Sequences Upstream of the Homologous *cis*-elements of the *Adh* Adult Enhancer of Drosophila Are Required for Maximal Levels of *Adh* Gene Transcription in Adults of *Scaptodrosophila lebanonensis*

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ABSTRACT

The evolution of *cis*-regulatory elements is of particular interest for our understanding of the evolution of gene regulation. The *Adh* gene of Drosophilidae shows interspecific differences in tissue-specific expression and transcript levels during development. In *Scaptodrosophila lebanonensis* adults, the level of distal transcripts is maximal between the fourth and eighth day after eclosion and is around five times higher than that in *D. melanogaster* Adh^S. To examine whether these quantitative differences are regulated by sequences lying upstream of the distal promoter, we performed *in vitro* deletion mutagenesis of the *Adh* gene of *S. lebanonensis*, followed by *P*-element-mediated germ-line transformation. All constructs included, as a cotransgene, a modified *Adh* gene of *D. melanogaster* (d*Adh*) in a fixed position and orientation that acted as a chromosomal position control. Using this approach, we have identified a fragment of 1.5 kb in the 5′ region, 830 bp upstream of the distal start site, which is required to achieve maximal levels of distal transcript in *S. lebanonensis*. The presence of this fragment produces a 3.5-fold higher level of distal mRNA (as determined by real time quantitative PCR) compared with the *D. melanogaster* d*Adh* cotransgene. This region contains the degenerated end of a minisatellite sequence expanding farther upstream and does not correspond to the *Adh* adult enhancer (AAE) of *D. melanogaster*. Indeed, the *cis*-regulatory elements of the AAE have been identified by phylogenetic footprinting within the region 830 bp upstream of the distal start site of *S. lebanonensis*. Furthermore, the deletions Δ-830 and Δ-2358 yield the same pattern of tissue-specific expression, indicating that all tissue-specific elements are contained within the region 830 bp upstream of the distal start site.

THE expression and regulation of the alcohol dehydrogenase (*Adh*) gene has been extensively studied in *Drosophila melanogaster* and other Drosophilidae species with different gene organizations. The ancestral gene organization, which is present in Scaptodrosophila (Juan *et al.* 1994), in Idiomya (Rowan *et al.* 1986; Rowan and Dickinson 1986), in the subgenus Sophophora, and in the *virilis* group of the subgenus Drosophila (Nurminsky *et al.* 1996), has two promoters, as initially described in *D. melanogaster* (Benyajati *et al.* 1983). In the *repleta* group of the Drosophila subgenus, some species possess two linked single-promoter *Adh* genes that are differentially expressed during development in a manner that parallels the differential promoter use in *D. melanogaster* (Batterham *et al.* 1983; Fischer and Maniatis 1985). Recently, it has been shown that the *Adh* gene of *D. funebris* has only one functional promoter active in both larvae and adults (Amador and Juan 1999; Amador *et al.* 2001).

In *D. melanogaster*, the two promoters are tissue specific and temporally regulated by the *Adh* adult enhancer (AAE; Posakony *et al.* 1985) and the *Adh* larval enhancer (ALE; Corbin and Maniatis 1990). The AAE drives transcription from the distal promoter with low levels of transcript accumulating transiently in midstage embryos and in late third instar larvae (Savakis *et al.* 1986; Heberlein and Tjian 1988). Upon eclosion, and also within the first hour of adult life, the level of distal transcript increases dramatically, and it remains high throughout most of adult life (Savakis *et al.* 1986). The *cis*-regulatory elements of the AAE have also been identified functionally in the *Adh-2* gene of *D. mulleri* and by phylogenetic footprinting (comparison of noncoding sequences) in other Drosophila species (Sullivan *et al.* 1990; Juan *et al.* 1994; Nurminsky *et al.* 1996).

The Drosophilidae species with a two-promoter organization share a common pattern of transcription of the *Adh* gene from the proximal and distal promoter in the larval and adult fat body, respectively. However, differences have been reported in the pattern of expression from each promoter in tissues other than the fat body in Idiomya (Rowan and Dickinson 1986) and in the mRNA profile throughout development in Scaptodrosophila (Juan *et al.* 1994).

The *Adh* gene of *Scaptodrosophila lebanonensis* is maximally transcribed from the proximal promoter in first

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instar larvae, in contrast to the *D. melanogaster Adh* gene, which shows maximal transcription in second and third instar larvae. In adults of *S. lebanonensis*, the *Adh* gene is transcribed mainly from the distal promoter and the maximal level of transcript is achieved between 4 and 8 days after eclosion. This level is five times higher (per microgram of total RNA) than that of a strain of *D. melanogaster* homozygous for the ancestral allele Adh^S (Juan *et al.* 1994). Differences between these two species are also observed in the pattern of *Adh* gene expression in several tissues.

These interspecific differences are likely caused by differences in the regulation of gene expression and our goal is to determine whether there have been evolutionary changes that result in quantitative interspecific differences at the transcriptional level. Interspecific comparisons show that regulatory sequences involved in chromatin organization and transcriptional regulation occupy a much larger fraction of the genome than the sequences coding for proteins (Fickett and Wasserman 2000; Pennachio and Rubin 2001; Hardison 2002).

Evolutionary changes leading to differences in the pattern of gene expression in tissues have been widely investigated using *P*-mediated gene transfer in *D. affinidisjuncta* and Hawaiian Drosophila species (Wu *et al.* 1990). However, studies of the evolutionary changes leading to quantitative differences in gene expression are often compromised by the possibility of genomic position effects. It has been reported that this difficulty can be overcome by analyzing a large number of transgenic lines or by using the method of transgene coplacement (Siegal and Hartl 1996). This method allows any two sequences to be positioned at the same site and orientation in the genome and greatly enhances the detection of quantitative differences in expression between transgenes (Siegal and Hartl 1998). Here, we report a different approach based on constructs that include the *S. lebanonensis Adh* gene, with a particular deletion in each construct, together with a cotransgene that acts as a chromosomal position control. The cotransgene is a modified *D. melanogaster Adh* gene (d*Adh*). The *Adh* mRNA content of *S. lebanonensis* is then normalized to the d*Adh* mRNA content in each transgenic line. This approach revealed quantitative differences in the mRNA level associated with the different constructs of the *S. lebanonensis Adh* gene. Furthermore, we have identified a region that is required for maximal transcript levels in adults of *S. lebanonensis*. This level is 3.5 times higher than that of a strain of *D. melanogaster* with the ancestral allele Adh^S. The region identified lies upstream of the *cis*-regulatory elements homologous to those of the AAE of *D. melanogaster* and other Drosophila species.

MATERIALS AND METHODS

Drosophilidae strains: Three strains of *D. melanogaster* homozygous for the *Adh* gene, Requena (Adh^F α$Gpdh^S$) and Artés [Adh^S α$Gpdh^F$, In(2L)t], kindly supplied by M. Aguadé, and the stock Canton-S (Adh^F) were used as controls. The highly inbred strain G323 of *S. lebanonensis* was obtained from a sample collected in Gandesa (Spain).

Plasmid construction: All constructs were generated using the *P*-element vector pUChsneo (Steller and Pirrotta 1985) or with a modified version lacking the *Sal*I restriction site, but containing the additional restriction sites *Xba*I, *Kpn*I, and *Not*I in the multiple-cloning site. Five deletions (Δ-6431, Δ-2358, Δ-1264, Δ-830, and Δ-93) upstream of the distal promoter of the *S. lebanonensis Adh* gene were constructed, using a 12-kb *Xba*I clone (accession nos. X53429 and X63716) and a 5986-bp *Hinc*II-*Hin*dIII clone (accession no. AJ300179) that contains the fragment *Hinc*II-*Xba*I upstream of the distal promoter. The different deletions were constructed by digesting with appropriate enzymes, isolating suitable fragments, modifying ends where necessary, and ligating the fragments to produce the different pUChsneo constructs used in *P*-element-mediated transformation. Deletion Δ-2358 was constructed using exonuclease ExoIII due to the lack of an appropriate restriction site in this region.

All constructs have, at the same position and in the same orientation, an *Xba*I-*Bal*I fragment isolated from the pRI4.8 plasmid (Benyajati *et al.* 1987), encompassing the Adh^F gene of *D. melanogaster* conveniently modified (d*Adh*) to be used as a chromosome position control. Its coding region has been shortened by a 165-bp deletion in the second exon, corresponding to amino acids 84–138, as confirmed by sequencing of the complete gene. This deletion does not alter the reading frame although it abolishes ADH activity in spite of the presence of the mRNA.

***P*-element-mediated transformation and isolation of transgenic lines:** Recombinant pUChsneo plasmids were injected, along with the transposase plasmid phsπ (Steller and Pirrotta 1985), into embryos of the Canton-S strain of *D. melanogaster* as described previously (Rubin and Spradling 1982). The DNA concentration of each plasmid was 500 and 100 μg/ml, respectively. G0 adults were crossed individually to Canton-S and the G1 progeny were selected on G418-containing medium. Resistant flies were backcrossed to Canton-S, and G2-resistant larvae were analyzed by *in situ* hybridization on polytene chromosomes (Montgomery *et al.* 1987) to identify the chromosome on which the transgene was inserted. Once the chromosomal position of the insertion was identified, adult G2 flies were individually crossed to appropriate stocks (using *FM6*, *CyO*, and *TM6B*, *Tb*/*MKRS* to balance insertions on the first, second, and third chromosomes, respectively). Transformed lines with insertions on chromosome 3 were made homozygous for a chromosome 2 carrying Adh^{fn6} *cn*, using the strain Adh^{fn6} *cn*; *TM6B*, *Tb*/*MKRS*. Adh^{fn6} is a defective allele in *Adh* mRNA processing that produces a very small amount of mRNA (Benyajati and Dray 1984; Corbin and Maniatis 1989) that is undetectable with the small distal riboprobe used in our experimental conditions. Insertions on chromosome 1 or 2 were mobilized with the transposase source *P*[ry^+ Δ2-3] (Robertson *et al.* 1988), using the stock *w*; Adh^{fn6} *cn*; *Sb*, Δ2-3/*TM6B*, *Tb*. Only insertions on chromosome 2 or 3 with a background Adh^{fn6} were used in the analyses. Adults from the homozygous transgenic lines were crossed to Adh^{fn6} *cn*; *TM6B*, *Tb*/*MKRS* flies to produce offspring heterozygous for the insertion, prior to analysis.

To verify that each transgenic line carried the expected construct, genomic DNA was isolated, digested with the appropriate restriction enzymes, transferred to a nylon membrane, and probed in independent hybridizations with the 6.359-kb fragment (*Hinc*II-*Bsm*I) upstream of the distal TATA or the 5.332-kb fragment (*Hinc*II-*Bam*HI) upstream of position −1264.

The lack of activity of the modified *D. melanogaster* d*Adh* gene was verified by starch gel electrophoresis (Johnson and

Denniston 1964) since the ADH enzymes of *D. melanogaster* and *S. lebanonensis* have different isoelectric points (Juan and Gonzalez 1980; Winberg *et al.* 1986) and, therefore, their different mobility can be easily distinguished.

RNA purification: Total RNA was isolated from 40 mg of 4- and 8-day-old adults according to the procedure of Chirgwin *et al.* (1970; scaled down to use the ultramicrocentrifuge Optima TL100; Beckman, Fullerton, CA) and was spectrophotometrically quantified.

RNase protection assay: Plasmids pT7AdhDmel and pT7AdhSleb were used to generate *D. melanogaster* and *S. lebanonensis Adh* riboprobes, respectively. Additionally, plasmid pT3rp 49Dmel was used to generate a riboprobe for the endogenous *rp49* gene. Plasmid pT7AdhDmel was constructed by cloning the fragment *Sal*I-*Hpa*I (−63/+327), which encompasses the distal transcription start site, in pBluescriptII KS(−). Plasmid pT7AdhSleb was obtained by cloning the fragment *Xba*I (−830/+1242), encompassing the distal and proximal transcription start sites, in pBluescriptII KS(+). Plasmid pT3rp49Dmel was obtained by cloning the fragment −60/+164 of the *rp49* gene. This fragment was amplified from a pBluescriptII KS(+)*rp49* with the oligonucleotides T7 and 5′-TCG AAT CGA TGC TTG GTG CGC TTC TTC ACG AT-3′, digested with *Eco*RI and *Cla*I and cloned in pBluescriptII KS(−). Prior to transcription, plasmids pT7AdhDmel, pT7AdhSleb, and pT3rp49Dmel were linearized with *Sal*I, *Hpa*I, and *Eco*RI, respectively. Riboprobes were synthesized using T7 RNA polymerase (Promega, Madison, WI) for *Adh* of *D. melanogaster* and *S. lebanonensis* and T3 RNA polymerase (Promega) for *rp49.* The resulting riboprobes [438, 402, and 260 nucleotides (nt) long, respectively] were gel purified. Hybridization of 5 μg of total RNA and 250,000 cpm of each riboprobe and digestion with 6 μg of RNase A and 450 units of RNase T_1 (Boehringer Mannheim, Indianapolis) were carried out as described by Ayer and Benyajati (1990). Undigested RNA was analyzed on a 7% acrylamide, 8-m urea gel. Autoradiographs were exposed for 2–3 hr at −80° with one intensifying screen. The sizes of protected fragments corresponding to distal and proximal *Adh* transcripts of *S. lebanonensis,* distal d*Adh* (Figure 1), and *rp49* of *D. melanogaster* were 114, 127, 87, and 61 nt, respectively. Preliminary experiments demonstrated that none of the *Adh* riboprobes cross-hybridized with the heterologous mRNA as expected from the interspecific nucleotide divergence of the leaders of the two genes. After careful alignment of the exposed films and the dried gels, the protected fragments were excised from the dried gels and quantified by scintillation counting.

The content of distal mRNA of *S. lebanonensis Adh* was normalized to the content of *D. melanogaster* d*Adh* distal mRNA in the same sample and in the same lane. *rp49* was used to normalize the expression of each transgene to study the correlation of their expression in the different transgenic lines. We performed at least three experiments of RNase protection with the mRNAs from 4- and 8-day-old adults of each of the transgenic lines since the mRNA content of the *Adh* gene in *S. lebanonensis* adults increases with time (Juan *et al.* 1994).

Real-time quantitative RT-PCR: The TaqMan probes and primers for the *Adh* distal transcript of *S. lebanonensis* (5′-CAA ACA AAC AGT TAG AGG CAC AAG ATG GAT TTG-3′; forward primer, 5′-CAG CAG CGA TCG AGA CCA A-3′; reverse primer, 5′-GCA ACG AAA ATA ACG TTC TTG TTG-3′) and *D. melanogaster* (5′-AGA AGT CAC CAT GTC GTT TAC TTT GAC CAA CAA-3′; forward primer 5′-GCT AAC GAG TAC TTG CAT CTC TTC A-3′; reverse primer, 5′-AGA CCG GCA ACG AAA ATC AC-3′), for the *D. melanogaster rp49* gene (5′-TTC CTG GTG CAC AAC GTG CGC-3′; forward primer, 5′-GCC CAC CGG ATT CAA GAA-3′; reverse primer, 5′-CAT GAG CAG GAC CTC CAG CT-3′), and for the *28S* gene of both species (5′-TGG AGT TTA CCA CCC ACT TAG TGC TGC ACT-3′; forward primer, 5′-TCC AAA GAG TCG TGT TGC TTG A-3′; reverse primer, 5′-TTA CTA TCG GTC TCA TGG TTA TAT TTA GTT TTA-3′) were designed using the Primer Express program (Applied Biosystems, Foster City, CA). The analyses were performed with the TaqMan one-step PCR master mix reagents kit and universal conditions in an ABI PRISM 7700 (Applied Biosystems). Total RNA samples from each transgenic line were diluted appropriately and two 15-ng replicates of total RNA were analyzed for each of the three genes. Relative quantification of the *Adh* transcripts was performed by the relative standard curve method (Applied Biosystems). The standard curves were constructed with five fourfold dilutions (from 25.6 to 0.01 ng) of total RNA from a Δ-830 transgenic line with a high expression of both transgenes. The real-time PCR analysis was performed twice.

Histochemical staining of tissues: ADH histochemical staining (Ursprung *et al.* 1970) was performed on tissues of at least 10 individuals of 4-day-old adults as described (Ashburner 1989). The null Adh^{fn6} stock of *D. melanogaster* was used as a negative control. The stocks Canton-S of *D. melanogaster* and G323 of *S. lebanonensis* were used as positive controls.

Statistical and sequence analysis: General linear models analysis was carried out using the Statgraphics Plus 5.0 software package. Nucleotide variation was estimated using the DnaSP program (Rozas and Rozas 1999). Phylogenetic footprinting was performed using the Bestfit program from the Genetics Computer Group sequence analysis software package (Devereux *et al.* 1984).

RESULTS

Identification of regulatory elements in the 830-bp region upstream of the distal *Adh* promoter of *S. lebanonensis* by phylogenetic footprinting: Phylogenetic footprinting attempts to identify regulatory DNA sequences, on the basis of their conservation, in interspecific comparisons. We used the Bestfit algorithm to align the 6431-bp region upstream of the distal promoter of *S. lebanonensis* with the functionally characterized regions that control the *Adh* distal promoter of *D. melanogaster* and the *Adh-2* promoter of *D. mulleri.* In *D. melanogaster,* the *Adh* adult enhancer (Figure 1A, AAE) was delimited between 660 and 470 bp upstream of the distal promoter and is sufficient for tissue specificity and maximal expression levels in adults (Posakony *et al.* 1985; Ayer and Benyajati 1990). In the AAE, the *cis*-elements that bind to the transcription factors BBF2, AEF1, cEBP, DHR39, and FTZ-F1 were characterized (see Figure 2 and references therein). In the *D. mulleri Adh-2* gene (Fischer and Maniatis 1986), the same *cis*-elements are localized between −2660 and −2550 (Figure 2 and references therein).

In *S. lebanonensis*, the sequences homologous to the *cis*-regulatory elements of the *D. melanogaster* AAE and *D. mulleri Adh*-2 enhancer were identified by phylogenetic footprinting between 691 and 232 bp upstream of the distal start site (Figure 2). Two sequences were identified at −691/−677 (complementary strand) and at −629/−620, which were 86.7 and 80% similar to the BBF2 site of *D. melanogaster* and to the BBF2 consensus, respectively. AEF1 and cEBP binding sites were identi-

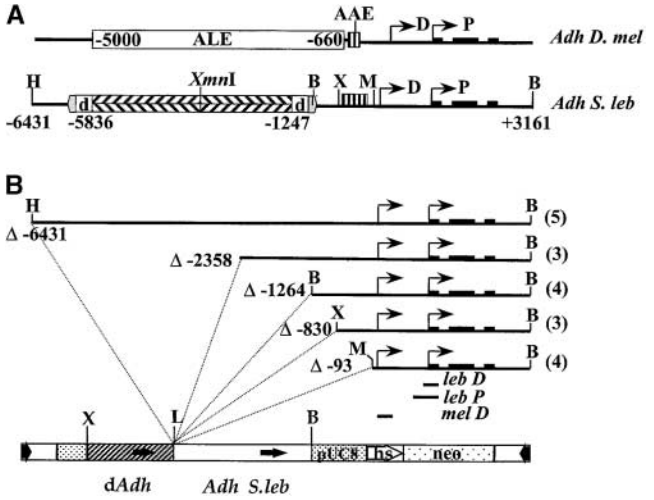

FIGURE 1.—Diagrams of the *Adh* gene upstream sequences and constructs used in the *P*-element-mediated transformation. (A) Comparison of the upstream *Adh* gene sequences of *D. melanogaster* and *S. lebanonensis*. ALE, *Adh* larval enhancer; AAE, *Adh* adult enhancer. In the *S. lebanonensis Adh* gene, a box with vertical lines indicates the AAE homologous sequences identified by phylogenetic footprinting. The arrowed box indicates a minisatellite sequence (4.5 kb), which is divided into two arrays separated by an *Xmn*I site (at −3195) and inverted with respect to each other; d indicates a region with increasing repeat degeneration (substitutions and deletions) observed in the 247 bp at 5′ and the 222 bp at 3′ of the 4.5-kb region. Furthermore, the 4.5-kb region is flanked by 47-bp imperfect inverted repeats. (B) The 5′ deletion mutations of the distal promoter of the *S. lebanonensis Adh* gene. Constructs were made using the pUChsneo plasmid. Each construct has the chromosome position control d*Adh* gene of *D. melanogaster* and the gene of *S. lebanonensis* (*Adh S. leb*) with one of the deletions upstream of the distal promoter named by the 5′ end point. The number of transgenic lines analyzed for each construct is given in parentheses. Below the deletion Δ-93 are the probe segments protected with distal and proximal transcripts of *S. lebanonensis* (*leb D* and *lebP*) and the fragment protected with the distal transcript of *D. melanogaster* (*mel D*). H, *Hinc*II; B, *Bam*HI; L, *Bal*I; M, *Bsm*I; X, *Xba*I; D, distal promoter; P, proximal promoter.

fied at −647/−629 and −257/−242, respectively, with a high similarity to the corresponding elements of *D. mulleri* (78 and 92%, respectively). A sequence 94.7% similar to the FTZ-F1/DHR39 binding site of *D. melanogaster* was identified at −252/−232. Interestingly, in *S. lebanonensis*, the putative cEBP site overlaps the FTZ-F1/DHR39 binding site, as in the *D. mulleri Adh-2* regulatory region.

The same sequences were also identified by the Bestfit algorithm in the comparisons between the *Adh* gene of *S. lebanonensis* and those of *D. affinidisjuncta* (between −660 and −146; MCKENZIE and BRENNAN 1998) and *D. virilis* (between −300 and −1; NURMINSKY *et al.* 1996). The spacing between the elements and their distance from the distal transcription start site vary among species.

Functional analysis of the 5′ region: Since the *Adh* mRNA content of *S. lebanonensis* adult flies is five times higher than that of a strain of *D. melanogaster* with the ancestral allele Adh^S, and the sequences homologous to the *cis*-regulatory elements of the AAE have been identified within the 830 bp upstream of the distal start site, we wanted to identify the 5′ flanking sequences that have a quantitative effect on the *Adh* mRNA content of *S. lebanonensis*. *D. melanogaster* flies transgenic for five different constructs were generated by *P*-element-mediated transformation. The constructs differ in the size of the 5′ region of the *S. lebanonensis Adh* gene. Figure 1B shows the five *S. lebanonensis Adh* deletions used in the constructs. Each deletion has been named according to the size of the *Adh* region upstream of the distal start site.

Deletion Δ-6431 contains the entire sequenced 5′ region (6431 bp from the distal start site) that includes a 4.5-kb minisatellite sequence composed of a 75% A-T rich, tandemly repeated dodecanucleotide sequence with the consensus 5′-GAATACAGAATA-3′ (ORENGO *et al.* 2004). This repetitive DNA is divided into two arrays, which are inverted with respect to each other and separated by an *Xmn*I site (Figure 1A). These two arrays, which are capable of forming a fold-back structure, contain 211 and 154 repeats, respectively. The dodecanucleotide sequence is highly conserved, since 40% of the repeats are identical to the consensus and 31% show changes in only one nucleotide. At both ends, after the last completely conserved repeat, we found degenerate repeats. Furthermore, this minisatellite is flanked by imperfect inverted repeats of 47 bp (Figure 1).

Deletion Δ-2358 includes only the 3′ end of the minisatellite sequence, encompassing 69 repeats, 26 of them with the consensus sequence and the rest with different substitutions. Neither repeats nor degenerated sequences are included in deletion Δ-1264. Deletion Δ-830 encompasses all the *cis*-regulatory elements identified by phylogenetic footprinting. Finally, deletion Δ-93 includes only the distal TATA and 61 nucleotides upstream.

Each construct also carries the modified *D. melanogaster Adh* gene (d*Adh*) that has been used as a chromosomal position control (Figure 1B). The d*Adh* version has the adult enhancer, the two promoters, and a coding region partially deleted in the second exon to obtain an inactive enzyme (see MATERIALS AND METHODS). These constructs allow us to quantify the mRNA of the *S. lebanonensis Adh* gene relative to the d*Adh* mRNA content in each transgenic line, to avoid potential variation due to position effects. Moreover, the lack of a *D. melanogaster* ADH enzyme activity allows us to determine the tissue specificity of the *Adh* gene of *S. lebanonensis* in a genetic background of *D. melanogaster* Adh^{fn6}. We also used the mRNA content of the *rp49* gene of *D. melanogaster* as an endogenous control.

The number of transgenic lines analyzed for each construct is given in Figure 1B. The transgenic lines were examined by Southern analysis to verify the construct they carried and by electrophoresis in starch gel

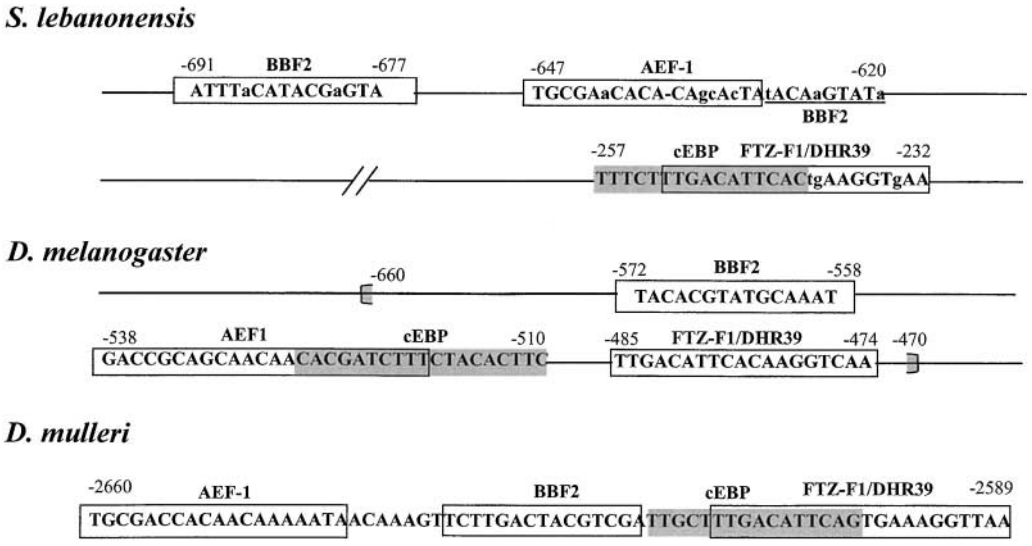

FIGURE 2.—Comparison of the regulatory regions of *S. lebanonensis* and *D. melanogaster Adh* distal promoters and *D. mulleri Adh-2* promoter. The binding sites for BBF2 (ABEL *et al.* 1992), AEF1/cEBP (FALB and MANIATIS 1992a,b), and FTZ-F1/DHR39 (AYER and BENYAJATI 1992; AYER *et al.* 1993) are the *cis*-regulatory elements functionally characterized in the AAE of *D. melanogaster* (between brackets) and in the *D. mulleri Adh-2* enhancer. These elements have been identified by Bestfit in *S. lebanonensis*. Small letters indicate nucleotide substitutions in *S. lebanonensis* relative to the *cis*-regulatory elements BBF2 and FTZ-F1/DHR39 of *D. melanogaster* and to the AEF-1/cEBP of *D. mulleri*. The element homologous to the consensus binding site of BBF2 is underlined.

to confirm that only the *S. lebanonensis* ADH active enzyme was detected (data not shown).

The level of distal transcripts from the *Adh* transgene of *S. lebanonensis* and the *D. melanogaster* d*Adh* cotransgene were quantified by RNase mapping. Figure 3 shows a representative RNase mapping experiment performed with some transgenic lines of each construct. The riboprobe used for *S. lebanonensis Adh*, which simultaneously maps transcripts from the distal and the proximal promoter, reveals that ~87% of the *Adh* transcripts in 8-day-old adults of this species originate from the distal start site. The riboprobe for *D. melanogaster Adh* maps only the transcript from the distal promoter and the riboprobe for *rp49* maps the endogenous transcripts. A transgenic line for the deletion Δ-830 without the d*Adh* gene (lane labeled Δ-830a in Figure 3) was used as a control and, as expected, shows no transcripts for the endogenous Adh^{fn6} of *D. melanogaster* when simultaneously probed with the *Adh* riboprobes of both species. In Figure 3, the brackets indicate the protected fragments that were quantified by scintillation counting to determine the amount of mRNA.

The contents of distal transcripts from *S. lebanonensis Adh* and *D. melanogaster* d*Adh* transgenes show a high, positive correlation with genomic position in transformed flies: Figure 4A shows the mean expression profile, in 8-day-old adults, of the *D. melanogaster* and *S. lebanonensis Adh* genes relative to the endogenous control *rp49* in the different constructs. A similar result was obtained for 4-day-old adults (data not shown). The large variance detected among lines carrying the same construct (long error bars) indicates that, as expected, chromosomal position has a very strong effect on the level of expression of both transgenes. No significant differences in the d*Adh* expression were observed among constructs, since the error bars of the different constructs overlap. In contrast, significant differences are observed in the expression of the *S. lebanonensis Adh* gene between Δ-93 and all the other constructs. These results indicate that some important *cis*-regulatory elements required for the expression of the *Adh* gene of *S. lebanonensis* are localized in the region between 93 and 830 bp upstream of the distal transcription start site. The effect of these *cis*-elements is strong enough to be independent of position effects. However, quantitative contributions of the

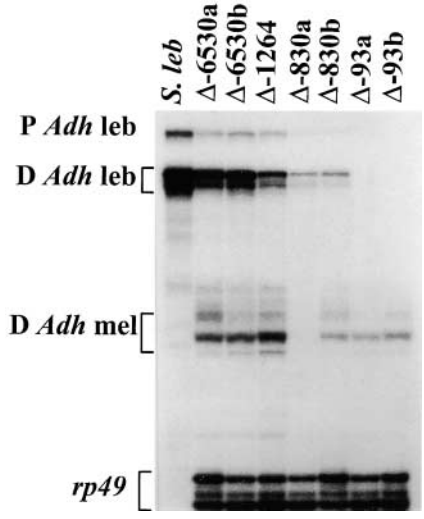

FIGURE 3.—Representative RNase mapping analysis of some transgenic lines. Total RNA from 8-day-old adults was analyzed with riboprobes designed to distinguish between proximal (P) and distal (D) *Adh* transcripts of *S. lebanonensis* (leb), distal (D) transcripts of *D. melanogaster* (mel), and the endogenous *rp49* of *D. melanogaster*. Brackets indicate the protected fragments that were excised from the gel to be quantified by scintillation counting. The lane Δ-830a shows the protected fragments from a line transformed with a Δ-830 plasmid that does not contain the d*Adh* gene of *D. melanogaster*.

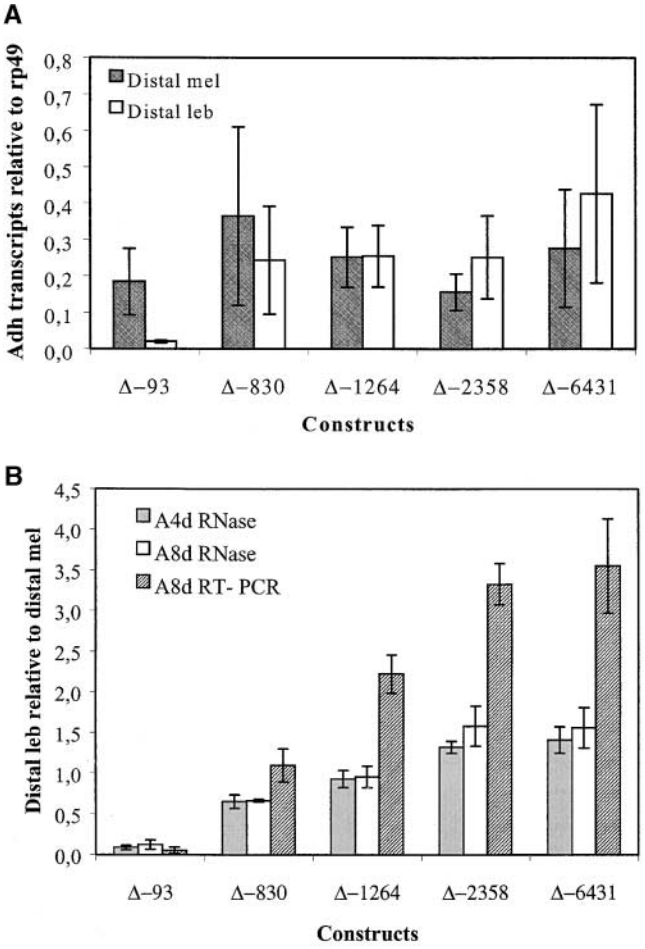

FIGURE 4.—Effect of the 5′ deletion mutations on the expression of the *Adh* gene of *S. lebanonensis*. (A) Quantitative analysis of the distal transcripts of *S. lebanonensis* and *D. melanogaster* relative to *rp49* obtained by RNase mapping in 8-day-old adults. (B) Quantitative analysis of *S. lebanonensis Adh* mRNA content relative to *D. melanogaster* d*Adh*, measured by RNase mapping in 4- (A4d RNase) and 8-day-old adults (A8d RNase) and by real-time quantitative RT-PCR in 8-day-old adults (A8d RT-PCR). In experiments of RNase mapping the numbers of transgenic lines analyzed for the different constructs were $n_{\Delta\text{-}93} = 4$, $n_{\Delta\text{-}830} = 3$, $n_{\Delta\text{-}1264} = 4$, $n_{\Delta\text{-}2358} = 3$, and $n_{\Delta\text{-}6431} = 5$. In real-time RT-PCR three lines of each construct were analyzed. Error bars represent standard deviations.

other regions are masked by the chromosomal position effect and thus cannot be elucidated without a chromosome position control.

To use the d*Adh* cotransgene as a position control gene, it is necessary to determine whether genomic position similarly affects the expression of both transgenes. It has been proposed that the expression of two genes at the same chromosomal position could be differently affected by unique interactions between each gene and the genomic milieu. This effect has been defined as lineage-specific position effect (LSPE; SIEGAL and HARTL 1998). LSPE would preclude the comparative analysis of genes located in the same chromosomal position. However, a high correlation in gene activity between transgenes at the same position would argue against LSPE and, therefore, the expression level of the cotransgene could be used as a position control.

For each construct except Δ-93, the mRNA content of *S. lebanonensis Adh* and *D. melanogaster* d*Adh* transgenes was strongly and significantly correlated ($r_{\Delta\text{-}6431} = 0.952$, $n = 15$; $r_{\Delta\text{-}2358} = 0.950$, $n = 6$; $r_{\Delta\text{-}1264} = 0.736$, $n = 17$; $r_{\Delta\text{-}830} = 0.951$, $n = 13$, $P < 0.001$; Figure 5). The lack of correlation in the Δ-93 construct ($r = -0.128$, $n = 12$, $P = 69.06$) is explained by the fact that the lines carrying this construct have only basal expression of the *Adh* gene of *S. lebanonensis* since there are only 61 nt upstream of the TATA box and most of the required *cis*-regulatory elements are missing. The strong correlation detected in the constructs longer than Δ-93 indicates that genomic position affects the expression of both transgenes in the same way and consequently validates our approach.

Quantitative effect of the different deletions on the expression of the *Adh* gene of *S. lebanonensis*: Figure 4B shows the expression profile of the *S. lebanonensis Adh* gene normalized to the mRNA content of the *D. melanogaster* d*Adh* cotransgene. The results in Figure 4B indicate that the deletion to 93 bp upstream of the TATA nearly abolishes the expression of the gene, whereas the addition of increasing lengths of DNA is accompanied by a significant increase in the *Adh* mRNA content of *S. lebanonensis*. The quantitative effect of the different constructs was statistically tested by fitting a general linear model relating the relative mRNA content of *S. lebanonensis Adh* to the predictive factors, construct, and lines nested within the constructs. Since no significant differences were found between adults of different ages ($P = 0.9213$), the statistical test was performed with the data set from 4- and 8-day-old adults (five constructs, 19 lines with several replicates, giving a total of 102 determinations). Significant differences were obtained among constructs ($F_{(4, 14)} = 57.18$, $P < 0.0001$) and among lines within constructs ($F_{(14, 83)} = 2.76$, $P = 0.002$). The 95% Fisher's least significant difference (LSD) intervals for each construct showed significant differences among the constructs except between Δ-2358 and Δ-6431, indicating that the region from 93 to 2358 bp upstream of the distal start site accounts for the maximal transcriptional activity of the *Adh* gene of *S. lebanonensis* from the distal promoter, at least in the background of *D. melanogaster*.

Since the two transgenes are included in the same construct, it can be argued that the regulatory regions of each transgene affect each other's expression simultaneously. However, different observations point away from this argument. First, the basal expression of the *S. lebanonensis* transgene in the construct Δ-93, which carries the TATA box and 61 nt upstream, argues against any effect of the *D. melanogaster* AAE on the *S. lebanonensis* promoter. Thus it seems reasonable to assume that the AAE does not affect the *S. lebanonensis Adh* expression in the

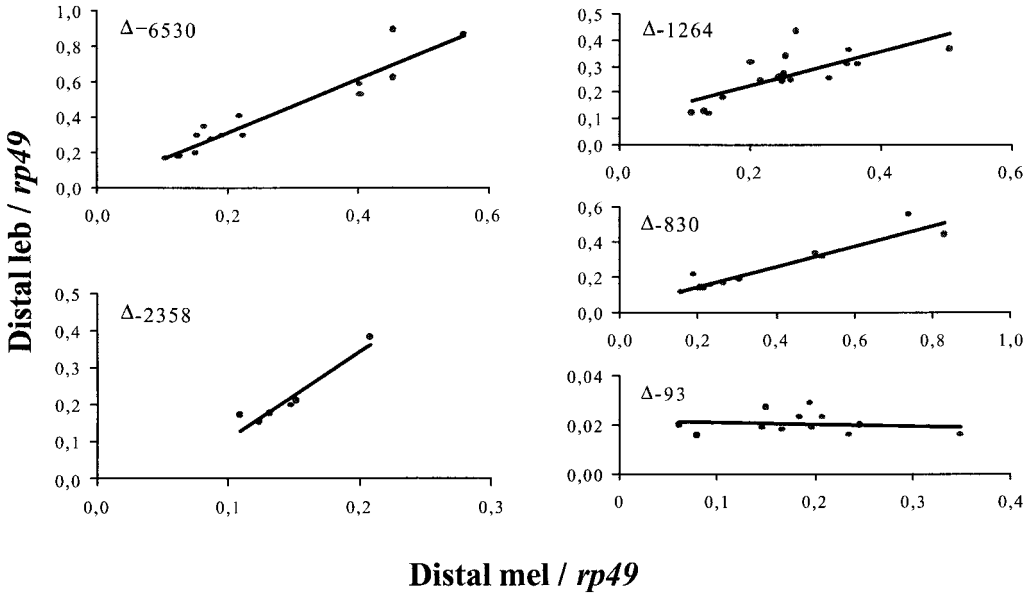

FIGURE 5.—Correlation between the amount of distal transcripts of *S. lebanonensis Adh* and that of *D. melanogaster* d*Adh* in the transgenic lines of each construct. The mRNA content of each transgene relative to the endogenous *rp49* was determined by RNase mapping in 8-day-old adults. The correlation coefficients are given in the text.

other constructs. Second, no construct differences in the relative expression of the *S. lebanonensis Adh* gene would be detected if the *S. lebanonensis* regulatory region (the construct) equally affected both genes. Finally, the significant difference observed in the expression of the *S. lebanonensis* transgene between constructs Δ-93 and Δ-830, together with the nonsignificant difference in the *D. melanogaster* d*Adh* expression (Figure 4A), argues against a putative positive or negative effect of the *S. lebanonensis Adh* regulatory region (the construct) on the expression of the d*Adh* cotransgene.

In the lines transgenic for the deletions Δ-6431 and Δ-2358, the ratio of *S. lebanonensis Adh* mRNA content to d*Adh* mRNA of *D. melanogaster* was only ~1.5 (Figure 4B, RNase mapping results). However, the total amount of *Adh* mRNA in wild-type adults is five times higher in *S. lebanonensis* than in *D. melanogaster* (JUAN *et al.* 1994). To confirm the former result, we quantified again the amount of mRNA by real-time quantitative PCR, using samples from the same RNA extractions from 8-day-old adults of 3 lines for each construct. The results are shown in Figure 4B. The statistical analysis (five constructs, 15 lines, and four replicates) showed significant differences among constructs ($F_{(4,10)} = 76.47$, $P < 0.001$) but not among lines within constructs ($F_{(10,45)} = 1.19$, $P = 0.322$). The 95% LSD intervals also showed significant differences among constructs, except between Δ-2358 and Δ-6431, which further supports the suggestion that deletion Δ-2358 encompasses the *cis*-acting elements necessary for the maximal expression of the gene.

When the results from both analyses are compared, we observe that the relative values of the *S. lebanonensis Adh* mRNA are higher in the real-time quantitative PCR than in RNase mapping. This difference may be explained by the fact that real-time quantitative PCR requires less manipulation of the samples and the measurements are directly obtained. So, real-time quantitative PCR seems a more accurate technique than RNase mapping in quantifying the level of transcripts.

Table 1 shows the expression of the *S. lebanonensis Adh* gene in each construct relative to Δ-93 as detected by both RNase mapping and real-time quantitative PCR. Again, when comparing the results from both analyses, we observe that the relative values are higher in the real-time quantitative PCR than in RNase mapping. This difference can be explained since in RNase mapping experiments there is a level of radioactive background that results in higher values of *S. lebanonensis* mRNA content for the lines Δ-93 than those observed in the experiments of quantitative PCR, where the values correspond only to the amount of transcript present. The accuracy of real-time quantitative PCR is the reason why the ratio of *S. lebanonensis Adh* mRNA content to d*Adh* mRNA of *D. melanogaster* is ~3.5 in the transgenic lines with the two longest constructs (Figure 4B).

TABLE 1

Effect of increasing the region upstream of the distal promoter on the transcriptional activity of the *S. lebanonensis Adh* gene

Constructs	RNase mapping	RT-PCR
Δ-93	1.00 ± 0.361	1.00 ± 1.301
Δ-830	6.85 ± 1.25	19.83 ± 3.572
Δ-1264	10.06 ± 2.762	40.28 ± 6.574
Δ-2358	14.65 ± 2.174	62.07 ± 10.456
Δ-6431	14.92 ± 3.017	68.86 ± 12.497

Adh mRNA content relative to Δ-93 ± standard deviation is shown. RT-PCR, real-time quantitative RT-PCR.

The higher expression of the *S. lebanonensis* transgene relative to the d*Adh* cotransgene indicates that there are sequences in its regulatory region that contribute to the differences in transcriptional activity between both species. It can be argued, however, that the d*Adh* gene is expressed at a lower level than the wild-type gene of *D. melanogaster* due to its modification. To address this point, we have compared the amount of distal d*Adh* mRNA relative to *rp49* mRNA in all transgenic lines (0.4 ± 0.19) with the relative amount of distal *Adh* mRNA in two strains homozygous for the fast allele (1.2 ± 0.19) and in one strain homozygous for the slow allele (0.5 ± 0.04). These results indicate that the mean level of d*Adh* mRNA in the transgenic lines is similar to that of the strain with the ancestral allele Adh^S and support the suggestion that the *Adh* gene of *S. lebanonensis* is transcribed at a higher rate than the gene of *D. melanogaster* due to differences in its regulatory regions.

We confirmed the difference in mRNA content between wild-type adults of both species, first determined by Northern analysis, using real-time quantitative PCR. We performed this analysis using the 28S gene as a reference, instead of the rp49 gene, because the sequence of the rp49 gene of *S. lebanonensis* is not available and the primers used for PCR amplification in *D. melanogaster* yielded no product in *S. lebanonensis*. The region of the 28S gene chosen to design the primers and Taqman probe for real-time quantitative PCR is 100% identical in both species. The result of this analysis shows that the content of distal *Adh* mRNA of *S. lebanonensis* is 5.85 ± 1.75 higher than that in *D. melanogaster* Canton-S, which is a similar value to that obtained by Northern analysis.

The fragment of 830 bp upstream of the distal transcription start site encompasses the *cis*-acting sequences required for the nearly wild-type pattern of tissue-specific expression: ADH activity was detected by histochemical staining of whole organs of 4-day-old adults (males and females) in three transgenic lines of each construct. The analyzed tissues were those that showed ADH activity in either of the two species (Ursprung *et al.* 1970; Juan *et al.* 1994).

The construct Δ-93 showed only basal activity in mid midgut and ovarioles (Figure 6) as expected if the *cis*-acting sequences of *S. lebanonensis* driving the tissue-specific expression have been deleted. The constructs Δ-6431 (Figure 6), Δ-1264, and Δ-830 showed activity in the fat body and in the tissues summarized in Figure 7. In the cardia, the pattern of *Adh* expression in transgenic flies is different from the wild-type pattern of both species, although more similar to that of *D. melanogaster* (Figure 7). Along the gut, ADH activity is always lower than that in either of the two species. The seminal vesicles of the male reproductive system showed activity, as observed in *D. melanogaster* but not in *S. lebanonensis*. We observed ADH activity in the testis of some transgenic individuals although it has not been described in either of the two species. In the female reproductive system there is activity in the anterior half of the ovarioles and in the oviduct. We did not detect differences in the pattern of the *S. lebanonensis Adh* expression among the constructs Δ-830, Δ-1264, and Δ-6431 (Figure 7). However, the number of individuals with ADH activity in each tissue increases with the length of 5′ flanking region.

These results indicate that the *cis*-elements required for tissue-specific expression are located in the 830 bp upstream of the distal transcription start site. The pattern of *S. lebanonensis Adh* expression in the cardia and in the seminal vesicles, which is more similar to that of *D. melanogaster*, might indicate differences in the chromatin accessibility or/and in the availability of transcription factors in both organs of the two species.

DISCUSSION

Enhancer sequences required for temporal and tissue specificity of *Adh* gene expression in adults have been delimited in several Drosophila species. Phylogenetic footprinting allowed us to identify several sequences in *S. lebanonensis*, between 692 and 232 bp upstream of the distal start site, homologous to the *cis*-regulatory elements previously characterized in *D. melanogaster* and *D. mulleri* (Abel *et al.* 1992; Ayer and Benyajati 1992; Falb and Maniatis 1992a,b; Ayer *et al.* 1993). The same sequences were also identified in the comparisons between the *Adh* gene of *S. lebanonensis* and those of *D. affinidisjuncta* (between −660 and −146) and *D. virilis* (between −300 and −1).

As expected, the deletion from −830 to −93 produces a decrease of 20-fold in the *S. lebanonensis* mRNA content of transgenic lines (Table 1). Furthermore, these sequences allow nearly wild-type tissue-specific expression (Figure 7). However, the expression of the *Adh* gene of *S. lebanonensis* relative to the cotransgene d*Adh* revealed that additional sequences farther upstream are required for maximal transcriptional activity from the distal promoter. Sequence elements from −830 to −1264 lead to a 2-fold increase in mRNA transcripts and the segment from −1264 to −2358 leads to an additional increase of 1.5-fold (Table 1). A detailed sequence analysis of the region from −1264 to −830 has not revealed any previously characterized element that may be responsible for the rise in *Adh* transcriptional activity. However, upstream of −1264 we have identified a minisatellite region (Orengo *et al.* 2004). Repetitive sequences are not present upstream of the *Adh* gene in the sequenced genomes of *D. melanogaster* and *D. pseudoobscura*, nor have they been described upstream of the *Adh* genes sequenced in other Drosophila species. The construct Δ-2358 encompasses 69 repeats; 26 of them show the consensus sequence, while the rest have different substitutions. Interestingly, the repeats degenerate as they reach

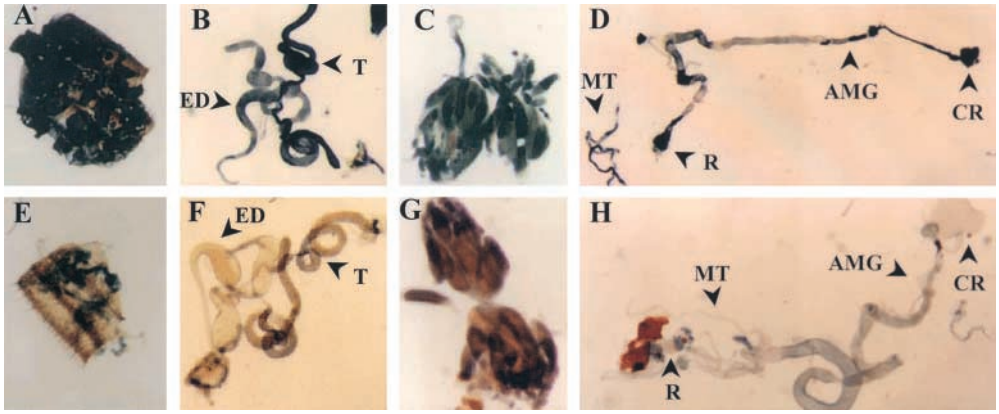

FIGURE 6.—Tissue-specific expression in 4-day-old adults of the transgenic lines. (A–D) Tissues of transgenic flies with deletion Δ-6431. (E–H) Tissues of transgenic flies with deletion Δ-93. (A and E) Fat body and carcass; (B and F) male reproductive system; (C and G) female reproductive system; (D and H) expression along the gut. CR, crop; AMG, anterior midgut; R, rectum; MT, Malpighian tubules; ED, ejaculatory duct; T, testis.

the 3′ end close to the *Bam*HI site (construct Δ-1264). A measure of this degeneration is given by the average of changes per nucleotide between the minisatellite sequence and a sequence of the same length composed of the consensus dodecanucleotide. The value for the stretch from −3195 (Figure 1A, *Xmn*I) to −2359 is 0.069 while the value for the stretch from −2358 to −1350 is 0.107. These two values are significantly different ($P < 0.01$). The most degenerated part of this minisatellite region confers the maximal transcriptional activity of the gene. One hypothesis could be that the higher substitution rate in this stretch of sequence might have been responsible for appearance of new binding sites for transcription factors and/or proteins that maintain an open chromatin configuration, increasing the transcriptional activity of the gene. We have also identified a T-box at −1380, described to be associated with scaffold-associated regions (SARs; GASSER and LAEMMLI 1986). It has been suggested that SARs would attract the putative D-proteins and facilitate the opening of the chromatin (LAEMMLI *et al.* 1992).

Although it remains to be proven which sequence elements within the region from −1264 to −2358 are associated with the increase of transcriptional activity, some cases have been reported where a minisatellite or a minisatellite-like sequence has positive or negative effects on the transcription of a gene. The difference in transcriptional activity between long and short variable number of tandem repeat minisatellite alleles is twofold in the human insulin gene (KRONTIRIS 1995) and in the nearby insulin-like growth factor (IGF2) the long alleles have nearly half the activity of the short alleles in human placenta both *in vivo* and *in vitro* (PAQUETTE *et al.* 1998). In addition, a minisatellite-like sequence contributes significantly to the enhancer activity of the mts/S100A4 mouse gene via interaction with abundant proteins described as minisatellite-binding proteins (COHN *et al.* 2001). In our case, the effect does not appear to depend on a number of repeats higher than 69 since no significant differences are observed between constructs Δ-6431 (365 repeats) and Δ-2358 (69 repeats). Moreover, the minisatellite sequence appears not to have any kind of barrier effect, since the expression of the *Adh* gene of *S. lebanonensis* is position dependent in all transgenic lines.

The sequences between −2358 and −830 might also produce the 3.5-fold difference in the level of distal transcripts between *S. lebanonensis* and *D. melanogaster* *Adh* genes. According to our experimental results, the control gene d*Adh* and the ancestral allele Adh^S are

	CA		CR	AMG	MMG	PMG	AHG	PHG	MT	R	OV	SV	ED	T
D. melanogaster			++	++	+	+	+	+	++	+	+	+	+	-
S. lebanonensis			++	++	+	+	+	+	++	+	++	-	+	-
Δ-6431		++	++	+	-	-	++	+	+	++	+	+	+	+
Δ-1264		+	++	+	-	-	++	+	+	+	+	+	+	+
Δ-830		+	++	+	-	-	+	+	+	+	+	+	+	+
Δ-93	-		-	-	b	-	-	-	-	-	b	-	-	-

FIGURE 7.—Histochemical staining of tissues from wild-type *D. melanogaster*, *S. lebanonensis*, and transformed adults. These results are a compilation of the histochemical staining of at least 10 adults, including both sexes, obtained from three transgenic lines of each construct. A "++" was scored when staining was present in all specimens, a "+" when more than one but not all specimens showed staining, and a "−" when no specimen showed staining; b, basal activity. The symbol in the CA column represents the *Adh* spatial expression pattern in cardia; CA, cardia; CR, crop; AMG, anterior midgut; MMG, mid midgut; PMG, posterior midgut; AHG, anterior hindgut; PHG, posterior hindgut; MT, Malpighian tubules; R, rectum; OV, ovarioles; SV, seminal vesicle; ED, ejaculatory duct; T, testis.

expressed at a similar level. If the different Adh^S strains had, on average, similar levels of *Adh* mRNA, our results would indicate that this region accounts for the quantitative differences between both species in the expression of the *Adh* gene. The difference in *Adh* mRNA content between strains Adh^S and Adh^F has not been unambiguously determined since some authors have found quantitative differences (Anderson and McDonald 1983) while others (Laurie and Stam 1988) have not. Nevertheless, taking into account that in the transgenic flies the *S. lebanonensis* gene may have some constraints on its expression due to the foreign genetic background, it easily explained that the actual difference between both species is higher than that observed in the transgenic flies. Our measurements of this difference gave always a higher value. In a previous work, a 5-fold difference between *S. lebanonensis* and an Adh^S strain of *D. melanogaster* was obtained by Northern analysis (Juan *et al.* 1994). In this work, a 5.8-fold difference was obtained by real-time quantitative PCR referred to the Canton-S strain (Adh^F) of *D. melanogaster* (used to inject the pUCchsneo constructs). Then, not only differences in the *cis*-regulatory elements account for the actual difference between both species. Among other causes we could consider differences in the *trans*-acting factors and in mRNA stability.

In contrast to the situation in *D. melanogaster*, where the AAE determines the wild-type levels of mRNA and the pattern of tissue-specific expression, in *S. lebanonensis* the region with the *cis*-regulatory elements homologous to the AAE determines the nearly wild-type pattern of tissue-specific expression, although a region farther upstream is necessary to achieve the highest level of mRNA content. Our results indicate that the region controlling the expression of the *Adh* gene in *S. lebanonensis* has been shaped by evolution in a way that has preserved some *cis*-regulatory elements, specifically those necessary for the tissue-specific expression, but other novel elements might have been acquired through the process of nucleotide substitution in the flanking region upstream of −830. This would suggest that some *cis*-regulatory elements have evolved *de novo* from random sequences, as has been proposed in a recent review on models for the evolution of functional noncoding DNA (Ludwig 2002). Only experimental work will determine which *cis*-elements in the region from Δ-2358 to Δ-830 are responsible for the increase in transcriptional activity.

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