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Trans-acting Factors Required for Inclusion of Regulated Exons in the *Ultrabithorax* mRNAs of *Drosophila melanogaster*

James M. Burnette,[1] Allyson R. Hatton[1] and A. Javier Lopez

Department of Biological Sciences, Carnegie Mellon University, Pittsburgh, Pennyslvania 15213

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ABSTRACT

Alternatively spliced *Ultrabithorax* mRNAs differ by the presence of internal exons mI and mII. Two approaches were used to identify *trans*-acting factors required for inclusion of these cassette exons. First, mutations in a set of genes implicated in the control of other alternative splicing decisions were tested for dominant effects on the *Ubx* alternative splicing pattern. To identify additional genes involved in regulation of *Ubx* splicing, a large collection of deficiencies was tested first for dominant enhancement of the haploinsufficient *Ubx* haltere phenotype and second for effects on the splicing pattern. Inclusion of the cassette exons in *Ubx* mRNAs was reduced strongly in heterozygotes for hypomorphic alleles of *hrp48*, which encodes a member of the hnRNP A/B family and is implicated in control of *P*-element splicing. Significant reductions of mI and mII inclusion were also observed in heterozygotes for loss-of-function alleles of *virilizer*, *fl(2)d*, and *crooked neck.* The products of *virilizer* and *fl(2)d* are also required for *Sxl* autoregulation at the level of splicing; *crooked neck* encodes a protein with structural similarities to yeast-splicing factors Prp39p and Prp42p. Deletion of at least five other loci caused significant reductions in the inclusion of mI and/or mII. Possible roles of identified factors are discussed in the context of the resplicing strategy for generation of alternative *Ubx* mRNAs.

ALTERNATIVE splicing of pre-mRNAs is a versatile regulatory mechanism that can achieve quantitative control of gene expression and functional diversification of gene products (reviewed by Lopez 1995, 1998; MacDougall *et al.* 1995; Cooper and Mattox 1997). Much progress has been made toward understanding the basic splicing reaction and recognizing exon/intron boundaries, but the mechanisms that regulate alternative splicing are only beginning to be elucidated. Recognition of the 5′ splice site by U1 snRNP and of the branchpoint near the 3′ splice site by U2 snRNP auxiliary factor (U2AF) are critical early steps that are regulated in cell- or stage-specific alternative splicing. The picture emerging from biochemical and genetic studies is that splice site selection results from the combined action of conserved consensus sequences that base-pair with the U snRNAs together with protein-protein and protein-RNA interactions that stabilize snRNP binding and mediate bridging interactions between snRNPs at the 5′ and 3′ splice sites (reviewed by Berget 1995; Black 1995; Reed and Palandjian 1997; Wang and Manley 1997; Lopez 1998). These interactions involve a growing list of non-snRNP factors (reviewed by Fu 1995; Krämer 1996; Manley and Tacke 1996; Cáceres and Krainer 1997), some of which may be responsible for developmental regulation of splice site selection.

Corresponding author: A. Javier Lopez, Department of Biological Sciences, Carnegie Mellon University, 4400 Fifth Ave., Pittsburgh, PA 15213. E-mail: jlaa@andrew.cmu.edu

[1] These authors contributed equally to this work.

Members of the SR family of RNA-binding proteins are required for multiple steps of the splicing reaction *in vitro* and their concentration can influence splice site competition both *in vitro* and in overexpression assays using cultured cells (reviewed in Fu 1995; Manley and Tacke 1996). SR proteins are required for the activity of at least some splicing enhancers that stimulate the use of weak 5′ or 3′ splice sites, and there is evidence for distinct specificities in these interactions (reviewed in Lopez 1998). Members of the hnRNP A/B family of RNA-binding proteins also influence splice site selection in a concentration-dependent manner *in vitro* and when overexpressed in cultured cells, and they can antagonize the action of SR proteins in these assays (reviewed in Fu 1995). These observations have suggested that SR proteins and hnRNP A/B proteins function *in vivo* as concentration-dependent regulators of alternative splicing. Another possibility is that members of these families serve as cofactors or targets for the actual regulators. Particular SR proteins have been proposed to interact with developmentally specific factors to promote regulation of splicing (Wu and Maniatis 1993; Heinrichs and Baker 1995; Lynch and Maniatis 1996), and so has hrp48, a member of the hnRNP A/B family in Drosophila (Siebel *et al.* 1994; Hammond *et al.* 1997).

Although a framework of hypotheses is evolving, we still know little about regulators of alternative splicing and how they function *in vivo.* Notable exceptions are SXL and TRA, proteins that control alternative splicing decisions during sex determination in Drosophila (reviewed in Cline and Meyer 1996), and PSI, a soma-specific factor that represses splicing of a *P*-element

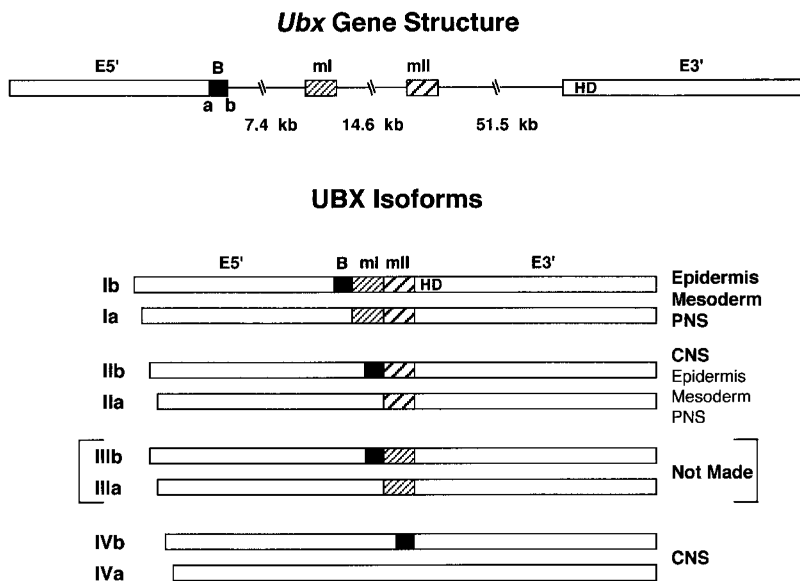

Figure 1.—Alternative splicing of *Ubx* RNAs. The structure of the *Ubx* transcription unit is shown at the top, not drawn to scale. E5′, common exon at 5′ end; B, B element (27 nt) defined between two alternative 5′ splice sites (a and b) for exon E5′; mI, microexon I (51 nt); mII, microexon II (51 nt); E3′, common exon at 3′ end; HD, homeodomain. The structures of the alternatively spliced *Ubx* isoforms and a summary of their expression are shown at the bottom (for detailed description, see Lopez *et al.* 1996). The b subtypes (which contain the B element) comprise a minor proportion of each major class (I, II, IV). Class I is also expressed in the CNS but at very low levels. Class II is expressed at highest levels in the CNS but also in other tissues. Classes II and IV are expressed in distinct but overlapping stage- and neuron-specific patterns in the CNS.

intron in somatic tissues, and also in Drosophila (Siebel *et al.* 1994, 1995; Adams *et al.* 1997). Because few developmentally specific regulators of alternative splicing have been identified, it is possible that many—if not most—alternative splicing decisions are regulated by relatively subtle variations in the levels of general, widely distributed factors, perhaps acting cooperatively or antagonistically as proposed for SR and hnRNP A/B proteins. This is consistent with much correlative evidence and many *in vitro* observations, but conclusive proof that either type of protein normally regulates an alternative splicing decision *in vivo* has yet to be obtained. Although null alleles of the Drosophila SR protein gene *B52* (homolog of human SRp55) show it to be essential for viability, examination of multiple constitutively and alternatively spliced RNAs have failed to reveal any alterations of splicing even in the absence of detectable protein (Ring and Lis 1994; Peng and Mount 1995; this article).

We are using the homeotic gene *Ultrabithorax* (*Ubx*) of *Drosophila melanogaster* as a model for regulation of alternative splicing in large and complex transcription units. The six alternative *Ubx* mRNAs share large protein-coding 5′ and 3′ exons but differ in the pattern of incorporation of three elements: B is comprised between two alternative donor sites at the end of the first common exon, whereas mI and mII are internal cassette exons (Figure 1). Alternative isoforms that differ by the presence of mI and/or mII are expressed with different stage-, tissue-, and cell-specific profiles (O'Connor *et al.* 1988; Kornfeld *et al.* 1989; Lopez and Hogness 1991; Lopez *et al.* 1996). Within the central nervous system (CNS), different neurons express distinct ratios of *Ubx* isoforms (Lopez *et al.* 1996). The complex and quantitative nature of this regulation is unlike that of other well-studied model systems in Drosophila (*e.g.*, sex-specific splicing in the sex determination hierarchy or germ line-specific splicing of *P*-element transcripts) but resembles that of many other genes in vertebrates and invertebrates (see Grabowski 1998). It seems most likely that this type of alternative splicing is controlled not by highly tissue- and gene-specific splicing regulators but by developmental variations in the concentration or activity of broadly distributed multifunctional factors that may act combinatorially (reviewed in Lopez 1998). Hence, *Ubx* should be a valuable model where genetic approaches can be used to dissect this type of regulation.

Strong reductions of function for the postulated type of regulatory factors would probably cause lethal phenotypes that would be uninterpretable in terms of effects on *Ubx* splicing. On the other hand, the *Ubx* splicing pattern should be sensitive to partial reductions in the concentration or activity of these regulatory factors. This may also be true for factors that play important accessory roles in regulation as targets or as constitutively expressed components of regulatory complexes. We used two approaches to identify such factors. First, we tested whether the *Ubx* alternative splicing pattern is altered in heterozygotes for strong loss-of-function mutations in a set of genes implicated in the control of alternative splicing in *Sxl* and *P*-element RNAs. To identify the location of additional genes involved in regulation of *Ubx* splicing, we tested a large collection of deficiencies for dominant enhancement of the haploinsufficient *Ubx* haltere phenotype; then we asked whether the *Ubx* splicing pattern is altered in heterozygotes for the interacting deficiencies, and we traced the phenotypic interaction and effect on splicing to specific genes when mutations existed in reasonable candidates.

MATERIALS AND METHODS

Strains: The wild-type reference strain was Oregon-R. The deficiency kit from the Drosophila Stock Center (Bloomington, IN) was used. Deficiencies that exhibited relevant interac-

tions with *Ubx* are described in the text. A complete list of deficiencies tested is available upon request. Except as noted, all deficiencies and mutations were balanced using *FM7c* [*In(1)FM7, y^{31d} sc^8 w^a B, sn^{X2} v^{Of}, g^4*], *CyO* [*In(2LR)O, Cy dp^{lvI} pr cn^2*], *TM6B* [*In(3LR)TM6B, Hu e Tb*], *TM3* [*In(3LR)TM3, ri p^p sep l(3)89Aa bx^{34e} e Sb*], *TM1* [*In(3LR)TM1, Me ri sbd^1*), or *MKRS* [*Tp(3;3)MKRS, M(3)76A kar ry^2 Sb*] (detailed descriptions in Lindsley and Zimm 1992).

Isolation of RNA: For each genotype analyzed, total RNA was isolated from five late third instar female larvae, using the RNeasy reagents from QIAGEN (Chatsworth, CA). Larvae were grown on standard medium containing bromphenol blue (0.05%) to facilitate visualization of gut contents, and individuals within 15 min of pupariation were identified by their cleared intestinal tracts (Maroni and Stamey 1983). All of the mutations tested were recessive lethal before this stage, as were all of the balancers used in reverse transcription polymerase chain reaction (RT-PCR) assays. Larval sex was identified by examining gonad size, as described by Ashburner (1989). For some mutations, total RNA was also isolated from five freshly eclosed heterozygous adult males or females, as appropriate, using the RNeasy reagents (QIAGEN).

Reverse transcription/amplification assays: Reverse transcription primed with oligo(dT) was performed with 1 μg total RNA in a volume of 20 μl using Superscript II reverse transcriptase (GIBCO-BRL Life Technologies, Gaithersburg, MD). After treatment with RNAse H, 2 μl of the cDNA products were diluted to 50 μl and amplified with Display Taq-FL polymerase (PGC Scientific) using 10 pmol of each primer. To amplify *Ubx* cDNAs, the primers were Ubx.5S1 (5′-TGGAATGCCAAT TGCACCATC-3′), which hybridizes to *Ubx* exon E5′ at nucleotides −133 through −113 relative to 5′ splice site "b," and Ubx.3A1 (5′-GCGGGTCAGATAATGATTCGT-3′), which hybridizes to nucleotides 78 through 98 relative to the 3′ splice site of exon E3′. To amplify *ras1* cDNAs as an internal standard, the primers were Ras1.5S1 (5′-GAGAGTAAGCATCGATC GCG-3′) and Ras1.3A1 (5′-GGCGGATGTCTCAATGTATGG-3′). Amplification was performed in the presence of 5 μCi [α-^{32}P]dCTP. The products were separated on a nondenaturing 8% polyacrylamide gel and quantitated on a Fuji BAS-2000 phosphorimager (Fuji Medical Systems USA/Bio Images, Stamford, CT) using MacBas image analysis software. The identities of the amplified *Ubx* cDNAs were confirmed by digestion with *Bgl*II and *Dde*I, which cleave in mI and mII, respectively. To ensure that the assays would accurately reflect the *Ubx* isoform ratios, we determined the extent of amplification of *Ubx* cDNAs and of the reference standard *ras1* between 16 and 28 cycles of the polymerase chain reaction. Under our conditions for RNA isolation, reverse transcription and PCR, amplification of both *ras1* and *Ubx* was exponential through the 26th cycle and the qualitative and quantitative aspects of the *Ubx* isoform pattern were highly reproducible within the same range. For subsequent experimental determinations, the quantitations of *Ubx* isoform ratios were performed on samples amplified through the 22nd cycle, which was well within the exponential range in all cases.

Screening protocol: We reasoned that the tissue-specific *Ubx* splicing pattern should be sensitive to the zygotic dose of critical regulatory factors. To bias the screen toward identification of such factors, we focused on zygotic effects whenever this was practical. This strategy also helped to avoid problems due to masking of enhancers or suppressors by maternal effect modifiers with opposing effects, and it allowed us to identify clear correlations between inheritance of the mutant chromosome and modification of the *Ubx* phenotype. For deficiencies or mutations on the autosomes and for X-linked lesions balanced with X;Y translocations, males of the corresponding strain were mated to virgin females of genotypes *Ubx^{195}/MKRS* and *$Ubx^{9.22}$/MKRS.* For lethal X-linked lesions balanced in females, virgin females of the corresponding strain were mated to male *Ubx^{195}/MKRS* and *$Ubx^{9.22}$/MKRS.* Control crosses between Oregon-R and *Ubx^{195}/MKRS* or *$Ubx^{9.22}$/MKRS* were performed in the appropriate orientation for each case. Two sets of paired replicates were established for each cross and the phenotypes of progeny in each set were evaluated by different individuals without knowledge of the specific lesion being tested. The number of pigmented bristles along the anterior "margin," base, and hinge was recorded for each haltere on intact, living flies, and qualitative appraisals of relative haltere size and shape were made. At least 50 halteres (over 100 in most cases) were scored for each genotype. The set of mutant strains included a very weak *Ubx* allele [associated with *Df(3R)C4*] and several known suppressors and enhancers belonging to the *Polycomb* and *trithorax* groups as positive controls. All crosses were performed on standard molasses/cornmeal/agar/yeast medium at 25°.

Statistical analysis: Data on bristle numbers and isoform ratios were analyzed using the Statistica software package (StatSoft, Tulsa, OK). There were no significant differences among the bristle counts recorded by different scorers when individual genotypes or the complete data set were considered. Bristle counts for experimental genotypes were compared with those of progeny from crosses to Oregon-R to identify cases of suppression or enhancement. A value of $P < 0.01$ (Student's *t*-test) was imposed as the upper limit for evidence of significant modification of the *Ubx* phenotype relative to the Oregon-R background.

RESULTS

Stability of the *Ubx* alternative splicing pattern: We used coupled RT-PCR assays to analyze the pattern of *Ubx* alternative splicing in heterozygous third instar larvae and in adults. To minimize variations between strains due to differences in developmental rate, the larvae used for these experiments were staged to be within 15 min of pupariation by monitoring the clearing of bromphenol blue-containing medium from the gut (Maroni and Stamey 1983); adults were collected and processed within 1 hr after they eclosed. To ensure that the assays would accurately reflect the *Ubx* isoform ratios, we determined the extent of amplification of *Ubx* cDNAs and of the reference standard *ras1* between 16 and 28 cycles of the polymerase chain reaction. Under our conditions for RNA isolation, reverse transcription and PCR, amplification of both *ras1* and *Ubx* was exponential through the 26th cycle and the qualitative and quantitative aspects of the *Ubx* isoform pattern were highly reproducible within the same range (data not shown). For subsequent experimental determinations, the quantitations of *Ubx* isoform ratios were performed on samples amplified through the 22nd cycle, which was well within the exponential range in all cases. These methods yielded highly reproducible results (Figure 2; Table 1). The isoform ratios in third instar larvae were in close agreement with those determined previously using nuclease protection assays (O'Connor *et al.* 1988; Kornfeld *et al.* 1989). Types Ia and IIa were the predominant *Ubx* mRNAs and those lacking both mI and mII (isoforms IVa and b) made up only a small fraction of the total (Figure 2; Table 1). Adults contained a

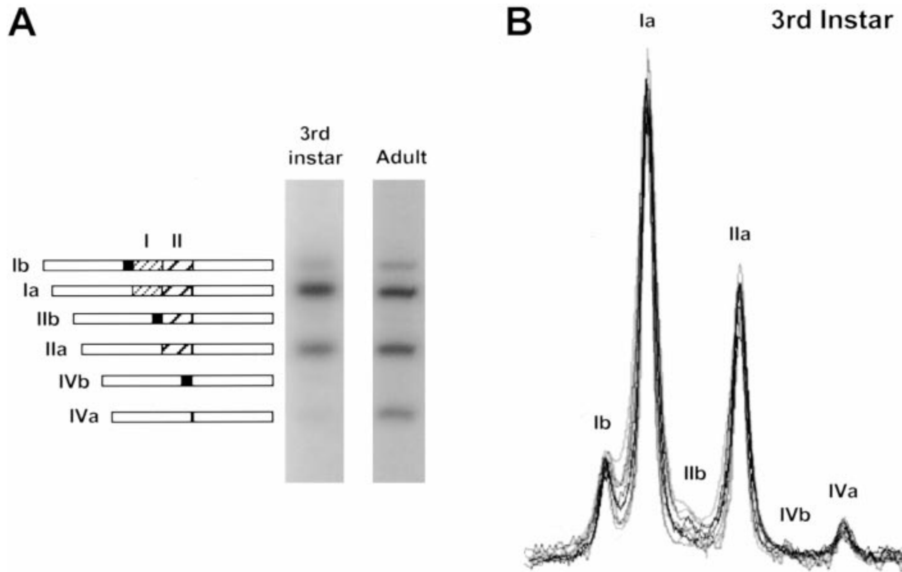

Figure 2.—Isoform ratios in control strains. (A) Electrophoretic separation of ^{32}P-labeled *Ubx* cDNA amplimers generated by quantitative RT-PCR using total RNA from late third instar larvae or adults of strain Oregon-R, as described in materials and methods. The images are phosphorimager scans of the polyacrylamide gels. (B) Superimposed gel profiles showing the proportion of radioactivity in amplimers representing each *Ubx* isoform, generated by quantitative RT-PCR using total RNA from late third instar larvae of six different strains: Oregon-R, Canton-S, *Df(1)JF5 f^1 car^1/FM7*, *Df(2R)NCX11 bw^D/CyO*, *red e Scr^{11}/TM3*, and *MKRS/TM6B*. The profiles were generated from primary phosphorimager scans like those in A, using MacBas image analysis software (Fuji).

significantly higher proportion of class IV mRNAs than larvae (Figure 2; Table 1); this differs from previous reports and probably reflects the very early and narrow age distribution of the adults used in our study.

It is important to note that the *Ubx* isoform ratios did not vary significantly between different wild-type strains nor between these and several control strains that carried different balancer chromosomes and irrelevant mutations (Figure 2; Table 1). The *Ubx* isoform ratios were also unaltered in heterozygotes for various *Minute* mutations that cause dominant eye and bristle abnormalities and reductions in developmental rate, viability, and fertility (below and data not shown). These results demonstrate that the mechanism that controls *Ubx* alternative splicing is robust, a conclusion that is consistent with the faithful conservation of *Ubx* isoform structure and expression among Drosophila species spanning 60 million years of evolution (Bomze and Lopez 1994). The fact that the quantitative isoform pattern revealed by our assay is insensitive to considerable variation in genetic background highlights the significance of the effects described below for specific mutations and deficiencies.

Although amplified *Ubx* cDNA fragments that contain mI but not mII (*i.e.*, hypothetical isoforms IIIa and IIIb, as illustrated in Figure 1) would have the same length as isoforms IIa and IIb, such amplimers exhibit distinctly slower mobility due to the difference in nucleotide sequence (Hatton *et al.* 1998). Class III amplimers can also be distinguished by the pattern of digestion with *Bgl*II (cleaves only in mI) and *Dde*I (cleaves only in mII). By both criteria, class III RNAs were not produced in any wild-type or mutant strain discussed above nor in subsequent sections.

***virilizer* and *fl(2)d* are required for inclusion of mI**

TABLE 1

***Ubx* isoform ratios in control late third instar larvae and adults**

Strain	N^a	B element^b		Class I^b		Class II^b		Class IV^b	
Larvae^c									
Oregon-R	7	17.90 (1.94)		58.34 (1.97)		36.06 (1.61)		5.59 (1.87)	
Canton-S	3	16.43 (2.27)	0.37	56.22 (2.59)	0.19	37.41 (1.37)	0.25	6.36 (1.30)	0.54
Df(1)JF5, f^1, car^1/FM7	2	17.12 (0.64)	0.62	59.87 (3.46)	0.43	34.89 (1.99)	0.41	5.25 (1.47)	0.82
Df(2R)NCX11, bw^D/CyO	4	19.28 (1.18)	0.26	58.91 (1.36)	0.62	34.80 (1.41)	0.23	6.29 (0.85)	0.50
red e Scr^{11}/TM3	4	16.17 (1.91)	0.22	58.99 (2.22)	0.63	35.84 (2.28)	0.85	5.18 (0.63)	0.68
MKRS/TM6B	1	14.87		59.84		33.52		6.64	
Adults									
Oregon-R	3	16.19 (1.77)		51.53 (0.71)		35.24 (0.27)		13.23 (0.58)	

The B element is calculated as Ib/Ib + Ia, class I as IB + Ia/total, class II as IIb + IIa/total, and class IV as IVa + IVb/total.

[a]The number of independent reverse transcription/amplification reactions analyzed, performed on two independent RNA samples (except MKRS/TM6B).

[b]The indicated ratios are expressed as a percentage, followed by the standard deviation (in parentheses) and the *P* value calculated from Student's *t*-test for the comparison of each strain with Oregon-R.

[c]Late third instar larvae were identified using the blue food method (materials and methods).

TABLE 2

***Ubx* isoform ratios in heterozygotes for mutations in candidate alternative splicing factors**

Strain	N^a	B element[b]	Class I[b]	Class II[b]	Class IV[b]
Late larvae					
Control[c]	17	17.10 (1.96)	58.75 (2.17)	35.52 (1.76)	5.74 (1.01)
vir³/CyO	3	16.41 (2.88) NS*	51.67 (0.42)****	40.97 (0.86)****	7.36 (1.11)**
fl(2)d²/CyO	4	15.94 (2.63) NS*	55.48 (1.66)**	36.01 (1.17) NS*	8.46 (1.30)****
l(2)49Db^TW6/CyO	4	17.17 (3.98) NS*	59.47 (1.86) NS*	34.03 (2.08) NS*	6.49 (0.87) NS
hrp48¹/CyO	5	17.97 (9.13) NS*	43.16 (3.93)****	41.03 (2.74)****	15.77 (5.48)****
Adults					
Oregon-R	3	16.19 (1.77)	51.53 (0.71)	35.24 (0.17)	13.23 (0.58)
vir³/CyO	3	16.57 (2.76) NS*	48.66 (1.78)**	37.75 (1.12)**	13.59 (1.07) NS*
fl(2)d²/CyO	2	14.03 (6.77) NS*	48.41 (5.39) NS*	36.66 (1.57) NS*	14.94 (3.81) NS*
hrp48¹/CyO	7	15.79 (2.04) NS*	36.50 (3.39)****	42.94 (3.40)***	20.56 (3.60)***

The B element is calculated as Ib/Ib + Ia, class I as Ib + Ia/total, class II as IIb + IIa/total, and class IV as IVa + IVb/total.

[a] The number of independent reverse transcription/amplification reactions analyzed.

[b] Ratios are expressed as a percentage, followed by the standard deviation (in parentheses) and the P value calculated from Student's t-test for the comparison of each strain with the control: *Not significant, $P > 0.05$; **$0.05 > P > 0.01$; ***$0.01 > P > 0.001$; ****$P < 0.001$.

[c] The control is the pool of wild-type and irrelevant mutant strains from Table 1.

and mII in *Ubx* mRNAs: The products of *Sxl*, *tra*, and *tra-2* are known regulators of alternative splicing decisions in Drosophila (reviewed in Cline and Meyer 1996) but they are not essential for processes other than sex determination (and dosage compensation, in the case of *Sxl*) because males that are null for these genes are viable and appear phenotypically normal. However, additional genes [*fl(2)d*, *virilizer*, and *l(2)49Db*] are required for correct control of alternative splicing decisions by SXL but are also essential for viability in both sexes (Granadino *et al.* 1992, 1996; Hilfiker *et al.* 1995; H. Salz, personal communication); hence, their products may also have roles in other alternative splicing events. To determine whether these include the control of *Ubx* alternative splicing, we asked whether the *Ubx* isoform ratios were altered in heterozygotes for mutations in these genes.

In contrast to the stability described in the preceding section, the *Ubx* splicing pattern was altered significantly when the expression or function of *virilizer* or *fl(2)d* was reduced. The strongest effect was observed with *virilizer*, using a loss-of-function allele (*vir³*) that is recessive lethal in both sexes. In heterozygous larvae the proportion of *Ubx* class I mRNAs declined while that of classes II and IV increased (Table 2; Figure 3). The proportion of class I that contained the B element was not altered. The increase in classes II and IV indicates that inclusion of both mI and mII was reduced but that the effect on mI exceeded that on mII (see Figure 1). Inclusion of mI was also reduced in adults (Table 2), although the effect was weaker than in larvae. More modest but statistically significant reductions of mI and mII inclusion were also observed in larvae heterozygous for the *fl(2)d²* mutation (Table 2), which is also a loss-of-function allele that is recessive lethal in both sexes. A null allele of *l(2)49Db* (TW6) had no significant dominant effect on the *Ubx* splicing pattern (Table 2).

hrp48 plays a critical role in the inclusion of mI and mII: hrp48 is a member of the hnRNP-A/B family of RNA-binding proteins (Matunis *et al.* 1992) and forms part of a protein complex that regulates splicing of intron 3 (IVS3) in *P*-element transcripts (Siebel *et al.* 1994; Hammond *et al.* 1997). Although repression of IVS3 splicing in somatic tissues is dictated by PSI, which is a soma-specific component of the regulatory complex, the hrp48 protein binds specifically to sequences within the *cis*-acting regulatory element in the RNA (Siebel *et al.* 1994) and is also required for effective inhibition of splicing (Hammond *et al.* 1997). hrp48 was originally identified as a general component of heterogeneous nuclear ribonucleoprotein particles (Matunis *et al.* 1992) and the *hrp48* gene is essential for viability (Hammond *et al.* 1997), so it must perform additional functions unrelated to *P*-element expression; these functions might include regulation of other splicing decisions. The five known mutant alleles of *hrp48* are all *P*-element insertions in the upstream regulatory region and are not null (Hammond *et al.* 1997; A. R. Hatton, and A. J. Lopez, unpublished results). Nevertheless, inclusion of mI and mII in *Ubx* mRNAs was reduced markedly in larvae and adults heterozygous for the strong recessive lethal allele *hrp48¹* (Table 2; Figures 3 and 4); weaker alleles, some of which are viable as homozygotes (Hammond *et al.* 1997), had similar but more modest effects (not shown). The effect of *hrp48* mutations resembled that of *vir* and *fl(2)* mutations in that inclusion of mII was affected more weakly than mI, and the proportion of isoform I that contains the B element was not altered. Heterozygosity for *hrp48¹* reduced inclusion of mI by 27%; this was the strongest effect observed for

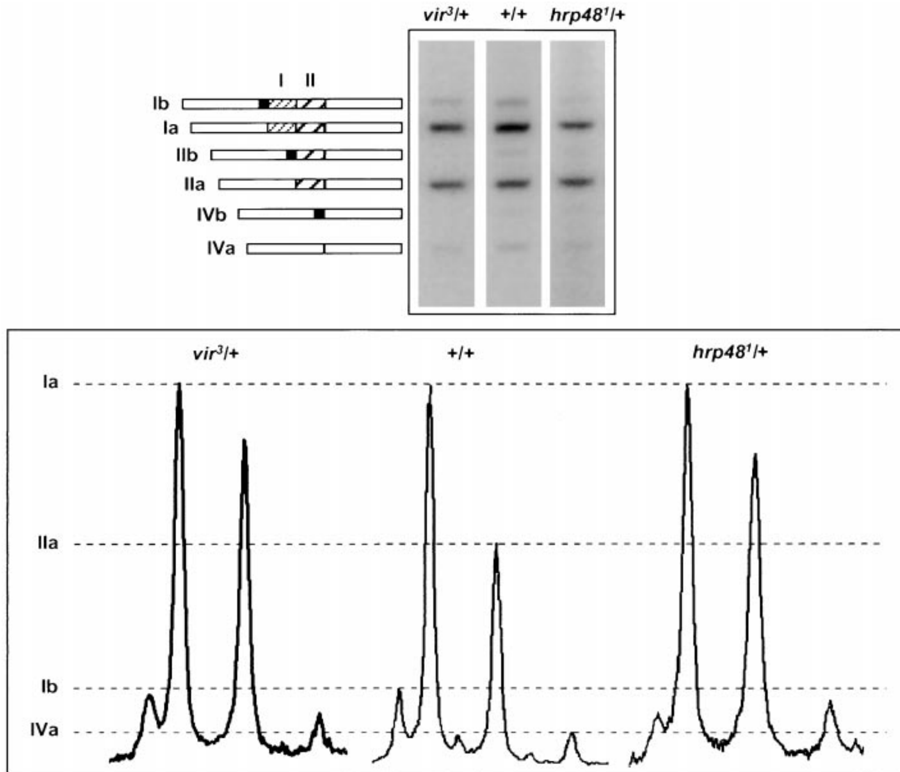

Figure 3.—Isoform ratios in larvae heterozygous for *vir*[3] or *hrp48*[1]. (Top) Electrophoretic separations of ^{32}P-labeled *Ubx* cDNA amplimers generated by quantitative RT-PCR using total RNA from late third instar larvae of strains *vir*[3]*/CyO* (*vir*[3]/+), Oregon-R (+/+), or *w; hrp48*[1]*/CyO* (*hrp48*[1]/+). The images are phosphorimager scans of the polyacrylamide gels. (Bottom) The corresponding gel profiles obtained as in Figure 2, normalized to the peak for isoform Ia in Oregon-R. The dashed lines mark the heights of peaks corresponding to specific isoforms (labeled at the left) in the Oregon-R control sample (+/+). Separate experiments (*e.g.*, Figure 1) showed that the *CyO* balancer itself has no significant effect on the *Ubx* splicing pattern.

any mutation or deficiency in this study, indicating that normal levels of hrp48 are critical for inclusion of the internal exons, especially mI, in *Ubx* mRNAs.

Strategy to identify additional modifiers of the *Ubx* splicing pattern: In heterozygotes for strong loss-of-function *Ubx* alleles the haltere is transformed weakly toward wing, exhibiting a slight increase in size and a small and variable number of bristles on the hinge, on the base, and on the region of the capitellum homologous to the anterior wing margin. The severity of this transformation increases with further reductions in *Ubx* function, for example, in heteroallelic combinations involving null and hypomorphic alleles. Several observations suggested that this phenotype might be enhanced by alterations in the *Ubx* splicing pattern. First, throughout development cells of the integument express UBX isoforms Ia/b (which contain exons mI and mII) and IIa/b (which contain exon mII) but not isoforms IVa/b, which lack exons mI and mII and are restricted to the central nervous system (Lopez *et al.* 1996). Second, the haploinsufficient *Ubx* haltere phenotype was enhanced in flies that were also heterozygous for *hrp48*[1], which was shown above to reduce the inclusion of exons mI and mII. This interaction was observed with two different *Ubx* alleles: *Ubx*$^{9.22}$, which makes no functional protein because it consists of a 1587-bp deletion com-

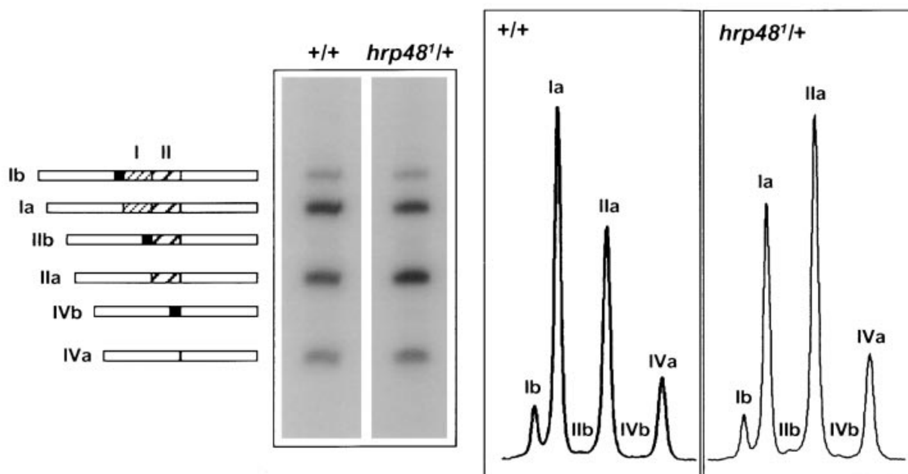

Figure 4.—Isoform ratios in adults heterozygous for *hrp48*[1]. (Left) Electrophoretic separations of ^{32}P-labeled *Ubx* cDNA amplimers generated by quantitative RT-PCR using total RNA from freshly eclosed adult males of strains Oregon-R (+/+) or *w; hrp48*[1]*/CyO* (*hrp48*[1]/+). The images are phosphorimager scans of the polyacrylamide gels. (Right) Corresponding gel profiles obtained as in Figure 2. The corresponding isoform is identified above each peak.

prising the last 1.4 kb of intron 3 through the first 48 codons of the homeobox (Weinzerl *et al.* 1987), and *Ubx*[195], which consists of a nonsense mutation in mII (Weinzerl *et al.* 1987). The size of the haltere was enlarged and the average number of bristles increased from 0.8 [±1.2 (SD); $N = 252$] for *Ubx*[195]/+ and 0.9 [±0.9 (SD); $N = 138$] for *Ubx*[9.22]/+ to 3.6 [±1.6 (SD); $N = 50$] and 2.6 [±1.5 (SD); $N = 46$] in the corresponding double heterozygotes with *hrp48*[1]/+. These enhancements were highly significant (*t*-test, $P < 0.001$). The *vir*[3] and *fl(2)d*[2] mutations produced more modest enhancements of bristle number (~50% increase, $P < 0.01$) that were consistent with their weaker effects on *Ubx* splicing.

Other factors required for inclusion of mI and/or mII might exhibit similar genetic interactions with *Ubx* mutations. To identify such factors, we tested a collection of 196 deficiencies (collectively deleting ~85% of the euchromatic genome) for dominant modification of the haltere phenotype in *Ubx*[195] or *Ubx*[9.22] heterozygotes (see materials and methods). We used both *Ubx* alleles to increase the versatility of the screen: depending on the degree to which the shift in splicing pattern reduces the function of the wild-type *Ubx* allele, factors required for mII inclusion might be identified as specific suppressors of *Ubx*[195]/+, as specific enhancers of *Ubx*[9.22]/+, or as enhancers of both. Suppressors of both genotypes are unlikely to reduce inclusion of mI and mII, because the function of the *Ubx*[9.22] allele cannot be increased by alterations in the splicing pattern. Subsequently, we determined whether the *Ubx* splicing pattern was altered in animals that were homozygous wild type for *Ubx* but were heterozygous for appropriately interacting deficiencies. Where possible, we traced the phenotypic interaction and the effect on splicing to specific genes using existing mutations. As described below, effects on the *Ubx* splicing pattern were identified among differential modifiers as well as enhancers of the haltere phenotype.

Deficiencies that interact differentially with *Ubx*[195] and *Ubx*[9.22]: Only two deficiencies enhanced *Ubx*[9.22]/+ significantly ($P < 0.01$) but suppressed or had no significant effect on *Ubx*[195]/+, and these deficiencies overlapped (Table 3). RT-PCR analysis revealed that inclusion of mI and (as predicted) mII was reduced significantly in larvae heterozygous for *Df(3R)l26c*, although not in adults (Table 4). A qualitatively similar result was observed with *Tp(3;Y)ry506-85C*, although reduction of mI was stronger and was also observed in adults (data not shown). Both deficiencies delete the interval 87E1–87F11. Among the genes known to reside in this region, four encode widely expressed RNA-binding proteins that might influence processing or stability of *Ubx* RNAs: *squid* (*sqd*; encodes hrp40, related to the hnRNP A/B family; Kelley 1993), *Next-to-squid* (*Nts*; Kelley 1993), *Hrb87Fa* (also related to the hnRNP A/B family; Haynes *et al.* 1991; Matunis *et al.* 1992), and *B52* (encodes an SR protein closely related to mammalian SRp55; Champlin *et al.* 1991; Roth *et al.* 1991). These genes are adjacent to each other within a 35-kb region of DNA in 87F4-10 (Haynes *et al.* 1997). Tests of available null mutations showed that deletion of *squid* or *B52* did not account for the effect of *Df(3R)l26c* or *Tp(3;Y)ry506-85C* on the *Ubx* splicing pattern (Table 5). A smaller deficiency that deletes all four genes (*B52*[R1]; Peng and Mount 1995) was subsequently tested by RT-PCR and was found to produce a statistically significant reduction in the proportion of mI- and mII-containing *Ubx* mRNAs, but the effect was very subtle (Table 5) and this deficiency did not have a dominant effect on the *Ubx* haltere phenotype. Thus, although one or more of these genes might contribute quantitatively to the effect of the deficiencies, their deletion even as a group does not account entirely for this effect, which must involve previously uncharacterized gene(s) within region 87E1–87F11. The suppression of *Ubx*[195]/+ by *Df(3R)l26c*, in contrast to its enhancement by *hrp48*[1], may be explained by the stronger effect of *Df(3R)l26c* on

TABLE 3

Differential modification of the *Ubx* haltere phenotype by deficiencies

		Bristles per haltere[a]							
		Ubx[195]/+				*Ubx*[9.22]/+			
Deficiency	Breakpoints	M	SD	*N*	*P*	M	SD	*N*	*P*
Oregon-R control	None	0.81	1.2	252		0.91	0.87	138	
Tp(3;Y)ry506-85C	87D1-2;88E5-E6	1.1	1.1	36	NS*	2.8	1.4	28	***
Df(3R)126c	87E1; 87F11	0.38	0.82	58	***	4.8	2.2	40	****

Oregon-R control males or males carrying a deficiency balanced over *MKRS* were mated with *Ubx*[195]/*MKRS* or *Ubx*[9.22]/*MKRS* females. The haltere phenotype was examined in female progeny that were *trans*-heterozygous for the deficiency and the *Ubx* mutation.

[a]M, mean; SD, standard deviation; *N*, number of halteres examined; *P*, probability derived from Student's *t*-test for comparison with the Oregon-R control: *Not significant, $P > 0.05$; **$0.05 > P > 0.01$; ***$0.01 > P > 0.001$; ****$P < 0.001$.

TABLE 4

***Ubx* isoform ratios in late third instar larvae heterozygous for selected interacting deficiencies**

Strain	N^a	B element[b]	Class I[b]	Class II[b]	Class IV[b]
Control[c]	17	17.10 (1.96)	58.75 (2.17)	35.52 (1.76)	5.74 (1.01)
Differential modifiers					
Df(3R)126c/MKRS	2	14.87 (0.78) NS*	51.98 (3.91)****	34.74 (1.57) NS	13.28 (2.33)****
Common enhancers					
Df(2L)TW158/CyO	4	15.78 (0.98) NS*	54.01 (1.37)****	38.12 (1.26)**	7.88 (1.01)****
Df(3L)pbl-NR/TM3	2	17.06 (4.30) NS*	60.61 (1.05) NS*	33.94 (1.87) NS*	5.44 (0.82) NS*
Df(3L)Rdl-2/TM3	2	16.43 (0.52) NS*	51.71 (0.035)****	38.68 (1.50)**	9.61 (1.54)****
Df(3L)st-f13/TM6B	2	16.74 (4.32) NS*	59.60 (1.12) NS*	32.40 (2.77) NS*	8.00 (1.66)**
Df(3L)fz-D21/TM6B	4	15.72 (1.63) NS*	59.18 (1.15) NS*	35.14 (0.94) NS*	5.68 (1.66) NS*
Df(3R)by62/TM1	3	14.81 (0.78) NS*	60.38 (0.36) NS*	32.87 (0.92)**	6.75 (0.57) NS*
Df(3R)M86D/TM3	3	18.19 (0.58) NS*	56.69 (1.50) NS*	36.56 (0.45) NS*	6.68 (1.20) NS*
Df(3R)M-Kx1/TM6B	2	15.29 (0.65) NS*	60.41 (0.57) NS*	30.46 (2.73)***	9.13 (2.16)****
Df(3R)crb87-4/TM3	4	17.40 (0.72) NS*	54.37 (2.09)***	39.24 (1.91)****	6.38 (1.22) NS*

Late third instar larvae were identified using the blue food method (materials and methods). The B element is calculated as Ib/Ib + Ia, class I as Ib + Ia/total, class II as IIb + IIa/total, and class IV as IVa + IVb/total.

[a] The number of independent reverse transcription/amplification reactions analyzed.

[b] The indicated ratios are expressed as a percentage, followed by the standard deviation (in parentheses) and the P value calculated from Student's t-test for the comparison of each strain with the control: *Not significant, $P > 0.05$; **$0.05 > P > 0.01$; ***$0.01 > P > 0.001$; ****$P < 0.001$.

[c] The control is the pool of wild-type and irrelevant mutant strains from Table 2.

mII (which contains the nonsense mutation in *Ubx*[195]) relative to mI (Tables 2 and 4).

Common enhancers: Forty deficiencies stood out for their strong enhancement of both *Ubx*[195]/+ and *Ubx*[9.22]/+; these effects consisted of an obvious increase in haltere size and a 4- to 18-fold enhancement in the average number of bristles per haltere that was significant at the $P < 0.01$ level (Table 6). A somewhat weaker enhancer, *Df(2R)OR-BR6*, deletes *virilizer* (located at 59D8–59D11), which was shown above to be required for inclusion of mI and mII. RT-PCR analysis confirmed that *Df(2R)OR-BR6* produces a dominant reduction of mI and mII inclusion similar to that caused by *vir*[3] in larvae and adults (data not shown).

***crooked neck*:** One enhancer, *Df(1)64c18g*, deletes the genes *crooked neck* (*crn*) and *kurz* (*kz*), which are located at 2F1 and are both candidate RNA-processing factors. The *crn* gene encodes a protein with 16 tetratrichopeptide repeats, a motif implicated in protein-protein interactions. Although CRN protein has been proposed to

TABLE 5

***Ubx* isoform ratios in late third instar larvae heterozygous for mutations in candidate genes**

Strain	N^a	B element[b]	Class I[b]	Class II[b]	Class IV[b]
Control[c]	17	17.10 (1.96)	58.75 (2.17)	35.52 (1.76)	5.74 (1.01)
B52[R2]*/TM6B*	3	16.53 (3.65) NS*	58.42 (2.09) NS*	36.30 (3.29) NS*	5.21 (1.55) NS*
sqd[ix50]*/ TM6B*	3	16.40 (2.29) NS*	56.80 (4.84) NS*	34.47 (3.08) NS*	8.73 (3.57)**
B52[R1]*/TM6B*	3	14.89 (2.54) NS*	55.28 (2.94)**	36.19 (3.93) NS*	7.54 (0.22)***
crn[EA130]/+[d]	2	14.91 (1.96) NS*	52.88 (2.17)***	41.91 (1.76)****	5.22 (1.01) NS*
kz[DF942]/+[d]	5	18.81 (2.43) NS*	59.07 (2.14) NS*	34.47 (1.62) NS*	6.42 (1.39) NS*

Late third instar larvae were identified using the blue food method (materials and methods). The B element is calculated as Ib/Ib + Ia, class I as Ib + Ia/total, class II as IIb + IIa/total, and class IV as IVa+ IVb/total.

[a] The number of independent reverse transcription/amplification reactions analyzed.

[b] The indicated ratios are expressed as a percentage, followed by the standard deviation (in parentheses) and the P value calculated from Student's t-test for the comparison of each strain with the control. *NS, not significant, $P > 0.05$; **$0.05 > P > 0.01$; ***$0.01 > P > 0.001$; ****$P < 0.001$.

[c] The control is the pool of wild-type and irrelevant mutant strains from Table 2.

[d] *crn*[EA130] and *kz*[DF942] were balanced in males over *T(1;Y)w*[+] *303* [females in stock were *C(1)DX/Y*]. Males were crossed to Oregon-R females and the female larvae were selected for analysis: all of these are heterozygous for the mutation.

function as a transcription factor involved in cell cycle control (Zhang *et al.* 1991), recent data show that it is closely related to the yeast splicing factors Prp39p and Prp42p, which associate with yeast U1 snRNP and are required for splicing (McLean and Rymond 1998). The *kz* gene encodes a protein with extensive homology to yeast ATP-dependent splicing factors Prp2p, Prp16p, and Prp22p (Tirronen and Roos 1992), which define a distinct subfamily of ATP-dependent putative RNA-helicases (Schwer and Guthrie 1991). Because mutant alleles of these genes were available, we tested directly whether deletion of one or both might be responsible for enhancement of the *Ubx* haltere phenotype and whether they affect the *Ubx* splicing pattern. Like the deficiency, two hypomorphic, recessive lethal alleles of *crn* (EA130 and RC63) acted as dominant zygotic enhancers of $Ubx^{195}/+$ and $Ubx^{9.22}/+$. RT-PCR analysis showed that inclusion of mI, but not mII, was reduced significantly in larvae heterozygous for crn^{EA130} (Table 5). The second allele, crn^{RC63}, had similar effects on the *Ubx* phenotype and splicing pattern. A recessive lethal allele of *kz* (DF942) behaved as a weak dominant enhancer of $Ubx^{195}/+$ and $Ubx^{9.22}/+$, but RT-PCR analyses did not reveal a significant dominant effect on the *Ubx* splicing pattern (Table 5).

Unknown factors: We defined priorities for detailed analysis of the remaining enhancers by excluding those deficiencies that delete genes known to be required for normal levels of transcription of *Ubx* and other homeotic genes. Among the remaining strong enhancers, we focused on cases where two or more overlapping deficiencies with similar effects defined a region likely to contain the interacting gene(s). These criteria defined eight distinct candidate regions (identified as paired deficiencies; see Table 6, footnote *b*). Both deficiencies covering each of these regions were tested by RT-PCR, and the results confirmed that deletion of two candidate regions altered the splicing pattern of *Ubx* mRNAs significantly (Table 4). The strongest effect was observed with *Df(3L) Rdl-2* (deletes 66F5); this effect matched that of *Df(3L)29A6* (deletes 66F5; 67B1; data not shown), confirming the location within 66F5 of genes required for inclusion of mI and mII. The effect of *Df(2L)TW158* (deletes 37B2-8; 37E2-F4) matched that of *Df(2L)TW203* (deletes 36E4-F1; 37B9-C1; data not shown), confirming that the region between 36E4 and 37C1 also contains genes required for inclusion of mI and mII. No significant effect on *Ubx* splicing was observed with overlapping deficiencies covering the regions 65F3 through 66B10, 70D1 through 71E4-5, 71F1-4 through 73A3-4, or 85D8-12 through 85F16 (Table 4; data not shown). *Df(3R)crb87-4* (deletes 95E8-F1; 95F15) had a weak effect on mI but this was not exhibited by *Df(3R)crb87-5* (deletes 95F7; 96A17-18; data not shown), suggesting that the relevant locus is between 95E8 and 95F7.

The last case is more complicated. RT-PCR experiments showed extremely variable effects of *Df(3R)M-Kx1* (deletes 86C1; 97B5) on *Ubx* splicing, ranging from almost complete exclusion of all three differential elements (two independent experiments, not included in Table 4) to subtle reduction of mII inclusion (Table 4). This variability may be due to the presence of additional strong modifiers in the genetic background. *Df(3R)M-Kx1* and *Df(3R)M86D* have dominant Minute phenotypes that may have favored the accumulation of modifiers that improve viability, fertility, or developmental rate. The effect of *Df(3R)M-Kx1* on *Ubx* splicing decreased upon extensive outcrossing, suggesting that it is caused or enhanced strongly by such modifiers. *Df(3R)M86D* (deletes 86D1; 86D4) produced no obvious change in the splicing pattern, but it has not been tested after extensive outcrossing. The gene for a Drosophila SR protein, Rbp1, has been mapped to region 86D by *in situ* hybridization (Kim *et al.* 1992). A possible role for Rbp1 in alternative splicing of *Ubx* cannot be tested at present because specific mutant alleles of this gene are not known, but such a role is suggested by the presence in mI of potential Rbp1-binding sites and by the effect on mI inclusion of mutations within these sequences (Hatton *et al.* 1998; see discussion).

DISCUSSION

We have conducted a partial survey of the Drosophila genome to identify genes with zygotic haploinsufficient effects on alternative splicing of *Ubx* mRNAs. These experiments have identified four specific factors and at least five regions that contain additional genes required for inclusion of exons mI and mII in *Ubx* mRNAs. In contrast to the effects of these mutations and deficiencies, null mutations in many other known or suspected splicing factors had no haploinsufficient effect on the *Ubx* phenotype or alternative splicing pattern; in addition to factors described above (the SR protein B52; the hnRNP-A/B-like proteins hrp40 and Hrb87F), this also included components of the basic splicing machinery (U2AF-38; SNF, which is a component of the U1- and U2-snRNPs), other factors implicated in control of splicing (Suppressor-of-sable and Suppressor-of-w^a), and other hnRNP proteins (hnRNP-L, encoded by *smooth*; zur Lage *et al.* 1997; data not shown). Furthermore, with the exception of deficiencies that delete 86D1–86D4 (which exhibit a Minute phenotype), heterozygosity for the mutations or deficiencies that altered the *Ubx* splicing pattern was not associated with any overt phenotype, indicating that the heterozygotes do not suffer large-scale disruptions of RNA processing.

An important feature of our approach is that splicing of *Ubx* RNAs was not compromised in any way to sensitize the screen. The Ubx^{195} mutation does not affect splice sites nor the splicing process, and $Ubx^{9.22}$ is null because of deletion of essential protein sequences, so that enhancement of $Ubx^{9.22}/+$ must result from effects on expression or function of the wild-type allele. Further-

TABLE 6

Enhancement of the *Ubx* haltere phenotype by deficiencies

		Bristles per haltere[a]					
		Ubx^{195}/+			$Ubx^{9.22}$/+		
Deficiency	Breakpoints	M	SD	*N*	M	SD	*N*
Oregon-R control	None	0.8	1.2	252	0.9	0.9	138
(1)64c18g	2E1-2;3C2	4.1	2.2	136	3.4	1.8	74
(1)JC19	2F6;3C5	3.9	2.3	114	7.4	3.5	38
(1)dm75e19	3C11;3E4	2.7	1.4	64	4.1	1.4	62
(1)HC244	3E8;4F11-12	4.8	2.7	36	ND		
(1)Sxl-bt	6E2;7A6	4.2	1.7	86	4.0	1.7	124
(1)KA14	7F1-2;8C6	5.4	2.6	46	4.5	1.9	68
(1)C246	11D-E;12A1-2	4.1	2.7	67	4.1	1.2	28
(1)sd72b	13F1;14B1	2.6	1.5	34	3.9	1.8	70
(1)N19	17A1;18A2	5.4	2.8	68	3.6	2.1	54
(1)HF396	18E1-2;20	5.2	2.4	80	4.3	1.7	116
(2L)dp-79b	22A2-3;22D5-E1	3.6	1.8	92	ND		
(2L)TE29Aa-11	28E4-7;29B2-C1	2.5	1.5	84	4.1	2.0	60
(2L)Mdh	30D-F;31F	2.3	1.5	160	3.8	2.2	138
(2L)Prl	32F1-3;33F1-2	11.7	3.3	61	14.6	1.7	63
(2L)TW203[b]	36E4-F1;37B9-C1	3.6	2.1	50	ND		
(2L)TW158[b]	37B2-8;37E2-F4	2.3	1.8	78	ND		
(2L)TW84	37F5-38A1;39D3-E1	4.6	1.9	85	2.9	1.1	80
(3L)pbl-X1[b]	65F3;66B10	3.0	2.1	114	3.1	1.6	120
(3L)pbl-NR[b]	66B1;66B2	1.9	1.3	106	3.4	1.5	134
(3L)Rdl-2[b]	66F5;66F5	7.3	2.3	98	5.8	2.5	236
(3L)29A6[b]	66F5;67B1	1.9	1.2	78	ND		
(3L)fz-D21[b]	70D1;70E7	5.5	2.3	108	8.9	2.1	125
(3L)fz-M21[b]	70D2-3;71E4-5	4.7	2.6	219	4.2	1.4	140
(3L)brm11[b]	71F1-4;72D1-10	10.9	4.2	82	13.4	5.4	78
(3L)st-f13[b]	72C1-D1;73A3-4	3.3	2.6	90	2.9	2.6	90
(3L)ri-79c	77B-C;77F-78A	4.5	1.5	124	5.2	1.9	116
(3R)ME15	81F3-6;82F5-7	4.6	1.1	86	4.6	1.7	54
(3R)9A99	83F2-84A1;84B1-2	5.6	2.0	180	3.6	1.6	94
(3R)Antp17	84B1-2;84D11-12	2.7	1.4	92	3.4	1.0	92
(3R)by10[b]	85D8-12;85E7-F1	4.1	2.8	226	2.4	1.6	108
(3R)by62[b]	85D11-14;85F16	7.7	3.0	78	5.0	2.2	86
(3R)M-Kx1[b]	86C1;87B1-5	4.0	1.8	102	5.2	2.9	30
(3R)M86D[b]	86D1-2;86D4	2.7	1.7	72	ND		
(3R)Dl-BX12	91F1-2;92D3-6	6.4	3.0	76	3.1	1.9	108
(3R)crb87-4[b]	95E8-F1;95F15	2.3	1.3	82	3.2	1.4	84
(3R)crb87-5[b]	95F7;96A17-18	4.0	1.8	258	ND		
(3R)X3F	99D	5.1	2.4	116	3.0	1.4	106
(3R)awd-KRB	100C;100D	5.3	2.1	84	6.3	2.2	68
(4)G	102E2;102E10	3.5	1.1	100	3.3	1.3	122

Oregon-R control males or males carrying a deficiency over an appropriate balancer were mated with Ubx^{195}/MKRS or $Ubx^{9.22}$/MKRS females. The haltere phenotype was examined in female progeny that were *trans*-heterozygous for the deficiency and the *Ubx* mutation. ND, not determined.

[a] M, mean; SD, standard deviation; *N*, number of halteres examined. All of the cases shown differed significantly from the Oregon-R control ($P < 0.01$; Student's *t*-test).

[b] Pairs of overlapping deficiencies (see breakpoints).

more, the RT-PCR experiments that revealed effects on splicing of *Ubx* RNAs were performed on larvae and adults whose *Ubx* genotypes were wild type for both alleles. Thus, all effects reported here reflect the sensitivity of the natural *Ubx* transcript to reductions in factors that are required for its correct processing during development.

Together, these considerations suggest strongly that the genes we have identified play important roles in the regulation of *Ubx* splicing by encoding regulatory factors, cofactors, or targets.

Possible roles of *trans*-acting factors: The inclusion of mI and mII in *Ubx* mRNAs is regulated by competition between 5′ splice sites that flank each of these exons

after they are joined to E5′ (Hatton *et al.* 1998). As the RNA is transcribed, mI and subsequently mII are spliced constitutively to the upstream exon but can then be removed, together with the downstream intron, using an upstream 5′ splice site within E5′ or at the junction with this exon. For the majority of nascent RNAs (those initially spliced using 5′ splice site a in E5′; Figure 1), a strong 5′ splice site is regenerated at the junction between E5′ and mI or mII that competes with the mI or mII 5′ splice site located 51 nt downstream. For a minority of nascent RNAs (those initally spliced using 5′ splice site b in E5′; Figure 1) the a site is still present in E5′ and can compete with the mI or mII 5′ splice site located 78 nt downstream; use of the a site then removes the B element along with mI or mII. Developmental regulation of mI and mII inclusion is achieved by modulating the competition between the upstream and downstream 5′ splice sites that flank these exons.

Reduction of function in all of the factors identified in this work leads to reduced inclusion of mI (and in most cases also mII). This suggests roles in suppression of the upstream sites (which strongly match the 5′ splice site consensus) or stimulation of the downstream sites (which match the consensus more weakly). It is interesting that three of the factors identified in this study that are required for inclusion of mI and mII in *Ubx* mRNAs may also be required for suppression of 5′ splice site utilization in other RNAs: the functions of *virilizer* and *fl(2)d* are required for SXL to repress splicing of the male-specific exon in its own RNA (Hilfiker *et al.* 1995; Granadino *et al.* 1996), and hrp48 is implicated as part of a complex that mediates repression of a 5′ splice site in *P*-element RNA (Siebel *et al.* 1994; Hammond *et al.* 1997). In addition, heterozygosity for a null allele of *sans-fille* (*snf*[1210]) did not alter the *Ubx* splicing pattern, but the antimorphic allele *snf*[e8H], which interferes with autoregulation of *Sxl* splicing (Salz and Flickinger 1996), enhanced the *Ubx* haltere phenotype and increased exclusion of mI and mII (A. R. Hatton and A. J. Lopez, unpublished results).

The products of *virilizer*, *fl(2)d*, and *snf* might function as parts of a complex that mediates active repression of 5′ splice site utilization through interactions with U1 snRNP. Formation or stabilization of this repression complex could be directed to different target splice sites through the action of distinct factors that, like SXL, bind to *cis*-acting regulatory signals and interact with components of the complex. A direct interaction between SXL and SNF to mediate 5′ and 3′ splice site repression in *Sxl* RNA has been proposed on the basis of genetic interactions, coimmunoprecipitation in RNA-dependent complexes from Drosophila extracts, and physical association of recombinant proteins *in vitro* (Deshpande *et al.* 1996; Salz and Flickinger 1996; Samuels *et al.* 1998). SNF protein is the structural and functional homolog of mammalian U1A and U2B″, closely related proteins that are integral components of vertebrate U1 and U2 snRNP, respectively, as is SNF in Drosophila (reviewed in Salz and Flickinger 1996). This has suggested that SNF protein incorporated in U1 and U2 snRNPs is engaged by SXL to block spliceosome assembly (Deshpande *et al.* 1996; Salz and Flickinger 1996).

An intriguing possibility is that hrp48 interacts (directly or indirectly) with a U1 snRNP/SNF/VIR/FL(2)D complex to target suppression of splicing at the upstream sites that are used to remove mI. The strong reduction of mI inclusion (27%) observed in *hrp48*[1] heterozygotes suggests a critical role for hrp48 in modulating competition between the regenerated and downstream 5′ splice sites that flank this exon. Although hrp48 is an hnRNP protein that probably binds nonspecifically to many RNAs, it is also known to form part of a specific complex that blocks use of the 5′ splice site for the third intron of *P*-element RNA in somatic cells (Siebel *et al.* 1994). This regulatory complex prevents U1 snRNP from binding at the 5′ splice site and recruits it instead, nonproductively, to the more upstream of two overlapping pseudo-5′ splice sites within the exon; hrp48 itself makes contact with the downstream pseudo-5′ splice site, F2 (Siebel *et al.* 1994). Splicing of *P*-element IVS3 in a reporter transgene is partially derepressed in adult escapers homozygous for a semilethal *hrp48* allele, indicating that hrp48 is necessary for efficient suppression of the 5′ splice site (Hammond *et al.* 1997). Hence, it may be significant that a sequence within mI that overlaps the regenerated 5′ splice site matches F2 and flanking nucleotides at 8 of 10 positions; this sequence is conserved among four Drosophila species that diverged up to 60 mya but maintain identical regulation of mI inclusion (Bomze and Lopez 1994). hrp48 might bind to this sequence and help to recruit U1 snRNP nonproductively to the regenerated 5′ splice site at the E5′/mI junction; in intermediates where mI has been spliced to the b site of E5′, this complex could also block access to the a site located 27 nt upstream (Figure 1). This would explain why the *hrp48*, *vir*, and *fl(2)d* mutations reduce mI inclusion but do not alter the proportion of class I mRNAs that contain the B element: failure to assemble the repression complex at the E5′/mI junction would allow inappropriate use of both the regenerated site (used to remove mI from E5′a/mI and E5′b/mI intermediates) and the a site (used to remove mI and the B element from E5′b/mI intermediates).

The effect of *hrp48*, *vir*, and *fl(2)d* mutations on inclusion of exon mII, which does not contain an F2-like element, may not be the result of resplicing at the E5′/mII junction. The reduction of mII inclusion (detected as an increase in class IV mRNAs rather than a decrease in class II) could be explained if the repression complex must remain assembled at the E5′/mI junction to prevent subsequent removal of mI and mII together during splicing of intron 3. Intermediates from which mI is

removed during splicing of intron 2 would retain mII. The net result would be an increase in both class II and class IV mRNAs, as observed.

In addition, we note that the effect of *hrp48* mutations on mI and mII inclusion is the opposite of what one would expect from the simple idea that hnRNP A/B proteins generally promote exon skipping (and use of upstream 5′ splice sites), antagonizing a general effect of SR proteins that promote exon inclusion (or use of downstream 5′ splice sites) (reviewed in Fu 1995; Chabot 1996). Our observations are more consistent with a specific role for hrp48 acting through *cis*-regulatory elements to prevent resplicing of mI.

It is more difficult to speculate on the roles of *crn* or the still-unidentified factors deleted by deficiencies that alter the *Ubx* splicing pattern. In principle, these could participate in repression of the regenerated 5′ splice sites or stimulation of the competing downstream site. They could also be involved in interactions between mI and mII that seem to be required for effective use of the downstream 5′ splice site located at the mI/intron 2 boundary (Hatton *et al.* 1998). Although a weak homology to the homeodomain led to the proposal that the *crooked neck* protein functions as a transcription factor, its 16 tetratrichopeptide repeats form a distinct subfamily with those of Prp39p and Prp42p, two splicing factors from yeast that interact with U1 snRNP but appear not to bind RNA directly (McLean and Rymond 1998). A third yeast member of this group has been identified that has more extensive homology to *crn* (McLean and Rymond 1998); it will be interesting to learn whether this also functions as a splicing factor.

Additional observations in our laboratory indicate that inclusion of mI is controlled by a complex regulatory switch employing multiple factors to balance positive and negative inputs acting on the upstream and perhaps downstream splice sites (Hatton *et al.* 1998; our unpublished results). A positive role for Rbp1 is suggested by studies of *cis*-acting elements within mI. Rbp1 is related to the mammalian SR proteins 9G8 and SRp20; it has been implicated in the control of *dsx* and *fru* RNA splicing as a component of exonic splicing enhancer complexes that assemble on *cis*-acting elements with the sex-specific factor TRA (Heinrichs and Baker 1995; Lynch and Maniatis 1996; Heinrichs *et al.* 1998). Rbp1 is expressed widely in both sexes and it is likely to play a role in the splicing of many RNAs. Mutations in mI at positions +11 and +14 downstream of the E5′/mI junction reduce the efficiency of the regenerated 5′ splice site *in vivo* (Hatton *et al.* 1998); these positions lie within a sequence that matches at 11 of 12 nucleotides a set of functionally important Rbp1-binding sites within the female-specific polypyrimidine tract of *dsx* RNA (Heinrichs and Baker 1995), suggesting that Rbp1 is required to stimulate use of the regenerated 5′ splice site in *Ubx.* These observations seem to be at odds with the effects of *Df(3R)M-KX1*, which deletes *Rbp1*, but it is possible that this deficiency also deletes factors required for mI retention or initial splicing to E5′, or that Rbp1 itself has multiple functions in *Ubx* splicing.

Additional factors: It is unlikely that the factors described here represent all of those with critical effects on *Ubx* splicing regulation. The analysis of deficiencies itself poses certain limitations: an effect on the *Ubx* haltere phenotype may be masked by the simultaneous deletion of a gene that encodes a negative regulator of *Ubx* expression or function or of two factors with opposite effect on the regulation of *Ubx* splicing. Furthermore, we have performed detailed molecular analyses by quantitative RT-PCR only for those regions whose phenotypic interactions with *Ubx* were confirmed by overlapping deficiencies, but another 22 regions were tentatively identified by single deficiencies as containing strong haploinsufficient enhancers of *Ubx* and might harbor genes with important effects on splicing; thus the regions described above are probably only a subset of those that can be identified with this approach. Using the positional information provided by the deficiencies plus RT-PCR assays of the *Ubx* splicing pattern, it should be possible to identify specific mutations in the relevant gene(s) within any region of interest.

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