MOLECULAR AND CELLULAR BIOLOGY, Aug. 1993, p. 4549–4555
0270-7306/93/084549-07$02.00/0

Vol. 13, No. 8

Tissue-Specific Alternative Splicing of the *Drosophila dopa decarboxylase* Gene Is Affected by Heat Shock

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Received 17 March 1993/Returned for modification 21 April 1993/Accepted 3 May 1993

The *Drosophila dopa decarboxylase* gene, *Ddc*, is expressed in the hypoderm and in a small number of cells in the central nervous system (CNS). The unique *Ddc* primary transcript is alternatively spliced in these two tissues. We investigated whether *Ddc* splicing in the CNS is a general property of the CNS or a unique property of the cells that normally express *Ddc* by expressing the *Ddc* primary transcript ubiquitously under the control of an *Hsp70* heat shock promoter. Under basal expression conditions, *Ddc* splicing shows normal tissue specificity, indicating that the regulation of *Ddc* splicing in the CNS is tissue specific rather than cell specific. Previous studies have shown that severe heat shock blocks mRNA splicing in cultured *Drosophila melanogaster* cells. Our results show that splicing of the heat shock-inducible *Hsp83* transcript is very resistant to heat shock. In contrast, under either mild or severe heat shock, the splicing specificity of the heat shock-induced *Ddc* primary transcript is affected, leading to the accumulation of inappropriately high levels of the CNS splice form in non-CNS tissues. The chromosomal *Ddc* transcript is similarly affected. These results show unexpected heterogeneity in the splicing of individual mRNAs as a response to heat shock and suggest that the *Ddc* CNS-specific splicing pathway is the default.

Alternative RNA splicing is an important mechanism by which animals control gene expression (21, 39). In animals from fruit flies to vertebrates, many genes that are alternatively spliced in different tissues or cell types or at different developmental stages have been identified. Alternative splicing often leads to the production of multiple protein isoforms that carry out distinct functions.

Many genes that are expressed in neural tissues of vertebrates and invertebrates are alternatively spliced in neural and nonneural tissues. Although there are examples of genes that are spliced into alternative mRNAs within neural tissues, there are also genes that are spliced into distinct mRNAs in neural versus nonneural tissues. Examples of these genes include the vertebrate genes encoding c-Src (4, 5, 22, 29), N-CAM (28, 34), calcitonin/CGRP (32), and substance P/K (26). There exist similar alternatively spliced genes in the fruit fly, including *neuroglian* (12), *antennapedia* (41), *Ultrabithorax* (17), and *dopa decarboxylase* (25). There is no common pattern in how the neural forms of these mRNAs are alternatively spliced; in some genes exons are skipped in neural tissues, whereas in others there are neural tissue-specific exons that are skipped in nonneural tissues.

Genes that are spliced into unique neural splice forms are often expressed in complex cell patterns that include only minor subsets of neural cells. This leads to the question of whether neuron-specific splicing is tissue specific or whether it is limited to selected populations of neural cells. If the former were to hold, this might imply the existence of a splicing regulator that distinguishes between neural and nonneural tissues.

We investigated the level at which the neural splicing pathway in *Drosophila melanogaster* is regulated by studying the alternative splicing of the *dopa decarboxylase* gene, *Ddc*. The *Ddc* primary transcript is expressed primarily in the central nervous system (CNS) and the hypoderm (25). In these tissues, *Ddc* is alternatively spliced into different mRNAs that encode distinct *Ddc* isoforms. The *Ddc* mRNA within the CNS contains four exons, whereas the hypodermal *Ddc* mRNA contains only three, skipping the second exon (Fig. 1). In the CNS, *Ddc* is only expressed in about 150 dopamine and serotonin neurons and in a subset of glial cells (3, 16). In the hypoderm, *Ddc* is expressed in most if not all hypodermal cells (8, and unpublished results). *Ddc* is regulated transcriptionally in the CNS via both tissue- and cell-specific regulatory elements (8, 14, 20, 35). To determine whether *Ddc* alternative splicing is regulated via a tissue- or a cell-specific regulatory mechanism, we expressed the *Ddc* primary transcript ubiquitously under the control of a basally expressed heat shock promoter from the *Drosophila Hsp70* gene (19). We abbreviate this fusion gene as *Hsp70/Ddc*.

Severe heat shock blocks splicing of particular mRNAs in *Saccharomyces cerevisiae* cells (46), *Drosophila* cells (44), and mammalian cells (15). Splicing is also inhibited in nuclear extracts prepared from heat-shocked mammalian cells, because of a defect in spliceosome assembly (7, 37). A complementing activity that promotes spliceosome assembly has been partially purified from a non-heat-shocked extract that consists of a protein fraction associated with the [U4/U6.U5] triple small nuclear ribonucleoprotein (43).

Under severe heat shock at 36.5°C or above, both *Hsp83* and *Hsp70/Alcohol Dehydrogenase* (*Adh*) precursor RNAs accumulate in *Drosophila* tissue culture cells (44). We investigated whether the same effect occurs in living flies by examining the splicing of *Hsp83* and *Hsp70/Ddc* transcripts in third-instar larvae under severe heat shock. We find that splicing of *Ddc* transcripts is regulated at a tissue-specific level within the CNS, and splicing specificity is strikingly affected by either mild or severe heat shock. Our results indicate heterogeneity in the splicing of individual mRNAs

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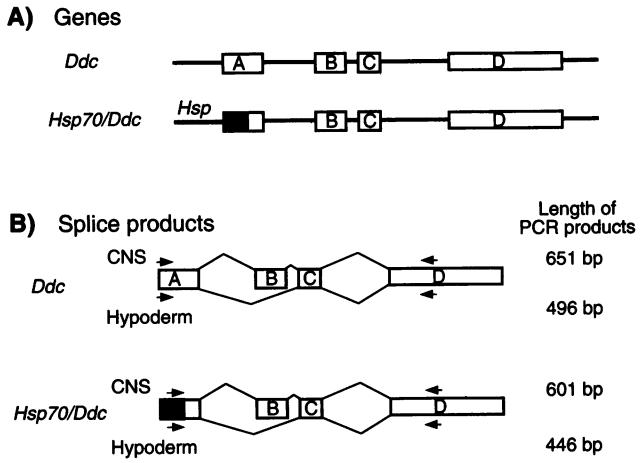

FIG. 1. Alternative splicing of *Ddc* and *Hsp70/Ddc*. (A) *Ddc* and *Hsp70/Ddc* genomic DNA. The *Hsp70* promoter and 79 bp of its transcription unit (shown shaded) were fused to *Ddc* at a site 96 bp from the *Ddc* transcription start point within exon A, to generate the gene labelled *Hsp70/Ddc*. Exons A, B, and C and introns AB and BC are drawn relatively to scale; their lengths are 192, 156, 86, 636, and 77 bp, respectively. Exon D and intron CD are not drawn to scale; their lengths are 1,644 and 1,027 bp, respectively (25). (B) Splice products of *Ddc* and *Hsp70/Ddc*. The CNS-specific splice form contains exons A, B, C, and D, whereas exon B is excluded from the hypodermal splice form. The arrows indicate the 5'→3' orientation and the locations of primers used for PCR analysis of transcripts. The *Hsp70* primer is located 50 bp downstream of the *Ddc* exon A primer, resulting in slightly smaller amplified PCR products than those from *Ddc*.

as a response to heat shock and lead to a model for alternative splicing of *Ddc*.

MATERIALS AND METHODS

Construction of the *Hsp70/Ddc* gene. A segment of *Hsp70* (1) from −440 to +79 was ligated to +96 of *Ddc*, incorporating a polylinker. The sequence, starting from +75 of *Hsp70*, is as follows, with the polylinker sequence underlined: ACAATGGTCGACTCTAGAGGGGCAAAC. This gene was incorporated into an Adh^+ P-element transformation vector and integrated into the germ line of a Ddc^{ts2} Adh^{fn23} strain (36).

Heat shock of third-instar larvae. Late-crawling third-instar larvae were placed in 25-mm shell vials containing moistened filter paper. The vials were plugged with foam stoppers and immersed in a water bath (at the appropriate temperature) such that the lower edge of the foam plug was below the water level. Although all experiments in this study were performed on larvae immediately after heat shock, results similar to those shown here have been obtained with larvae allowed to recover for 30 min following heat shock (unpublished data).

Reverse transcription-linked polymerase chain reaction (RT-PCR). All tissues were hand dissected from late third-instar larvae under a dissecting microscope on an ice-chilled block. The amount of tissue used per reverse transcription was 20 CNSs, 2 carcasses (lacking the CNS), 4 hypoderms, and 4 larvae for the internal organ fractions. The tissue was homogenized in a Teflon homogenizer in 0.5 ml of proteinase K buffer (50 mM NaCl, 50 mM Tris-HCl [pH 7.5], 5 mM EDTA [pH 8.0], 0.5% sodium dodecyl sulfate [SDS], and 0.2 mg of proteinase K per ml), incubated at 37°C for 1 h, and extracted with 1 volume of phenol-chloroform three times. The nucleic acid was precipitated with ethanol and resuspended in 10 mM Tris–1 mM EDTA (pH 7.5).

Reverse transcription was carried out with Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories) according to the manufacturer's instructions, except that 15 pmol of primer and 100 U of reverse transcriptase were used per 20-μl reaction. A total of 1/20 of the reverse transcription product (1 μl) was used for PCR. PCR was performed in the following buffer (41a): 20 mM morpholinepropanesulfonic acid (MOPS; pH 8.1), 5 mM $MgCl_2$, 5 mM Na isocitrate, 0.4 mM deoxynucleoside triphosphates, 15 to 75 pmol of primers, and 2.5 U of *Taq* polymerase (Boehringer Mannheim Biochemicals [BMB]) in a total volume of 50 μl. The thermal cycling was performed on a Coy model 60 cycler, using the following program: 94°C for 1 min and 69°C for 1 min (or 1 min 20 s or 2 min) for 30 to 35 cycles and then 72°C for 7 min. These conditions yield products that give a quantitative ratio of the *Ddc* CNS and hypodermal mRNAs but underamplify the longer precursors over 1 kb. Additionally, the absolute level of amplification is not a good indicator of the absolute amount of starting RNA (unpublished data). Before loading on an agarose gel, 15 μl of PCR products was treated with 5 U of mung bean nuclease (BMB) for 10 min at room temperature to digest single-stranded DNA.

Oligonucleotide primers were synthesized on a Milligen Cyclone Plus synthesizer and used without purification following deprotection.

Primers for PCR analyses. The following primers were used for PCR analysis: *Ddc* exon A primer, 5'GCTCTA GAGTTAAGAGGAGAACGCCAAGCG; *Hsp70* primer, 5'GAGCTCAAACAAGCGCAGCGAACAAGC; and *Ddc* exon D primer, 5'GAGCTCCACTCAGCATGTCCGCAAC.

Sequencing of PCR products. Single-stranded DNA was synthesized by thermal cycling in the presence of a single primer to selectively amplify one strand of the double-stranded PCR products. A total of 1 to 100 ng of double-stranded DNA was used as a template. The thermal cycling program used was 94°C for 1 min, 55°C for 3 min, and 72°C for 2 min for 35 cycles and then 72°C for 7 min. The linear PCR products were extracted with phenol-chloroform and chloroform and were then passed through a G-50 spin column. The single-stranded DNA product was sequenced by using the Sequenase 2.0 kit (U.S. Biochemicals).

Sequencing of the PCR products determined that the exon A, C, and D junctions were as previously reported (25). However, the 5' edge of *Ddc* exon B was 15 bp 5' relative to that given in previously published data (25), in which the junction was estimated from the lengths of primer extension products. The actual exon B 5' junction is tcaataatcgcacat tctttcatatt/AGCTCT, where exon sequences are in uppercase and introns are in lowercase. In addition, we have determined the 3' edge of exon B. The junction sequence is CAAGGTTTCG/gtatgt. This junction sequence was found previously in one *Ddc* cDNA clone (11).

Northern (RNA) analysis. Total RNA was isolated as described above except that it was extracted with phenol-chloroform once. The Poly(A)$^+$ Tract mRNA Isolation System III (Promega) was used to isolate poly(A)$^+$ RNA. Northern transfer was done by the method of Sambrook et al. (33). The blot was hybridized with single-stranded DNA probes which were synthesized by asymmetric PCR as described above, except that [^{32}P]dCTP was used instead of cold dCTP. After overnight hybridization at 42°C, the blot

was washed twice in 500 ml of 0.2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS for 20 min at 42°C. Exposure times were 10 min to 1 h. The blot was stripped of probe between hybridizations by boiling in 1 liter of 0.1% SDS–1 mM EDTA for at least an hour.

Primers used for probe synthesis. Primers used for probe synthesis are numbered from the start of transcription. All primers are given in a 5′→3′ orientation: primer 131, CACGCGGCCGCTATGAGCCACATACCC, *Ddc* exon B, nucleotides 886 to 900, starting with a synthetic *Not*I site; primer 139, GATCCAGCTTATCCG, *Ddc* exon B, 973 to 959; primer 141, AAGTCGACCATTGTCTTGGC, *Ddc* exon C, 1114 to 1095; primer 162, GCAGATCTCCGGCATAG GCAGCGTCCACATGG, *Ddc* exon D, 2920 to 2896, starting with an *Xba*I site; primer 169, CCCATGGAG ATCTAAGTCGACCATTGTCTTGGC, *Ddc* exon C, 1114 to 1095, starting with *Nco*I and *Bgl*II sites; primer 184, GACACGAGTTTGCACACAGCAGG, *Hsp83* intron, 945 to 967; primer 190, CCGGCCATGTAGCCC, *Hsp83* exon 2, 3124 to 3110; primer 191, CCAGCATACAGGCCC, *rp49* exon 1, 432 to 446; primer 192, GGGGTTGGTGAGCGG, *rp49* exon 2, 846 to 832; primer 193, GTATGTCTATTG GGT, *Ddc* intron BC, 985 to 999; and primer 194, CTTGAT GTGGATGCA, *Ddc* intron BC, 1061 to 1047.

Preparation of probes for Northern analysis. (i) *Ddc* exon D probe. The *Sac*I-*Hpa*I fragment in exon D was used as a template, and primer 162 was used to prime asymmetric PCR.

(ii) *Ddc* exon B probe. Two primers, 131 and 141, from within exon B and exon C were used to PCR amplify double-stranded DNA, with genomic *Ddc* DNA as a template. This product was then used as a template during a second round of amplification with a single exon B primer, 139, to synthesize a single-stranded DNA probe specific for exon B.

(iii) *Hsp83* probe. A double-stranded PCR product was synthesized with the *Hsp83* primers 184 and 190 with cloned *Drosophila Hsp83* plasmid DNA as a template (6). The product was cut with *Nco*I, and the resulting 630-bp fragment was used as a template for a single primer amplification with primer 190.

(iv) *Ddc* intron BC probe. Primers 193 and 169 from within intron BC and exon C, respectively, were used to synthesize the double-stranded DNA with *Ddc* genomic DNA as a template. This product was then used as a template, and single intron BC primer 194 was used to generate a single-stranded intron BC probe.

(v) *rp49* probe. Total *Drosophila* larval RNA was used as a template, priming reverse transcription with primer 192. The resulting cDNA product was used as a template for PCR with primers 191 and 192. The PCR-generated double-stranded DNA fragment was used as a template for asymmetric PCR with primer 192 (27).

RESULTS

Basal expression of the *Hsp70/Ddc* gene: the CNS-specific alternative splicing of *Ddc* is regulated in a tissue-specific manner. *Ddc* is normally expressed in the hypoderm and in specific cells of the CNS. The normal *Ddc* primary transcript is spliced into a four-exon mRNA (exons A, B, C, and D) in the CNS and into a mRNA that includes only three of these exons (A, C, and D) in the hypoderm (Fig. 1). To distinguish whether the CNS-specific splicing of *Ddc* is restricted to the cells that normally express *Ddc*, we induced expression of the *Ddc* primary transcript in all cells of the fly by expressing

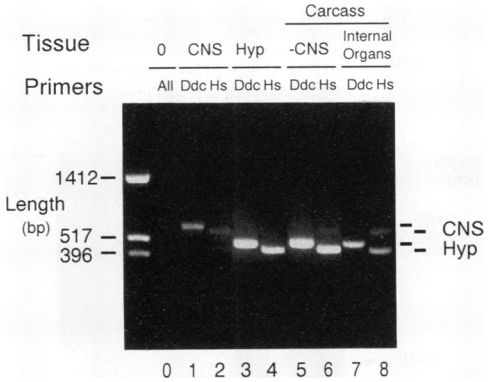

FIG. 2. RT-PCR analysis of splicing in different tissues at room temperature. Total RNA was made from the indicated third-instar larval tissues. The specific mRNAs of both chromosomal *Ddc* and *Hsp70/Ddc* were converted into cDNAs by reverse transcription with a common *Ddc* exon D primer (Fig. 1). *Ddc* exon A- and *Hsp70* exon-specific primers were then used as second primers during PCR to specifically amplify chromosomal *Ddc* and *Hsp70/Ddc* cDNAs, respectively. The hypodermal (Hyp) and CNS splice products are indicated at the right. All major *Ddc*-amplified products were confirmed by sequencing. Note that the PCR conditions in this and in subsequent figures used short extension times that yield an accurate ratio of the 400- to 600-bp mature *Ddc* mRNA species but underamplify the larger precursor species. Lanes with both primers specific for *Ddc* are labelled Ddc, whereas lanes using primers specific for the *Hsp70/Ddc* gene are labelled Hs. Lane 0, negative control in which the RNA was omitted. Tissue sources for RNA: lanes 1 and 2, larval CNS; lanes 3 and 4, hypoderm; lanes 5 and 6, carcass lacking CNS; lanes 7 and 8, carcass consisting of internal organs lacking both CNS and hypoderm.

Ddc under the control of the *Hsp70* heat shock promoter (Fig. 1).

The *Hsp70/Ddc* gene was introduced into a Ddc^{ts2} temperature-sensitive mutant line by P-element-mediated germ line transformation. Even in the absence of heat shock, this gene is expressed at a significant level, since strains containing this hybrid gene show a full suppression of the pupal cuticle coloration phenotype seen in Ddc^{ts2} (not shown).

To study *Ddc* transcripts in the CNS, we utilized an RT-PCR assay. As shown in Fig. 1, oligonucleotides that allow specific amplification between *Hsp70* and *Ddc* sequences to detect the *Hsp70/Ddc* hybrid transcripts or entirely within *Ddc* to detect the chromosomal Ddc^{ts2} transcripts were synthesized. The sensitivity of this method allows detection of *Ddc* transcripts from a single larval CNS. As shown in Fig. 1, an exon D-specific primer was used to prime reverse transcription, and then *Ddc* exon A or *Hsp70* exon-specific primers were used as second primers during PCR to specifically amplify chromosomal *Ddc* or *Hsp70/Ddc* mRNAs, respectively (Fig. 2).

Normal splicing specificity of *Hsp70/Ddc* transcripts is maintained in the predominant *Ddc*-expressing tissues. The CNS mRNAs of both the chromosomal *Ddc* and the *Hsp70/Ddc* genes are the only mature splice products visible in the CNS fractions (Fig. 2, lanes 1 and 2), and the hypodermal mRNAs are the only products detectable in the hypodermal fraction (Fig. 2, lanes 3 and 4). These results demonstrate the stringent tissue-specific splicing of the chromosomal *Ddc* transcripts and also show that most if not all of the CNS cells that do not normally express *Ddc* share the same splicing capability as the cells that normally express *Ddc*. Similar data have been obtained with a second strain containing the

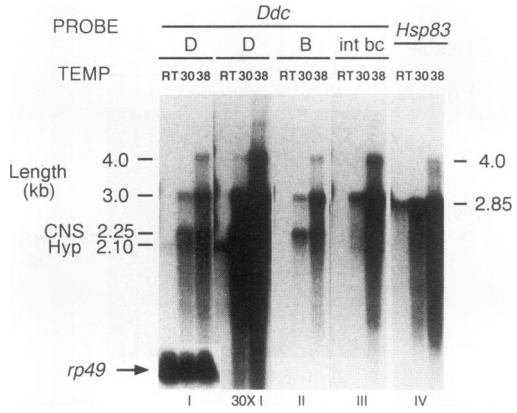

FIG. 3. Effects of heat shock on splicing of *Ddc* and *Hsp83*. Results of Northern blot analysis of poly(A)$^+$ RNA from third-instar larvae grown at room temperature (RT) or exposed to 30 or 38°C heat shock for 60 min are shown. A single blot was hybridized sequentially with exon D (panel I), exon B (panel II), and intron BC (int bc; panel III) probes from *Ddc* and with an *Hsp83* probe (panel IV). The 30X I panel is a 30-fold overexposure of panel I. The blot was also hybridized with an *rp49* probe as a control for loading (27). The RNAs hybridized by the *Ddc* probes include the following: 2.1 kb, *Ddc* hypodermal (Hyp) mRNA; 2.25 kb, *Ddc* CNS mRNA; 3 kb, *Ddc* precursor RNA, presumably containing introns AB and BC; 4 kb, *Ddc* primary transcript; 5 kb, a novel *Ddc* product, potentially a result of imprecise termination due to severe heat shock.

Hsp70/Ddc gene at a different chromosomal location (unpublished data).

Ddc is expressed in carcass fractions consisting of larvae from which CNSs were removed (Fig. 2, lanes 5 and 6) or in carcass fractions from which CNS and hypoderm were removed, consisting of the remaining internal organs (Fig. 2, lanes 7 and 8). In both of these fractions, chromosomal *Ddc* primary transcripts are spliced exclusively into the hypodermal mRNA. However, the *Hsp70/Ddc* transcripts are spliced with reduced specificity, showing significant splicing into the CNS mRNA (Fig. 2, lanes 6 and 8). In these fractions, the *Hsp70/Ddc* gene must certainly be expressed in cell types in which chromosomal *Ddc* is not normally expressed. It is likely that these cells have the capability to splice via the CNS pathway.

Effects of heat shock on splicing. Severe heat shock disrupts splicing of the *Hsp70/Adh* and *Hsp83* genes in cultured *Drosophila* cells (44). To investigate whether live *D. melanogaster* responds to heat shock in the same manner, larvae containing the *Hsp70/Ddc* fusion gene were subjected to heat shock, and *Hsp70/Ddc* and *Hsp83* RNAs were analyzed on a Northern blot. Polyadenylated RNA was prepared from third-instar larvae grown at room temperature or exposed to mild (30°C) or severe (38°C) heat shock for 60 min (Fig. 3). This Northern blot shows that the *Hsp70/Ddc* (panels I to III) and *Hsp83* (panel IV) genes are induced by a mild 30°C heat shock and that a severe 38°C heat shock further increases this induction. Panel IV shows that greater than 99% of the 4-kb *Hsp83* primary transcripts are spliced into mRNA even under severe heat shock. This indicates that splicing of this transcript in *Drosophila* larvae is far more resistant to heat shock than observed previously with cultured cells (44).

The blot (Fig. 3) was hybridized with a *Ddc* exon D-specific probe to detect all *Hsp70/Ddc* and *Ddc* transcripts (panel I), with an exon B probe to detect the CNS mRNA splice forms and their precursors (panel II), and with an intron BC probe to detect splicing intermediates retaining this intron (panel III). Under non-heat shock conditions, a 2.1-kb species is the only transcript visible even on a 30-fold overexposure (panel 30X I). This band presumably contains both the chromosomal *Ddc* and the *Hsp70/Ddc* hypodermal mRNAs, since the probe will not distinguish between these two similar-length species. After mild or severe heat shock, there is a significant increase in the intensity of this RNA and of additional higher-molecular-weight bands of 2.25, 3.0, 4.0, and 5 kb. This heat shock-induced expression must represent transcription from the *Hsp70/Ddc* gene. The 2.25-kb RNA is the appropriate length for the *Hsp70/Ddc* CNS mRNA and represents 50% or more of the mature transcripts induced by either mild or severe heat shock. To confirm the identity of the 2.25-kb species, the blot was rehybridized with a *Ddc* exon B probe to detect this CNS-specific exon. As expected, only the 2.25 kb and its precursor RNAs are hybridized, but the 2.1-kb RNA does not hybridize. The 3.0-kb RNA is a splicing intermediate that is hybridized by the exon B probe. It is the appropriate length for an intermediate retaining both the AB and the BC introns (panel III). The 4.0-kb RNA is the *Ddc* primary transcript (25). The appearance of these high-molecular-weight *Ddc* precursor RNAs is normally associated with expression of the CNS *Ddc* mRNA (3). The 5-kb *Ddc* RNA found after severe heat shock has not been reported previously. Since it is larger than the normal *Ddc* primary transcript, it may represent defective transcriptional termination during severe heat shock.

The appearance of the 2.25-kb *Ddc* CNS mRNA in whole larvae under conditions of heat shock is surprising, indicating that the primary effect of heat shock is on the specificity of *Ddc* splicing. To study this effect with greater precision and to examine simultaneously splicing of chromosomal *Ddc* transcripts, we used the reverse transcription-linked PCR. As shown in Fig. 4, both the chromosomal *Ddc* and the heat-induced *Hsp70/Ddc* transcripts are spliced exclusively into the CNS mRNA in the CNS during mild and severe heat shock (lanes 1 and 2). These results reconfirm the results, shown previously, that most if not all of the cells in the CNS contain the capability to splice via the CNS-specific *Ddc* splicing pathway.

In all tissues that normally express the hypodermal *Ddc* mRNA, there is accumulation of the CNS mRNA during heat shock. The heat-induced *Hsp70/Ddc* transcript is spliced into both the hypodermal and the CNS mRNAs in all non-CNS tissue fractions (Fig. 4, lanes 4, 6, and 8). At 30°C, the larval carcass fractions show strong expression of the CNS mRNA, whereas the hypodermal fraction expresses only a small amount of this mRNA. At 38°C, all the non-CNS tissue fractions express predominantly the CNS isoform.

These results demonstrate that non-CNS tissues which at normal temperatures have the capability to splice *Ddc* transcripts into the hypodermal mRNA shift their splice specificity during heat shock. During mild heat shock, the hypoderm continues to splice predominantly into the hypodermal isoform, whereas other larval tissues that do not normally transcribe *Ddc* show more extensive splicing into the CNS pathway. During severe heat shock, almost all *Hsp70/Ddc* mature transcripts found in the larva are the CNS isoform.

Despite these marked shifts in the splice specificity of *Hsp70/Ddc* transcripts, there are only subtle shifts in the splicing of the chromosomally derived *Ddc* transcripts. During severe heat shock, the hypodermal and carcass fractions (Fig. 4, lanes 3 and 5) express a very low level of

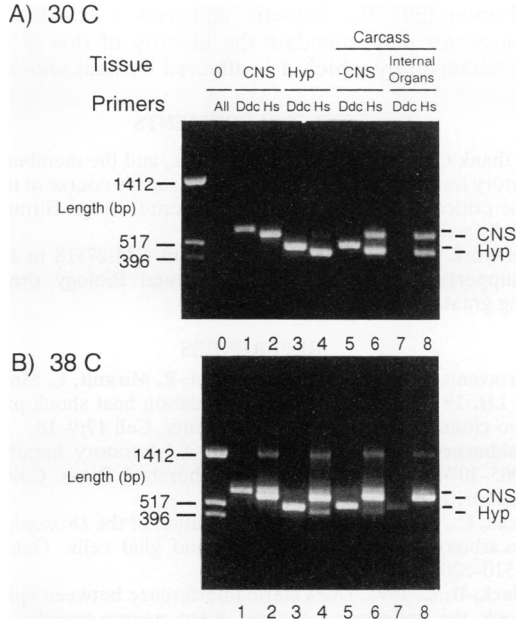

FIG. 4. The hypodermal splice pathway is selectively affected by heat shock. PCR analyses were performed as for Fig. 2, from third-instar larvae heat shocked for 60 min at 30°C (A) or 38°C (B). The internal organ fractions (lanes 7) contain a very low level of *Ddc* mRNA, such that this cDNA product is not consistently detected following PCR (A, lane 7). Note that during severe heat shock, intermediates containing intron BC accumulate (Fig. 3, panel III), resulting in the appearance of a splice intermediate retaining this 77-base intron. This results in the appearance of a band 77 bp larger than the normal CNS PCR product (B, lanes 2, 4, 6, and 8). The 1.4-kb band in panel B, even-numbered lanes, is the length expected for the precursor containing the introns AB and BC. The appearance of this product exclusively in the lanes which detect *Hsp70/Ddc* transcripts is likely due to the high levels of these transcripts relative to levels of transcripts from chromosomal *Ddc*.

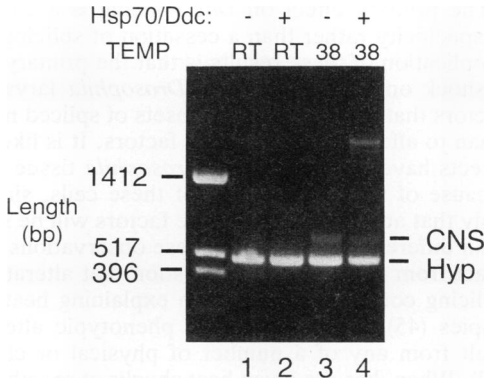

FIG. 5. Heat-induced alterations in chromosomal *Ddc* splicing are independent of the presence of the *Hsp70/Ddc* transgene. RNA was prepared from third-instar larval hypoderm (Hyp) fractions and then subjected to PCR analyses as previously described. The primers used are the *Ddc* exon A and D primers, which specifically amplify transcripts from the chromosomal *Ddc* gene. RNA was isolated from a strain lacking (lanes 1 and 3) or containing (lanes 2 and 4) the *Hsp70/Ddc* transgene. Lanes 1 and 2, room temperature (RT) control larvae; lanes 3 and 4, 38°C heat-shocked larvae, subjected to a 1-h heat shock.

the CNS splice form, levels far lower than are seen for *Hsp70/Ddc* under comparable conditions. These differences may well be because *Hsp70/Ddc* transcripts accumulate primarily during heat shock. In contrast, the *Ddc* transcripts result largely from transcription prior to heat shock, such that only a small fraction of the observed *Ddc* transcripts would have been synthesized during the time of heat shock. Additionally, heat shock suppresses transcription of many chromosomal loci (24, 40, 42), which might further reduce the number of *Ddc* transcripts that could be alternatively spliced.

There are two possible explanations for the observed changes in splice specificity during heat shock. First, it is possible that the high level of heat-induced expression of the *Hsp70/Ddc* gene is overloading a limiting component of the splicing machinery, resulting in a "spillover" into the CNS mRNA. However, it is also possible that heat or heat shock is leading to a real change in splicing specificity within larvae, a change that would be independent of the *Hsp70/Ddc* transgene.

To distinguish between these possibilities, we examined splicing of chromosomal *Ddc* transcripts in the hypoderm of strains containing or lacking the *Hsp70/Ddc* transgene, either in control room temperature larvae or in larvae exposed to severe heat shock. As shown in Fig. 5, both strains show a small amount of the *Ddc* CNS mRNA following severe heat shock (lanes 3 and 4). No detectable CNS *Ddc* mRNA is found in either strain in the absence of heat shock (lanes 1 and 2). These results demonstrate that there is an inherent change in *Ddc* splice specificity following heat shock that does not depend on the presence of the *Hsp70/Ddc* transgene.

DISCUSSION

By expressing the *Ddc* primary transcript ubiquitously under the control of an uninduced heat shock promoter, we show that the regulation of *Ddc* splicing in the CNS is at the level of the whole tissue. The CNS splice form of this transcript is the only form detectable within the CNS, even though this promoter is expressed in many more CNS cells than the normal *Ddc* promoter. *Ddc* transcriptional CNS regulatory elements act both at the whole tissue and at the cellular level (8, 13, 14, 20, 23, 35, 36). Apparently, transcriptional regulatory elements are better suited to the fine structure cellular regulatory decisions within the CNS than splicing regulators. It is difficult to generalize this observation at present. Although many vertebrate genes are differentially spliced in cells from specific tissues, in most cases they are studied in homogeneous cultured cells (4, 5, 9, 10, 22, 26, 28, 29, 32, 34). In these cases, it remains to be determined whether the whole of the tissues from which they were isolated retains similar splicing specificity.

Our data show that the effects of heat shock on splicing in living flies are much more heterogeneous than has been appreciated. We show that splicing of the *Hsp83* primary transcript is much more resistant to heat shock than has been observed previously with *Drosophila* tissue culture cells (44). Under conditions of severe heat shock at 38°C, only small amounts of *Hsp83* precursor RNAs accumulate, indicating that splicing of *Hsp83* is only slowed marginally relative to the rate of transcription. Splicing of introns from either *Hsp83* or the alcohol dehydrogenase gene, *Adh*, is largely inhibited at 36.5°C in *Drosophila* tissue culture cells (44). In contrast, splicing of the *Hsp70/Ddc* primary transcript is altered even at 30°C, conditions of very mild heat

shock. The primary effect on *Ddc* splicing is a change in splicing specificity rather than a cessation of splicing.

The implication of these results is that the primary action of heat shock on RNA splicing in *Drosophila* larvae is to affect factors that are specific for subsets of spliced mRNAs rather than to affect general splicing factors. It is likely that these effects have been missed in *Drosophila* tissue culture cells because of the homogeneity of these cells, since our data imply that at least some of these factors will be specific for certain differentiated tissues. These observations should not detract from the previous suggestion that alterations of RNA splicing could be important in explaining heat shock phenocopies (45). Phenocopies are phenotypic alterations that result from any of a number of physical or chemical agents (2). When flies are given heat shocks at specific times during development, a number of phenocopies that are highly reproducible depending on the time of heat shock can be produced. These phenocopies presumably result from alterations in the expression of specific genes. Accordingly, the splicing specificity of many genes might be affected by heat shock, resulting in even more complex developmental abnormalities than might result merely from a general inhibition of transcription and/or splicing.

The effects of heat shock on *Ddc* splicing lead to a model for regulation of *Ddc* alternative splicing. We find that heat shock leads to splicing in non-CNS tissues of the four-exon *Ddc* mRNA normally found exclusively within the CNS. These tissues include those that normally synthesize the three-exon hypodermal *Ddc* mRNA and those that never normally express *Ddc* at significant levels. In contrast, the *Ddc* primary transcript is spliced within the CNS exclusively into the four-exon CNS mRNA at all temperatures tested. The simplest explanation for these results is that the four-exon CNS *Ddc* mRNA is the default splice form, whereas the three-exon hypodermal mRNA is formed as a result of an active regulatory process. According to this model, non-CNS tissues contain a repressor that normally prevents splicing of the *Ddc* CNS-specific exon, exon B, and this repressor is inactivated either directly by heat or by some component of the heat shock response. In the presence of this repressor of exon B splicing, this exon would be excluded from the mature transcript, leading to the accumulation of the *Ddc* hypodermal mRNA retaining exons A, C, and D.

Our model for splicing of *Ddc* is strikingly similar to that proposed to explain the germ line specificity of P-element splicing, in that somatic tissues contain a negatively acting splicing regulator of the transposase mRNA (38). This factor prevents splicing of an intron, whereas the proposed *Ddc* splicing regulator leads to accumulation of an alternative splice form. P-element transposase–heat shock fusion constructs analogous to those presented in this paper show functional transposase only in the germ line (18). Data showing the effects of heat shock on transposase splicing have not been published.

We suspect that the regulator of *Ddc* splicing whose presence is inferred from data in this study is not used solely for *Ddc* but will also act on other genes that are spliced into distinct mRNAs within the CNS. This factor could act as a general determinant of CNS tissue identity. The gene encoding this factor might be expected to encode an RNA binding protein that is expressed widely in most tissues with the exception of the CNS. There is currently no good candidate for this gene. Although several genes that encode proteins containing RNA binding motifs have been isolated from *D. melanogaster*, none of these shows the expected tissue distribution (30, 31). Genetic approaches feasible for *D. melanogaster* may elucidate the identity of this factor and the mechanism by which it is affected by heat shock.

ACKNOWLEDGMENTS

We thank Claire Cronmiller, Paul Adler, and the members of our laboratory for helpful discussions throughout the course of this work and for critical comments on the manuscript. Serge Birman made especially valuable suggestions.

This work was supported by NIH grant GM-27318 to J.H. J.S. was supported by an NIH Developmental Biology predoctoral training grant.

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