Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 1822–1825, March 1995
Biochemistry

Exon skipping by overexpression of a *Drosophila* heterogeneous nuclear ribonucleoprotein *in vivo*

(alternative splicing/dopa decarboxylase)

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Communicated by Oscar L. Miller, Jr., University of Virginia, Charlottesville, VA, November 28, 1994 (received for review September 15, 1994)

ABSTRACT **Heterogeneous nuclear ribonucleoproteins (hnRNPs) are abundant RNA-binding proteins that are implicated in splicing regulation. Here we investigate the role of a *Drosophila* hnRNP in splicing regulation in living animals. We find that overexpression of the *Drosophila* hnRNP HRB98DE leads to skipping of all internal exons in the *Drosophila* dopa decarboxylase (*Ddc*) pre-mRNA *in vivo*. These results indicate that HRB98DE has a splicing activity that promotes use of terminal splice sites. The effect of excess HRB98DE on *Ddc* splicing is transient, even though high levels of HRB98DE persist for at least 24 hr. This suggests that *Drosophila* larvae can induce a compensating mechanism to counteract the effects of excess HRB98DE.**

Nascent pre-mRNAs associate with a specific group of proteins to form heterogeneous nuclear ribonucleoprotein (hnRNP) complexes (1). The major protein components of hnRNP complexes from HeLa cells are the A1, A2, B1, B2, C1, and C2 polypeptides (2, 3). Several lines of indirect evidence suggest that hnRNPs might be involved in pre-mRNA splicing. Four hnRNPs (A1, C, D, and I/PBT) have been found to bind specifically to the conserved polypyrimidine tract located upstream of most 3′ splice sites (4–6). The hnRNPs A1 and I/PBT can also associate with 5′ splice sites (7–9). The hnRNP A1 protein has RNA-annealing activity (10–13) and can bind to the U2 small nuclear ribonucleoprotein (snRNP) (10). Efficient UV crosslinking of hnRNP A1 to pre-mRNA requires both U1 and U2 snRNPs (14).

Recent *in vitro* studies provide direct evidence that hnRNP A1 might be actively involved in regulating alternative splicing. An excess of hnRNP A1 promotes the use of distal 5′ splice sites in model pre-mRNAs containing duplicated 5′ splice sites (15). This preferential use of distal 5′ splice sites can be counteracted by members of the SR protein family (16), such as SF2/ASF and SC35 (15, 17, 18). An excess of hnRNP A1 can also promote skipping of some alternatively spliced exons in model genes *in vitro*, although it does not cause inappropriate exon skipping in a natural constitutively spliced pre-mRNA (19). Recent studies show that hnRNP A1 can similarly influence alternative splice site choice in mammalian tissue culture cells. Transient overexpression of hnRNP A1 shifts 5′ splice site selection to the most distal 5′ splice site in the adenovirus E1A pre-mRNA (20, 21). Whether these results obtained from studies *in vitro* or in tissue culture cells are relevant in living animals remains to be determined. *Drosophila melanogaster* provides an excellent system to address this question.

Several major protein components of hnRNP complexes in *D. melanogaster* have been characterized. The *Drosophila* HRB98DE, HRB87F, hrp36, hrp40, and hrp48 proteins and the vertebrate hnRNP A/B proteins share a common overall primary structure: two amino-terminal RNA-binding domains and a glycine-rich carboxyl-terminal domain (22–27). However, none of these *Drosophila* hnRNPs display sufficient sequence similarity to any vertebrate hnRNPs to determine whether they are homologues of distinctive vertebrate hnRNPs. The HRB98DE protein, which has 58% sequence identity with the rat A1 protein in the RNA-binding domains, is probably the closest relative of A1 in *D. melanogaster* (25). The HRB98DE gene encodes four protein isoforms derived from alternative splicing and alternative use of 5′ exons 1A or 1B. However, these HRB98DE isoforms share identical RNA-binding domains.

In this report, we investigate the role of the *Drosophila* hnRNP HRB98DE in splicing regulation in living larvae. We tested whether overexpression of HRB98DE affects splice site choice of the endogenous dopa decarboxylase (*Ddc*) pre-mRNA (28). The *Ddc* pre-mRNA is expressed primarily in the central nervous system (CNS) and the hypoderm, and it is alternatively spliced in these two tissues (29). The *Ddc* CNS-specific mRNA contains all four exons, whereas the hypodermal mRNA contains only three exons, skipping the second exon B (see Fig. 2*B*) (29). Our previous results suggest that the *Ddc* hypodermal-specific splicing is negatively regulated by a blockage mechanism preventing splicing to exon B and indicate that the sequences of *Ddc* exon B and the adjacent upstream intron are sufficient for this negative regulation (30, 31). Here, we show that *in vivo* overexpression of a *Drosophila* hnRNP, HRB98DE, causes profound changes in *Ddc* splicing.

MATERIALS AND METHODS

Plasmid Construction. The cDNAs of *Hrb98DE* 1A and *Hrb98DE* 1B, *ov12* and *L3* (25), were cloned first into plasmid pBHS (32) and then into the PW8 *P*-element transformation vector that contains *w*+ as a marker (33). The resulting constructs were injected into *yw* embryos with a helper plasmid that encodes transposase but is incapable of transposition itself (34).

Western Blot Analysis. Ten third-instar larvae were homogenized in 100 μl of sample buffer [60 mM Tris·HCl, pH 6.8/2% (wt/vol) SDS/1% (vol/vol) 2-mercaptoethanol/0.5% (wt/vol) bromophenol blue]. The homogenates were clarified by centrifugation at ≈12,000 × *g* for 15 min. Samples of homogenates (5 μl, 15 μg of total protein) were electrophoresed in an SDS/10% polyacrylamide gel at 15 mA for 7 hr, and the separated proteins were transferred to nitrocellulose at 14 V overnight.

Analysis of Splicing Products. Total RNA was isolated from late third-instar larvae or from hand-dissected larval CNS or hypoderm tissue (30). The endogenous *Ddc* transcripts were converted into cDNAs by reverse transcription with a primer specific for *Ddc* exon D (indicated as arrows in Fig. 2). *Ddc* cDNAs were amplified by PCR using primers specific for exons A and D. Conditions and primers for reverse transcription and

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Abbreviations: hnRNP, heterogeneous nuclear ribonucleoprotein; CNS, central nervous system.

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PCR were the same as described previously (31). Under these conditions, PCR amplification yields a valid estimate of the abundance of the *Ddc* AD, ACD, and ABCD splice forms. The identities of the splicing products were confirmed by sequencing the PCR products.

RESULTS

Overexpression of HRB98DE in *D. melanogaster* Larvae. To overexpress the *Drosophila* hnRNP HRB98DE, we introduced *Hrb98DE* cDNAs into flies under the control of the *Drosophila Hsp70* heat shock promoter (35) by *P*-element-mediated germline transformation (36). In the presence of either *Hsp70/Hrb98DE*(1A) or *Hsp70/Hrb98DE*(1B) transgenes, which contain the alternative 5′ exons 1A or 1B, respectively (25), HRB98DE protein is induced by a 1-hr 38°C heat shock (Fig. 1, lanes 4 and 6–11). Low levels of HRB98DE can be detected in the absence of either transgene (Fig. 1, lanes 1 and 2) or heat shock (Fig. 1, lanes 3 and 5). A time course of HRB98DE(1A) protein expression shows that the HRB98DE protein is induced during heat shock (Fig. 1, lane 6), and that protein levels remain high throughout 24 hr of recovery at room temperature (Fig. 1, lanes 7–11).

High Levels of HRB98DE Affect *Ddc* Splicing, and This Effect Is Transient. We tested splicing of the endogenous *Ddc* pre-mRNA in late third-instar larvae containing the *Hsp70/Hrb98DE* transgenes. The *Ddc* primary transcript is alternatively spliced in the hypoderm and the CNS (29). The *Ddc* CNS splice form includes all four exons, ABCD, whereas the hypodermal splice form contains only three exons, ACD (Fig. 2*B*). We analyzed splicing of the endogenous *Ddc* primary transcript by reverse transcription-linked PCR, using primers specific for *Ddc* exons A and D. Since *Ddc* transcription is induced in the hypoderm during the late third-instar larval stage (28), and the CNS consists of a small portion of the body mass, the hypodermal splice form (ACD) is the predominant *Ddc* mRNA in these larvae. The *Ddc* hypodermal splicing specificity is selectively affected by heat shock, so that the endogenous *Ddc* CNS mRNA is found in the hypoderm during heat shock (30). Heat shock does not block splicing in *Drosophila* larvae (30, 37), though previous studies have shown that heat shock prevents splicing in *Drosophila* tissue culture cells (38).

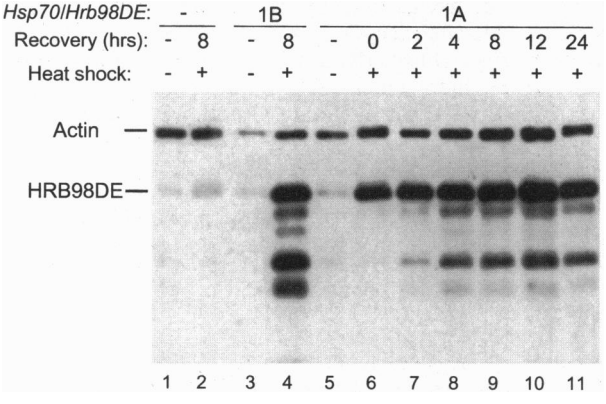

FIG. 1. High levels of HRB98DE induced by heat shock are maintained throughout 24 hr of recovery. Two *Hrb98DE* cDNAs containing two alternative 5′ exons, 1A or 1B, were introduced into flies under the control of the *Hsp70* promoter. Given the similar effects of the HRB98DE(1A) and -(1B) proteins on splicing, only the 1A isoform was studied extensively. Total protein from late third-instar larvae was separated by SDS/polyacrylamide gel electrophoresis. Larvae were allowed to recover at room temperature for various times following heat shock at 38°C for 1 hr as described (30). The immunoblot was stained with monoclonal anti-actin antibody (Amersham) as a loading control and rabbit polyclonal anti-HRB98DE serum (27). The sizes of HRB98DE and actin are 39 and 43 kDa, respectively. Bands below the 39-kDa HRB98DE band are degradation products of HRB98DE.

In the absence of induced HRB98DE proteins (Fig. 1, lanes 1–3 and 5), the ACD splice form is the only detectable spliced product (Fig. 2, lanes 1, 9, 11, and 12). However, in the presence of excess HRB98DE(1A) or HRB98DE(1B) protein, an aberrant splice form accumulates (Fig. 2, lanes 3–8 and 10). This aberrant splice form, labeled AD, skips both *Ddc* internal exons B and C and has not been detected previously. The identity of this AD splice form has been confirmed by sequencing of the PCR amplification product. The appearance of this AD splice form results from high levels of HRB98DE, rather than from heat shock alone. Under the same heat shock and recovery conditions, high levels of the AD splice form are found in larvae containing the *Hsp70/Hrb98DE* transgenes (Fig. 2, lanes 5 and 10), but not even trace amounts of this splice form can be detected in larvae lacking the transgene (Fig. 2, lane 12). These results indicate that overexpression of HRB98DE can influence both 5′ and 3′ splice site selection, causing skipping of internal exons by promoting use of the terminal *Ddc* splice sites.

The *Ddc* AD splice form appears during the first 2 hr of recovery (Fig. 2, lane 3) and becomes the predominant *Ddc* spliced product after about 4 hr of recovery at room temperature (Fig. 2, lanes 4, 5, and 10). These results indicate that the *Ddc* hypodermal mRNA turns over rather rapidly with a half-life of about 4 hr. Although the induced levels of

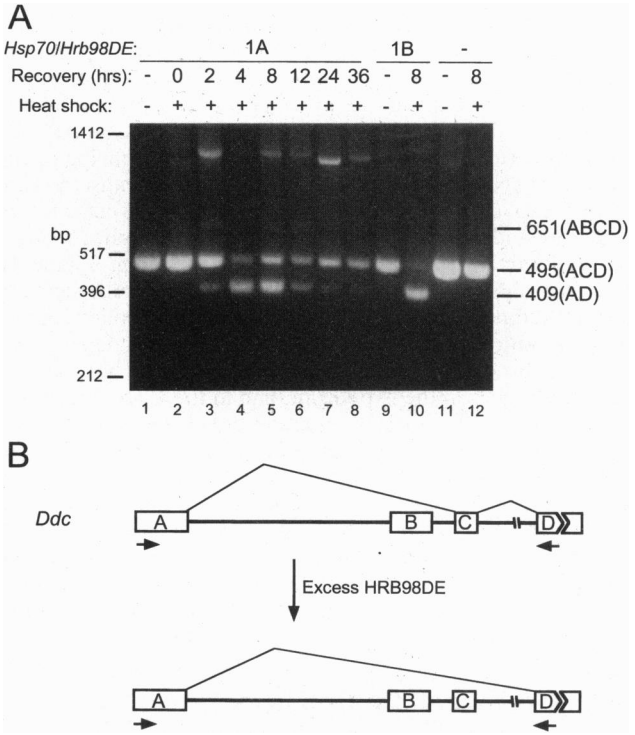

FIG. 2. (*A*) Time course of the effects of heat shock-induced HRB98DE on *Ddc* splicing. The figure shows reverse transcription-linked PCR amplification products from larval RNA with *Ddc*-specific primers. The identities of the splicing products are indicated on the right of the ethidium bromide-stained agarose gel, and size standards are indicated on the left. Low levels of the *Ddc* CNS splice form are found following heat shock (30). The high molecular weight bands are *Ddc* splicing intermediates, whose PCR amplification is not quantitative under the PCR conditions used here. (*B*) Sketch of the effects of HRB98DE on *Ddc* splicing. *Ddc* is drawn to scale except for *Ddc* intron cd and exon D. *Ddc* exons are shown as open boxes labeled A, B, C, or D. Lines joining exons indicate splicing. Arrows indicate the locations and the 5′ → 3′ orientation of primers.

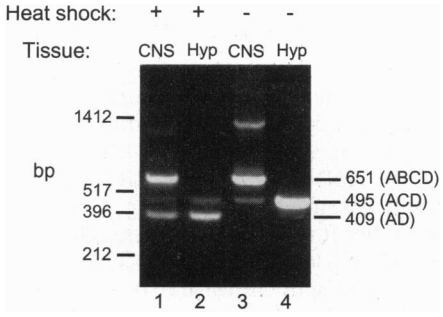

FIG. 3. Excess HRB98DE affects both the CNS and the hypodermal *Ddc* splicing pathways. RNA was isolated from either larval CNS or hypoderm (Hyp) tissue as described (31). Reverse transcription–PCR analyses were carried out using primers specific for *Ddc* exons A and D. Small amounts of *Ddc* hypodermal mRNAs in the CNS lanes could be due to contaminating non-CNS tissue.

HRB98DE proteins remain relatively constant throughout 24 hr of recovery (Fig. 1, lanes 6–11), levels of the aberrant AD splice form decrease gradually after 8 hr of recovery. By the end of 24 hr of recovery, only low levels of the AD splice form can be detected (Fig. 2, lane 7). These results suggest that high levels of HRB98DE proteins cause *Drosophila* larvae to induce a compensating mechanism which counteracts the effects of high levels of HRB98DE on splicing. This counteractive effect could be explained by induction of factors that have activities antagonistic to HRB98DE or that affect cellular localization and/or activity of HRB98DE.

Splicing of *Ddc* in the CNS Is Similarly Affected. We have shown that excess HRB98DE(1A) or -(1B) leads to preferential use of the *Ddc* exon D 3′ splice site relative to the exon C 3′ splice site in whole larvae, which normally express primarily the *Ddc* hypodermal mRNA containing exons A, C, and D. We tested the effects of excess HRB98DE(1A) on splicing of the *Ddc* pre-mRNA in the CNS, where *Ddc* primary transcripts are spliced into the ABCD form (Fig. 2*B*). In the absence of overexpressed HRB98DE protein, the ABCD splice form is the predominant spliced product in the CNS (Fig. 3, lane 3). After heat shock induction of HRB98DE for 1 hr with recovery at room temperature for 8 hr, the aberrant AD splice form is found in both the CNS and the hypoderm (Fig. 3, lanes 1 and 2). The ratio of the aberrant AD splice form to normal *Ddc* mRNA is higher in the hypoderm than in the CNS. This could be explained by the normal induction of *Ddc* expression in the hypoderm during the third-instar larval development, in contrast to constant *Ddc* transcription in the CNS (28, 39, 40). Alternatively, the *Ddc* CNS mRNA could be more stable than the hypodermal mRNA, so that more normal *Ddc* CNS mRNAs would be retained in the CNS during this time period. Nonetheless, these results demonstrate that overexpression of HRB98DE promotes skipping of the *Ddc* internal exons, regardless of the normal *Ddc* splice site choice.

DISCUSSION

Previous studies on the role of hnRNPs in regulating alternative splicing have focused on the human hnRNP A1 and its effects on splicing of both natural and model pre-mRNAs *in vitro* and in tissue culture cells. The predominant activity that has been seen for hnRNP A1 is a shift to use of the most distal 5′ splice site in model pre-mRNAs with duplicated and closely spaced alternative splice sites (15). Skipping of an internal exon has been observed in model substrates with small internal exons, but this activity was critically dependent on the size of the exon (19). The function of *Drosophila* hnRNPs in regulating alternative splicing has also been studied. The hnRNP A1-related protein hrp48 shows specific binding activity to the pseudo-5′ splice sites located upstream of the normal 5′ splice site of the third intron in the *P*-element pre-mRNA (41). This suggests a role for hrp48 in somatic inhibition of splicing by shifting U1 snRNP binding from the accurate 5′ splice site to the upstream inactive pseudo-5′ splice sites (41, 42). Here, our results demonstrate that another hnRNP A1-related protein, HRB98DE, can affect the normal splicing pathway of an endogenous pre-mRNA in a living animal and that the shift is very dramatic. When HRB98DE expression is at an elevated level, most of *Ddc* pre-mRNAs are spliced into this AD splice form, so that >90% of *Ddc* mRNAs can be this species after several hours of accumulation.

It has been proposed that internal exons are recognized as a unit and that additional mechanisms are required to recognize terminal exons (43). Our results support this model, since high levels of HRB98DE cause skipping of the *Ddc* internal exons, favoring processing to the terminal *Ddc* exons. This could be explained by either promotion of the terminal 5′ and 3′ splice sites or inhibition of the internal splice sites. HRB98DE may have a splicing activity like SF7 (19) that can promote use of terminal 3′ splice sites. Alternatively, high levels of HRB98DE could lead to a failure in 5′ splice site recognition of both *Ddc* internal exons. Mutations within the 5′ splice sites of internal exons can cause exon skipping *in vitro* (44, 45), and facilitated recognition of 5′ splice sites of internal exons can promote exon inclusion (45–47).

Whether HRB98DE is normally involved in regulating alternative splicing of *Ddc* is not clear. *Ddc* alternative splicing is normally regulated in the hypoderm, where a factor acts near the 3′ splice site of exon B to prevent splicing of this exon (31). It is possible that HRB98DE at lower concentrations could interact with other *Ddc* splicing factors to regulate the exclusion of exon B in the hypoderm.

We expect that the *Ddc* primary transcript is not the sole target whose splicing is affected by high levels of HRB98DE and that HRB98DE will play a role in regulating alternative splicing of many primary transcripts. Consistent with this is the poor viability observed in flies following HRB98DE induction (unpublished results). This poor viability is unlikely to be due to the altered expression of *Ddc*, since *Ddc* enzyme activity is present in large excess over what is required for survival (48). However, detection of these transcripts showing altered splicing may not be trivial, since genes encoding stable transcripts may show little net accumulation of altered products in the ≈10 hr during which HRB98DE maximally affects splicing specificity. Further studies will be required to define the global role of HRB98DE in regulating alternative splicing in *Drosophila*.

We thank S. Birman, N. Lewis, and M. Tian for discussions and comments on the manuscript. We thank M. Anzivino for sequencing of PCR products. This work was supported by grants from the National Institutes of Health (GM27318 and GM39271) and by a National Institutes of Health Developmental Biology predoctoral training grant.

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