

Research article

Characterization of the mouse *Dazap1* gene encoding an RNA-binding protein that interacts with infertility factors DAZ and DAZL

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Abstract

Background: DAZAPI (DAZ Associated Protein 1) was originally identified by a yeast two-hybrid system through its interaction with a putative male infertility factor, DAZ (Deleted in Azoospermia). *In vitro*, DAZAPI interacts with both the Y chromosome-encoded DAZ and an autosome-encoded DAZ-like protein, DAZL. DAZAPI contains two RNA-binding domains (RBDs) and a proline-rich C-terminal portion, and is expressed most abundantly in the testis. To understand the biological function of DAZAPI and the significance of its interaction with DAZ and DAZL, we isolated and characterized the mouse *Dazap1* gene, and studied its expression and the subcellular localization of its protein product.

Results: The human and mouse genes have similar genomic structures and map to syntenic chromosomal regions. The mouse and human DAZAPI proteins share 98% identity and their sequences are highly similar to the *Xenopus* orthologue Prrp, especially in the RBDs. *Dazap1* is expressed throughout testis development. Western blot detects a single 45 kD DAZAPI protein that is most abundant in the testis. Although a majority of DAZAPI is present in the cytoplasmic fraction, they are not associated with polyribosomes.

Conclusions: DAZAPI is evolutionarily highly conserved. Its predominant expression in testes suggests a role in spermatogenesis. Its subcellular localization indicates that it is not directly involved in mRNA translation.

Background

Spermatogenesis is a complex developmental process in which male germ cells progress through mitotic proliferation, meiotic division and dramatic morphological changes to form mature sperm. This process is vital for the propagation of a species, and involves a large portion of the genome of an organism to ensure the quality and quantity of the final products. It is estimated that muta-

tions in up to 11% of all genes in *Drosophila* might lead to male sterility [1]. This is likely to be true for humans also, considering the extremely high incidence (4–5%) of infertility in men [2]. Among the genes associated with male infertility is the *DAZ* (Deleted in Azoospermia) gene family. The family includes the Y-linked *DAZ* genes that are present only in great apes and old world monkeys [3], and the autosomal *DAZL1* (DAZ-like 1) and

BOULE genes [4,5] in all mammals. Deletion of the *DAZ* genes is found in about 10% of infertile males with idiopathic azoospermia [2], and disruption of *Dazl1* causes infertility in both male and female mice [6]. Mutations in the *DAZ* family members of *Drosophila* [7], *C. elegans* [8], and *Xenopus* [9] also affect the fertility in either males, females, or both sexes.

The *DAZ* gene family encodes RNA binding proteins that are expressed specifically in germ cells. *DAZ* and *DAZL* are expressed in the nucleus and cytoplasm of primordial germ cells and spermatogonia, and in the cytoplasm of meiotic spermatocytes [6,10]. *BOULE* is expressed later, in the cytoplasm of pachytene spermatocytes [5]. Genetic and biochemical studies suggest a role for the *DAZ* family in the regulation of mRNA translation. *Drosophila* *Boule* mutants was defective in the translation of the meiosis-specific *CDC25* homologue, *Twine* [11], and *DAZL* was found to be associated with polyribosomes in mouse testes [12]. More recently, *DAZL* was shown both *in vitro* and in a yeast three-hybrid system to bind specifically to oligo(U) stretches interspersed by G or C residues, including a U-rich segment in the 5' UTR of mouse *Cdc25C* mRNA [13].

In an attempt to elucidate the function of the *DAZ* gene family and to understanding the mechanisms of its action, we used a yeast two-hybrid system to isolate two human genes encoding *DAZ* associated proteins (*DAZAPs*) [14]. One of them, *DAZAP1*, is expressed predominantly in testes. It encodes a protein with two RNA

binding domains and a proline rich C-terminal portion. The *DAZAP1* protein interacted with both *DAZ* and *DAZL in vitro*. It also bound to RNA homopolymers. We now report our characterization of the mouse *Dazap1* gene and its protein product. The subcellular localization of *DAZAP1* suggests that it is not involved directly in mRNA translation.

Results

Characterization of the mouse *Dazap1* cDNA

Mouse *Dazap1* cDNA clones were isolated by library screening, and the 5' end of the cDNA was isolated by 5' RACE [15]. The near full length cDNA consists of a 53 bp 5' untranslated region (UTR), an open reading frame for a protein of 405 amino acid residues, and a 362 bp 3' UTR (GenBank Accession No: AF225910). The coding region shares 89% similarity with that of the human orthologue. The 3' UTR sequence is remarkably conserved. It contains three segments of 35 bp, 133 bp and 90 bp that share 85%, 90%, and 97% similarity with segments in the human 3' UTR, respectively. These segments probably contain regulatory elements.

The *DAZAP1* protein contains two RNA-binding domains (RBDS) and a C-terminal portion that is rich in proline (Figure 1). It is highly conserved evolutionarily. The mouse and the human proteins differ in 9 amino acids only, with 7 substitutions and two deletions/insertions. The mammalian proteins shares 89% similarity and 81% identity with *Xenopus* Prpp (for proline-rich RNA binding protein) [16]. The two RBDs are especially

Table 1: Exon-intron Organization of the Mouse and Human *DAZAP1* genes

Exon	Exon Size (bp)		Intron insertion sites ^a		Intron size (kb)	
	Mouse	Human	Mouse	Human	Mouse	Human
1	>82	>137	82	137	?	9.70
2	41	41	123	178	0.40	0.66
3	167	167	290	345	0.19	0.30
4	66	66	356	411	2.7	2.42
5	108	111	464	522	0.7	1.09
6	49	49	513	571	2.6	3.44
7	83	83	596	654	2.1	2.88
8	154	154	750	808	1.2	0.97
9	30	30	780	838	0.28	0.22
10	141	141	921	979	0.95	2.15
11	177	177	1098	1156	2.2	2.0
12	535	564				

a: Introns are inserted after the indicated nucleotide positions of *DAZAP1* cDNA sequences. GenBank accession numbers for mouse and human cDNAs are AF225910 and AF181719, respectively.

Human	MNNSGADEIGKLFVGGLDWSTTQETLRSYFSQYGEVVDCVIMKDKTNNQSRGFGFVKFKD	60		
Mouse	MNSAGADEIGKLFVGGLDWSTTQETLRSYFSQYGEVVDCVIMKDKTNNQSRGFGFVKFKD	60		
Xenopus	MNNQGGDEIGKLFVGGLDWSTTQETLRSYFSQYGEVVDCVIMKDKTNNQSRGFGFVKFKD	60		
	## *			
Human	PNCVGTVLASRPHTLDGRNIDPKPCTP	RGMQPERTRPKEGWQ-KG	PRSDYSKSNKIFVGG	119
Mouse	PNCVGTVLASRPHTLDGRNIDPKPCTP	RGMQPERTRPKEGW--KG	PRSDSSKSNKIFVGG	118
Xenopus	PNCVGTVLASRPHTLDGRNIDPKPCTP	RGMQPERSRPREGWQQKE	PRTENSRSNKIFVGG	120
		* * # * *	**# *	
Human	IPHNCGETELREYFKKFGVVTEVVMYDAEKQRPRGFGFITFEDEQSVDQAVNMHFHDIM			179
Mouse	IPHNCGETELREYFKKFGVVTEVVMYDAEKQRPRGFGFITFEDEQSVDQAVNMHFHDIM			178
Xenopus	IPHNCGETELKEYFNRFVGVTEVVMYDAEKQRPRGFGFITFEDEQSVDQAVNMHFHDIM			180
		* **		
Human	GKKVEVKRAEP	RDSKSQAPGQPGASQWGSRVVNAANGWAGQPPPTWQQGYGPGQGMWVPA	239	
Mouse	GKKVEVKRAEP	RDSKNQAPGQPGASQWGSRVAPSAANGWAGQPPPTWQQGYGPGQGMWVPA	238	
Xenopus	GKKVEVKRAEP	RDSKSQTPGPPGSNQWGSRAMQSTANGWTGQPPQTW-QGYSPQGMWMP	239	
		# * * ** *##* * * * *		
Human	GQAIGGYGPPPAGRGAPPPPPFTSYIVSTPPGGFPPPQGFPPQGYGAPPQFSFGYGPPPP			299
Mouse	GQAIGGYGPPPAGRGAPPPPPFTSYIVSTPPGGFPPPQGFPPQGYGAPPQFSFGYGPPPP			298
Xenopus	GQTIGGYG-QPAGRGGPPPPPSFAPFLVSTTPGFPPPPQGFPPGYATPPPFYGYGPPPP			298
		* ** * * **** * *	* ** * **	
Human	PPDQFAPPGVPPPPATPGAAPLAFPPPPSQAAPDMSKPPTAQPDPFYGYAGYQDLSGF			359
Mouse	PPDQFAPPGVPPPPATPGAAPLAFPPPPSQAAPDMSKPPTAQPDPFYGYQY-GYQDLSGL			357
Xenopus	PPDQFVSSGVPPPPGTPGAAPLAFPPPPGQSAQDLSKPPSGQDFPFSQFGNACFVKLSE			358
		*** *	* * * * ** * ** #*****##	
Human	GQGFSDPSQQPPSYGGPSVPGSGPPAGGSGFGRGQNHNVQGFHPYRR			407
Mouse	GQGFSDPSQQPPSYGGPSVPGSGPPAGGSGFGRGQNHNVQGFHPYRR			405
Xenopus	WI			360
		**		

Figure 1
Evolutionary conservation of the DAZAP1 proteins. The amino acid sequences of the human and mouse DAZAP1s and the *Xenopus* Prp are compared. The two RNA binding domains are boxed. Differences between the human and the mouse sequences, and between the mouse and *Xenopus* sequences are marked by #'s and *'s, respectively.

highly conserved. They share 98% and 97% similarity and 97% and 92% identity, respectively, between DAZAP1 and Prp. These proteins may therefore have a similar RNA binding specificity. The C-terminal proline-rich portions of DAZAP1 and Prp are less conserved (81% similarity and 71% identity). There is an insertion of a 58 bp segment in Prp cDNA that causes a change of reading frame and results in a shorter Prp with a different C-terminal end sequence.

Genomic structure of Dazap1 and chromosomal mapping
Several overlapping lambda clones containing mouse Dazap1 genomic sequences were isolated. The locations

of exons were determined by PCR amplification across exon-intron boundaries following by sequencing. All but the first exon were isolated and mapped. The genomic structure of the human DAZAP1 gene was also determined by blasting the human genome sequence at National Center for Biotechnology Information with the human DAZAP1 cDNA sequence. The mouse and the human genes have very similar structures, consisting of 12 exons spanning about 28 kb. All intron insertion sites are conserved (Table 1). The two RBDs are encoded by exons 1-4 and 5-8, respectively.

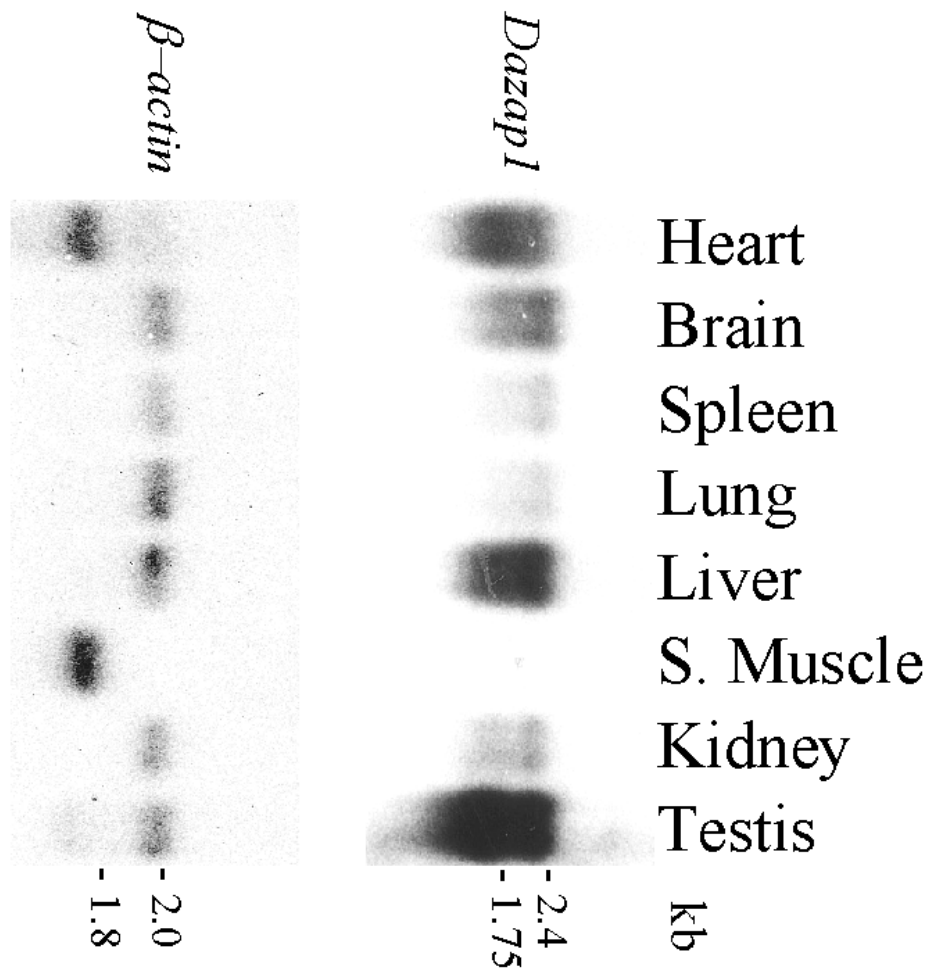


Figure 2

Expression of *Dazap1* in adult mouse tissues. A mouse multiple-tissue Northern blot was hybridized with a *Dazap1* cDNA probe, stripped, and rehybridized with a β -actin probe. *Dazap1* is expressed most abundantly in the testis.

A pair of PCR primers was designed from *Dazap1* intronic sequences that amplified mouse but not hamster genomic sequences. Using a panel of mouse-hamster radiation hybrids, the mouse *Dazap1* gene was mapped to chromosome 10 placed 27.84 cR from D10Mit260 (lod > 3.0) (data not shown). This region is syntenic to human 19p13.3 where the human *DAZAP1* gene is located [14,17]. It contains no known mutant alleles that are associated with infertility.

Expression of *Dazap1*

Northern analyses of adult mouse tissues showed the presence of two *Dazap1* transcripts of 1.75 kb and 2.4 kb, respectively (Figure 2). Only the shorter transcript has been isolated in cDNA clones. *Dazap1* was expressed most abundantly in the testis, much less in liver, heart and brain, and even less in other tissues. This pattern of

expression is similar to that of the human *DAZAP1* [14]. RT-PCR analyses showed that *Dazap1* mRNA was already present in fetal testes at embryonic day 15, similar to *Dazl1* mRNA (Figure 3). The expression of both *Dazl1* and *Dazap1* persisted throughout testis development, in both the prenatal and postnatal periods. *Dazl1* and *Dazap1* transcripts were also present in the testes of W^v/W^v mutant mice which contained diminished number of germ cells [18]. However, only *Dazap1* was expressed in a mouse germ cell line GCl-spg [19] and a Sertoli cell line MT4. The results suggest that *Dazap1* is expressed in both somatic and germ cells in the testis.

To study the expression of the *DAZAP1* protein, two antibodies against mouse *DAZAP1* were generated. The anti *DAZAP1*-C antibody was raised against a recombinant protein containing the C-terminal proline-rich

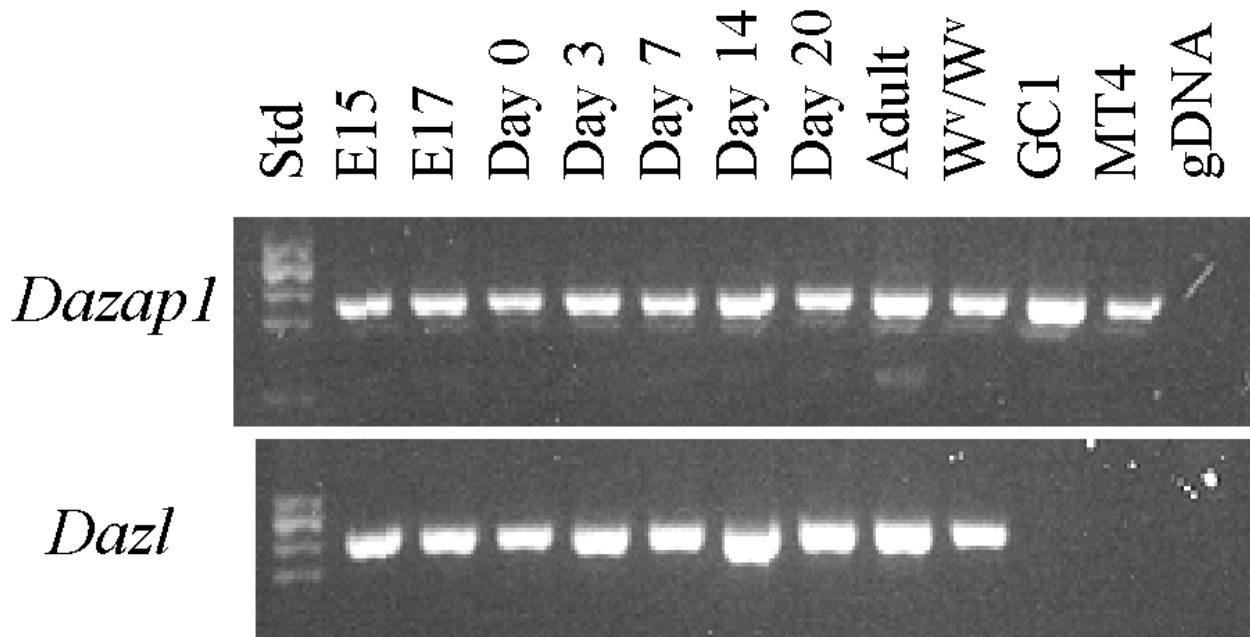


Figure 3

Developmental expression of *Dazap1* and *Dazl* in mouse testes. RT-PCR was performed on total testicular RNAs isolated from day 15 (E1 5) and day 17 (E1 7) embryos, new born mice (Day 0), and mice at various days after birth. W^v/W^v testes contain diminished germ cell population due to a mutated *W* (*White spotted*) gene. GCI and MT4 are mouse germ cell and Sertoli cell lines, respectively, and gDNA is mouse genomic DNA. The PCR primers span over introns and produce much larger (if any) fragments from genomic DNA.

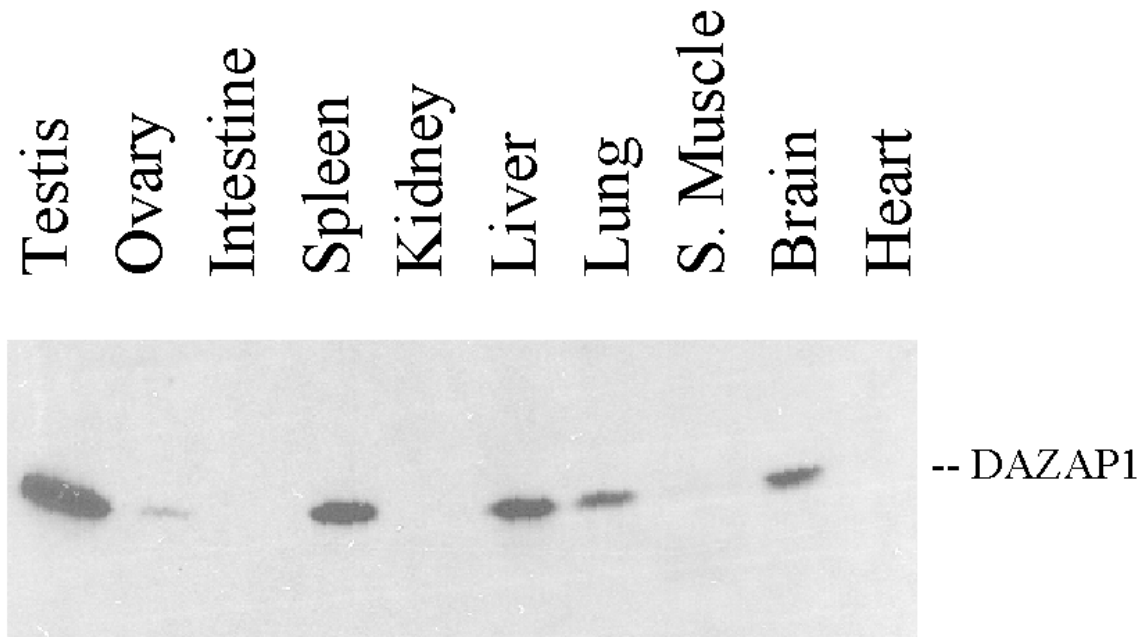
portion, and the anti DAZAP1-P antibody was raised against an oligopeptide containing the last 19 amino acid residue at the C-terminus. Both antibodies recognized *in vitro* synthesized DAZAP1 in an immunoprecipitation assay (data not shown). Western blotting of mouse tissue extracts detected a 45 kD protein that was present most abundantly in the testis, and to a lesser degree in spleen, liver, lung and brain (Figure 4). The protein was also present in the ovary. The expression of DAZAP1 during germ cell development paralleled that of DAZL (Figure 5). It is present at a low level in the testes of 6 days old mice which contained only primitive type A spermatogonia. The expression of DAZAP1 increased afterward, as the testes contained increasing number of proliferating and meiotic germ cells.

Subcellular localization of DAZAP1

Our previous fractionation of mouse testis extracts showed that most DAZL were present in the post mitochondrial fraction, and some of them were associated with polyribosomes [12]. Similar analyses showed that a majority of DAZAP1 in adult mouse testes was also present in the cytoplasmic fraction (data not shown). However, sucrose gradient analyses of the post-mitochondria fraction showed that, unlike DAZL, DAZAP1 did not co-sediment with polyribosomes (Figure 6).

Discussion

RNA-binding proteins have been found to participate in many cellular functions, including RNA transcription, pre-mRNA processing, mRNA transport, localization,

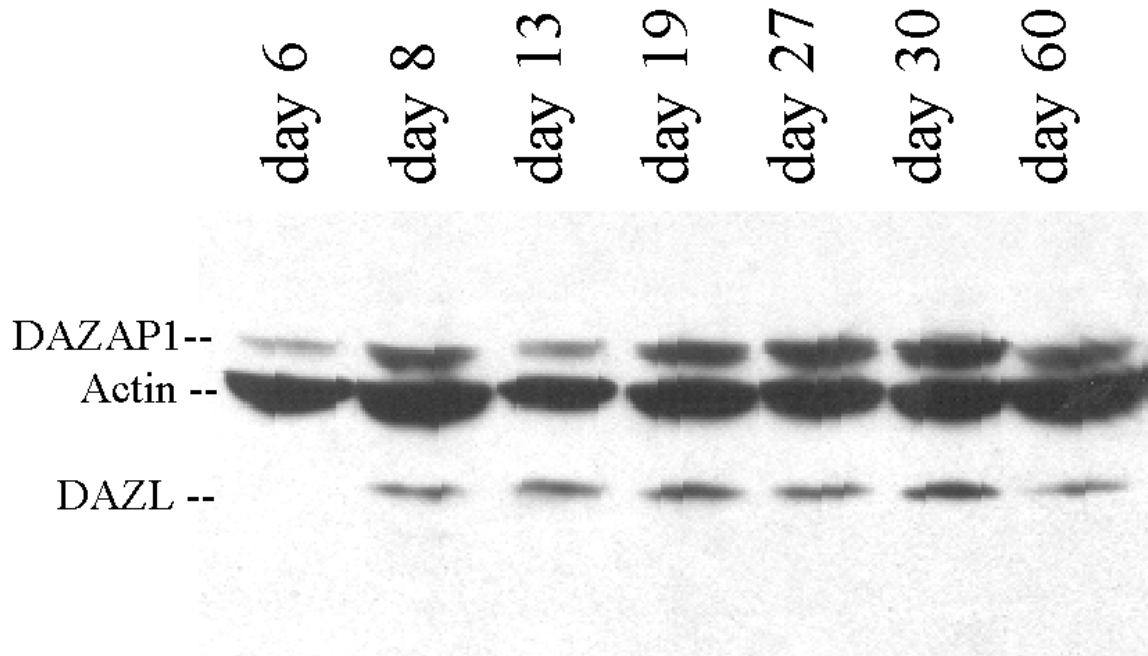
**Figure 4**

Expression of the DAZAP1 protein in adult mouse tissues. Equal amounts of total protein from various tissue extracts were applied to a 10% SDS-polyacrylamide gel and western blotted with the anti-DAZAP1-P antibody.

translation and stability [20]. A role for the DAZ family in the regulation of mRNA translation is supported by lines of circumstantial evidence, including the association of DAZL with polyribosomes [12]. The absence of DAZAP1 from polyribosomes indicates that it is not directly involved in protein synthesis. This finding is different from two RNA-binding proteins, FXR1P and FXR2P, that were identified through their interaction with another polysomal-associated RNA-binding protein, the fragile X mental retardation protein [21]. Both FXR1P and FXR2P are associated with the polyribosomes [22].

The significance of the interaction between DAZAP1 and DAZL/DAZ remains to be defined. These proteins may act together to facilitate the expression of a set of genes in germ cells. For example, DAZAP1 could be involved in

the transport of the mRNAs of the target genes of DAZL. Alternatively, DAZL and DAZAP1 may act antagonistically to regulate the timing and the level of expression. Such an antagonistic interaction between two interacting RNA-binding proteins is exemplified by the neuron-specific nuclear RNA-binding protein, Nova-1. Nova-1 regulates the alternative splicing of the pre-mRNAs encoding neuronal inhibitory glycine receptor $\alpha 2$ (GlyR $\alpha 2$) [23]. The ability of Nova-1 to activate exon selection in neurons is antagonized by a second RNA-binding protein, brPTB (brain-enriched polypyrimidine tract-binding protein), which interacts with Nova-1 and inhibits its function [24]. DAZAP1 could function in a similar manner by binding to DAZL and inhibiting its function. Comparing the phenotypes of *Dazl1* and *Dazap1* single and double knock-out mice may provide some clues to the significance of their interaction. *Dazl1* knock-out mice

**Figure 5**

Western blot analyses of the expression of DAZAP1 and DAZL in mouse testes during postnatal development.

have already been generated and studied [6]. The spermatogenic defect in the male becomes apparent only after day 7 post partum when the germ cells are committing to meiosis (H. Cooke, personal communication). The genomic structure of *Dazap1*, delineated here, should facilitate the generating of *Dazap1* null mutation.

DAZAP1 was shown to bind RNA homopolymers *in vitro*, with a preference for poly U and poly G. Its natural substrates have not been identified. Recently, the *Xenopus* orthologue of DAZAP1, Prrp, was identified and characterized [16]. Prrp binds to a 340 nt sequence in the 3' UTR of *Xenopus Vg1* mRNA. This *Vg1* localization element (VLE) is sufficient for the migration and clustering of *Vg1* mRNA to the vegetal cortex of mature oocyte. Prrp also interacts through its proline-rich domain with two microfilament-associated proteins profilin and Mena, which may facilitate the anchoring of *Vg1* mRNA to the

vegetal cortex. The *Vg1* RNA encodes a member of the transforming growth factor- β family that is required for generating dorsal mesoderm at the blastula stage of *Xenopus* embryogenesis [25]. Sequence conservation between the RBDs of DAZAP1 and Prrp suggests that these proteins may bind to similar RNA sequences. However, a BLAST search of the GenBank for the 340 nt VLE sequence failed to identify any mammalian sequences with significant homology. Further mapping of the RNA sequence within VLE that binds Prrp, and possibly DAZAP1, may help to identify the natural substrates of DAZAP1.

Conclusions

DAZAP1 is an evolutionarily conserved RNA-binding protein. It is present at variable levels in many tissues. Its predominant expression in testes suggests a role in spermatogenesis. In mouse testes, DAZAP1 was found both

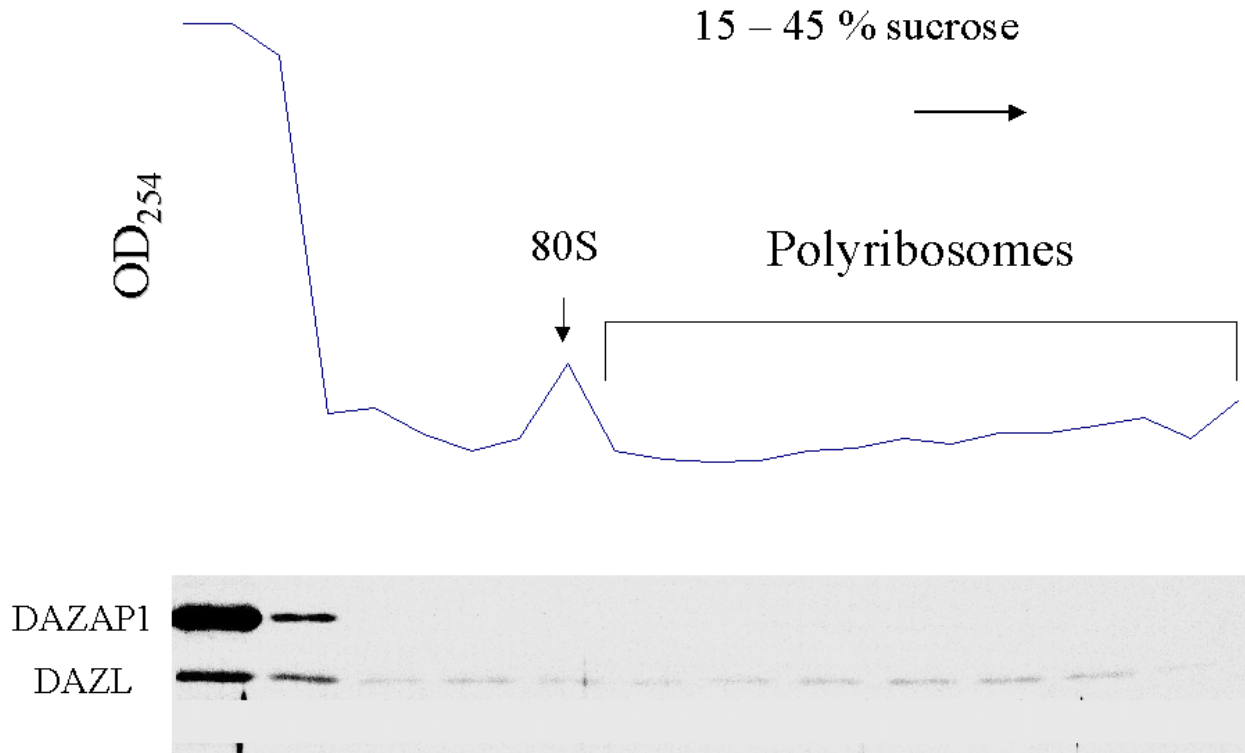


Figure 6

Sucrose gradient analyses shows that DAZAPI is not associated with polyribosomes. The post-mitochondrial supernatant of mouse testis extracts was analyzed on a 15–45% sucrose gradient. Sedimentation was from left to right. The presence of DAZAPI and DAZL in each fractions was analyzed by Western blotting.

in the nuclei and in the cytoplasm. Its absence from polyribosomes indicates that it is not directly involved in mRNA translation.

Materials and methods

Isolation of mouse *Dazap1* cDNA clones

Dazap1 cDNA clones were isolated from a mouse testis cDNA library (#937308, Stratagene, La Jolla, CA) using a human *DAZAP1* cDNA as a probe. The 5' end of the cDNA was isolated by 5' RACE [15], using *prdap11* (ttgcg-ggccatctcttg, #749–732) as the primer for cDNA synthesis from mouse testis RNA, and *prdap37* (ttgtgccacgtggcg, #734–718) and an adaptor primer as the primers for PCR amplification. The PCR products were cloned into a TA cloning vector pCR2.1-TOPO (Invitrogen, Carlsbad CA). *Dazap1* clones were identified by

colony hybridization and sequenced. The 5' RACE clone with the longest 5' UTR region and the cDNA clone P21 were ligated together through a unique *Pm*II site at # 722 to generate a cDNA clone (*Dazap1*-C) with a near full-length insert.

Chromosomal mapping of *Dazap1*

Dazap1 genomic clones were isolated from a mouse 129SV genomic library (#946305, Stratagene), and sequences flanking each exons were determined. PCR primers (*prdap25*: cactccaggatgtgttagc and *prdap26*:gtcaccaagggtgtctgaag) were designed from intronic sequences flanking *Dazap1* exon 8. These primers amplified a 271 bp fragment from mouse but not hamster genomic DNA. DNA samples of a panel of 100 radiation hybrids containing mouse chromosome fragments in a

hamster background were purchased from Research Genetics (Huntsville, AL). The presence of mouse *Dazap1* in the radiation hybrids was determined by PCR and the results were sent to the MIT server [www.genome.wi.mit.edu/mouse_rh/index.html] for computerized physical mapping of the gene.

Expression of *Dazap1* transcripts

Northern hybridization was carried out according to standard procedures [26] using a mouse Multiple Tissue Northern Blot #7762-1 from Clontech (Palo Alto, CA). The blot was hybridized sequentially with *DAZAP1* and β -actin cDNA probes, with stripping of the bound probes in between.

Reverse transcription-polymerase chain reaction (RT-PCR) was carried out as previously described using an annealing temperature of 54°C [27]. The primers were prdap35: agctcaggagtagtactcaaga and prdap24 :ggagcttgatttctgtctec for *Dazap1* which generated a product of 211 bp, and prdaz71: atcgaactgggtgtcgaagg and prdaz72: ggagctgcacatgaagtcca for *Dazl1* which generated a product of 245 bp. Both primer pairs annealed across intron insertion sites.

Generation of anti-*DAZAP1* antibodies

Antibodies were generated against both a recombinant protein produced in *E. coli* and an oligopeptide synthesized *in vitro*. The insert of a *Dazap1* cDNA clone P21, which encoded the C-terminal portion of *DAZAP1* (starting from aa #197), was cloned in-frame into the *EcoRI/XhoI* sites of an expression vector pET32b (Novagen, Madison, WI). Sequences at the junctions were verified by DNA sequencing. Milligrams of fusion proteins between thioredoxin and *DAZAP1* were prepared and purified on His-Bind metal chelation resins (Novagen, Madison, WI). The proteins were mixed with Freund's adjuvant and injected into rabbits to generate the anti-*DAZAP1*-C antibody. An oligopeptide containing the last 19 amino acid residues of the mouse *DAZAP1* was synthesized *in vitro* using the services of Bethyl Laboratories (Montgomery, TX). The peptide was conjugated to KLH as carrier and injected into a goat. The anti-*DAZAP1*-P antibody thus produced was purified on an affinity column containing the oligopeptide antigen.

Western blotting

Mouse tissues were homogenized in the RIPA lysis buffer (150 mM NaCl, 1.0% NP-40, 0.5% DOC, 0.1% SDS, 50 mM Tris, pH 8.0) at a concentration of 0.2 g tissue per ml of buffer. The homogenized samples were cleared of debris by centrifugation at 10,000 \times g for 10 minutes. Protein concentration of the tissue extracts was determined by the Bradford method using the Bio-Rad Protein Assay system (Bio-Rad, Hercules, CA). About 50 μ g of tissue

extracts were separated on 10% SDS-polyacrylamide gels and blotted with either the anti-*DAZAP1*-C antibody (at a 1/2,000 dilution) or the anti-*DAZAP1*-P antibody (at a 1/5,000 dilution). After incubation with horseradish peroxidase-conjugated secondary antibodies, the binding of antibodies was detected using the ECL Western Blotting System (Amersham Pharmacia Biotech, Piscataway, NJ).

Fractionation of mouse testicular extracts

Adult mouse testes were homogenized in a buffer containing 20 mM Tris, pH 7.5, 100 mM KCl, 5 mM MgCl₂, 0.3% NP-40, 40 U/ml of Rnasin ribonuclease inhibitor (Promega, Madison, WI), and a mixture of 10 protease inhibitors provided in the Protease Inhibitors Set (Roche Molecular Biochemicals, Indianapolis, IN). Homogenates were centrifuged at 1,000 \times g for 10 minutes to pellet cell debris and nuclei. After an additional centrifugation at 10,000 \times g for 10 minutes to pellet the mitochondria, aliquots of the supernatant were applied to 15–45% sucrose gradients in 20 mM Tris, 100 mM KCl and 5 mM MgCl₂ and centrifuged in a Beckman SW41 rotor at 39,000 rpm for 2 hours at 4°C. Fractions of 0.5 ml were collected from the bottom of the tubes and analyzed by western blotting.

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