Drosophila Hormone Receptor 38 Functions in Metamorphosis: A Role in Adult Cuticle Formation

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ABSTRACT

DHR38 is a member of the steroid receptor superfamily in Drosophila homologous to the vertebrate NGFI-B-type orphan receptors. In addition to binding to specific response elements as a monomer, DHR38 interacts with the USP component of the ecdysone receptor complex *in vitro*, in yeast and in a cell line, suggesting that DHR38 might modulate ecdysone-triggered signals in the fly. We characterized the molecular structure and expression of the *Dhr38* gene and initiated an *in vivo* analysis of its function(s) in development. The *Dhr38* transcription unit spans more than 40 kb in length, includes four introns, and produces at least four mRNA isoforms differentially expressed in development; two of these are greatly enriched in the pupal stage and encode nested polypeptides. We characterized four alleles of *Dhr38:* a *P*-element enchancer trap line, *l(2)02306*, which shows exclusively epidermal staining in the late larval, pre-pupal and pupal stages, and three EMS-induced alleles. *Dhr38* alleles cause localized fragility and rupturing of the adult cuticle, demonstrating that *Dhr38* plays an important role in late stages of epidermal metamorphosis.

METAMORPHOSIS in *Drosophila melanogaster* occurs over a four-day period and leads to a drastic transformation of the entire body pattern whereby most larval structures are histolyzed and replaced by new adult structures. Larval epidermal cells are totally replaced as the adult integument is formed by cells that originate in the imaginal discs (for the head and thorax), or the imaginal histoblast nests (for the abdominal-integument; reviewed in Fristrom and Fristrom 1993). Two cuticles are sequentially synthesized during metamorphosis: The pupal cuticle is deposited by both larval and adult epidermal cells during prepupal and early pupal stages, whereas the adult cuticle is deposited only by the latter epidermis 35–80 hr after puparium formation (Fristrom and Fristrom 1993). There are notable differences in the cuticle ultrastructure in pupae and adults, as well as regional differences within each stage (reviewed by Fristrom and Fristrom 1993). From the outside in, the adult cuticle consists of cuticulin, a protein epicuticle and a lamellate procuticle. The deposition of the inner cuticle layers also exhibits regional differences in timing and is associated with the synthesis of adult cuticle proteins of low molecular mass (Chinara *et al.* 1982; Roter *et al.* 1985). During tanning and hardening of the adult cuticle after fly eclosion, the adult cuticle proteins are thought to be cross-linked to each other and chitin using dopamine and β-alanine as the linking agents (Andersen 1979). Deposition, sclerotization and melanization of adult cuticle effectively conclude metamorphosis in the adult integument.

Metamorphosis is propelled by the action of ecdysteroids, mediated by members of the steroid hormone receptor superfamily. These receptors are ligand-dependent transcription factors that regulate expression of a large number of genes, which in turn effect the appropriate responses to the hormonal stimuli. Half of the known members of the steroid receptor superfamily in Drosophila appear to be regulated by ecdysone at the transcriptional level and, in turn, to participate in relaying the hormonal signal during early stages of metamorphosis (reviewed in Thummel 1995). Far less is known about late stages of metamorphosis, even though the highest peaks of ecdysone are achieved during this period (Riddiford 1993). It is widely accepted that the action of ecdysone in Drosophila is mediated by a functional ecdysone receptor consisting of a heterodimer of Ecdysone receptor (EcR) and ultra-spiracle (USP) proteins (Koelle 1992; Thomas *et al.* 1993; Yao *et al.* 1993). It is now known that other members of the steroid receptor superfamily in Drosophila can modulate the action of this ecdysone receptor complex, either by competing for binding on certain DNA EcREs (ecdysone response elements) or by interacting at the protein level with one of the receptor subunits (reviewed in Thummel 1995; Thummel 1997). We have shown previously that DHR38 is a potential modulator, as it is able to interact with USP *in vitro*, in yeast and in Drosophila cell lines (Sutherland *et al.* 1995). However, our results also demonstrated that DHR38 is able to bind DNA as

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a monomer *in vitro*, raising the possibility that both mechanisms of action are relevant *in vivo* in particular tissues or stages of Drosophila development. It is interesting that DHR38 homologues in mammals, the NGFI-B type receptors, can act as both monomers (Zetterström *et al.* 1996) and heterodimers with the RXR homologues of USP (Forman *et al.* 1995; Perlmann and Jansson 1995).

To gain insight into the mechanisms of DHR38 action we have initiated an *in vivo* analysis of *Dhr38* gene functions in Drosophila development. Elucidation of the *Dhr38* genomic organization revealed that *Dhr38* spans at least 40 kb and encompasses alternative promoters and polyadenylation sites. *Dhr38* transcripts are present throughout fly development with clear variations in quantity, being particularly enriched at the pupal stage. We report the isolation of mutations in the *Dhr38* gene which appear to result in fragility of the adult cuticle, at least in some areas. One of the alleles is represented by a pre-existing *P*-element insertion, while three others are new EMS-induced alleles. The three weaker alleles result in adult lethality shortly after eclosion with flies displaying haemolymph loss and melanization in the leg joints, while the strongest EMS allele causes earlier lethality.

MATERIALS AND METHODS

Recombinant DNA manipulations: A *D. melanogaster* genomic library prepared in λDASH II from a stock isogenic for the second chromosome (*dp*, *cl*, *cn*, *bw*) was a gift of W. Gelbart. A cDNA library made from third instar larval organs treated with ecdysone and cycloheximide was generously provided by C. Thummel. Phage DNA purification, digestion with restriction endonucleases, subcloning, agarose gel-electrophoresis, and transfer of nucleic acids to a nylon membrane (GeneScreen, NEN, Boston, MA) were performed as described (Sambrook *et al.* 1989). Inserts from genomic clones were subcloned into a pBluescript plasmid vector and sequenced on both strands by the dideoxy chain termination technique (Sanger *et al.* 1977). The ^{35}S dATP (NEN) and reagents from a standard sequencing kit (United States Biochemicals) were used according to manufacturer's instructions. For cTK61, exon 3 was sequenced on both strands and exon 1 on one strand; this allowed the determination of the splice sites between exons 1, 2, and 3 by comparing the sequence with our and available [Berkeley Drosophila Genome Project (BDGP), unpublished data] genomic sequences in 38E. The composite cTK61 sequence was generated using exon 1 and 2 sequences of BDGP and our sequences of exons 3–5.

To clone genomic DNA sequences, flanking the site of *P*-element insertion, genomic DNA from heterozygous adult flies bearing the *l(2)02306* insertion was digested with *Xba*I. After heat inactivation of the enzyme at 65° for 15 min, 2 fly equivalents of DNA were ligated overnight in 200 μl ligation buffer containing rATP (NEN) in the presence of 2 units of T4 DNA ligase at 14°. After precipitation and resuspension in 10 μl of H_2O, the samples were used to transform by electroporation XL1-Blue competent cells, which were plated on kanamycin-containing agar plates.

RNA manipulations: Total RNA was prepared from staged whole organisms using Trisolv™ (Biotecx Laboratories Inc., Houston, TX). For preparation of poly(A)$^+$RNA the Poly(A)-Tract mRNA Isolation System (Promega, Madison, WI) was used according to the manufacturer's protocol. Approximately 2 μg of poly(A)$^+$RNA were loaded per lane. Formaldehyde-containing agarose gel electrophoresis and nucleic acid transfer to Hybond-N membrane (Amersham) were performed as described (Sambrook *et al.* 1989). Antisense riboprobe corresponding to the DNA-binding and part of the ligand-binding domain of *Dhr38* was stringently hybridized and washed at 68° as described by Church and Gilbert (1984). No cross-hybridization was observed to a plasmid bearing an insertion of the CF1 cDNA clone of *usp*, a close relative of *Dhr38* (Shea *et al.* 1990). The blot was reprobed with an rp49 control (O'Connell and Rosbash 1984) to monitor mRNA integrity and loading.

For developmental reverse transcription PCR (RT-PCR) analysis poly(A)$^+$RNA purification and cDNA synthesis was carried out as in Barrio *et al.* (1996). PCRs were done in 50 μl with standard buffer conditions (1.5 mm $MgCl_2$) and AmpliTaq polymerase (Perkin Elmer, Norwalk, CT), using 20 pmoles of *Dhr38* specific primers. Three sets of such primers were used: (A) Flanking an intron in LBD and amplifying *ca.* 150 bp presumably from all the *Dhr38* mRNA species (T31 5′-AACGACATCATGGAGTTCAGC-3′ and T71 5′-CATCTGG AGCTGCTCCACCTT-3′); after 9 cycles (1 min steps at 94°, 60°, and 72°) the rp49A and rp49B primers (Barrio *et al.* 1996) corresponding to *D. melanogaster* ribosomal protein 49 (O'Connell and Rosbash 1984) were added at 10 pm each, and reactions were allowed to continue for 23 additional cycles. (B) Primers specifically amplifying a fragment of *ca.* 160 bp from the cTK61 isoform (61KS4 5′-GATCGGCTTGCTCG CTGATT-3′ and 61-VV10 5′-GCATTGAGGTTCTGTCTGTA-3′). The conditions were 1 min steps at 94°, 54°, and 72° for 8 cycles without rp49 primers and 23 cycles after rp49 primers were added. (C) Primers specifically amplifying a fragment of *ca.* 330 bp from the cTK11 isoform (TD31T32 5′-AGCTGGAG GACCTGGTAC-3′ and 11T36 5′-ATGCATGTCCGTAATT ATCG-3′); the conditions were 1 min steps at 94°, 54°, and 72° for 8 cycles without rp49 primers and 24 cycles after rp49 primers were added. Aliquots of the reactions were normalized with respect to rp49 amplified fragments and analyzed in a 2% agarose gel.

Histochemical staining for β-galactosidase: Dissected larval and pupal tissues were stained with X-gal by a standard procedure (Mlodzik and Hiromi 1992) with some modifications. Briefly, the tissues were dissected in PBX (PBS + 0.3% Triton X-100) and fixed in PBX/4% formaldehyde for 10–15 minutes. Staining reactions were allowed to develop overnight at 37°.

Fly stocks and EMS mutagenesis: Complete insertions, containing 5′ and 3′ UTR sequences, were subcloned from both cTK11 and cTK61, into the *Eco*RI site of the pCaSpeR-hs transformation vector under control of heat shock *hsp70* promoter (Thummel and Pirrotta 1992). Transgenic fly stocks were established and genetically mapped according to standard procedures.

Fly stocks bearing chromosome rearrangements with breakpoints in polytene division 38 and known mutations residing in this interval were obtained from S. Paine-Saunders (*Df(2)DS9 cn, pr, b* and *Df(2)DS6 cn, pr, b*), H. Jäckle (*caudal* alleles), M. Erdelyi (*Ketel* alleles and *Df(2)Ketel^{RX32}*) and the Bloomington Stock Center. The *P*-element insertion *l(2)02306, P[lacZ,ry$^+$], cn/CyO* was kindly provided by A. Spradling. In the course of genetic mappings we realized that both *Df(2)DS6* and *Df(2)DS9* extend distally much further than previously reported (Moore *et al.* 1983), uncovering *caudal*, *Ketel*, and *l(2)02306* located in 38E. To isolate recessive lethal mutations in the 38D-E region, we performed an F_2

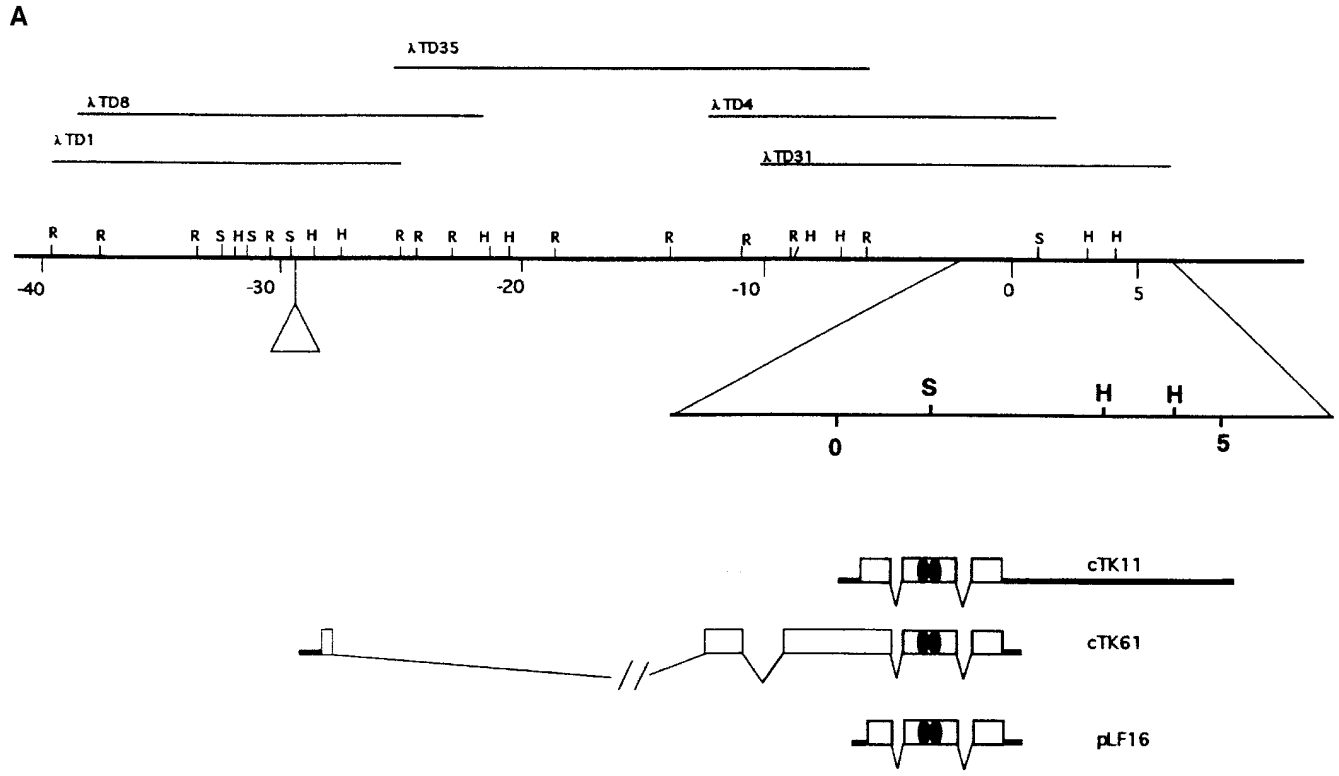
standard lethal screen using 0.025 M EMS essentially as described by Grigliatti (1986). The mutagenized second chromosome was marked with the recessive visible mutations *dp, bw*. In the first experiment *Df(2)DS9* was used as a selective system and the EMS 2, 8, 9, 15, 18, 27, 31, 36 and 38 mutations were recovered from 2,500 chromosomes analyzed. In a second experiment the remaining EMS mutations were recovered from 2600 chromosomes analyzed against *Df(2)Ketel*^{RX32}. The mutations were subjected to complementation testing and mapped with the deficiencies. For lethal phase determination the mutations were marked with *yellow* (*y*), the stocks being established in *yw* background and balanced against a *CyO, y*$^{+}$ chromosome. Collections of 200–400 eggs were done on apple juice-agar plates and allowed to develop for 24 hr at 25°. Live first instar *y*$^{-}$ larvae were transferred to vials with yeast paste and allowed to develop for 10 more days. The sample size for each genotype was 50–100 first instar larvae. The pupal cases and adult animals were scored. For the rescue experiments, heat shock was administered for the indicated periods of time to cultures in plastic vials in a water bath at 37°.

RESULTS

Genomic organization of *Dhr38*: We have performed a genomic walk of approximately 50 kb in the chromosomal 38E region where *Dhr38* resides. We partially sequenced a second *Dhr38* cDNA clone, cTK61, which overlaps with cTK11 (Sutherland *et al.* 1995), and mapped both clones with respect to genomic DNA from the region using hybridization and PCR scanning of the genomic DNA with cDNA sequencing primers to identify potential introns. Genomic sequences in 38E region corresponding to part of intron 2 of *Dhr38* and further upstream are available (BDGP, unpublished results) and were supplemented with our partial sequencing of genomic subclones (data not shown; see below). Genomic and cDNA sequences were compared. Taken together these experiments showed that the *Dhr38* gene extends over at least 40 kb and includes at least 5 exons (Figure 1A). The first intron is more than 20 kb in length, whereas the second through fourth introns are short (approximately 600 bp, 117 bp, and 184 bp, respectively). The first and second exons are unique to cTK61, and exon four is shared in its entirety by the two cDNAs; exon three is complete in cTK61 and incomplete in cTK11, and conversely exon five is shorter in cTK61 relative to cTK11. As previously reported (Sutherland *et al.* 1995) the cTK11 clone contains an open reading frame capable of encoding the DHR38 protein with a calculated molecular mass of 61 KDa, and more than 3 kb of 3′ untranslated region (3′ UTR). Using BDGP genomic and our cDNA sequences, a composite sequence of cTK61 was generated (see materials and methods). Computer analysis of this sequence showed a long open reading frame of 1071 amino acids beginning at nucleotide position 1121 with double methionine codons; the third methionine of this open reading frame is also unique to cTK61, whereas the fourth corresponds to the initiation codon of cTK11 (Figure 1B). Translation beginning with the first methionine codon of cTK61 would produce a polypeptide of 1071 residues and a calculated molecular mass of 117 KDa, which includes a transcription activation domain 522 amino acids longer than in the polypeptide encoded by cTK11 (Figure 1B); the DNA and ligand binding domains of the two polypeptides appear identical. A polypeptide of *ca.* 120 KDa is indeed detected immunochemically upon overexpression of the hsTK61 transgene (data not shown). As discussed below, expression studies using RNA blots and RT-PCR, together with the fact that both cDNA clones are terminated with a poly(A) stretch, suggest that these clones correspond to distinct RNA isoforms and that the gene contains alternative promoters and polyadenylation sites. This conclusion is reinforced by the findings of Fisk and Thummel (1995) who described another, shorter cDNA clone of *Dhr38*, pLF16 (Figure 1A; see below).

Expression of *Dhr38* in Drosophila development: We analyzed the expression of the *Dhr38* gene in Drosophila embryogenesis using RNA (Northern) blots, and observed multiple mRNA species even in high stringency hybridization experiments (Figure 2A). A prominent 2-kb band (sometimes resolved as a doublet of *ca.* 1.8 and 2.0 kb) is present in all embryonic mRNA preparations. A *ca.* 4.0-kb species is very abundant in the late embryos (19–23 hr post-egg laying) but is also detectable at lower levels earlier, especially in 15–19-hr embryos. A *ca.* 5.0 species is the least abundant in embryogenesis but is clearly present in 15–19-hr embryos. Of the multiple developmentally regulated transcripts of *Dhr38*, the *ca.* 4.0- and 5.0-kb species correspond in size and might be represented by the cDNA clones described above (cTK61 and cTK11, respectively). The pLF16 cDNA clone described by Fisk and Thummel (1995) might be represented by the 1.8- or 2.0-kb transcript, depending on the length of the poly(A)$^{+}$ tail. We have detected the 4.0-kb species and the 1.8–2.0-kb doublet in Schneider's S2 cell line as well (Figure 2B). All mRNAs of the *Dhr38* gene are of low abundance; blotting of purified poly(A)$^{+}$ RNA and probing by antisense riboprobes was required to detect them. Moreover, it appears that at least the 4.0-kb species is unstable because it is enriched in S2 cells treated with cycloheximide; the *ca.* 2.0-kb bands are unaffected by cycloheximide (Figure 2B).

To analyze the expression of the *Dhr38* gene during all stages of Drosophila development, we took advantage of a more sensitive technique, RT-PCR, and designed primers that would specifically amplify fragments corresponding to either the cTK61 or the cTK11 cDNA isoforms. We also used a pair of common primers, flanking the fourth intron in the ligand binding domain, which amplify a fragment present in all three cDNA clones described so far. The results of these experiments are summarized in Figure 3. The *Dhr38* gene is expressed during most of Drosophila development but with some notable variations in quantity. The common fragment



B

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MMRDRLASLIVVKQEGGSNTSISHHQATAIKCEASLYTESSLFQEINNNSCYRQNLNAPT   60
HQQSHTSHLQHAQQHQTHQQHPLLPPPLPTLPLIYPCRNLFPDGCDINHLACCSSSNSNS  120
NCNSDSNSTSSSPGNSHFFANGNTCAAALTPAPPATEPRKIKPLGAGKLKVGKTDSNSDS  180
NSNCDSRAAAAASTSATSATSATTLAATAAATAAAAEAGGAASAAAAAKISQVRLTNQAT  240
TSMLLLQPNSSFSSLSPFDNFSTQTASTTTTTSASAAGHHQHHNHLLHQQHHNQQQQQQQ  300
QQQQQQQQQQQQEHLQQQHQQQLVSPQQHLLKSETLLSHEEDQLISNLTDSSVVSHSELF  360
SDLFFPSDSNNSLLSPTTSGYPDNPAEDLTSSIENLTKLTCLRDKRLSSIPEQQLSSEQE  420
QQLCLLSLRSSSDPAIALHAQQQQQQQQQQQQQQQQHQQQQQHLQLQLISPIGGPLSCGS  480
SLPSFQETYSLKYNSSSGSSPQQASSSSTAAPTPTDQVLTLKMDEDCFPPLSGGWSASPP  540
APSQLQQLHTLQSQAQMSHPNSSNNSSNNAGNSHNNSGGYNYHGHFNAINASANLSPSSS  600
ASSLYEYNGVSAADNFYGQQQQQQQQSYQQHNYNSHNGERYSLPTFPTISELAAATAAVE  660
AAAAATVGGPPPVRRASLPVQRTVLPAGSTAQSPKLAKITLNQRHSHAHAHALQLNSAPN  720
SAASSPASADLQAGRLLQAPSQL                                        743
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C

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TAGCCGGGCCGCAAAGGTAGGTCGTGTCCCGCTCGCTTGCCGTCGCCCCTCCCTCTCCATCGCACACT

CACTCTCGCTCTCACTCGCTCTCGCGAGCGCAGCACGGCGCCAGCTGAGGCAGCGCTGTCGACGCGC

GCGTTGAATATAGTCGCGTGTAACCGTAAAGATAAGTTCAGTCGCGTCACAGACGCCGCATTGTTAAC
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Figure 1.—Genomic organization of *Dhr38*. (A) Positions of genomic λ clones are shown above the composite restriction map. Three cDNA clones, cTK11 (Sutherland *et al.* 1995), cTK61 (this report) and pLF16 (Fisk and Thummel 1995) are depicted below the enlarged map with protein coding regions represented by open boxes, untranslated regions by solid lines and introns by angled thin lines; positions of Zn fingers are denoted with two ovals. Distances are given in kb, and 0 corresponds to the start of the cTK11 clone; *Eco*RI, *Hin*dIII and *Sal*I restriction sites are indicated as R, H, and S, respectively. The site of the *l(2)02306* *P*-element insertion is shown as an open triangle. (B) Protein sequence of the putative activation domain encoded by the cTK61 isoform. The sequence shared by cTK11 and cTK61 is in bold. The locations of introns 1, 2, and 3 are indicated by arrows (following nucleotides 1553, 1867, and 3013 of cTK61), and methionines are underlined; the fourth methionine corresponds to the apparent initiation codon of cTK11, and the fifth to that of pLF16. (C) DNA sequence near the *P* insertion site (marked by an open triangle). The putative TATA box is underlined and the cap-site consensus (Cherbas and Cherbas 1993) is boxed; the start and direction of transcription in the cTK61 clone are shown with an arrow.

(Figure 3A) indicates that the combined *Dhr38* mRNAs are present in 0–8-hr embryos at very low levels, which are significantly elevated in late embryogenesis and through the larval stages. They become notably enriched in pre-pupal and especially pupal stages, and are again somewhat reduced in adult flies. The mRNAs are absent from the ovaries, but relatively concentrated in third instar larval imaginal discs and brain complexes. The expression profiles for individual isoforms (Figure 3B,C) are consistent with the profile of the common fragment (Figure 3A), but show some interesting variations: the pupal enrichment is most dramatic for the cTK11 isoform, and the adult has a substantial amount of cTK11 but virtually no cTK61 transcript. In overall

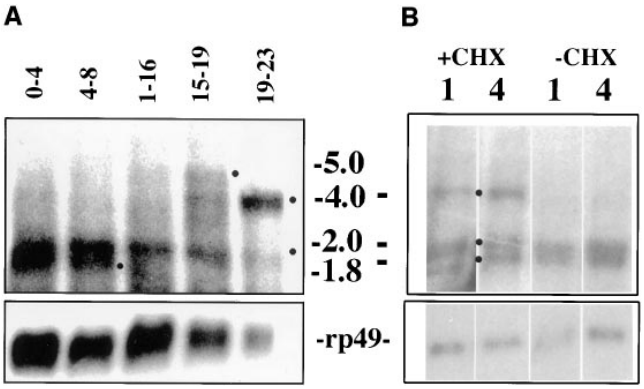

Figure 2.—RNA blot analysis of *Dhr38* expression. (A) *Dhr38* expression in Drosophila embryogenesis. Four mRNA species of *ca.* 5.0, 4.0, 2.0 and 1.8 kb, indicated with dots, hybridize with Dhr38 antisense probe (upper panel). The same blot was hybridized with the control rp49 probe to monitor RNA integrity and loading (lower panel). Poly(A)$^+$RNA was prepared from *yw* embryos collected at the indicated hours after egg laying. (B) *Dhr38* expression in the S2 cell line (upper panel). Two predominant transcripts of *ca.* 2.0 and 1.8 kb are detected in S2 cells incubated in Scheider's medium for 1 or 4 hr in the absence of cycloheximide (right). A low abundance transcript of *ca.* 4.0 kb which is detectable in untreated cells upon much longer exposure (not shown) is greatly enriched in the cells treated with 10 μg/ml cycloheximide for 1 or 4 hr (left). The same blot was hybridized with the control rp49 probe to monitor poly(A)$^+$RNA integrity and loading (lower panel).

terms, the cTK11 (*ca.* 5.0 kb) isoform is enriched in pupae and adults relative to the cTK61 (*ca.* 4.0 kb) isoform, which is more characteristic of the larvae.

The nature and significance of the *ca.* 2.0-kb transcript(s) merit further attention. Fisk and Thummel (1995) described a *ca.* 1.9-kb transcript as predominant at larval and pre-pupal stages, and this is clearly true for the *ca.* 2.0-kb doublet in S2 cells in the absence of cycloheximide (Figure 2B), as well as in early embryos at 0–16 hours (Figure 2A). However, the *ca.* 4.0-kb isoform clearly becomes dominant in the late embryos (19–23 hr; Figure 2A), and is enriched in cycloheximide-treated samples unlike the *ca.* 2.0-kb forms (Figure 2B). We cannot exclude the possibility that at least one or both of the *ca.* 2.0-kb bands correspond to alternative *Dhr38* RNA isoforms that have not yet been isolated and may not be amplifiable with our common primers. It is also possible that one or both bands correspond to a closely related gene, although we have hybridized our blots at high stringency (see materials and methods).

Analysis of the lethal *l(2)02306 P*-element insertion in the vicinity of the *Dhr38* gene: To find a mutation affecting *Dhr38* we screened the available *P*-element insertion collections and discovered one candidate mapping to polytene division 38E1-2, within the limits of a chromosomal deletion, *Df(2)Ketel^{RX32}*. We cloned the genomic DNA sequences flanking the site of *l(2)02306* *P*-element insertion and by hybridizing the rescued construct to cloned genomic sequences derived from the 38E chromosomal walk we mapped the site of the insertion very close (less than 1.5 kb) to the 5′ end of cTK61 (data not shown; Figure 1A). Subsequent sequence comparisons actually showed that the distance is only 34 bp (Figure 1C). The insertion site is found 8 bp downstream of a potential TATA box and 17 bp upstream of a TCAGT motif which is commonly associated with transcription start sites (± 10 bp; Cherbas and Cherbas 1993). Thus, it would not be surprising if this *P* insertion affects at least some aspects of *Dhr38* expression.

The *l(2)02306* insertion line is listed as homozygous lethal. We determined the effective lethal phase to be at the adult stage, for both homozygous and hemizygous individuals in combination with *Df(2)Ketel^{RX32}* (Table 1). Homozygous insertion mutants develop normally through embryonic, larval and pupal stages, and the majority of the flies eclosing from the pupal cases look phenotypically normal. However, the cuticle in the leg joints becomes ruptured (occasionally during eclosion but predominantly later), as evidenced by leakage of haemolymph. The flies subsequently die within a few hours, displaying heavy melanization in the leg joints and occasionally in the proboscis, antennae and wing hinge (Figure 4D). In combination with *Df(2)Ketel^{RX32}* the flies exhibit a slightly stronger phenotype, showing rupture of the cuticle and melanization predominantly during eclosion from the pupal case. Our interpretation is that the adult cuticle in mutant flies is not formed properly and is fragile at least in some areas, where it ruptures by mechanical stress when the flies start moving; this leads to leakage of haemolymph and melanization of the damaged spots, and eventual death possibly from desiccation. This phenotype is reverted and the viability is restored after precise excision of the *P* element (data not shown).

We performed histochemical staining for β-galactosidase at different stages of development using animals bearing the *P*-element insertion. No staining was detected in first and second larval stages in any tissue; staining first appears in the epidermis of feeding third instar larvae (Figure 4A), becomes much stronger in wandering third instar larvae (Figure 4B) and persists in pre-pupal and pupal stages. In early pupal stages the imaginal epidermis is stained homogeneously (data not shown), but about 70 hr after puparium formation (Bainbridge and Bownes 1981) the staining becomes localized to the leg joints, hinge region of the wing, proboscis and antennae (Figure 4C), locations which show the melanization phenotype with variable frequency (leg joints always, the rest sporadically). The same staining pattern persists in newly eclosed flies (data not shown). At all stages the staining is predominantly epidermal, with no internal staining observed in larvae and only very weak, presumably background, staining in pupae. Therefore the *l(2)02306* enchancer trap line is under the control of and may interfere with mostly

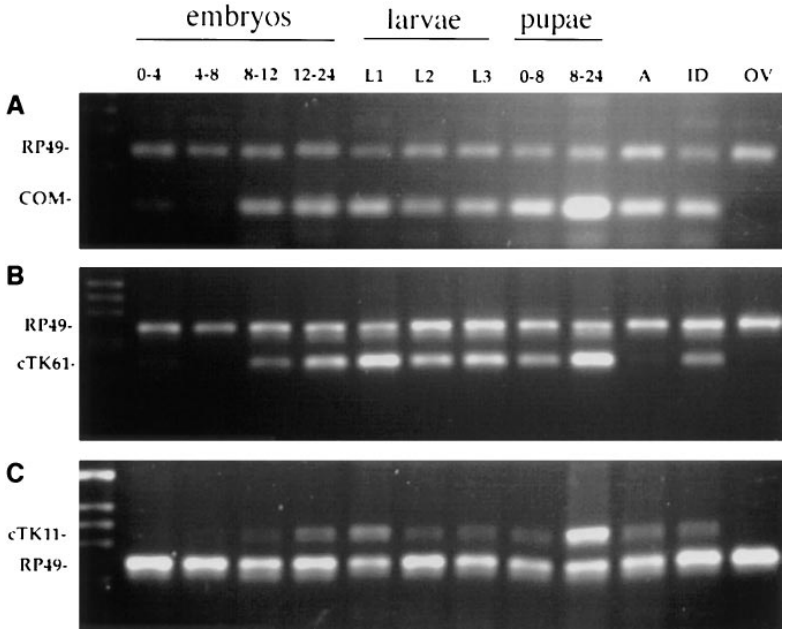

Figure 3.—RT-PCR analysis of *Dhr38* expression during Drosophila development. (A) Expression profile corresponding to all known *Dhr38* species at the indicated stages and hours of development; A = adult, ID = imaginal discs and brain complexes of third instar larvae, OV = ovary. (B) specific expression profile of the cTK61 isoform. (C) specific expression profile of the cTK11 isoform. Rp 49 represents a semiquantitative control of amplification and loading.

epidermis-specific enchancer(s), which presumably regulate the *Dhr38* gene. However, as assayed by RT-PCR, *Dhr38* mRNA is still detected in homozygous mutant animals at levels comparable to controls (data not shown). This is not surprising considering the localized nature of the phenotypic defects observed and the complexity of the *Dhr38* transcription unit, which apparently includes alternative promoters (Figure 1A).

TABLE 1

Effective lethal phase of *Dhr38* alleles

Genotype	First instar larvae (%)	Pupal cases (%)	Eclosed adults (%)	
			Dead[a]	Live[b]
56/56	100	0	0	0
56/RX32	100	66	0	0
43/43	100	80	51	6
43/RX32	100	75	66	0
57/57	100	80	50	17
57/RX32	100	85	65	4
P/P	100	77	75	0
P/RX32	100	84	80	0
P/CyO, y$^+$	100	84	0	84

The respective genotypes of animals homozygous or hemizygous in combination with the deficiency *Df(2)Ketel*^{RX32} (abbreviated as *RX32*) are shown in the left column; EMS alleles are symbolized by numbers, *P* represents the insertion allele *l(2)02306*, and *P/CyO,y*$^+$ is the control. Homozygous or hemizygous mutant first instar larvae, pupal cases and eclosed adults were scored.

[a] The eclosed adults designated dead displayed melanization in appendage joints by 12 hr after eclosion at the latest, varying from very weak in *57/57* and *43/43* homozygotes to very strong in *P/RX32*.

[b] Eclosed adults designated as live in *43/43*, *57/57* and *57/RX32* are viable for 2–3 days but are weak and could not be kept as homozygous stocks at 25°.

Generation of EMS-induced alleles in the *Dhr38* gene: A standard F_2 EMS lethal screen was performed on 5,000 mutagenized chromosomes to isolate mutations in the 38E region that do not complement the *l(2)02306* *P*-element insertion and potentially correspond to *Dhr38*. The induced EMS mutations were subjected to complementation analysis and mapped genetically with respect to deficiencies with breakpoints in 38E, as summarized in Figure 5. *In situ* hybridization to polytene chromosomes of the *Df(2)DS9* stock and PCR on genomic DNA prepared from homozygous *Df(2)Ketel*^{RX32} first instar larvae had shown that *Dhr38* sequences are removed in both deficiences (data not shown). We identified five complementation groups, each with multiple alleles, in the region of overlap of these two deficiencies. Two of these correspond to previously known genes, *Ketel* (Lindsley and Zimm 1992; Erdelyi *et al.* 1997) and *diaphanous* (Castrillon and Wasserman 1994), and two others, *l(2)38Ea* and *l(2)38Eb*, are new. Most importantly, the fifth group includes three EMS-induced alleles that do not complement *l(2)02306*. Two of them, 43 and 57, are weak alleles which show a delayed melanization similar to that of *l(2)02306* when crossed to *Df(2)Ketel*^{RX32}, but produce some viable escapers when homozygous (Table 1). The third allele, 56, behaves as an earlier, pupal lethal in combination with *Df(2)Ketel*^{RX32}. Two thirds of the hemizygous animals develop normally until the late pupal stage (85–90 hr after pupariation [AP]; Bainbridge and Bownes 1981), and pharate adults look externally normal when dissected from pupal cases (data not shown). However, when the pharate flies start moving shortly before eclosion (around 90 hr AP at 25°), the adult cuticle is ruptured

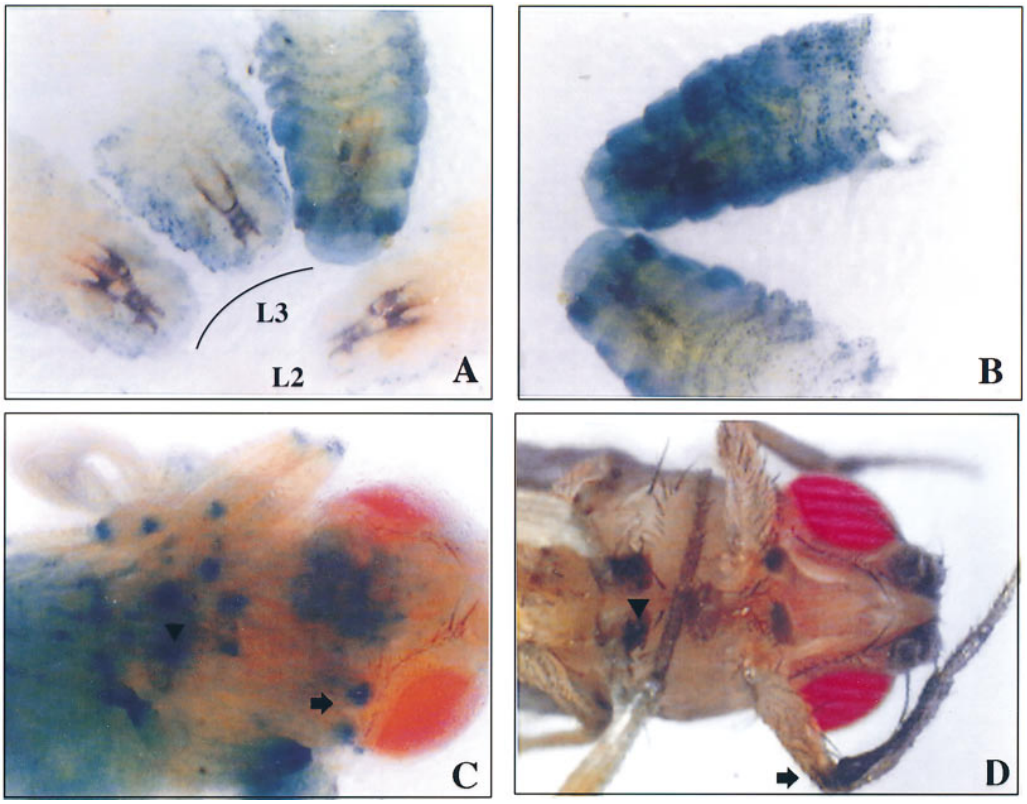

Figure 4.—Epidermal staining and mutant phenotype of the *l(2)02306 P* insertion line. (A) Histochemical X-gal staining is essentially absent in second instar larvae (L2) but is clearly detectable in the epidermis of mid-third instar larvae (marked L3). (B) The staining becomes very strong in wandering third instar animals. (C) Staining persists in imaginal epidermis, becoming localized to the regions of leg joints in the late pupae. (D) Mutant phenotype caused by the *P* insertion in the vicinity of the *Dhr38* gene. Note the heavy melanization in the leg joints. In (C) and (D), corresponding body regions of the fly are marked with an arrow (leg joint between femur and tibia) and arrowhead (the base of the coxae).

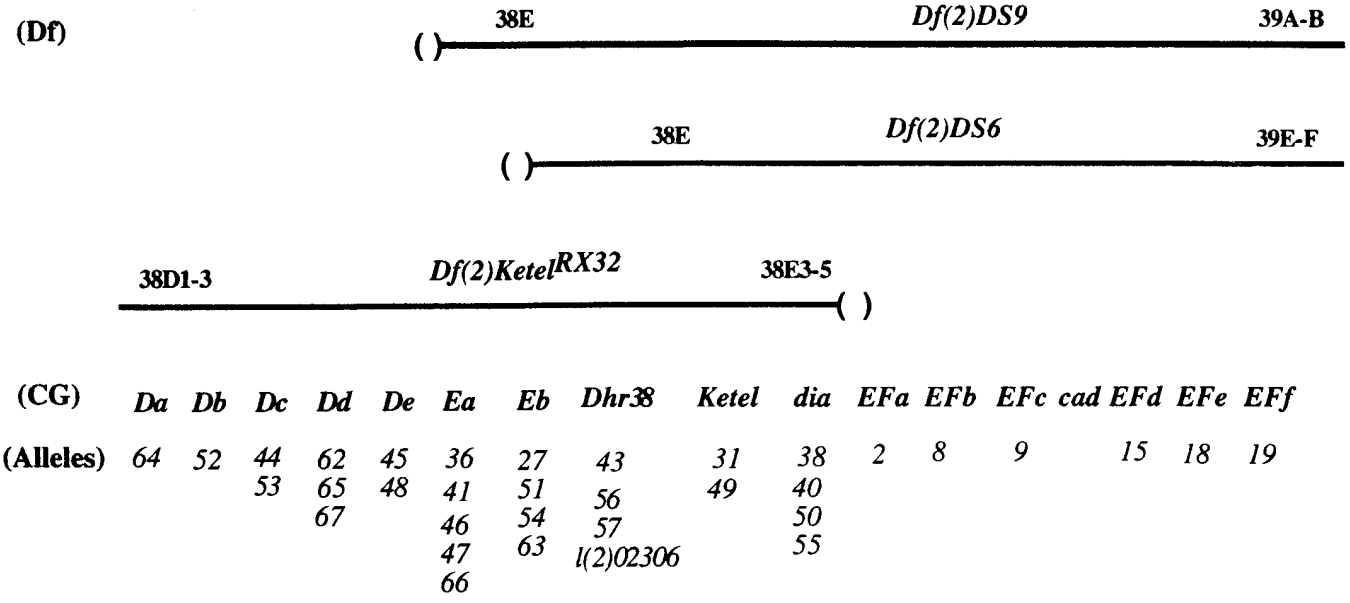

Figure 5.—Lethal mutations in the 38D-E region. The extent of the deficiencies in the region is shown by solid lines in the upper part of the figure (Df). The complementation groups (CG) and gene symbols (*Dhr38*, *Ketel*, *dia* and *cad*) are shown in bold italics. Complete names of the complementation groups are abbreviated [eg. *l(2)38Da* is represented by *Da*, and so on]. New EMS-induced alleles assigned to complementation groups are designated by plain numbers (Alleles). The *Da-De* cluster is defined by not being uncovered by *Df(2)DS9* and the *EFa-EFf* by not being uncovered by *Df(2)Ketel*^{RX32}; within each of these clusters the order of complementation groups is unknown because of the absence of relevant chromosomal rearrangements. Within the *Ea-dia* cluster, *Ea* is most distal because it is uncovered by *Df(2)DS9* but not *Df(2)DS6*; *Eb* and *Dhr38* are uncovered by all three deficiencies. *Ketel* and *dia*, placed in 38E cytologically (Lindsley and Zimm 1992; Castrillon and Wasserman 1994), were positioned proximally to *Dhr38* by sequence analysis (Berkeley Drosophila Genome Project, as reported in FLYBASE).

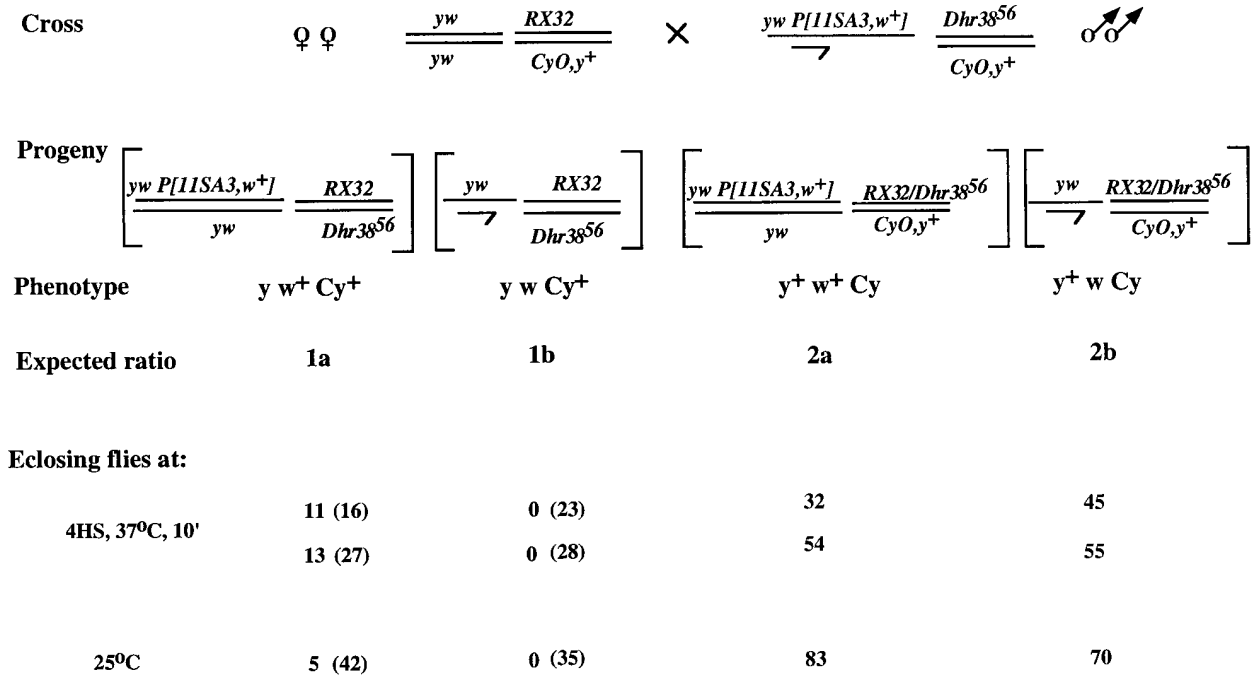

Figure 6.—Transgenic rescue of the *Dhr38*^{56} mutation with a *P[11SA3, w*$^{+}$*]* construct. Females of a balanced stock bearing a deficiency *Df(2)Ketel*^{RX32} (hereafter referred to as *RX32*) were crossed to males bearing the *P[11SA3, w*$^{+}$*]* transgene on the *X* chromosome and the EMS-induced *Dhr38*^{56} mutation on the second chromosome balanced against a *CyO,y*$^{+}$ chromosome. The resultant F_1 progeny genotypes (italics), respective phenotypes (Roman type) and expected ratio for flies of particular phenotype are shown; since all the stocks are in a *yw* background, the w^+ phenotype is caused by the presence of the transgene. The outcome of the rescue crosses with a mild heat shock treatment (four heat shocks of 10 min at 37°) at the pupal stage and at 25° is shown. The numbers represent observed eclosed adults, and those in parentheses represent the expected number of flies as calculated by comparison to the corresponding control class (a or b, respectively; note that the controls are expected at double the number of the experimentals).

followed by leakage of haemolymph and melanization within the pupal case. Effectively all pupariated hemizygous mutant flies exhibit this pupal lethal phenotype at 25° (Table 1). Homozygous 56/56 mutants die at larval stages, possibly because of a second-site lethal mutation present elsewhere on the chromosome or because *Dhr38*^{56} is a neomorphic mutation.

To prove that the *l(2)02306/EMS*^{43,56,57} complementation group corresponds to the *Dhr38* gene, we used transgenic copies of *Dhr38* to rescue the pupal lethality of the most extreme mutation, 56. We created transgenic fly stocks bearing the inserts of the cTK11 and cTK61 cDNA clones under the control of an *hsp70* heat shock promoter. Figure 6 diagrams the final rescue cross using the hsTK11 construct in the transgenic line, *P[11SA3, w*$^{+}$*]*, and reports the numbers of eclosing adult progeny in each phenotypic marker class. One of the progeny classes (male ywCy^{+}) is the *56/Df(2)Ketel*^{RX32} hemizygous mutant control, which shows absolutely no adult eclosion. In contrast, the hemizygous mutant class bearing the *P[11SA3, w*$^{+}$*]* insert (female yw^{+}Cy^{+} class) is represented by a substantial number of eclosed adults, documenting convincing rescue of the pupal lethality. Quantitative comparison with the number of control female heterozygotes (y^{+}w^{+}Cy class), in which the deficiency or *Dhr38*^{56} chromosome is balanced with the *Curly* chromosome, showed that the rescue is partial but robust: after a mild heat shock, 48–69% of the expected eclosed yw^{+}Cy^{+} adults were observed in two independent experiments. A weaker (12%) rescue at 25° was observed and can be explained by the known low-level leakage of the *hsp70* promoter at room temperature documented in similar experiments (Bayer *et al.* 1997; van de Wetering *et al.* 1997). The transgene-dependent, heat-shock-induced rescue is also partial, in that the adult yw^{+}Cy^{+} flies die within a few hours after eclosion, displaying the melanotic phenotype described earlier for *l(2)02306*. More vigorous heat shock treatments for obtaining fuller rescue were precluded by the deleterious effect of massive DHR38 overexpression observed with the hsTK11 inserts, even in wild-type backgrounds (T. Kozlova and F. C. Kafatos, unpublished results). For the same reason, we chose to test rescue of pupal lethality with the *56/Df(2)Ketel*^{RX32} hemizygotes, rather than larval lethality with *56/56* homozygotes; the latter would have required repeated heat shocks in both the larval and pupal stages, as well as being subject to complications from possible second site lethals on the mutagenized chromosome. Despite these limitations, the reality of the rescue is verified by similar results obtained

with a second independent hsTK11 insert, 11SC1 (between 40 and 60% rescue of pupal lethality in several independent experiments; data not shown). However, even the simultaneous presence of both hsTK11 and hsTK61 constructs in the same animal is not sufficient to produce viable rescued flies in similar experiments (data not shown).

DISCUSSION

***Dhr38* has a complex genomic organization and produces developmentally regulated isoforms:** In terms of its large size and complex structure, the *Dhr38* gene is a typical member of the steroid receptor superfamily in Drosophila (Segraves and Hognesss 1990; Koelle *et al.* 1992; Stone and Thummel 1993; Talbot *et al.* 1993; Russell *et al.* 1996). It spans at least 40 kb in length and includes four introns varying in size from 117 bp to more than 20 kb. At least four developmentally regulated mRNA species are detected, apparently generated by alternative promoters and polyadenylation sites. Since only three of the four putative mRNA isoforms have been characterized, each by a single cDNA clone, the transcriptional structure of the gene may be more complex than is currently known. The *Dhr38* mRNA isoforms corresponding to cTK11 and cTK61 differ in the untranslated regions, suggesting a possible complex post-transcriptional regulation. In particular, cTK61 has a 5′ UTR of 1.1 kb, while cTK11 has more than 3 kb of 3′ UTR containing multiple ATTTA motifs which presumably affect mRNA stability (Decker and Parker 1994). The isoforms also differ in the encoded putative transcription activation domains, but not the domains corresponding to DNA binding, dimerization and possible ligand binding. In particular, the cTK61 clone encodes a putative polypeptide containing 522 N-terminal amino acids in addition to those encoded in cTK11. This difference is unlikely to affect the DNA binding or dimerization properties described for DHR38 (Sutherland *et al.* 1995), but might well alter the transcriptional activation properties. Similarly, the polypeptide encoded by the pLF16 clone (Fisk and Thummel 1995) appears to differ only in the putative transcriptional activation domain; this clone is missing the first initiation codon of cTK11 and, if it is complete, presumably utilizes a second in-frame methionine as a translation start site, resulting in an N-terminal domain truncated by 34 amino acids relative to cTK11. Three *Dhr38* homologues exist in well-characterized vertebrate species such as mouse or human. Two of them, NGFI-B/Nur77/N10 and Nurr1, are relatively short (*ca.* 14 kb) and include six introns (Ryseck *et al.* 1989; Watson and Milbrandt 1989; Zetterström *et al.* 1997). The third member of the family, NOR-1, spans at least 35 kb and contains seven introns (Ohkura *et al.* 1996). There is no correlation in the sizes of introns between the fly gene and its vertebrate homologues, and the positions of the first three introns are not conserved. However, the last intron in the putative ligand binding domain of DHR38 does correspond in location to that of intron six of NGFI-B/Nur77/N10 (Ryseck *et al.* 1989; Watson and Milbrandt 1989) and intron seven of NOR-1 (Ohkura *et al.* 1996).

The *Dhr38* gene is expressed broadly during fly development but at a relatively low level, requiring for detection poly(A)$^+$ RNA selection (for RNA blot analysis) or more than 30 amplification cycles (for RT-PCR). The level of expression sharply increases in pre-pupal and pupal stages, suggesting participation in metamorphic events. Of the two best characterized mRNA species, the one corresponding to cTK61 is relatively more larval-specific while cTK11 shows the most dramatic enchancement in pupae and persists into the adult stage. Neither of these isoforms is significantly expressed in the ovary or early embryos. The *l(2)02306* enchancer trap insertion located very close to *Dhr38* exhibits epidermal-specific expression in late larval, pre-pupal and pupal stages. Undoubtedly this only corresponds to a sub-pattern of *Dhr38* expression; the regulatory regions of *Dhr38* must be quite complex judging from genomic structure analysis. We detected DHR38 protein by immunostaining in the epidermis of third instar larvae but also in additional tissues and stages (J. D. Sutherland, T. Kozlova and F. C. Kafatos, unpublished results).

Mutations in *Dhr38* result in localized fragility of the adult cuticle: The lethal phases of available EMS and *P*-element induced mutations indicate that *Dhr38* is important for late stages of metamorphosis; the haemolymph leakage and melanization phenotype suggest that all presently available alleles affect adult cuticle formation, possibly leading to incomplete sclerotization. In the three weaker alleles the defects appear to be specific to the thoracic cuticle of the leg joints, as abdominal and head structures are not visibly affected. Overall morphology of the mutant flies bearing the stronger EMS allele, including tanning of the bristles, is normal in $Dhr38^{56}/Df(2)Ketel^{RX32}$ hemizygotes at 80–90 hr after puparium formation. It is unlikely that these mutations represent complete loss-of-function alleles. The weak $Dhr38^{43}$ and $Dhr38^{57}$ alleles behave as hypomorphs in genetic assays, and both mRNA and DHR38 protein are still present in the $Dhr38^{56}/Df(2)Ketel^{RX32}$ hemizygous mutant animals (T. Kozlova and F. C. Kafatos, unpublished results). Therefore either a specific epidermal function of *Dhr38* is affected in these mutants, specific epidermal cells are most sensitive to altered levels of *Dhr38* expression, or *Dhr38* is dispensable in tissues other than epidermis.

We proved by the genetic rescue experiments that the mutations generated indeed correspond to *Dhr38*. Transgenic copies of *Dhr38* under the control of the *hsp70* promoter permit a robust, albeit not complete, rescue under various heat shock regimens. At least four reasons could explain the incomplete rescue. First, we

may not have generated sufficient DHR38 protein, although we know that DHR38 is produced in excess under these conditions as compared to endogenous protein (J. D. Sutherland, T. Kozlova and F. C. Kafatos, unpublished results). Second, we were not able to precisely adjust the timing of inductions to reproduce the complex dynamics of *Dhr38 in vivo* expression, which were crucial in similar experiments described for the *Broad-Complex* (Bayer *et al.* 1997). Third, it is formally possible that a *Dhr38* isoform which we have not tested is necessary to rescue the later adult lethality; Bayer *et al.* (1997) showed that the rescue of particular *Broad-Complex* alleles requires isoform-specific transgenes which, separately, are unable to rescue a complete loss-of-function allele. Fourth, a great excess of DHR38 protein results in dominant lethality in many developmental stages, making it practically impossible to reconstitute the *in vivo Dhr38* expression pattern with heat shock constructs.

What is the mechanism of DHR38 action in the cuticle? Since *Dhr38* mutations have a phenotype suggesting localized effects on the adult cuticle, they might interfere directly or indirectly with important structures or enzymatic components of the cuticle. The first adult specific cuticle protein, *Dacp-1* (Drosophila adult cuticle protein 1), has been recently characterized molecularly and shown to be expressed exclusively in the epidermis underlying the head and thoracic cuticle; it is believed to participate in a specialized function such as cuticle sclerotization (Qiu and Hardin 1995). DOPA decarboxylase (DDC) is a key enzyme in the biochemical pathway responsible for cuticle sclerotization and tanning (reviewed in Wright 1996). It is well documented that *Ddc* is regulated by ecdysone during development (Kraminsky *et al.* 1980; Clark *et al.* 1986), and the phenotypes of particular *Ddc* alleles retaining 5–10% of the enzyme activity closely resemble the phenotype of the *l(2)02306* mutation (Lindsley and Zimm 1992; Wright 1996). We analyzed the expression of both *Dacp-1* and *Ddc* in *Dhr38* mutants. Assays were performed on RNAs extracted from $Dhr38^{56}$ / $Df(2)Ketel^{RX32}$ hemizygous or *l(2)02306* homozygous pupae 80–90 hr after puparium formation, as compared to the heterozygous sibling controls bearing a balancer chromosome. However, no significant effect on the level of either *Dacp-1* or *Ddc* transcripts was detected in these preliminary experiments. This is not altogether surprising, considering that both the late pupal expression pattern of the insertion line and the apparent cuticle defects are highly localized.

Of course we cannot exclude that structural or enzymatic cuticle constituents other than those tested are affected, for example phenol oxidase or tyrosine hydroxylase, as well as some of the genes clustered in the *Ddc* region, which act in cuticle sclerotization and melanization (Wright 1996). Any one of the biochemically described 15–20 adult-specific cuticle proteins (Chinara *et al.* 1982), or larval and pupal cuticle proteins reused to synthesize adult cuticle may also be targets of *Dhr38*. It is interesting that regulatory regions of two of the pupal cuticle genes, *EDG-78* and *EDG-84*, contain a response element to which DHR38 can potentially bind albeit not with highest affinity (Apple and Fristrom 1991).

Deposition of larval and pupal cuticles is known to be under hormonal control in various insects including Drosophila (Hiruma *et al.* 1991; reviewed in Fristrom and Fristrom 1993) and expression of pupal cuticle genes is regulated by ecdysone in a complex way (Fechtel *et al.* 1988; Apple and Fristrom 1991). It is less clear if this is also true for adult cuticle deposition, although the highest ecdysone peak is achieved between 24 and 48 hr after pupariation (reviewed in Riddiford 1993). Bainbridge and Bownes (1988) described two ecdysone peaks during late pupal stages (20–55 hr and approximately 70 hr after pupariation). Thus, considering *Dhr38* mRNA enrichment in pupae and the observed mutant phenotypes, it is tempting to speculate that DHR38 might be one of the transcriptional regulators participating in relaying the ecdysone signal in epidermis and possibly in other tissues. Further tests of this hypothesis and of possible interaction with *usp* awaits future experimentation, including clonal analysis.

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