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Restricted expression of the human DAZ protein in premeiotic germ cells

William J. Huang[1,2], Yi-Wen Lin[3,4], Kuang-Nan Hsiao[3], Karyn S. Eilber[5,9], Eduardo C. Salido[6,7] and Pauline H. Yen[3,4,8,10]

[1]*Department of Urology, School of Medicine, National Yang-Ming University, Taipei 11221, Taiwan;* [2]*Division of Urology, Department of Surgery, Taipei Veterans General Hospital, Taipei 11217, Taiwan;* [3]*Institute of Biomedical Sciences, Academia Sinica, 128 Academia Road, Sec. 2, Taipei 11529, Taiwan;* [4]*Graduate Institute of Life Sciences, National Defense Medical Center, Taipei 11490, Taiwan;* [5]*Division of Urology, Department of Surgery, Harbor-UCLA Medical Center, Torrance, CA 90505, USA;* [6]*CIBERER, Hospital Universitario de Canaries, Spain;* [7]*Department of Pathology, Universidad de La Laguna, E-38071 Tenerife, Spain;* [8]*Division of Medical Genetics, Harbor-UCLA Medical Center, Torrance, CA 90505, USA;*
[9]*Present address: Century Urology Medical Group, 2080 Century Park East, Suite 1407, Los Angeles, CA 90067, USA.*

[10]Correspondence address. Tel: +886-2-2652-3912; Fax: +886-2-2782-9224; E-mail: pyen@ibms.sinica.edu.tw

BACKGROUND: The role of the Y chromosome-encoded Deleted in Azoospermia (*DAZ*) gene family in spermatogenesis remains unclear. The ability of men without the *DAZ* gene to produce sperm, as well as the lack of selective pressure on *DAZ* exon sequences during evolution, casts doubts on its functional significance. Most men have four *DAZ* genes encoding protein isoforms that differ significantly in size. However, published western blots showed only a single "DAZ" band, raising the possibility that not all four *DAZ* genes are expressed. METHODS: RT–PCR, western blotting and immunostaining were used to study the expression of the four *DAZ* genes and the autosomal *DAZL* gene in human testes and in tissue culture cells. RESULTS: RNA transcripts of all four *DAZ* genes were found in the testis, but at much lower levels than that of the *DAZL* transcripts. Expression in cultured somatic cells showed that *DAZ* transcripts encoding multiple DAZ repeats were translated inefficiently. No DAZ proteins could be unambiguously identified on western blots when the testicular samples from three patients without the *DAZ* genes were used as negative controls. Nonetheless, low levels of DAZ were detected in the cytoplasm of spermatogonia by immunostaining. CONCLUSIONS: The expression of DAZ proteins in adult human testes is restricted to the spermatogonia and suggests a premeiotic role.

Keywords: DAZ; DAZL; male infertility; spermatogenesis

Introduction

The Deleted in Azoospermia (*DAZ*) gene was one of the first "azoospermia factors" to be isolated from the *AZF* regions on the human Y chromosome and is frequently deleted in infertile men with non-obstructive azoospermia (Reijo *et al*., 1995). Originally thought to be a single-copy gene, *DAZ* was later found to be a gene family with most men having four copies (Saxena *et al*., 2000). *DAZ* orthologues are found only on the Y chromosomes of great apes and Old World monkeys (Shan *et al*., 1996; Gromoll *et al*., 1999). Nonetheless, *DAZ* has two autosomal paralogues, *DAZL* and *BOULE*, which are present as single-copy genes in all vertebrates and some lower organisms (Cooke *et al*., 1996; Eberhart *et al*., 1996; Saxena *et al*., 1996; Shan *et al*., 1996; Yen *et al*., 1996; Seboun *et al*., 1997; Houston *et al*., 1998; Maegawa *et al*., 1999; Karashima *et al*., 2000; Johnson *et al*., 2001; Xu *et al*., 2001). It is thought that *DAZ* arose from an ancient *DAZL* gene through transposition and amplification (Saxena *et al*., 1996). *DAZ*, *DAZL* and *BOULE* encode a family of RNA-binding proteins that are expressed exclusively in the germ cells and play a role in the regulation of mRNA translation (reviewed in Reynolds and Cooke, 2005). The requirement of *DAZL* and *BOULE* in gametogenesis is well documented (Eberhart *et al*., 1996; Ruggiu *et al*., 1997; Houston and King, 2000; Karashima *et al*., 2000), but the role of *DAZ* in spermatogenesis remains unclear. *DAZ* is definitely not essential for spermatogenesis. Many men with the *AZFc* deletion can still produce mature sperm, though at a significantly reduced number, and some of them have passed the deletion to their sons (Chang *et al*., 1999; Saut *et al*., 2000; Calogero *et al*., 2002; Kuhnert *et al*., 2004). Because the *AZFc* region contains several genes in

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addition to *DAZ*, it was questioned whether deletion of the *DAZ* genes alone would impair fertility (Saut *et al*., 2000). It was even suggested that *DAZ* represents an evolutionary byproduct with no functional significance because its exons were not subjected to selective pressures during evolution (Agulnik *et al*., 1998).

DAZ and *DAZL* share extensive homology, but their protein products have different C-terminal sequences due to frame shifting in the middle of the genes. DAZL contains an RNA recognition motif (RRM) and a DAZ repeat of 24 amino acid residues. The four DAZ protein isoforms consist of 1–3 copies of the RRM and a DAZ repeat region that contains from 8 to 24 copies of the DAZ repeat (Fig. 1; Yen *et al*., 1997; Saxena *et al*., 2000). DAZ2 and DAZ3 each have only one RRM, whereas DAZ1 and DAZ4 contain three and two RRMs, respectively. The four DAZ isoforms in an individual therefore differ significantly in size. However, previously published western blot analyses of DAZ in human testis extracts showed only a single band (Habermann *et al*., 1998; Reijo *et al*., 2000). Thus our aim was to investigate whether all four *DAZ* genes are transcribed and translated, using *DAZL* as an internal control.

Materials and Methods

Human testicular samples

Human testicular samples were obtained from 10 patients with their consents and IRB approval. Testicular biopsy was performed for infertility (HT-18, HT-41 and HT-42), and orchiectomy for prostate cancer (HT-1, HT-6, HT-8, HT-13, HT-14 and HT-21) and benign testicular tumour (HT-20), at Harbor-UCLA Medical Center and Taipei Veterans General Hospital. HT-1, HT-6 and HT-20 had normal spermatogenesis; HT-8, HT-13, HT-14 and HT-21 had normal spermatogenesis with atrophic change due to hormone treatment; HT-18 and HT-41 had early maturation arrest; HT-42 had late maturation arrest. The samples were snap frozen in liquid nitrogen before RNA purification or protein analysis, or fixed in Bouin's solution or Formalin for sectioning and immunostaining.

RT–PCR analyses of the DAZ transcripts

Total RNA was purified from human testicular samples using TRIzol Reagents (Invitrogen, Carlsbad, CA, USA), and the quality of the RNA preparation was evaluated by the ratio of the 28S and 18S rRNA on agarose gels stained with ethidium bromide. To study the expression of the various *DAZ* transcripts, *DAZ* cDNA was reverse transcribed from total testis RNA using primer P1 (PrDAZ82: 5′-gacatccagtgatgacctgac) derived from the first DAZ repeat. The

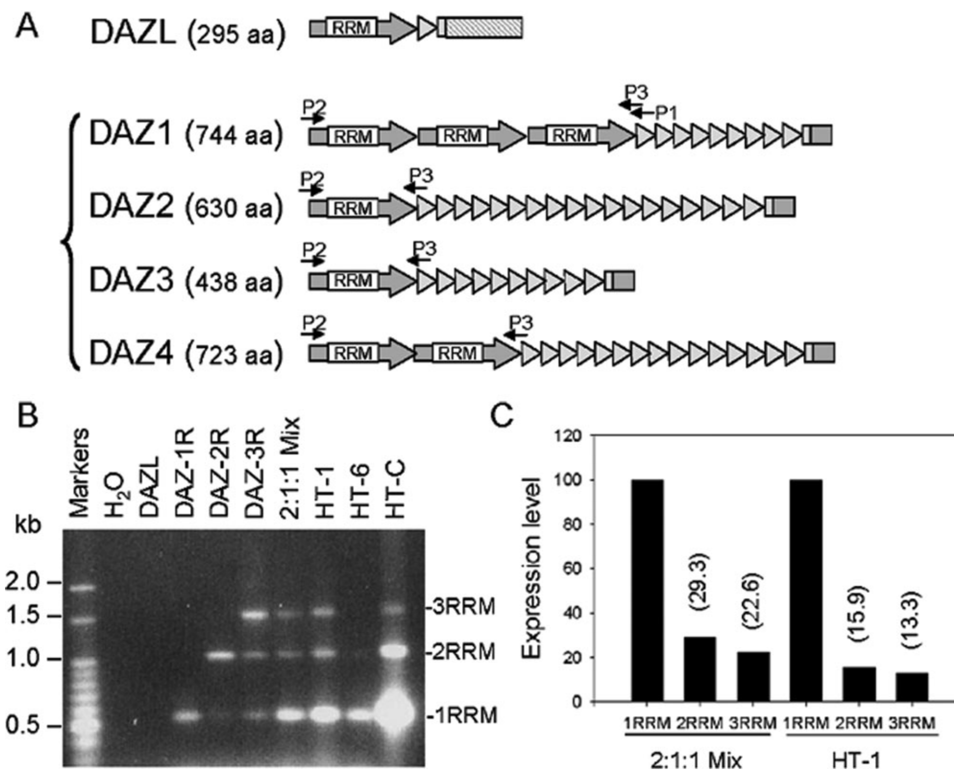

Figure 1: Expression of the *DAZ* transcripts in human testes.
(**A**) Structures of the protein coding regions of the DAZL and the *DAZ* transcripts. The copy numbers of the DAZ repeat in the *DAZ* transcripts are taken from the individual RPCI-11 (Kuroda-Kawaguchi *et al*., 2001) and the sizes of the encoded proteins are indicated. The RNA recognition motifs (RRMs) and the DAZ repeats are depicted as horizontal arrows and triangles, respectively. The locations of primers for reverse transcription of the *DAZ* transcripts (P1) and PCR amplification of the RRM regions (P2 and P3) are indicated. (**B**) PCR amplification of the RRM regions. DAZ-1R, DAZ-2R and DAZ-3R are *DAZ* cDNA clones encoding one, two and three RRMs, respectively. The 2:1:1 Mix lane contains a mixture of the three cDNA clones in a 2:1:1 molar ratio. HT-1, HT-6 and HT-C are cDNAs reverse-transcribed from human testis RNAs. A *DAZL* cDNA clone was included as a negative control. The PCR products were analysed on a 1% agarose gel. The 1RRM fragments in the DAZ-2R and the DAZ-3R lanes as well as the 2RRM fragment in the DAZ- 3R lane could be the results of partial extension products acting as primers or amplification of shorter templates that had lost some RRM repeats during propagation in *E. coli*. (**C**) Quantitative analyses of the signals of the PCR products of the 2:1:1 mix and HT-1 in (B). The signal of 1RRM was arbitrarily set at 100.

RRM region within the *DAZ* cDNA was then PCR amplified using primers P2 (PrDAZ101: cctgccaccaccatgtctg; spanning exons 1 and 2) and P3 (PrDAZ102: agcagaataagcctgaacgtg; spanning exons 6 and 7). The products were analysed on 1% agarose gels and the intensities of the bands were measured using Gel-Pro ANALYZER™ version 3.1., Media Cybernetics, Bethesda, MD, USA.

To determine the relative levels of the *DAZ* and the *DAZL* transcripts, total testis RNA was reverse transcribed using primer P4 (PrDAZ18:tatccagtgatgacctga) and PCR amplified using primers P4 and P5 (PrDAZ113:gccaaacactgtttttgttgg). The products were digested with PstI and analysed on 2% agarose gels.

Generation of anti-DAZ antibodies

An expression vector encoding a TRX fusion protein containing amino acid residues #25–#153 of DAZ, spanning the entire RRM region, was constructed by cloning a 383 bp PstI fragment of *DAZ* cDNA clone e-11 in-frame into pET32b (Novagen, Madison, WI, USA). A second vector encoding a TRX fusion protein containing the C-terminal portion of DAZ, including 12 DAZ repeats, was constructed by cloning a 1.1 kb PstI + BamH I fragment of another *DAZ* cDNA clone e-4 in-frame into pET32b. The recombinant proteins were produced in *Escherichia coli*, affinity purified on nickel columns, and injected into rabbits to generate the anti-DAZ-RRM and anti-DAZ-repeat antibodies, respectively. In addition, antibodies against oligopeptides DAZ-R1 (HGKKLKLGPAIRKQKL, amino acids #104–119) and DAZ-C end (CPVGEQRRNLWTEAYK, near the C-terminus of DAZ) were generated in rabbits and in a goat, respectively, and affinity purified using the services of Bethyl Laboratories, Inc (Montgomery, TX, USA).

Construction of DAZ expression vectors

The *DAZ* cDNA clone e11 (DAZ-1R) that encodes one RRM and nine DAZ repeats was used as the starting material (Yen *et al*., 1997). The cDNA contains a single ApaI site that is located inside RRM. A 495 bp ApaI fragment spanning two adjacent RRMs was RT-PCR amplified (using primers PrDAZ-Apa-F: gggccctgcaatcaggaaac and PrDAZ-Apa-R: gggcccagcttcagcttttta) from *DAZ* transcripts in human testis RNA, and inserted into the ApaI site of DAZ-1R to generate the *DAZ* cDNA clone (DAZ-2R) encoding two RRMs. Dimers of the ApaI fragment were isolated from the self-ligation products and PCR amplified using primers PrDAZ-BsaF: ggtctcgggccctgcaatcaggaa and PrDAZ-BsaR: ggtctcgggcccagcttcagcttt. After cloning and sequencing to confirm the correct joining of the RRM repeats, the RRM dimer was excised from the cloning vector using BsaI, which recognizes the ggtctc sequence but cut within the ApaI site, and inserted into the ApaI site of DAZ-1R to generate DAZ-3R encoding three RRMs. DAZ-1R, DAZ-2R and DAZ-3R were digested with EcoRV and re-ligated to generate DAZ-1RD, DAZ-2RD and DAZ-3RD, respectively, encoding only two DAZ repeats. Segments containing the open-reading frames of the various *DAZ* cDNA clones as well as a *DAZL* cDNA clone were isolated by PCR amplification and cloned in-frame into the NotI and BamH1 sites of pcDNA3.1/myc-His(-)A (Invitrogen) and p3xFlag-CMV14 (Sigma, St Louis, MO, USA) for expression in tissue culture cells, or blunt-ended and cloned into the PvuII site of pRSET A (Invitrogen) for expression in *E. coli*.

Expression of DAZ and DAZL proteins in cultured cells

Human 293 cells or COS7 cells were transfected with the various expression vectors using lipofectamine 2000 (Invitrogen) according to the manufacturer's manual. Twenty-four hours after transfection, the cells were harvested and lysed in a cell lysis buffer containing 50 mM Tris, pH 7.8, 150 mM NaCl, 1% Nonidet P-40 and 1× protease inhibitor cocktail (Sigma). The protein concentration was determined using the Bio-Rad Protein Assay Kit (Bio-Rad, Hercules, CA, USA) with bovine serum albumin as the standard. Aliquots containing 50 μg of protein were subjected to 10% SDS–polyacrylamide gel electrophoresis (PAGE) and western blotted with various antibodies. Bands on the blots were boxed and quantified using the software Metamorph Offline, version 6.3r1 (Universal Imaging, Molecular Devices Corp., Downingtown, PA, USA).

Degradation of DAZ and DAZL proteins in cultured cells

Twenty-four hours after COS7 cells were transfected with the various Flag-tagged *DAZ* or *DAZL* expression constructs, cycloheximide (Sigma) was added to the media to a final concentration of 100 μg/ml to inhibit further protein synthesis. Cells were harvested 0, 1, 2, 4 and 8 h afterward and lysed in a buffer containing 50 mM Tris, pH7.5, 150 mM NaCl, 0.5% Triton X-100, 1 mM PMSF and 1× protease inhibitor cocktail, followed by three cycles of freeze-thawing. Aliquots containing ∼100 μg of protein were subjected to 10% SDS–PAGE and western blotted with the anti-Flag M2 monoclonal antibody (Sigma) and anti-β-actin antibody (Sigma).

Translation efficiency of DAZ and DAZL

Twenty-four hours after COS7 cells were transfected with the various Flag-tagged *DAZ* or *DAZ*L expression constructs, the cells were incubated with 50 μM proteasome inhibitor MG132 (*N*-benzyloxycarbonyl-Leu-Leu-leucinal, International Peptides) (Calbiochem, San Diego, CA, USA) and 100 μM lysosome inhibitor chloroquine (Sigma). Cells were harvested 0, 0.5, 1, 2, 4 and 6 h afterward and analysed by western blotting as described above.

Western blot analysis of DAZ proteins in human testes

Human testicular samples were homogenized in RIPA buffer (120 mM NaCl, 10 mM Tris, pH 6.8, 1% NP-40, 0.1% SDS, 1% deoxycholate and 1× protease inhibitor cocktail) followed by centrifugation at 10 000*g* for 30 min to remove cell debris. Approximately 100 μg of protein was subjected to 10% SDS–PAGE and electrophoretically transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). The membranes were then submerged in NET solution (0.1 M Tris–HCl, pH 7.5, 0.9% NaCl, 0.2% NP-40 and 0.25% gelatin) supplemented with 5% milk powder, and incubated overnight at 4°C with the anti-DAZ-R1 antibody that had been pretreated with 0.5% mouse liver acetone powder with or without the DAZ-R1 oligopeptide (peptide:antibody =1:1) at 4°C for 16 h. At the end of incubation, the membranes were washed with 1× PBST (phosphate-buffered saline with 0.1% Tween-20), and incubated with a 1:2000 dilution of the anti-rabbit IgG/peroxidase conjugate (Amershan, Arlington Heights, IL, USA) at room temperature for 2 h. The membranes were washed three times with 1× PBST at room temperature for a total of 15 min. Peroxidase was then detected using the chemiluminescence system (Millipore).

Detection of AZF deletions

The presence of Y chromosome microdeletion in patient HT-18, HT-41, HT-42 was determined using a multiplex PCR reaction that amplifies markers within the three *AZF* regions, the Sex-determining Region Y (*SRY*) gene, and the Zinc-Finger X (*ZFX*) and Zinc-Finger Y (*ZFY*) gene pair (Simoni *et al*., 2004).

Immunostaining of DAZ and DAZL

In order to test whether the anti-DAZ antibodies were suitable for immunostaining of DAZ in fixed cells, COS7 cells were transfected with expression vectors for Flag-tagged DAZ or DAZL and harvested 24 h later. The cells were fixed with 4% paraformaldehyde and immunostained using various antibodies according to Lin and Yen (2006) except that Alexa Fluor 488 conjugated secondary antibodies (Sigma) were used. The images were taken using a Bio-Rad Radiance 2100 laser scanning confocal microscope.

Human testis samples were fixed in Bouin's solution, embedded in paraffin and sectioned at 5 μm. The sections were deparaffinized in 100% xylene for 10 min twice and sequentially rehydrated in 100, 100, 95, 85 and 70% ethanol for 5 min each. After two 5 min washes in water, the slides were treated with 3% hydrogen peroxide in methanol for 10 min to remove endogenous peroxidase activity, and washed once more in water for 5 min. The slides were then heated at 97°C for 40 min in Target Retrieval Solution (Dakocytomation, Glostrup, Denmark), and cooled for 20 min at room temperature. After three 5 min washes in 1× PBS, the slides were blocked with 5 mg/ml bovine albumin (Sigma) for 1 h at room temperature before incubation with various primary antibodies (1:20 dilution in 1× PBS) at 4°C overnight. The slides were then washed with 1× PBS, and incubated with a 1:50 dilution of anti-rabbit IgG/peroxidase conjugate (Amershan) at room temperature for 2 h. Finally the slides were washed three times with 1× PBS for 5 min each, followed by reaction with diaminobenzidin tetrachloride/hydrogen peroxide (Dakocytomation). Sections were subsequently counterstained with haematoxylin (Merck, Darmstadt, Germany), dehydrated, mounted and examined.

Results

The DAZ transcripts in human testes

We first determined whether transcripts of all four *DAZ* genes are present in human testes.

To distinguish the transcripts of these genes, we selectively RT–PCR amplified the RRM regions in the *DAZ*, but not the *DAZL* transcripts using primers P2 and P3 (Fig. 1A). *DAZ* transcripts with one, two and three RRMs produced PCR products of 0.5, 1.0 and 1.5 kb, respectively. To correct for the difference in PCR amplification efficiency for the various products, we constructed and mixed cDNA clones encoding one RRM (DAZ-1R), two RRMs (DAZ-2R) and three RRMs (DAZ-3R) in a 2:1:1 ratio to mimic the RRM regions contributed by the four *DAZ* genes. PCR amplification of this mixture produced the expected three fragments with the signal ratio of 1:0.29:0.22, indicating that, as predicted, the larger fragments were amplified less efficiently than the smaller fragments (Fig. 1B and C). Under the same PCR condition, cDNA samples reverse transcribed from the RNAs of two human testis specimens and a commercial sample (HT-C) gave the same three fragments. The relative ratio of these fragments varied significantly between samples, depending on the quality of the RNA preparation. The 1RRM fragments in the DAZ-2R and the DAZ-3R lanes as well as the 2RRM fragment in the DAZ-3R lane could be the results of partial extension products acting as primers or amplification of shorter templates that had lost some RRM repeats during propagation in *E. coli*. The result of the best sample HT-1 was comparable to that of the cDNA mixture, suggesting that all four *DAZ* genes are transcribed and that they are transcribed at comparable rates.

We next compared the level of the *DAZ* transcripts to that of the *DAZL* transcript in the human testis. To distinguish the two transcripts, we PCR amplified a 432 bp fragment from both *DAZ* and *DAZL* transcripts using a pair of common primers P4 (from exon 2) and P5 (from the first DAZ repeat), and digested the products with PstI which cut the *DAZ*, but not the *DAZL* fragment, into 342 and 90 bp fragments (Fig. 2). As standards, we mixed *DAZ*-1R and *DAZL* cDNA clones in different molar ratios and subjected them to the same PCR amplification and restriction digestion procedures. A comparison of the relative intensities of the *DAZ* and the *DAZL* fragments in three human testis samples with the standards indicates that in the human testis the level of the *DAZ* transcripts is less than one quarter that of the *DAZL* transcripts.

Expression of DAZ in tissue culture cells

In order to detect the DAZ proteins on western blots, we generated four anti-DAZ antibodies using both recombinant proteins and synthetic oligopeptides as antigens (Table I). Oligopeptides DAZ-Cend and DAZ-R1 are identical to peptides 133 and 146, respectively, used by Reijo *et al*. (2000) to raise anti-DAZ antibodies. To test whether these antibodies could recognize DAZ on western blots, we constructed expression vectors for DAZ isoforms with two (DAZ-1RD, DAZ-2RD and DAZ-3RD) or nine DAZ repeats (DAZ-1R, DAZ-2R and DAZ-3R), as well as DAZL in the cloning vector pcDNA3.1/myc-His (Fig. 3A). Expression of these proteins under the direction of the CMV promoter in human kidney epithelial 293 cells and monkey COS7 cells gave similar results. Western blot analyses of the transfected cells showed that of the four antibodies only the anti-DAZ-R1 and the anti-DAZ-RRM antibodies recognized the DAZ proteins on the blots (Fig. 3B and C). The anti-DAZ-R1 antibody had a higher sensitivity, and detected DAZL as well as DAZ. Both antibodies detected additional bands at smaller molecular weights (marked with asterisks) in cells transfected with

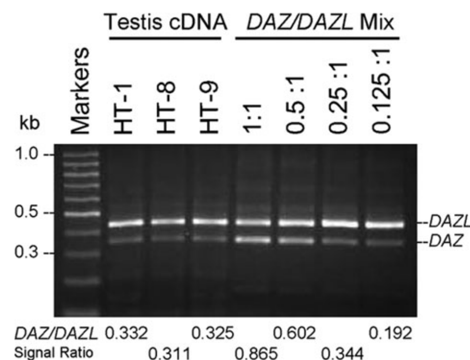

Figure 2: Determination of the relative amounts of the *DAZ* and the *DAZL* transcripts in human testes.
Testis cDNAs and mixtures of *DAZ*-1R and *DAZL* cDNA clones at the indicated molar ratios were subjected to PCR amplified using primers P4 and P5. The PCR products were digested with PstI, which cut the *DAZ* but not the *DAZL* fragment, and analysed on a 2% agarose gel. The signal ratios between the two larger fragments were determined and indicated at the bottom.

Table I. Characteristics of the anti-DAZ antibodies.

Antibody	Antigen[a]	Animal	Purification	Dilution
Anti-DAZ-RRM	Recombinant protein containing the RRM domain (#25–153)	Rabbit	No	1:1000
Anti-DAZ-repeat	Recombinant protein containing the DAZ repeats and C-terminus (#153–534)	Rabbit	No	1:500
Anti-DAZ-Cend	Oligopeptide close to the C-terminus (#507–522)	Goat	Yes	1:500
Anti-DAZ-R1	Oligopeptide within the RRM domain (#104–119)	Rabbit	Yes	1:500

[a]The positions of the amino acid residues are taken from the protein sequence AAL24 502 that consists of 534 amino acids, including one RRM and 14 DAZ repeats.

DAZ-3R or DAZ-3RD. These bands probably represent degradation products. Further blotting of known amounts of DAZ and DAZL, produced in *E. coli* and affinity purified, showed that the anti-DAZ-R1 antibody had similar sensitivity towards DAZ and DAZL, and could detect as little as 30 ng of the proteins (Fig. 3D). The antibody may have higher sensitivity towards DAZ proteins with two or three RRMs, since it recognizes an epitope within the RRM.

We noticed that in the transfected cells, the levels of DAZ-1R, DAZ-2R and DAZ-3R were much lower than those of DAZL and DAZ isoforms with only two DAZ repeats. To investigate further, we cotransfected equal moles of *DAZ* and *DAZL* expression vectors into 293 cells and compared the levels of the *DAZ* and the *DAZL* transcripts as well as their protein products 24 h after transfection. RT–PCR analyses showed similar levels of the *DAZL* and the *DAZ* transcripts except for the *DAZ-2R* transcript (Fig. 3E). The much lower level of the *DAZ-2R* transcript is unexplainable since DNA sequencing of the CMV promoter of its expression vector failed to identify any mutations. On western blots, DAZ isoforms with 9 DAZ repeats, but not those with only two DAZ repeats, were present at much lower levels than DAZL in the same cells (Fig. 3F). To better quantify the expression levels, we constructed Flag-tagged expression vectors and detected the proteins using an anti-Flag antibody that produced western blots with cleaner background (Fig. 3G). Quantification of the signal intensities indicated that the amounts of both DAZ-1R and DAZ-2R were about 5-folds less than that of DAZL, whereas the levels of DAZ-1RD and DAZ-2RD were comparable to that of DAZL.

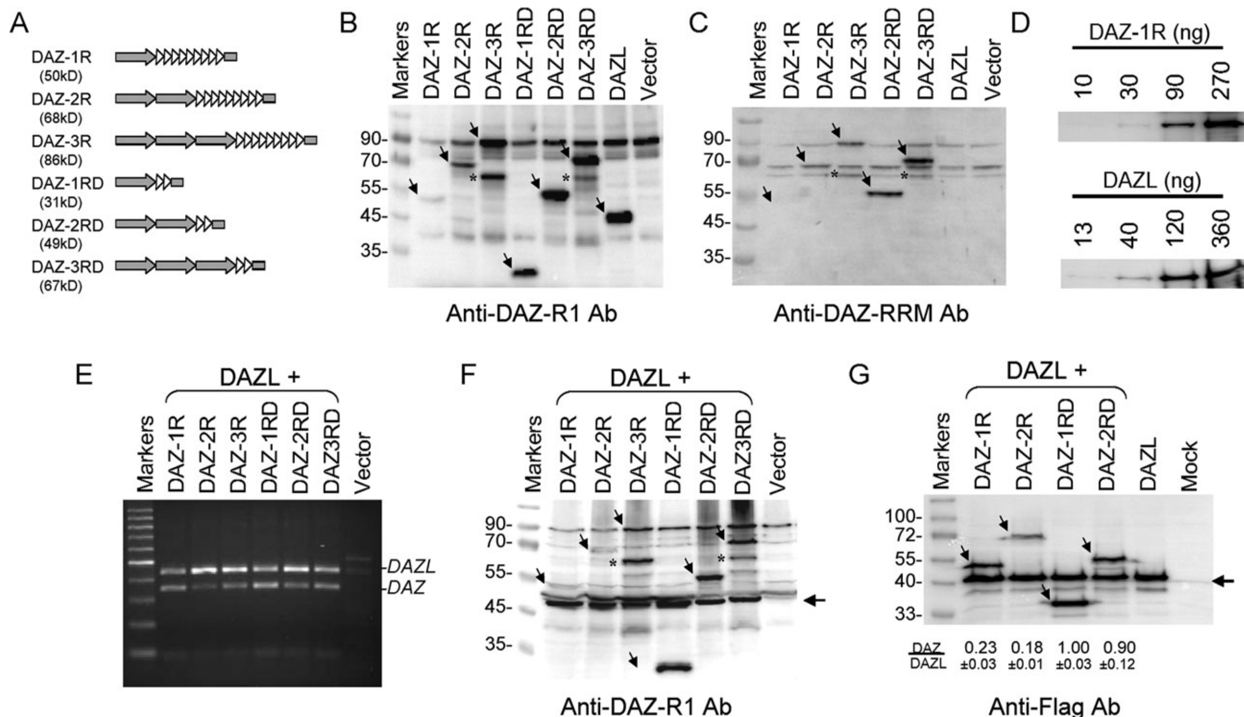

Figure 3: Expression of DAZ and DAZL in cultured cells.
(**A**) Structures and molecular weights of the various proteins used in the expression studies. Human 293 cells (**B**) or COS7 cells (**C**) were transfected with expression vectors for the indicated proteins and harvested 24 h afterward. The cell lysates were subjected to 10% SDS–PAGE and western blotted with the anti-DAZ-R1 (B) or the anti-DAZ-RRM (C) antibody. Proteins expressed from the expression vectors are marked with arrows, and the degradation products are marked with asterisks. (**D**) Western blot detection of known amounts of recombinant DAZ-1R and DAZL using the anti-DAZ-R1 antibody. (**E**) RT–PCR analyses of the *DAZ* and the *DAZL* transcripts in 293 cells transfected with equal moles of the expression vectors for DAZL and the various DAZ isoforms as indicated. The PCR fragments were digested with PstI and analysed as in Fig. 2. (**F**) Western analyses of the same cells in (E) using the anti-DAZ-R1 antibody. The horizontal arrow points to the location of DAZL. (**G**) Lysates of 293 cells transfected with equal moles of expression vectors for Flag-tagged DAZL and the various DAZ isoforms were western blotted with an anti-Flag antibody. The ratio of the DAZ and DAZL signals is listed underneath each lane.

To investigate whether the low expression of DAZ was due to protein instability, we expressed Flag-tagged DAZ isoforms and DAZL in COS7 cells and followed their degradation after inhibiting new protein synthesis with cycloheximide (Sun *et al.*, 2005). The results showed that both DAZL and DAZ-1R were quite stable, whereas DAZ-1RD was degraded more rapidly (Fig. 4A and B). Thus the low level of DAZ-1R cannot be explained by protein instability. We next looked at the rate of protein synthesis by inhibiting proteasomal and lysosomal degradation pathways by MG132 and chloroquine, respectively (Robben *et al.*, 2005). The results showed that DAZ-1R was synthesized at a rate about one-fifth that of DAZL or DAZ-1RD (Fig. 4C and D). The slow synthesis of DAZ-1R could not be due to its large size since DAZ-2RD (49 kDa), which is of similar size as DAZ-1R (51 kDa) but has only two DAZ repeats, was synthesized at a rate comparable to that of DAZL. It appears that increasing copy number of the DAZ repeat has a negative effect on protein synthesis.

Detection of the DAZ proteins in human testis lysates by western blotting

We used western blotting to study the expression of DAZ and DAZL in human testicular samples, including some with normal spermatogenesis and some with spermatogenic arrest (Fig. 5A and data not shown, see Materials and Methods for details on the samples). Of particular importance are three samples from patients lacking the *DAZ* genes (Fig. 5B). Both HT-18 and HT-41 had the *AZFb+AZFc* deletion, and their testes showed early maturation arrest with no post-meiotic germ cells. HT-42 had the *AZFc* deletion, and testis sections showed some tubules with normal spermatogenesis and others with late maturation arrest at the spermatid stage. The anti-DAZ-RRM antibody produced dirty western blots with no consistent protein bands (data not shown). We therefore used the anti-DAZ-R1 antibody for the remaining studies (Fig. 5C and D). Due to variation in the copy numbers of both the RRM and the DAZ repeats, the DAZ proteins are expected to be of different sizes, ranging from 43 kDa (one RRM and eight DAZ repeats) to 120 kDa (3 RRMs and 24 DAZ repeats), in different individuals. This makes the identification of DAZ on western blots quite challenging, especially when the expression level of DAZ is probably very low and the affinity-purified antibody detects additional non-specific bands. Nonetheless, an authentic DAZ band should meet the following criteria: it migrates slower than DAZL; its signal can be abolished by the immunogen DAZ-R1 peptide, and it is absent in HT-18, HT-41 and HT-42 who lack the *DAZ*

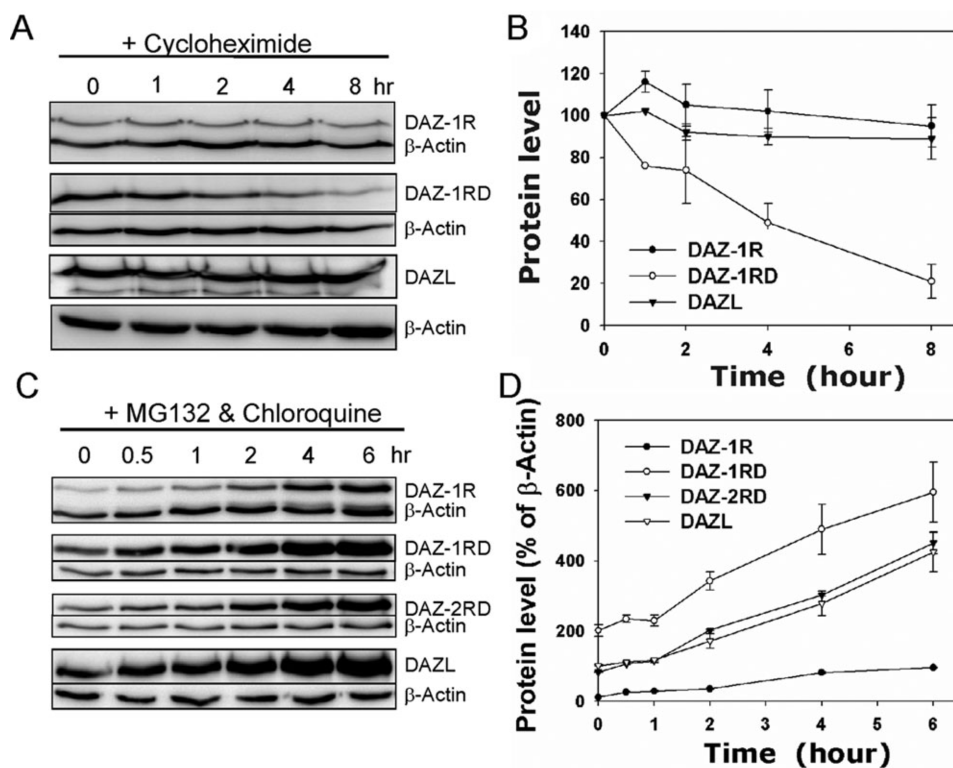

Figure 4: Synthesis and degradation of DAZL and DAZ isoforms in cultured cells.
(**A**) Protein degradation. COS7 cells expressing the various Flag-tagged proteins were harvested after treatment with cycloheximide (an inhibitor of protein synthesis) for the indicated time periods and western blotted with an anti-Flag or anti-β-actin antibody. (**B**) Quantification of the results shown in (A). The signals of the various DAZ/DAZL proteins were normalized with β-actin signals and compared to signals at time 0 that were arbitrarily set as 100. The data shown are the averages of three independent experiments. (**C**) Protein synthesis. COS7 cells expressing the various Flag-tagged proteins were harvested after treatment with both MG132 (a proteasome inhibitor) and chloroquine (a lysosome inhibitor) for the indicated time periods and western blotted with an anti-Flag or anti-β-actin antibody. (**D**) Quantification of the results shown in (C). The signals of the various DAZ/DAZL proteins were compared with those of β-actin and plotted. The data shown are the averages of three independent experiments.

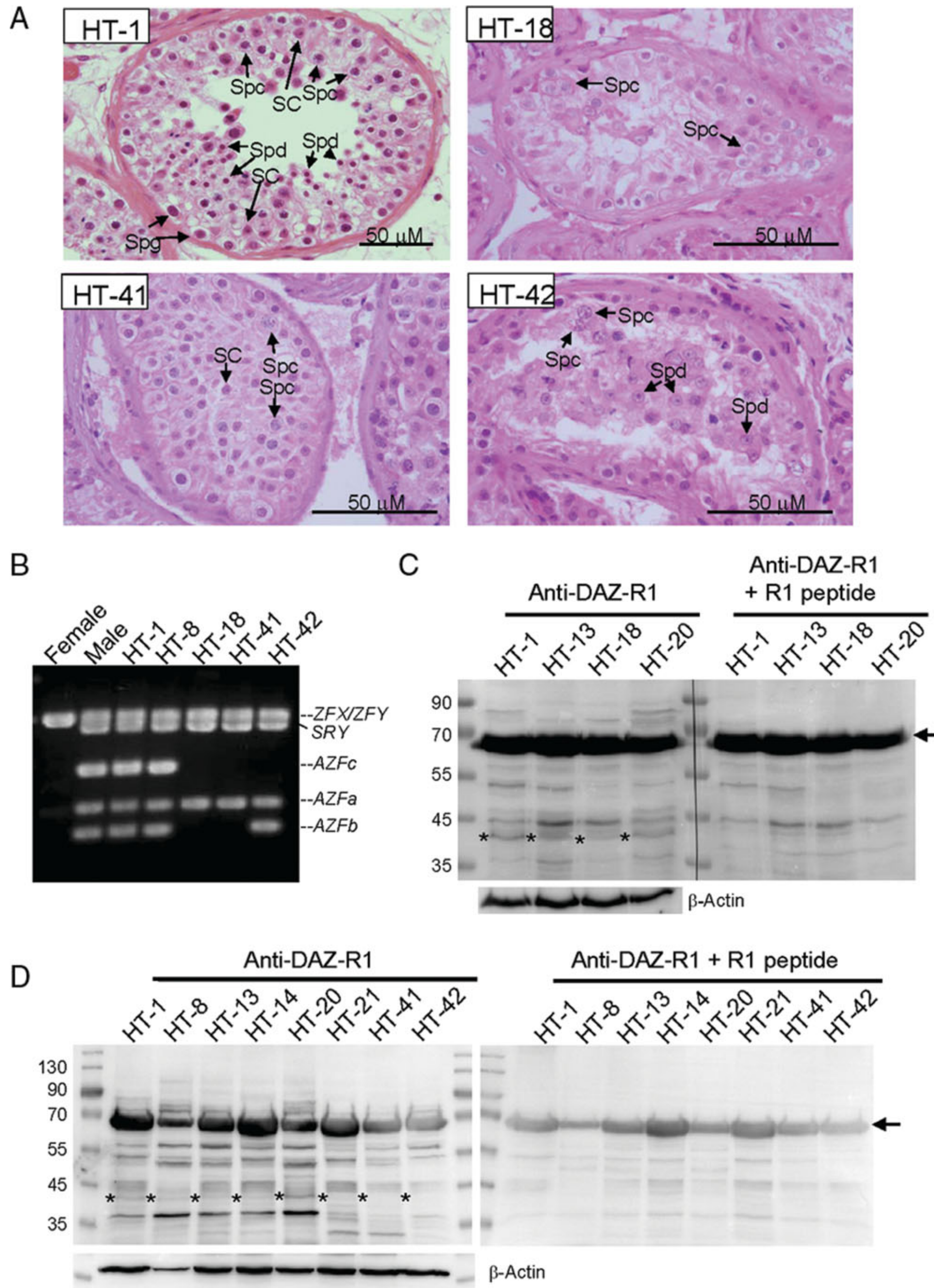

Figure 5: Western blot analyses of DAZL and DAZ in human testis lysates.
(**A**) Testis histology of some patients. HT-1 has normal spermatogenesis, HT-18 and HT-41 have early maturation arrest, and HT-42 has late maturation arrest. Cell types are SC: Sertoli cell; Spg: spermatogonium; Spc: spermatocyte; Spd: spermatid. (**B**) Multiplex PCR analyses of the Y chromosome AZF regions. Leukocyte DNAs from patients and male and female controls were genotyped for the various Y chromosome markers. HT-18 and HT-41 lack both AZFb and AZFc whereas HT-42 lacks AZFc. (**C** and **D**) Western blot analyses. About 100 mg of total protein in human testicular lysates were subjected to 10% SDSPAGE and western blotted using the anti-DAZ-R1 antibody with (right panel) and without (left panel) preincubation with the DAZ-R1 peptide. The DAZL bands in the left panels are indicated with asterisks. The horizontal arrows point to the signals caused by the non-specific binding of a highly abundant 68 kDa protein. The blots were stripped and reincubated with an anti-β-actin antibody to show equal loading of the samples.

genes. We had no problem detecting DAZL which co-migrated with the mouse DAZL (data not shown) and the signal of which was abolished by preincubation of the antibody with the DAZ-R1 peptide. However, after repeated experiments using different testicular samples and conditions we could not positively identify any DAZ band that met the above criteria. There were some polymorphic bands between 70 and 90 kDa that could be abolished with the DAZ-R1 peptide. Nonetheless, their presence in HT-18 and HT-42 precludes them from being DAZ. We thus conclude that DAZ is expressed at a level much lower than that of DAZL in the human testis.

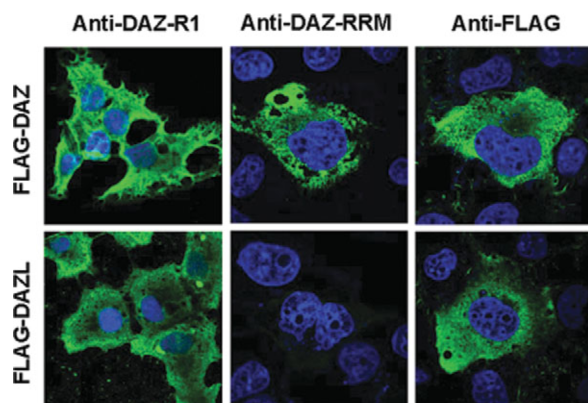

Figure 6: Immunofluorescence staining of DAZ in transfected cells. COS7 cells were transfected with expression vectors for Flag-tagged DAZ-1R (top) or Flag-tagged DAZL (bottom) and immunostained with the various antibodies as indicated at the top.

Detection of the DAZ proteins in human testis section by immunostaining

Our inability to detect DAZ on western blots using total human testis lysates could be due to restricted expression of DAZ in specific cells. Previous *in situ* hybridization and *in situ* RT–PCR detected *DAZ* transcripts mainly in spermatogonia (Menke *et al*., 1997; Szczerba *et al*., 2004). We therefore tested whether our anti-DAZ antibodies could detect DAZ/DAZL in immunostaining by using transfected COS7 cells expressing Flag-tagged DAZ or DAZL as the substrates (Fig. 6). The anti-DAZ-R1 antibody generated signals in both the nuclei and the cytoplasm of all cells, indicating non-specific binding. However, the anti-DAZ-RRM antibody detected signals in the cytoplasm of a subset of cells transfected with the DAZ, but not the DAZL expression vector. The fraction of cells which stained positive for DAZ was approximately half that which stained positive for the Flag epitope, indicating that the detection sensitivity of the anti-DAZ-RRM antibody is lower than that of the anti-Flag antibody. Thus the anti-DAZ-RRM antibody is able to detect DAZ, but not DAZL in cells fixed on slides.

Immunostaining of sections of HT-1 testis with normal spermatogenesis, using the anti-DAZ-RRM antibody, detected signals in the cytoplasm of spermatogonia near the basal lamina of the seminiferous tubules (Fig. 7A and B). Similar patterns were observed for tissues fixed in Bouin's solution or Formalin. The patchy distribution of the DAZ signals around the peripherals of the tubules is similar to that detected by *in situ* hybridization (Menke *et al*., 1997). Confocal microscopy on immunofluorescence stained sections showed that the signals were present predominantly, if not exclusively, in the cytoplasm (Fig. 7B, inset). No signals were detected when the same antibody was used on a testis section of HT-18 who lacked the *DAZ* genes (Fig. 7C), or when a preimmune antiserum was used on a HT-1 section (Fig. 7D). Our results indicate that in adult human testes DAZ is expressed primarily in the cytoplasm of premeiotic spermatogonia.

Discussion

Our study analysed the expression of the *DAZ* genes in adult human testes using *DAZL* as an internal reference. Our

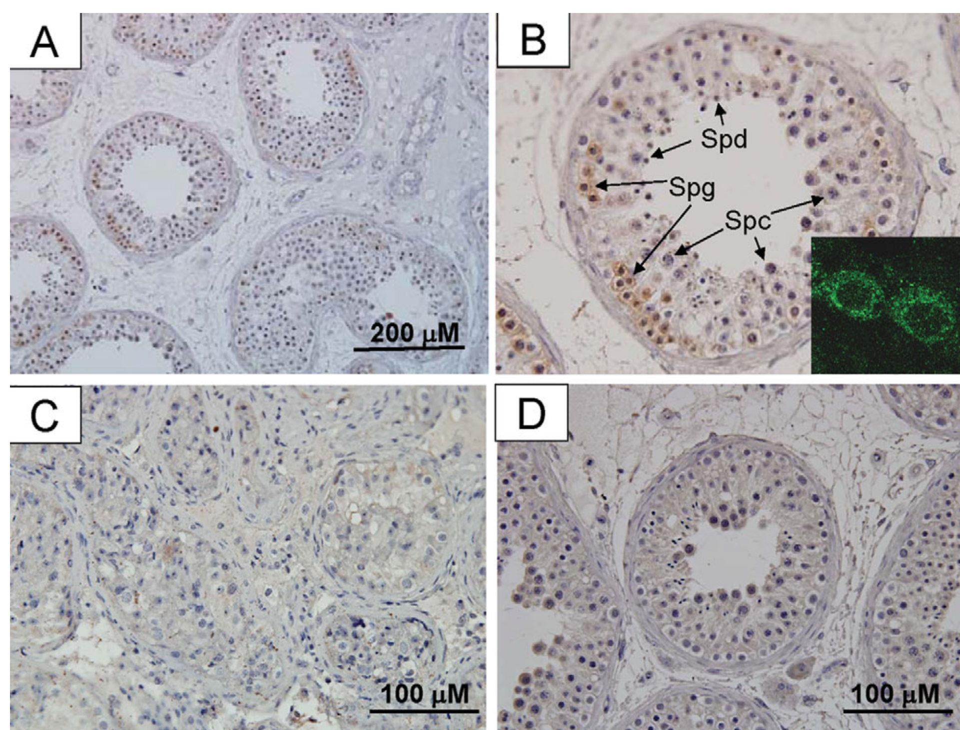

Figure 7: Immunocytochemical detection of DAZ in human testis sections.
Human testis sections of HT-1 who has normal spermatogenesis (**A**, **B** and **D**) or HT-18 who lacks the DAZ genes (**C**) were immunostained with the anti-DAZ-RRM antibody (A–C) or a preimmune antiserum (D). The inset in (B) shows a confocal immunofluorescence image of spermatogonia to illustrate the cytoplasmic localization of the signals. Cell types are Spg: spermatogonium; Spc: spermatocyte; Spd: spermatid.

results show that all four *DAZ* genes are transcribed, but at levels much lower than that of the *DAZL* gene. We had difficulties identifying the DAZ proteins on western blots, but were able to detect DAZ in the cytoplasm of spermatogonia by immunostaining of testis sections. Additional studies in tissue culture systems indicate that DAZ proteins containing multiple copies of DAZ repeats are synthesized inefficiently. Our results show restricted expression of DAZ in spermatogonia and suggest a premeiotic role for the protein.

The promoters of the four *DAZ* genes are nearly identical in sequence, but they share only ~90% similarity with that of the *DAZL* gene. It is therefore not surprising to find similar transcription activities of the four *DAZ* genes but a higher transcription level of *DAZL*. The transcription of more than one *DAZ* gene in the human testis is also supported by a northern blot of *DAZ* that showed additional bands above the major 3.5 kb *DAZ* transcript (Reijo *et al*., 1995).

Our inability to identify positively DAZ on western blots is at variance with two previous studies reporting the detection of a single DAZ band on western blots of human testis lysates (Habermann *et al*., 1998; Reijo *et al*., 2000). However, those studies were carried out before the realization that there are in fact four *DAZ* genes (instead of the originally reported single *DAZ* gene) encoding isoforms of very different size (Saxena *et al*., 2000). The antibodies used in the earlier studies were not tested for their abilities to detect authentic DAZ on western blots, and the assignment of the DAZ band was based solely on its size. Antibodies are known to bind non-specifically to other proteins and do not always locate their target proteins on western blots or immunostaining (Wilson *et al*., 1996; Szot *et al*., 2003). We have established the ability and the sensitivity of our anti-DAZ-R1 antibody to detect both DAZ and DAZL on western blots, and have included appropriate controls including testicular samples from patients without the *DAZ* genes and preincubation of the antibody with the DAZ-R1 peptide to test the authenticity of a putative DAZ band. Our failure to identify DAZ on western blots thus reflects the low concentration of DAZ in human testis lysates. This may be due partially to low translation efficiencies of mRNAs encoding high copy number of DAZ repeats, as suggested by our *in vitro* studies. The reason(s) for the slow synthesis rate for DAZ remains unclear. Examination of the secondary structure free energies of *DAZL* and the various *DAZ* mRNAs, predicted by three programs (Gene Bee, Mfold and Vienna RNA Secondary Structure Prediction), as well as their codon usages failed to identify any features associated with the DAZ repeats that would impair their translation (Nackley *et al*., 2006).

Despite the failure in identifying DAZ using western blotting, we were able to detect DAZ in the cytoplasm of spermatogonia by immunostaining human testis sections using a different antibody. We found little evidence for the presence of DAZ either in late spermatids or exclusively in the nuclei of spermatogonia as reported previously by others (Habermann *et al*., 1998; Reijo *et al*., 2000). The antibodies used in those studies were not tested for their abilities to detect DAZ in immunostaining and may have been subject to non-specific binding. The distribution pattern of DAZ in the seminiferous tubules is similar to that of the *DAZ* transcripts, indicating concomitant transcription and translation of the *DAZ* mRNAs (Menke *et al*., 1997; Szczerba *et al*., 2004). Our *in vitro* results showed similar translation efficiencies for DAZ isoforms containing different copies of RRMs. We therefore propose that all four *DAZ* genes are transcribed and translated in spermatogonia. The differential expression of DAZ and DAZL, which is expressed predominately in primary spermatocytes, suggests that these two proteins have distinct functions (Ruggiu *et al*., 1997; Lin *et al*., 2001). There are about four times as many spermatocytes as spermatogonia in the testis. The lower expression level of DAZ compared with that of DAZL could be due to both the lower number of cells in which DAZ is expressed and the lower translation efficiency of *DAZ* mRNAs encoding multiple copies of the DAZ repeat. The restricted expression of DAZ in spermatogonia suggested that DAZ plays a role mainly in the premeiotic stage of spermatogenesis.

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