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The Concentration of B52, an Essential Splicing Factor and Regulator of Splice Site Choice In Vitro, Is Critical for *Drosophila* Development

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B52 is a *Drosophila melanogaster* protein that plays a role in general and alternative splicing in vitro. It is homologous to the human splicing factor ASF/SF2 which is essential for an early step(s) in spliceosome assembly in vitro and also regulates 5′ and 3′ alternative splice site choice in a concentration-dependent manner. In vitro, B52 can function as both a general splicing factor and a regulator of 5′ alternative splice site choice. Its activity in vivo, however, is largely uncharacterized. In this study, we have further characterized B52 in vivo. Using Western blot (immunoblot) analysis and whole-mount immunofluorescence, we demonstrate that B52 is widely expressed throughout development, although some developmental stages and tissues appear to have higher B52 levels than others do. In particular, B52 accumulates in ovaries, where it is packaged into the developing egg and is localized to nuclei by the late blastoderm stage of embryonic development. We also overexpressed this protein in transgenic flies in a variety of developmental and tissue-specific patterns to examine the effects of altering the concentration of this splicing factor in vivo. We show that, in many cell types, changing the concentration of B52 adversely affects the development of the organism. We discuss the significance of these observations with regard to previous in vitro results.

In eukaryotes, an important means of gene regulation is the process of pre-mRNA splicing. Many genes are interrupted by noncoding sequences, or introns, and the production of a functional mRNA requires the removal of these introns. For many pre-mRNAs, the splicing reaction produces only a single type of processed transcript from its pre-mRNA precursor. However, a number of genes also undergo alternative splicing, a process by which a single pre-mRNA is spliced in two or more ways to produce two or more different transcripts (1, 6, 28, 39). In *Drosophila melanogaster*, for example, the sex-determination pathway is modulated by a cascade of alternatively spliced genes that are processed into unique transcripts in males and females (2, 41).

A large body of work has been done to mechanistically define the splicing process, and many of the components required have been identified. The splicing process is carried out by a large ribonucleoprotein (RNP) complex called the spliceosome. Components of this complex include the small nuclear RNPs (snRNPs) U1, U2, U4/U6, and U5 (42). A number of other non-snRNP proteins that play roles in the general splicing pathway have been identified and characterized. These include SC35, an essential splicing factor that can commit certain pre-mRNA substrates to the splicing pathway (13, 40); SF2, an essential splicing factor that is required early in the splicing pathway for 5′ splice site cleavage and lariat formation (23); and U2AF, required for the binding of U2 snRNP to the branch site (48, 49). Also required for the general splicing event are the heterogeneous nuclear RNPs (hnRNPs), a set of conserved nonspliceosomal proteins also involved in the packaging of bulk heterogeneous nuclear RNA (hnRNA) (9).

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A number of genes that encode proteins that regulate alternative splice site usage have also been identified. In *D. melanogaster*, for example, Sex-lethal, transformer, and transformer-2 proteins regulate sex-specific splice site choice in the sex-determination pathway (2, 3). The *suppressor-of-white-apricot* gene encodes a protein that regulates splicing of its own pre-mRNA as well as that of the *white* gene (3, 10). In humans, the best characterized alternative splicing factor is ASF/SF2, which functions in a concentration-dependent manner to promote the use of 5′- and 3′-proximal splice sites in alternatively spliced pre-mRNAs (14–16, 22–24). Additionally, in vitro studies have indicated that the ratio of ASF/SF2 to hnRNP A1 protein determines which alternative 5′ splice site is used; increasing the ratios of hnRNP A1 to ASF/SF2 promotes usage of the distal 5′ splice site (29).

Recently, ASF/SF2 has been shown to be a member of the SR proteins, a family of proteins conserved from *D. melanogaster* to humans. This family consists of at least five different proteins that all contain a domain rich in serine and arginine (the SR domain) (46). Additionally, most members of the family contain a conserved domain called the RNA recognition motif (RRM) that has been shown to bind RNA and single-stranded DNA (21, 32, 37, 43). The different SR proteins have been purified and can functionally substitute for ASF/SF2 general and alternative splicing activities in in vitro assays (30, 46). B52, also called SRp55, is the 52-kDa member of the *D. melanogaster* SR family. It contains an N-terminal RRM, a second, degenerate RRM, and an extended SR domain at the C terminus (7, 35, 46). In vitro it functions as a general splicing factor in human splicing extracts and also promotes the use of proximal 5′ splice sites in alternatively spliced pre-mRNAs in a concentration-dependent manner (30, 50).

Despite the similarities in sequence and function among the different SR family members, these proteins are not likely to be functionally redundant in vivo. Recent work has shown some tissue-specific differences in levels of SR proteins, suggesting that varying levels of a given SR protein may regulate tissue-

specific alternative splicing events in vivo (29, 47). Additionally, in vitro the different SR family members are not functionally redundant; in fact, individual SR proteins have different activities on different substrates (13, 47).

It is important to note that most of the work done to date on SR proteins has been done in vitro. In vitro systems provide excellent means of studying mechanistically the process of splicing, and they clearly identify the potentials of different proteins to participate in general and alternative splicing. However, the functional similarities among the SR proteins seen in vitro will make the determination of the roles of the family members in the developing organism difficult, if not impossible, without examining their distribution and functions in vivo. Additionally, the splicing process occurs within the context of the nuclear architecture and involves levels of regulation, including chromatin structure and component compartmentalization, that are not present in in vitro systems. These factors will likely influence the activities of the SR proteins.

In this study, we characterize the expression of B52 in a developmental and tissue-specific manner. With this information, we examine in vivo the effects of overexpressing this protein in a variety of different tissues at different times. We find that B52 is widely expressed throughout development and that the concentration of this protein in vivo is critical to the viability and proper development of the organism. Overexpression of B52 creates a number of phenotypes, ranging from lethality to reproducible visible phenotypes.

MATERIALS AND METHODS

Western blot (immunoblot) analysis. Whole animals of the strain z^1w^{11E4} (alleles described in reference 26) at various stages of development were homogenized in 0.15 M NaCl. The homogenate was loaded onto 10% sodium dodecyl sulfate (SDS)-polyacrylamide gels. The protein concentration of each sample was determined by the Bradford assay (4). Proteins were transferred to nitrocellulose (Schleicher & Schuell), and blots were processed by the chemiluminescence technique according to the directions of the manufacturer (ECL; Amersham), with the following exceptions.

Filters were blocked in 3% gelatin containing 10% calf serum in Tris-buffered saline. Primary antibody incubations were done in 1% gelatin containing 10% calf serum in Tris-buffered saline with a 1:200 dilution of affinity-purified B52 antibody (anti-RRM). For quantitation, X-ray films (Kodak XAR) were scanned by densitometry.

Nuclei of different developmental stages were prepared essentially as described previously (33). Briefly, animals were homogenized in a mixture of 0.15 M NaCl, 10 mM Tris (pH 8.0), 5 mM EDTA, 0.2% Nonidet P-40, and 2 mM phenylmethylsulfonyl fluoride (PMSF) in a Dounce homogenizer. The homogenate was filtered through nylon mesh and then spun through a sucrose cushion consisting of 0.8 M sucrose layered over 1.6 M sucrose for 20 min at 10,000 rpm in an SW50.1 rotor at 4°C. The resulting pellet was resuspended in SDS loading buffer, loaded onto SDS-polyacrylamide gels, and processed as described above.

Anti-RRM antibody production and purification. To raise antibodies, a glutathione *S*-transferase (GST)–partial B52 fusion protein was made as follows. The *Xho*I-*Eco*RI fragment of the B52 cDNA encoding amino acids 4 to 41 was subcloned in frame into pGEX-2T (Pharmacia). GST-B52 fusion protein expression was induced, and the protein was excised and eluted from an SDS-polyacrylamide gel. This gel-purified protein was injected into rabbits.

For affinity purification, the B52 cDNA from the endogenous *Xho*I site to the C terminus was subcloned in frame into pET-16b (Novagen). This construct produces a histidine-tagged B52 fusion protein. The fusion protein was expressed and purified according to the directions of the manufacturer. The purified fusion protein was coupled to CNBr-Sepharose 4B (Pharmacia) according to the directions of the manufacturer. Crude serum was then loaded onto the column and eluted with 0.1 M glycine (pH 2.7). Fractions containing antibody were then dialyzed against three changes of Tris-buffered saline.

Whole-mount assay. *D. melanogaster* z^1w^{11E4} larvae at first instar (24 to 29 h), second instar (84 to 88 h), third instar (112 to 116 h), or late crawling third instar were dissected in Ringer's solution (110 mM NaCl, 4 mM KCl, 1 mM $MgCl_2$, 5 mM $CaCl_2$, 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES] [pH 7.2]), fixed for 20 min in 2% paraformaldehyde in phosphate-buffered saline (PBS), and treated essentially as previously described (12). Briefly, tissues were rinsed with 0.1% Triton X-100 (Sigma) in PBS and permeabilized for 15 min in 1% Triton X-100 in PBS. The tissues were then rinsed in 0.1% Triton X-100 in PBS and were blocked for 1 h at room temperature in a mixture containing 10% calf serum, 0.1% Triton X-100, and 0.1% deoxycholate in PBS. The tissues were again rinsed in 0.1% Triton X-100 in PBS and incubated in a 1:10 or 1:100 dilution of affinity-purified anti-RRM antibody in a mixture of 10% calf serum, 0.1% Triton X-100, and 0.1% deoxycholate in PBS overnight at 4°C. Control experiments used either no primary antibody in this incubation mix or 12CA5 antibody (Berkeley Antibody Co.) against the influenza virus hemagglutinin (HA) epitope at dilutions of 1:20 or 1:40. Control experiments with either undiluted preimmune serum or a 1:10 dilution of preimmune serum were performed in the above incubation buffer. Experiments on larvae expressing HA-tagged B52 proteins and control parents used 12CA5 antibody at a 1:4 dilution.

Following primary antibody incubations, tissues were washed three times for 10 min in PBS containing 0.1% Triton X-100. Tissues were blocked for 1 h at room temperature as described above and were then incubated overnight at 4°C in a 1:200 dilution of tetramethyl rhodamine isothiocyanate-conjugated donkey anti-rabbit immunoglobulin G (Jackson ImmunoResearch Laboratories, Inc.) in a mixture of 10% calf serum, 0.1% Triton X-100, and 0.1% deoxycholate in PBS. Tissues were washed three times for 10 min in PBS containing 0.1% Triton X-100, blocked for 1 h at room temperature as described above, washed twice for 15 min in PBS containing 0.1% Triton X-100, and mounted in glycerol with 2.5% *n*-propyl gallate added. Samples stained with Hoechst dye were treated as described above except that a 5-min incubation with Hoechst dye preceded the final wash steps.

Embryos were collected and allowed to age at room temperature for 0 to 2 h, 2 to 4 h, or 3 to 6 h. They were then dechorionated and fixed as described previously (18). Briefly, embryos were dechorionated in 50% Clorox and washed with 0.7% NaCl–0.002% Triton X-100. They were fixed for 20 min at room temperature in a 1:1 mix of heptane and 4% paraformaldehyde in PBS. Embryos were then devitellinized as described previously (31) and processed for immunofluorescence as described above. Unfertilized eggs were treated in the same manner as were embryos. Slides of larval tissues and 3 to 6 h embryos were examined and photographed with a Bio-Rad MRC-600 laser scanning confocal microscope. Slides of 0- to 3-h embryos stained with Hoechst dye were photographed with a Zeiss Universal fluorescence microscope.

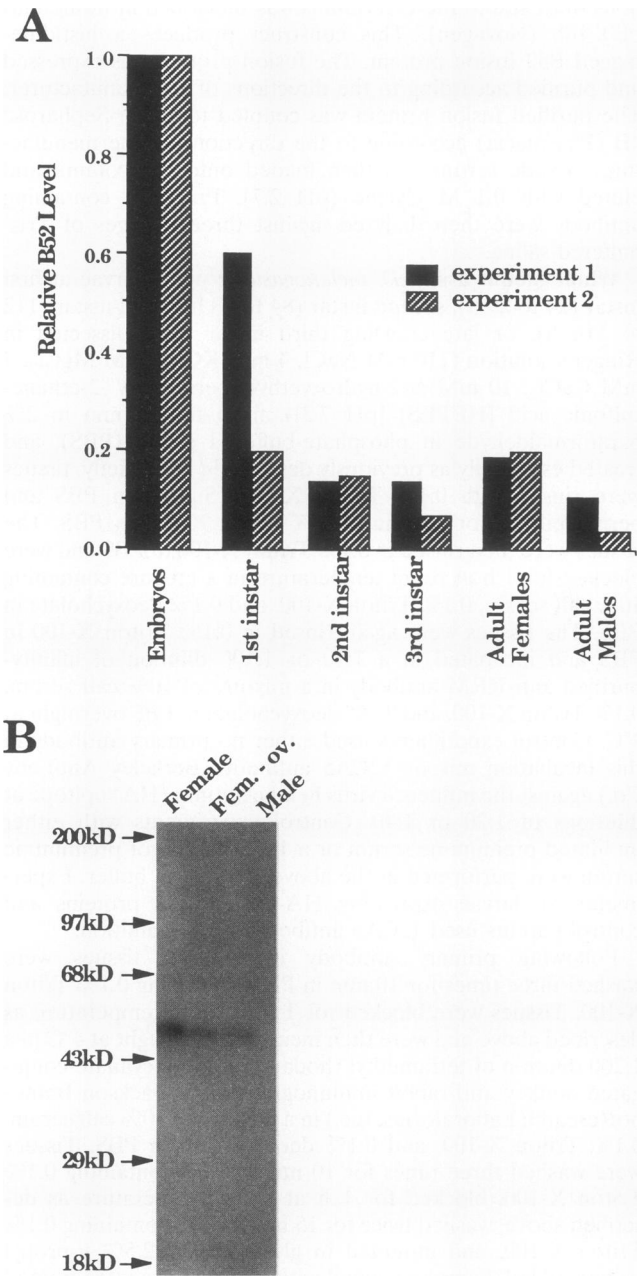

FIG. 1. B52 levels throughout *D. melanogaster* development. (A) Total protein from animals at the specified stages was analyzed by Western blotting using chemiluminescence as described in Materials and Methods. B52 levels were quantitated by scanning densitometry and were standardized to the total protein loaded per lane. The results are presented as the amount of B52 relative to total protein at each developmental stage. The value for each stage has been normalized to the B52 levels in embryos. Shown are the results of two experiments. B52 protein is detected in pupae as well; however, the background on Western blots of whole pupae is significantly higher than that on blots done at any other developmental stage, making quantitation of the relative level of B52 in pupae impossible by this method. (B) Whole adult females, adult females from which the ovaries had been dissected (Fem.-ov.), and adult males were examined by Western blots probed with anti-RRM antibody. Comparable levels of protein were loaded in each lane. All flies were 2 days posteclosion.

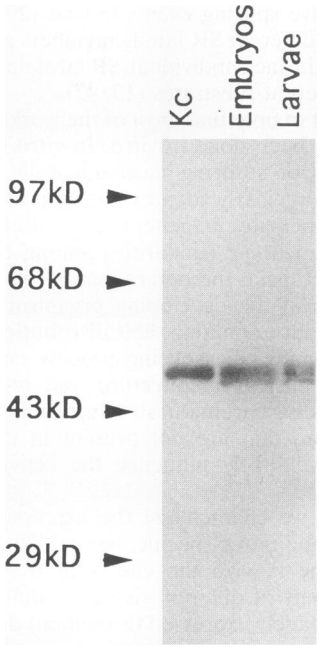

FIG. 2. Anti-RRM antibody specifically recognizes B52 in embryos and *D. melanogaster* nuclei. *D. melanogaster* Kc nuclei, whole embryos, and third-instar larval nuclei (Larvae) were analyzed by Western blot probed with affinity-purified anti-RRM antibody.

***D. melanogaster* transformation and B52 overexpression.** The B52 cDNA and the B52 cDNA with the HA tag sequences subcloned at the N terminus at the *Xho*I site were subcloned into the P-element transformation vector pUAST (5), and plasmid DNA was injected into embryos (36, 38). Germ line transformants were obtained by using a genomic source of transposase as described previously (34). At least two independent transformant lines were established for each construct. To induce expression of GAL4-controlled constructs, male GAL4 transgenic flies were mated to virgin female flies carrying GAL4-controlled transgenes. Progeny were examined for phenotype.

The B52 cDNA used is one of two cDNAs for B52 cloned and sequenced in our laboratory (7, 8). It is identical to that previously published (7) except that it lacks the coding sequence for amino acids 317 to 337. Therefore, the sequence is nearly identical, with only a few amino acid changes, to the sequence of SRp55 (35).

β-Galactosidase assays. Male GAL4 transgenic flies were mated to virgin female UASLacZ flies carrying a GAL4-controlled *lacZ* transgene. Progeny at the same developmental stages as those described above for the whole-mount assay were examined for tissues with β-galactosidase activity by using the 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (Bethesda Research Laboratories) assay (17, 27).

RESULTS

B52 is widely expressed throughout development. In vitro work has shown that changing the ratio of the human SR protein ASF/SF2 to hnRNP A1 alters the usage of alternative 5′ splice sites (29). Since B52 has striking homology to ASF/SF2 (46) and can functionally substitute for ASF/SF2 in vitro (30), the ratio of B52 to other splicing factors in vivo might be an important means of regulating alternative splicing. Previous developmental Northern blot (RNA) results indi-

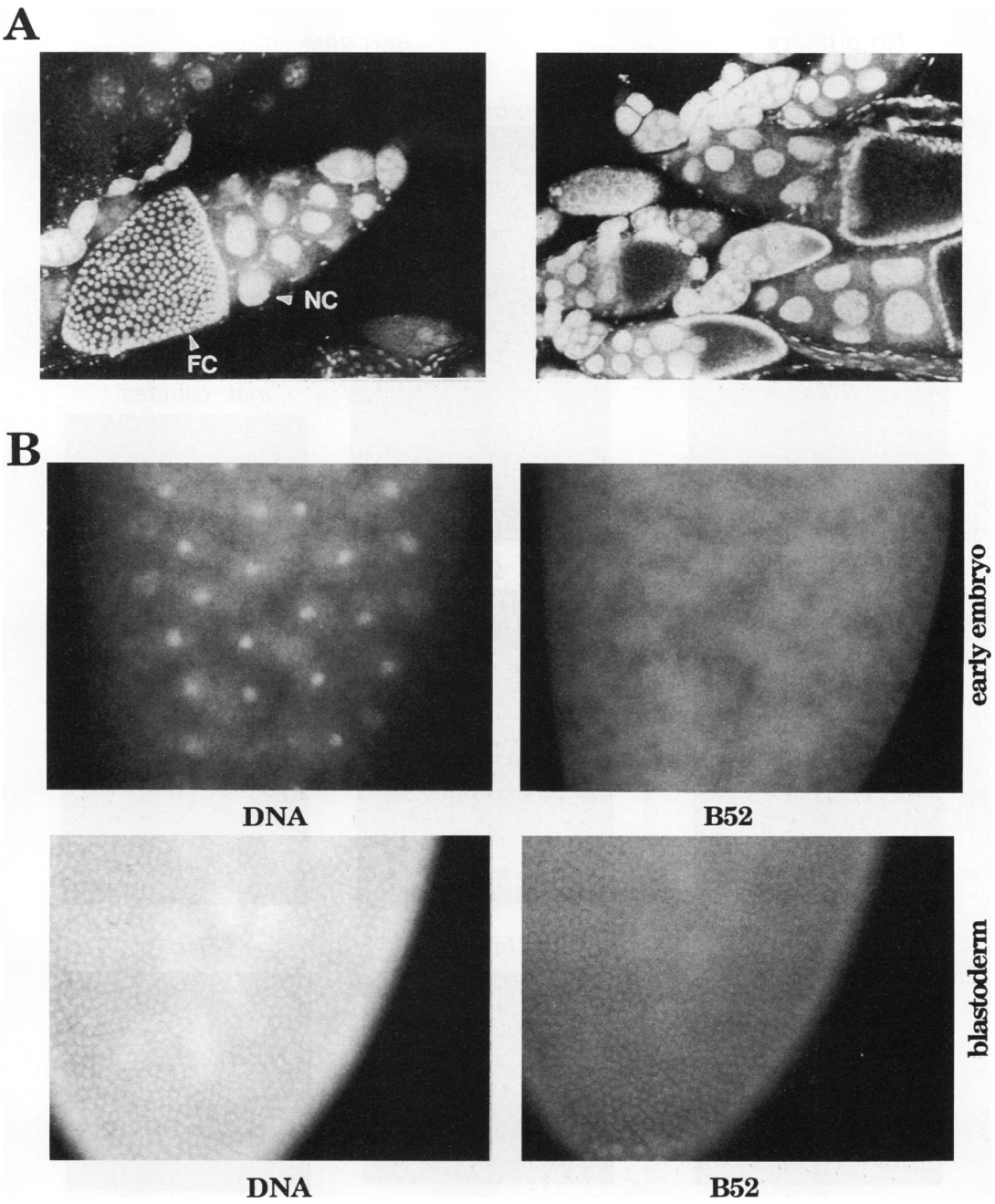

FIG. 3. B52 is present in *D. melanogaster* egg chambers and early embryos. Ovaries from adult female flies and early embryos were examined by whole-mount analysis as described in Materials and Methods. (A) A surface view of a stage 10 egg chamber (left) and a cross-section of an ovariole (right), showing the various stages of oogenesis, are presented. NC, nurse cells; FC, follicle cells. (B) In early embryos, nuclei, visualized with Hoechst dye, are visible in the interior of the embryo (top left) and B52 is not localized exclusively to nuclei (top right). Once embryos have reached the late blastoderm stage, nuclei are closely packed at the surface of the embryo (bottom left) and B52 is localized to nuclei (bottom right).

cated that the B52 gene is expressed throughout development (7); however, the levels of individual SR proteins in mammals appear to vary from tissue to tissue (47). Therefore, we decided to examine different developmental stages and tissues for changes in the level of B52 protein.

Embryos, each of the three larval instars, pupae, and adult flies were ground in 150 mM NaCl and were loaded onto SDS-polyacrylamide gels. The level of B52 at each stage was determined by Western blotting with an affinity-purified antibody to B52. The total protein loaded per lane was quantitated, and the amount of B52 was standardized to the total amount of protein. A summary of the results of two such experiments is shown in Fig. 1A. In embryos, the levels of B52 are elevated relative to later developmental stages, but the levels decrease through the first instar in such a way that second-instar larvae have only about 15 to 20% of the B52 (relative to total protein) that embryos contain. B52 levels drop slightly (at most twofold) through the remainder of develop-

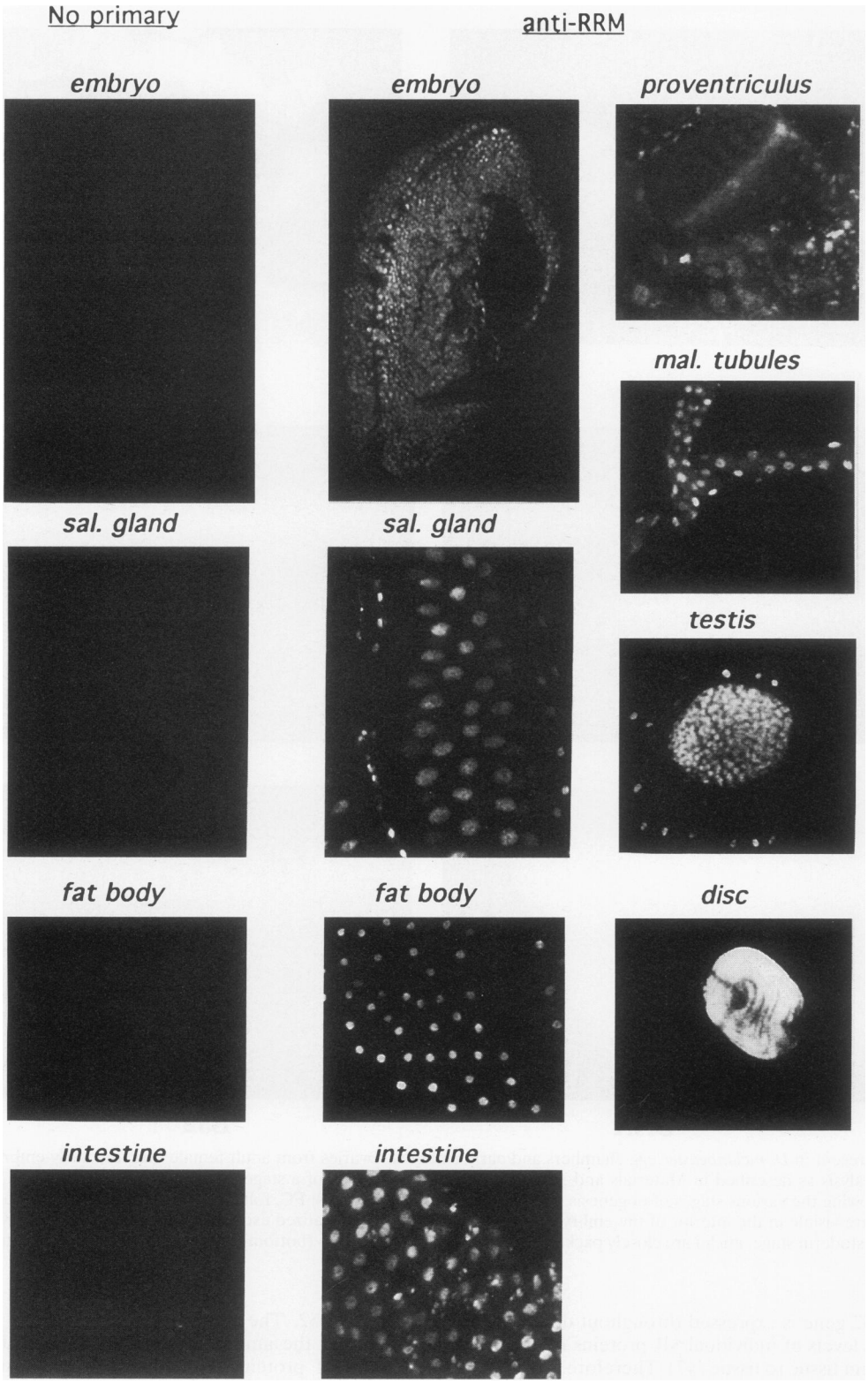

FIG. 4. B52 is generally expressed in embryos and larvae. Three- to six-hour embryos and dissected late third-instar larvae were examined by whole-mount analysis as described in Materials and Methods. Samples were treated in the absence of primary antibody (left column) as a control or with affinity-purified anti-RRM antibody (center, right). Tissues shown are as labeled and represent a selection of all larval tissues examined. Other developmental stages were examined (Materials and Methods) and exhibited staining similar to that shown for late-third-instar larvae. Abbreviations: sal. gland, salivary gland; mal. tubules, Malpighian tubules; disc, imaginal disc.

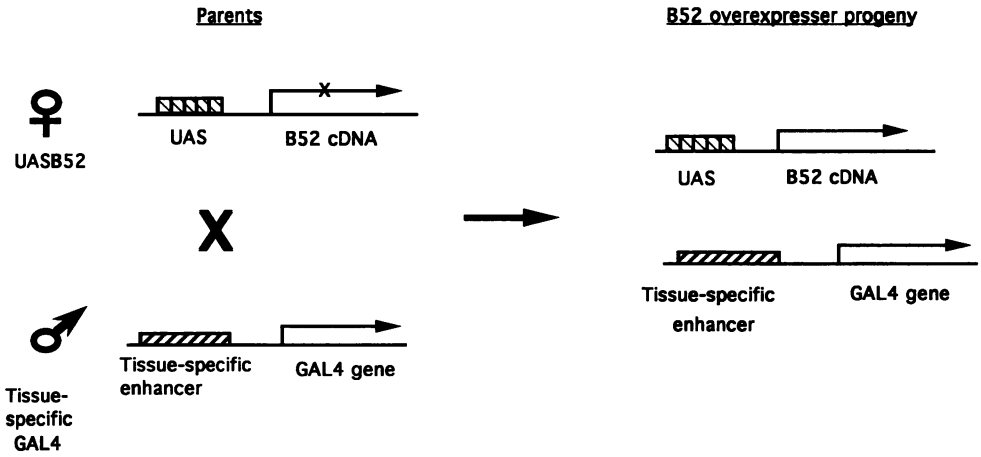

FIG. 5. B52 overexpression using the GAL4 system. Virgin female flies carrying a transgene in which the B52 cDNA was placed downstream of five GAL4 binding sites (UAS) were mated to males carrying a GAL4 gene downstream of a genomic enhancer. In the progeny of this cross, tissues in which the enhancer is active will express GAL4 protein. In turn, the GAL4 activator in these tissues will bind the UAS in the UASB52 transgene and induce expression of the B52 cDNA, resulting in B52 overexpression.

ment. Adult males have severalfold less B52 than do adult females, yet this difference appears to be due exclusively to B52 found in the ovaries of adult females (Fig. 1B). In general, B52 levels appear to be fairly constant throughout development with the exception of the relatively high levels in embryonic and early first-instar stages. These Western blot results agree well with previous Northern blot results showing that B52 mRNA is present at all stages of development (7).

Next, we examined the distribution of B52 in different tissues at different developmental stages, using a whole-mount procedure in which tissues are incubated with a B52-specific antibody and a tetramethyl rhodamine isothiocyanate-conjugated secondary antibody and are examined by confocal microscopy. This assay allows all tissues in the organism to be compared in a single assay (12). This assay required a highly specific antibody that would not recognize any of the other SR family members. We generated rabbit polyclonal sera against a GST fusion to amino acids 4 to 41 from the N terminus of B52 that, by Western blot, does not recognize any of the other SR family members (Fig. 2). Note that when nuclei are examined by Western blot, the B52 signal is very strong and the anti-RRM antibody is extremely specific. When whole animals are examined by Western blot (Fig. 1B), B52 is a more minor component of the cellular proteins, and the ratio of signal to noise is reduced. However, since B52 is a nuclear protein, it is the specificity of the anti-RRM antibody when nuclei are examined that is of relevance for the following whole-mount experiments. We frequently observe B52 running as a doublet on SDS-polyacrylamide gels (Fig. 2); the significance of this doublet is unclear but may be related to the minor differences in the two cDNAs that have been sequenced (7, 35). Alternatively, the doublet may be the result of phosphatase activity during sample preparation, as the mobility of B52, known to be a phosphoprotein, in SDS gels is dramatically increased upon phosphatase treatment (35).

Having observed that B52 protein levels appear to be high in adult ovaries (Fig. 1B), we examined the distribution of B52 during oogenesis and the early stages of embryonic development. B52 protein is found in the nuclei of all cell types in the adult ovaries and developing egg chambers, with the nurse cell nuclei exhibiting very strong signals (Fig. 3A). The cytoplasm of the nurse cells also shows a signal. These results suggested that the B52 protein itself might be packaged into the developing egg. In fact, Western blots of unfertilized eggs indicate that B52 protein is present in quantities similar to the quantity seen in later stages of embryonic development, thus confirming the above hypothesis. Whole-mount analysis of early embryos indicates that B52 protein is not predominantly nuclear until late blastoderm stage (Fig. 3B); prior to this time, B52 is found in both nuclei and the cytoplasm surrounding these nuclei in the preblastoderm embryo. Additionally, B52 is found in the nuclei of postblastoderm embryos; it was also found in every tissue examined in each of the larval stages (Fig. 4). Double immunofluorescence assays using Hoechst, a DNA dye, to identify each nucleus showed that the B52 distribution is indistinguishable at this resolution from that of DNA. Control assays in which tissues were incubated in the absence of primary antibody (Fig. 4), with 12CA5 primary antibody to influenza virus hemagglutinin (see Fig. 7) or with preimmune serum, showed no nuclear staining, indicating that the nuclear fluorescence is specific to our B52 antibody.

In general, the B52 nuclear signal was strong in most tissues examined at all postblastoderm developmental stages. One exception occurred in the intestines of the larvae. The proventriculus typically showed a very weak B52 signal (Fig. 4), although the remainder of the intestine, including the gastric cecae, exhibited quite a strong signal. Additionally, the imaginal discs, the brain and ventral ganglion, and the larval testes and ovaries reproducibly showed extremely intense B52 immunofluorescence. Although this in situ immunofluorescence is not extremely quantitative, it appears that some differences in B52 concentration between tissues and within cell types of a given tissue exist. With the exceptions of the weak B52 signal in the proventriculus and very strong signal in the imaginal tissues, however, these differences are not very dramatic. Although we did not notice any tissues in which B52 protein is absent, we cannot exclude the possibility that, during development, some cell types may not express B52. Nonetheless, B52 appears to be quite generally expressed throughout the major stages of *D. melanogaster* development. We have also observed general expression of B52 in adult flies.

The level of B52 protein is critical in vivo. Having determined the B52 levels in tissues at different times during development, we began to characterize the effects of overpro-

Gal4 Line	*Stage*	*Tissues expressing Gal4*	*Phenotype*
A-25	1st instar	SG (++)	3rd instar/ Early
	2nd instar	SG(++); ESO (+); TRC (+); INT (++)	pupal lethality. A few adults
	3rd instar	SG (++); TRC (++)	survive.
	Early pupa	+	
	Late pupa	++	
A-27	1st instar	SG (++)	Late larval/ Early
	2nd instar	SG (++); INT (+)	pupal lethality.
	3rd instar	SG (++); BRN (+); VG (+)	
	Early pupa	+	
C-70	1st instar	no blue	Late larval/ Early
	2nd instar	SG (++)	pupal lethality.
	3rd instar	SG (++); AS (++); INT (+)	
	Early pupa	+	
G-17	1st instar	SG (++); EPI (+/-)	Some late larval
	2nd instar	SG (++); EPI (+)	lethality. Adults
	3rd instar	SG (++); EPI (++); FB (+)	have abdominal sternite defects.
	Early pupa	++	
	Late pupa	++	
I-65	1st instar	SG (++); EPI (++)	Early larval
	2nd instar	SG (++); EPI (++); TRC (++); PV (++); BRN (+); VG (+)	lethality.
I-86	1st instar	SG (++)	Adults have
	2nd instar	SG (++); FB (+)	abdominal sternite
	3rd instar	SG (++); ESO (+)	defects.
	Early pupa	++	
	Late pupa	++	
O-96	1st instar	SG (++); INT (+/-)	Some lethality
	2nd instar	SG (+); INT (+)	at each larval stage.
	3rd instar	SG (++); INT (++)	
	Early pupa	++	
	Late pupa	++	
hsGal4	1st instar	SG (++); INT (+/-)	Adults exhibit:
	2nd instar	SG (+); FB (+)	Bristle defects
	3rd instar	SG (+); FB (+)	Wing defects
	Early pupa	++	Slower egg prod'n.
	Late pupa	++	

FIG. 6. Phenotypes of tissue-specific B52 overexpression. The estimated levels of GAL4 are indicated in parentheses. When a particular developmental stage is indicated as the point of lethality, the majority of the progeny died at that stage, although some may have been able to proceed further in development and some died sooner. Abbreviations: AS, anterior spiracles; BRN, brain; EPI, epidermis; ESO, esophagus; FB, fat body; INT, intestine; PV, proventriculus; SG, salivary gland; TRC, tracheae; VG, ventral ganglion; prod'n, production.

ducing B52 in different tissues at different developmental stages. We predicted that, if the ratio of B52 to other splicing factors regulates splice site choice in vivo as it does in vitro, changing the levels of B52 in vivo should affect the development of the organism in some manner. Initially we attempted to generate germ line transformants carrying the B52 cDNA under transcriptional control of the *D. melanogaster hsp70* promoter. However, the only transformants we obtained died before we could establish a stable fly line. This result suggested to us that the level of B52 overexpression resulting from the constitutive expression of the *hsp70* promoter might be lethal in vivo. Therefore, we switched to a recently developed transformation system (5). In this system, the B52 cDNA is placed under the transcriptional control of the *Saccharomyces cerevisiae* activator GAL4 (Fig. 5). Since GAL4 is not naturally present in *D. melanogaster*, this construct will not be expressed and it should be possible to generate viable germ line transformants. To induce overexpression of B52, these transformants are mated to a transgenic fly line that expresses the GAL4 activator. The progeny of this cross are then scored for phenotype.

In this study, we crossed our B52 cDNA (UASB52) transgenic line to 33 fly lines that express GAL4 in various tissues at different developmental stages. These fly lines were generated by integrating the GAL4 coding sequence into various sites of the *D. melanogaster* genome (19) in such a way that GAL4 expression was dependent upon the coding sequence inserting near a genomic enhancer (45). Each GAL4 transgenic line will express the GAL4 activator only in tissues in which the genomic enhancer is active. To examine the tissue-specific expression of GAL4 in each cross, each line was crossed to a transgenic line carrying the β-galactosidase gene under the control of the GAL4 activator (UASLacZ). The progeny were scored at different developmental stages for β-galactosidase activity by incubating dissected animals with X-Gal (Fig. 6). To ascertain that there was a correlation between tissues that expressed β-galactosidase in the progeny of the UASLacZ cross and tissues that overexpressed B52 in the progeny of the

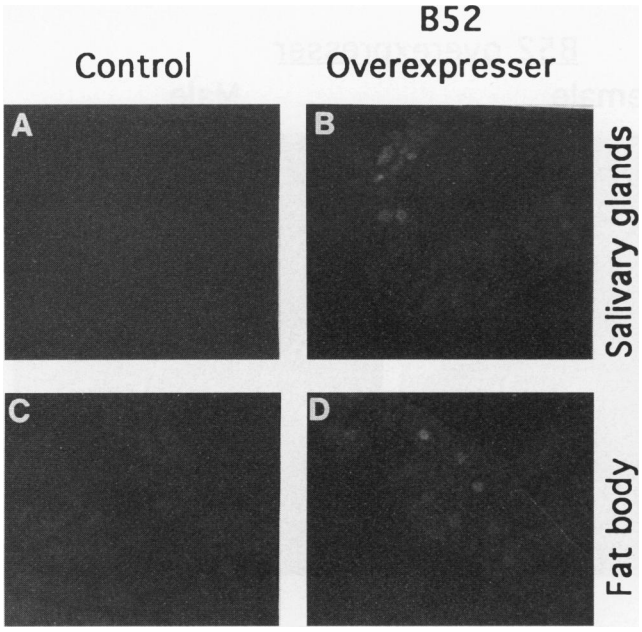

FIG. 7. B52 is overexpressed in the same tissues in which the GAL4 activator is expressed. Third-instar UASHB52 control larvae carrying an inactive HA-tagged UASB52 transgene (A and C) and B52 overexpresser larvae carrying both an hsGAL gene and a UASHB52 transgene (B and D) were examined by whole-mount analysis using the 12CA5 antibody specific for the HA epitope. Tissues shown are the only ones that exhibit staining. Note that the salivary gland in panel B is extremely small, with only the anterior nuclei distinguishable.

cross between the same GAL4 parents and UASB52 parents, we crossed a transgenic fly line carrying a GAL4-controlled B52 cDNA with the HA epitope from influenza virus hemagglutinin inserted at the N terminus (UASHB52) to the *D. melanogaster* hsGAL4 line. This GAL4 line carries the GAL4 gene under the control of the *D. melanogaster hsp70* promoter. In our hands, this line has high constitutive tissue-specific expression of the hsGAL4 transgene, and the experiments in this study were all done in the absence of heat shock. We performed whole-mount assays on larvae by using the 12CA5 monoclonal antibody specific for the HA epitope and compared the immunofluorescence pattern with that of X-Gal staining in the progeny of hsGAL4 and UASLacZ parents. We observed a good correlation between the two assays (Fig. 6 and 7). X-Gal staining showed GAL4 activity in the larval salivary glands and fat body. Likewise, the 12CA5 antibody specifically labeled nuclei in the salivary glands and fat body. In our hands, the 12CA5 antibody is not as sensitive as our B52 polyclonal antibody, so we were forced to use much more antibody in these whole-mount assays. The whole-mount assay was not a feasible means, economically, of determining which tissues overexpressed B52 in all of the crosses we examined subsequently. However, the above result and the phenotypes of a number of our B52 overexpressers suggested that the X-Gal assay was an accurate reflection of which tissues overexpressed B52.

Figure 6 lists a subset of the different GAL4 transgenic lines used, the tissues in which the GAL4 activator is expressed, the relative levels of GAL4 produced as determined by X-Gal staining for β-galactosidase activity, and the phenotypes of progeny carrying both the GAL4 gene and the UASB52 construct. Only three of the crosses produced adult progeny. The progeny of the remaining 30 crosses died at different developmental stages from embryo to late pupa, depending upon the GAL4 line used to generate the cross. That we observed a range of phenotypes from these crosses was not unexpected, because each GAL4 line will drive B52 overexpression in distinct tissues and developmental stages. Note that, in most cases, the overexpression of B52 is lethal. It is difficult to determine the direct cause of this lethality; however, there are some correlations that are interesting and should be investigated further. First, in larvae overexpressing B52, the salivary glands are extremely small (Fig. 8B). This phenotype was observed in all crosses between the GAL4 lines and UASB52 flies and correlates with the expression of GAL4 in the salivary glands in each of our GAL4 lines. Second, in most cases, if B52 was overexpressed in tissues other than the salivary glands, larvae could not survive to the pupal stage. However, some tissues may be more sensitive to B52 overexpression than others are. For instance, in three crosses, the progeny survived to the adult stage, and in each of these crosses, B52 was expressed in the larval fat body (Fig. 6, GAL4 [Gal4] lines G-17, I-86, and hsGAL4). Notably, in one of these crosses (G-17), high GAL4 expression was also observed in the larval epidermis. It may be that the larval fat body and epidermis can tolerate B52 overexpression better than other tissues can. Alternatively, these tissues may be of lesser importance to the development of the larva, so that defects in these tissues do not affect the viability of the organism as severely.

We studied the overexpression of B52 driven by the hsGAL4 gene in more detail, because it resulted in viable progeny with reproducible phenotypes (Fig. 8). As before, these experiments were performed with non-heat-shocked animals. Larvae exhibit the salivary gland phenotypes discussed previously. The progeny have a decreased survival rate, eclose later than control flies, have wing defects (with the wings either being easily ripped or curling or wrinkling at the ends), and also exhibit defects in the long bristles or macrochaetes on the thorax, head, and legs. The bristles tend to be shorter, thinner, or absent altogether. Additionally, the females have ovaries that take longer to produce eggs than controls do. This range of effects is similar to that seen in a number of *D. melanogaster* mutants, such as ribosomal protein mutants (11, 20, 44), that have defective protein synthesis machinery.

In these B52 overexpresser progeny, the males tend to have significantly higher (up to 10-fold) overexpression than females do, as determined by Western blotting, and virtually all males die before they reach the adult stage. Many males die as pharate adults; Western blot analysis demonstrates that B52 levels in these flies are very high at the pharate adult stage. The males that do survive have very high levels of B52 overexpression, and the phenotypes are more severe; for instance, in most cases most of the bristles are missing altogether (Fig. 8A). Therefore, a correlation exists between the level of B52 overexpression and the severity of this phenotype. Although we cannot at present explain the mechanism of lethality observed in this study, we have shown that the lethality is specific to overexpression of B52, since the expression of the C-terminal serine-arginine-rich domain of B52 at levels comparable to the levels of B52 overexpression in this study results in no visible phenotypes (25).

DISCUSSION

B52 is the *D. melanogaster* 52-kDa member of the SR protein family and is a homolog of ASF/SF2 (7, 35, 46). In vitro it can act as an essential splicing factor that is required for an

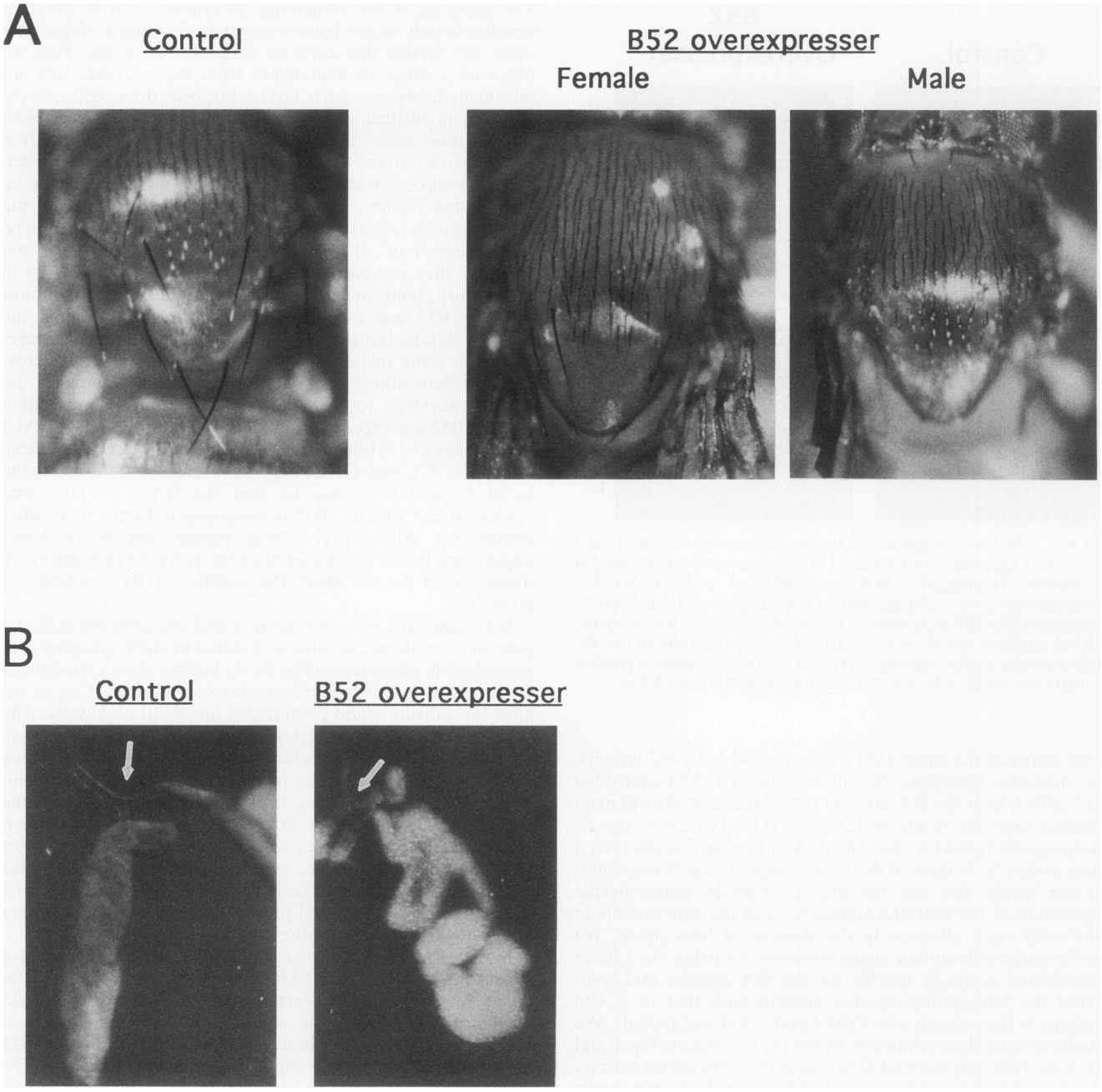

FIG. 8. B52 overexpression causes bristle and salivary gland defects. (A) Control flies carrying the hsGAL4 gene show normal bristle morphology (left). B52 overexpresser flies carrying the hsGAL4 gene as well as the UASB52 transgene have shorter, thinner, or absent bristles (center and right). Females (center) generally show a less severe phenotype than do males (right). (B) Wild-type salivary glands of late third-instar larvae (left) and salivary glands from progeny carrying the hsGAL4 and UASB52 transgenes (right) are shown. Arrows mark the point at which the common salivary gland duct branches into ducts leading into the right and left glands. In each panel the fat body is the white tissue, while the gland from the control larva is translucent.

early step in the splicing pathway (30). B52 and other members of the SR family also function as alternative splicing factors, influencing alternative 5′ splice site usage in a concentration-dependent manner (29, 46). Despite the sequence and functional similarities between the SR family members, it has recently been shown that the family members exhibit different activities in vitro on different pre-mRNA substrates (13, 47). These results suggest that the SR proteins most likely have distinct functions in vivo. One means of regulating general and alternative splicing of the pre-mRNA substrates of an individual SR protein in vivo could be through regulation of the spatial and temporal concentrations of that splicing factor. Here, we examine the tissue-specific expression and levels of the SR protein, B52, throughout *D. melanogaster* development

and examine the effects of changing the levels of B52 in various tissues at various times in development, to determine how the organism is affected.

B52 protein is maternally packaged into eggs and becomes a predominantly nuclear protein by the late blastoderm stage of embryonic development. B52 levels relative to total protein are approximately 5- to 10-fold higher in embryos than in later stages of development. Although the B52 levels decrease throughout the first larval instar, B52 remains widely expressed throughout development. It is present in the nuclei of every tissue examined from late blastoderm embryos and from the three larval stages. We have noticed some differences in intensity of the signal from tissue to tissue; in particular, the larval imaginal discs, brain, ventral ganglion, and reproductive primordia reproducibly stain very strongly with B52 antibody relative to other tissues. Also, within a tissue we have noticed varying degrees of intensity, particularly throughout the larval intestine.

The concentration of SR family proteins, of which B52 is a member, is of importance in regulating 5′-alternative splice site usage in vitro (16, 22, 24, 30). Perhaps the high level of B52 in imaginal discs and reproductive primordia is important in determining which cells will differentiate into adult tissues and which are solely larval tissues. Alternatively, higher levels of B52 may be required in cells undergoing rapid cell division. The higher levels of B52 during embryonic development may regulate alternative splice site choice in transcripts that produce embryonic and larval messages; therefore, this stage is a reasonable point to search for RNAs whose splicing may be modulated by B52. Additionally, it is important to note that it is not necessarily the absolute concentration of an SR protein that determines splice site usage but the ratio of that SR protein to another splicing factor, such as homologs to mammalian hnRNP A1, that may be critical (29). Therefore, even though the B52 levels in the whole-mount assays did not show drastic changes in many tissues and Western blot analysis demonstrated that B52 levels are relatively constant during development, B52 may be involved in regulation of alternative splicing events in various cell types or developmental stages if the level of a B52 antagonist changes.

Overexpressing B52 in a variety of tissues is detrimental to *D. melanogaster* development. Greater than 90% of the crosses set up to overexpress B52 in different tissue-specific patterns in this study were unable to produce viable progeny. Each cross produced progeny overexpressing B52 in a small number of tissues, making it difficult to determine the direct cause of lethality. However, the extremely low number of crosses producing progeny that reached the adult stage strongly indicates that the absolute level of B52 protein is critical to the survival of a range of different cell types. However, some tissues appear to be less sensitive to B52 overexpression than others are. In particular, the larval fat body and epidermis do not exhibit obvious defects from B52 overexpression, even when the levels of overexpression are similar to that which results in salivary gland defects (Fig. 8). This insensitivity may be a result of levels of B52 normally lower in the salivary glands than in the fat body. Therefore, to cause phenotypic effects in the fat body, higher levels of B52 overexpression may be required than are required to cause phenotypic effects in the salivary glands. Alternatively, either a B52 substrate or an antagonist may be expressed in a tissue-specific distribution, so that tissues not expressing the substrate or antagonist will be less sensitive to B52 overexpression.

In this study we have shown that B52 is widely expressed during *D. melanogaster* development and that the level of this protein in a number of cell types (with the apparent exception of the fat body and the larval epidermis) drastically affects the survival of the organism. All of these results are consistent with current hypotheses, surmised from in vitro studies, to explain the function of SR proteins in splicing. We cannot, at this time, prove that the phenotypes we observed are actually a result of splicing defects, although the large body of in vitro work makes this a likely conclusion. Identification of the in vivo substrates of B52 is likely to be a laborious process involving a combination of biochemical and genetic approaches. However, having observed that the in vivo level of B52 is critical to the development of *D. melanogaster*, we are developing a more tightly controlled system of overexpression, that will allow us to introduce and test in vivo substrates that in vitro studies have shown to be sensitive to different levels of B52. Additionally, since the overexpression of B52 in vivo results in a highly reproducible phenotype, it is possible to use *Drosophila* genetics to search for suppressors that may identify substrates for B52 activity or proteins that functionally interact with B52.

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