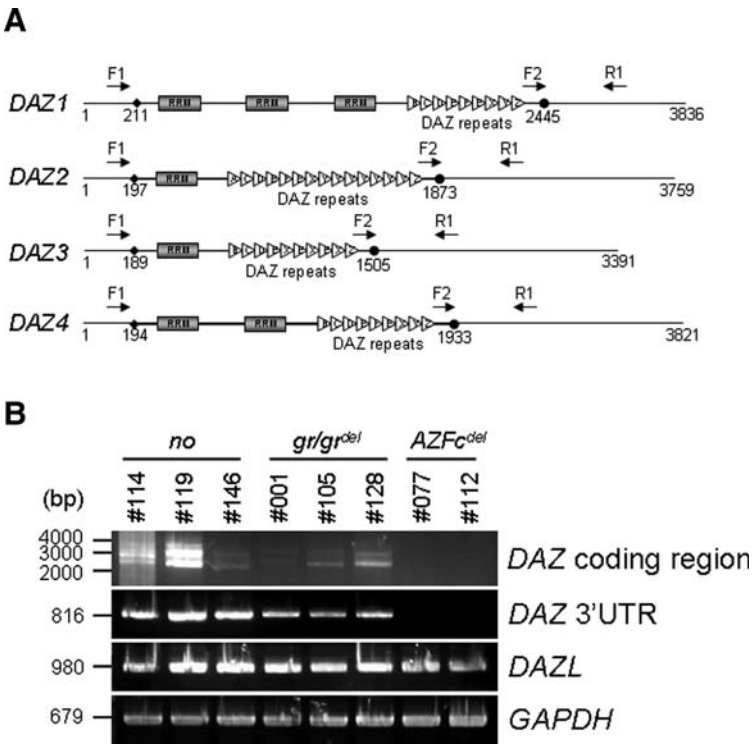

Figure 4 Cloning of polymorphic *DAZ* transcripts.
(**A**) PCR primer sets for cloning *DAZ* transcripts from human testis samples. F1 and R1 primers were used for cloning the full-length coding sequence of *DAZ*, whereas F2 and R1 were used for the 3′UTR of *DAZ*. The primer sequences are common to all seven transcripts from four *DAZ* genes available in GenBank. Only the largest transcripts from each *DAZ* gene are depicted. The start and stop codons are marked with dots. The RRM and DAZ repeats are indicated. (**B**) RT–PCR analysis of *DAZ* expression in the human testis. Deletions at the *AZFc* locus are listed in Table I. Total RNA extracted from the testes of the 8 individuals was reverse-transcribed and subsequently amplified with the *DAZ* primer sets. PCR fragments for the *DAZ* coding regions were heterogeneous in size, whereas those for the *DAZ* 3′UTR were discrete at 816 bp. *DAZL* and *GAPDH* cDNAs were also amplified. Size markers are shown on the left.

of four *DAZ* genes. Two DAZ-specific bands in *gr/gr*-deleted individuals originated from *DAZ1/DAZ2* or *DAZ3/DAZ4*; these pairings were expected from intrachromosomal recombination (Repping *et al.*, 2003).

Previous reports have shown that *DAZ* mRNA is predominantly detected in premeiotic germ cells (Menke *et al.*, 1997; Szczerba *et al.*, 2004). Several studies have attempted to detect DAZ proteins in the human testis. Habermann *et al.* (1998) reported the presence of DAZ proteins in late spermatids and in the tails of spermatozoa. Reijo *et al.* (2000) did not detect DAZ proteins in the human spermatozoa. Rather, they observed DAZ signals in nuclei of the early, premeiotic phases of male germ cells. Huang *et al.* (2008) recently reported the presence of DAZ proteins in the cytoplasm of spermatogonia, but failed to identify DAZ-specific bands in the immunoblot analysis. In the present work, we were able to detect four discrete DAZ bands in the intact testis samples. The same DAZ antibody immunostained the cytoplasm of spermatogonia and spermatocytes. Differences in the DAZ expression pattern may be largely due to the antibodies used in the experiments. We believe that our DAZ antibody is specific enough to distinguish individual DAZ proteins in the immunoblot analysis.

One of the interesting observations in this study is that among multiple transcripts originating from a single *DAZ* gene, only the largest transcripts are translated into the DAZ proteins (Fig. 5; Supplementary Fig. S1). It remains to be determined whether the smaller transcripts are expressed in the male germ cells or are artificially generated during PCR amplification. It has been shown that PCR amplification of the *DAZ* repeat region of a single *DAZ* cDNA clone would produce a ladder of fragments with increments of one repeat length due to highly repetitive nature of the sequence (Yen *et al.* 1997). When we used the same primers to amplify *DAZ* cDNAs, we also observed a faint ladder of PCR products (data not shown). Furthermore, we detected a smaller *DAZ* transcript that was PCR-amplified from a single *DAZ* cDNA clone, suggesting that the smaller clones in our study may be artifacts that were generated during PCR amplification (data not shown). Nevertheless, we do not rule out the possibility that the multiple transcripts were results of an alternative splicing mechanism. In fact, alternative splicing has been reported in other members of *DAZ* gene family, including human *BOULE* (Kostova *et al.*, 2006) and bovine *DAZL* (Liu *et al.* 2007).

Our immunoblot analysis revealed the polymorphic nature of DAZ proteins expressed in the human testes. From the 6 testicular samples in this study, we identified 18 different *DAZ* cDNA clones that are translated into proteins. Different *DAZ2*, *DAZ3* and *DAZ4* transcripts

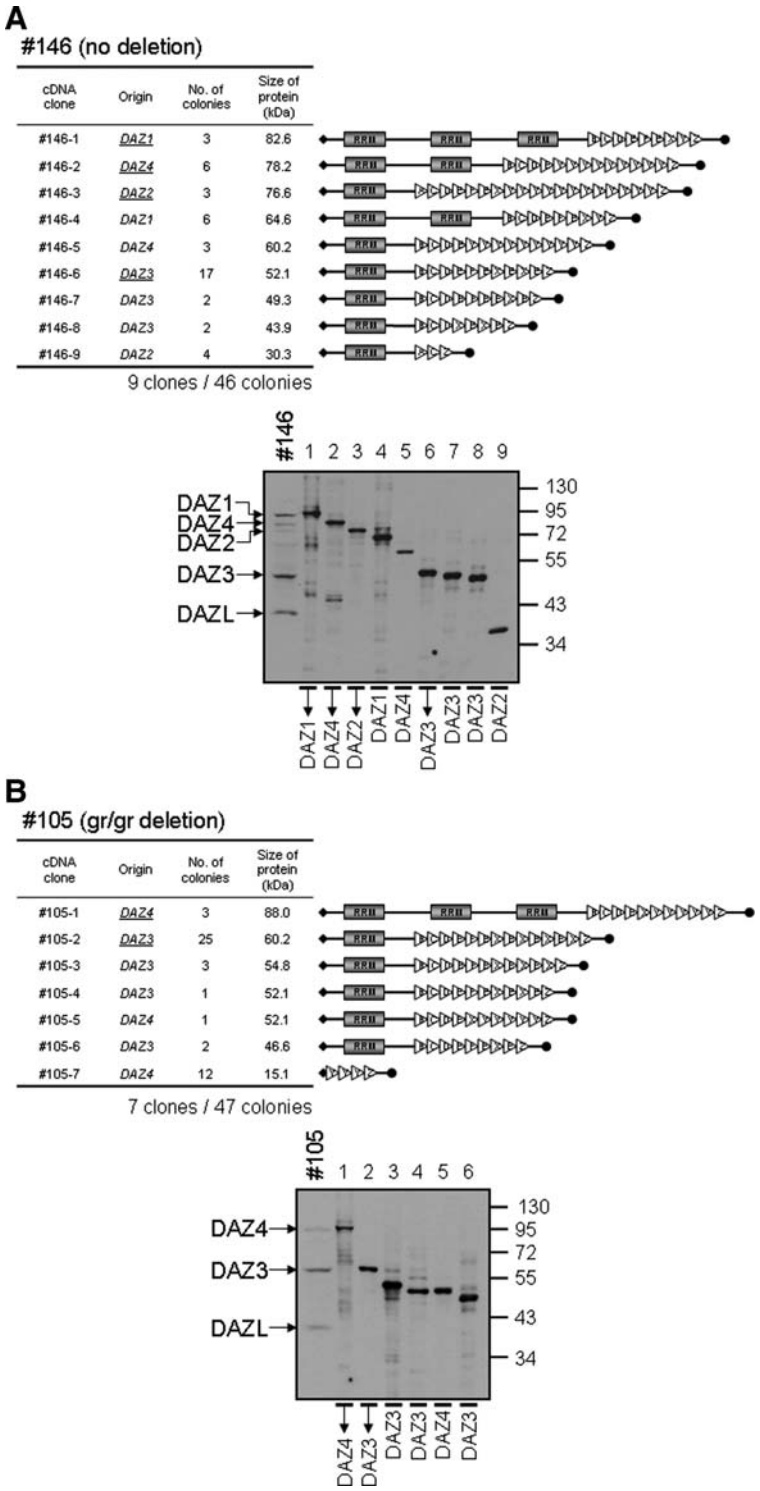

Figure 5 (**A** and **B**) Identification of polymorphic DAZ proteins expressed in human testis.
DAZ cDNAs were cloned from an individual with intact *AZFc* loci (#146) and an individual with *gr/gr* deletion (#105) by RT–PCR using primers for the *DAZ* coding region. Characteristic domain structures (RRM and DAZ repeats) of the cloned *DAZ* cDNA are indicated. Immunoblot analysis of DAZ was carried out in human testis lysates as well as with *DAZ* clones expressed in 293T cells by transient transfection. By comparing the protein size, we identified *DAZ* cDNA clones matching DAZ proteins expressed in human testes (underlined).

were translated into proteins in testis samples, whereas the same *DAZ1* transcript was translated in all samples. We hypothesized that such variation might arise from polymorphic *DAZ* gene structures. In support of this, we were not able to detect any *DAZ* transcripts larger than those that were translated; this finding suggests that the translated *DAZ* transcripts include all exons in the *DAZ* genes. Polymorphism of *DAZ* genes has been reported previously. Lin *et al.* (2005) characterized the *DAZ* genes of 82 individuals by Southern blotting and found that *DAZ* genes were polymorphic in men. The two currently available genomic sequences of *DAZ* regions show sequence identity to *DAZ1* but not to *DAZ2*, *DAZ3* or *DAZ4* (Vogt and Fernandes, 2003). The sequence of the largest *DAZ1* transcript in our study is identical to that of *DAZ1* exons. However, we cannot rule out the possibility that this polymorphism also results from alternative splicing of the *DAZ* genes. Analysis of *DAZ* gene sequences and expression in one man may elucidate this mechanism.

The current study showed that individuals possess unique sets of DAZ proteins. It remains to be investigated whether these diverse forms of DAZ protein share a common biological activity. It is also important to examine whether DAZ polymorphisms can be genetically delivered to progeny.

Supplementary data

Supplementary data are available at http://humrep.oxfordjournals.org/.

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