Rearrangement of Upstream Regulatory Elements Leads to Ectopic Expression of the *Drosophila mulleri* *Adh-2* Gene

Dean Falb, Janice Fischer¹ and Tom Maniatis

Department of Biochemistry and Molecular Biology, Harvard University, Cambridge, Massachusetts 02138

Manuscript received June 3, 1992

Accepted for publication September 3, 1992

ABSTRACT

The *Adh-2* gene of *Drosophila mulleri* is expressed in the larval fat body and the adult fat body and hindgut, and a 1500-bp element located 2–3 kb upstream of the *Adh-2* promoter is necessary for maximal levels of transcription. Previous work demonstrated that deletion of sequences between this upstream element and the *Adh-2* promoter results in *Adh-2* gene expression in a novel larval tissue, the middle midgut. In this study we show that the upstream element possesses all of the characteristics of a transcriptional enhancer: its activity is independent of orientation, it acts on a heterologous promoter, and it functions at various positions both 5' and 3' to the *Adh-2* gene. Full enhancer function can be localized to a 750-bp element, although other regions possess some redundant activity. The ectopic expression pattern is dependent on the proximity of at least two sequence elements. Thus, tissue-specific transcription can involve complex proximity-dependent interactions among combinations of regulatory elements.

THE *Drosophila* alcohol dehydrogenase (*Adh*) genes are expressed in the fat body of larvae and adults and in a variety of other tissues (URSPRUNG, SOFER and BURROUGHS 1970; DICKINSON 1980a; GOLDBERG, POSAKONY and MANIATIS 1983; BATTERHAM *et al.* 1983; FISCHER and MANIATIS 1986). The *Adh* locus of *Drosophila mulleri* consists of two closely linked *Adh* genes and a pseudogene (FISCHER and MANIATIS 1985; Figure 1). *Adh-1* is expressed mainly in larvae, *Adh-2* in late larvae and in adults, and pseudogene transcripts are detected in adults (FISCHER and MANIATIS 1985, 1986).

The *D. mulleri* *Adh-1* and *Adh-2* genes are regulated in *D. melanogaster* P element transformants almost exactly as they normally are in *D. mulleri*, and each gene is regulated independently when introduced into the *D. melanogaster* genome on separate DNA fragments (FISCHER and MANIATIS 1986). A detailed analysis of the *Adh-1* regulatory elements has been carried out, and promoter-enhancer interactions involved in the tissue-specific expression of the gene in larvae have been characterized (FISCHER and MANIATIS 1988).

Adh-2 is normally expressed in the larval and adult fat body, and in the adult hindgut (BATTERHAM *et al.* 1983; FISCHER and MANIATIS 1986). However, in P element transformants containing an *Adh-2* gene with 2.3 kb of 5'- and 0.6 kb of 3'-flanking sequences *Adh-2* is expressed in the appropriate stage- and tissue-specific manner, but at 10–20-fold reduced levels

(FISCHER and MANIATIS 1986). Thus, a 1.5-kb regulatory element located 2.3 kb upstream of the *Adh-2* transcription start site is required for normal levels of *Adh-2* gene expression (FISCHER and MANIATIS 1986).

Surprisingly, high levels of expression in a novel tissue, the larval middle midgut, were observed when the *Adh-2* regulatory sequences upstream of the pseudogene were placed 1.2 kb upstream of the *Adh-2* transcription start site, 1.1 kb closer than normal to the *Adh-2* promoter (FISCHER and MANIATIS 1986). Here we report an investigation of the basis for this novel expression and an analysis of the *cis*-sequences required for maximal levels of expression in larvae and adults. We find that the region upstream of the pseudogene contains a tissue-specific enhancer. Moreover, we find that the *cis*-acting sequence requirements for the novel expression of *Adh-2* are complex. The high level of *Adh-2* expression observed in the larval middle midgut is a consequence of proximity-dependent interactions among at least two regulatory elements: one region of the enhancer and additional DNA sequences between –1.2 and –0.8 kb upstream of the *Adh-2* transcription start site. Thus, *Adh-2* transcription can be activated in a novel tissue through the juxtaposition of existing regulatory elements.

MATERIALS AND METHODS

Construction of P element-transformant lines: Test constructs were microinjected into *ry*³⁰⁶ or *Adh*^{tr6}*cn*; *ry*³⁰⁶ (*FCR*) embryos (SPRADLING and RUBIN 1982; RUBIN and SPRADLING 1982) and transformant lines were established in an *FCR* background exactly as described previously (FISCHER and MANIATIS 1988). *Adh*^{tr6} is a mutant *Adh* allele that

¹ Present address: Whitehead Institute for Biomedical Research, Nine Cambridge Center, Cambridge, Massachusetts 02142.

produces low levels of unspliced mRNA but no ADH protein (BENYAJATI *et al.* 1982). All *P* element constructs were injected into *FCR* embryos except for *123adh2*, *234adh2*, *12adh2*, *32adh2*, *34adh2* and *enh6*, which were injected into *ry³⁰⁶* embryos. The *FCR* strain was abandoned as a microinjection host because of serious problems associated with its viability, fertility and transformation efficiency in our hands. *P* element transformed lines were maintained over balancer chromosomes (FISCHER and MANIATIS 1988) or as homozygotes for the introduced genes. *Drosophila* strains were grown on standard cornmeal food at 25°.

Plasmid constructions: All test constructs were ligated into the *Hpa*I site of the *P* element transformation vector Carnegie 20 (RUBIN and SPRADLING 1983) or into the *Xba*I site of C20X, a derivative of Carnegie 20 containing an *Xba*I linker in the *Hpa*I site (provided by Paul Macdonald). The *hs1*, *hs2*, *1234adh2*, *enh1*, *enh5*, *enh6*, *12adh2*, *adh2* and *4321adh2* constructs were in the same transcriptional orientation in Carnegie 20 as the *ry* gene, and *123adh2*, *234adh2*, *enh2*, *enh3* and *enh4* were in the opposite orientation. The orientations of the remaining constructs were not determined. The DNA fragments within the different test gene constructs are described in the legends to Figures 2, 3 and 4. Standard procedures were employed for all enzymatic reactions and cloning manipulations (see MANIATIS, FRITSCH and SAMBROOK 1982). The constructions of pSP6-*atub* and pSP6-*Ad2* were previously described (FISCHER and MANIATIS 1985, 1986).

ψ1234adh2 is a 2.5-kb *Bam*HI fragment from pSP6 ψ *Bam* cloned into the *Bam*HI site of pucAdh2. pucAdh2 is the 2.8-kb *Nru*I-*Eco*RI fragment of *Adh-2* blunted into the *Sma*I site of puc12. pSP6 ψ *Bam* is the 2.5-kb *Sac*I-*Nru*I *Adh-2* fragment blunted and ligated into the *Bam*HI site of pSP64 with *Bam*HI linkers. *4321ψadh2* is the 5.5-kb *Sac*I-*Eco*RI *Adh-2* genomic fragment.

4321adh2 is a 1.5-kb *Sac*I-*Xmn*I fragment blunted into the blunt *Bam*HI site of pucAdh2. *1234adh2* was constructed in the same way, except that the blunt 1.5-kb *Sac*I-*Xmn*I fragment is ligated into pucAdh2 in the opposite orientation.

Histochemistry: ADH histochemical staining (URSPRUNG, SOFER and BURROUGHS 1970) was performed exactly as previously described (FISCHER and MANIATIS 1988). At least 10 individuals from each transformant line were tested. The larval and adult tissues were stained for approximately 30 min. Histochemical staining allows a qualitative determination of the tissue distribution of ADH expression and is not used as a quantitative measure of ADH expression levels.

RNA analysis: Total nucleic acid preparations, RNase protection experiments (ZINN, DIMAIO and MANIATIS 1983), and transcription of ³²P-labeled SP6 RNA probes (MELTON *et al.* 1984) were performed exactly as previously described (FISCHER and MANIATIS 1985, 1986, 1988). Prior to transcription, pSP6-*atub* was linearized with *Hinf*I, resulting in a 100-nucleotide (nt) probe, and pSP6-*Ad2* was linearized with *Hpa*II, resulting in a 270-nt probe (Figure 1). Visible α -tubulin protected bands vary with hybridization and digestion conditions and with autoradiograph exposure time. Expression levels can vary between different transformant lines of the same DNA construct due to chromosomal position effects, and between individuals of the same transformant line due to slight differences in age and in the mRNA preparations. To control for these differences, RNase protection assays were carried out at least three times with different mRNA preparations. Densitometry values were determined by the use of an LKB Bromma Ultros-can XL laser densitometer from autoradiographs exposed

A

B

FIGURE 1.—Diagram of the *D. mulleri* *Adh* locus. (A) Shown is a 9.3-kb region containing the *D. mulleri* *Adh* locus. The black regions indicate exons, the open areas within exons introns, and the white regions intergenic sequences. The striped regions indicate exons of the pseudogene. Arrows indicate transcription start sites and the direction of transcription. In *D. melanogaster* *P* element transformants, *Adh-1* is expressed primarily in the larval stages, and *Adh-2* primarily in third instar larvae and adults (FISCHER and MANIATIS 1986). In addition, there is a pseudogene (ψ -*Adh*) upstream of *Adh-2* which is transcribed in adults, but not translated. (B) Restriction map of the *Adh-2* gene and flanking sequences. Shown is a diagram of the 5.5-kb *Sac* I-*Eco*RI fragment of the *D. mulleri* *Adh* locus, containing the *Adh* pseudogene and *Adh-2*, used to transform the *4321ψadh2* lines (Figure 2). Striped boxes represent the exons of the pseudogene, black boxes are *Adh-2* exons, open boxes within exons are introns, and white regions are intergenic sequences. The numbers in the white boxes at the left end indicate the four subregions into which the *Adh-2* enhancer has been divided (Figure 4). The direction of transcription is from left to right. Restriction endonuclease recognition sites used to construct the various test genes are indicated and referred to elsewhere. Note that the figure is not drawn to scale. The extent (*Nru*I-*Hpa*II) of the RNA probe (SP6-*Ad2*) complementary to *Adh-2* is indicated by the leftward pointing arrow. The 130-nt fragment of the first exon of *Adh-2* protected by the SP6-*Adh-2* probe is indicated by the black bar.

within the linear range of the film. *Adh* message levels were normalized to α -tubulin levels in the same samples and in the same lanes.

RESULTS

Previous studies demonstrated that the 5.5-kb *Sac*I-*Eco*RI fragment containing *Adh-2* with 3.7 kb of 5'-flanking sequences (including the pseudogene) and 0.6 kb of 3'-flanking sequences (Figure 2; *4321ψadh2*) is sufficient for high levels of *Adh-2*

FIGURE 2.—*Adh-2* gene constructs. The expression patterns of *4321Ψadh2*, *adh2* and *4321adh2* were reported previously (FISCHER and MANIATIS 1986). (Here, *4321Ψadh2* and *4321adh2* are named *Ψadh2* and *5Ψadh2*, respectively.) The numbers of independent transformant lines bearing each construct are indicated in the parentheses. The boxes containing the numbers represent the 1.5-kb *SacI-XmnI* fragment containing the *Adh-2* enhancer, normally located upstream of the pseudogene. The order of the numbers in the boxes indicates the orientation of the enhancer fragment, with *4321Ψadh2* representing the genomic configuration. The arrows indicate the start sites of transcription and the boxes labeled *Ψ-Adh* and *Adh-2* represent the respective transcription units. The *4321Ψadh2* construct is a 5.5-kb *SacI-EcoRI* fragment of the *D. mulleri Adh* locus (Figure 1) containing the pseudogene and 1.5 kb of 5'-flanking sequences. *Adh-2* was constructed with 1.2 kb of 5'- and 0.6 kb of 3'-flanking sequences. The *adh2* structure is a 2.9-kb *NruI-EcoRI* fragment containing the *Adh-2* gene with 1.2 kb of 5'- and 0.6 kb of 3'-flanking sequences. The four remaining constructs all contain the 2.9-kb *NruI-EcoRI* of *Adh-2* and the additional DNA sequences indicated upstream or downstream of this *Adh-2* gene fragment. Message levels were quantitated with densitometry, with detectable message levels indicated by a +, and very low or undetectable levels by a -. + levels are 16–20-fold greater than - levels (data not shown).

expression in the larval and adult fat body, and in the adult hindgut (FISCHER and MANIATIS 1986). Analysis of two additional *Adh-2* gene constructs showed that sequences upstream of the pseudogene are required for normal levels of *Adh-2* transcription (FISCHER and MANIATIS 1986). First, transformants containing an *Adh-2* gene truncated at -1200 from the transcription start site (*adh2*, Figure 2) showed an approximately 20-fold lower level of *Adh-2* transcripts than the *4321Ψadh2* transformants. Second, when 1.5 kb of sequences (referred to as the *Adh-2* upstream regulatory region) normally located upstream of the pseudogene were added 5' to the *adh2* construct (*4321adh2*, Figure 2), normal levels of *Adh-2* expression were restored.

A tissue-specific enhancer is upstream of the pseudogene: We performed several experiments to determine if the *Adh-2* upstream regulatory region has the properties of a tissue-specific enhancer. First, we constructed genes similar to *4321adh2* and *4321Ψadh2*, except that the region upstream of the pseudogene was placed in reverse orientation (*1234adh2* and

FIGURE 3.—*hs* constructs. The *hsp70/Adh^F* hybrid gene is a 2.5-kb *XhoI-XbaI* fragment of the plasmid R68 (DUDLER and TRAVERS 1984), containing 0.6 kb of *Xenopus* 5S "spacer" DNA, the *hsp70* promoter and 5'-untranslated region from -68 to +198 from the transcription start site, and *Adh^F* gene sequences from +9 of the proximal promoter transcription start site to the *XbaI* site 0.7 kb downstream of the *Adh-2* polyadenylation site. In *hs1*, the 2.6-kb *SacI-NruI* of *Ψ1234adh2* (Figure 2), and in *hs2*, the 1.5-kb *SacI-XmnI* fragment in *1234adh2* (Figure 2), are ligated via *Bam*HI linkers upstream of the *hsp70/Adh^F* gene. The numbers in parentheses indicate the number of independent transformed lines of each construct analyzed. Expression of the *hsp70/Adh^F* gene in larval and adult tissues was assayed by histochemical staining. Detectable expression in the larval and adult fat body (FB) and in the adult hindgut (HG) is indicated by a + sign, and undetectable expression in the larval middle midgut (MMG) is indicated by a - sign.

Ψ1234adh2, Figure 2) or 3' to the *Adh-2* gene (*adh24321Ψ*, Figure 2).

The levels of *Adh-2* and tubulin control messages in RNA prepared from larval and adult transformants were determined by quantitative RNase protection. As summarized in Figure 2, the expression levels of the *Adh-2* genes in *1234adh2* and *adh24321Ψ* transformants are similar to those in *4321adh2*. Thus, the upstream regulatory region of *Adh-2* can function as an enhancer in either orientation 5' to the *Adh-2* promoter, and also downstream of the *Adh-2* gene.

To determine if the *Adh-2* upstream regulatory region could activate transcription from a heterologous promoter, we placed the 2.5-kb *SacI-NruI* fragment containing the pseudogene and the upstream regulatory region (*hs1*, Figure 3) or just the 1.5-kb upstream regulatory region (*hs2*, Figure 3) 5' to an *hsp70/Adh^F* hybrid gene (DUDLER and TRAVERS 1984). The hybrid gene contains the *hsp70* promoter and 5'-untranslated sequences (-68 to +198) joined to the *D. melanogaster Adh^F* gene (at +9 from the proximal transcription start site) and thus lacks its own promoter and regulatory sequences (FISCHER and MANIATIS 1988). The *hsp70* sequences provide a "neutral" promoter for our experiments, as the *hsp70* gene is not normally expressed in a stage- or tissue-specific manner (MASON, HALL and GAUSZ 1984). Consistent with these observations, the *hsp70/Adh^F* gene used in these experiments is not expressed in *P* element trans-

formants without heat shock (DUDLER and TRAVERS 1984; FISCHER and MANIATIS 1988).

In *hs1* and *hs2* transformants, ADH activity was detected by histochemical staining in the larval and adult fat body and in the adult hindgut (Figures 3, 6, and data not shown). These results, taken together with those above, show that there is a tissue-specific enhancer within the 1.5-kb *SacI*-*XmnI* fragment upstream of the pseudogene.

Localization of the enhancer: We attempted to localize the *Adh-2* enhancer within the 1.5-kb *SacI*-*XmnI* fragment by placing various fragments of this region 1.2 kb upstream of the *Adh-2* transcription start site (4321 constructs, Figure 4), and assaying the level of *Adh-2* transcripts relative to tubulin in adult transformants by quantitative RNase protection.

We found that the 750-bp *BsmI*-*XmnI* fragment in *12adh2* (Figure 4) is the smallest region tested that retains the ability to confer wild type levels of transcription on the *Adh-2* promoter. However, the DNA fragments in the *34adh2*, *32adh2*, and *1adh2* transformants also display some enhancer activity. Notably, the *34adh2* construct does not contain any enhancer DNA sequences in common with the *12adh2* construct. Thus, while the 750-bp *BsmI*-*XmnI* fragment in *12adh2* can restore normal levels of *Adh-2* expression, the 750-bp *SacI*-*BsmI* fragment upstream also contains sequences sufficient to elevate the *Adh-2* transcription level.

Deletion of the pseudogene results in high levels of transcription in a novel tissue: Preliminary experiments demonstrated that the 1.5 kb upstream region confers wild-type levels of expression on the -1200 *Adh-2* construct (FISCHER and MANIATIS 1986). Surprisingly, ADH staining activity was observed not only in the fat body but also in a novel tissue, the middle midgut of *4321 Ψ adh2* transformant larvae.

To determine if the ADH histochemical staining activity observed in the middle midgut represents a significant change in the level of transcription in this tissue, we analyzed RNA prepared from isolated fat body and middle midgut tissues of *4321 Ψ adh2* and *4321adh2* transformants by quantitative RNase protection. As shown in Figure 5, while *Adh-2* transcripts are detected only in the fat body of *4321 Ψ adh2* larvae, similar levels of *Adh-2* transcripts are detected in the fat body and middle midguts of *4321adh2* transformants. Thus, deletion of the pseudogene, which juxtaposes the *Adh-2* upstream regulatory region with sequences 1.2 kb upstream of the *Adh-2* transcription start site results in high levels of ectopic *Adh-2* transcription in the middle midgut.

To determine if the novel expression seen in the *4321adh2* transformants is dependent on the orientation of the enhancer, we investigated the ADH histochemical staining patterns and *Adh-2* transcript

FIGURE 4.—*Adh-2* mRNA transcripts in adults transformed with *4321* genes. (A) *Adh-2* transcripts in adult flies of various *4321* transformants were analyzed by quantitative RNase mapping using the SP6-*Adh2* and SP6-*atub* probes (see MATERIALS AND METHODS). Relevant bands are marked to the left of the autoradiograms. (B) The *4321* genes were constructed by ligating various portions of the 1.5 kb *SacI*-*XmnI* fragment located upstream of the pseudogene to the -1200 *adh2* construct. The boxes containing the numbers indicate the regions which the 1.5-kb element is divided into, and the numbers over the boxes indicate the approximate length of each segment. The normalized levels of *Adh-2* mRNA in total adults were determined by RNase protection analysis, as described in MATERIALS AND METHODS. The normalized level of *Adh-2* message in lane 6 (part A) was arbitrarily designated "1" in order to calculate the relative transcription level (RTL) in each of the other lines. Each bar in the histogram represents the RTL calculated for an independent transformant line. The RTLs mainly reflect the levels of *Adh-2* transcripts in the fat body, as it is the largest of the *Adh-2*-expressing tissues.

distributions in *Ψ1234adh2* and *1234adh2* larvae. ADH activity staining (Figure 6C and data not shown) and *Adh-2* transcripts (see Figure 8) were detected in both the fat body and middle midgut of *Ψ1234adh2* and *1234adh2* larvae. Thus, the novel middle midgut expression is independent of enhancer orientation.

FIGURE 5.—*Adh-2* transcripts in the larval fat body and middle midguts of *4321 Ψ adh2* and *4321adh2* transformants. *Adh-2* transcripts in early third instar larval fat body (FB) and middle midgut (MMG) of two independent lines each of the *4321 Ψ adh2* and *4321adh2* transformants were detected by RNAse protection assays. Total nucleic acid preparations from dissected fat body and middle midguts were hybridized separately with the RNA probes SP6-*Adh2* (Figure 1) and SP6- α tub (MATERIALS AND METHODS) which protect 130- and 90-nt segments of the *Adh-2* and α -tubulin genes, respectively. The tissues from which the total nucleic acid was prepared were from the same larvae (approximately 10). The tubulin probe served as an internal control to show that there are similar amounts of mRNA in all of the preparations, as equal amounts of each RNA preparation were assayed with each of the two probes. Consistent with the histochemical staining results (FISCHER and MANIATIS 1986), *Adh-2* transcripts were detected in the middle midguts of *4321adh2*, but not *4321 Ψ adh2* transformed larvae.

These results demonstrate that the novel expression cannot be due to the fortuitous creation of a small sequence element that induces middle midgut expression by the deletion of the pseudogene sequences, as the construction of the *Ψ 1234adh2* and *1234adh2* genes resulted in a different junction sequence than in the *4321adh2* gene (Figure 2).

Enhancer region 4 is necessary for middle midgut expression: In the course of localizing the enhancer, we found that although *23adh2* and *12adh2* transformants express high levels of ADH in the larval fat body (Figures 4 and 7 and data not shown), no ADH histochemical staining was observed in the middle midgut (Figure 7). RNAse protection analysis of isolated fat body and middle midguts of *1234adh2* and *123adh2* transformant larvae show that expression of *Adh-2* in the middle midgut is dependent on the presence of region 4 (Figure 7). Middle midgut expression was also observed with *234adh2* and *34adh2*. As summarized in Figure 7, the presence of region 4 in conjunction with regions 3 and 2, or region

3 alone results in middle midgut expression. The level of expression with region 4 alone was too low to assay by histochemical staining, so we do not know whether region 4 alone is sufficient for middle midgut expression. Thus, it appears that various combinations of regions 1, 2 and 3 result in fat body expression, while the addition of region 4 results in both fat body and middle midgut expression.

***Adh-2* expression in the larval middle midgut depends on the position of the enhancer upstream of the *Adh-2* promoter:** Next we addressed the question of why middle midgut expression of *Adh-2* is not observed when the enhancer is present in its genomic context upstream of the pseudogene. We designed several experiments to distinguish between the following two alternative explanations. First, the failure of the wild type *Adh-2* gene to be expressed in the larval middle midgut could be due to a blocking effect of the pseudogene sequences. Alternatively, larval middle midgut expression may depend solely on the distance between the enhancer and the *Adh-2* promoter and upstream sequences.

To determine if the pseudogene sequences prevent the enhancer from activating transcription in the larval middle midgut, we constructed *Adh-2* genes in which the 1.1 kb of pseudogene sequences were replaced by either a 1.0-kb fragment of *esc* gene coding region (*enh1*; Figure 8) or a 1.0-kb fragment of the α -tubulin coding region (*enh2*; Figure 8). ADH activity (data not shown) and *Adh-2* transcripts (Figure 8) were detected in the larval fat body, but not in the middle midguts of *enh1* and *enh2* transformant larvae. Thus, the failure to see larval middle midgut expression in larvae transformed with the *4321 Ψ adh2* (genomic) construct, in which the pseudogene sequences were present, was not because the pseudogene sequences blocked the action of the enhancer in this tissue. This conclusion is also supported by the observation that in *adh24321 Ψ* transformants, which contain an *Adh-2* gene with the enhancer 3' to the gene (Figure 2), ADH activity in larvae is observed only in the fat body (data not shown).

The above observations suggest that the novel expression may be a consequence of placing the enhancer at -1.2 kb from the *Adh-2* transcription start site. To test the stringency of this position requirement, we constructed *enh3*, *enh4* and *enh5*, in which the 1.5-kb *SacI-XmnI* fragment containing the enhancer was located at -1800 , -800 and -200 bp, respectively, from the *Adh-2* transcription start site (Figure 8). ADH activity (data not shown) and *Adh-2* transcripts were detected in the larval fat body, but not in the middle midguts of the *enh3*, *enh4* and *enh5* transformants. Thus, the expression of *Adh-2* in the larval middle midgut requires that the enhancer be

FIGURE 6.—ADH activity in larval tissues. Third instar larval tissues were dissected, fixed, and histochemically stained for ADH activity (MATERIALS AND METHODS). The drawing in the upper left corner is oriented similarly to the dissections in the photographs. The labeled tissues are the gastric caecae (gc), anterior midgut (amg), middle midgut (mmg), hindgut (hg), Malpighian tubules (mt) and fat body (fb). Each of these cell types has a different embryologic origin, with the possible exception of the Malpighian tubules and hindgut, which may arise from the same cells (POULSEN 1950; CAMPOS-ORTEGA and HARTENSTEIN 1985). Most of the fat body, a large structure which runs the entire length of the larva, was removed to reveal the other tissues. The *Adh^{fn6}cn; ry⁵⁰⁶ (fn6)* strain produces no ADH protein and does not stain (A). The transformatant lines in (B) and (C) are indicated in the lower left corner of each photograph. The arrows in each photograph point to the fat body, and the stained middle midgut (mmg) is indicated in (C).

positioned at a fairly precise location relative to the *Adh-2* promoter.

Sequences between -1.2 and -0.8 kb of the *Adh-2* transcription start site are required for expression in the larval middle midgut: We next determined whether the juxtaposition of the enhancer with specific sequences at -1.2 kb from the *Adh-2* transcription start site is necessary for larval middle midgut expression, or if this effect is simply due to the spacing between the enhancer and sequences near the *Adh-2* transcription start site. We constructed *enh6* (Figure 8), in which the 1.5-kb enhancer fragment was placed at -1.2 kb upstream of the *Adh-2* transcription start site, with the sequences between -1.2 and -0.8 kb replaced by a 0.4-kb fragment of spacer DNA. *Adh-2* transcripts were detected in the fat body, but not in the middle midgut of *enh6* transformatant larvae. Thus, the enhancer must be positioned at a certain distance from regulatory elements in the -1.2 - and -0.8 -kb region upstream of the *Adh-2* transcription start site to activate transcription in the larval middle midgut.

DISCUSSION

We have shown that the *Adh-2* gene of *D. mulleri*, normally expressed in the larval and adult fat body and in the adult hindgut, is regulated by a tissue-specific enhancer that can function in association with the *hsp70* promoter in these tissues. We have also localized the activity of the *Adh-2* enhancer to a 750-bp element which activates wild type levels of transcription from the *Adh-2* promoter. Furthermore, this enhancer can function in either orientation upstream

of the *Adh-2* gene, and also when placed 3' to the *Adh-2* coding region.

In addition to the activities mentioned above, the *Adh-2* enhancer can also activate transcription in the larval middle midgut. This activity requires three conditions to be satisfied. The *Adh-2* enhancer must contain region 4, the enhancer must be positioned at -1200 , and the sequences between -1200 and -800 must be present. This conclusion is based on the following observations. First, replacing the pseudogene with DNA segments of similar lengths does not result in ectopic expression of *Adh-2*, indicating that sequences within the pseudogene are not responsible for repressing expression in the middle midgut. Second, when the enhancer is placed at different distances from the *Adh-2* transcription start site or when sequences between -800 and -1200 are replaced by heterologous sequences, *Adh-2* expression is seen only in the fat body of third instar larvae. Finally, when region 4 of the enhancer is deleted, expression in the middle midgut is abolished, while fat body expression is unchanged. Interestingly, expression in the middle midgut is observed regardless of the orientation of the enhancer at -1200 , even though this changes the distance between individual enhancer elements and the *Adh-2* promoter. Thus, the enhancer can be considered a regulatory unit, and the relative position of elements within this unit with respect to the *Adh-2* promoter does not appear to be important, as long as the unit is located at -1200 .

These results provide two important conclusions. First, transcriptional activation in the middle midgut can be achieved by combining a fat body specific

FIGURE 7.—Expression levels in the middle midgut. (A) *Adh-2* transcripts in early third instar larval fat body (F) and middle midgut (M) of several 1234*adh2* and 123*adh2* transformant lines were detected by RNase protection (MATERIALS AND METHODS). Total nucleic acid prepared from dissected fat body and middle midguts was hybridized simultaneously with the SP6-*Adh2* and SP6- α -tub RNA probes, which protect 130- and 90-nt RNA fragments of the *Adh-2* and α -tubulin genes, respectively. The tissues from which the total nucleic acid was prepared were from the same larvae (approximately 10). The autoradiograms were deliberately overexposed to show that there is no *Adh-2* mRNA detectable in the middle midguts of several of the transformant lines. Relevant bands are marked to the left of the autoradiogram. (B) ADH histochemical staining in 4321 gene transformant larvae. Third instar larval tissues were dissected, fixed, and histochemically stained for ADH activity. Detectable staining is indicated by a + and undetectable staining by a -. The numbers in parentheses following the construct names indicate the number of independent transformant lines analyzed. Note that this is a qualitative measurement and does not indicate the quantitative differences in *Adh* expression levels shown in Figure 4.

enhancer (regions 1, 2 and 3) and an additional element (region 4). Other examples of this phenomenon are the expression of the *D. mulleri Adh-1* gene (FISCHER and MANIATIS 1988) and the *D. melanogaster Adh* gene (CORBIN and MANIATIS 1989; FALB 1991). Second, the tissue specific activity of an enhancer can be profoundly affected by its proximity to other regulatory elements, and its distance from the promoter.

Novel cell-type specificities have previously been reported in cases where a tissue-specific enhancer is linked to a heterologous promoter (SWANSON *et al.* 1985; SHERMOEN *et al.* 1987). In contrast, our studies

demonstrate that expression in novel tissues can be generated by rearranging the existing regulatory elements of a single gene.

One interpretation of these results is that transcriptional specificity is determined by a combinatorial mechanism (SWANSON *et al.* 1985). Support for this idea comes from experiments on the dopa decarboxylase (*Ddc*) gene. *Ddc* expression in neurons requires the combination of one *cis*-acting element used also in glial cells and another element used only in neurons (BEALL and HIRSH 1987). Similarly, the combination of the *Adh-1* larval fat body specific enhancer (box B) and a particular promoter element (box A) activates transcription in three larval tissues in addition to the fat body (FISCHER and MANIATIS 1988).

There is much species variation in the tissue specificity of expression of *Adh* genes, even among closely related species (DICKINSON 1980a; BATTERHAM *et al.* 1983; RABINOW and DICKINSON 1986; FISCHER and MANIATIS 1986). All *Drosophila* species examined so far express *Adh* in the fat body, but expression in a range of other cell types varies, and some species express *Adh* only in the fat body (DICKINSON 1980a). Evidence from genetic crosses (DICKINSON and CARSON 1979; DICKINSON 1980b) and *P* element transformation experiments (FISCHER and MANIATIS 1986; BRENNAN and DICKINSON 1988) suggests that the variations in tissue specificity are due to differences in the *Adh cis*-acting regulatory sequences, rather than to the presence of different *trans*-acting *Adh* transcription factors in each species. In fact, based on the results presented here, these variations could be due, at least in part, to rearrangement of existing regulatory elements during evolution, rather than to alterations in the binding sites of transcription factors.

These observations, taken together with the finding that *Adh-1* is regulated by larval fat body specific enhancers (FISCHER and MANIATIS 1986), suggested that the enhancers which regulate *Adh* are under evolutionary pressure to accommodate the levels and type of transcription factors present in the fat body (FISCHER and MANIATIS 1986). Thus, it was hypothesized that *Adh* genes in general are regulated by fat body specific enhancers (FISCHER and MANIATIS 1986). Indeed, the proximal and distal promoters of the *D. melanogaster Adh* gene are regulated by fat body specific enhancers (CORBIN and MANIATIS 1989; FISCHER and MANIATIS 1988; FALB 1991). The additional tissue specificity (adult hindgut) of the *Adh-2* enhancer is probably due to pseudogene promoter sequences contained within the 1.5-kb enhancer fragment. These promoter sequences in combination with the 1.5-kb enhancer may facilitate the activation of the *Adh-2* gene in the adult hindgut. This is consistent with the other *Adh* genes mentioned above, where the tissue specificities of fat body specific enhancers are

A

B

MMG

FIGURE 8.—*enh* constructs. (A) The *enh1*–*enh6* constructs all contain the 2.9-kb *NruI*–*EcoRI* fragment in *Adh-2*, and the additional DNA sequences indicated upstream or downstream of this gene fragment. In *enh1* and *enh2* the 1.5-kb enhancer fragment is separated by, respectively, a 1.0-kb *Bam*HI fragment of the coding of the *Drosophila esc* gene (provided by GARY STRUHL), or a 1.0-kb *ScaI*–*Bam*HI fragment of pDmta1 (KALFAYAN and WENSINK 1982), containing coding sequences of the *Drosophila* α_1 -tubulin gene. In the *enh3*–*enh5* constructs, the *Adh-2* enhancer fragment is upstream of a 3.5-kb *SphI*–*EcoRI* fragment, a 2.5-kb *NdeI*–*EcoRI* fragment, or a 1.9-kb *SnaBI*–*EcoRI* fragment, respectively, containing *Adh-2* and different extents of 5'-flanking sequences as indicated. In *enh6*, the *Adh-2* enhancer fragment is separated from the *Adh-2* gene fragment in *enh4* by a 0.4-kb fragment of the *cad* gene coding sequence (provided by PAUL MACDONALD). (B) *Adh-2* transcripts in early third instar larval fat body (F) and middle midgut (M) of the indicated transformant lines were detected by RNAse protection as described in Figure 7.

broadened in conjunction with *Adh* promoter elements.

We thank T. ABEL and A. MICHELSON for helpful discussions, advice, and critical reading of the manuscript. This work was supported by a grant from the National Institutes of Health to T.M.

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Communicating editor: P. CHERBAS