Drosophila DHR38 Nuclear Receptor Is Required for Adult Cuticle Integrity at Eclosion

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DHR38 is the only *Drosophila* member of the NR4A subclass of vertebrate nuclear receptors, which have been implicated in multiple biological pathways, including neuronal function, apoptosis, and metabolism. Although an earlier study identified three point mutations in *DHR38*, none of these were shown to be a null allele for the locus, leaving it unclear whether a complete loss of *DHR38* function might uncover novel roles for the receptor. Here we show that a specific *DHR38* null allele, $DHR38^{Y214}$, leads to fully penetrant pharate adult lethality, similar to the most severe phenotype associated with the EMS-induced mutations. $DHR38^{Y214}$ mutants display minor effects on ecdysone-regulated transcription at the onset of metamorphosis. In contrast, cuticle gene expression is significantly reduced in $DHR38^{Y214}$ mutant pupae. These studies define the essential functions of *DHR38* and provide a genetic context for further characterization of its roles during development. *Developmental Dynamics 238:701–707, 2009.* © 2009 Wiley-Liss, Inc.

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INTRODUCTION

Nuclear receptors (NRs) comprise a family of ligand-regulated transcription factors that play central roles in growth, metabolism, and development (Chawla et al., 2001). They are defined by a highly conserved DNA-binding domain that consists of two zinc-fingers, and a less conserved ligand binding domain (LBD) that can regulate receptor activity, often in response to small lipophilic compounds. We use the fruit fly, *Drosophila melanogaster*, as a model system to characterize the regulation and function of NRs during development. *Drosophila* offers several unique advantages in these studies (King-Jones and Thummel, 2005). First, the *Drosophila* genome encodes only 18 canonical NRs in contrast to the 48 receptors found in humans and 284 receptors in *Caenorhabditis elegans* (Maglich et al., 2001; Escriva Garcia et al., 2003). Despite their small number, the *Drosophila* NRs are distributed among all major subclasses of vertebrate NRs, often with a single fly receptor representing multiple vertebrate paralogs. Furthermore, in contrast to the complexity of vertebrate hormone signaling pathways, *Drosophila* has only one known physiologically active steroid hormone, 20-hydroxyecdysone (referred to here as ecdysone), which directs the major developmental transitions in the life cycle, including molting and metamorphosis. Ecdysone initiates transcriptional hierarchies through binding and activating an NR heterodimer composed of the Ecdysone Receptor (EcR) and its RXR partner, Ultraspiracle (USP; Riddiford et al., 2000). The late larval ecdysone pulse triggers puparium formation and the prepupal stage of development, terminating the juvenile growth phase and initiating adult maturation. A second ecdysone pulse approximately 10 hr after pupariation triggers adult head eversion and marks the prepupal-to-pupal transition. Detailed functional studies have shown that ecdysone exerts its effects through multiple pri-

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mary-response transcription factors. These include the zinc finger proteins encoded by the *Broad-Complex* (*BR-C*), the E74 ETS-domain proteins, and the E75A nuclear receptor (Burtis et al., 1990; Segraves and Hogness, 1990; DiBello et al., 1991). These transcription factors, in turn, regulate batteries of downstream secondary-response effector genes that direct the appropriate stage- and tissue-specific biological responses to the hormone (Thummel, 2001; Henrich, 2005).

Here we characterize the *Drosophila* DHR38 NR, which has three vertebrate orthologs, NGFI-B, Nurr1, and NOR1, all members of the NR4A class of NRs. Studies in cultured cells and mouse models have shown that these receptors are widely expressed and function in multiple biological pathways (Maxwell and Muscat, 2006). These include critical roles in the central nervous system (Zetterstrom et al., 1997; Saucedo-Cardenas et al., 1998; Ponnio and Conneely, 2004), inflammatory responses (Pei et al., 2006a), T-cell apoptosis (Hsu et al., 2004), metabolism (Pei et al., 2006b), vascular disease (Bonta et al., 2007), and cancer (Mullican et al., 2007). Although X-ray crystallographic studies have shown that the Nurr1 and DHR38 LBDs lack a classic hydrophobic ligand-binding pocket, they remain subject to posttranslational control by cofactor recruitment and/or posttranslational modifications (Baker et al., 2003; Wang et al., 2003). DHR38, NGFI-B, and Nurr1 can heterodimerize with RXR (USP in *Drosophila*) to exert their regulatory functions (Sutherland et al., 1995; Maxwell and Muscat, 2006).

Our understanding of NR4A function has been complicated by redundancy between the three vertebrate family members (Maxwell and Muscat, 2006). This complexity, however, is overcome in *C. elegans* or *Drosophila*, where only a single NR4A receptor is present. In *C. elegans*, *nhr-6* is expressed in chemosensory neurons and the developing somatic gonad, and plays a critical role in fertility through its effects on spermatheca and spermathecal valve development (Gissendanner et al., 2008). The molecular basis for these functions, however, remains unknown.

The *DHR38* gene in *Drosophila* is ~30 kb in length and consists of at least two mRNA isoforms, defined by the cTK11 and cTK61 cDNAs (Sutherland et al., 1995; Kozlova et al., 1998) (Fig. 1). *DHR38* appears to be expressed at a relatively constant low level throughout development, requiring the use of riboprobes on northern blots or quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) for mRNA detection (Fisk and Thummel, 1995; Kozlova et al., 1998). Three EMS-induced point mutations have been used to define *DHR38* functions during development (Kozlova et al., 1998). Homozygous mutants for each allele survive normally through early stages, but die late in development. $DHR38^{43}$ and $DHR38^{57}$ homozygotes and hemizygotes display rupturing of the cuticle at leg joints and consequent hemolymph leakage during the first 12 hr of adult life, followed by death a few hours later. A few homozygotes can survive as adults for several days, but are weak and cannot be maintained as a stock. A more severe phenotype is seen in $DHR38^{56}$ hemizygotes, which develop normally until the end of metamorphosis. These mutants then display cuticle rupturing and hemolymph leakage inside the pupal case as the adult fly begins the movements that would normally lead to eclosion. Both $DHR38^{43}$ and $DHR38^{57}$ behave as hypomorphic alleles, with less severe phenotypes seen in homozygous mutants than when the mutation is combined with a deficiency for the *DHR38* locus. In contrast, the nature of the $DHR38^{56}$ allele cannot be determined because homozygotes die during larval stages (Kozlova et al., 1998). Although this early lethality might be due to a second-site mutation on the chromosome, it also raises the possibility that a defined *DHR38* null mutation might uncover novel functions for this receptor before metamorphosis.

Here, we describe the isolation of a specific null mutation in *DHR38* and demonstrate that it has a lethal phenotype identical to that of $DHR38^{56}$ hemizygotes. Consistent with this, we map the EMS-induced lesions in each *DHR38* mutant allele and show that $DHR38^{56}$, but not $DHR38^{43}$ or $DHR38^{57}$, disrupts sequences common to both *DHR38* mRNA isoforms, accounting for the relative strength of its phenotype. The *DHR38* null mutation has only a minor effect on the expression of ecdysone-regulated genes at the onset of metamorphosis. In contrast, several cuticle gene transcripts are significantly reduced in *DHR38* mutant pupae, consistent with the lethal phenotype of these animals. These studies define an essential role for *DHR38* in cuticle gene expression during pupal stages and adult cuticular integrity.

RESULTS AND DISCUSSION

Molecular Mapping of EMS-Induced Mutations in *DHR38*

Each of the three EMS-induced point mutations in *DHR38* was molecularly mapped in an effort to define their effect on gene function. PCR was used to amplify the *DHR38* protein coding regions in genomic DNA isolated from homozygous or hemizygous mutants, and each fragment was subjected to DNA sequencing. These studies revealed premature stop codons upstream from sequences encoding the DNA-binding domain in all three alleles (Fig. 1). In $DHR38^{43}$, the codon encoding glutamine 309 of the cTK61 isoform (Kozlova et al., 1998) is converted into a stop codon (CAG>TAG). In $DHR38^{57}$, the codon encoding glutamine 318 of cTK61 is converted into a stop codon (CAA>TAA). Finally, in $DHR38^{56}$, the codon encoding tyrosine 607 in cTK61 is converted into a stop codon (TAT>TAA). It is interesting to note that, although all three mutations map to the same general location in *DHR38*, only the $DHR38^{56}$ allele disrupts coding sequences common to both *DHR38* transcripts (Fig. 1). The other two alleles affect only the longer *DHR38* mRNA isoform. In addition, it is possible that *DHR38* translation can reinitiate from either of two methionine codons that are located downstream from the $DHR38^{43}$ and $DHR38^{57}$ nonsense mutations (Fig. 1). These observations are consistent with the report that $DHR38^{56}$ mutants display a more severe phenotype than either $DHR38^{43}$ or $DHR38^{57}$ mutants (Kozlova et al., 1998).

Generation and Characterization of a *DHR38* Null Mutation

Although the $DHR38^{56}$ mutant has a more severe phenotype than the other two EMS-induced mutations, it remains unclear whether it represents a

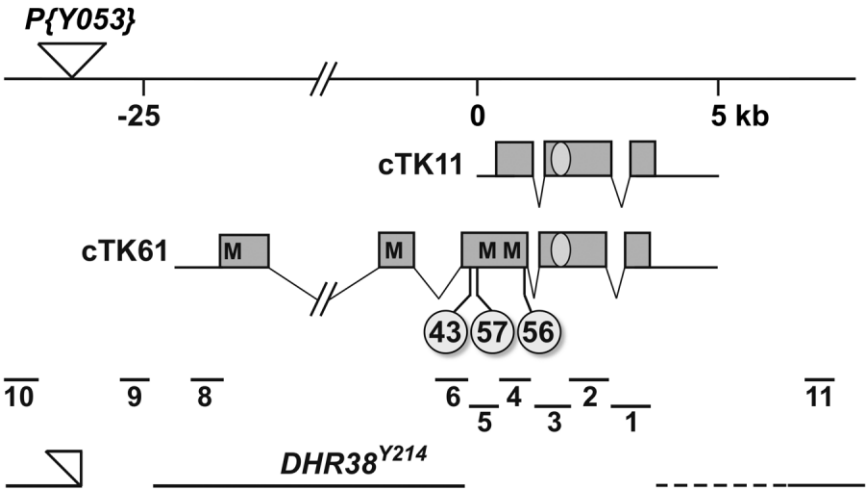

Fig. 1. Genomic organization of the *DHR38* locus and molecular nature of the mutations. The *DHR38* genomic region is adapted from Kozlova et al. (1998). Distances are given in kilobases (kb) relative to the 5′ end of the cTK11 cDNA clone (Sutherland et al., 1995). The site of the *P{Y053}* viable P-element insertion is marked with an inverted triangle. The maps of cTK11 and cTK61 (Kozlova et al., 1998) are depicted below, with protein coding regions represented by boxes, untranslated regions by solid lines, and introns by angled thin lines. The DNA-binding domain is denoted by an oval, and the positions of the in-frame methionine codons in cTK61 are marked (M) as well as the locations of the three EMS-induced mutations, $DHR38^{43}$, $DHR38^{56}$, and $DHR38^{57}$, labeled with circles. The locations of the 10 polymerase chain reaction (PCR) -amplified regions that were used to map the imprecise excision mutations are shown below the cDNA maps. At the bottom is depicted the molecular structure of $DHR38^{Y214}$, with the genomic sequences present in the mutant represented by solid lines, deletions by an absence of the line, and uncertainty of the mapping by a dotted line. Part of the original *P{Y053}* insertion, bearing the w^+ marker, is still present in this mutant (open triangle).

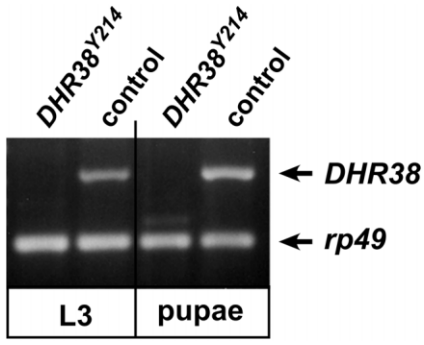

Fig. 2. No *DHR38* expression is detected in $DHR38^{Y214}$ mutants. Reverse transcriptase-polymerase chain reaction (RT-PCR) was used to detect *DHR38* transcripts in homozygous $DHR38^{Y214}$ mutants or *y, w* control animals, as wandering third instar larvae (L3) or 1- to 2-day-old pupae (pupae). RT-PCR to detect the *rp49* ribosomal protein transcript was used as a control for amplification and loading.

true null allele (Kozlova et al., 1998). To address this issue, we used P-element imprecise excision to generate deletion mutations in *DHR38*. A homozygous viable P-element insertion, *P{Y053}*, was selected for this purpose, which maps approximately 2 kb upstream from the 5′ end of the cTK61 cDNA (Fig. 1). This P-element was mobilized and potential deletion mutants were recovered by scoring for lethality in combination with $Df(2)Ketel^{RX32}$, a large deletion that removes the 38D1-38E6 interval encompassing the *DHR38* locus. Several resulting lethal mutations were characterized in more detail by PCR and DNA sequencing, using genomic DNA isolated from homozygous or hemizygous mutants. One mutation was identified, $DHR38^{Y214}$, that lacks all protein coding sequences contained within cTK11, including sequences encoding the entire DNA-binding and ligand binding domains of the receptor, while sequences 3′ of *DHR38* appear to be unaffected (Fig. 1). In addition, $DHR38^{Y214}$ carries a small deletion that removes part of the *P{Y053}* P-element and adjacent 3′ sequences that lie immediately upstream from the 5′ end of cTK61 (Fig. 1). Part of the original P-element, however, remains in place, including the w^+ marker gene along with genomic sequences that lie upstream from *DHR38*. This mutation thus appears to specifically affect the *DHR38* locus and is a good candidate for a complete loss of function allele. Consistent with this, the $DHR38^{Y214}$ allele fully complements lethal mutations that map on either side of *DHR38*, *l(2)38Ea*, *l(2)38Eb*, and *Ketel* (Kozlova et al., 1998). In addition, whereas the coding sequence for the C-terminal 113 amino acids of the DHR38 protein could be detected by RT-PCR using RNA isolated from control third instar larvae or early pupae, no RNA was detectable in $DHR38^{Y214}$ mutants (Fig. 2). This region is shared by all known *DHR38* mRNA isoforms (Fisk and Thummel, 1995; Sutherland et al., 1995; Kozlova et al., 1998), supporting the conclusion that $DHR38^{Y214}$ represents a null allele.

We next determined the lethal phenotype of the $DHR38^{Y214}$ mutation in combination with the $Df(2)Ketel^{RX32}$ deficiency (Fig. 3). Approximately the same number of $DHR38^{Y214}/Df(2)Ketel^{RX32}$ mutant embryos hatched into first instar larvae as either *y w*/$Df(2)Ketel^{RX32}$ or *P{Y053}*/$Df(2)Ketel^{RX32}$ controls, indicating that there is no embryonic lethality associated with this mutation (Fig. 3). Similarly, equivalent numbers of control or $DHR38^{Y214}/Df(2)Ketel^{RX32}$ mutants collected as first instar larvae developed normally through the end of pupal development (P15). All mutants, however, died as pharate adults, within the pupal case before eclosion. $DHR38^{Y214}/Df(2)Ketel^{RX32}$ mutant pupae have differentiated adult structures and appear normal except for a cuticle melanization phenotype and hemolymph leakage, indistinguishable from that seen in $DHR38^{56}$ mutants (Kozlova et al., 1998). Penetrant pharate adult lethality was also observed for $DHR38^{Y214}$ homozygotes and for $DHR38^{56}/DHR38^{Y214}$ mutants (data not shown). These observations indicate that $DHR38^{Y214}$ represents a specific null allele for the locus.

Analysis of Ecdysone-Regulated Transcription in *DHR38* Mutants

To date, only one gene has been shown to be dependent on *DHR38* for its proper expression, *Acp65A* (Kozlova et al., 1998; Bruey-Sedano et al., 2005; Davis et al., 2007). This is, most likely, because hypomorphic alleles were used for those studies. In an effort to identify more DHR38 target genes, we examined the transcriptional cascade

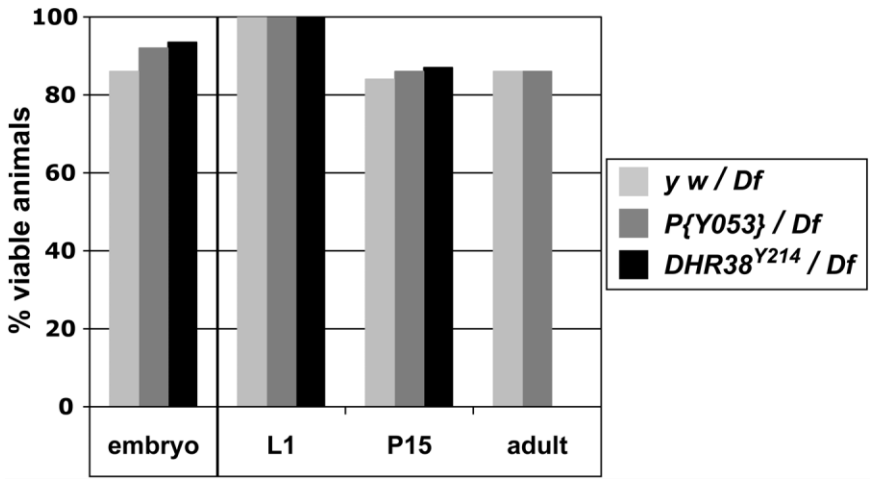

Fig. 3. *DHR38*^{Y214} mutants die as pharate adults. Males of three genotypes, *y w*, *y w*; *P{Y053}/CyO*, or *y w*; *DHR38*^{Y214}*/CyO* were crossed with females carrying a deletion for the 38E interval, *y w*; *Df(2)Ketel*^{RX32}*/CyO* (*Df*), and viability was scored for each genotype at the indicated developmental stages. Embryonic viability was assayed by using a green fluorescent protein (GFP) -marked *CyO* balancer chromosome and determining the percentage of *GFP*$^{-}$ embryos that hatched into first instar larvae. Later viability was scored by using a *CyO* y^{+} balancer chromosome in a *y w* genetic background and scoring for the percent of y^{-} first instar larvae (L1) that survived to either a late pupal stage (P15) or adulthood (adult).

triggered by the steroid hormone ecdysone at the onset of metamorphosis. DHR38 has been proposed to impact ecdysone signaling through its interaction with USP (Sutherland et al., 1995). In addition, some mutations that result in pharate adult lethality are known to have a role in the ecdysone transcriptional cascade at puparium formation (e.g., *E74A*, Fletcher and Thummel, 1995). We thus decided to examine the effect of the *DHR38* null allele on genes regulated by the late larval and prepupal pulses of ecdysone. RNA was isolated from both control and *DHR38*^{Y214}*/Df(2)Ketel*^{RX32} mutants staged as either mid-third instar larvae (−18 hr), late third instar larvae (−4 hr), newly formed prepupae (0 hr), or at 2-hr intervals thereafter until 12 hr after puparium formation. These RNA samples were analyzed by northern blot hybridization to detect the expression of genes that are induced directly by the hormone/receptor complex, *BR-C*, *E74*, *E75A*, *DHR4*, *FTZ-F1*, and *Fbp1*, as well as ecdysone-inducible secondary-response genes *Sgs-4, Ddc*, *IMP-L1*, *L71-1* (Fig. 4). No significant effect was seen on *E74*, *E75A*, *DHR4*, or *FTZ-F1* transcription. The apparent increase in *E74A* and *E75A* mRNA in 10 hr mutant prepupae is likely due to a sampling artifact caused by a few animals that had experienced the brief high titer prepupal ecdysone pulse (Richards, 1981). Similarly, there is little or no effect on *Sgs-4*, *IMP-L1*, *L71-1*, and *Fbp1* expression in the *DHR38* mutant. The increased level of *Sgs-4* mRNA in -4 hr mutant larvae is likely due to the inaccuracy of staging third instar larvae by gut clearance (Andres and Thummel, 1994). *L71-1* mRNA displays a 2–4 hr temporal shift to earlier times in the *DHR38* mutant, while *Fbp1* displays a more narrowed peak of expression in mid-prepupae. These results are consistent with our observation that developmental events associated with puparium formation and the prepupal-to-pupal transition, such as the destruction of the larval midgut and salivary glands, occur normally in *DHR38* mutants (data not shown).

In contrast, the *BR-C* and *Ddc* appear to display significant changes in expression level in the *DHR38* mutant. All *BR-C* mRNA isoforms are reduced in the mutant (Fig. 4). This is interesting because ectopic expression of *BR-C* can lead to misregulation of pupal cuticle genes, although no effect of *BR-C* has been reported on cuticle function in adult flies (Zhou and Riddiford, 2002). In addition, levels of *Ddc* mRNA appear to be elevated in *DHR38* mutants. Whereas the -4 hr time point is difficult to interpret, as mentioned above, puparium formation (0 hr) provides an accurate time to synchronize the developmental stage of both control and mutant animals. This apparent effect on *Ddc* expression is of interest because this gene encodes dopa decarboxylase, and mammalian NR4A family members play a critical role in dopaminergic neuronal development (Zetterstrom et al., 1997; Saucedo-Cardenas et al., 1998). In addition, the increased expression of *Ddc* mRNA in *DHR38* mutants is consistent with a recent study by Davis et al. (2007), which showed that ectopic *DHR38* expression can down-regulate *Ddc* promoter activity. This may not, however, be a specific effect because we have found that ectopic *DHR38* expression at puparium formation can efficiently repress several ecdysone-inducible genes, including *E74A*, *E75A*, and *DHR4* (data not shown). Down-regulation of these genes might be an indirect effect caused by ectopic DHR38 protein removing USP from functional EcR/USP receptor complexes (Sutherland et al., 1995). Further work is required to determine the significance of these effects of the *DHR38* mutation on *BR-C* and *Ddc* expression.

Reduced Levels of Cuticle Gene Expression in *DHR38* Mutant Pupae

As part of a microarray study that will be reported elsewhere, three cuticle genes were identified among the most significantly down-regulated genes in *DHR38* mutant pupae: *Cpr92A* (*CG6240*), *Cpr49Ae* (*CG8505*), and *Acp65A* (*CG10297*) (A.-F. Ruaud, T.K. and G.L., unpublished data). Northern blot analysis was used to validate these results. RNA was isolated from control and *DHR38*^{Y214}*/Df(2)Ketel*^{RX32} mutant pupae staged at 10-hr intervals during the final half of metamorphosis, and equal amounts of RNA were analyzed by northern blot hybridization to detect *Cpr92A* and *Acp65A* transcription (Fig. 5). We also examined the expression of *Acp1* (*CG7216*), which was reduced in some, but not all of the *DHR38* mutant datasets. This study revealed that these three cuticle genes are transiently expressed in late stage control pupae, preceding the deposition of adult cuticle. The expression of all three genes is also significantly reduced in the *DHR38* mu-

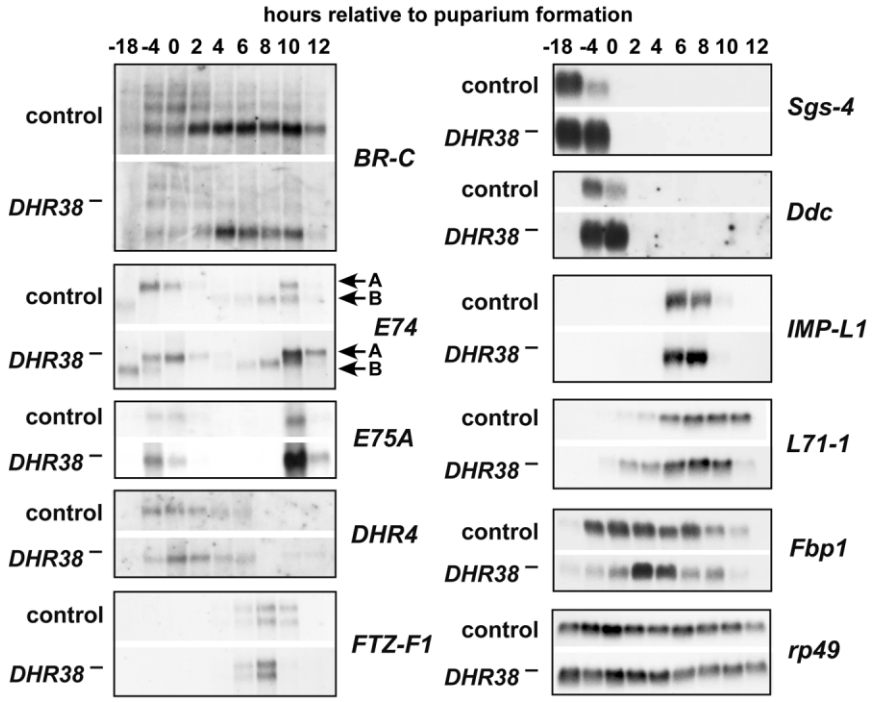

Fig. 4. The *DHR38* mutation has minor effects on ecdysone-regulated gene expression at the onset of metamorphosis. RNA was isolated from *DHR38*^{Y214}/*CyO* and *Df(2)Ketel*^{RX32}/*CyO* heterozygotes (control) or *DHR38*^{Y214}/*Df(2)Ketel*^{RX32} mutants (*DHR38*$^{-}$) and analyzed by northern blot hybridization. RNA was extracted from mid-third instar or late third instar larvae (-18 or -4 hr relative to puparium formation), newly-formed prepupae (0 hr), or at 2-hr intervals after puparium formation until 12 hr. Northern blots were probed to detect *BR-C*, *E74A*, *E74B*, *E75A*, *DHR4*, *FTZ-F1*, *Sgs-4*, *Ddc*, *IMP-L1*, *L71-1*, and *Fbp1*. Hybridization to detect *rp49* mRNA was performed as a control for loading and transfer.

tant (Fig. 5). The earliest effect is seen at 60 hr after puparium formation, more than a day before the mutants die as pharate adults with ruptured cuticle and hemolymph leakage. In contrast, another gene expressed in late pupae, the adult-specific isoform of *Adh*, is only slightly affected in the mutant.

These results are consistent with earlier work by Bruey-Sedano et al. (2005), who showed that *Acp65A* expression is reduced in the hypomorphic *DHR38*^{02306} mutant (Kozlova et al., 1998). *Acp65A* is expressed in a specific pattern in the adult epidermis, underlying regions of flexible cuticle at the joints, suggesting that it is a critical target for *DHR38* function (Bruey-Sedano et al., 2005). Like *Acp65A*, *Cpr49Ae* also encodes a protein with an RR-1 motif, associated with flexible cuticle (Karouzou et al., 2007). In contrast, *Cpr92A* encodes a protein with the RR-2 motif, associated with more rigid cuticle (Karouzou et al., 2007), and *Acp1* appears to encode rigid cuticle that provides strength to the exoskeleton of the animal (Qiu and Hardin, 1995). *DHR38* thus appears to regulate genes that direct the deposition of both flexible and rigid components of the exoskeleton. An attempt to rescue the lethality of *DHR38*^{Y214} mutants by epidermal expression of wild type *DHR38* using the GAL4/UAS system was unsuccessful, due to the lethality associated with *DHR38* overexpression (Kozlova et al., 1998) (A.-F. Ruaud, unpublished results). Future studies that attempt to control *DHR38* expression more precisely may allow us to test the hypothesis that the essential functions for this gene reside in its expression in the epidermis of late pupae.

In this study, we demonstrate that a complete loss of *DHR38* function results in pharate adult lethality, apparently as a result of reduced cuticle gene expression in late pupae and consequent loss of cuticular integrity, with death by exsanguination. This observation eliminates the possibility that a *DHR38* null mutation might uncover an earlier essential function for the receptor. In addition, it provides an ideal genetic context for further characterization of *DHR38* activity. Given that this receptor is the only member of the NR4A subclass in *Drosophila*, further studies of *DHR38* mutants should provide insights into the ancestral roles of these receptors during development, with implications for better understanding the regulatory functions of NR4A family members in all higher organisms.

EXPERIMENTAL PROCEDURES

Generation and Characterization of *DHR38* Mutants

A homozygous viable P-element insertion was molecularly mapped ~2 kb upstream from the 5′ end of the cTK61 cDNA clone (Genbank AJ002073; Fig. 1). This P-element was mobilized using standard genetic protocols and imprecise excision mutations were recovered by scoring for lethality in combination with the *Df(2)Ketel*^{RX32} deletion. Lethal excisions were molecularly mapped by extracting genomic DNA from 10–20 homozygous or hemizygous mutant third instar larvae (in combination with *Df(2)Ketel*^{RX32}) and scoring for the presence or absence of eleven short regions spanning the *DHR38* locus by PCR and DNA sequencing (Fig. 1).

DHR38 transcripts were detected by RT-PCR analysis as described (Kozlova et al., 1998) using primers that are specific to a 407-bp region present in all known *DHR38* mRNA isoforms (5′-ACATCATGGAGTTCAGCCGCA-3′ and 5′-GGAGGCATAACTTAGGGGCTA-3′). PCR was performed in 30-sec steps at 94°, 61°, and 72°C for 10 cycles without *rp49* primers, and 22 cycles after *rp49* primers were added.

Embryonic lethality was assayed by crossing stocks that carried the mutations over a *CyO {actin-GFP}* balancer chromosome and selecting for embryos that did not express green fluorescent protein (GFP). A *CyO*, *y*+ balancer chromosome in a *y w* genetic background was used to assay for later lethal phases. Approximately 200–400 eggs were collected on grape juice-agar plates and allowed to develop for 24 hr at 25°C. First instar *y*$^{-}$

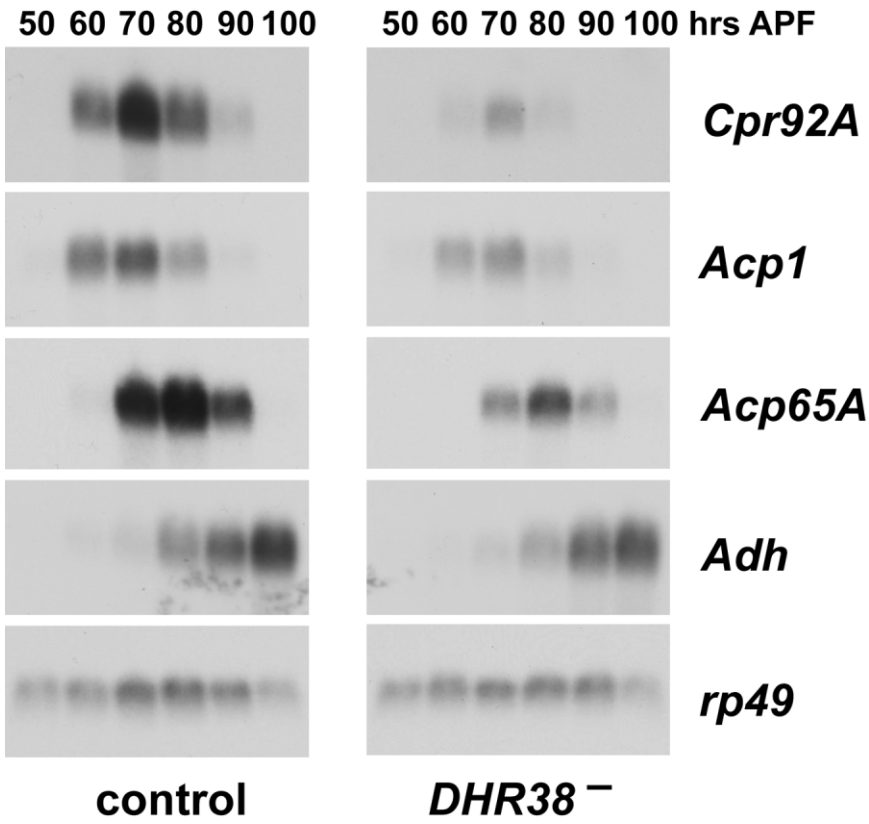

Fig. 5. Cuticle gene expression is reduced in *DHR38* mutant pupae. RNA was isolated from *DHR38*^{Y214}/*CyO* and *Df(2)Ketel*^{RX32}/*CyO* heterozygotes (control) or *DHR38*^{Y214}/*Df(2)Ketel*^{RX32} mutants (*DHR38*$^{-}$) staged at either 50, 60, 70, 80, 90, or 100 hr after puparium formation (APF). Northern blot hybridization was used to detect transcripts from the *Cpr92A*, *Acp1*, and *Acp65A* cuticle genes, as well as the adult-specific *Adh* mRNA. Hybridization to detect *rp49* mRNA was performed as a control for loading and transfer.

larvae were selected, transferred to vials with yeast paste, and allowed to develop. The sample size for each genotype was at least 100 animals.

Northern Blot Analysis

Total RNA was isolated from third instar larvae grown on food containing 0.05% bromophenol blue, selecting for either blue gut (−18 hr relative to puparium formation) or clear gut (−4 hr) animals (Andres and Thummel, 1994). Prepupae were staged by collecting newly-formed white prepupae (0 hr) and harvesting animals at 2-hr intervals. Late pupae were staged by collecting animals that pupariated within a 1-hr interval and allowed to develop to the desired stage at 25°C. RNA was extracted, fractionated by formaldehyde gel electrophoresis, transferred to nylon membranes, and hybridized with radioactively labeled probes, as described (Andres et al., 1993).

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