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Altered Levels of the *Drosophila* HRB87F/hrp36 hnRNP Protein Have Limited Effects on Alternative Splicing In Vivo

Kai Zu,* Martha L. Sikes,* Susan R. Haynes,† and Ann L. Beyer*‡

*Department of Microbiology, University of Virginia, Charlottesville, Virginia 22908; and †Laboratory of Molecular Genetics, National Institute of Child Health and Human Development, Bethesda, Maryland 20892

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The *Drosophila melanogaster* genes *Hrb87F* and *Hrb98DE* encode the fly proteins HRB87F and HRB98DE (also known as hrp36 and hrp38, respectively), that are most similar in sequence and function to mammalian A/B-type hnRNP proteins. Using overexpression and deletion mutants of *Hrb87F*, we have tested the hypothesis that the ratio of A/B hnRNP proteins to SR family proteins modulates certain types of alternative splice-site selection. In flies in which HRB87F/hrp36 had been overexpressed 10- to 15-fold above normal levels, aberrant internal exon skipping was induced in at least one endogenous transcript, the *dopa decarboxylase* (*Ddc*) pre-mRNA, which previously had been shown to be similarly affected by excess HRB98DE/hrp38. In a second endogenous pre-mRNA, excess HRB87F/hrp36 had no effect on alternative 3′ splice-site selection, as expected from mammalian hnRNP studies. Immunolocalization of the excess hnRNP protein showed that it localized correctly to the nucleus, specifically to sites on or near chromosomes, and that the peak of exon-skipping activity in *Ddc* RNA correlated with the peak of chromosomally associated hnRNP protein. The chromosomal association and level of the SR family of proteins were not significantly affected by the large increase in hnRNP proteins during this time period. Although these results are consistent with a possible role for hnRNP proteins in alternative splicing, the more interesting finding was the failure to detect significant adverse effects on flies with a greatly distorted ratio of hnRNPs to SR proteins. Electron microscopic visualization of the general population of active genes in flies overexpressing hnRNP proteins also indicated that the great majority of genes seemed normal in terms of cotranscriptional RNA processing events, although there were a few abnormalities consistent with rare exon-skipping events. Furthermore, in a *Hrb87F* null mutant, which is viable, the normal pattern of *Ddc* alternative splicing was observed, indicating that HRB87F/hrp36 is not required for *Ddc* splicing regulation. Thus, although splice-site selection can be affected in at least a few genes by gross overexpression of this hnRNP protein, the combined evidence suggests that if it plays a general role in alternative splicing in vivo, the role can be provided by other proteins with redundant functions, and the role is independent of its concentration relative to SR proteins.

INTRODUCTION

HnRNP (heterogeneous nuclear ribonucleoprotein) proteins are a family of RNA-binding proteins comprising >20 polypeptides ranging from 34 to 120 kDa (reviewed, Dreyfuss *et al.*, 1993). These proteins associate with nascent RNA polymerase II transcripts (pre-mRNAs) to form RNP complexes, which become the substrate for subsequent nuclear RNA processing and transport activities. Although hnRNP proteins are not

‡ Corresponding author: Department of Microbiology, Box 441 UVA HSC, Charlottesville, VA 22908.

spliceosomal components (Bennett *et al.*, 1992), some of them seem to have a role in splice-site selection. HnRNP I/PTB may regulate alternative splicing by competing with $U2AF^{65}$ for certain polypyrimidine tract/3′ splice sites (Mulligan *et al.*, 1992; Lin and Patton, 1995; Singh *et al.*, 1995). The *Drosophila* hnRNP hrp48 acts as part of a protein complex that recognizes an inactive pseudo-5′ splice site in a tissue-specific manner, resulting in retention of a downstream intron (Siebel *et al.*, 1992, 1994). HnRNP F is involved in the tissue-specific splicing of Src pre-mRNA as part of a complex that specifies alternative exon inclusion in neurons (Min *et al.*, 1995).

The closely related A/B type hnRNP proteins, major components of mammalian hnRNP complexes, are perhaps the best-studied hnRNPs. These proteins (e.g., A1, A2, B1, B2) are basic proteins of 30–40 kDa with a domain structure consisting of two copies of ~90 amino acid RNA-binding domain (RBD) (Dreyfuss *et al.*, 1988), followed by a domain very rich in glycine (~50%) (reviewed, Dreyfuss *et al.*, 1993). They are abundant and ubiquitous in eukaryotic nuclei, and although binding preferences can be identified in vitro (Swanson and Dreyfuss, 1988; Buvoli *et al.*, 1990), immunocytological studies indicate that they bind to nascent pre-mRNA at essentially all sites of Pol II transcription in vivo in amounts that correlate with the RNA mass at the site (Wu *et al.*, 1991; Amero *et al.*, 1992, M.J. Matunis *et al.*, 1992; E.L. Matunis *et al.*, 1993). They are thought to coat completely the transcript in a cooperative manner as it is synthesized (Cobianchi *et al.*, 1988; Conway *et al.*, 1988; reviewed, Dreyfuss *et al.*, 1993), yet they seem to be displaced easily at splice sites by more specific splicing factors (Bennett *et al.*, 1992; Sun *et al.*, 1993; Staknis and Reed, 1994a). The nuclear abundance and helix destabilizing/RNA annealing activities of A1 hnRNP (Kumar and Wilson, 1990; Munroe and Dong, 1992; Pontius and Berg, 1992; Portman and Dreyfuss, 1994) may, in fact, facilitate the binding of more specific factors, such as snRNPs, to splice sites. Recent studies have focused on the ability of these hnRNPs to shuttle between the nucleus and cytoplasm, perhaps as a major component of the mRNA export machinery (Michael *et al.*, 1995 and references therein), and also on their ability to influence splice-site selection (Mayeda and Krainer, 1992; also see below). It is the latter property that has been investigated in a living organism in the present study.

The hnRNP A1 protein has been shown to influence alternative 5′ splice-site selection in cell-free systems and in transiently transfected cell cultures. Its activity is opposite to that of certain SR proteins, a family of nuclear phosphoproteins containing one or more RBDs as well as a domain rich in alternating serine and arginine residues in which many of the serine residues are phosphorylated (Zahler *et al.*, 1992). For example, the SR protein SF2/ASF, which is an essential splicing factor, promotes use of proximal 5′ splice sites in some pre-mRNAs tested in vitro in which there are alternative 5′ splice sites (Fu *et al.*, 1992; Mayeda and Krainer, 1992; Horowitz and Krainer, 1994; but Wang and Manley, 1995). HnRNP A1 counteracts the activity of SR proteins in the selection of alternative 5′ splice sites in certain natural and model pre-mRNAs, leading to the activation of distal 5′ splice sites (Mayeda and Krainer, 1992). However, hnRNP A1 does not affect the constitutive splicing and alternative 3′ splice-site selection activities of SF2/ASF and SC35 (Mayeda and Krainer, 1992). Additional studies show that overexpression of hnRNP A1 in mammalian tissue-culture cells has a similar effect on alternative 5′ splice-site selection. Specifically, transient overexpression of hnRNP A1 promotes the use of the most distal 5′ splice site in the adenovirus E1A pre-mRNA (Cáceres *et al.*, 1994; Yang *et al.*, 1994). Addition of hnRNP A1 can also promote skipping of some small, alternatively spliced internal exons in model genes in vitro (Mayeda *et al.*, 1993). Finally, HnRNP A1 promotes alternative intron retention in a bovine growth hormone pre-mRNA in vitro, again counteracting the opposite activity of SF2/ASF (Sun *et al.*, 1993). Whereas the SR proteins typically act as enhancers of splice-site selection (reviewed, Fu, 1995), the activities of A1 hnRNP (distal 5′ splice-site selection, internal exon skipping, and intron retention) can be interpreted as a general repressive activity on splice-site selection and perhaps specifically on 5′ splice-site recognition (compare Talerico and Berget, 1990; Kuo *et al.*, 1991). On the basis of the studies reviewed above, it has been proposed that the amounts of specific hnRNP proteins and specific SR proteins, or their amounts relative to each other in a given nucleus, may regulate the alternative splicing patterns of many genes (Mayeda and Krainer, 1992; Mayeda *et al.*, 1993; Cáceres *et al.*, 1994). A prediction of this proposal is that a significant change in the concentration of either type of protein will have adverse effects on normal gene expression and thus on normal development and viability in an animal. Indeed, this has been borne out for the *Drosophila* SR protein B52 (Kraus and Lis, 1994; Ring and Lis, 1994), which is an essential protein involved in splicing (Peng and Mount, 1995).

For hnRNP proteins, it is important to extend the current studies (done in vitro and by transient expression in tissue culture cells) to studies in living animals in which pre-mRNA metabolism occurs in the context of normal development, tissue differentiation, and nuclear organization. *Drosophila melanogaster* provides an excellent test system. *Drosophila* hnRNP proteins similar to the vertebrate hnRNP A/B proteins recently have been characterized (Haynes *et al.*, 1990, 1991; E.L. Matunis *et al.*, 1992; M.J. Matunis *et al.*, 1992). These

proteins, like the A/B hnRNP proteins, have a modular structure consisting of two RNP consensus (RNP-CS) RBDs and a carboxyl-terminal glycine-rich domain. Among these *D. melanogaster* hnRNPs, the hrp40 protein was shown to be encoded by the squid gene, which is required for dorsoventral axis formation during oogenesis (Kelley, 1993; Matunis *et al.*, 1994), and the hrp48 protein was shown to be involved in P-element pre-mRNA alternative splicing (Siebel *et al.*, 1992, 1994). The *Drosophila* hnRNP proteins most similar in sequence to mammalian A1 and A2/B1 proteins are encoded by two genes, *Hrb98DE* and *Hrb87F* (Haynes *et al.*, 1990, 1991; E.L. Matunis *et al.*, 1992).[1] HRB98DE/hrp38 and HRB87F/hrp36 have ~60% sequence identity with the A1 protein in the RBDs. Despite this low-sequence similarity, they are identical to the A/B proteins in certain conserved motifs in the RBDs that tend to be characteristic of protein types (Haynes *et al.*, 1991; E.L. Matunis *et al.*, 1992), and an evolutionary analysis places them closest to the A/B proteins (Birney *et al.*, 1993). HRB87F/hrp36 and HRB98DE/hrp38 are basic in charge, like the mammalian A/B proteins (with pIs in the 8.5–10 range) (Beyer *et al.*, 1977; Haynes *et al.*, 1990, 1991), whereas hrp40/squid and hrp48 are acidic proteins (pIs 6–7) (M.J. Matunis *et al.*, 1992). Antibodies raised to the RBDs of HRB98DE/hrp38 also recognize HRB87F/hrp36 as well as the A and B hnRNP proteins of HeLa cells (Raychaudhuri *et al.*, 1992). In all characteristics examined to date, HRB98DE/hrp38 and HRB87F/hrp36 are similar to the A/B hnRNP proteins. These include formation of monomer hnRNP complexes with bulk poly(A)+ nuclear pre-mRNA (Beyer *et al.*, 1977; Raychaudhuri *et al.*, 1992); general association with essentially all Pol II transcripts (Wu *et al.*, 1991; Amero *et al.*, 1992; Matunis *et al.*, 1993); nuclear localization mediated via a short M9-like sequence in the C-terminal glycine-rich domain (Siomi and Dreyfuss, 1995; Weighardt *et al.*, 1995; K. Zu and A.L. Beyer, unpublished observation), and ability to promote exon skipping (Mayeda *et al.*, 1993; Shen *et al.*, 1995; this report) but not alternative 3′ splice-site selection (Mayeda and Krainer, 1992; this report) when overexpressed. Furthermore, neither the human A1 gene (Ben-David *et al.*, 1992) or the *Drosophila HRB87F* gene (this report) is essential, presumably because of functional redundancy among the various members of this abundant, ubiquitous protein family.

The HRB87F/hrp36 and HRB98DE/hrp38 proteins are 76% identical in the RNP motifs and 67% identical in the glycine-rich region (Haynes *et al.*, 1991). Both genes are expressed robustly throughout all stages of development, although there is some quantitative variation in the level of expression (Haynes *et al.*, 1990, 1991). In this study we examined some of the consequences of HRB overexpression and absence in living flies. Although overexpression of HRB87F/hrp36 does alter the splicing of at least one pre-mRNA (*Ddc*), indicating that the overexpressed protein can reproduce the in vitro effect of excess protein, the bulk of the evidence calls into question the simplest interpretation of a model in which the absolute amount of the hnRNP protein or the ratio of hnRNP proteins to SR family proteins plays a general role in the regulation of alternative splicing.

MATERIALS AND METHODS

D. melanogaster Transformation and HRB87F/hrp36 Overexpression

The plasmid expressing FLAG-tagged *Hrb87F* cDNA was constructed by replacing a *Sty*I fragment at the N terminus of the wild-type gene (−11 to +822) with a corresponding PCR-generated fragment bearing the FLAG epitope (Eastman Kodak, Rochester, NY) at the N terminus plus the original *Sty*I fragment. PCR amplification was performed with the Hrb87F N-terminal oligonucleotide (5′ GACTGAACCCAAGGATGGACTACAAAGACGATGACGATAAAATGGCGGAACAAAACGA 3′) and C-terminal oligonucleotide (5′ACCCTGGTTATTCCATGG 3′). The correct sequence was confirmed by sequencing. The *Sty*I fragment of *Hrb87F* cDNA was replaced with the same PCR-generated fragment containing the FLAG epitope. The *Nco*I/*Kpn*I fragment containing epitope-tagged *Hrb87F* under the control of the *Drosophila* hsp70 promoter (Lis *et al.*, 1983) and ending with the SV40 polyadenylation signal was subcloned into the P-element transformation vector pW8 (Klemenz *et al.*, 1987). The resulting construct was injected into embryos with a helper plasmid encoding transposase (Spradling and Rubin, 1982). Germ line transformants were obtained, and homozygous fly stocks were established. The FLAG epitope-tagged *Hrb87F* gene was induced by heat shock by placing organisms of the desired developmental stage into a 37° incubator for 30–60 min (as indicated for appropriate figures).

Generation of an Hrb87F Null Mutation

Southern blots of digests of genomic DNA from nine fly strains with P elements in the 87F region were hybridized to a *Hrb87F* probe, and one line, ry^{506} *P[lacW]Y1217/TM3*, showed an altered restriction pattern. PCR amplification and sequencing of the junctions between the P element and *Hrb87F* showed that this line has a P element located in the 5′ untranslated region of the gene. Imprecise excisions of this P element were generated by standard genetic techniques

[1] A word about nomenclature is required here. Haynes *et al.* (1990, 1991) named the genes *Hrb* for *h*nRNA *b*inding, followed by their genetic locus, on polytene chromosomes 98DE or 87F. When E.L. Matunis *et al.* (1992) and M.J. Matunis *et al.* (1992) isolated a larger spectrum of hnRNP proteins from *Drosophila*, they named the series of proteins according to apparent molecular weight, from hrp36 to hrp75. The *Hrb98DE* gene encodes the HRB98DE protein, which corresponds to hrp38, and the *Hrb87F* gene encodes the HRB87F protein, which corresponds to hrp36. We use both names in this report. It should be noted, however, that the major protein product of *Hrb87F* (HRB87F/hrp36) is slightly larger than the *Hrb98DE* products (HRB98DE/hrp38). The 36-kDa isoform is described by E.L. Matunis *et al.* (1992) as an alternatively spliced form that is missing 60 amino acids from the glycine-rich domain as compared with the protein studied in this report (Haynes *et al.*, 1991). The HRB87F/hrp36 protein was also identified as the *Drosophila* nuclear antigen P11 (Hovemann *et al.*, 1991). The original designation for a cDNA clone of HRB98DE/hrp38 was p9 (Haynes *et al.*, 1987).

with the Δ 122–3 P transposase (Robertson *et al.*, 1988). Of 78 potential excision lines tested, one had a deletion that began in the *P[lacW]* element and removed the entire *Hrb87F* coding region and a portion of the adjacent gene 3′ of *Hrb87F*. This mutant has been named *Df(3R)Hrb87F*.

Western Blot Analysis

Ten third-instar larvae were homogenized in 100 μl of sample buffer containing 60 mM Tris-HCl (pH 6.8), 2% SDS, 1%-mercaptoethanol, and 0.5% bromophenol blue. The homogenates were clarified by centrifugation at 12,000 rpm for 15 min. Protein concentrations were determined by the Bicinchoninic Acid-Protein Assay (BCA-Protein; Pierce, Rockford, IL). Fifty micrograms of total larval protein of each sample were loaded onto 10% SDS-polyacrylamide gels. Proteins were transferred to nitrocellulose (Schleicher and Schuell, Keene, NH), and blots were detected according to the directions of the manufacturer (enhanced chemiluminescence; Amersham, Arlington Heights, IL), with a 1:1000 dilution of anti-FLAG antibody (Eastman Kodak) and horseradish peroxidase-conjugated secondary antibody.

Analysis of Splicing Products

The RNA preparation and RT-PCR were performed as described previously (Shen *et al.*, 1993).

Immunofluorescence on Polytene Chromosomes and Salivary Glands

Polytene chromosome squashes (Figure 2, c and d) were performed as described (Zink and Paro, 1989). After the glands were squashed, slides were incubated in a 1:300 dilution of anti-FLAG immunoglobulin G (IgG) in Tris-buffered saline (TBS) with 5% dry milk overnight at 4°C, followed by three 5 min washes with TBS. Then slides were incubated at room temperature for 1 h in a 1:100 dilution of fluorescein-conjugated anti-mouse IgG (Vector Laboratories, Burlingame, CA) in TBS with 5% dry milk, followed by three 5 min washes with TBS, and mounted in Vectashield mounting medium (Vector Laboratories).

For immunostaining of whole salivary glands (Figure 2, a and b), dissected salivary glands were fixed in 4% formaldehyde in phosphate-buffered saline (PBS) for 30–45 min at room temperature. Glands were washed three times for 5 min each in PBS and two times for 5 min each in PBT (PBS with 0.1% BSA and 0.3% Triton X-100). Glands were then incubated in a 1:300 dilution of anti-FLAG IgG in PBT with agitation for 6–18 h at room temperature and washed five times for 5 min each with PBT. Glands were incubated in a 1:100 dilution of fluorescein-conjugated anti-mouse IgG in PBT for 8 h at room temperature, followed by 2–4 washes for 6–24 h in PBT. The slides were examined and photographed with a Zeiss Axioplan microscope.

For immunostaining of partially squashed salivary glands (Figures 6 and 7), glands were dissected in 45% acetic and 3.7% formaldehyde and were squashed between a siliconized coverslip and a slide until the desired degree of dispersal was obtained (elapsed time 2–4 min). The slides were immersed in liquid nitrogen until the bubbling stopped, after which time the coverslip was flicked off and the slide was immersed in 4% paraformaldehyde in PBS for 10 min. The slides were rinsed in Tris-buffered saline with Tween (TBST; 20 mM Tris-HCl pH 7.7, 1.7% NaCl, and 0.1% Tween-20), and then the tissue samples were permeabilized by incubating them for 10 min in a drop of 1% Triton-X 100 in TBST. After two 5 min washes in TBST, the primary antibody was added (FLAG antibody diluted 1:300 in TBST plus 5% nonfat dry milk), and incubation occurred overnight at 4°C in a humid box. Slides were then washed two times for 10 min in TBST, and the secondary antibody (anti-mouse IgG fluorescein diluted 1:100 in TBST plus 5% nonfat dry milk) was added for 4–5 h in a humid box at room temperature. Slides were washed three times for 10 min and were either mounted for viewing or stained for 2 min in freshly diluted 4,6-diamidino-2-phenylindole (DAPI; 0.5 μg/ml in 180 mM Tris-HCl pH 7.5) and then washed and mounted in Vectashield for viewing.

Electron Microscopy

Miller chromatin spreading of *Drosophila* embryo chromatin for electron microscopic (EM) visualization of active genes was performed as described (Beyer and Osheim, 1988; Beyer *et al.*, 1994). Before EM preparation, the hnRNP protein was induced by heat shock of 3–4-h-old embryos at 37°C for 30 min followed by 2.5–14 h recovery at room temperature.

RESULTS

Overexpressed HRB87F Protein Is Stable and Localizes Correctly within the Nucleus

To investigate the in vivo function of the *Drosophila* A/B-like hnRNP protein HRB87F/hrp36, we introduced FLAG-epitope–tagged *Hrb87F* cDNAs under the control of the *Drosophila* heat-shock Hsp70 promoter into flies by P-element–mediated transformation. The FLAG-tagged HRB87F/hrp36 protein began to accumulate 2 h after a 1 h 38°C heat-shock induction (Figure 1, lane 5) and reached a peak at 8–12 h of recovery (Figure 1, lanes 7–8) to ~10–15 times the level of the endogenous HRB87F/hrp36 protein. The 10- to 15-fold induction level was estimated from visual inspection of a Western blot (stained with a polyclonal anti-HRB antibody) of a dilution series of the larval extract from induced animals 8 h after induction, as compared with undiluted extract from control animals (our unpublished results). A time course after heat shock shows that the induced FLAG/HRB87F remained at high levels throughout 24 h of recovery at room temperature (Figure 1, lanes 5–9).

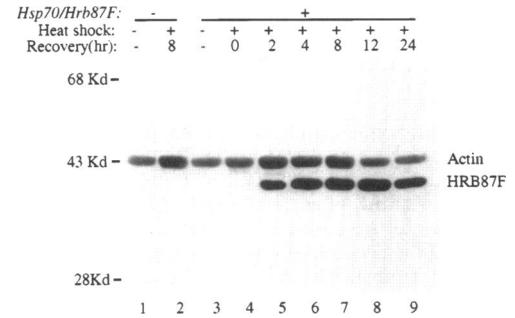


Figure 1. Heat-shock induction of HRB87F/hrp36 protein. FLAG epitope-tagged *Hrb87F* cDNA was introduced into flies under the control of the Hsp70 promoter. Total third-instar larval protein from parental wild-type flies (lanes 1 and 2) or *Hrb87F* transgenic flies (lanes 3–9) was resolved by SDS-PAGE and probed with anti-FLAG antibody. Larvae were allowed to recover at room temperature for periods of time as indicated after heat shock at 38°C for 1 h, as described (Shen *et al.*, 1993). The blot was also probed with anti-actin mAb as a loading control. Molecular weights of the protein standards are indicated.

Previous studies have shown that HRB87F/hrp36 is a non-nucleolar nuclear protein and is present at sites of transcription on polytene chromosomes (Hovemann *et al.*, 1991; Amero *et al.*, 1992; M.J. Matunis *et al.* 1992; E.L. Matunis *et al.*, 1993). (HRB87F/hrp36 is termed P11 in the Hovemann study.) We tested the subcellular localization of HRB87F/hrp36 before and after heat-shock induction of the transgene by indirect immunofluorescent staining in salivary gland tissue with the use of anti-FLAG monoclonal antibody (mAb). Because of the basal activity of the Hsp70 promoter at room temperature, FLAG/HRB87F/hrp36 could be detected in the salivary gland nuclei without heat-shock induction (Figure 2a). (This basal activity is particularly noticeable in salivary glands.) The nuclear staining increased significantly after heat shock, and the signal was still predominantly nuclear, excluding nucleoli, after 8 h of recovery at room temperature (Figure 2b). The protein was seen in a banded pattern on polytene chromosomes at room temperature (Figure 2c), presumably corresponding to sites of RNA synthesis and processing, as shown previously (Amero *et al.*, 1992; Matunis *et al.*, 1993). The polytene-chromosome staining increased dramatically after induction and was distributed throughout the chromosomes at 8 h after heat shock (compare Figure 2d with 2c, both shown at the same exposure). The correct localization of the epitope-tagged, overexpressed protein within the nucleus suggests that it behaves similarly, if not identically, to the wild-type protein.

The animals in which HRB87F/hrp36 had been overexpressed showed no obvious developmental abnormalities other than somewhat-slowed development relative to wild type. A test of excess HRB87F/hrp36 on viability was conducted by inducing hnRNP expression (38°, 1 h) at 24 h intervals throughout development and with 100 experimental and control animals at each time point. Viability was typically 70% or higher and was independent of HRB87F/hrp36 induction.

Excess HRB87F/hrp36 Protein Induces Exon Skipping in Ddc Pre-mRNA

We tested endogenous *Dopa decarboxylase* (*Ddc*) pre-mRNA splicing in late third-instar larvae to see whether excess HRB87F/hrp36 was capable of inducing exon skipping. The *Ddc* transcript shows significant splicing abnormalities when the related hnRNP protein HRB98DE/hrp38 is overexpressed (Shen *et al.*, 1995), whereas four other transcripts tested do not (C. Cass and A.L. Beyer, unpublished observation). The *Ddc* transcript is a particularly favorable substrate for this in vivo assay, because flies are tolerant to gross alterations in the normal patterns of isoform expression and also to significantly reduced protein levels (Morgan *et al.*, 1986).

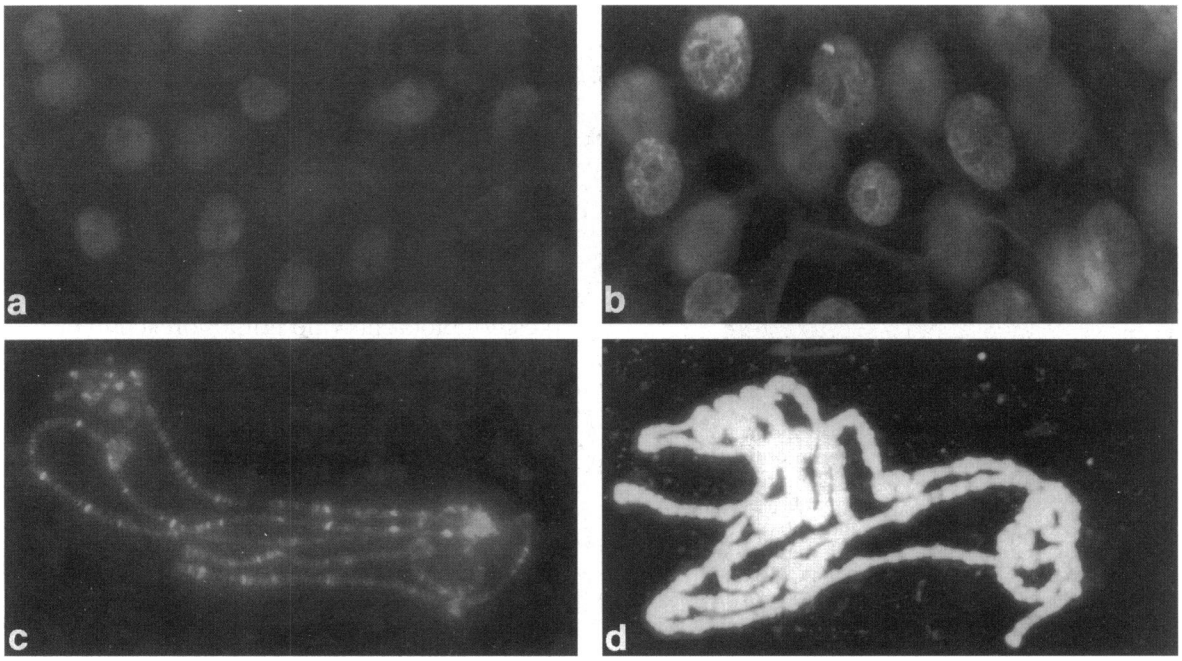


Figure 2. Immunofluorescent staining of whole-mount salivary gland and polytene chromosomes with anti-FLAG mAb. Salivary glands from third-instar larvae of *Hrb87F* transformants were examined by whole-mount analysis (a and b) or were squashed (c and d) and probed with anti-FLAG mAb as described in MATERIALS AND METHODS. (a and c) Room temperature samples; (b and d) heat-shocked samples with 8 h room temperature recovery.

Ddc is expressed in the hypoderm and the central nervous system (CNS), and *Ddc* pre-mRNA is spliced alternatively in these two tissues (Morgan *et al.* 1986). The *Ddc*–CNS splice form includes all four exons ABCD, whereas the hypodermal splice form contains only three exons, ACD, as shown in the schematic in Figure 3A. We analyzed splicing of the *Ddc* pre-mRNA by reverse transcription-linked polymerase chain reaction (RT-PCR) with the use of primers specific for *Ddc* exons A and D. Because *Ddc* transcription is induced in the hypoderm during the late third-instar larval stage (Hirsh and Davidson, 1981) and the CNS represents a small portion of the body mass, the hypodermal splice form (ACD) is the predominant *Ddc* mRNA in total larvae. In the absence of the HRB87F/hrp36 transgene (Figure 3B, lanes 1 and 2) or with an uninduced HRB87F/hrp36 transgene (Figure 3B, lane 3), the hypodermal splice form was the only detectable spliced product. However, after heat-shock induction as excess HRB87F/hrp36 protein accumulated, an aberrant *Ddc* splice form accumulated (Figure 3B, lanes 6–9). The induction of this aberrant splice form during 0–12 h after heat shock (Figure 3B, lanes 4–8) in general paralleled the induction of the excess HRB87F/hrp36 protein (Figure 1, lanes 4–8). However, at 2 h after induction, the splicing shift was not yet detected (Figure 3B, lane 5), although there was significant protein present (Figure 1, lane 5). We presume this lag is due in part to pre-existing *Ddc* mRNA, such that at this early time point the majority of the mRNA was still unaffected by the accumulating hnRNP protein. Furthermore, as shown in Figure 6, the epitope-tagged hnRNP protein is not yet generally distributed on the chromosomes at 2 h after induction and thus may not yet be accessible to *Ddc* transcripts. (Figure 6 also addresses the discordance between protein amount and splicing shift in the 24-h time points.) The aberrant splice form comigrated with the AD splice form produced when the other basic hnRNP protein HRB98DE/hrp38 was overexpressed (Shen *et al.*, 1995), and restriction mapping indicated that these two splicing products were the same (our unpublished observation). Thus the overexpression of either HRB98DE/hrp38 or HRB87F/hrp36 had the same effect on the *Ddc* hypodermal pattern splicing, i.e., promotion of internal exon skipping (ACD to AD). Interestingly, the effect on *Ddc*–CNS splicing is much more modest for both proteins, with most *Ddc* mRNA in the CNS representing the expected ABCD form but with some shift to the AD form (Shen *et al.*, 1995; our unpublished results).

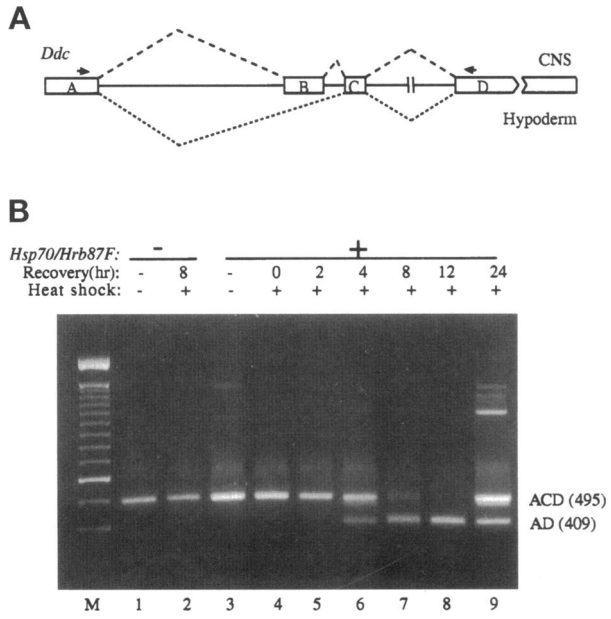


Figure 3. Overexpression of HRB87F/hrp36 induces exon skipping in *Ddc* pre-mRNA. (A) Schematic diagram of the structure of *Ddc* transcripts. Open boxes, labeled A, B, C, D, represent *Ddc* exons. The central nervous system (CNS) splicing pattern is shown at the top of the diagram, and the hypodermal splicing pattern is shown at the bottom of the diagram. Arrows indicate the locations and the 5′-3′ orientation of primers used in the RT-PCR assay. (B) Time course (0–24 h) of the effect of excess HRB87F/hrp36 on *Ddc* splicing in flies with the *Hrb87F* transgene (lanes 3–9). Control lanes (1 and 2) display the same analysis in parental flies with no transgene. Third-instar larval RNA was reverse transcribed and PCR amplified with *Ddc* exons A- and D-specific primers. The amplified material was separated on a 2% agarose gel. The identities of the spliced products are shown on the right. Lane M displays molecular weight standards.

HRB87F/hrp36 Is Not Required for Ddc Splicing Regulation

If the HRB87F/hrp36 protein is required for the normal regulation of *Ddc* alternative splicing, we might expect that the absence of HRB87F/hrp36 would affect *Ddc* splicing in the CNS and hypoderm. To test this, we generated a *Hrb87F* null mutation as described in MATERIALS AND METHODS. The mutation is a null because it deletes the entire *Hrb87F* coding region; Western blots show no immunoreactive protein at the expected position (our unpublished results). Lack of HRB87F/hrp36 does not impair the viability of the flies, because the survival rate of homozygous adults is nearly identical to that of their heterozygous siblings. Similarly, in an otherwise wild-type genetic background, male and female fertility seem unaffected. (However, our preliminary data suggest that genetic interactions between two hnRNP protein genes can affect fertility.) To evaluate *Ddc* splicing, RNA was prepared from the CNS and hypoderm of *Hrb87F* null flies and analyzed by RT-PCR. In the absence of the HRB87F/hrp36 protein, *Ddc* pre-mRNA was spliced normally. The ABCD splice form was the predominant spliced product in the CNS, and the ACD form was the predominant product in the

hypoderm (Figure 4). This indicates that although HRB87F/hrp36 can induce aberrant *Ddc* splicing when overexpressed, it is not required for normal *Ddc* splicing regulation. However, other *Drosophila* hnRNP proteins, especially the closely related HRB98DE/hrp38, may have overlapping functions with HRB87F/hrp36, so it is difficult to rule out a requirement for an A/B-type hnRNP protein in *Ddc* splicing.

Overexpression of HRB Proteins Does Not Affect the Choice of Alternative 3′ Splice Sites in the Hrb98DE Pre-mRNA

Next we tested whether an excess of *Drosophila* hnRNP proteins had any effect on an alternative 3′ splice-site choice in vivo. Mammalian hnRNP A1 does not affect the use of an alternative 3′ splice site in a model pre-mRNA in vitro (Mayeda and Krainer, 1992). *Hrb98DE* pre-mRNA, which is ubiquitously expressed (Haynes *et al.*, 1990), was used as the substrate to test the effect of excess hnRNP protein (i.e., the effect of its own protein product HRB98DE/hrp38 as well as that of HRB87F/hrp36) on the use of specific alternative 3′ splice sites. Transcripts from the *Drosophila Hrb98DE* locus generate four protein isoforms by the use of two alternative first exons and two alternative 3′ splice sites at the end of the first intron (Figure 5A). In wild-type flies, alternative exons 1A and 1B are used at all stages of development. Both 3′ splice sites upstream of exon 2 are also used at approximately equal frequency with both exons 1A and 1B in what seems to be a balanced competition rather than a highly regulated choice (Haynes *et al.*, 1990). Use of alternative 3′ splice sites in the *Hrb98DE* pre-mRNA in flies overexpressing either the HRB98DE/hrp38 protein or the HRB87F/hrp36 protein was assayed by RT-PCR with primers specific to exon 2 and either exons 1A or 1B. The sizes of the expected products are 200 and 188 base pairs (bp) for exon 1A and 170 and 158 bp for exon 1B. In the absence of overexpression of either HRB98DE/hrp38 or HRB87F/hrp36, both 3′ splice sites were used equally, as expected (Figure 5B, lanes 1–3 and 10–12). After heat-shock induction of either HRB98DE/hrp38 or HRB87F/hrp36 for 1 h with recovery at room temperature for either 0 or 8 h, there was no reproducible change in the usage of 3′ splice sites (Figure 5B, lanes 7–9 and 13–18). This suggests that overexpression of neither HRB98D/hrp38 nor HRB87F/hrp36 affects the 3′ splice-site choice of *Hrb98DE* pre-mRNA. (Lanes 4–6 should be disregarded. Note that the apparent change in these lanes was due to heat-shock induction of transcription of that particular *Hrb98DE* transgene. These lanes do, however, give an indication of the induced mRNA level relative to the normal mRNA level.)

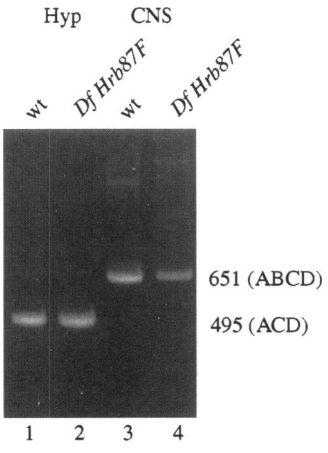


Figure 4. HRB87F/hrp36 is not required for *Ddc* splicing regulation. Total RNA was prepared from larval hypoderm (lanes 1 and 2) or larval CNS (lanes 3 and 4) of *Hrb87F* null mutant flies, as described (Shen and Hirsh, 1994). RT-PCR was performed with primers specific to *Ddc* exons A and D. Wt, wild type; Df, *Hrb87F*-deficient. The size and identity of the spliced products are shown on the right.

Exon Skipping Activity Is Correlated with the Amount of Excess hnRNP Protein Associated with Polytene Chromosomes

As noted above, the induction of the aberrant splice form of *Ddc* pre-mRNA paralleled the induction of HRB87F/hrp36 protein (compare Figure 1, lanes 4–8, and Figure 3B, lanes 4–8). However, the decrease in exon-skipping activity noted at 24 h after HRB87F/hrp36 induction (Figure 3B, lane 9) was not accompanied by so significant a decrease in HRB87F/hrp36 protein as that detected by Western blot analysis (Figure 1, lane 9). We noted this same phenomenon in our earlier report on overexpression of the HRB98DE/hrp38 hnRNP protein in which, at 24 h, *Ddc* pre-mRNA splicing had returned to normal even though HRB98DE/hrp38 protein levels had not fallen significantly (Shen *et al.*, 1995). We speculated at that time that the larvae may counteract the effects of unusually high hnRNP protein levels by inducing factors with antagonistic activity to hnRNPs (e.g., SR family proteins) and/or by changing the cellular location or activity of the excess hnRNP.

A test of the latter hypothesis is shown in Figure 6, in which the location of the epitope-tagged hnRNP protein is shown in salivary gland cells at the same time points after induction as those monitored in Figures 1 and 3. Each pair of micrographs in Figure 6 shows the location of the epitope-tagged HRB87F protein (a–f) compared with the location of DNA in the same cells as monitored by DAPI staining (a'–f'). Before heat-shock induction there was a low level of tagged HRB87F/hrp36 protein in nuclei (Figure 6a). Exon skipping was first seen at 4 h after heat shock (Figure 3B, lane 6), which represents the time at which the protein was first generally distributed on the chromosomes (Figure 6d). The period of maximal exon skipping (i.e., 8–12 h as seen in Figure 3B) correlated with the period in which the excess hnRNP protein was present in highest amounts in a general distribution on polytene chromosomes (Figure 6, e and f). At

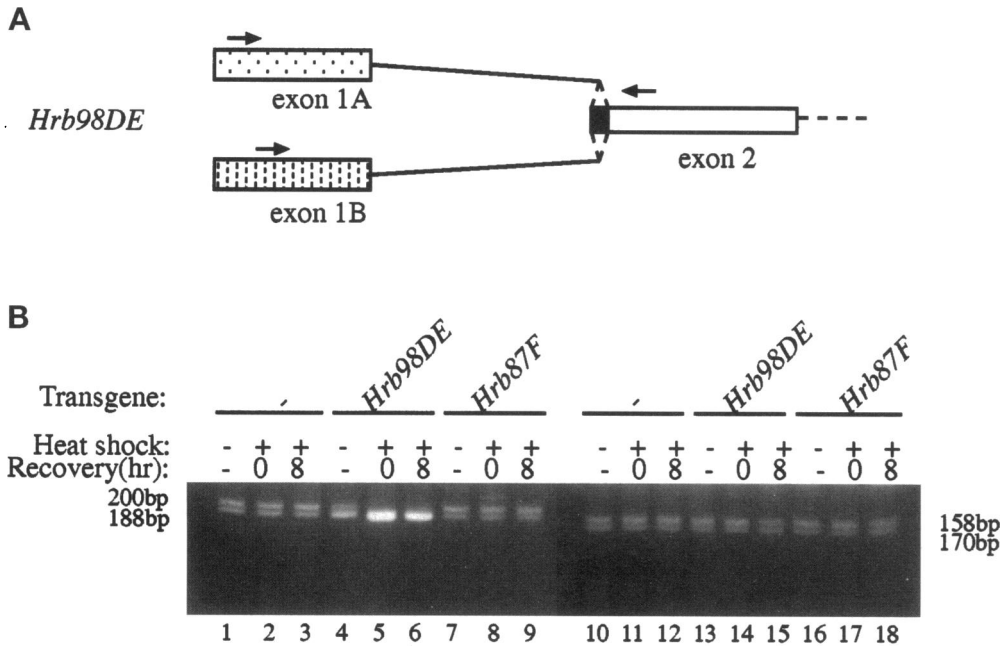


Figure 5. Overexpression of either HRB98DE/hrp38 or HRB87F/hrp36 proteins does not affect 3′ splice-site selection at the end of the first intron in *Hrb98DE* pre-mRNA. (A) Schematic diagram of the structure of the first two exons of *Hrb98DE* transcripts (total of six exons) (Haynes *et al.*, 1990). Open boxes represents *Hrb98DE* exons 1A, 1B, and 2. Arrows indicate the locations and the 5′-3′ orientation of primers used in the RT-PCR assay. (B) RT-PCR analysis of *Hrb98DE* 3′ splice-site choice. RNA samples from third-instar larvae before and after 1 h 38°C heat shock (0 or 8 h recovery) were reverse transcribed with a primer specific to exon 2. Aliquots of the RT product were PCR amplified with the RT primer and a primer specific for either exon 1A (lanes 1–9) or 1B (lanes 10–18). The PCR products were separated on a 2% agarose gel. The sizes of PCR products are indicated. The apparent increase in the 188-bp product in lanes 5 and 6 is not due to a splicing change but rather to the fact that this version of the *Hrb98DE* cDNA is present as the heat-shock–induced transgene in these flies.

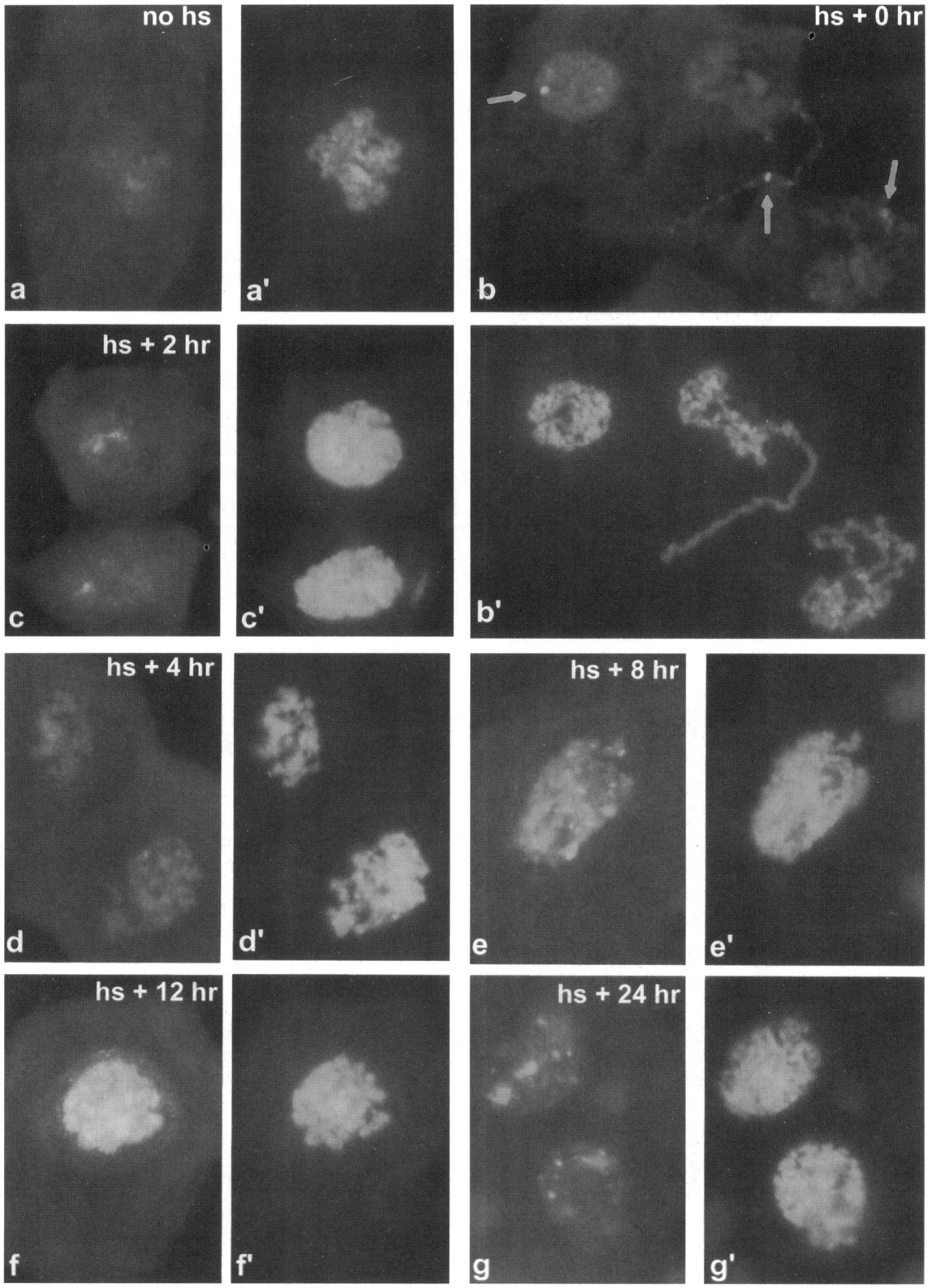
24 h after induction when exon skipping represented a minor proportion of the splicing pattern (Figure 3B, lane 9), the general distribution pattern of HRB87F/hrp36 was replaced by one to a few local accumulations of the protein (Figure 6g). Although we do not know the nature of this aggregation phenomenon, it has been observed in several different larval cell types from diploid to fully polytene nuclei. Thus, the loss of hnRNP protein from chromosomal sites accompanied the return to a normal splicing pattern.

It is interesting to note that, immediately after heat shock, the majority of the protein accumulated at a single chromosomal locus, detectable as a bright focus in intact nuclei and mappable to the heat-shock puff at position 93D on the right arm of the third chromosome arm (Figure 6b, arrows). This heat-shock–induced localization has been reported previously for HRB87F/hrp36 (Schuldt *et al.*, 1989; Hovemann *et al.*, 1991) and is further evidence that the tagged, overexpressed protein behaves like the native protein. It is also worth noting that at all time points the protein was nuclear and located on or in close proximity to the chromosomes, which seem to be able to accommodate whatever level of hnRNP protein is induced.

SR Protein Distribution on Polytene Chromosomes Is Not Significantly Affected by hnRNP Protein Overexpression

The results of Figure 6 suggested that the induction and duration of the splicing shift were due to the amount of hnRNP protein at chromosomal sites of transcription. Because SR proteins have been shown to oppose the activity of hnRNP proteins in alternative splice-site choice (see INTRODUCTION), we took advantage of our in vivo system to test whether SR proteins were affected by overexpression of hnRNP proteins. For example, in theory the displacement of hnRNP proteins from chromosomal sites at 24 h (Figure 6g) could be due to a competition with SR proteins induced or recruited by excess hnRNPs. We used mAb104 (Roth *et al.*, 1990) for immunofluorescent localization of the proteins on polytene chromosomes. This antibody has been a very important tool in the study of the SR protein family and has been shown to recognize the shared phosphorylated arg-ser–rich domain on SR proteins from many species, including *Drosophila* (Roth *et al.*, 1991). Previous studies have shown that the *Drosophila* SR proteins RBP1 (SRp20) and B52 (SRp55) bind to sites of Pol II transcription on polytene chromosomes (Kim *et al.*, 1992; Champlin and Lis, 1994). As shown in Figure 7, the amount and general chromosomal distribution of SR proteins did

Figure 6 (facing page). Immunofluorescent localization of the epitope-tagged HRB87F/hrp36 protein in larval polytene nuclei. The FLAG antibody and a fluorescein-conjugated secondary antibody were used to localize HRB87F/hrp36 in salivary gland cells (a–g). DAPI was used to localize DNA in the same cells (a′–g′). To detect possible cytoplasmic staining, the glands were broken apart and flattened somewhat, but not completely "squashed," so that the cytoplasm remained around the nuclei. The protein was detected in



(Figure 6 cont.) conditions of no heat-shock induction (a) or with a 1-h heat induction followed by various times of recovery from heat shock, as indicated in panels b–g. Immediately after heat shock, the protein localized to a single bright focus in each nucleus (arrows in b) corresponding to the heat-shock puff at 93D on the right arm of the third chromosome.

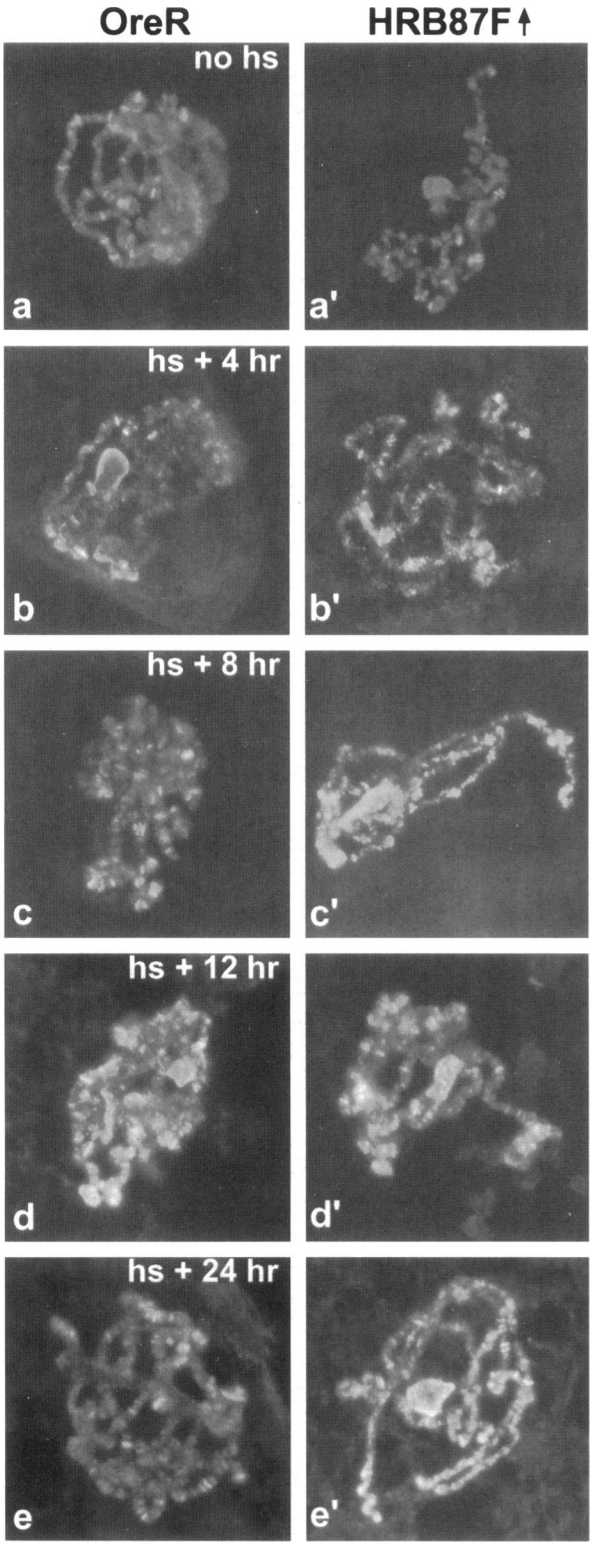


Figure 7. Immunofluorescent localization of SR family proteins in larval polytene nuclei. The mAb 104 and a fluorescein-conjugated secondary antibody were used to localize SR proteins in salivary gland squash preparations. Wild-type Ore-R flies were used (a–e) to control for possible heat-shock effects on SR protein amount and/or distribution. *Hrb87F* transgenic flies were used (a'–e') at the same time points as shown in the corresponding Ore-R panels (a–e) to test for possible hnRNP overexpression effects on SR protein amount and/or distribution.

not change significantly during the 24 h period after heat-shock induction of HRB87F/hrp36 (Figure 7, a'–e'). As a control for possible heat-shock effects, (which were not detected between 4–24 h after heat shock), the same time points were tested for wild-type Oregon-R (Ore-R) flies (Figure 7, a–e). Western blot analysis of SR protein levels in hnRNP overexpressors also revealed no changes in detectable SR proteins for 24 h after hnRNP protein induction (our unpublished results). Combined with Figure 6, these results indicate that, at 8–12 h after induction of HRB87F/hrp36, there was a significant increase in HRB87F/hrp36 on chromosomes, accompanied by little change in SR family proteins on chromosomes, and thus a significant increase in the ratio of this hnRNP protein to SR proteins at or near chromosomal sites of RNP assembly.

EM Visualization of Active Genes in hnRNP Overexpressors Reveals Rare Ultrastructural Anomalies Consistent with Exon Skipping

In previous studies we have used Miller chromatin-spreading methods to analyze RNP packaging and RNA splicing events that occur on nascent pre-mRNA transcripts (Beyer and Osheim, 1988; Beyer *et al.*, 1994). We have found that splicing frequently occurs cotranscriptionally and is represented by a series of ultrastructural events (RNP particle deposition at 5′ and 3′ splice sites, intron loop formation, and intron removal) that is quite reproducible in structure and kinetics. Because this approach provides a view of the general population of genes, we used it to evaluate the generality and types of aberrant splicing seen in HRB98DE/hrp38 and HRB87F/hrp36 overexpressors. After viewing >200 genes from flies with excess hnRNPs, we found that almost all pre-mRNA genes seemed perfectly normal in terms of spliceosome deposition and intron removal.

Of a few rare anomalies seen (~3% of genes), one of them could be interpreted as possibly representing exon skipping. An example is shown in Figure 8. The anomaly is the occurrence of large RNP particles within looped-out segments of the RNA. In all previous studies (reviewed, Beyer *et al.*, 1994) these RNA loops have been shown to represent intron loops and to form between nearest-neighbor RNP particles (5′ and 3′ splice sites), resulting in a smooth RNP morphology in the loop (intron) section (see example of the normal situation in the inset in Figure 8). In the unidentified gene in Figure 8, although a reproducible loop occurred on more than one-half of the transcripts

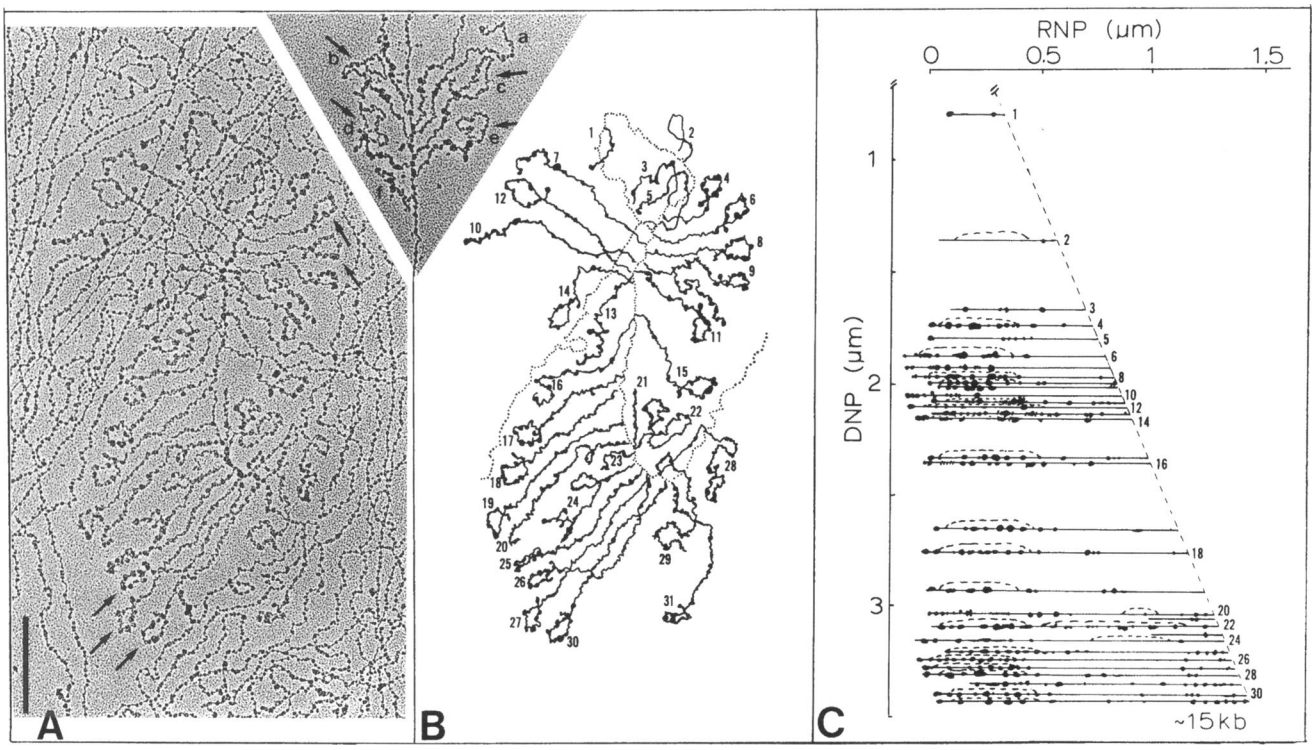


Figure 8. *Drosophila* gene exhibiting ultrastructural abnormalities on its RNA transcripts in the presence of excess hnRNP protein. (A) Electron micrograph of an unidentified embryo gene 3 h after induction of HRB98DE/hrp38. Arrows indicate the unusual beaded loops seen on this gene. There are many overlying inactive chromatin strands near the gene; these are eliminated in the interpretive tracing in B. Bar, 0.5 μm. (B) Interpretive tracing of the gene in A. Dotted line is the DNA template. RNA transcripts are shown as solid lines, including the RNP particles and loops seen on the transcripts. (C) RNP fibril gene map. The sloped, dashed line represents the DNA template; its slope was determined by linear regression analysis of transcript length (Beyer and Osheim, 1988). Linearized transcripts are aligned by abutting their 3′ ends to this "template" line. RNP particles are shown as they appear on the transcripts. Looped structures on the transcripts are shown as dashed lines connecting the two appropriate loop base sites. There is no obvious cotranscriptional splicing on this gene. (Inset) Portion of an unidentified control gene from a wild-type embryo that had been heat shocked and prepared for EM concurrently with the experimental embryo. The vertical fibril is the DNA template. Six complete transcripts are shown (a–f) as well as two transcripts that are cropped at the top of the figure. Arrows indicate the typical unbeaded loop structures on transcripts b–e, which correspond to intron loops. (f) The last transcript shown is missing the loop; it has been spliced.

(as mapped in Figure 8C), the RNA within the loop was not smooth but, rather, was studded with several particles in the general size range of spliceosomes. This morphology is consistent with several possibilities, including exon skipping by the looping out of small internal exons, as occurs in the Ddc transcript. Alternatively, it may represent excess hnRNP protein deposition at these sites because of the presence of particularly favorable binding sites. Although this beaded-loop anomaly was seen only rarely in flies expressing excess hnRNP proteins (~2% of genes), it has never been seen in the genes of wild-type flies in our 15-year experience. Another unusual aspect of these loops, if they indeed represent introns, is that their splicing was slower than is typical. Intron loop removal is detected, on average, within 3 min of 3′ splice-site synthesis (Beyer and Osheim, 1988). On this gene, no loop removal was seen before transcription termination, which represents 5 min after synthesis of the sequence at the 3′ end of the RNA loop.

DISCUSSION

We have analyzed the in vivo function of the *Drosophila* hnRNP protein HRB87F/hrp36 by examining the effects of either increasing or decreasing normal protein levels. Protein induced by overexpressing the *Hrb87F* gene under the control of a heat-inducible promoter was quite stable and remained at high levels for 24 h after heat shock. This allowed us to separate possible heat-shock effects on splicing (Yost and Lindquist, 1986; Shen *et al.*, 1993) from hnRNP protein effects on splicing. When overexpressed, the protein localized to its typical endogenous sites—in the nucleus (Figure 2b) and, more specifically, on the chromosomes (Figures 2d and 6). In a previous study we

reported that overexpression of the other major *Drosophila* A/B-type hnRNP HRB98DE/hrp38 resulted in aberrant splicing of *Ddc* pre-mRNA (Shen *et al.*, 1995). Here we show that excess HRB87F/hrp36 also induced inappropriate skipping of the same internal exon in the endogenous *Ddc* pre-mRNA (Figure 3). In vitro mammalian splicing studies have shown that, although excess A1 hnRNP protein is not able to induce exon skipping in two natural pre-mRNA substrates (the alternatively spliced β-tropomyosin internal exons and the constitutively spliced β-globin internal exon), it is able to induce exon skipping in model substrates with short internal exons and weak upstream polypyrimidine tracts (Mayeda *et al.*, 1993). *Ddc* pre-mRNA is the first natural substrate to show exon skipping in hnRNP excess, although the pattern induced is not a natural one. It is the *Ddc* hypodermal splicing pattern and thus the constitutively used exon C that is primarily affected by excess hnRNP (i.e., ACD to AD). The C exon is short (86 nt), but the upstream polypyrimidine tract is quite reasonable (CTTTTGCATCCACATCAAG/A) (Morgan *et al.*, 1986). It is not obvious why this exon is particularly susceptible to repression by excess hnRNPs. If there are specific in vivo functions of HRB98DE/hrp38 and HRB87F/hrp36, they are not distinguishable by this assay. Previous studies have noted that different A/B–hnRNP isoforms act similarly in alternative splicing modulation, although with different efficiencies (Mayeda *et al.*, 1994; Yang *et al.*, 1994).

Several reports have proposed a model in which the ratio of SF2/ASF-type SR proteins to A/B-type hnRNPs plays a role in the regulation of alternative splicing through the antagonistic effects of the two protein types on splice-site selection. The available data, however, suggest that large variations in at least some A/B-type hnRNP proteins are tolerated, suggesting that the many genes regulated at the level of alternative splicing are not adversely affected by significant changes in hnRNP protein concentration (Ben-David *et al.*, 1992; Mayeda *et al.*, 1993; Yang *et al.*, 1994; this study). At the low end of hnRNP protein concentration, our data show that a null mutant in the *Drosophila Hrb87F* gene is healthy and viable, as is a mouse erythroleukemia cell line in which the A1 hnRNP protein is at least 200-fold lower than normal levels and essentially absent (Ben-David *et al.*, 1994). Similar proteins must substitute for required functions, but we have seen no evidence in flies for altered expression levels of these similar proteins in response to higher- or lower-than-normal levels of HRB98DE/hrp38 or HRB87F/hrp36. At the high end, the 10- to 15-fold overexpression of HRB87F/hrp36 in this study produced no significant adverse effects, although the excess protein was present on the chromosomes and was capable of affecting particularly sensitive substrates, such as *Ddc* mRNA. Because the SR protein level was not significantly altered by hnRNP overexpression (Figure 7), the ratio of these proteins on the chromosomes was changed considerably.

Although previous reports have noted the ability of excess A/B-type hnRNPs to modulate alternative splicing, these reports also include considerable evidence that several pre-mRNA substrates are resistant to effects of excess hnRNP, such as the SV40 early region pre-mRNA (Mayeda and Krainer, 1992) and the β-tropomyosin and β-globin pre-mRNAs (Mayeda *et al.*, 1993). In fact, the only mammalian target identified (in both in vitro and cell culture studies) is the adenovirus E1A transcript, and the magnitude of the E1A splicing shift by excess hnRNP A1 is relatively small (Mayeda and Krainer, 1992; Cáceres *et al.*, 1994; Yang *et al.*, 1994). This result, combined with the tight regulation of A1 hnRNP level in their cell lines, prompted Yang *et al.* (1994) to question whether modulation of A1 hnRNP levels is a genuine strategy used by cells to regulate alternative splicing. Furthermore, studies of actual adenovirus infection indicate that E1A splicing regulation can be accounted for by modulation of SR protein availability, whereas hnRNP protein levels are not regulated (Gattoni *et al.*, 1980; Himmelspach *et al.*, 1995).

In *Drosophila*, of six pre-mRNAs tested, *Ddc* was the only one to be affected by excess hnRNP, and our discovery of this substrate was fortuitous. In addition, because a null mutation in *Hrb87F* does not affect *Ddc* splicing (Figure 4) and because the induced exon-skipping pattern does not occur naturally, it is questionable whether HRB87F/hrp36 protein is typically involved in *Ddc* splicing. Our EM observations of the general population of genes in hnRNP excess revealed very few abnormalities in nascent transcript packaging and processing. Most important, absence or overexpression in the whole animal of HRB87F/hrp36 resulted in no obvious tissue or developmental stage-specific adverse effects that may have gone undetected in tissue culture studies. These effects are far milder than absence or overexpression of the *Drosophila* SR protein B52. B52 is one isoform of the *Drosophila* SRp55 family (Roth *et al.*, 1991) and is involved in the splicing regulation of specific transcripts in vivo (Peng and Mount, 1995). A nearly identical variant of this protein can replace both the constitutive and regulated splicing functions of ASF/SF2 in a human cell-free splicing system (Mayeda *et al.*, 1992). Null mutations in B52 are lethal (Ring and Lis, 1994), and modest overexpression results in severe developmental defects and poor viability (Kraus and Lis, 1994). This is exactly the behavior expected of a protein whose concentration is critically important in the regulation of specific splice-site selection, although the results with hnRNP A1 and HRB87F/hrp36 argue otherwise for these proteins.

Previous studies have demonstrated important differences between these two classes of proteins. For

example, although neither SR family proteins or A/B hnRNP proteins are detected in purified spliceosomes (Bennett *et al.*, 1992), SR proteins are required for constitutive splicing (Krainer *et al.*, 1990) and are found in the prespliceosomal commitment complex E, which excludes hnRNP proteins (Staknis and Reed, 1994b). The intimate involvement of SR family proteins in splice-site selection seems to be much more extensive (reviewed, Fu, 1995) than the involvement of hnRNP proteins, including A/B type hnRNPs. Although A1 hnRNP is up-regulated in proliferating versus quiescent cells (LeStourgeon *et al.*, 1977; de Koch *et al.*, 1981; Celis *et al.*, 1986; Planck *et al.*, 1988), there is as yet no evidence that this is responsible for alterations in splicing patterns in these cells; other roles, such as altered nuclear export of mRNAs, are also possible.

It is important to consider that the A/B hnRNP proteins are among the most abundant in the nucleus and are already in stoichiometric excess over SR proteins and pre-mRNA; current models of hnRNP packaging of pre-mRNA propose a complete coverage of the RNA by the proteins (Conway *et al.*, 1988; Dreyfuss *et al.*, 1993). It seems likely that a combination of their abundance, their strand-annealing properties, and their low affinity for pre-mRNA relative to splicing factors makes them valuable participants in nuclear RNA metabolism. The combined evidence suggests that A/B hnRNP proteins bind nascent pre-mRNA rapidly and promiscuously and probably play a role in pre-mRNA "packaging" (which may include both length compaction and presentation in an accessible form to specific splicing components) and in mRNA export from the nucleus. However, for most transcripts, they may have no direct role in the splicing reaction and typically are readily displaced by splicing components at splice-junction sequences (Bennett *et al.*, 1992; Staknis and Reed, 1994a). The mechanism of their ability to modulate splice-site selection when present in unusual excess is not known. Their location on chromosomes when overexpressed (Figures 2 and 6) is consistent with the possibility that the mechanism involves a shift in RNA-binding equilibrium such that they are less likely to be displaced or more likely to bind, especially at high-affinity binding sites. It is also possible, however, that the mechanism involves no direct interaction with the RNA substrate but rather, for example, sequestration of a factor involved in certain 5′ splice-site recognitions. Given the paucity of evidence in the electron micrographs for excess protein bound tightly to nascent transcripts and the paucity of evidence for deleterious effects on the flies, it seems reasonable that the chromosomal binding, which did not seem to be saturable even at the highest overexpression levels, may not represent direct binding to chromosomal RNA but rather protein aggregation mediated through the previously defined protein interaction domain in the glycine-rich C terminus (Cobianchi *et al.*, 1988; Kumar *et al.*, 1990). Cells typically deal with abundant hnRNP proteins, and we conclude that increasing or decreasing that abundance is taken in stride, at least in *Drosophila*. Clearly, the results presented and summarized here concerning the tolerance by the cell of large variations in hnRNP protein levels are inconsistent with the behavior expected for a general alternative splicing regulator whose activity is dependent on its concentration.

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