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Mutations in the *hrp48* Gene, Which Encodes a *Drosophila* Heterogeneous Nuclear Ribonucleoprotein Particle Protein, Cause Lethality and Developmental Defects and Affect P-Element Third-Intron Splicing In Vivo

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The *Drosophila melanogaster* hnRNP protein, hrp48, is an abundant heterogeneous nuclear RNA-associated protein. Previous biochemical studies have implicated hrp48 as a component of a ribonucleoprotein complex involved in the regulation of the tissue-specific alternative splicing of the P-element third intron (IVS3). We have taken a genetic approach to analyzing the role of hrp48. Mutations in the *hrp48* gene were identified and characterized. *hrp48* is an essential gene. Hypomorphic mutations which reduce the level of hrp48 protein display developmental defects, including reduced numbers of ommatidia in the eye and morphological bristle abnormalities. Using a P-element third-intron reporter transgene, we found that reduced levels of hrp48 partially relieve IVS3 splicing inhibition in somatic cells. This is the first direct evidence that hrp48 plays a functional role in IVS3 splicing inhibition.

The hnRNP (heterogeneous nuclear ribonucleoprotein particle) proteins are abundant eukaryotic nuclear RNA-binding proteins that bind nascent transcripts (heterogeneous nuclear RNAs or pre-mRNAs) as they emerge from the transcriptional machinery. hnRNP complexes from vertebrates, where there are over 20 abundant, highly conserved, nuclear hnRNP proteins, designated A to U (10), have been extensively studied. These proteins are important for the packaging, processing, and intracellular transport of cellular mRNAs (5). hnRNP proteins are also found in invertebrates, such as *Drosophila melanogaster*. In *Drosophila*, several hnRNP proteins have been isolated by immunopurification and characterized by molecular cloning (20, 21). There are at least 10 major hnRNP proteins in *Drosophila*, the most abundant being hrp36, hrp40, and hrp48. These proteins have been shown to associate with the majority of nascent transcripts (19, 21).

All the abundant *Drosophila* heterogeneous nuclear RNA-binding proteins characterized so far are similar in structure to the vertebrate A and B hnRNP proteins, containing two N-terminal RBD-type RNA-binding domains and a glycine-rich carboxyl-terminal domain (21). The RBD (or RNP-CS) type is a distinct class of RNA recognition motif (5). The RBD domains are necessary for RNA binding, and the glycine-rich region is thought to mediate RNA-binding, RNA-RNA-annealing, or protein-protein interactions (10). Proteins with these motifs are referred to as 2× RBD-Gly proteins (5). Alignment of RNA-binding domains from evolutionarily diverse 2× RBD-Gly proteins reveals a high degree of conservation of these domains among members of this protein family and establishes a specific consensus sequence for 2× RBD-Gly proteins (5).

Early evidence for a role for hnRNP proteins in pre-mRNA splicing came from antibody inhibition experiments implicating the mammalian hnRNP C protein as a splicing factor (9). More recent evidence for the role of an hnRNP protein in pre-mRNA splicing was obtained through the purification of an activity that influences alternative 5′ splice site selection in vitro and its identification as hnRNP A1 (22). By using a pre-mRNA with duplicated 5′ splice sites as a substrate for in vitro splicing assays, hnRNP A1 has been shown to promote splicing to the upstream (distal) 5′ splice site (22). hnRNP A1 has also been shown to promote RNA-RNA annealing (reviewed in reference 27). This activity is required for splice site switching (23) and may function to facilitate U1 small nuclear RNA annealing to the 5′ splice site. In vivo evidence for a role in splice site selection for hnRNP proteins comes from overexpression studies. Transient overexpression of hnRNP A1 in mammalian tissue culture cells caused 5′ splice site shifting in the adenovirus E1A transcript (7). An increase in the selection of the proximal 5′ splice site of the adenovirus E1A transcript was observed in an hnRNP A1 mutant cell line (39). Transient expression of hnRNP A1 in this mutant cell line promoted a shift toward distal 5′ splice site selection. Overexpression of *Drosophila* 2× RBD-Gly general hnRNP proteins, Hrb87F/hrp36 or Hrb97DE/hrp38, transiently affected the splice site selection of the dopa decarboxylase (Ddc) pre-mRNA in *Drosophila* larvae (32, 40). These overexpression studies provide the first glimpses of the importance of hnRNP proteins in RNA processing in vivo.

Subsequent to its identification as a *Drosophila* hnRNA-binding protein (20, 21), the abundant general hnRNP protein, hrp48, was implicated in the tissue-specific regulation of P-element third-intron splicing (35). In *Drosophila*, the tissue specificity of P-element transposition is regulated at the level of pre-mRNA splicing (15). In germ line cells, all three introns of the P-element transcript are accurately spliced and the resulting mRNA encodes transposase. In somatic cells, the third intron (IVS3) is retained, and as a result a truncated protein that represses transposition is expressed (25). Genetic and biochemical studies have revealed that regulation of this tissue-specific splicing event involves inhibition of IVS3 removal in

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TABLE 1. Quantitation of RT-PCR products from wild-type and *hrp48* mutant flies

Fly	IVS3[a]		Cryptic splice[a]		Total RNA[a]	Accurately spliced IVS3/total RNA	All spliced products/total RNA
	Unspliced (346 bp)	Spliced (156 bp)	1 (277 bp)	2 (186 bp)			
Control (+/+)	1,123	29	28	20	1,200	0.024	0.064
Experimental (*hrp48*^{4}/*hrp48*^{4})	1,016	52	74	78	1,220	0.043	0.1672

[a] Data are given in relative units, as determined with a phosphoimager.

the soma (8, 16, 36). Somatic inhibition requires a discrete regulatory sequence in the 5′ exon RNA adjacent to the IVS3 5′ splice site (8, 34).

A multiprotein complex has been identified from *Drosophila* somatic cell nuclear splicing extracts that binds to the exonic regulatory element. U1 snRNP was also found to bind to one of two pseudo-5′ splice sites (termed F1) in this regulatory element (34). Biochemical and genetic experiments have provided strong evidence that the P-element somatic-inhibitor (PSI) protein, one of the four proteins in this complex, plays a direct role in IVS3 splicing inhibition. Antibodies directed against PSI relieve IVS3 splicing inhibition in *Drosophila* somatic splicing extracts (33). Reduction of PSI expression in somatic embryonic cells, using a ribozyme targeted against PSI mRNA, activates splicing of an IVS3 reporter transgene (1). PSI has recently been identified as the tissue-specific regulator of IVS3 splicing. PSI levels are high in the soma, where IVS3 splicing is inhibited, and low in the germ line, where IVS3 is accurately spliced (33). Ectopic expression of PSI in the female germ line is sufficient to repress splicing of an IVS3 reporter transgene (1). Another protein identified in the multiprotein complex is the general hnRNP protein, hrp48. hrp48 binds the second pseudo-5′ splice site (termed F2) within the exon regulatory element (34, 35). Mutations in the F2 pseudo-5′ splice site relieve IVS3 splicing inhibition in somatic cells in vivo (8) and in vitro (34). These experiments strongly implicate hrp48 in IVS3 splicing regulation.

Here, we examine the phenotypes of mutations affecting the *Drosophila hrp48* gene. *hrp48* is an essential gene. Hypomorphic, recessive, P-element-induced *hrp48* mutations with reduced levels of hrp48 protein exhibit larval lethality and developmental defects, including reduced numbers of ommatidia in the eye and bristle abnormalities. These mutant phenotypes can be rescued by P-element-mediated germ line transformation with an *hrp48* cDNA controlled by the actin 5C or *hsp70* promoters. Finally, using a P-element third-intron (IVS3) reporter transgene, we show that reduced expression of hrp48 partially relieves third-intron splicing inhibition in somatic tissues. This activation is identical to that observed with *cis*-acting mutations in the IVS3 5′ exon negative regulatory element (8). These genetic data provide the first direct evidence that hrp48 plays a functional role in somatic inhibition of P-element IVS3 splicing.

MATERIALS AND METHODS

Mapping the P-element insertions relative to the *hrp48* gene. Genomic DNA was isolated from the heterozygous mutant fly lines l(2)k16203, l(2)k10413, l(2)10280, l(2)rF680, and l(2)2647 and a wild-type control, w^{1118} (Berkeley *Drosophila* Genome Project [BDGP]). The P elements were mapped relative to the *hrp48* coding sequence by PCR with primers complementary to the P-element inverted repeat (5′ GCTGCCCTGGTGGAATACAATAAAGTAGTAC 3′) and to the 5′ end of the *hrp48* coding sequence (5′ GTTTGCCCCTCTCGTCT TCCTCC 3′).

Immunoblots. Whole-fly extracts (one-half an adult fly equivalent) were separated on a 10% sodium dodecyl sulfate-polyacrylamide gel, electroblotted to nitrocellulose, and probed with affinity-purified anti-hrp48 rabbit polyclonal antibody (35). To control for loading, the blot was reprobed with affinity-purified anti-dU2AF^{50} rabbit polyclonal antibody. Antibody to recombinant hrp48 (36) was prepared in rabbits and affinity purified as described previously (12).

Construction of P-element transgenes. A 1.4-kb *Eco*RI-*Bam*HI fragment containing the *hrp48* cDNA was cloned into pCaSpeRhs (2) to generate P[w^+; *hsp70-hrp48* cDNA]. A 2.6-kb *Eco*RI-*Bam*HI fragment containing the actin 5C promoter, a 1.3-kb *Bgl*II-*Xba*I fragment containing the *hrp48* cDNA, and a 0.8-kb *Xba*I-*Not*I fragment containing the simian virus 40 3′ untranslated region were cloned into pw8 between *Eco*RI and *Not*I in a four-way ligation to create P[w^+; actin 5C-*hrp48* cDNA]. cDNA constructs and helper DNA (2) were microinjected into w^{1118} embryos as described previously (37, 38).

Complementation crosses. w^{1118}; l(2)k16203 or l(2)10280/Sm6β; P[w^+; *hrp48*-cDNA]/+ males were crossed to w^{1118}; l(2)k16203 or l(2)10280/Sm6β virgin females. Viabilities of homozygous mutant progeny with and without the *hrp48* cDNA transgene were compared to the viabilities of their heterozygous mutant siblings. Crosses with the *hsp70-hrp48* cDNA transgene were maintained at 25°C and heat shocked at 38°C for 30 min every 6 h. This heat shock regimen was maintained until eclosion. All other crosses were maintained at 25°C.

Isolation of *hrp48* genomic clones. Genomic clones were isolated from a *Drosophila* lambda Fix genomic library by using a random hexamer-primed *hrp48* cDNA fragment as a probe as described previously (4).

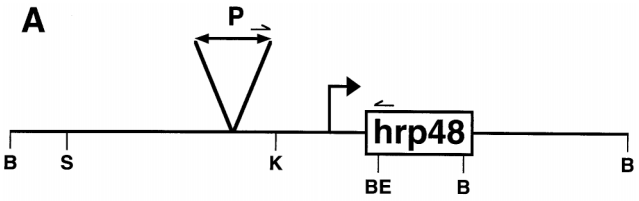
RNA isolation and reverse transcription-PCR (RT-PCR) analysis. Male flies from strains w^{1118}; l(2)k16203/l(2)k16203; P[w^+; *hsp70-IVS3-LacZ*]/+ and w^{1118}; P[w^+; *hsp70-IVS3-LacZ*] were heat shocked at 38°C for 1 h to induce expression of the IVS3 reporter. Following heat shock, the flies were immediately frozen in liquid nitrogen. Total RNA was isolated with guanidinium thiocyanate followed by a CsCl step gradient as previously described (4). DNase I-treated total RNA (2 μg) was reverse transcribed with 25 pmol of the downstream *lacZ* primer (29). Two percent of the reverse transcriptase reaction mixture was amplified in a standard PCR mixture containing both the downstream (3′ β-galactosidase) and upstream (5′ nuclear localization signal) primers, as previously described (29). The PCR mixtures included 2 μCi of [α-^{32}P]dCTP (10 mCi/ml; 3,000 Ci/mmol of stock). cDNA amplification products were analyzed on an 8% native polyacrylamide gel. Amplified products were quantitated with a Fuji BAS1500 phosphorimager, and values obtained after background subtraction for each time point were plotted semilogarithmically. Time points that fell within the linear portion of the curve were assumed to be undergoing exponential amplification and were used to calculate the ratio of spliced to total RNA within each sample lane. These data are provided in Table 1 (phosphorimager values from Fig. 5). Products were typically analyzed between cycles 18 and 22. Analysis of IVS3 splicing was performed six times on two independent RNA samples. The ratio of cDNA products was dramatically altered when amplification was no longer log-linear.

Analysis of PCR-amplified cDNA products. α-^{32}P-labeled cDNA products from amplification cycle 20 (as described above) were eluted out of a native 8% polyacrylamide gel and reamplified for 30 cycles. Gel-purified reamplification products were subjected to cycle sequencing with the 3′ β-galactosidase primer.

β-Galactosidase staining. Fly lines w^{1118}; +/+; P[w^+; *hsp70-hrp48*]/+ and w^{1118}; l(2)k16203/l(2)k16203; P[w^+; *hsp70-hrp48*]/+ were heat shocked at 38°C for 1 h. Flies were immediately dissected in fix solution (10 mM Na^+ phosphate [pH 7.2], 150 mM NaCl, 1 mM $MgCl_2$, and 1% glutaraldehyde) and then incubated in fix solution for 15 min. The fixed flies were preincubated in staining solution (10 mM Na^+ phosphate [pH 7], 150 mM NaCl, 3.1 mM K_4[FeII(CN)$_6$], 3.1 mM K_3[FeIII(CN)$_6$], 0.3% Triton X-100) for 5 to 10 min (3, 29). The flies were then incubated in staining solution containing 0.2% X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). Staining was terminated after 30 min to 4 h by washing the flies in 1× phosphate-buffered saline.

RESULTS

P-element insertions near the *hrp48* gene that reduce hrp48 protein expression cause lethality and developmental defects. The cytological location of the *hrp48* gene had been determined by in situ hybridization to polytene chromosomes to be at position 27C on the left arm of the second chromosome (21). Through a search of the BDGP database, five independent P-element insertion strains were identified that mapped to the same cytological location as *hrp48*. Three of these P-



ALLELE	STRAIN	DISTANCE
$hrp48^{1}$	l(2)10280	1.5 kb
$hrp48^{2}$	l(2)rF680	1.8 kb
$hrp48^{4}$	l(2)k16203	2.2 kb
$hrp48^{5}$	l(2)k10413	2.2 kb
$hrp48^{3}$	l(2)2647	3.3 kb

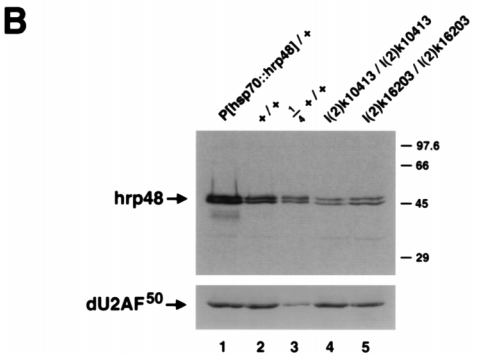


FIG. 1. Genomic *hrp48* locus and hrp48 protein expression levels in the homozygous P-element mutants. (A) Schematic diagram of the *hrp48* genomic locus. One of the P elements is indicated as well as the primers used to map the proximity of the insertion site to the *hrp48* coding sequence. *Bam*HI (B), *Sal*I (S), *Kpn*I (K), and *Bst*EII (Be) restriction enzyme sites are indicated. The distances of the P elements from the *hrp48* coding sequence in the various strains are indicated in the table. (B) hrp48 protein expression levels were determined by immunoblotting. Equal whole-fly equivalents were loaded in each lane except lane 3, which contained 25% of the amount of the wild-type extract (lane 2). Overexpression of hrp48 was observed in the heat-shocked flies containing the P[w^+; *hsp70-hrp48* cDNA] transgene (lane 1). A reduced hrp48 protein level was observed in the homozygous mutant escapers l(2)k16203/l(2)k16203 (lane 5) and l(2)k10413/l(2)k10413 (lane 4). hrp48 protein has previously been detected as a doublet band of ~48 kDa which arises from alternative splicing or posttranslational modifications (20, 21). The molecular masses of markers in kDa are indicated at the right. The *Drosophila* U2AF^{50} (dU2AF^{50}) expression level was used to control for variability in sample loading.

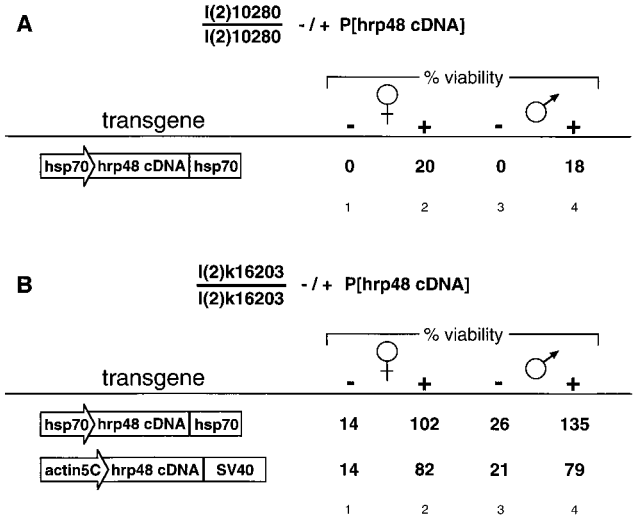


FIG. 2. *hrp48* cDNA transgenes can rescue the lethality associated with the P-element insertions. (A) The P[w^+; *hsp70-hrp48* cDNA] transgene can partially rescue the fully penetrant recessive lethality associated with the l(2)k10280 P-element insertion. (B) Both the P[w^+; *hsp70-hrp48* cDNA] transgene and the P[w^+; actin 5C-*hrp48* cDNA] transgene can rescue the reduced viability associated with the l(2)k16203 P-element insertion. Percent viability was determined by comparing the number of homozygous mutant flies containing or lacking the cDNA transgene to their respective heterozygous mutant siblings (see Materials and Methods). The failure of the P[w^+; actin 5C-*hrp48* cDNA] transgene to rescue the l(2)k10280 lethal insertion may reflect quantitative, temporal, or tissue-specific variations in expression of hrp48 from the transgene.

element insertion strains were identified as fully penetrant recessive lethal. Two were found to be semilethal; a low percentage of homozygous mutants survived when grown under optimal, noncrowding conditions. Complementation tests performed by the BDGP and by us (L.E.H., R.K., and D.C.R.) demonstrated that the five P-element insertion alleles did not complement and thus were affecting the same gene.

The proximity of these five lethal P-element insertions to the *hrp48* coding sequence was determined by PCR and DNA blot hybridization. Genomic DNA was isolated from the five P-element insertion strains and amplified by PCR with primers complementary to the P-element terminal inverted repeat and to the 5′ end of the *hrp48* coding sequence. The P-element insertions were found to be located at a distance of between 1.5 and 3.3 kb from the coding sequence of the *hrp48* gene (Fig. 1A). These results were confirmed by DNA blot hybridization (data not shown).

The effect of these P-element insertions on hrp48 protein expression levels was assessed by immunoblotting. The hrp48 protein level in homozygous, hypomorphic mutant survivors was compared to that in wild-type flies (Fig. 1B). There was a significant reduction (about fourfold) in hrp48 protein expression in the homozygous P-element insertion flies (Fig. 1B; compare lanes 4 and 5 to lane 3). We conclude that the P-element insertions are affecting expression of the *hrp48* gene.

All five P-element insertion strains were characterized genetically. Quantitative crosses were performed to assess the effect of these insertions on viability. Balanced, heterozygous mutant flies were raised under optimal, noncrowded conditions, and the resulting progeny were scored. We confirmed the original observations of the BDGP: the recessive lethal lines l(2)10280, l(2)rF680, and l(2)2647 were fully penetrant recessive lethal (Fig. 2A, columns 1 and 3, and data not shown). Homozygous mutant progeny were larval lethal (data not shown). The viability of homozygous hypomorphic mutant flies [l(2)k16203 and l(2)k10413] ranged from 10 to 30% (Fig. 2B, columns 1 and 3, and data not shown), and there was a 1.5- to 10-fold-higher viability of males than females (Fig. 2B, columns 1 and 3, and data not shown). In addition, the homozygous hypomorphic mutant flies were found to have several developmental abnormalities. A small-eye phenotype was observed in the majority of the homozygous females (Fig. 3). This small-eye phenotype was due to reduced numbers of ommatidia and varied in its severity (Fig. 3). A large bristle identified as the posterior supra alar macrochaeta was absent in a large percentage of both males and females (Fig. 4b). For $hrp48^{4}$ [l(2)k16203], 53% of the rescued females and 20% of the rescued males had restored the missing bristle. None of the $hrp48^{4}$ mutants lacking the transgene had the bristle. These data indicate that the P-element insertions mapping close to the *hrp48* coding sequence cause lethality and developmental defects. The reduction in hrp48 protein levels in the homozy

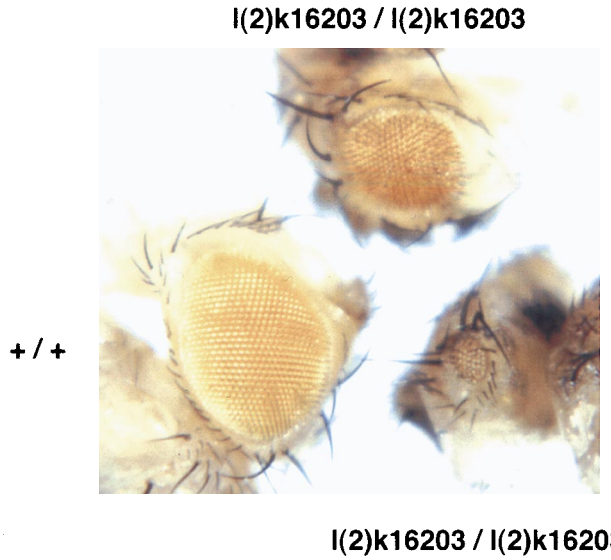


FIG. 3. *hrp48* mutant flies exhibit a small-eye phenotype. A wild-type fly eye contains approximately 800 ommatidia. In the *hrp48* mutant survivors, the number of ommatidia is reduced, resulting in a small-eye phenotype. Fly eyes from wild-type and homozygous [l(2)k16203/l(2)k16203] mutant females are shown. The variable severity of the mutant defect can be seen in the two homozygous mutant sisters.

gous hypomorphic mutant flies suggests that these phenotypes are due to an effect on hrp48 expression.

***hrp48* cDNA transgenes rescue the lethality and developmental defects caused by P-element-induced *hrp48* mutations.** To unambiguously determine whether the phenotypes associated with these P-element insertions were due to the reduction in *hrp48* gene expression, germ line transformation rescue experiments were performed. The full-length cDNA encoding hrp48 under the control of the constitutively active actin 5C or the heat-inducible *hsp70* promoters (see Materials and Methods) were used to generate P-element-mediated germ line transformants (38). The *hsp70-hrp48* cDNA transgene was shown by immunoblotting to overexpress hrp48 (Fig. 1B, lane 1). The cDNA transgenes were crossed into the P-element insertion strains l(2)k10280 and l(2)k16203 and assayed for their ability to rescue the phenotypes associated with the homozygous insertion mutants. The *hsp70-hrp48* transgene, but not the actin 5C-*hrp48* cDNA transgene, was capable of rescuing the recessive lethality associated with the l(2)k10280 insertion (Fig. 2A, columns 2 and 4). Both transgenes were found to rescue the lethality of the hypomorphic P-element insertion allele in strains l(2)k16203 and l(2)k10463 (Fig. 2A, columns 2 and 4, and data not shown). Partial rescue of the small-eye phenotype (Fig. 4a) and partial rescue of the missing-bristle phenotype were also observed (Fig. 4b and data not shown). Since the *hrp48* cDNA transgenes can rescue the phenotypes associated with the P-element insertions we have renamed the insertions as alleles of *hrp48* (Fig. 1A). These data indicate that the mutant phenotypes associated with the P-element insertions were the direct result of reduced hrp48 protein levels and that hrp48 provides an essential function in vivo.

Somatic P element IVS3 splicing from a reporter transgene is activated in *hrp48* mutants. Previous biochemical and genetic studies implicated hrp48 in the somatic splicing inhibition of the P-element third intron (IVS3) (8, 34, 35). The identification of viable hypomorphic *hrp48* mutants (*hrp48*^{4} and *hrp48*^{5}) that have reduced levels of hrp48 protein (Fig. 1B, lanes 4 and 5) allowed us to assess the role of hrp48 in the regulation of the splicing of the P-element third intron. If hrp48 is required for somatic IVS3 splicing inhibition, reduction in hrp48 protein levels should activate third-intron splicing. A reporter transgene, in which IVS3 and flanking regulatory exon sequences were placed upstream of *LacZ* under the control of the heat-inducible *hsp70* promoter (Fig. 5A), was used to assess third-intron splicing in vivo. Wild-type and homozygous *hrp48*^{4} mutant males carrying the splicing reporter were heat-shocked at 38°C for 1 h. Total RNA was isolated, and the levels of spliced and unspliced IVS3 RNAs were determined by RT-PCR. No products were observed in the absence of reverse transcription (Fig. 5B, lanes 3 and 4). As expected for this reporter, the majority of the IVS3-containing transcripts were unspliced, yielding the expected 346-bp cDNA product (Fig. 5B, lanes 1 and 2). Three additional products of 277, 188, and 156 bp were observed (Fig. 5B, lanes 1 and 2). The identities of these spliced products were confirmed by direct DNA sequencing of the RT-PCR products (see Materials and Methods and the legend to Fig. 5). The 156-bp product corresponds to the expected accurately spliced IVS3 mRNA (15, 16); the other two products of 277 and 188 bp correspond to use of the accurate IVS3 5′ splice site at nucleotide 1911 (8, 15) and cryptic 3′ and 5′ splice sites within IVS3 at P-element nucleotides 2017 and 2048. As previously described (8), the double splice with inclusion of a small microexon within IVS3 corresponds to the 188-bp product. The 277-bp product, corresponding to the removal of the first small intron within IVS3 (Fig. 5B), had not been observed previously (8), but our ability to detect this species may be due to the more sensitive RT-PCR conditions used here (see Materials and Methods).

Consistent with a role for hrp48 in somatic inhibition of IVS3 splicing, an activation of accurate IVS3 splicing was observed in the *hrp48* mutant (Fig. 5A, compare lanes 1 and 2). The spliced products utilizing the accurate 5′ splice site and cryptic 5′ and 3′ splice sites within IVS3 were also increased in the *hrp48* mutant. All activated splicing products from the *hrp48* mutant used the accurate IVS3 5′ splice site (Fig. 5B). There was a reproducible 2 (±0.3)-fold increase in the ratio of accurately spliced IVS3 message to total RNA in the *hrp48* mutant compared to that in the wild type. If all spliced products that use the accurate 5′ splice site and either the accurate or the internal cryptic 3′ splice site are considered, then the activation of splicing is 3 (±0.4)-fold greater. The spliced products that were activated in the *hrp48* mutant arise from the use of splice sites previously observed in vivo when *cis*-acting mutations in the IVS3 5′ exon regulatory element were shown to activate IVS3 splicing in somatic cells (8). In particular, a *cis*-acting mutation in the IVS3 5′ exon regulatory element that alters the hrp48 binding site (F2) resulted in the same spliced products in vivo. These results are also in agreement with in vitro studies in which *cis*-acting mutations in the IVS3 5′ exon abolish hrp48 binding and activate IVS3 splicing (34). Thus, a reduction in hrp48 protein levels partially relieves IVS3 splicing inhibition in vivo and the splicing products observed are identical to those found when somatic activation occurs due to inactivation of the F2 pseudo-5′ splice site in the 5′ exon negative regulatory element both in vivo (8) and in vitro (34). While the observed activation of IVS3 splicing is modest, this detectable shift in splicing pattern could affect transposase production in the maternal P-element mRNA, which is processed to yield only low levels of spliced transposase mRNA in the germ line (29).

In order to determine the tissue specificity of IVS3 splicing

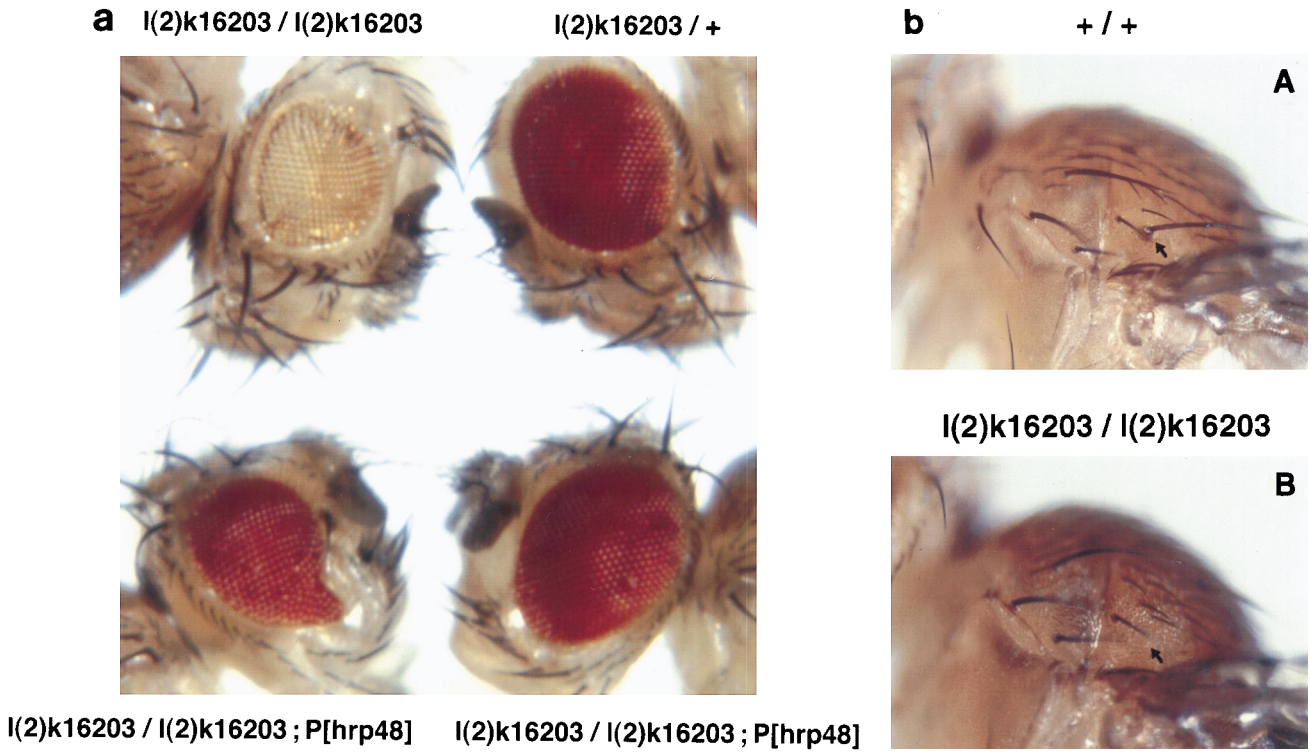


FIG. 4. *hrp48* cDNA transgenes can rescue the small-eye and missing-bristle phenotypes associated with the homozygous P-element insertion. (a) Eyes from homozygous and heterozygous l(2)k16203 females are shown at the top, and eyes from two homozygous mutant females carrying the P[w^+; actin 5C-*hrp48* cDNA] transgene are shown below. Rescue was not complete, but the eyes of homozygous mutants carrying the *hrp48* cDNA transgene were always larger and more like wild-type eyes than those from siblings lacking the transgene. (b) Shown are the posterior supra alar macrochaeta of the wild type (A) and the absence of this bristle in *hrp48* mutants [l(2)k16203/l(2)k16203] (B). Arrows indicate the location of the bristle.

in the *hrp48* mutant, a histochemical staining assay for β-galactosidase (LacZ) activity was used. Similar assays have been used previously to examine IVS3 splicing in vivo (1, 16). Following heat induction of the IVS3 reporter RNA, adults were dissected, fixed, and stained for β-galactosidase activity (see Materials and Methods). Several somatic tissues exhibit weak β-galactosidase activity in wild-type flies (Fig. 6A). However, in the *hrp48* mutant flies a variety of somatic tissues, including the gut and Malpighian tubules, exhibit strong β-galactosidase activity (Fig. 6B). The LacZ gene is only in frame when the third intron is accurately removed. Thus, the observed LacZ activity represents accurately spliced IVS3 mRNA product in these somatic tissues. These histochemical staining results are consistent with the RT-PCR data (Fig. 5A) and provide direct evidence that hrp48 is involved in somatic inhibition of IVS3 splicing.

DISCUSSION

Previous biochemical studies have implicated hrp48 in inhibition of third-intron splicing in somatic cell nuclear splicing extracts. In these studies hrp48 and three other proteins were found to bind to the *cis*-acting regulatory sequences in the upstream exon in the P element transposase pre-mRNA. hrp48 specifically recognized one of the two pseudo-5′ splice sites (F2) in the upstream regulatory element. Mutations in the F2 pseudo-5′ splice site relieved IVS3 splicing inhibition in somatic cells in vivo (8) and in somatic splicing extracts in vitro (34). These experiments provided indirect evidence for the involvement of hrp48 in somatic inhibition. Here, we demonstrate that hypomorphic mutations in *hrp48* that reduce hrp48 protein levels result in a twofold increase in accurate IVS3 splicing and that the spectrum of IVS3 splicing products observed in the *hrp48* mutant is similar to that identified from the F2 mutant pre-mRNA transcripts. Perhaps a further reduction in hrp48 protein levels beyond the fourfold found in the mutants examined here would result in even higher levels of IVS3 splicing. It has been previously shown that low levels of spliced IVS3 mRNA in the germ line were sufficient to give detectable transposase activity (29). Interestingly, both with the IVS3-LacZ reporter RNA (Fig. 6) and the natural P-element pre-mRNA, a small activation of accurate IVS3 splicing is sufficient to generate biologically detectable amounts of protein product (β-galactosidase or P-element transposase) from the spliced form of the mRNA. This is the first direct evidence that hrp48 plays a functional role in IVS3 splicing inhibition.

This report also provides a detailed genetic characterization of hrp48. Unlike the other hnRNP A1-like proteins in *Drosophila*, Hrb87F/hrp36 and Hrb98DE/hrp38, which are redundant in vivo (13, 40), hrp48 performs an essential function. Additionally, the *Drosophila* squid gene, which encodes the hrp40 hnRNP protein, exhibits dorsoventral patterning defects and is essential for viability (18). Hypomorphic *hrp48* mutations cause developmental defects, including reduced ommatidia in the eye and morphological bristle defects. The cause of these defects is not understood, though it seems that both bristle morphology and eye development are particularly sensitive to perturbations in splicing factors. Bristle and eye defects have also been observed in *Drosophila* U2AF small-sub-

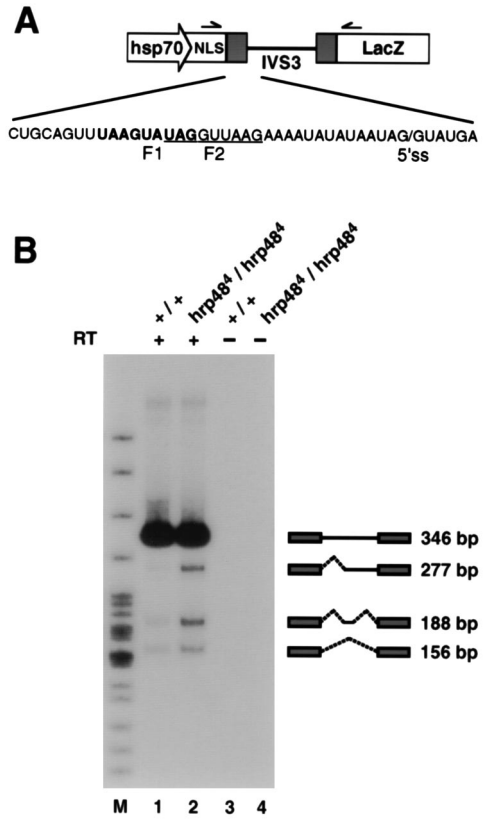


FIG. 5. Activation of P-element IVS3 splicing in *hrp48* mutants. (A) Schematic diagram of the third-intron (IVS3) reporter construct used to assess efficiency of third-intron splicing in vivo. A nuclear localization signal (NLS) was fused to the P-element IVS3 and flanking exon regulatory sequences (shaded). This fragment was fused to the bacterial *lacZ* gene such that *lacZ* is in frame only if the third intron is accurately removed. This reporter is under the control of the heat-inducible *hsp70* promoter. The primers used for RT-PCR are indicated schematically above the construct. ss, splice site. (B) RT-PCR products of the IVS3 reporter from wild-type and *hrp48* mutant flies. Amplified cDNA products are shown from w^{1118}; +/+; P[w^+; *hsp70-IVS3-lacZ*] (lane 1) and w^{1118}; l(2)k16203/l(2)k16203; P[w^+; *hsp70-IVS3-lacZ*]/+ (lane 2). Control reactions without reverse transcriptase (RT) are also shown (lanes 3 and 4). A schematic diagram of the cDNA products is on the right. The markers (M) are end-labeled *Msp*I-cleaved pBR322 DNA.

unit mutants (30, 31), and transgenic flies overexpressing the *Drosophila* SR splicing factor B52/SRp55 exhibit severe bristle defects or lethality (14, 28). It is possible that the processing of some transcripts involved in bristle formation and eye development is particularly sensitive to levels of splicing factors. We have also found that the viability of homozygous *hrp48* hypomorphic mutant males is reproducibly higher than that of females (1.5- to 10-fold higher). These skewed sex ratios have also been observed for hypomorphic mutations in the *Drosophila* U2AF small-subunit (31) and U1 70K genes (17). In all cases, viability of mutant male progeny was higher than that of mutant female progeny. We cannot account for this differential viability, but we think it is unlikely to be due to perturbations in the sex determination pathway, because we found no sexual transformations of any dimorphic body parts in the hypomorphic *hrp48* mutant progeny.

It has previously been proposed that the general hnRNP protein, hnRNP A1, and the SR protein ASF/SF2 are dose-dependent regulators of 5′ splice site choice and act antagonistically to each other (22). Increasing the concentration of hnRNP A1 promotes distal 5′ splice choice in a substrate containing duplicated 5′ splice sites. Proximal splicing can be restored by increasing the concentration of the SR protein ASF/SF2. If hnRNP proteins and SR proteins are dose-dependent regulators of alternative splicing, overexpression of these proteins should have dramatic consequences in vivo. Consistent with a role as a dose-dependent alternative splicing factor, ectopic overexpression of the *Drosophila* SR protein B52/SRp55, a member of the SR family of splicing factors, caused lethality and severe developmental defects (14). These defects are presumed to be due to aberrations in pre-mRNA processing and are exactly the phenotypes expected of a protein whose concentration is critically important in the regulation of splice site selection. Ectopic overexpression of hrp48, however, had no effect on viability or development (Fig. 2) (11). Similarly, overexpression of Hrb87F/hrp36, another abundant 2× RBD-Gly mammalian hnRNP A1-like protein, also had no detectable effects on viability and development (40). Viability and development were also unaffected when the general splicing factors U2AF (large or small subunit) (30) or U1 70K (17) were overexpressed. Splice site shifting was observed in one transcript (dopa decarboxylase) when Hrb98DE or Hrb87F were overexpressed (32, 40). This effect was transient, and no splice site shifting was detected in five other transcripts analyzed. Using a duplicated 5′ splice site reporter carrying the *fushi tarazu* (*ftz*) 5′ splice site, we did not detect any splice site switching when hrp48 was overexpressed (11). These in vivo observations are at odds with the model that hnRNP proteins are dose-dependent regulators of splice site selection. One possibility is that hnRNP proteins, like general splicing factors, are in excess in the nucleus. Increasing the protein level of these general factors would be expected to have only modest effects on RNA processing. hnRNP proteins, like general splicing factors, may not themselves be splicing regulators; rather, they might be acted upon or modulated by dose-dependent or tissue-specific alternative splicing factors.

Previous biochemical and genetic experiments have implicated the PSI in somatic inhibition of P-element third-intron splicing. These experiments demonstrated that PSI is the tissue-specific regulator of third-intron splicing. The results reported here support a role for the general hnRNP protein, hrp48, in somatic inhibition. U1 snRNP was found to bind to the F1 pseudo-5′ splice site in the regulatory element in *Drosophila* somatic extracts. Mutations in the F1 site relieve splicing inhibition in vitro and in vivo, implicating U1 snRNP in IVS3 splicing regulation. The activities associated with hnRNP A1 suggest possible roles for hrp48 in somatic inhibition. Studies on mammalian hnRNP A1 have shown that the A1 protein can influence U1 snRNP–pre-mRNA interactions (23, 24). A series of biochemical experiments showed that mammalian A1 protein binds with high affinity to the core sequence UAGG(U/G)(U/A) (6). This sequence closely resembles the hrp48 binding site, F2 (UAGGUU), in the IVS3 5′ exon (34). The juxtaposition of the F1 and F2 sites in the P-element pre-mRNA might allow hrp48 to stabilize U1 snRNP bound to the F1 site. Additionally, localization studies have revealed that hnRNP A1 shuttles from the nucleus to the cytoplasm, implicating the protein in nucleocytoplasmic mRNA transport (26). Interestingly, immunolocalization of hrp48 in *Drosophila* embryos showed both a nuclear and a cytoplasmic distribution (35). In the case of IVS3 splicing inhibition, the unspliced mRNA needs to exit the nucleus, and perhaps hrp48 plays a role in the transport of the unspliced P-element message that retains IVS3 and encodes the transpositional repressor. Future biochemical and genetic experiments will further dissect the role hrp48 plays in inhibition of third-intron splicing.

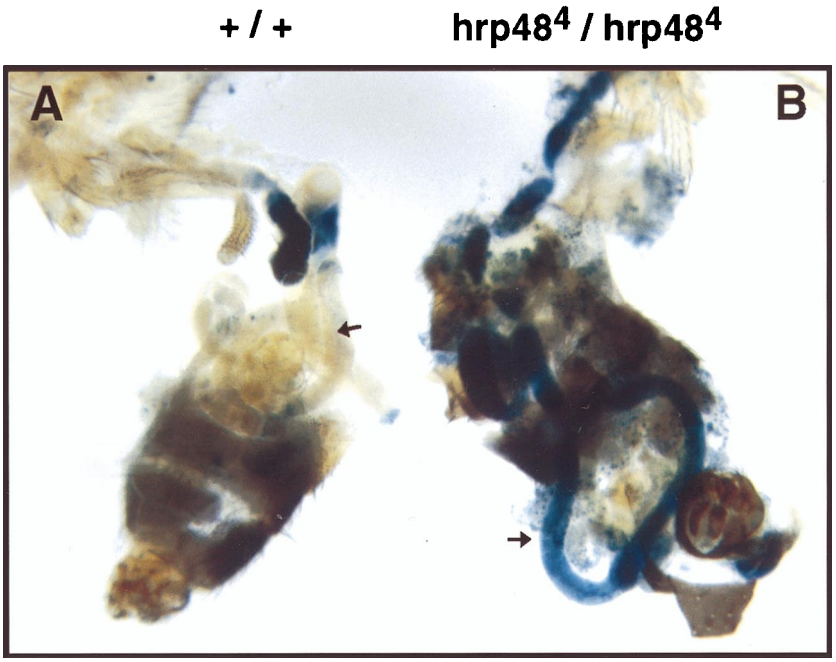


FIG. 6. Activation of IVS3 splicing in the *hrp48* mutants occurs in somatic tissues. Wild-type w^{1118}; +/+; P[w^+; *hsp70-IVS3-lacZ*]/+ and *hrp48* mutant w^{1118}; l(2)k16203/l(2)k16203; P[w^+; *hsp70-IVS3-lacZ*]/+ flies were dissected and stained for β-galactosidase activity. (A) Weak staining of the wild-type fly can be seen in part of the gut (indicated by an arrow). (B) Strong and extensive nuclear LacZ staining in the *hrp48* mutant fly can be seen in the gut (indicated by an arrow) as well as other unidentified somatic tissues. In other preparations and with direct observation, staining of the somatic Malpighian tubules was also evident.

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