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Involvement of a Tissue-Specific RNA Recognition Motif Protein in *Drosophila* Spermatogenesis

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RNA binding proteins mediate posttranscriptional regulation of gene expression via their roles in nuclear and cytoplasmic mRNA metabolism. Many of the proteins involved in these processes have a common RNA binding domain, the RNA recognition motif (RRM). We have characterized the Testis-specific RRM protein gene (*Tsr*), which plays an important role in spermatogenesis in *Drosophila melanogaster*. Disruption of *Tsr* led to a dramatic reduction in male fertility due to the production of spermatids with abnormalities in mitochondrial morphogenesis. *Tsr* is located on the third chromosome at 87F, adjacent to the nuclear pre-mRNA binding protein gene *Hrb87F*. A 1.7-kb *Tsr* transcript was expressed exclusively in the male germ line. It encoded a protein containing two RRMs similar to those found in HRB87F as well as a unique C-terminal domain. TSR protein was located in the cytoplasm of spermatocytes and young spermatids but was absent from mature sperm. The cellular proteins expressed in premeiotic primary spermatocytes from *Tsr* mutant and wild-type males were assessed by two-dimensional gel electrophoresis. Lack of TSR resulted in the premature expression of a few proteins prior to meiosis; this was abolished by a transgenic copy of *Tsr*. These data demonstrate that TSR negatively regulated the expression of some testis proteins and, in combination with its expression pattern and subcellular localization, suggest that TSR regulates the stability or translatability of some mRNAs during spermatogenesis.

Proper control of gene expression involves not only transcriptional but also posttranscriptional events, which are mediated through RNA-RNA and RNA-protein interactions. In addition to nuclear processes such as splicing, which are required for the production and transport of mature mRNA, cytoplasmic processes can play significant regulatory roles. For example, under conditions in which transcriptional activity is low or absent, regulation of mRNA stability or translatability can provide an effective mechanism for controlling protein expression (for recent reviews, see references 11 and 44). These circumstances are found during gametogenesis and early embryogenesis in several organisms. In *Xenopus laevis*, a precisely choreographed pattern of cytoplasmic adenylation and deadenylation of mRNAs is an important regulatory event in oogenesis and early embryonic development (reviewed in references 51 to 53). These alterations in poly(A) tail length control the translatability of several mRNAs that are critical for oocyte maturation and embryogenesis.

Similar types of regulatory events are important in postmeiotic cells during spermatogenesis (see references 7, 21, and 42 and references therein). The mouse testis contains a large proportion of nonpolysomal poly(A)$^+$ RNA (16), which may represent a large pool of mRNA stored in a translationally repressed form. Furthermore, studies of individual genes encoding several mouse germ cell-specific proteins have shown that transcription can precede translation by several days. For example, the mRNAs for transition proteins and protamines are transcribed but not translated in early postmeiotic round spermatids. These mRNAs are stored in a translationally inactive form for up to 7 days before becoming associated with polysomes and undergoing poly(A) tail shortening (27). Translational repression of testis mRNAs has also been demonstrated in *Drosophila melanogaster*. In *D. melanogaster*, the development of mature sperm from a stem cell takes about 10 days. For most genes, transcription ceases at meiosis (17, 35), although there is evidence for postmeiotic expression of one gene (2). Members of the *Mst(3)CGP* gene family, and the *janB* and *dhod* genes, are transcribed in premeiotic spermatocytes but not translated until after meiosis (reviewed in reference 42). For some of these mRNAs, translation is delayed for up to 3 days, until very late stages of spermatid development. The RNA sequence elements required for translational repression are known for several of these genes, and testis-specific proteins bind to them (25). However, as yet there is no direct evidence that these proteins are responsible for translational regulation.

Many of the RNA binding proteins involved in posttranscriptional regulation contain a common RNA binding domain, termed the RNA recognition motif (RRM) or RNP motif (4, 8). Structural studies have shown that the RRM can form a discrete domain capable of sequence-specific RNA binding (33). Although the RNA binding properties and structures of only a few RRMs have been characterized in any detail, it is generally believed that all proteins with RRMs are capable of RNA binding, albeit with different degrees of sequence specificity for their RNA targets. Sequence comparisons of RRMs have shown that they can be grouped into families (4). Sometimes, members of a family also have additional protein domains, termed auxiliary domains, in common. These auxiliary domains may be involved in protein-protein or protein-RNA interactions (3). For example, the pre-mRNA binding protein hnRNP A1 belongs to a family of closely related proteins, the A/B hnRNP proteins (reference 13 and references therein). Each of these proteins has two well-conserved RRMs and a C-terminal auxiliary domain rich in glycines and interspersed with aromatic amino acids.

We have been interested in understanding the functions of several *Drosophila* RRM proteins, including the pre-mRNA

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binding proteins HRB98DE and HRB87F (19, 20), which are closely related to hnRNP A1. To that end, we initiated a genetic analysis of the *Hrb87F* locus, which is located at 87F on the third chromosome, near two other RRM proteins, the hnRNP protein SQD (24) and the splicing factor B52 (24, 40). We generated a small chromosomal deletion by imprecise excision of a P element inserted in *Hrb87F* (56). Characterization of this deletion led to the identification of an adjacent gene that we have named *Tsr* (for Testis-specific RRM protein), which is the subject of this report. Many RRM proteins in higher eukaryotes are expressed in multiple cell types or during much of development. In contrast, the TSR protein was expressed in only the male germ line, in the cytoplasm of spermatocytes and elongating spermatids. A mutation in *Tsr* drastically reduced male fertility due to abnormal spermatid development. Analysis of proteins expressed in primary spermatocytes in *Tsr* mutant males indicated that TSR negatively regulated the expression of some proteins prior to meiosis. Our data suggest that TSR plays a role in mRNA stability or translation during spermatogenesis.

MATERIALS AND METHODS

***Drosophila* strains.** Fly stocks and mutations are described in the text or in reference 31. Flies were cultured on standard molasses-cornmeal-agar medium, and crosses and assays were performed at 25°C. The *Df(3R)Hrb87F* mutation, which deletes portions of *Hrb87F* and *Tsr*, was generated by imprecise excision of a P element in the *Hrb87F* gene (56). A small portion of the P element remained at the insertion site. Tsr^{-} denotes flies that are homozygous for the *Df(3R)Hrb87F* chromosome. Transformants carrying the *Hrb87F* or *Tsr* gene were generated by standard P-element transformation techniques using genomic DNA fragments from the cos sqd2 genomic clone (24) inserted into the pCasper vector (36). A single *Hrb87F* transformant on chromosome 2 was obtained; this line is homozygous lethal, presumably due to the disruption of an essential gene. Multiple *Tsr* transformants were obtained; two homozygous viable lines with insertions on chromosome 2 were used in the experiments described in this report and gave essentially identical results.

Male fertility assays. Male fertility was assessed by determining the number of adult progeny produced by males of a given genotype. Individual males were placed with 5 virgin Oregon R wild-type females in a yeasted food vial; at least 10 males were tested for each genotype. The flies were allowed to lay eggs for 2 weeks, with periodic transfers to fresh food vials. The adults emerging from each vial were counted. Vials from which few adults emerged showed little evidence of larval activity and contained large numbers of unhatched eggs that showed no evidence of embryonic development. These observations indicate that there was no significant embryonic or larval lethality, and they validate the use of adult progeny counts as a measure of male fertility.

Nucleic acid analyses. Southern and Northern blot analyses were performed as previously described (22). The *Tsr* cDNA clone was isolated from a pupal library (37) and sequenced by using a Sequenase kit (U.S. Biochemical). Total RNA for reverse transcription (RT)-PCR analyses was prepared from 10 sexed larvae, pupae, or adults and treated with RNase-free DNase (Promega) to remove contaminating DNA. Each RT reaction mixture contained 1 μg of total RNA, primers specific for both *Tsr* and *Hrb87F*, and reagents from Perkin-Elmer's RNA PCR kit. The RT reaction products were precipitated with ethanol, redissolved, and split into two samples before amplification of *Tsr* and *Hrb87F* sequences in separate standard PCRs.

Antibodies and immunostaining. PCR amplification of the *Tsr* coding region was used to introduce an *Nde*I site at the beginning of the coding region and a *Bcl*I site in the 3′ untranslated region. The amplified fragment was cloned into the pET15b expression vector (Novagen), expressed in *Escherichia coli*, and purified by standard procedures as described previously (46). After elution from a His-Bind metal chelation resin column (Novagen) and dialysis into RNA binding buffer, purified TSR was partially insoluble. The protein was resolubilized in sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) loading buffer and separated on preparative gels. Crushed gel slices containing TSR protein were injected into rabbits for the production of polyclonal antibodies. Purified TSR was also dialyzed into a buffer consisting of PBS and 1 M urea and coupled to Affigel-10 beads as described by the manufacturer (Bio-Rad). Anti-TSR antibodies were affinity purified from the crude antiserum by using this column and used for Western blotting of testis extracts as described previously (46). Immunostaining of testes was done according to the protocol of Bopp et al. (5), with modifications suggested by Salz (40a; described in reference 46), using a 1:500 dilution of affinity-purified antibody and horseradish peroxidase-conjugated secondary antibody.

Two-dimensional gel electrophoresis. Two-dimensional gel electrophoresis was performed by isoelectric focusing in the first dimension and SDS-PAGE in the second. For the first dimension, Immobiline Drystrips were used as described by the manufacturer (Pharmacia). Briefly, 10 testes from males of the genotypes given in the text were homogenized in isoelectric focusing buffer, and focusing was performed overnight. The samples were then equilibrated in SDS-PAGE buffer, followed by overnight electrophoresis through SDS–10% polyacrylamide gels. The gels were fixed and stained by using the Bio-Rad enhanced silver stain kit and then dried. Differences in the patterns of protein spots on two-dimensional gels can arise from several causes. There is an inherent variability in the running and staining of two-dimensional gels, even though all samples are handled as identically as possible. In addition, the different fly stocks used were not isogenic with respect to other genes. This can lead to variability due to protein polymorphisms between strains. To minimize these factors, the dried gels were analyzed by performing a careful spot-by-spot comparison of the different genotypes, superimposing the dried gels on a light box to detect faint spots in gels with weaker staining. We used conservative criteria to identify spots affected by TSR: (i) the presence, absence, or intensity of the spot correlated with the *Tsr* genotype; (ii) based on comparison with nearby spots, the spot should have stained strongly enough for detection in all gels analyzed; and (iii) the effect of TSR on the presence, absence, or intensity of the spot was reproducible in separate experiments done with independently prepared protein samples. The second criterion was particularly important, as there were differences in overall staining intensity within and between gels.

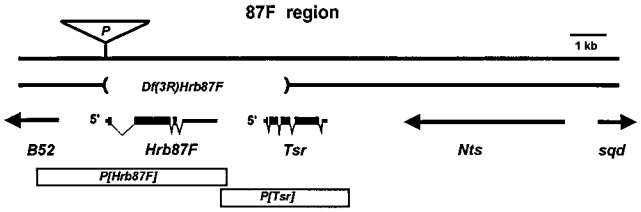

FIG. 1. Map of the 87F region. The locations of genes and their transcriptional orientations are indicated. The arrows for the *sqd* and *B52* genes indicate the direction of transcription but do not reflect the sizes of the transcription units. Thick lines in the *Hrb87F* and *Tsr* transcripts represent coding regions, and thin lines represent untranslated regions. The locations of *Df(3R)Hrb87F* and the original P-element mutation are shown relative to the transcription units; the P element is not drawn to scale. The genomic fragments used for transformation experiments are shown as boxes. The map is based in part on the data of Kelley (24).

Electron microscopy. Dissected testes were fixed in 2.5% glutaraldehyde in phosphate buffer and then sectioned and stained by standard methods (12).

Nucleotide sequence accession number. The *Tsr* sequence has been deposited in the sequence databases under accession number U18401.

RESULTS

Isolation and phenotypic analysis of a *Tsr* mutation. The *Tsr* gene was identified during studies of the *Hrb87F* gene, which encodes a *Drosophila* pre-mRNA binding protein related to vertebrate hnRNP A1 (19, 56). *Df(3R)Hrb87F* is a small deletion that removes *Hrb87F* and some adjacent DNA. Homozygous *Df(3R)Hrb87F* flies of both sexes were viable and the females were fertile, but male fertility was greatly reduced. Homozygous males produced only a few percent of the progeny produced by heterozygous or wild-type males, and most were sterile (Table 1). Since a transgenic copy of *Hrb87F* (*P[Hrb87F]*) did not restore fertility to *Df(3R)Hrb87F* males, we looked for other genes that might be affected by the deletion. Northern blots revealed a transcript hybridizing to DNA immediately 3′ of *Hrb87F*, and we isolated a cDNA clone from this region. Figure 1 shows the location of this gene, called *Tsr*, relative to other genes in the region and to *Df(3R)Hrb87F. Tsr* is separated from *Hrb87F* by ~1.2 kb, and *Df(3R)Hrb87F* deleted approximately 0.6 kb of the *Tsr* transcription unit. A *Tsr* transgene, *P[Tsr]*, increased the fertility of *Df(3R)Hrb87F* males to near wild-type levels (Table 1). In fact, having *P[Hrb87F]* in addition did not further increase fertility. (The slight reduction in male fertility in the flies with both transgenes compared to *P[Tsr]* alone may be due to differences in genetic background and has not been investigated further.) The rescue experiments with *P[Tsr]* demonstrate that the poor

TABLE 1. Male fertility

Male genotype[a]	Progeny/male
Oregon R (wild type)	464
Df(3R)Hrb87F/TM3	442
Df(3R)Hrb87F	5
P[Hrb87F]/+;Df(3R)Hrb87F	5
P[Tsr]/+;Df(3R)Hrb87F	349
P[Tsr]/P[Hrb87F];Df(3R)Hrb87F	281

[a] *TM3* is a third-chromosome balancer; + refers to a wild-type second chromosome.

fertility of *Df(3R)Hrb87F* males was due largely, if not completely, to the disruption of *Tsr*. For this reason, *Df(3R)Hrb87F* mutants will hereafter be referred to as Tsr^- for simplicity.

The testes of adult flies normally contain all stages of spermatogenesis, from stem cells to mature sperm, which are easily distinguishable in the light microscope (for reviews of spermatogenesis, see references 15 and 30). The testes of Tsr^- males contained both premeiotic spermatocytes and postmeiotic spermatids, including cysts of elongated spermatids. However, spermatid nuclei were frequently found in aberrant locations within the cysts (18), as has been observed in other male sterile mutants (10). Most spermatids were unable to complete morphogenesis, for the base of the testis was filled with degenerating spermatids. Occasionally, nonmotile sperm were found in the seminal vesicles. Thus, the mutation in *Tsr* appears to disrupt spermatid maturation. However, since some Tsr^- males produced some progeny, a few spermatids must escape this developmental block and complete morphogenesis.

Elongating spermatids from Tsr^- males are highly abnormal at the subcellular level. Figure 2 shows electron micrographs of sections through elongating spermatids from wild-type and Tsr^- males. Normally, spermatids develop in cysts of 64 interconnected cells. Prominent structures in the spermatid tail include an axoneme and two mitochondrial derivatives, one of which accumulates a dark paracrystalline material (Fig. 2A). At the end of spermatid maturation, the cysts form compact bundles of mature, individualized sperm. Such bundles were never observed in Tsr^- males. Instead, the cysts frequently contained fewer spermatids than expected, and most of the spermatids had aberrant mitochondrial derivatives (Fig. 2B). Three or more mitochondrial derivatives were often associated with a single axoneme or developed in the absence of a nearby axoneme. Two or more mitochondrial derivatives in a single spermatid accumulated paracrystalline material, even when only two were present. These defects are severe enough to account for the failure of final maturation of most spermatids.

***Tsr* encodes a testis-specific RRM protein.** To begin our analysis of the role of *Tsr* in spermatogenesis, we sequenced cDNA and genomic clones containing the *Tsr* gene (Fig. 3). The transcription unit consisted of five exons separated by four small introns ranging in size from 61 to 104 nucleotides (nt). Since the size of the cDNA clone (~1.5 kb) was about the size of the transcript detected on Northern blots [assuming a 100- to 200-nt poly(A) tail; see below], it is likely to be nearly full length. A single large open reading frame encoded a protein of 428 amino acids (M_r, 46.6×10^3) with a calculated pI of ~10.

The TSR protein can be divided into three parts: a short N-terminal region, a portion containing two RRM-type RNA binding domains, and the C-terminal half. Database searches revealed that part of the first RRM had previously been cloned in a PCR-based screen by Kim and Baker (26); this is their clone RRM4. Based on the sequence of the RRMs and the overall organization of domains within the protein, TSR is most closely related to the A/B group of hnRNP proteins, although it is clearly not an hnRNP protein (see below). The N-terminal half of TSR has two RRMs that show the greatest overall sequence homology (48 to 54%) to those of the RB97D protein (22), a *Drosophila* RRM protein also required for male fertility. The two RRMs in TSR are separated by a 30-amino-acid spacer containing a short glycine-rich region, which may provide a flexible linker between them. This spacer is longer than that typically found between the RRMs of the A/B hnRNP proteins (~17 amino acids). This spacing could have a significant effect on TSR's nucleic acid binding affinity and whether the RRMs bind the same or different RNA molecules, as has been shown for other RRM proteins (43).

Many RRM proteins have auxiliary domains rich in one or a few amino acids (e.g., the glycine-rich C-terminal domains of the HRB hnRNP proteins). In contrast, the C-terminal portion of TSR is distinguished by its more average amino acid composition. Although it has short stretches that are somewhat rich in glycine or proline, there was no extensive homology with auxiliary domains of other RRM proteins. Furthermore, sequence database searches found only patchy homologies of

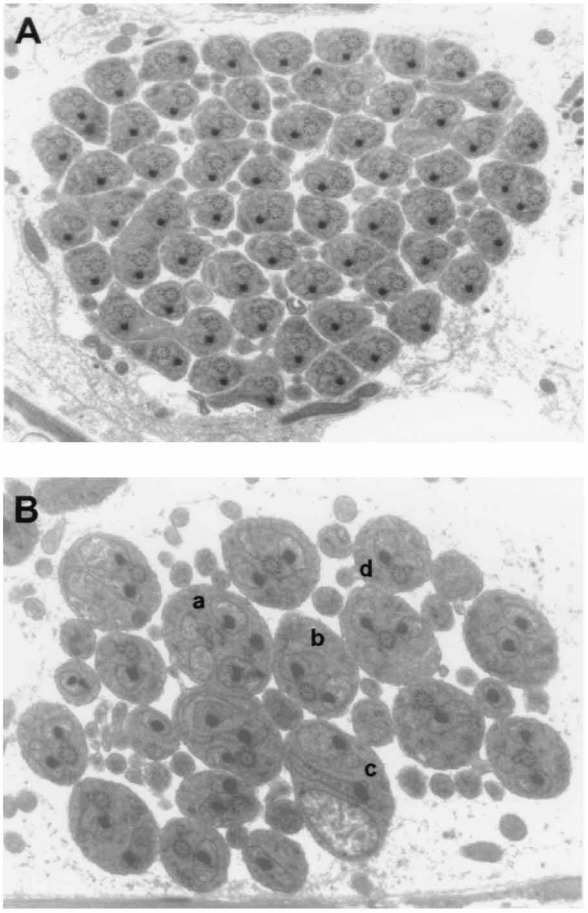

FIG. 2. Electron micrographs of testis sections. The panels show cysts of developing spermatids of testes from wild-type (A) and Tsr^- (B) males. Most spermatid tails in panel A show regularly organized axonemes and mitochondrial derivatives. In contrast, the spermatid tails in panel B show a range of abnormalities, including five mitochondrial derivatives and one axoneme (a), two mitochondrial derivatives accumulating paracrystalline material (b), and four mitochondrial derivatives and no axoneme (c). Note that there are a few apparently normal spermatid tails (d). The phenotype of the Tsr^- males was not influenced by lack of *Hrb87F*, since the males carried a *P[Hrb87F]* transgene.

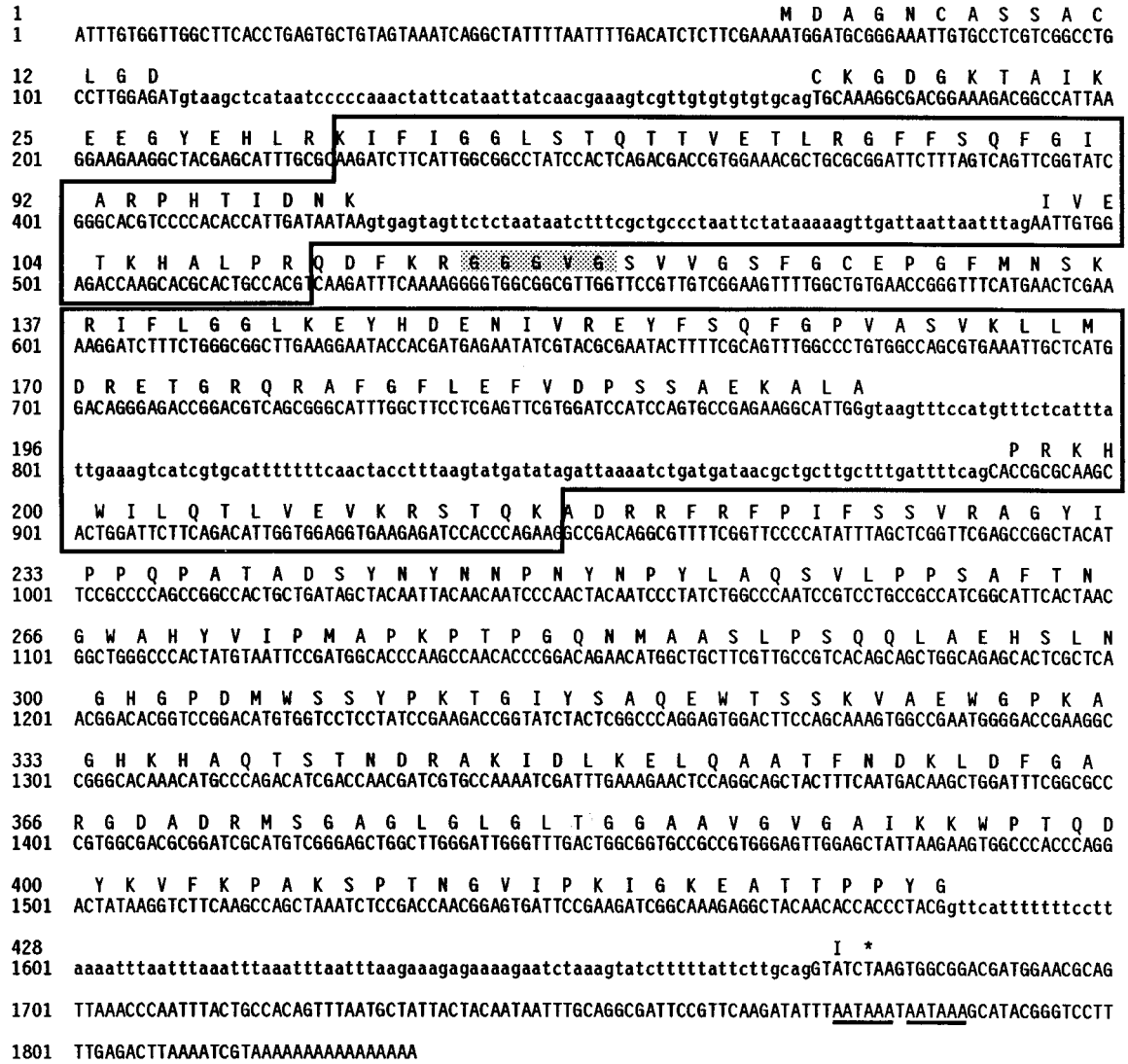

FIG. 3. Sequence of *Tsr*. The nucleotide sequence of *Tsr* is shown with the conceptual amino acid translation above it. Uppercase letters represent exons, and lowercase letters represent introns. Nucleotide 1 is the first nucleotide of the cDNA; the transcription start site has not been mapped. Two potential polyadenylation signals are underlined. The two RRMs are boxed, and the glycine-rich portion of the spacer between the RRMs is shaded. The RRM4 clone of Kim and Baker (26) is identical to a portion of the first RRM of TSR.

limited significance. Interestingly, the short N-terminal region (amino acids 1 to 32) contains three of the four Cys residues in the protein, with a His residue near the end of the region. This could indicate a potential metal binding domain, as the spacing of the residues resembles that seen in some class II metallothioneins (23).

The *Tsr*$^-$ mutation deleted sequences through nt 597 of *Tsr*, removing the first RRM and the linker. Although some *Tsr* coding sequences remain, it is unlikely that a truncated protein is expressed in testes. The deletion fused part of the *E. coli bla* gene contained in the P element to the remainder of the *Tsr* gene. Even if the fusion were transcribed from promoter sequences 5′ of *Hrb87F*, which were not deleted, there are stop codons in all three reading frames upstream of the *Tsr* coding sequences. In addition, Western blot experiments (see below) showed no evidence for a novel protein species in testes from *Tsr*$^-$ males.

To examine the transcription pattern of *Tsr*, a blot containing poly(A)$^+$ RNA from different developmental stages was hybridized with a *Tsr* probe (Fig. 4A). A single ~1.7-kb transcript was detected in third-instar larvae, pupae, and adult males but not in adult females. The pattern of expression in adults suggested that *Tsr* might be a sex-specific transcript. To test this, we used RT-PCR to examine *Tsr* expression in male and female third-instar larvae, pupae, and adults (Fig. 4B). As a control for the integrity of the RNA, *Hrb87F* transcripts were amplified from the same samples. The primers for each gene spanned an intron, so that amplification of contaminating genomic DNA would produce a larger band. The *Tsr* amplification product was present only in the lanes containing RNA from male animals, whereas the control *Hrb87F* band was amplified from all samples. We used a similar approach to examine whether *Tsr* transcription was dependent on the presence of germ cells. Females homozygous for the *tud*^{1} mutation produce offspring that lack germ cells (6). RNA was prepared from adult males derived from either heterozygous (*tud*^{1}/+) or

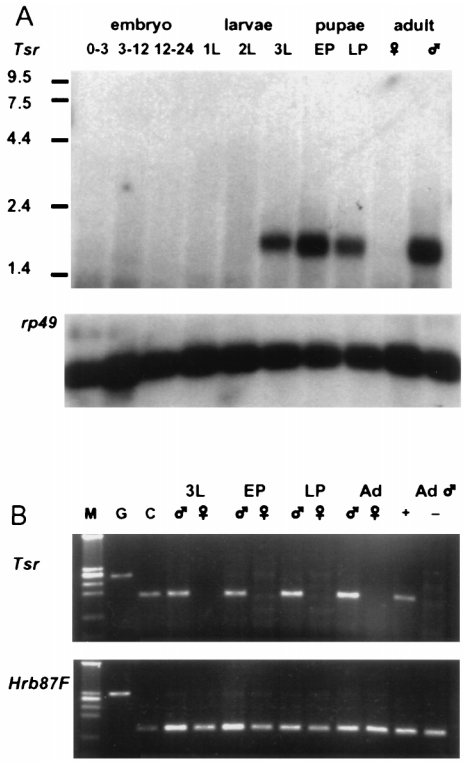

FIG. 4. Expression of *Tsr* transcripts. (A) A Northern blot containing 1 μg of poly(A)$^+$ RNA from embryos (0 to 3, 3 to 12, and 12 to 24 h), larvae (first, second, and third instar [1L, 2L, and 3L]), early (EP) and late (LP) pupae, and adult males (♂) and females (♀) was hybridized with a probe from the *Tsr* cDNA clone and a probe for the ribosomal protein gene *Rp49* [also known as *M(3)99D*], which serves as a loading control (34). The bottom section shows a short exposure of the blot in the region of the *Rp49* transcripts, which has been cut off the longer exposure shown in the top section. The locations of RNA molecular weight marker bands are shown in kilobases on the left. (B) RT reactions were done with 1 μg of total RNA from male and female third-instar larvae, early and late pupae, adults (Ad), and adult male progeny of *tud*^{1}/+ (+) and *tud*^{1} (−) mothers. The upper section shows the PCR products obtained with *Tsr*-specific primers, and the lower section shows products obtained with control *Hrb87F*-specific primers. Lanes: M, ϕX174 *Hae*III digest; G, PCRs with a genomic DNA clone as the template (*Tsr*, 262 nt; *Hrb87F*, 303 nt); C, PCRs with a cDNA clone as the template (*Tsr*, 175 nt; *Hrb87F*, 139 nt); remaining lanes, products of the RT-PCRs. The prominent bands are the same sizes as the bands from the cDNA template controls, indicating that they are not due to amplification of contaminating genomic DNA.

homozygous (*tud*^{1}) mothers. The RT-PCR experiments (Fig. 4B) showed that the *Tsr*-specific band was detectable only in progeny of heterozygous mothers (lane +) and not in progeny of homozygous mothers (lane −). Again, the *Hrb87F*-specific band was present in both samples. Therefore, *Tsr* expression in adult males depends on the presence of germ cells and is likely to be testis specific.

TSR is a cytoplasmic RNA binding protein. Sequences encoding TSR were cloned into an *E. coli* expression system, and the protein was overexpressed and purified. The purified protein was used to generate polyclonal antibodies and to test for RNA binding activity. A UV cross-linking assay (45) demonstrated that TSR, but not a bovine serum albumin control, could be cross-linked in vitro to two different labeled RNAs (47). The binding presumably reflects general, non-sequence-specific RNA binding, since TSR bound similar amounts of both samples. In addition, TSR bound to ribonucleotide homopolymers and single-stranded DNA cellulose (38) with affinities similar to those of other RRM proteins (48). We conclude that TSR, like many RRM proteins, binds single-stranded nucleic acids.

Testis proteins were separated on SDS-polyacrylamide gels, blotted, and probed with affinity-purified anti-TSR antibodies (Fig. 5A). The antibodies detected a protein in wild-type testes (lane wt) that is about the same size as recombinant TSR (lane rec). This protein was absent from testes of *Tsr*$^-$ males (lane *Df*), but was restored in transgenic males (lane *[Tsr*$^+$*];Df*). There were other faint bands that reacted with the antibody; these are unlikely to be aberrant forms of TSR since they were present in all genotypes tested. They may correspond to other related RRM proteins that cross-react weakly with the antibody.

Testes from wild-type (Fig. 5B and C) and *Tsr*$^-$ (Fig. 5D) males were stained with anti-TSR antibodies to determine the subcellular localization and expression pattern of TSR. TSR was absent from the germ line stem cells at the apical tip of the testis (Fig. B) and was first detectable as faint staining in young primary spermatocytes. Protein continued to accumulate as the primary spermatocytes developed, and the highest levels were seen in cells immediately following meiosis. TSR persisted until later stages of spermatid elongation, but little or no protein was detectable in fully elongated spermatids (Fig. 5B). This finding suggests that TSR is no longer required by late stages of spermatogenesis. Figure 5C shows a higher-magnification view of cysts of mature primary spermatocytes (right) and early postmeiotic spermatids (left). When the stained testes were viewed under the microscope, it was clear that TSR was located mostly, if not exclusively, in the cytoplasm. Three of the spermatocytes in the cyst on the right are in the appropriate focal plane to illustrate this, as are several of the spermatids in the cyst on the left. Note that the spermatocyte nuclei occupy much of the cell volume, so that the staining appeared as a narrow ring at the cell periphery. The darker line over the nuclei is from spermatid tails that are out of the plane of focus. The arrowhead points to elongating spermatid tails that still expressed TSR. We also examined TSR expression from the transgene. Consistent with the ability of *P[Tsr]* to rescue the fertility defect of *Tsr*$^-$ males, TSR expression from the transgene was very similar to that from the endogenous gene (18).

Mutation in TSR affects the expression of other testis proteins. The identification of TSR as a cytoplasmic RRM protein suggested that it might affect mRNA localization, polyadenylation, stability, or translatability during spermatogenesis. To begin to assess these possibilities, we examined whether TSR affected the expression of proteins in primary spermatocytes. Since the small size of *Drosophila* testes made it impractical to attempt a biochemical purification of different cell types, we used a genetic approach. Testes from homozygous *bol*^{1} males contain spermatocytes that fail to divide meiotically and show only very limited postmeiotic differentiation (14). We used two-dimensional PAGE to compare the proteins present in testes from males of four genotypes: wild type, *bol*^{1} mutants, *bol*^{1} *Tsr*$^-$ mutants, and *bol*^{1} *Tsr*$^-$ mutants carrying a *Tsr*$^+$ transgene. Only young males (within a day of eclosion) were used, to minimize the number of degenerating cells present in the testis. Overall, the testis protein profiles from the strains containing the *bol*^{1} mutation were quite similar; the majority of the observed differences could be attributed to differences in staining intensity of the gels or to protein polymorphisms unrelated to the *Tsr* genotype. (See Materials and Methods for a discussion of how the gels were analyzed and sources of variability in these experiments.) However, we reproducibly found a few proteins that appeared to be negatively regulated by TSR. Sections of the gels showing one of these proteins are presented in Fig. 6. This protein was weakly expressed in *bol*^{1}

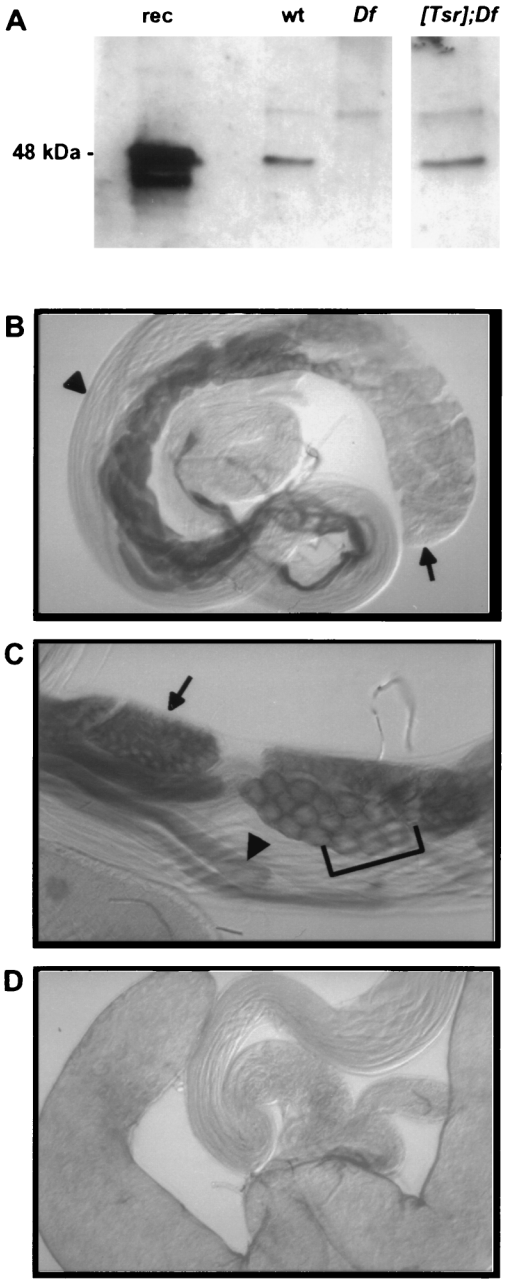

FIG. 5. Expression of TSR protein. (A) A Western blot of testis extracts was probed with anti-TSR antibodies. Lane rec contains 0.1 μg of purified recombinant TSR. The remaining lanes contain 5 μg of testis protein extracts from males of the indicated genotypes: wt, wild type; *Df*, Tsr^-; *[Tsr];Df*, *P[Tsr];*Tsr^-. All lanes were taken from the same film and thus represent the same reaction conditions and exposure time. (B to D) Immunohistochemical staining of testes with anti-TSR antibodies. (B and C) Wild type; (D) Tsr^- (only background staining is seen). The arrow in panel B points to the stem cells at the apical tip of the testis, which do not stain with the antibody, and the arrowhead indicates fully elongated spermatids which no longer express TSR. Panel C is a high-magnification view of cysts of primary spermatocytes and spermatids. The bracket encloses three spermatocytes with nuclei that are in the plane of focus of the photograph. The arrow indicates a cyst of spermatids shortly after meiosis, and the arrowhead points to the end of a cyst of elongating spermatids that still express TSR. Note that other nearby cysts do not.

primary spermatocytes but was strongly induced in the absence of TSR (bol^1 Tsr^- panel). The extent of induction of this protein is evident when the intensity of its staining is compared to that of nearby proteins in the two panels. Importantly, if the Tsr^- mutant was complemented with the Tsr^+ transgene ($P[Tsr^+]/+;bol^1$ Tsr^- panel), expression of this protein was repressed, while the majority of the other proteins were unchanged (compare to the bol^1 Tsr^- panel). This finding demonstrates that the aberrant protein expression was due to the lack of TSR and not to some other mutation(s) present in this genetic background. Additionally, this protein was present in wild-type testes, which contain cells in all stages of spermatogenesis (WT panel), indicating that it is not an aberrant protein produced only in bol^1 testes. These data strongly implicate TSR as having a role in the expression of at least one protein during spermatogenesis.

DISCUSSION

We have identified and characterized the *Tsr* gene, which encodes a tissue-specific RRM protein required for normal male fertility in *Drosophila*. Most Tsr^- males were sterile, although a few produced some progeny. Spermatids in mutant males failed to complete the final stages of morphogenesis, and examination by electron microscopy revealed that their mitochondria developed abnormally. Northern blot and RT-PCR analyses indicated that *Tsr* was transcribed solely in the male germ line. Although its RRMs are very similar to those found in some nuclear pre-mRNA binding proteins, TSR was found in the cytoplasm of primary spermatocytes and developing spermatids. Spermatocytes from Tsr^- males expressed several testis proteins prematurely, suggesting that TSR negatively regulated the expression of these proteins.

The RRM motifs of TSR suggest that it is an RNA binding protein, and our data indicate that it can bind RNA and single-stranded DNA, although we do not know if it has a preferred target sequence(s). Given its cytoplasmic localization, it could bind mRNA and potentially affect its polyadenylation, translatability, stability, or localization. A role for TSR in mRNA localization is unlikely, however, as TSR appeared to be distributed uniformly in spermatocytes and spermatids. The data shown in Fig. 6, comparing testis protein profiles of bol^1 Tsr^- and bol^1 Tsr^+ males, provide some clues regarding how and when TSR might act. Since TSR represses the expression of a few proteins in primary spermatocytes, it must have some premeiotic function. This eliminates strictly postmeiotic roles, such as the degradation of spermatocyte mRNA after meiosis. Also, TSR's negative effect on protein expression rules out a role in the activation of translationally repressed mRNAs. Rather, TSR is more likely to act to destabilize specific mRNAs or repress their translation. Although we cannot rule out either possibility, we favor the view that TSR is involved in translational repression. The proteins affected by TSR are present in low levels in bol^1 spermatocytes. Assuming that this accurately reflects the normal spermatocyte protein levels, if TSR acts to destabilize mRNAs, protein levels should be low in whole testes, since transcription is shut down after meiosis. Instead, Fig. 6 shows that one of the TSR-regulated proteins was relatively abundant in whole testes. While more complex scenarios are possible, the simplest model that accounts for our results is that TSR is required for the translational repression of a limited number of mRNAs prior to meiosis. These mRNAs might remain translationally inactive for part of spermatid development as well, since TSR is present until mid-elongation stages. The apparent turnover of TSR protein at this time could be required to release the mRNA for translation. Alternatively, TSR might undergo a posttranslational modification that results in release of the mRNA, and protein turnover is a subsequent event.

Translational regulation is known to be an important regu-

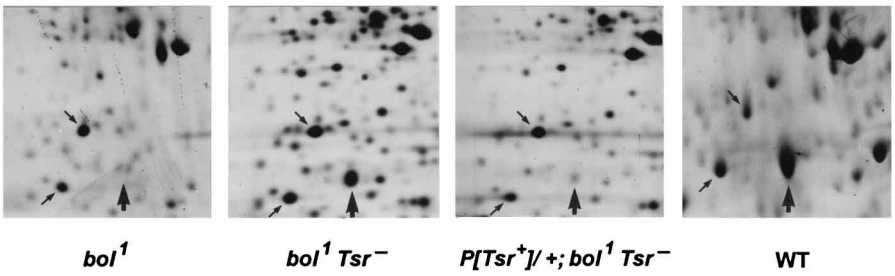

FIG. 6. Effects of TSR expression on testis proteins. Testis extracts from males of the indicated genotypes were separated by two-dimensional gel electrophoresis. The panels show portions of the gels containing a protein (large arrow) whose expression is affected by lack of TSR; small arrows indicate nearby proteins that are expressed similarly in the three mutants. This protein has an M_r of 26.5×10^3 and a pI of ~6.5. Testes were dissected from males within about 1 day of eclosion to minimize protein degradation due to degenerating spermatocytes, and care was taken to ensure that all samples contained only testes and seminal vesicles. Note that the panel with proteins from wild-type males is the least similar to the others; this is not unexpected, as these testes contained cells in all stages of spermatogenesis.

latory mechanism in spermatogenesis, in both *Drosophila* and vertebrates (42). Proteins implicated in these processes have been identified in mouse germ cells. Members of the Y-box family of nucleic acid binding proteins can be isolated from nonpolysomal fractions of mouse testis extracts, which contain stored untranslated mRNAs, and can be shown to bind mRNA in vitro and in vivo (28, 49). These proteins (MSY1 and the 48/52-kDa proteins) are related to the *Xenopus* Y-box protein FRGY2, which acts as a translational repressor in *Xenopus* oocytes (50). Recently, another mouse testis protein (Prbp) containing double-stranded RNA binding motifs has been implicated in translational repression during spermatogenesis, as it can repress translation of mRNAs in vitro (29). These mouse proteins are thought to have little or no target sequence specificity, and they may act as general translational regulators. In contrast, our results indicate that TSR is unlikely to be a global regulator of mRNA translation. We do not yet know the identities of the proteins whose expression is affected by TSR. *cis*-regulatory sequences important for translational regulation have been identified for only a few *Drosophila* genes expressed in the testis, e.g., the *Mst87F*, *janB*, and *dhod* genes (41, 54, 55). It is unlikely that TSR plays an important role in their regulation, since the translation of reporter constructs containing these regulatory sequences is similar in wild-type and Tsr^- males (38, 42). However, other genes may employ different translational control sequences, for which TSR might be required. Experiments are in progress to isolate TSR-containing RNP complexes and identify the RNA targets of TSR.

Tsr is a member of a small group of *Drosophila* RRM protein genes that have important roles in spermatogenesis. One member of the group, *tra-2*, plays a crucial role in additional developmental processes, while *Tsr*, *bol*, and *Rb97D* are required only during spermatogenesis (1, 14, 22). The importance of RRM proteins to male fertility is not unique to *Drosophila*. The disruption of regions of the human Y chromosome that include genes encoding testis-specific RRM proteins is associated with infertility, although which genes correspond to *Azoospermia Factor* remains controversial (9). The YRRM/RBM proteins (32) and DAZ (a *bol* homolog [14, 39]) each have a single N-terminal RRM and a C-terminal domain unlike that of TSR. Similar to TSR, the human DAZ and the mouse YRRM/RBM proteins may not be absolutely required for spermatogenesis. DAZ/*bol* has a different function from that postulated for *Tsr*, but the role of YRRM/RBM is unknown. It is clear, however, that continued study of testis-specific RNA binding proteins in a variety of organisms should lead to further insights into the importance and mechanisms of posttranscriptional regulation in spermatogenesis.

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