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Deleted in Azoospermia Associated Protein 1 Shuttles Between Nucleus and Cytoplasm During Normal Germ Cell Maturation

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ABSTRACT: DAZAP1 (Deleted in Azoospermia Associated Protein 1) was originally identified through its interaction with a putative male azoospermia factor, DAZ (Deleted in Azoospermia). It contains 2 RNA-binding domains (RBDs) and a proline-rich C-terminal portion and is expressed most abundantly in testes. We used RNA in situ hybridization and immunocytochemistry to study the expression of *Dazap1* in mouse testes. *Dazap1* messenger RNA (mRNA) was present predominantly in immature germ cells, between the intermediate spermatogonia and preleptotene spermatocyte stages. The DAZAP1 protein was more abundant in germ cells of later stages of development and showed a dynamic subcellular distribution. High expression of DAZAP1 was first detected in midpachytene spermatocytes in stage VII tubules. In these cells, DAZAP1 was present in both the cytoplasm and the nuclei and was clearly excluded from the sex vesicles. In round spermatids, DAZAP1 was localized mainly in the nuclei, whereas in elongated spermatids, it redistributed to the cytoplasm. The subcellular distribution of DAZAP1 suggests that it shuttles between the nucleus and the cytoplasm and may play a role in mRNA transport and/or localization.

Key words: Spermatogenesis, RNA-binding protein, gene expression.

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RNA-binding proteins play important roles in the post-transcriptional regulation of gene expression (Siomi and Dreyfuss, 1997). They participate in the processing, transport, localization, and translation of messenger RNA (mRNA). An increasing number of mammalian RNA-binding proteins that are expressed specifically or predominantly in the testis have been identified and studied (Ma et al, 1993; Reijo et al, 1995; Cooke et al, 1996; Lahn and Page, 1997; Venables et al, 1999; Elliott et al, 2000). Among them, the DAZ (Deleted in Azoospermia) family has evoked a great deal of interest (Reijo et al, 1995). The family consists of 3 members, DAZ, DAZL (DAZ-Like), and BOULE, encoded by genes derived from the same ancestor (Xu et al, 2001). The *DAZ* genes are present only on the Y chromosomes of great apes and Old World monkeys (Reijo et al, 1995; Shan et al, 1996). The autosomal *DAZL* gene is present in all vertebrates studied (Cooke et al, 1996; Shan et al, 1996; Houston et al, 1998; Maegawa et al, 1999), and *BOULE* orthologs have been isolated from *Drosophila* and *Caenorhabditis elegans* in addition to mice and humans (Eberhar et al, 1996; Karashima et al, 2000; Xu et al, 2001). The DAZ RNA-binding proteins contain an RNA-binding domain (RBD) and 1 or multiple copies of a DAZ repeat unit. They play a pivotal role in germ cell development, and their deficiency results in infertility in males, females, or both sexes (Reijo et al, 1995; Eberhar et al, 1996; Ruggiu et al, 1997; Karashima et al, 2000). Genetic and biochemical studies suggest their involvement in the regulation of mRNA translation (Maines and Wasserman, 1999; Tsui et al, 2000b).

DAZAP1 (Deleted in Azoospermia Associated Protein 1) is an RNA-binding protein identified through its interaction with DAZ in a yeast 2-hybrid system (Tsui et al, 2000a). It binds to both DAZ and DAZL in vitro and is expressed most abundantly in the testis. DAZAP1 contains 2 RBDs in the N-terminal portion and a proline-rich domain in the C-terminal portion. It binds to RNA homopolymers in vitro, with a preference for poly-uridylic acid and poly-guanylic acid. In an attempt to elucidate the function of DAZAP1, we studied its expression and subcellular localization in mouse testes using the techniques of RNA in situ hybridization and immunocytochemistry. Our results indicate that DAZAP1 is expressed

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mainly in germ cells and appears to shuttle between the nucleus and the cytoplasm. In the nuclei of pachytene spermatocytes, DAZAP1 is clearly excluded from the sex vesicles that contain transcriptionally inactive X and Y chromosomes.

Materials and Methods

RNA In Situ Hybridization

A plasmid (Dazap1–5′) containing the first 510 bp of the mouse *Dazap1* complementary DNA (cDNA) (GenBank accession AF225910) was generated by digesting a full-length *Dazap1* cDNA clone in Bluescript (Dai et al, 2001) with the restriction enzyme *Xho*I, followed by self-ligation. After linearization of Dazap1–5′ with restriction enzyme *Xho*I (for the sense probe) or *Bam*HI (for the antisense probe), digoxigenin-labeled sense and antisense RNA probes were prepared by in vitro transcription from the T3 and T7 promoters, respectively, using the Riboprobe System (Promega, Madison, Wis) in the presence of digoxigenin-uridine triphosphate.

Three adult mice were perfused with Bouin solution immediately after euthanasia. Testes were removed, fixed in the same solution overnight, dehydrated in a graded series of ethanol, embedded in paraffin, and sectioned at 5-μm thickness (Sinha Hikim and Swerdloff, 1993). RNA in situ hybridization was carried out according to Millar et al (1993). The hybridization solution contained 50% formamide, 4× saline Tris-EDTA, 1× Denhardt solution, 125 μg/mL each of salmon sperm DNA (sheared and denatured) and yeast transfer RNA, and the probe at a concentration of 50 ng/mL. After hybridization at 54°C overnight, the slides were washed extensively. Bound probes were detected by incubation with alkaline phosphatase–conjugated sheep anti-digoxigenin antibodies (Roch Molecular Biochemicals, Indianapolis, Ind), followed by the addition of 5-bromo-4-chloro-3-indolylphosphate toluidinium and nitroblue tetrazolium for color development. The slides were counterstained with Nuclear Fast Red. The entire testicular sections were scanned at 400× magnification under a Nikon Eclipse E800 microscope, and pictures were taken at 200×, 400×, and 1000× magnifications. Animal handling and experimentation were in accordance with the recommendation of the American Veterinary Medical Association and were approved by the Harbor-University of California, Los Angeles Research and Education Institute animal care and use review committee.

Western Blotting

A goat anti-DAZAP1 antibody was generated against the last 19 amino acid residues of mouse DAZAP1 and affinity purified (Dai et al, 2001). Western blotting of mouse tissues was carried out as described previously (Dai et al, 2001), and consistent results were obtained using tissues from more than 3 animals. For the competition studies, an aliquot of anti-DAZAP1 antibody was preincubated with the oligopeptide antigen at 100 μg/mL at 4°C for 1 hour before being diluted 5000-fold and incubated with the Western membranes.

Immunocytochemical Detection of DAZAP1 in Mouse Testes

Immunostaining of DAZAP1 was carried out on Bouin-fixed paraffin-embedded testicular sections as previously described (Yamamoto et al, 2000). Binding of the primary antibody to the tissue sections was detected using a goat avidin-biotin complex staining system (Santa Cruz Biotechnology, Santa Cruz, Calif). The slides were counterstained with Harris hematoxylin. As in the RNA in situ hybridization, the entire testicular sections were scanned, and pictures were taken at various magnifications.

Results

Expression of the Dazap1 Transcripts

RNA in situ hybridization was performed on adult mouse testicular sections using an antisense RNA probe containing the 5′ portion of the mouse *Dazap1* cDNA. Hybridization signals were detected in all seminiferous tubules, mainly in the peripheral regions close to the basal lamina (Figure 1a). No signals were observed when the sense RNA was used as a probe (Figure 1b). The stages of germ cell development were determined by the morphology of the germ cells and their associations according to Russell et al (1990). Analyses of the signals in tubules at various stages of the cycle (Figure 1c through h) indicated that type B spermatogonia and preleptotene and leptotene spermatocytes contained high levels of *Dazap1* transcripts. The level of expression decreased rapidly as spermatogenesis proceeded. Pachytene spermatocytes retained some signals of hybridization, whereas spermatids lacked any detectable signals. Sertoli cells contained a very low level, if any, of *Dazap1* transcripts.

Expression of the DAZAP1 Protein

An anti-DAZAP1 antibody was generated against an oligopeptide containing the last 19 amino acid residues of mouse DAZAP1, outside the proline-rich domain. The antibody was able to precipitate in vitro synthesized S^{35}-labeled DAZAP1 (data not shown). On Western blots, the antibody detected a single band at a molecular weight of 50 kd in mouse liver and testis extracts (Figure 2). Preincubation of the antibody with the oligopeptide antigen abolished the signals (Figure 2, panel C), demonstrating the specificity of the antibody.

Immunostaining of adult mouse testicular sections with the anti-DAZAP1 antibody showed a dynamic distribution of DAZAP1 (Figure 3). No specific staining of the germ cells was observed using the preimmune antiserum (Figure 3b) or the anti-DAZAP1 antibody preincubated with the oligopeptide antigen (Figure 3c). Spermatogonia and early premeiotic spermatocytes exhibited weak nuclear staining. High levels of DAZAP1 expression were detected after midpachynema, with pachytene spermato-

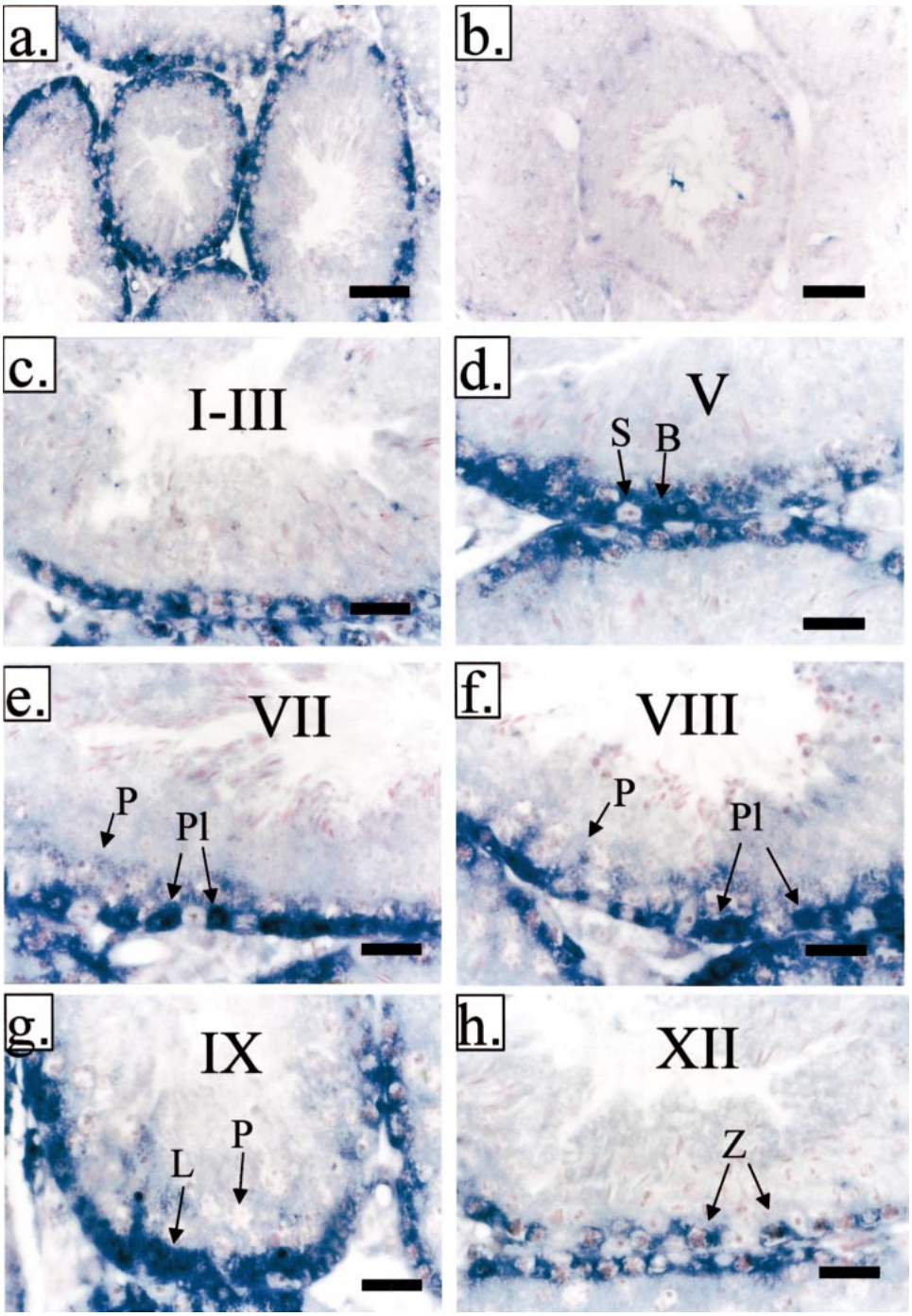

Figure 1. The expression of *Dazap1* (Deleted in Azoospermia Associated Protein 1) messenger RNA (mRNA) in adult mouse testes as detected by RNA in situ hybridization. Testicular sections were hybridized with antisense **(a, c–h)** and sense **(b)** RNA probes of mouse *Dazap1* complementary DNA (cDNA). The stages of the tubules in **(c–h)** are indicated, and the cells are indicated as S, Sertoli cells; B, type B spermatogonia; P, pachytene spermatocytes; Pl, preleptotene spermatocytes; L, leptotene spermatocytes; and Z, zygotene spermatocytes. Bar = 0.1 mm in **(a, b)** and 20 μm in **(c–h)**.

cytes in stage VII–stage X, but not those in stage V, tubules being strongly stained (Figure 3e through l). In these cells, the signals were present mainly in the nuclei but also in the cytoplasm, as evident in sections without the counterstaining (Figure 3h and j, insets). The nuclear staining had a patchy appearance and was clearly excluded from the sex vesicles (Figure 3i, inset). During meiosis, as seen in stage XII tubules, the DAZAP1 signal spread evenly in the anucleated meiotic cells and relocalized to the nuclei of secondary spermatocytes (Figure

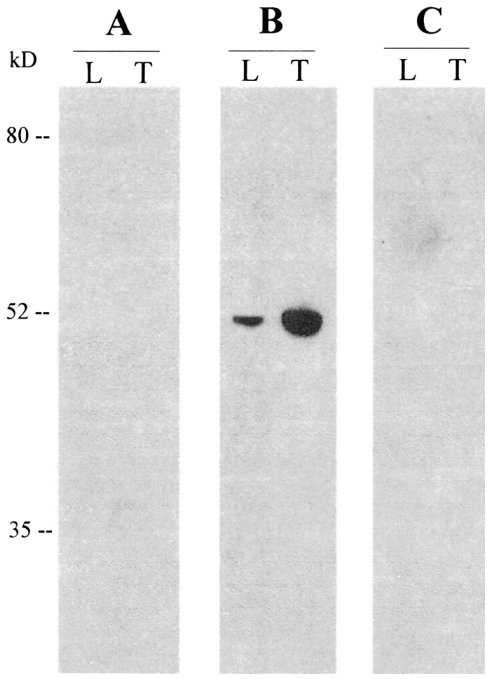

Figure 2. Specificity of the anti-DAZAP1 (Deleted in Azoospermia Associated Protein 1) antibody. Mouse liver (L) and testis (T) extracts underwent Western blotting with **(Panel A)** preimmune antiserum, **(Panel B)** anti-DAZAP1 antibody, and **(Panel C)** anti-DAZAP1 antibody preincubated with the oligopeptide antigen.

3m). In round spermatids, the signals were found predominantly in the nuclei (Figure 3d through j). However, in late-stage IX tubules (Figure 3k), there was a clear redistribution of the signals from the nucleus to the cytoplasm, with the nuclei of some spermatids remaining darkly stained, whereas others were only slightly stained. In elongated spermatids in stage X tubules, the signals were found predominantly in the cytoplasm (Figure 3l). The immunostaining remained in the cytoplasm of spermatids as spermiogenesis proceeded and finally disappeared from the spermatids in stage VII tubules shortly before spermiation (Figure 3g).

The expression of DAZAP1 in prepubertal mice was also studied. In the testes of 10-day-old mice, very weak signals were detected in some cells, presumably preleptotene spermatocytes (Figure 3n). In the testes of 18-day-old mice, much stronger signals were observed in pachytene spermatocytes (Figure 3o). The signals were found in both the cytoplasm and the nucleus and were similar to those observed in adult testes.

Discussion

Our immunostaining results showed a dynamic distribution of DAZAP1 in male germ cells. Early premeiotic cells contain a low level of DAZAP1 in the nuclei. In late pachytene spermatocytes, high levels of DAZAP1 are found in both the nuclei and the cytoplasm. Round spermatids have the protein mainly in the nuclei. Between step 8 and step 9 spermatids, there is a rapid exodus of DAZAP1 from the nucleus when the germ cells undergo nuclear elongation and progressive transcription inactivation (Kierszenbaum and Tres, 1974). Afterward, DAZAP1 is found only in the cytoplasm of elongated spermatids and disappears shortly before spermiation. The nuclear localization of DAZAP1 appears to correlate with the transcriptional activities of the cells. The exclusion of DAZAP1 from the sex vesicles of pachytene spermatocytes, which contain transcriptionally inactive X and Y chromosomes (Kierszenbaum and Tres, 1974), also supports the association of DAZAP1 with active transcription. In the nucleus, DAZAP1 could bind to newly synthesized heterogeneous nuclear RNAs as a component of the heterogeneous nuclear ribonucleoprotein (hnRNP) particles (Dreyfuss et al, 1993). Over 20 hnRNP proteins associated with the hnRNP particles have been identified. DAZAP1 does have an overall structure similar to those of the hnRNP A/B proteins, which shuttle between the nucleus and the cytoplasm and are involved in the biogenesis and transport of mRNA (Pinol-Roma and Dreyfuss, 1992). All these proteins have 2 RBDs at the N-terminal portions. However, the C-terminal portions of the hnRNP A/B proteins are rich in glycine, but that of DAZAP1 is rich in proline. The hnRNP A/B proteins are both expressed in testes, with hnRNP A1 present only in spermatogonia (Kamma et al, 1995) and hnRNP A2/B1 being expressed throughout spermatogenesis (Kamma et al, 1999). These RNA-binding proteins with different expression patterns may be associated with different sets of transcripts in various germ cells.

A role of DAZAP1 in mRNA transport is supported by a recent study on the *Xenopus* ortholog of DAZAP1, Prrp (proline-rich RNA-binding protein) (Zhao et al, 2001). Prrp shares 89% similarity and 81% identity with DAZAP1 (Dai et al, 2001). It binds to a localization element in the 3′-untranslated region of *Vg1* mRNA that is essential for proper movement of the *Vg1* mRNA to the vegetal cortex of mature oocytes (Mowry and Melton, 1992). It also interacts with 2 microfilament-associated proteins, profilin and mena, through its proline-rich domain (Zhao et al, 2001). Prrp therefore appears to be involved in the transport and anchorage of the *Vg1* mRNA to the vegetal cortex. The localization of DAZAP1 in both the nucleus and the cytoplasm is consistent with a role in mRNA transport. Whether DAZAP1 shuttles between nucleus and cytoplasm, especially in pachytene spermatocytes in which DAZAP is present in both cellular compartments, remains to be determined. Shuttling proteins typically have both a nuclear localization signal and a nuclear export signal (Gama-Carvalho and Carmo-Fonseca, 2001).

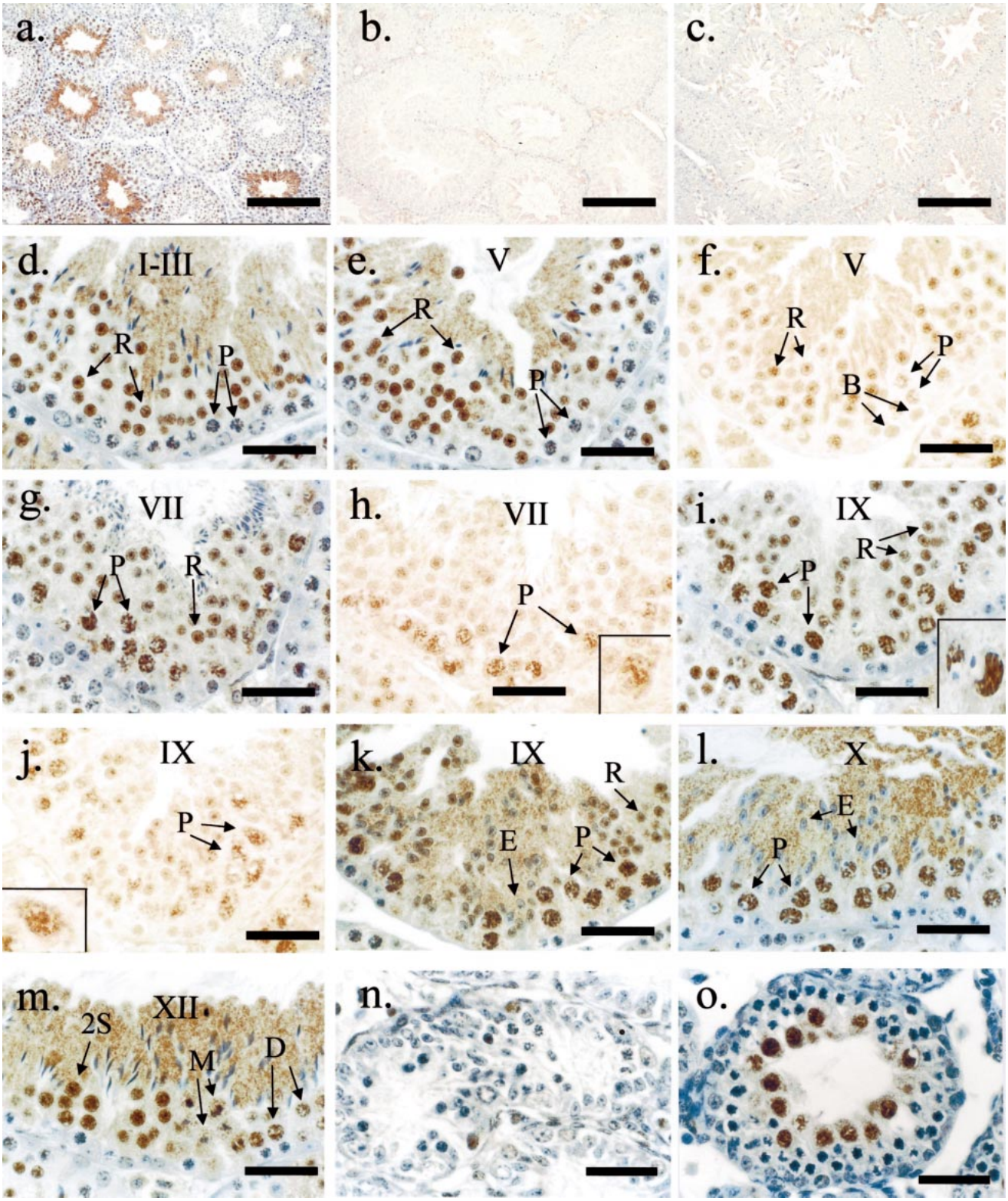

Figure 3. Immunocytochemical detection of DAZAP1 (Deleted in Azoospermia Associated Protein 1) in mouse testes. Testicular sections were prepared from adult **(a–m)** and prepubertal mice at day 10 **(n)** and day 18 **(o)**. Immunostaining was carried out with the anti-DAZAP1 antibody **(a, d–o)**, the preimmune antiserum **(b)**, or the anti-DAZAP1 antibody preincubated with the oligopeptide antigen **(c)**. No counterstaining was applied to sections **(f)**, **(h)**, or **(j)**. The stages of the tubules in **(d–m)** are indicated. The cells are B, type B spermatogonia; D, diplotene spermatocytes; E, elongated spermatids; M, meiotic cells; P, pachytene spermatocytes; R, round spermatids; and 2S, secondary spermatocytes. Bar = 0.2 mm in **(a)**, 0.1 mm in **(b, c)**, and 30 μm in **(d–m)**.

DAZAP1 does not contain any recognizable nuclear import/export signals. Nonetheless, it contains a glycine-rich segment near its carboxy terminus. The M9 protein domain sequence in the hnRNP A/B proteins that is required for the nucleocytoplasmic shuttling of the proteins is also very rich in glycine (Michael et al, 1995). Another RNA-binding protein with testis-predominant expression, testis/brain-RBP (translin), is present in both the nuclei and the cytoplasm of male germ cells and uses a highly conserved nuclear export signal to exit nuclei (Morales et al, 1998; Chennathukuzhi et al, 2001). In addition to proteins that are involved in mRNA transport, other proteins that are involved in signal transduction and cell cycle regulation also shuttle between the nucleus and the cytoplasm. The zinc finger transcription factor GLI1 was shown recently to move between cytoplasm and nucleus during spermatogenesis (Kroft et al, 2001).

The temporal and spatial expression of DAZAP1 in mouse testes partially overlaps that of DAZL. DAZL is present mainly in premeiotic cells (Ruggiu et al, 1997; Reijo et al, 2000). It is present most abundantly in the cytoplasm of pachytene spermatocytes and less so in the nuclei of type B spermatogonia and early primary spermatocytes. Therefore, only a fraction of the total DAZAP1 in testes colocalizes with DAZL in the same compartments of the same cells, and the interaction between DAZAP1 and DAZL could be transient. Initial attempts to coimmunoprecipitate DAZAP1 and DAZL with antibodies against DAZL or DAZAP1 have not been successful.

There appears to be a relative discordance between the expression of *Dazap1* mRNA and that of the DAZAP1 protein in mouse testes. The highest level of *Dazap1* mRNA is found in type B spermatogonia and preleptotene spermatocytes, yet these cells produce relatively low levels of DAZAP1. Early pachytene spermatocytes contain low levels of both the transcript and the protein. The amounts of protein increase significantly in late pachytene spermatocytes, which contain only a very low level of the transcript. Spermatids also contain high levels of DAZAP1 but undetectable amounts of *Dazap1* mRNA. The expression patterns indicate that *Dazap1* mRNA is either translated inefficiently for a prolonged period or is not translated at the same efficiency in all cells and that DAZAP1 is a stable protein with a long half-life. Such peculiar patterns of gene expression are quite common for mammalian spermatogenic cells, and many proteins are detected at high levels much later than their mRNAs (reviewed by Kleene, 2001). One plausible explanation for such patterns of gene expression is delayed translation, which is well studied for the mouse *Prm1* gene encoding one of the protamines (Braun, 1998). *Prm1* is transcribed in round spermatids and translated in elongated spermatids. Premature translation of *Prm1* mRNA in round spermatids causes precocious condensation of nuclear DNA and dominant male sterility (Lee et al, 1995). Because elongated spermatids no longer synthesize RNA, it makes great biological sense for round spermatids to transcribe genes for later use. The necessity of translational delay for proteins needed in germ cells with active transcription is less clear. In addition to *Dazap1, Dazl1* also shows a similar pattern of expression, with the highest level of *Dazl1* transcripts in type B spermatogonia (Niederberger et al, 1997) and the majority of DAZL present in pachytene spermatocytes (Ruggiu et al, 1997; Reijo et al, 2000). Other genes, such as *Tenr* and *Spnr*, were found to be actively transcribed in midpachytene spermatocytes and translated in round spermatids (Schumacher et al, 1995a,b). It is possible that the high demand of a specific protein at a certain developmental stage of the germ cells exceeds their transcriptional capacity, necessitating the precocious transcription of a gene. It is also possible that the late appearance of the protein products reflects an overall inefficiency in protein translation. Additional studies on polysomal loading of *Dazap1* mRNA may determine whether the mRNA undergoes developmental changes in translational activity.

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