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Genetic Enhancement of RNA-Processing Defects by a Dominant Mutation in *B52*, the *Drosophila* Gene for an SR Protein Splicing Factor

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SR proteins are essential for pre-mRNA splicing in vitro, act early in the splicing pathway, and can influence alternative splice site choice. Here we describe the isolation of both dominant and loss-of-function alleles of *B52*, the gene for a *Drosophila* SR protein. The allele $B52^{ED}$ was identified as a dominant second-site enhancer of *white-apricot* (w^a), a retrotransposon insertion in the second intron of the eye pigmentation gene *white* with a complex RNA-processing defect. $B52^{ED}$ also exaggerates the mutant phenotype of a distinct *white* allele carrying a 5′ splice site mutation (w^{DR18}), and alters the pattern of sex-specific splicing at *doublesex* under sensitized conditions, so that the male-specific splice is favored. In addition to being a dominant enhancer of these RNA-processing defects, $B52^{ED}$ is a recessive lethal allele that fails to complement other lethal alleles of *B52*. Comparison of $B52^{ED}$ with the $B52^{+}$ allele from which it was derived revealed a single change in a conserved amino acid in the β4 strand of the first RNA-binding domain of B52, which suggests that altered RNA binding is responsible for the dominant phenotype. Reversion of the $B52^{ED}$ dominant allele with X rays led to the isolation of a *B52* null allele. Together, these results indicate a critical role for the SR protein B52 in pre-mRNA splicing in vivo.

SR proteins are members of a family of related non-snRNP splicing factors that are highly conserved from *Drosophila melanogaster* to humans (82). All members of this family can complement an S100 splicing-deficient extract and alter splice site selection in templates with competing 5′ splice sites (23, 24, 36, 83), implying that they perform related functions in splicing. SR proteins also function in the recognition of exonic splicing enhancers (40, 70, 74), participate in the formation of ATP-independent prespliceosomal complexes under standard splicing conditions (20, 34, 69), and interact with other early-acting components of the splicing machinery including the U1 small nuclear ribonucleoprotein particle (snRNP) (34, 78) and U2AF (78). Furthermore, specific SR proteins have been shown to be sufficient to commit specific pre-mRNAs to splicing in vitro (22), and high concentrations of SR proteins can even compensate for the absence of intact U1 snRNP, an otherwise essential splicing factor (17, 72).

Structurally, SR proteins are characterized by one or two RNA-binding domains at the amino terminus and an auxiliary domain rich in arginine-serine dipeptide repeats (the RS domain). The RNA-binding domains conform to the RNP-type consensus (CS-RBD) (19) known as the RNA recognition motif (RRM) (32). RRMs have been found in a wide variety of RNA-binding proteins, including snRNPs, essential non-snRNP splicing factors, and the splicing regulatory proteins Sex lethal and Transformer 2 (for a review, see reference 19; for an extensive list, see reference 10). Structural information on members of this family includes a high-resolution X-ray crystal structure of the U1A snRNP protein RRM, both alone (54) and bound to RNA (57); and similar nuclear magnetic resonance solution structures of the same domain (29), of the heterogeneous nuclear RNP (hnRNP) C RRM (77), and of the second RRM of Sex lethal (41). The consensus RRM structure is a compact βαββαβ domain which binds RNA on the surface of a four-stranded β-sheet. The degree of amino acid identity among SR proteins within the RRM (roughly 35 to 50% [33]) is higher than that observed among other proteins containing RRMs (typically less than 20%). Some SR proteins, including B52 (16, 64), have a second RNA-binding domain adjacent to the amino-terminal consensus RNA-binding domain. This second RNA-binding domain shows less agreement with the consensus, but its ability to bind RNA has been verified in the case of ASF/SF2 (13, 85).

SR proteins invariably have a region rich in serine-arginine dipeptides of variable length at their carboxyl termini (the RS domain). In the case of B52, this domain includes 130 amino acids of which 74 are arginine or serine residues in alternation. RS domains are also found in U2AF and in proteins encoded by the *Drosophila* genes *transformer*, *transformer-2*, and *suppressor-of-white-apricot* (8). Similar domains of shorter length or greater variability in sequence are also found in the *Drosophila* suppressor-of-sable protein (76) and in the U1 70K snRNP protein (46, 61). The RS domain is required for ASF/SF2 to function as an essential splicing factor (13, 85). However, no RS domains have been found in yeast-splicing proteins, and in the case of yeast homologs to metazoan proteins with RS domains, the RS domain is either reduced or entirely absent (1, 30, 60, 67).

Because SR proteins act early in the splicing pathway, are directly involved in the determination of which splice sites will be used, and mediate the action of differentially expressed regulators of alternative splicing (73), it is expected that they play a central role in the regulation of splicing in vivo. Biochemical results cited above reveal distinct but overlapping activities for individual SR proteins (see reference 48 for a

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review), and we favor the hypothesis that while SR proteins are functionally redundant for many splicing events in vivo, the accurate splicing of a subset of pre-mRNAs is critically dependent on a particular SR protein or combination of SR proteins. Support for this hypothesis comes from examination of the distribution of SR proteins among mammalian tissues, which indicates considerable variation in their relative abundance (35, 83). Furthermore, altered levels of ASF/SF2 activity in extracts from normal WI38 and transformed WI38VA13 cells correlate with altered in vivo and in vitro splicing activities (14), and the rat SR protein HRS (SRp40 [82]) is highly induced in regenerating liver and in liver-derived insulin-treated cells (18), suggesting that it may be an important mediator of growth regulation.

The *Drosophila* SR protein B52 (also known as SRp55) was identified by Champlin et al. (16) as the antigen recognized by a monoclonal antibody that stained boundaries of transcriptionally active chromatin and by Roth et al. (64) as one of several proteins carrying the antigen recognized by the SR protein-specific monoclonal antibody MAb104. B52 was subsequently found to provide the essential in vitro splicing function conferred by SR proteins; i.e., it complements HeLa S100 splicing-deficient extract (49). In vivo, B52 is an abundant protein whose critical role in *Drosophila* development is indicated by the fact that severe developmental defects result from B52 overexpression (38) and by the lethality of a small deletion that removes part of the gene for B52 (*B52*) and the flanking, nonessential gene *Hrb87F* (63). However, no splicing defects were observed in five endogenous pre-mRNAs in larvae deficient for *B52* (63). The absence of overt splicing defects is consistent with the hypothesis that B52 is one member of a partially redundant family of splicing regulators if essential genes whose splicing depends on B52 were not among those examined.

Genetic screens for regulatory genes with essential functions are made problematic by the fact that loss-of-function mutations in such genes are expected to cause lethality. However, essential regulatory genes have been successfully identified by exploiting the effect of viable allelic combinations on sensitized phenotypes (see, for example, reference 66). We have searched for genes whose protein products play pivotal roles in splicing decisions by evaluating the mechanism of action of mutations isolated as modifiers of w^a, an allele of the eye color gene *white* carrying a *copia* insertion in the second intron. The phenotype caused by w^a is particularly sensitive to disruptions of RNA processing because of competition between RNA-processing events in the expression of w^a (11, 39) and because of the exquisite sensitivity of *Drosophila* eye color to the precise level of *white* expression. Indeed, molecular characterization of genetic modifiers of w^a has already identified several genes that act in RNA processing. For example, *suppressor-of-white-apricot* [*su*(w^a) or *DmSWAP*] has been shown to regulate the splicing of its own transcript (80, 81) and that of the segmentation gene *hedgehog* (52). The *DmSWAP* protein contains an arginine-serine-rich domain and shares a conserved domain with the widely conserved yeast splicing factor *PRP21* (68). Similarly, *suppressor-of-forked*, a recessive enhancer of w^a, is homologous to a human polyadenylation factor (CstF) subunit (71) and to *Saccharomyces cerevisiae RNA14* (51), which is itself a component of the yeast polyadenylation machinery (50).

Here we describe the isolation of mutations in the SR protein gene *B52* by this approach. $B52^{ED}$ was isolated as a dominant enhancer of w^a and also enhances a number of other phenotypes attributable to alterations in the regulation of splicing. This original dominant $B52^{ED}$ allele carries a single amino acid substitution in the amino-terminal RNA-binding domain of B52, and an X-ray screen for revertants of this dominant allele led to the isolation of null alleles of *B52*.

MATERIALS AND METHODS

Nomenclature. We follow Ring and Lis (63) in using the gene name *B52*. $B52^{ED}$ was originally given the allele designation *E726* (39) and has been referred to in unpublished communications as *Enhancer-of-Deformed* [or $E(Dfd)^1$]. Thus, ED refers to the interim name *Enhancer-of-Deformed*, which is an allusion to our observation that $B52^{ED}$ is an enhancer of the *Dfd* dominant allele; it can also be remembered as enhancer dominant. Similarly, the revertants $B52^{R1}$ and $B52^{R2}$ have been referred to as $E(Dfd)^{XP28}$ and $E(Dfd)^{XP2}$, respectively. The allele referred to here as $Hrb87F^{\#7}$ is also known as $B52^7$ or $Df(3R)B52^7$ (27).

Phenotypic effect of $B52^{ED}$. The response of various *white* alleles to $B52^{ED}$ was tested by crossing w^a; $B52^{ED}/TM3$ males to females homozygous for the *white* allele and comparing the phenotypes of $B52^{ED}$ and *TM3* male progeny. In the case of w^{DR18}, females of the genotype w^{1118}; P[w^{DR18}] were used.

Hoyer's preparations of adult heads were made as described previously (3, 15), except that fixation was carried out at 45 rather than 60°C.

Meiotic recombinational mapping. Preliminary mapping involved the multiply marked third chromosomes *ru h th st cu sr* e^s *ca* and *Gl Sb H*. Then, $B52^{ED}$ was mapped relative to *squid* by mating w^a/+; $B52^{ED}/ry^{506}$ sqd^{ix50} *cv-c sbd* females to *ru h th st cu Df(3R)urd st* e^s *ca/TM3*, *Sb* males [ry^{506} sqd^{ix50} *cv-c sbd* was obtained from Richard Kelley, and *Df(3R)urd* was obtained from Susan Haynes].

Strains with the deficiencies depicted in Fig. 2 were obtained from Susan Haynes. Strains with the following *rosy* deficiencies were obtained from the Mid-America Drosophila Stock Center and tested for viability in combination with $B52^{ED}$: $Df(3R)ry^{27}$, $Df(3R)ry^{36}$ (87E), $Df(3R)ry^{75}$ (87D1-2, 87D14-E1), $Df(3R)ry^{81}$ (87C1-3, 87D14-E2), $Df(3R)ry^{614}$ (87D2-4, 87D11-14), and $Df(3R)ry^{619}$ (87D7-9, 87E12-F1). All were found to be viable. Cytology was performed as described by Lindsley and Zimm (43). The *Dfd cu kar* $Df(3R)ry^{27}$ strain was obtained from Susan Haynes and from the Mid-America Drosophila Stock Center.

Reversion of the $B52^{ED}$ dominant allele. w^a; $B52^{ED}/TM3$, *Sb* males were irradiated with an Astrophysics Torrex 120D X-ray instrument for 620 s (approximately 4,500 R) and crossed to w^a; *TM3*, *Sb*/*CxD* females. A total of 136,132 progeny were examined for eye pigmentation.

Lethal phase analysis. The lethal phase was determined by counting animals at various stages of development as described previously (59). All combinations (except those involving $B52^{28}$) were tested in reciprocal crosses, and symmetric results were obtained, indicating a lack of maternal effect. In every case, control crosses were performed to determine the mortality of genotypes other than the experimental one. In the case of $B52^{ED}$ homozygotes, recombination with flanking markers was carried out first, and the results of reciprocal crosses between w^a; $B52^{ED}$ *cv-c sbd*/ry^{506} sqd^{ix50} *cv-c* and w^a; *ri* tub^4 $B52^{ED}/TM3$, *Sb* were observed to address the possibility that these chromosomes carry unrelated lethal alleles that may have resulted from mutagenesis or accumulated during maintenance of the stock. In some cases [including all crosses among $B52^{ED}$, $B52^{R1}$, and $B52^{R2}$ or between these alleles and *Df(3R)urd*)], larval and pupal viability of the experimental genotype was further assessed by crossing flies heterozygous for *TM6*, *Tb Hu*. In these crosses, all animals except the experimental animals show the Tubby phenotype. $B52^{28}$ was obtained from John Lis, and $Hrb87F^{\#7}$ was obtained from Susan Haynes.

Consistent results were observed in series of large experiments carried out under identical conditions within a period of a few months at Columbia University. All combinations of *B52* null alleles or deficiencies caused embryonic death in those experiments. In contrast, Ring and Lis (63) reported larval lethality with the *B52* null allele $B52^{28}$. This difference was further investigated in additional but smaller experiments performed by the *TM6*, *Tb Hu* method (53). In these experiments, carried out at the University of Maryland a year later, larval lethality was observed for $B52^{28}$, $B52^{R2}$ and $Hrb87F^{\#7}$ homozygotes and for $B52^{28}/B52^{ED}$, $B52^{28}/Df(3R)urd$, $B52^{28}/B52^{R1}$, $B52^{28}/B52^{R2}$, and $B52^{28}/Hrb87F^{\#7}$ transheterozygotes. It is possible that an experimental variable such as humidity or the type of *Drosophila* medium used accounted for the difference between results obtained at different times and locations. We wish to emphasize that all combinations of putative *B52* null alleles gave consistent results within each experiment. Furthermore, the difference between development to form morphologically normal embryos that fail to hatch (observed at Columbia University in an original large experiment) and larval death (observed at Cornell and at the University of Maryland) is unlikely to be of great developmental significance.

Northern blot analysis. Northern (RNA) blot analyses were performed as described previously (59). *B52* (16), *rp49* (55), and *doublesex* (12) cDNA clones were used as probes, and the signals were quantified on a Molecular Dynamics PhosphorImager with ImageQuant software.

PCR amplification and sequencing. Oligonucleotides used in PCR amplifications were as follows: B52-1, 5′ TTATCATATGGTGGGATCTCGAGTGTA 3′; B52-2, 5′ CGCGACGATCCTCGACCAAG 3′; B52-3, 5′ TTGGATGACAC CGAGCTAAAC 3′; B52-4, 5′ AATCAAGCTTTTAATATTAATGGGACAG 3′; and B52-5, 5′ GGCATCACGATAGTCTTCGAATTC 3′. DNA was prepared as described by Ballinger and Benzer (5). Genomic DNA was amplified with oligonucleotides B52-1 and B52-5 and subcloned by using the natural *Xho*I

and *Eco*RI sites spanning positions 11 to 435 (see Fig. 2). The sequence was determined from multiple, independently amplified M13 clones.

$B52^{ED}$ and $B52^{+}$ RNAs were made from Tb^{+} larvae carrying only $B52^{ED}$ or its $B52^{+}$ parental allele, respectively, following a cross between $B52^{ED}$/*TM6*, *Tb Hu* or $B52^{+}$ Doa^{E755}/*TM6*, *Tb Hu* and *ru h th st cu Df*(*3R*)*urd* st^{s}-*eca*/*TM6*, *Tb Hu*. The Doa^{E755} chromosome is wild type for *B52* and was used here to provide the parental $B52^{+}$ chromosome; Doa^{E755} is a *Doa* allele isolated in the same screen as $B52^{ED}$ (39) and maintained in a balanced condition. RNA was prepared as described previously (59); the cDNA sequence was determined from multiple M13 clones of independently amplified PCR fragments generated following reverse transcription of RNA. Primer pairs used were B52-1/B52-2 and B52-3/B52-4. Multiple clones of both $B52^{ED}$ and $B52^{+}$ were sequenced between the *Xho*I and *Ssp*I sites (positions 11 to 1078, including all but 10 nucleotides of the coding region).

Only a single difference was observed between $B52^{ED}$ and its $B52^{+}$ parental allele. In particular, these two alleles are identical at all positions where the two previously published sequences differ (16, 64) (accession number X58720). The only position in which their sequence does not match one of the two previously published cDNA sequences is position 279, a silent substitution (GGC versus GGG) that does not affect the amino acid sequence. In six of the eight cases in which the two previously published sequences differ from each other, the sequence of $B52^{ED}$ and its $B52^{+}$ parental allele is identical to that of the dSRp55 cDNA (64). In the other two cases (positions 222 to 223 and 864 to 865), our alleles are identical to the B52 cDNA (16). For two positions where nucleotides are found in the B52 cDNA of Champlin et al. (16) but not that of Roth et al. (64), we found evidence in the genomic sequence consistent with the hypothesis that the differences are generated by the use of alternative 3′ splice sites (49a) but were unable to detect inclusion of these nucleotides in mRNA by the reverse transcription (RT) PCR method (58).

Quantitative RT-PCR assay. Total RNA was isolated from adult flies and examined by RT-PCR as previously described (44). Radioactivity in the gels was quantitated and log-linear amplification was verified by direct quantitation of RT-PCR products with a PhosphorImager and ImageQuant software (version 3.1). In the case of *doublesex*, primers specific for male and female *doublesex* RNA described by Amrein et al. (2) were used. Male and female amplifications were carried out in a single reaction vessel, and the ratio of the two products was constant for each sample throughout the log-linear range of amplification. We have observed that the ratio between products is more reproducible than their absolute levels (53, 58). The same RNA samples were examined by Northern blotting (53, 58), and roughly equivalent RNA recovery was verified by probing for both *dsx* and *rp49* (55).

RESULTS

$B52^{ED}$ is a dominant enhancer of splicing defects. $B52^{ED}$ was isolated in a screen for dominant second-site modifiers of w^{a} (39). Acting as a single copy in *trans*, $B52^{ED}$ enhances w^{a}, causing reduced eye pigmentation (Fig. 1A). A comparison of this effect with differences in pigmentation caused by varying *white* gene dosage indicates that $B52^{ED}$ causes a roughly 50% reduction in expression of the w^{a} allele.

The screen that yielded $B52^{ED}$ (39) also led to the isolation of four intragenic mutations at *white*, three alleles of *Darkener-of-apricot* (*Doa*), and nine additional novel dominant *trans*-acting modifiers of w^{a}. To identify candidate mutations in pre-mRNA splicing factors, we examined these modifiers for their ability to alter the expression of other alleles that are sensitive to defects in the RNA splicing machinery. Two other transposable-element insertion alleles in the same intron as w^{a}, *white-blood* (7) and *white-apricot-4* (42), were affected by $B52^{ED}$ in a manner similar to w^{a} (Fig. 1B and data not shown). However, $B52^{ED}$ is not a general modifier of *white* expression; it has no effect on the pigmentation of the *white* point mutations *white-apricot-2* and *white-apricot-3* (Fig. 1C and data not shown), and also has no effect on the phenotype encoded by wild-type *white* alleles (data not shown).

We also examined modifiers of w^{a} for effects on w^{DR18}, a *white* transgene carrying nucleotide substitutions at positions −1 and +6 of the 5′ splice site of the second intron (ACG | **GT**GAGTT to ACC | **GT**GAGCT [44]). This mutation, which results in an apricot eye color and the accumulation of RNA that retains the second *white* intron, can be partially suppressed by a suppressor U1-3G transgene (44). Among five previously described modifiers of w^{a} (43) (they are *suppressor-of-white-apricot* [80], *suppressor-of-forked* [51], *mottler-of-white* [9], *Darkener-of-apricot* [62], and *Enhancer-of-white-apricot* [59]) and the nine novel dominant modifier alleles described by Kurkulos et al. (39), only $B52^{ED}$ significantly decreased the expression of w^{DR18} (Fig. 1D). Significantly, $B52^{ED}$ had an even greater phenotypic effect on this defined splice site mutation than on the w^{a} allele against which it was isolated (compare Fig. 1A and D).

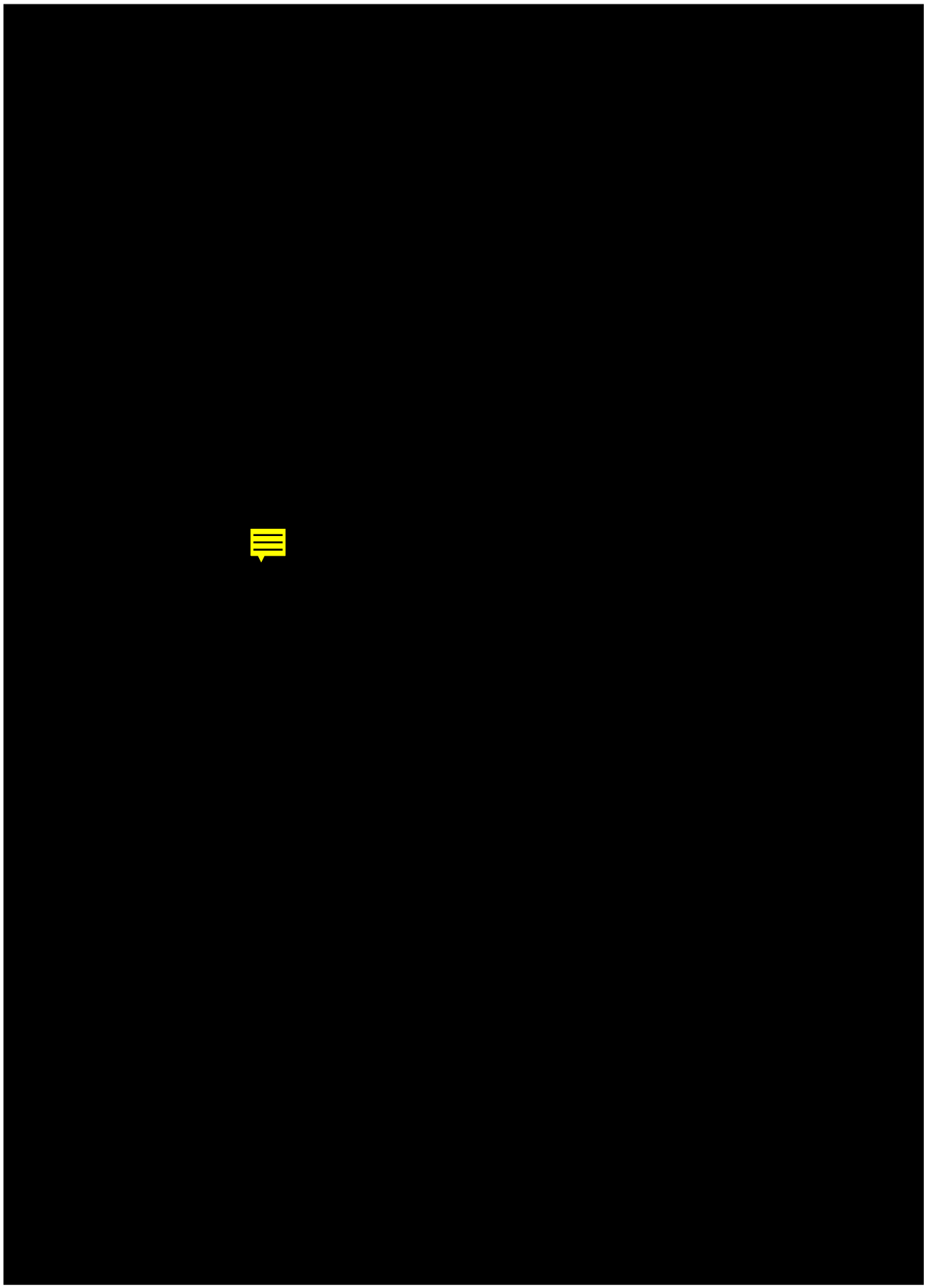
Synthetic lethality and enhancement of *Deformed*. In the process of mapping $B52^{ED}$ (described below), we set up a cross that should have generated $B52^{ED}$/*Dfd cu kar Df*(*3R*)ry^{27} progeny. However, very few adult progeny of this genotype were observed, and those that survived (typically less than 1% of the expected number) had head defects that resembled those observed in extreme cases of the *Dfd* dominant phenotype (15) (compare Fig. 1E and F). In addition to *Dfd* itself, this effect requires both $B52^{ED}$ and an uncharacterized genetic element on the *Dfd cu kar Df*(*3R*)ry^{27} chromosome [i.e., the combination $B52^{ED}$/*Dfd cu kar Df*(*3R*)ry^{27} is fully viable if the uncharacterized genetic element is removed from the *Dfd cu kar Df*(*3R*)ry^{27} chromosome by recombination (53)]. Although $B52^{ED}$ enhances *Dfd* in the absence of any other genetic elements (58), the severity of this effect is much less than that observed in the context of the *Dfd cu kar Df*(*3R*)ry^{27} chromosome. The semilethal interaction between $B52^{ED}$ and the *Dfd cu kar Df*(*3R*)ry^{27} chromosome, although complex and not understood, is useful as another dominant phenotype of $B52^{ED}$.

The dominant modifier is an allele of *B52*. We determined that our dominant modifier ($B52^{ED}$, originally designated *E726* [39]) is an allele of *B52* in several different ways.

First, we found that $B52^{ED}$ is lethal when homozygous and when in *trans* to deficiencies [such as *Df*(*3R*)*urd*] that include the chromosomal region 87F (Fig. 2). Lethality could not be recombinationally separated from the dominant enhancement of w^{a} and so was considered likely to be due to $B52^{ED}$. The lethality of $B52^{ED}$ could be complemented by sqd^{ix50}, a lethal allele of the hnRNP protein gene *squid* (31) (Fig. 2), but not by $Hrb87F^{\#7}$, a small deletion that extends from the 5′ end of *Hrb87F* into the 5′ end of *B52* (coordinates −14 through −16 on the map in Fig. 2) (27, 58). $Hrb87F^{\#7}$ is similar to $B52^{28}$, a small deletion described by Ring and Lis (63); both $Hrb87F^{\#7}$ and $B52^{28}$ were generated by imprecise excision of the *P* element in $Hrb87F^{2}$, and both remove the 5′ terminus of *Hrb87F* and extend into *B52* (27, 58, 63). Because *Hrb87F* is not essential (27), these results imply that $B52^{ED}$ is indeed an allele of *B52*.

To localize $B52^{ED}$ more precisely within this region, we sought recombinants between $B52^{ED}$ and the lethal allele sqd^{ix50} (31) (Fig. 2) that were viable in *trans* to *Df*(*3R*)*urd*. Only one such recombinant was isolated in an experiment that generated 13,980 control flies. These results indicate that $B52^{ED}$ lies extremely close to *squid*, as is expected of a *B52* allele. The exchange of closely spaced flanking markers (*ry* at 52.0 and *cv-c* at 54.1) indicates that this was indeed a recombination event rather than a reversion and allowed orientation of the genetic map with respect to the previously determined physical map of the region as shown in Fig. 2.

We next carried out a reversion analysis. We had observed that heterozygosity for deficiencies that uncover the $B52^{ED}$ lethality [such as *Df*(*3R*)*urd*] has little or no effect on the eye color phenotype of w^{a} flies. From this, we reasoned that the $B52^{ED}$ dominant allele was not a loss-of-function allele but that loss-of-function alleles could be isolated by reversion. A total of 136,132 progeny of X-irradiated w^{a}; $B52^{ED}$/*TM3* males were examined for eye pigmentation, and 20 phenotypic revertants were recovered. Of these, 13 were identified as new alleles of



the dominant suppressor *Doa* (62, 79) and an additional 5 phenotypic revertants appear to have mutations in novel genes. Two revertant alleles from this screen, $B52^{R1}$ and $B52^{R2}$, were identified as bona fide reversions of the $B52^{ED}$ dominant allele on the basis of our inability to recover nonrevertant $B52^{ED}$ by recombination and the reversion of other dominant phenotypes [enhancement of w^{DR18}, w^{a4}, and the *Dfd cu kar Df(3R)ry*^{27} synthetic lethality]. Simultaneous reversion of these dominant phenotypes shows that they are indeed due to a single genetic element mutated in both reversion events. Neither $B52^{R1}$ nor $B52^{R2}$ is associated with a chromosomal rearrangement that could be detected by examination of polytene chromosomes (58). However, molecular defects were observed when DNA from $B52^{R1}$ and $B52^{R2}$ was examined with probes from the region between coordinates −31 and +14 on the map shown in Fig. 2. In the case of $B52^{R2}$, small differences in the mobility of restriction fragments on Southern blots were traced to a 25-nucleotide deletion beginning within the ninth codon of the B52 coding region (Fig. 2). This deletion not only removes the nucleotides that encode conserved amino acids in the RNP-2 motif of the first RRM of B52 but also introduces a frameshift that almost certainly prevents expression of the remaining coding region. We conclude that $B52^{R2}$ is a null allele of *B52*. Analysis of the $B52^{R1}$ allele revealed a deletion that removed sequences including the first exon encoding B52 and extended to the left, including all of the *squid* gene (Fig. 2). Thus, two independent mutations detected as revertants of the dominant effect of $B52^{ED}$ on w^a expression both have alterations that preclude expression of *B52*, and the lesion detected in the $B52^{R2}$ allele affects only *B52*.

That our dominant mutation maps to *B52* is also supported by complementation tests among six chromosomes carrying mutations in this region. As expected, $B52^{R1}$ and $B52^{R2}$, both of which carry deletions that preclude production of wild-type B52 protein, fail to complement $Hrb87F^{\#7}$ or $B52^{28}$, with death occurring during the embryonic or larval stages. $B52^{ED}$ also fails to complement $Hrb87F^{\#7}$ or $B52^{28}$, but death occurs later, during the larval or pupal stages. The observation that the revertants $B52^{R1}$ and $B52^{R2}$ are associated with an earlier lethal stage than the original $B52^{ED}$ allele when in *trans* to bona fide *B52* null alleles or deficiencies is expected from our molecular evidence that they are more severe mutations in this same gene and is consistent with our strategy for isolating null alleles from a dominant allele. Significantly, each of the three alleles $B52^{R1}$, $B52^{R2}$ and $B52^{ED}$ shows the same lethal phase in combination with the small $Hrb87F^{\#7}$ and $B52^{28}$ deficiencies as in combination with the much larger *Df(3R)urd*. Since *Hrb87F* is not lethal mutable and since both $B52^{28}$ and $Hrb87F^{\#7}$ can be rescued by a *B52* transgene (53, 63), these results demonstrate that the original $B52^{ED}$ allele carries a defect in the *B52* gene.

Finally, the lethality of $B52^{R2}/B52^{ED}$ is also rescued by the same *B52* transgene (53), which shows that there are no lethal alleles on the $B52^{ED}$ chromosome outside of the *B52* region.

As an allele of *B52*, the $B52^{ED}$ dominant allele might be expected to carry a mutation that affects the B52 protein. In the next section, we show that the $B52^{ED}$ dominant allele indeed carries a mutation in a conserved residue of the B52 RNA-binding domain.

The $B52^{ED}$ dominant allele carries a glutamate-to-lysine change in the amino-terminal RNA-binding domain of B52. Because we have maintained in the laboratory a number of lines carrying the wild-type chromosome on which $B52^{ED}$ was derived, it was possible to identify the molecular basis of this mutation by directly comparing $B52^{ED}$ with its parental wild-type allele. Rather than sequencing the entirety of the *B52* gene from both $B52^{ED}$ and its wild-type parent, we chose to sequence cDNA from these two alleles. First, Northern blot analysis was carried out on RNA from adults heterozygous for either $B52^{ED}$, $B52^{R2}$, or $B52^{R1}$ (58). Flies heterozygous for either $B52^{R2}$ or $B52^{R1}$ have levels of B52 RNA that are reduced approximately twofold relative to those in the wild type, implying that the two null alleles contribute little RNA to steady-state levels in adults. In contrast, RNA levels were unaltered in the case of $B52^{ED}$. This suggested that the $B52^{ED}$ allele produces normal levels of *B52* mRNA. RNA was then isolated from larvae hemizygous for $B52^{ED}$ [$B52^{ED}/Df(3R)urd$] or its parental B52$^+$ allele [$B52^+/Df(3R)urd$], reverse transcribed, and used for amplification and sequencing.

The sequence of cDNA from $B52^{ED}$ is identical to that from the parental $B52^{ED+}$ allele in every position but one, position 208, where the dominant allele carries an A and the wild type carries a G (Fig. 2). This change results in substitution of lysine for glutamic acid at position 70 of the B52 protein, within the amino-terminal RNA-binding domain. Alignment with other RRM proteins (10, 32) indicates that this position is highly conserved among SR proteins (where it is glutamic acid in the *Drosophila* proteins B52 and RBP1 and in the human proteins ASF/SF2, SRp20, and SRp75 but glutamine in SC35) but only moderately conserved among all RRMs. Examination of RRMs whose three-dimensional structure is known suggests that the altered amino acid projects outward from the surface of the protein (Fig. 3). Furthermore, in the recently published crystal structure of the RNA-binding domain of the U1A protein complexed with RNA (57), the corresponding amino acid (Gln-85) makes hydrogen-bonding contact with a C residue that is stacked on a conserved tyrosine residue within RNP-2 (on the adjacent β1 strand). Görlach et al. (26) have monitored the binding of hnRNP C to U_8 by nuclear magnetic resonance spectroscopy. In this case, the corresponding amino acid is Asn-83, and its chemical shift upon oligonucleotide binding is one of the highest in the protein. By analogy to these structurally similar proteins, we consider it very likely that Glu-70 makes contact with RNA. Because the mutation to lysine would reverse the charge on the side chain, it follows that the $B52^{ED}$ mutation is likely to alter the interaction between B52 and RNA, and a reduced affinity or altered specificity of binding would be expected.

The $B52^{ED}$ dominant mutation alters somatic sex determination and the sex-specific splicing of *doublesex*. The sexual differentiation of somatic tissues is under the control of *doublesex* (*dsx* [6]). Male- and female-specific splicing leads to the production of sex-specific proteins (DSX^M and DSX^F), which share a DNA-binding domain (21) and are believed to act directly to regulate downstream genes to bring about sex-specific phenotypes (12). The regulation of sex-specific splicing at *doublesex* is critically dependent upon SR proteins (see refer-

FIG. 1. Enhancement of mutant phenotypes by $B52^{ED}$. (A) Effect of one copy of $B52^{ED}$ on *white-apricot*, a *copia* retrotransposon insertion in the second intron of the eye pigmentation gene *white* with a complex RNA-processing defect. Genotypes: w^a (left) and w^a; $B52^{ED}/TM3$ (right). (B) Effect of one copy of $B52^{ED}$ on *white-blood*, an independent *BEL* retrotransposon insertion in the same intron as in panel A. Genotypes: w^{bl}; *TM3*/+ (left) and w^{bl}; $B52^{ED}$/+ (right). (C) Effect of one copy of $B52^{ED}$ on *white-apricot-2*, a point mutation in *white*. Genotypes: w^{a2}; *TM3*/+ (left) and w^{a2}; $B52^{ED}$/+ (right). (D) Effect of one copy of $B52^{ED}$ on w^{DR18}, a 5′-splice-site mutation. Genotypes: w^{118}; P[w^{DR18}]/+; *TM3*/+ (left) and w^{1118}; P[w^{DR18}]/+; $B52^{ED}$/+ (right). (E) A wild-type *Drosophila* head. (F) Head from a *Dfd cu kar Df(3R)ry*$^{27}/B52^{ED}$ pharate adult. (G) Effect of one copy of $B52^{ED}$ on the abdominal phenotype of $tra2^B$/+; *tra*/+ transheterozygotes. Genotypes: $tra2^B$/+; tra/+ (left) and $tra2^B$/+; $tra/B52^{ED}$+ (right).

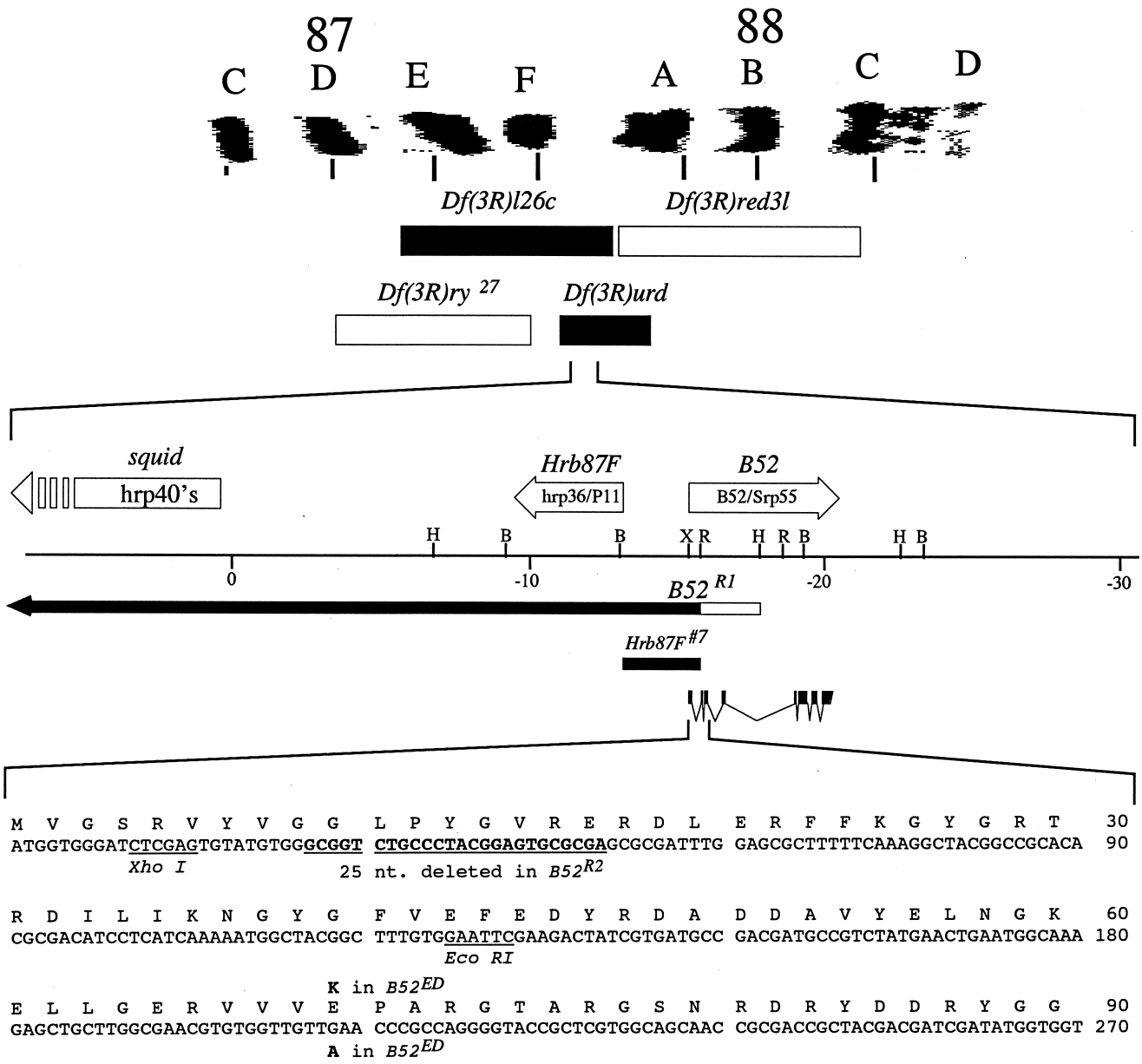
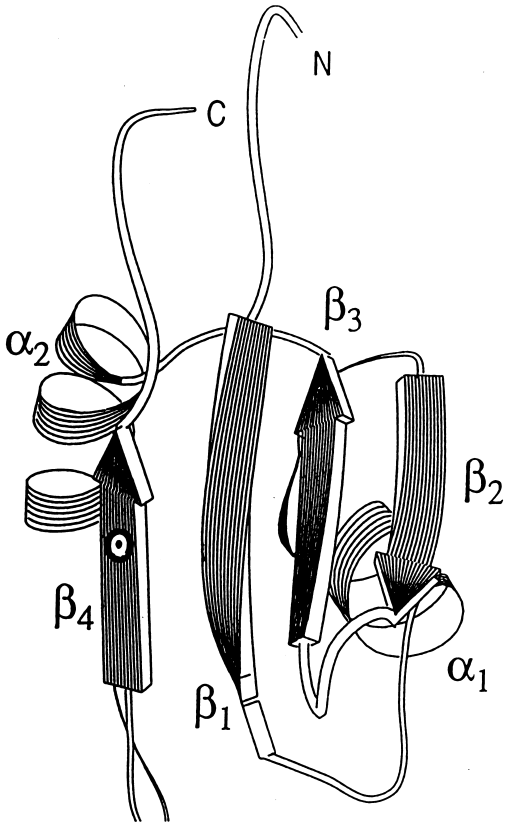


FIG. 2. Genetic and molecular map of the region surrounding *B52*. The region surrounding the $B52^{ED}$ mutation is shown at three levels of resolution. First, the approximate extents of deficiencies used to map $B52^{ED}$ are shown relative to a schematic polytene chromosome of this region (43). Deficiencies that fail to complement the lethality of $B52^{ED}$ are shown in black, while those that complement are shown in white. Below the polytene map is a partial restriction map of the 87F RNA-binding protein gene cluster. The relative orientation of the chromosome and restriction maps is inferred from the exchange of flanking markers observed in a single recombination event between $B52^{ED}$ and sqd^{ex50} (see the text). The numerical coordinates (in kilobases), as well as the locations of *squid* and *Hrb87F*, are taken from reference 31. Only restriction sites that we have confirmed by Southern blot analysis in the course of this study are shown. H, *Hin*dIII; B, *Bam*HI; E, *Eco*RI; X, *Xho*I). A white region at the right indicates uncertainty in boundary of the $B52^{R1}$ deletion as determined by Southern analysis. The cDNA sequence shown was derived from both $B52^{ED}$ and $B52^{+}$ RNA as described in Materials and Methods; only a portion is shown here. The only difference observed between $B52^{ED}$ and its $B52^{+}$ parental allele is indicated in boldface type (G-208→A; E-70→K).

ence 45 and references therein) and requires the expression of *transformer* in females but not males. The *transformer* (*tra*) and *transformer 2* (*tra2*) genes encode proteins with RS domains that stimulate use of a female-specific exon in preference to male-specific 3′ exons (12, 28, 65). The RS domain proteins Tra and Tra2, encoded by *tra* and *tra2*, respectively, are not SR proteins, as that term is used here, because they do not carry SR-type RRMs (Tra has no RRM, and the Tra2 RRM is not of the SR protein type) and cannot complement splicing-deficient S100 extract. However, these proteins do contain RS domains, and stimulation of female-specific splicing by Tra and Tra2 appears to be mediated by their ability to attract constitutively expressed SR proteins to the vicinity of the 3′ splice site (74). Monitoring *dsx* expression in females is advantageous because *dsx* is a natural gene in which the alteration of splicing leads not to defects but to an alternative wild-type state (maleness) that is easily detected.

To sensitize the expression of *doublesex* to $B52^{ED}$, we examined animals with reduced levels of the proteins Tra and Tra2. Females of the genotype $tra2^{B}$/+; *tra*/+ are sometimes inter-



ß4 strand of selected RNA binding domains:

Orientation	**--⇓⇑⇓⇑⇓---**
dro70K	**KRVLVDVERA**
dSRp55/B52	**ERVVVEPARG**
hSRp75	**ERVIVEHARG**
ASF/SF2	**YRLRVEFPRS**
U1A	**KPMRIQYAKT**
hnRNPC1/C2	**QVLDINLAAE**

FIG. 3. Location of the $B52^{ED}$ (Glu-70→Lys) mutation in the B52/SRp55 amino-terminal RNA-binding domain. The site of the glutamate-to-lysine change associated with the $B52^{ED}$ allele is indicated by a bull's-eye on a ribbon diagram of the RNA-binding domain and by underlining in the alignment of β4 strands, which is shown below the ribbon diagram and was used to place Glu-70 on the structure. Note the prominent location of this amino acid on the RNA-binding face of the β-sheet. This alignment follows those of Kenan et al. (32) and Zahler et al. (82). The ribbon diagram was provided by G. Dreyfuss and is based on a set of coordinates for hnRNP C amino acids 15 to 86 (corresponding to B52/SRp55 amino acids 3 to 73) determined by nuclear magnetic resonance spectroscopy (77). Very similar structures have been determined for the first RNA-binding domain of human U1 snRNP A by both nuclear magnetic resonance spectroscopy (29) and X-ray crystallography (54, 57). Aligned β4 strands from selected RNA-binding domains include *Drosophila* U1 70K (46), two other SR proteins (ASF/SF2 [25, 37] and hSRp75 [84]), and the two RNA-binding domains whose structures have been determined (54, 77). ⇓ indicates amino acid residues that project into the interior of the domain and contribute to the hydrophobic core of the protein, while ⇑ indicates residues that project outwards from the RNA-binding surface.

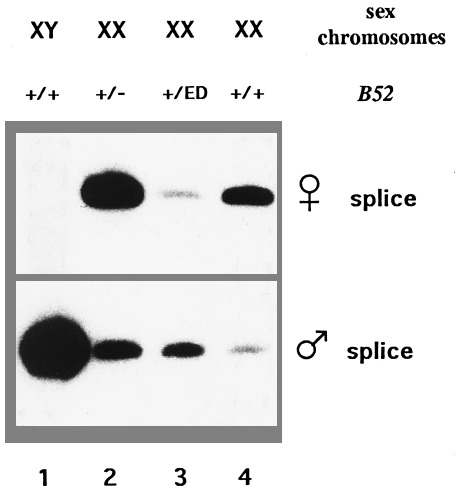


FIG. 4. One copy of the dominant $B52^{ED}$ allele alters the sex-specific splicing of *doublesex*. Male- and female-specific forms of *doublesex* RNA were reverse transcribed and amplified with the oligonucleotide primers of Amrein et al. (2) as described by Lo et al. (44). All lanes show amplification products derived from flies transheterozygous for *transformer* and *transformer 2*. Males of the genotype $tra2^{B}$/+; *tra*/+ (designated XY and +/+; lane 1) show no detectable female RNA. Females homozygous for $B52^{+}$ (genotype $tra2^{B}$/+; *tra*/+, designated XX and +/+; lane 4) have a very low ratio of male *dsx* mRNA- to female *dsx* mRNA-derived amplification products, while females heterozygous for $B52^{ED}$ (genotype $tra2^{B}$/+; *tra*/$B52^{ED}$, designated XX and +/ED; lane 3) have a significantly increased ratio of male *dsx* mRNA- to female *dsx* mRNA-derived amplification products. Reversion of the $B52^{ED}$ dominant reverses the effect on ratios between male and female RT-PCR products (genotype $tra2^{B}$/+; *tra*/$B52^{R2}$, designated XX and +/−; lane 2).

sexual, particularly at higher temperatures and in response to alterations in the genetic background, including mutations in other sex determination genes (6, 56). We have observed that such doubly heterozygous females are overtly intersexual no more than 3% of the time at 29°C. To examine the effect of $B52^{ED}$ on this genotype, we compared $tra2^{B}$/+; *tra*/$B52^{ED}$ triply heterozygous animals with $tra2^{B}$/+; *tra*/$B52^{+}$ and $tra2^{B}$/+; *tra*/$B52^{R2}$ controls. We found a significant number of intersexual animals among those carrying the dominant enhancer, even at 25°C. In one experiment, we observed that 337 of 586 of all females with the genotype $tra2^{B}$/+; *tra*/$B52^{ED}$ (58%) had intersexual features (primarily genital defects [Fig. 1G]), while females with the genotype $tra2^{B}$/+; *tra*/$B52^{R2}$ show this phenotype no more frequently than do $tra2^{B}$/+; *tra*/+ controls (0 of 374 versus 0 of 883 in this experiment, which was carried out at 25°C). This phenotype is also dependent upon both *tra* and *tra2* being heterozygous (53, 58). Since $B52^{R2}$ is a null derivative of $B52^{ED}$, this experiment indicates that the $B52^{ED}$ dominant allele acts, in the context of reduced amounts of the *transformer* and *transformer 2* regulatory proteins, to interfere with the activation of the female *doublesex* 3′ splice site.

To confirm that the splicing of *doublesex* RNA is indeed affected in these $tra2^{B}$/+; *tra*/$B52^{ED}$ females, we examined RNA from these flies by RT-PCR with primers specific for the male- and female-specific exons of *doublesex* RNA (2). These experiments were conducted by RT and quantification of the incorporation of labeled nucleotides into PCR products during the exponential phase of the amplification. We observed that $tra2^{B}$/+; *tra*/$B52^{ED}$ females indeed contain an increased ratio of *dsx* RNA spliced in the male mode to *dsx* RNA spliced in the female mode when compared with $tra2^{B}$/+; *tra*/+ or $tra2^{B}$/+; *tra*/$B52^{R2}$ controls (Fig. 4). Thus, the phenotypic effect observed is indeed correlated with the expected effect on *dsx* splicing.

DISCUSSION

We have described the isolation of both dominant and loss-of-function mutations in the gene encoding the *Drosophila* SR protein B52. $B52^{ED}$, identified as a dominant second-site en-

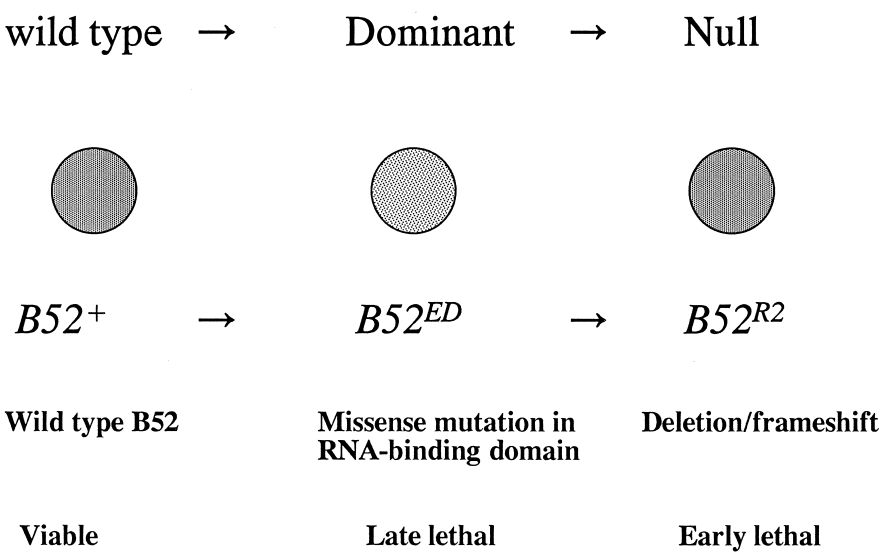


FIG. 5. Summary of the strategy followed here for the isolation of mutations in *B52*. The strategy followed in this paper, and the results obtained, are schematically summarized. First, the $B52^{ED}$ allele was isolated as a dominant enhancer of *white-apricot*. This allele carries a missense mutation in *B52*, resulting in death during larval or pupal stages when homozygous or in *trans* to a deficiency. In a second step, a loss-of-function *B52* allele was isolated by screening for inactivation of the dominant allele. The resulting allele, $B52^{R2}$, carries a 25-bp deletion/frameshift which precludes expression of B52 protein and has an earlier lethal phase when homozygous or in *trans* to a deficiency. Shading within the circles schematically indicates the level of eye pigmentation observed in w^a flies heterozygous for the indicated *B52* allele.

hancer of w^a, also exaggerates the mutant phenotype of a number of mutant alleles with splicing defects, including a distinct *white* allele carrying a 5′ splice site mutation (w^{DR18}). The $B52^{ED}$ dominant allele carries a single amino acid change in the β4 strand of the first RNA-binding domain of B52, indicating that the RNA-binding domain of this SR protein is critical for pre-mRNA splicing decisions in a multicellular organism. The strategy for isolating loss-of-function alleles of *B52* from the $B52^{ED}$ dominant allele is summarized in Fig. 5.

Possible molecular bases of the $B52^{ED}$ dominant phenotype. The $B52^{ED}$ allele carries a Glu-70→Lys change within the first RRM, at a position on the RNA-binding surface that is highly conserved among SR proteins. Expression of mutant B52 protein from the $B52^{ED}$ allele could lead to a dominant phenotype in either of two ways. An alteration in the specificity of RNA binding could result in the formation of inappropriately positioned splicing enhancer complexes and could redirect splicing factors to inappropriate splice sites. This hypothesis is appealing because of the demonstrated role of SR proteins in modulating 5′ splice site choice in vitro and because of the role of SR proteins in the recognition of exonic splicing enhancers (40, 70, 74). Alternatively, the mutation could simply reduce the affinity of B52 for RNA. In this case, the mutant protein might still participate in the formation of complexes with other proteins involved in splicing, thereby preventing those proteins, and possibly an entire preformed complex, from making appropriate interactions with RNA. This hypothesis is supported by recent data showing that the SR proteins SC35 and ASF/SF2 bind U1-70K and U2AF (34, 78).

The question of dominance can be considered specifically in the case of *doublesex*, whose alternative splicing has been examined in detail by Maniatis and coworkers (2, 45, 73–75). Sequences in the *doublesex* female-specific exon that are responsible for sex-specific activation of the splice (28, 65) are bound by a complex of Tra, Tra2, and SR proteins in vitro (74, 75). Of particular relevance to our results is the observation that Tra, Tra2, and B52 (SRp55) proteins bind cooperatively to a splicing enhancer sequence in the female-specific exon (45). We think it likely that B52 is normally a part of an in vivo complex and that the Glu-70→Lys mutation confers a dominant negative activity because the mutant protein is recruited to the *dsx* splicing enhancer complex by Tra and Tra2 but is unable to make the appropriate contacts with *dsx* pre-mRNA. In this specific case, either altered RNA-binding specificity or reduced affinity for RNA could account for the negative effect on inclusion of the female-specific *dsx* exon.

Essential and redundant genes as regulators of splicing. *B52* provides a contrast to the paradigm established by earlier examples of *Drosophila* genes encoding *trans*-acting regulators of splicing, such as *Sex lethal*, *transformer*, and *transformer 2*. The products of these genes act on specific well-defined targets (4, 47), and the specificity of their action is underscored by their genetic properties; null alleles in all three of these genes are viable in males (43). Furthermore, other potential splicing regulators identified by genetic screens, *suppressor-of-sable* and *suppressor-of-white-apricot*, are also nonessential (43). It is known that SR proteins provide a function essential for splicing in vitro. Ring and Lis have shown that null alleles of *B52* confer a lethal phenotype when homozygous (63), and we have confirmed this result. Thus, *B52* is an essential gene encoding a protein that can function in splicing. However, it has not been possible to observe splicing defects in *B52* null animals (58, 63). One explanation for this observation is possible instability of unspliced or aberrantly spliced RNAs. Another possibility is that only a subset of splicing events require *B52*; although other SR proteins can substitute for B52 in the splicing of most substrates, an unknown number of substrates may require *B52* in particular for accurate splicing. Even if one such substrate exists, the aberrant splicing of that substrate could account for developmental failure.

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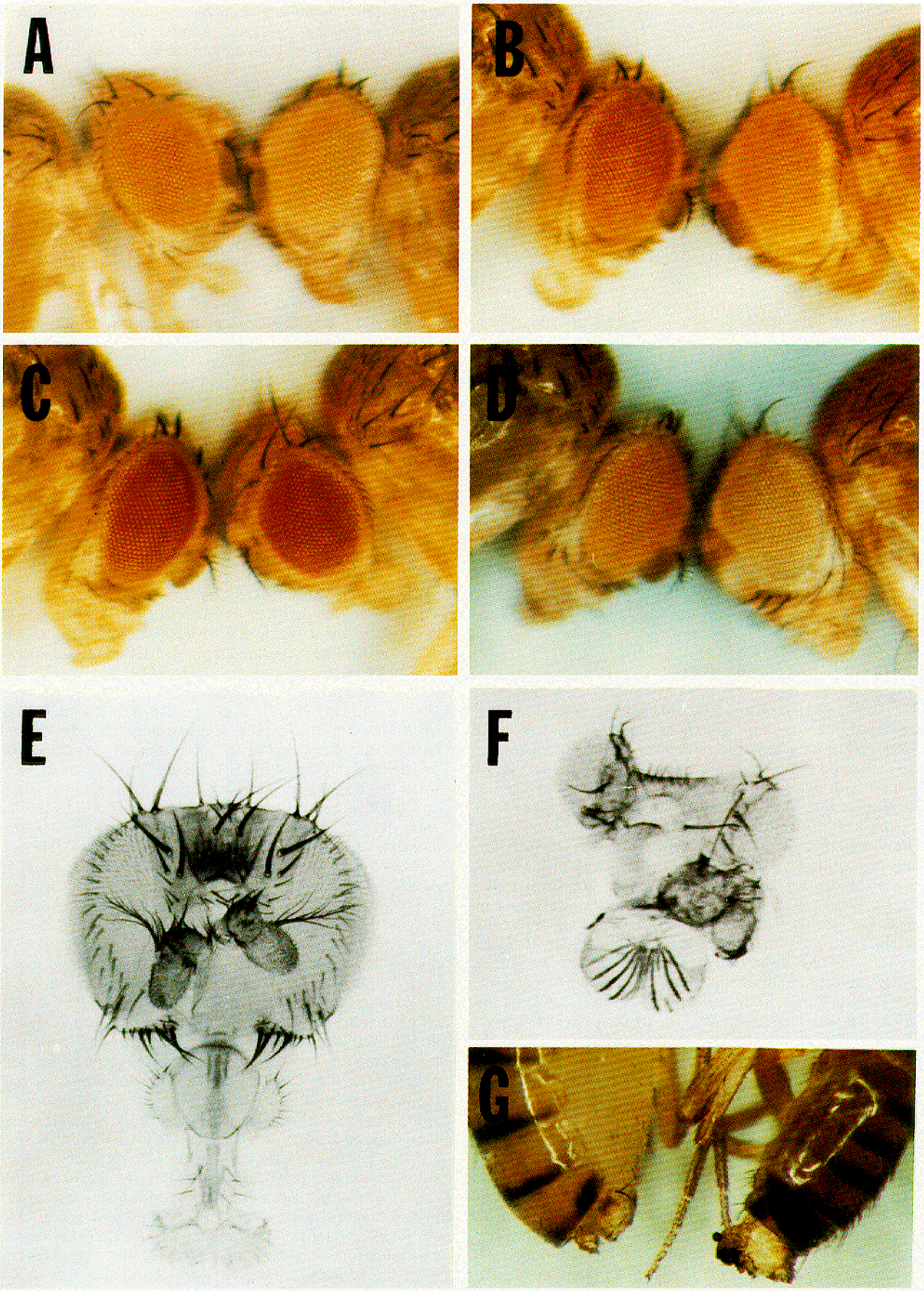
REFERENCES

1. **Abovich, N., X. C. Liao, and M. Rosbash.** 1994. The yeast MUD2 protein: an interaction with PRP11 defines a bridge between commitment complexes and U2 snRNP addition. Genes Dev. **8:**843–854.
2. **Amrein, H., M. L. Hedley, and T. Maniatis.** 1994. The role of specific protein-RNA and protein-protein interactions in positive and negative control of pre-mRNA splicing by *transformer 2*. Cell **76:**735–746.
3. **Ashburner, M.** 1989. *Drosophila*: a laboratory manual, p. 247–307. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
4. **Baker, B. S.** 1989. Sex in flies: the splice of life. Nature (London) **340:**521–524.
5. **Ballinger, D. G., and S. Benzer.** 1989. Targeted gene mutations in *Drosophila*. Proc. Natl. Acad. Sci. USA **86:**9402–9406.
6. **Belote, J. M., M. B. McKeown, D. J. Andrew, T. N. Scott, M. F. Wolfner, and B. S. Baker.** 1985. Control of sexual differentiation in *Drosophila melanogaster*. Cold Spring Harbor Symp. Quant. Biol. **50:**605–614.
7. **Bingham, P. M., and C. H. Chapman.** 1986. Evidence that *white-blood* is a novel type of temperature-sensitive mutation resulting from temperature-dependent effects of a transposon insertion on formation of *white* transcript. EMBO J. **5:**3343–3351.
8. **Bingham, P. M., T.-B. Chou, I. Mims, and Z. Zachar.** 1988. On-off regulation of gene expression at the level of splicing. Trends Genet. **4:**134–138.
9. **Birchler, J. A., J. C. Hiebert, and L. Rabinow.** 1989. Interaction of the *mottler-of-white* with transposable element alleles at the *white* locus in *Drosophila melanogaster*. Genes Dev. **3:**73–84.
10. **Birney, E., S. Kumar, and A. R. Krainer.** 1993. Analysis of the RNA-recognition motif and RS and RGG domains: conservation in metazoan pre-mRNA splicing. Nucleic Acids Res. **21:**5803–5816.
11. **Braaten, D., M. Kurkulos, and S. Mount.** Unpublished data.
12. **Burtis, K. C., and B. S. Baker.** 1989. *Drosophila doublesex* gene controls somatic sexual differentiation by producing alternatively spliced mRNAs encoding related sex-specific polypeptides. Cell **56:**997–1010.
13. **Cáceres, J. F., and A. R. Krainer.** 1993. Functional analysis of pre-mRNA splicing factor SF2/ASF structural domains. EMBO J. **12:**4715–4726.
14. **Chabot, B. F., D. Frappier, and H. La-Branche.** 1992. Differential ASF/SF2 activity in extracts from normal WI38 and transformed WI38VA13 cells. Nucleic Acids Res. **20:**5197–5204.
15. **Chadwick, R., B. Jones, T. Jack, and W. McGinnis.** 1990. Ectopic expression from the *Deformed* gene triggers a dominant defect in *Drosophila* adult head development. Dev. Biol. **141:**130–140.
16. **Champlin, D. T., M. Fräsch, H. Saumweber, and J. T. Lis.** 1991. Characterization of a *Drosophila* protein associated with boundaries of transcriptionally active chromatin. Genes Dev. **5:**1611–1621.
17. **Crispino, J. D., B. J. Blencowe, and P. A. Sharp.** 1994. Complementation by SR proteins of pre-mRNA splicing reactions depleted of U1 snRNP. Science **265:**1866–1869.
18. **Diamond, R. H., K. Du, V. M. Lee, K. L. Mohn, B. A. Haber, D. S. Tewari, and R. Taub.** 1993. Novel delayed-early and highly insulin-induced growth response genes: identification of HRS, a potential regulator of alternative pre-mRNA splicing. J. Biol. Chem. **268:**15185–15192.
19. **Dreyfuss, G., M. J. Matunis, S. Piñol-Roma, and C. G. Burd.** 1993. hnRNP proteins and the biogenesis of RNA. Annu. Rev. Biochem. **62:**289–321.
20. **Eperon, I. C., D. C. Ireland, R. A. Smith, A. Mayeda, and A. R. Krainer.** 1993. Pathways for selection of 5′ splice sites by U1 snRNPs and SF2/ASF. EMBO J. **12:**3607–3617.
21. **Erdman, S. E., and K. C. Burtis.** 1993. The *Drosophila doublesex* proteins share a novel zinc finger related DNA binding domain. EMBO J. **12:**527–535.
22. **Fu, X.-D.** 1993. Specific commitment of different pre-mRNAs to splicing by single SR proteins. Nature (London) **365:**82–85.
23. **Fu, X.-D., and T. Maniatis.** 1992. General splicing factors SF2 and SC35 have equivalent activities in vitro, and both affect alternative 5′ and 3′ splice site selection. Proc. Natl. Acad. Sci. USA **89:**11224–11228.
24. **Ge, H., and J. L. Manley.** 1990. A protein factor, ASF, controls cell-specific alternative splicing of SV40 early pre-mRNA in vitro. Cell **42:**355–367.
25. **Ge, H., P. Zuo, and J. L. Manley.** 1991. Primary structure of the human splicing factor ASF reveals similarities with *Drosophila* regulators. Cell **66:**373–382.
26. **Görlach, M., M. Wittekind, R. A. Beckman, L. Mueller, and G. Dreyfuss.** 1992. Interaction of the RNA-binding domain of the hnRNP C proteins with RNA. EMBO J. **11:**3289–3295.
27. **Haynes, S. R.** Personal communication.
28. **Hedley, M. L., and T. Maniatis.** 1991. Sex-specific splicing and polyadenylation of dsx pre-mRNA requires a sequence that binds specifically to TRA2 protein in vitro. Cell **65:**579–586.
29. **Hoffman, D. W., C. C. Query, B. L. Golden, S. W. White, and J. D. Keene.** 1991. RNA-binding domain of the A protein component of the U1 small nuclear ribonucleoprotein analyzed by NMR spectroscopy is structurally similar to ribosomal proteins. Proc. Natl. Acad. Sci. USA **88:**2495–2499.
30. **Kao, H., and P. G. Siliciano.** 1992. The yeast homolog of the U1 snRNP protein 70K is encoded by the *SNP1* gene. Nucleic Acids Res. **20:**4009–4013.
31. **Kelley, R. L.** 1993. Initial organization of the *Drosophila* dorsoventral axis depends on an RNA-binding protein encoded by the *squid* gene. Genes Dev. **7:**948–960.
32. **Kenan, D. J., C. C. Query, and J. D. Keene.** 1991. RNA recognition: towards identifying determinants of specificity. Trends Biochem. Sci. **16:**214–220.
33. **Kim, Y.-J., P. Zuo, J. L. Manley, and B. S. Baker.** 1992. The *Drosophila* RNA-binding protein RBP1 is localized to transcriptionally active sites of chromosomes and shows a functional similarity to human splicing factor ASF/SF2. Genes Dev. **6:**2569–2579.
34. **Kohtz, J. D., S. F. Jamison, C. L. Will, P. Zuo, R. Lührmann, M. A. Garcia-Blanco, and J. L. Manley.** 1994. Protein-protein interactions and 5′ splice site recognition in mammalian mRNA precursors. Nature (London) **368:**119–124.
35. **Kohtz, J. D., and J. L. Manley.** Personal communication.
36. **Krainer, A. R., G. C. Conway, and D. Kozak.** 1990. Purification and characterization of pre-mRNA splicing factor SF2 from HeLa cells. Genes Dev. **4:**1158–1171.
37. **Krainer, A. R., A. Mayeda, D. Kozak, and G. Binns.** 1991. Functional expression of cloned human splicing factor SF2: homology to RNA-binding proteins, U1 70K, and *Drosophila* splicing regulators. Cell **66:**383–394.
38. **Kraus, M. E., and J. T. Lis.** 1994. The concentration of B52, an essential splicing factor and regulator of splice site choice, is critical for *Drosophila* development. Mol. Cell. Biol. **14:**5360–5370.
39. **Kurkulos, M., J. M. Weinberg, M. E. Pepling, and S. M. Mount.** 1991. Polyadenylation in *copia* requires unusually distant upstream sequences. Proc. Natl. Acad. Sci. USA **88:**3038–3042.
40. **Lavigueur, A., H. L. Branch, A. R. Kornblihtt, and B. Chabot.** 1993. A splicing enhancer in the human fibronectin alternate ED1 exon interacts with SR proteins and stimulates U2 snRNP binding. Genes Dev. **7:**2405–2417.
41. **Lee, A. L., R. Kanaar, D. C. Rio, and D. E. Wemmer.** 1994. Resonance assignments and solution structure of the second RNA-binding domain of sex-lethal determined by multidimensional heteronuclear magnetic resonance. Biochemistry **33:**13775–13786.
42. **Levis, R., K. O'Hare, and G. M. Rubin.** 1984. Effects of transposable element insertions on RNA encoded by the *white* gene of *Drosophila*. Cell **38:**471–481.
43. **Lindsley, D. L., and G. G. Zimm.** 1992. The genome of *Drosophila melanogaster*. Academic Press, Inc., New York.
44. **Lo, P. C. H., D. Roy, and S. M. Mount.** 1994. Suppressor U1 snRNAs in *Drosophila*. Genetics **138:**365–378.
45. **Lynch, K., and T. Maniatis.** 1995. Synergistic interactions between two distinct elements of a regulated splicing enhancer. Genes Dev. **9:**284–293.
46. **Mancebo, R., P. C. H. Lo, and S. M. Mount.** 1990. Structure and expression of the Drosophila melanogaster gene for the U1 small nuclear ribonucleoprotein particle 70K protein. Mol. Cell. Biol. **10:**2492–2502.
47. **Maniatis, T.** 1991. Mechanisms of alternative pre-mRNA splicing. Science **251:**33–34.
48. **Manley, J. L.** 1993. Question of commitment. Nature (London) **365:**14.
49. **Mayeda, A., A. M. Zahler, A. R. Krainer, and M. B. Roth.** 1992. Two members of a conserved family of nuclear phohoproteins are involved in general and alternative splicing. Proc. Natl. Acad. Sci. USA **89:**1301–1304.

49a. **Meier, E., and X. Peng.** Unpublished data.

50. **Minvielle-Sebastia, L., P. J. Preker, and W. Keller.** 1994. RNA14 and RNA15 proteins as components of a yeast pre-mRNA 3′-end processing factor. Science **266:**1702–1705.
51. **Mitchelson, A., M. Simonelig, C. Williams, and K. O'Hare.** 1993. Homology with *Saccharomyces cerevisiae RNA14* suggests that phenotypic suppression in *Drosophila melanogaster* by *suppressor of forked* occurs at the level of RNA stability. Genes Dev. **7:**241–249.
52. **Mohler, J., and K. Vani.** 1992. Molecular organization and embryonic expression of the *hedgehog* gene involved in cell-cell communication in segmental patterning in *Drosophila*. Development **115:**957–971.
53. **Mount, S. M.** Unpublished data.

54. **Nagai, K. C., C. Oubridge, T. H. Jessen, J. Li, and P. R. Evans.** 1990. Crystal structure of the RNA-binding domain of the U1 small nuclear ribonucleoprotein A. Nature (London) **346:**515–520.
55. **O'Connell, P., and M. Rosbash.** 1984. Sequence, structure, and codon preference of the Drosophila ribosomal protein 49 gene. Nucleic Acids Res. **12:**5495–5513.
56. **Oliver, B., and B. Baker.** Personal communication.
57. **Oubridge, C., N. Ito, P. R. Evans, C.-H. Teo, and K. Nagai.** 1994. Crystal structure at 1.92Å resolution of the RNA-binding domain of the U1A spliceosomal protein complexed with an RNA hairpin. Nature (London) **372:** 432–438.
58. **Peng, X.** Unpublished data.
59. **Peng, X., and S. M. Mount.** 1990. Characterization of *Enhancer-of-white-apricot* and its effects on the copia insertion allele *white-apricot* in *Drosophila melanogaster*. Genetics **126:**1061–1069.
60. **Potashkin, J., K. Naik, and K. Wentz-Hunter.** 1993. U2AF homolog required for splicing in vivo. Science **262:**573–575.
61. **Query, C. C., R. C. Bently, and J. D. Keene.** 1989. A common RNA recognition motif identified within a defined U1 RNA binding domain of the 70K U1 snRNP protein. Cell **57:**89–101.
62. **Rabinow, L., and J. A. Birchler.** 1989. A dosage-sensitive modifier of retrotransposon-induced alleles of the *Drosophila white* locus. EMBO J. **8:**879–890.
63. **Ring, H. Z., and J. T. Lis.** 1994. The SR protein B52/SRp55 is essential for *Drosophila* development. Mol. Cell. Biol. **14:**7499–7506.
64. **Roth, M. B., A. M. Zahler, and A. M. Stalk.** 1991. A conserved family of nuclear phosphoproteins localized to sites of polymerase II transcription. J. Cell Biol. **115:**587–596.
65. **Ryner, L. C., and B. S. Baker.** 1991. Regulation of *doublesex* pre-mRNA splicing occurs by 3′ splice site activation. Genes Dev. **5:**2071–2085.
66. **Simon, M. A., D. D. L. Bowtell, G. S. Dodson, T. R. Laverty, and G. M. Rubin.** 1991. Ras1 and a putative guanine nucleotide exchange factor perform crucial steps in signaling by the sevenless tyrosine kinase. Cell **67:**701–716.
67. **Smith, V., and B. Barrell.** 1991. Cloning of a yeast U1 small nuclear RNP 70K protein homologue: functional conservation of an RNA-binding domain between humans and yeast. EMBO J. **10:**2627–2634.
68. **Spikes, D. A., J. Kramer, P. M. Bingham, and K. Van Doren.** 1994. SWAP pre-mRNA splicing regulators are a novel, ancient protein family sharing a highly conserved sequence motif with the *prp21* family of constitutive splicing proteins. Nucleic Acids Res. **22:**4510–4519.
69. **Staknis, D., and R. Reed.** 1994. SR proteins promote the first specific recognition of pre-mRNA and are present together with the U1 snRNP in a general splicing enhancer complex. Mol. Cell. Biol. **14:**7670–7682.
70. **Sun, Q., A. Mayeda, R. K. Hampson, A. R. Krainer, and F. M. Rottman.** 1993. General splicing factor SF2/ASF promotes alternative splicing by binding to an exonic splicing enhancer. Genes Dev. **7:**2598–2608.
71. **Takagaki, Y., and J. L. Manley.** 1994. A polyadenylation factor subunit is the human homolgue of the *Drosophila suppressor of forked* protein. Nature (London) **372:**471–474.
72. **Tarn, W.-Y., and J. A. Steitz.** 1994. SR proteins can compensate for functions of U1 snRNP functions in vitro. Genes Dev. **8:**2704–2717.
73. **Tian, M., and T. Maniatis.** 1992. Positive control of pre-mRNA splicing in vitro. Science **256:**237–240.
74. **Tian, M., and T. Maniatis.** 1993. A splicing enhancer complex controls alternative splicing of *doublesex* pre-mRNA. Cell **74:**105–114.
75. **Tian, M., and T. Maniatis.** 1994. A splicing enhancer exhibits both constitutive and regulated activities. Genes Dev. **8:**1703–1712.
76. **Voelker, R. A., W. Gibson, J. P. Graves, J. F. Sterling, and M. Eisenberg.** 1991. The *Drosophila suppressor-of-sable* gene encodes a polypeptide with regions similar to those of RNA-binding proteins. Mol. Cell. Biol. **11:**894–905.
77. **Wittekind, M., M. Görlach, M. Friedrichs, G. Dreyfuss, and L. Mueller.** 1992. ^{1}H, ^{13}C, and ^{15}N NMR assignments and global folding pattern of the RNA-binding domain of the human hnRNP C proteins. Biochemistry **31:** 6254–6265.
78. **Wu, J. Y., and T. Maniatis.** 1993. Specific interactions between proteins implicated in splice site selection and regulated alternative splicing. Cell **75:**1061–1070.
79. **Yun, B., R. Farkas, K. Lee, and L. Rabinow.** 1994. The *Doa* locus encodes a member of a new protein kinase family and is essential for eye and embryonic development in *Drosophila melanogaster*. Genes Dev. **8:**1160–1173.
80. **Zachar, Z., T.-B. Chou, and P. M. Bingham.** 1987. Evidence that a regulatory gene autoregulates splicing of its own transcript. EMBO J. **7:**4105–4111.
81. **Zachar, Z., T.-B. Chou, J. Kramer, I. P. Mims, and P. M. Bingham.** 1994. Analysis of autoregulation at the level of pre-mRNA splicing of the *suppressor-of-white-apricot* gene in *Drosophila*. Genetics **131:**139–150.
82. **Zahler, A. M., W. S. Lane, J. A. Stolk, and M. B. Roth.** 1992. SR proteins: a conserved family of pre-mRNA splicing factors. Genes Dev. **6:**837–847.
83. **Zahler, A. M., K. M. Neugebauer, W. S. Lane, and M. B. Roth.** 1993. Distinct functions of SR proteins in alternative pre-mRNA splicing. Science **260:**219–222.
84. **Zahler, A. M., K. M. Neugebauer, J. A. Stolk, and M. B. Roth.** 1993. Human SR proteins and isolation of a cDNA encoding SRp75. Mol. Cell. Biol. **13:**4023–4028.
85. **Zuo, P., and J. L. Manley.** 1993. Functional domains of the human splicing factor ASF/SF2. EMBO J. **12:**4727–4737.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