Nucleic Acids Research, 1993, Vol. 21, No. 7 1619–1627

Activation and repression of *Drosophila* alcohol dehydrogenase distal transcription by two steroid hormone receptor superfamily members binding to a common response element

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Received December 10, 1992; Revised and Accepted March 1, 1993 GenBank accession nos L07549–L07551 (incl.)

ABSTRACT

Developmental activation of the *Drosophila* alcohol dehydrogenase (*Adh*) distal promoter is controlled by the *Adh* adult enhancer (AAE). Within this 150 bp, complex enhancer is a small (12 bp) positive cis-acting element that is required for high levels of distal transcription in adult flies and ADH-expressing tissue culture cells. We previously reported that the steroid receptor superfamily member FTZ-F1 binds to this site. We have identified a second steroid receptor superfamily member, DHR39, which also binds to this site. *DHR39* is expressed throughout development in transcripts of several sizes. In situ hybridization to embryos has shown that DHR39 RNA is found primarily in the central nervous system, and not in embryonic tissues that express ADH. FTZ-F1 RNA, however, shows temporal-specific patterns similar to those of the distal promoter. FTZ-F1 and DHR39 have identical amino acids in the 'P-box' of the DNA binding domain, suggesting that they have identical DNA recognition characteristics. By electrophoretic mobility shift analysis we show that a DHR39 fusion protein binds specifically to two FTZ-F1 binding sites. By over expressing the full length DHR39 protein in a transient co-transfection assay we have shown that it represses distal *Adh* expression in a dosage- and binding site-dependent manner. Over expression of an alternative DHR39 open reading frame that lacks part of the putative ligand binding domain does not alter *Adh* expression. In contrast, over expression of FTZ-F1 specifically activates distal *Adh* expression.

INTRODUCTION

The steroid hormone receptor superfamily includes a large number of ligand activated transcription factors as well as a number of orphan receptors for which no ligand is known (reviewed in ref 1). These nuclear receptors share several structural features as well as mechanisms of controlling gene activity. The most highly conserved region shared by this superfamily is the DNA binding domain (DBD). The second evolutionarily conserved region is referred to as the ligand binding domain (LBD). This domain appears to serve additional functions such as dimerization and transactivation or repression. Generally the members of this superfamily bind DNA as homo- or heterodimers. The response elements usually consist of small inverted or direct repeats. Subfamily members within the nuclear receptor superfamily can bind to the same response elements and bring about a differential response. These interactions make it possible for a small number of proteins to regulate diverse biological activities.

A number of hormone receptor superfamily members are known to play important roles in *Drosophila* development (2, 3, 4, 5, reviewed in ref 6), however little is known about their mechanism of action. Studies on the receptor for the molting hormone ecdysone (EcR) have begun to reveal complex interaction among *Drosophila* receptors. Ecdysone response elements consist of palindromic sequences similar to mammalian steroid hormone response elements (7, 8). Recently it has been shown that the *Drosophila* RXRα homolog ultraspiracle will form a heterodimer with EcR, and is required for ecdysone responsiveness in heterologous cells (9). It has also been suggested that various *Drosophila* receptors with similar DNA binding domains might bind to common target sites and generate different responses during development (6), but this has not been shown previously.

The *Drosophila* alcohol dehydrogenase distal (adult) promoter is transcribed briefly in mid to late third instar larvae and at a high level in adults (10). This temporal pattern of expression is controlled by a 150 bp developmentally activated enhancer, the *Adh* adult enhancer (AAE, 11, 12). To better understand the molecular basis for this developmental control of transcription we have identified both positive and negative cis-acting elements

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within the AAE (13), and characterized two proteins that bind to a positive regulatory element within the AAE (14). This palindromic sequence with similarity to steroid hormone response elements is required for normally high levels of *Adh* distal transcription in adult flies (14), and mid to late third instar larvae (15). Transformed flies lacking this element fail to exhibit the rapid burst of *Adh* distal transcription normally seen immediately after adult emergence from the pupal case (10, 15). One of the proteins which binds to this positive cis-acting element has been identified (14) as the steroid hormone receptor superfamily member FTZ-F1 (16). The binding site of the second protein shares nucleotide contacts with FTZ-F1 (14).

Here we report the cloning of a family of cDNAs encoding a second steroid receptor protein that also binds specifically to this positive cis-acting element within the AAE. This new receptor gene is located at cytological position 39B-C on the left arm of the second chromosome and we refer to it here as *Drosophila* Hormone Receptor 39 (*DHR39*). The developmental expression patterns of *FTZ-F1* coincide with the temporal activation of the AAE, whereas *DHR39* is expressed throughout development. Transient co-transfection studies in the *Drosophila* cell line, 1006-2, in which the AAE is active (13) confirmed that FTZ-F1 protein activates and DHR39 represses distal *Adh* expression. FTZ-F1 and DHR39 bind to the same site in the AAE, suggesting they may compete for binding in cells where both are present, such as the tissue culture cells used in this study. Competition for binding by an activator and a repressor to the same DNA binding site might play a role in modulating the level of *Adh* expression.

MATERIALS AND METHODS

Cloning and Sequencing

To isolate cDNA clones on the basis of their ability to recognize the AAE positive cis-acting sequences, an ovarian cDNA expression library in λgt11(gift of Laura Kalfayan) was probed with a ^{32}P-end-labeled, concatenated, complementary oligonucleotides

5′gatcCTATGCTTGACATTCACAAGGTCA3′
3′ GATACGAACTGTAAGTGTTCCAGTctag5′

corresponding to the binding sites of DEP1 and DEP2 (14). 10^6 plaques were screened by the method of Vinson (17) and recombinant proteins of three plaques bound the probe specifically when compared with the mutant oligonucleotide (Fig. 1C). Phage inserts were subcloned into pBluescript SK$^+$ (Stratagene) and sequenced using the USB Sequenase kit. Two clones were identical and encode the *FTZ-F1* gene (16), these clones have been described previously (14). The third clone (encoding DHR39) was sequenced and found to contain a region homologous to the zinc finger region of steroid receptor proteins. To isolate full length *DHR39* cDNA clones, 0−4 hr and 4−8 hr embryonic cDNA libraries (18) were screened. 2×10^6 colonies from each library were screened, 11 positives clones were purified from the 0−4 hr library and 35 positives were purified from the 4−8 hr library.

The NW1 cDNA was obtained following a screen of a partial *Eco*RI *Drosophila* genomic library in λ Charon 4A. The probes used were ^{32}P end labeled 42 and 44-mer oligonucleotides, derived from a consensus sequence for the DNA binding domains of the steroid/thyroid receptor superfamily members and modified according to *Drosophila* codon usage (19). The oligomers represent the antisense strand, containing part of the first finger domain and the region between the two fingers. Their sequence is: 5′accctcsacggcgcgcttgaagaasaccttcagswxccgcaggt3′ and 5′ctggatgswgcggcggaagaaxcccttrcaxcccgcaggt3′ (s=c or g; r=a or g; w=a or t; x=either a, c, g or t). Hybridization was performed under low stringency conditions: 3× SSC, 4× Denhardt's solution, 0.2 mg/ml salmon sperm DNA, 0.1% SDS, incubation was overnight at 50°C. Hybridized filters were washed in 2× SSC, 0.1% SDS at 45°C. To isolate cDNA clones an embryonic 3−12 hr cDNA library in λgt10 phage (20) was screened with a 0.9 kb genomic *Pst*I fragment containing the coding region for the two zinc fingers. Sequencing was carried out according to the standard dideoxy chain termination procedure, using the USB Sequenase kit.

Northern blotting

Two methods were used for RNA preparation. In the first method poly(A$^+$) RNA was prepared from appropriate developmental stages as described (21). Briefly, dechorionated embryos, washed larvae, pupae and adult flies were first frozen in liquid nitrogen and ground with mortar and pestle in cold 6 M guanidine hydrochloride, 0.2 M sodium acetate buffer, pH 5.2. The supernatant fraction of the homogenized suspension was precipitated after overnight incubation with 0.5 vol. ethanol at −20°C. The pellet was solubilized in buffered solution (6 M urea, 1 mM EDTA, 0.1% SDS, 10 mM Tris HCl, pH 7.4) and underwent several rounds of phenol/chloroform extraction. RNA was precipitated with ethanol. Poly(A$^+$) RNA was separated from total RNA by oligo-dT chromatography.

Poly(A$^+$) RNA (15 μg/lane) was separated on an agarose formaldehyde gel and blotted to a nylon membrane (Hybond-N, Amersham). The membrane was hybridized in a mixture containing 50% formamide, 5× SSCPE, 2× Denhardt's, 0.5 mg/ml salmon sperm DNA, at 58°C. The probe used was a ^{32}P labeled, random primed fragment, containing the 2.5 kb region from the 3′ end of the NW1 cDNA clone. The membrane was washed in 0.1× SSC, 0.1% SDS at 68°C.

In the second protocol total RNA was isolated by homogenizing whole animals from appropriate developmental stages in 7 M urea, 2% SDS, 0.35 M NaCl, 0.01 M EDTA, 0.1 M Tris HCl, pH 7.5, and extracted several times in phenol:chloroform:isoamyl alcohol (25:24:1), followed by 2 chloroform:isoamyl (24:1) extractions and ethanol precipitated. 25 μg of total RNA was dissolved in 10 μl 50% formamide, 6.5% formaldehyde, 20 mM MOPS, pH 7.0, 8.8 mM ammonium acetate, 1 mM EDTA and electrophoresed on a 1% agarose, 6.5% formaldehyde gel in 20 mM MOPS, pH 7.0, 8.8 mM ammonium acetate, 1 mM EDTA.

The gels were transferred to nylon (Nytran, S&S), and hybridized in 50% formamide, 5× SSPE, 2× Denhardt's, 1% SDS, 0.1 mg/ml denatured salmon sperm DNA, with nick translated C15 (see Fig. 2) or *FTZ-F1*-early cDNA at 42°C. The filter was washed in 0.1× SSC, 1% SDS at 65°C and exposed to Kodak XAR X-ray film with a Du Pont Cronex Lightning-plus intensifying screen at −70°C. The filter was stripped in 25% deionized formamide, 1% SDS, 0.1× SSPE at 70°C and reprobed as above with nick translated *rp49* plasmid, pHR0.6 (19).

Whole mount RNA hybridization

Embryos from different developmental stages were collected, fixed and hybridized to random primed cDNA fragments labeled with digoxygenin-dUTP as probes. Probes generated from either

the 5′ or the 3′ end of the NW1 cDNA gave similar results. The protocol was based on the Tautz and Pfeifle procedure (22), using the Boehringer Manneheim kit. Hybridizations were carried out at 48°C.

Construction of expression plasmids

The metal inducible expression plasmids were constructed by inserting the open reading frame downstream of the *Drosophila* metallothionein gene promoter in the vector pRmHa3 (23). The *DHR39* long open reading frame plasmid, pMT-DHR39 was constructed by ligating a 2918 bp *Dra*I fragment from the C10 cDNA (see Fig. 2) to *Sma*I digested pRmHa3. The *DHR39*-short open reading frame plasmid pMT-DHR39-short was constructed from a 2344 bp *Dra*I fragment from the C12 cDNA. The *FTZ-F1*-late isoform expression plasmid pMT-FTZF1-late was constructed with a 2.5 kb EcoRI fragment from a *FTZ-F1*-late cDNA. This cDNA was purified from a 12−24 hr embryonic cDNA library (18) using a partial *FTZ-F1*-early cDNA (14) as a probe.

A *malE-DHR39* gene fusion was constructed by inserting an EcoRI fragment from the *DHR39* C10 cDNA, containing the DNA binding domain and ligand binding/dimerization domain, into the EcoRI site of pMAL-c2 (New England Biolabs). This created an in-frame fusion of amino acids 293−808 downstream of the *malE* gene. The *E.coli* strain DH5α containing the *malE-DHR39* fusion plasmid was grown at 30°C, induced with IPTG and the fusion protein was purified on amylose resin (New England Biolabs) according to the manufacturer's recommendations.

Culture and transfection of cell lines

1006-2 cells (24) were grown in M3 media (Sigma) supplemented with 10% fetal calf serum (Intergen) and 50 μg/ml gentamicin (GIBCO) at 23°C. Transient transfections were performed by calcium phosphate as previously described (13), all transfections contained 10 μg total DNA per 25 cm^2 flask containing $\sim 8\times10^6$ cells. pBluescript-KS$^+$ was used to bring total DNA to 10 μg. Induction of the expression vectors containing the metallothionein promoter was performed by adding cupric sulfate, to a final concentration of 0.7 mM, 24 hr after transfection. After an additional 24 hr, cells were harvested and ADH and β-Galactosidase assays were performed as described previously (13).

Electrophoretic mobility shift analysis of malE-DHR39 fusion protein

Electrophoretic mobility shift analysis was performed as described (14). 1 μg of purified *malE-DHR39* fusion protein was incubated with 0.5 ng of ^{32}P-labeled AAE (−662 to −446 from the *Adh* distal promoter) and appropriate competitor DNA. The binding reaction was electrophoresed on a 5% acrylamide gel in 0.5× TBE at room temperature, the gel was dried and exposed to X-ray film. The oligonucleotides used as competitors have been described (14).

RESULTS

Cloning of *DHR39* cDNAs

Previous analysis of the *Adh* adult enhancer (AAE, Fig. 1A) by transient transfection in cultured cell lines and by germline transformation has identified a 12 bp positive cis-acting element (14). Transfection of the ADH-expressing cell line 1006-2 (24),

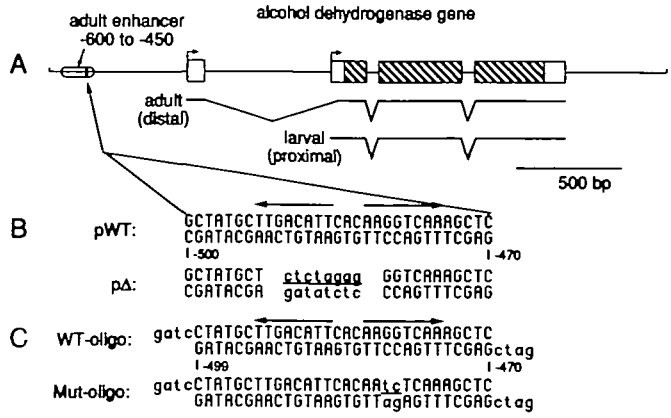

Figure 1. A map of the *Adh* gene. **A.** A map of the *Adh* gene showing the *Adh* adult enhancer at −600 to −450 relative to the distal start site (+1), the adult (distal) and larval (proximal) promoters as well maps of the two transcripts. The open rectangles represent the 5′ and 3′ untranslated region, and the hatched rectangles represent the protein coding region. **B.** The sequence of wild type (pWT) and mutant (pΔ) cis-acting element within the *Adh* adult enhancer. **C.** The wild type oligo (WT-oligo) and mutated oligo (Mut-oligo) that were used to characterize and purify clones. The imperfect palindrome with similarity to steroid/thyroid/retinoic acid response elements are indicated by arrows.

in which the AAE is active (13, 14), with a plasmid containing the intact *Adh* gene and a wild type AAE (pWT, Fig. 1B) showed high levels of *Adh* distal transcription (14, also see Fig. 7). Transfection of these cells with a similar plasmid containing a 12 bp linker replacement deletion within the AAE (pΔ, Fig. 1B) exhibited reduced levels of *Adh* distal transcription (14). Similarly, transgenic fly lines transformed with the pWT construct showed a normally high level of *Adh* distal transcription in adults, whereas flies that received the pΔ construct showed only a low level of distal transcription in adults (14).

To identify proteins that bind to the positive cis-acting element we screened a λgt11 cDNA expression library with a concatenated, labeled oligonucleotides corresponding to the positive element (WT-oligo; Fig. 1C). We identified two different cDNA clones encoding proteins that bound specifically to the wild type oligo probe (WT-oligo; Fig. 1C) but not to the mutated oligo (Mut-oligo; Fig. 1C). These clones were sequenced and one was identified as encoding FTZ-F1 (14, 16). The second clone (*DHR39*) was sequenced (Fig. 2, bp 306 to 1624, where +1 is the first nucleotide of the initiation codon) and found to contain amino acid sequence similar to the DNA binding domain of FTZ-F1 and other members of the steroid hormone receptor superfamily. The partial *DHR39* cDNA clone was used to screen 0−4 hr and 4−8 hr embryonic cDNA libraries (18) to identify full length clones. Positive clones were restriction mapped and appeared to represent four types of full length cDNAs of 4.8, 3.3, 3.2, and 2.7 kb (Fig. 3A) all with poly(A$^+$) tails suggesting functional cytoplasmic mRNAs. An additional *DHR39* cDNA (NW1) was cloned by homology to the DNA binding domain of other steroid/thyroid receptor proteins. Individual cDNAs representing the 4.8 kb (C15), 3.3 kb (C10) and the 2.7 kb (C12) cDNAs as well as the 3.1 kb NW1 cDNA were completely sequenced and found to encode 2 different open reading frames (ORF, Fig. 2 and Fig. 3). Both ORFs contain the same N-terminal region, the DNA binding domain, and part of the putative ligand binding domain. However, the ORF of the C12 class of cDNAs lacks the C-terminal half of the putative ligand

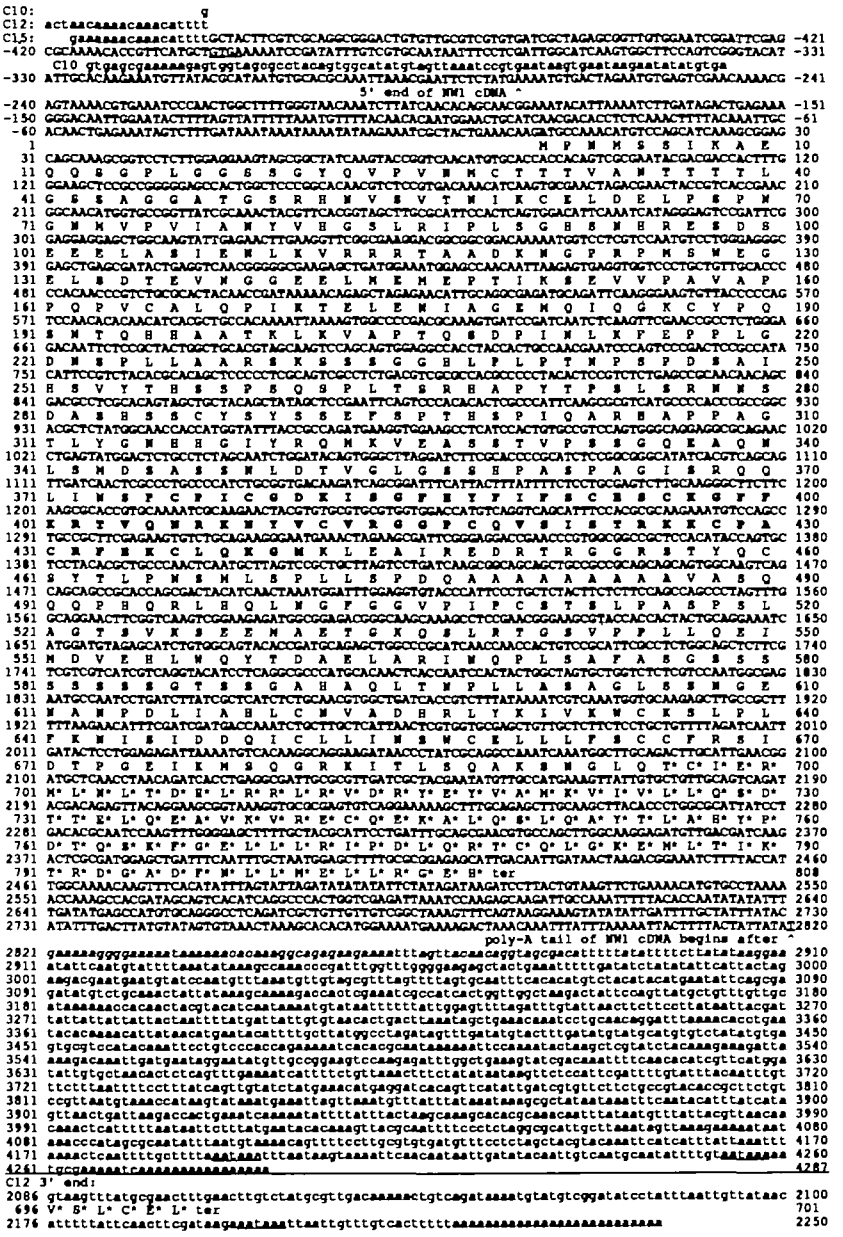

Figure 2. The sequence of the *DHR39* cDNA clones. The DNA sequence shared by cDNAs C10, C12, and C15 is indicated by upper case letters. The open reading frame is represented by one letter amino acid codes beneath each codon. Variations at the 5′ end are indicated by lower case letters at the beginning of the sequence. Additional 5′ untranslated sequence found in the C10 cDNA is shown beneath the underlined GTGA (−400 to −397) that it replaces. Nucleotide +1 (bold) indicates the first base of the initiation codon. The amino acids that are found only in the long open reading frames of the C10, C15, and NW1 cDNA are indicated by asterisk. The beginning and end of the NW1 cDNA is marked with a caret (^). The bold amino acid codes indicate the zinc finger DNA binding domain. The poly-A tail of cDNA C10 and NW1 is located after position 2820, however the NW1 cDNA contains an additional T, which is underlined after position 2820. The extended 3′ untranslated sequence of the C15 cDNA (2821−4271) is shown as lower case letters. The 3′ sequence and short open reading frame of the C12 cDNA is shown at the bottom of the figure. Polyadenylation signals at the end of the C15 and C12 cDNAs are underlined.

binding domain. The sequence of cDNA NW1 is identical to bp −273 to 2820 of cDNA C15 with an additional T-residue immediately prior to the poly-A tail (Fig. 2).

In situ hybridization to salivary gland polytene chromosomes localized this gene to the 39B-C region of the second chromosome (data not shown). Based on its cytologic location we have named this gene *Drosophila* Hormone Receptor 39 (*DHR39*). The localization to a single site on polytene chromosomes as well as genomic southern analysis (not shown) suggest that it is a single copy gene.

Developmental expression of *DHR39* and *FTZ-F1*

DHR39 and FTZ-F1 bind to a positive cis-acting element in the *Adh* adult enhancer. This site has been shown to be required for high levels of distal transcription in the adult fly and tissue culture cells (14). If either FTZ-F1 or DHR39 is responsible for this transcriptional enhancement it would be expected to be present at the appropriate times in development. To address this question the developmental expression patterns of *DHR39* and *FTZ-F1* were analyzed by developmental northerns (Fig. 4). A 2.5 kb probe to the 3′ end of the NW1 cDNA was hybridized to a blot

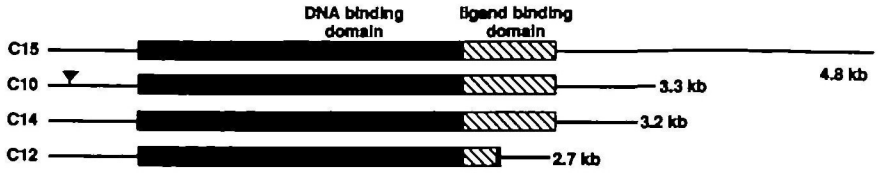

Figure 3. Maps of the four classes of *DHR39* cDNAs. The structure of four classes of cDNAs are illustrated, all four have similar 5′ ends (see fig. 2), the presence of additional sequences in the 5′ untranslated region of cDNA C10 is represented by a shaded triangle. The open reading frames are indicated by shaded or hatched rectangles. The 5′ and 3′ untranslated regions are shown as lines. The location of the DNA binding domain (darkly shaded rectangle) and ligand binding domain (hatched rectangles) are indicated. Poly(A+) tails are found at the 3′ ends of all cDNA classes.

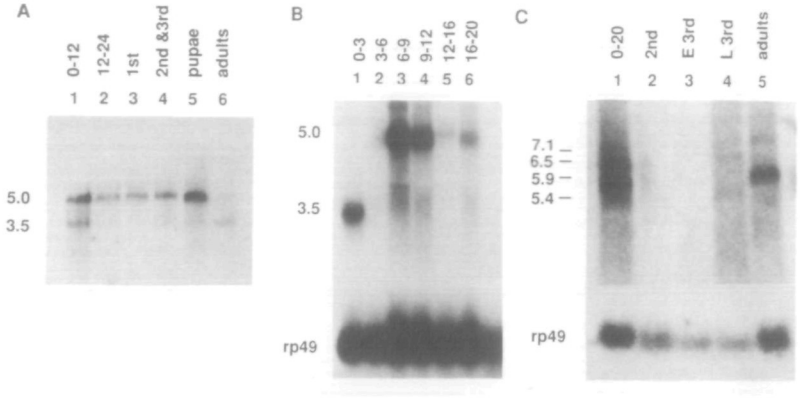

Figure 4. Developmental Northerns. **A.** Poly (A^+) RNA from different developmental stages was probed with a 2.5 kb fragment from cDNA NW1 containing the DNA and ligand binding domains. Lanes 1) 0−12 hr embryos; 2) 12−24 hr embryos; 3) 1st instar larvae; 4) 2nd and 3rd instar larvae; 5) pupae; and 6) adult flies. Note that in lane 6 the amount of RNA loaded was lower than the amount loaded on the other lanes, and therefore the hybridization signal appears less prominent. **B.** Total RNA (25 μg) from staged embryos was probed with the entire 4.8 kb C15 cDNA. Lanes 1) 0−3 hr embryos; 2) 3−6 hr embryos; 3) 6−9 hr embryos; 4) 9−12 hr embryos; 5) 12−16 hr embryos; 6) 16−20 hr embryos. **C.** Total RNA (25 μg) from different developmental stages was hybridized with a 2.5 kb *FTZ-F1* probe from the early *FTZ-F1* ORF (amino acids 202−1043; Lavorgna et al., 1991). Lanes 1) 0−20 hr embryos; 2) second instar larvae; 3) early third instar larvae; 4) late third instar larvae; 5) adults.

of poly(A^+) RNA from different developmental stages (Fig. 4A). Two different size messages were detected. A 5 kb message is seen at all developmental stages and a smaller 3.5 kb message is seen in 0−12 hr embryos (Fig. 4A, lane 1), adults (Fig. 4A, lane 6), and to a lesser extent in pupae (Fig. 4A, lane 5) and larvae (Fig. 4A, lanes 3−4). Embryonic expression of *DHR39* was further analyzed by a second developmental northern (Fig. 4B). The 3.5 kb message is most abundant in 0−3 hr embryos (Fig. 4B, lane 1) and may represent maternally inherited transcripts. At this early developmental stage there is very little of the 5 kb message, which appears at a high level during 6−9 hr of development (Fig. 4B, lane 3) and continues at a lower level in 12−20 hr embryos (Fig. 4B, lanes 5−6).

Another set of staged total RNA was used to investigate the developmental patterns of *FTZ-F1* expression (Fig. 4C). High levels of *FTZ-F1* expression were detected in 0−20 hr embryos (Fig. 4C, lane 1) as 4 different messages of 7.1, 6.5, 5.9, and 5.4 kb, and in adults (Fig. 4C, lane 5) as 7.1 kb and 5.9 kb. A low level of *FTZ-F1* transcripts of 6.5 and 5.4 kb were detected in late third instar larvae (Fig. 4C, lane 4), whereas no message can be detected in second and early third instar larvae (Fig. 4C, lanes 2 and 3). The 5.9 kb RNA is likely the early *FTZ-F1* message, encoding the early FTZ-F1 isoform, and the 6.5 and 5.4 kb RNAs encode the late FTZ-F1 isoform (16, 25). The 7.1 kb transcript may represent heterogeneous nuclear RNA present in our total RNA preparations. During early embryogenesis FTZ-F1 RNA and protein are uniformly distributed (25).

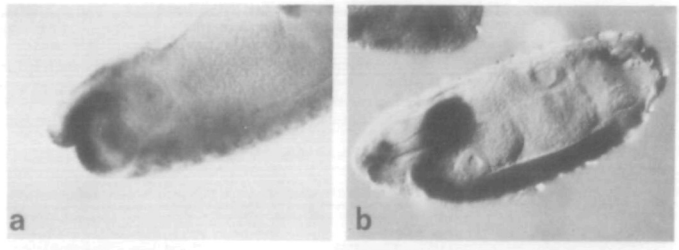

Figure 5. Whole mount embryo *in situ* hybridization. Whole mount RNA in situ hybridization was carried out with probes representing the 5′ or 3′ end of the cDNA. **a.** In a stage 13 embryo hybridization to the stomodeum is observed. **b.** In a stage 16 embryo, prominent hybridization to the fully developed central nervous system and brain lobes is seen. Staining in the pharynx can also be observed.

The tissue distribution of *DHR39* transcripts was examined by in situ hybridizations to whole mount embryos (Fig. 5). In stage 13 embryos (9.3−10.3 hr of development, 26) *DHR39* RNA was present in the stomodeum (Fig. 5A), an invagination that will develop into the foregut. In stage 16 embryos (13−16 hr) hybridization was most prominent in the central nervous system and brain lobes (Fig. 5B). No hybridization was seen to the embryonic fat body, or to other tissues where ADH expression is seen in embryos. In contrast, proximal and distal *Adh* transcripts are first detected in fat body cells of stage 14 embryos

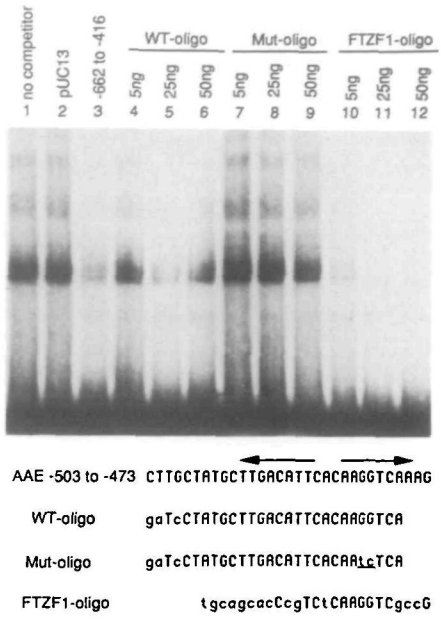

Figure 6. Electrophoretic mobility shift/competition assay of DHR39 binding to wild type and mutant FTZ-F1 binding sites. Binding of a DHR39 fusion protein to 0.5 ng ^{32}P-labeled *Adh* adult enhancer (AAE) DNA was analyzed in the absence of competitor DNA (lane 1), or in the presence of 50 ng of nonspecific pUC13 DNA (lane 2), 50 ng of unlabeled AAE (lane 3), 5,25, or 50 ng of unlabeled wt-oligo (lanes 4−6), 5,25, or 50 ng of unlabeled mut-oligo (lanes 7−10), 5,25, or 50 ng of unlabeled FTZF1-oligo (lanes 10−12).

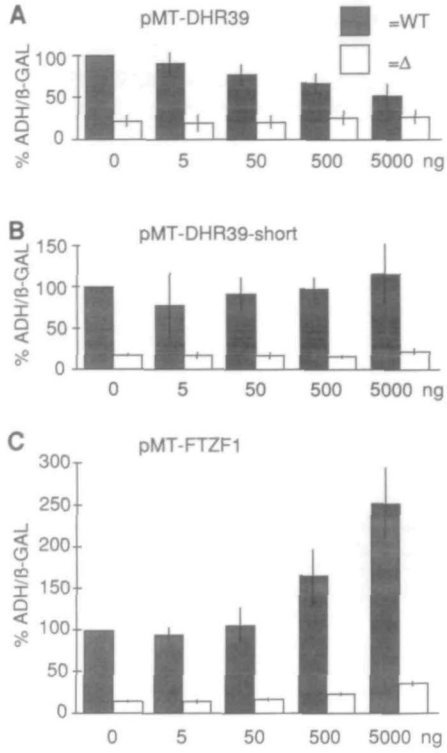

Figure 7. Co-transfection of *DHR39* and *FTZ-F1* with *Adh*-containing plasmids. Increasing amounts (0−5000 ng) of a plasmid which contained the ORF of: **A.** the long DHR39 isoform, **B.** the short DHR39 isoform or **C.** the late isoform of FTZ-F1 downstream of the inducible metallothionein promoter were transfected along with 5 μg of either the wild type *Adh* gene or an intact *Adh* gene which lacks the overlapping binding sites of DHR39 and FTZ-F1. 1 μg of the control plasmid pPAcLacZ (28) was also included in the transfection. The ADH activity relative to β-GAL activity was measured and is presented as a percentage of the activity seen in the absence of an expression plasmid (0 ng). The results are averages of 6 (A) or 3 (B and C) experiments, and the error bars indicate ± one standard deviation.

(10.3−11.3 hr of development); distal transcripts then disappear by stage 16 (27).

In summary *DHR39* RNAs are present throughout development. The 4.8 kb cDNA (Fig. 3,C15) probably represents the 5 kb message, whereas the 3.3 kb cDNA represents the 3.5 kb message. These two cDNAs share identical ORFs (Fig. 2 and 3) suggesting that identical DHR39 protein is present throughout development. In contrast, the developmental expression pattern of *FTZ-F1* more closely correlates with AAE activation and distal *Adh* transcription. *FTZ-F1* is expressed primarily in embryos, late third instar larvae, prepupae (not shown), and adults.

DHR39 binds to two different FTZ-F1 sites

DHR39 was cloned from an expression library based on its ability to specifically bind to a site in the AAE (−499 to −476), however the resulting clone lacked a ligand binding/dimerization domain. To test the ability of the DHR39 protein to bind to this site we expressed a fusion protein in *E.coli* and performed an electrophoretic mobility shift/competition assay (Fig. 6). When incubated with ^{32}P-labeled AAE bands of decreased mobility are observed (Fig. 6, lane 1). Multiple bands of decreased mobility are detected, although the significance of these are at present unknown. In the fusion protein preparation 80% of the product ran as a single band on an SDS-polyacrylamide gel (data not shown). The fusion protein specifically binds the AAE as the shifted bands are not competed by non-specific pUC13 DNA (Fig. 6, lane 2), whereas they are competed away by a 100 fold excess of unlabeled AAE DNA (Fig. 6, lane 3). Furthermore, the shifted nucleoprotein complexes decrease with increasing amounts of a 24 bp oligonucleotide from the AAE (wt-oligo, Fig. 6, lanes 4−6) and with a 25 bp oligonucleotide from the *fushi tarazu* zebra element (16) (FTZF1-oligo, Fig. 6, lanes 10−12). A 24 bp oligonucleotide containing a 2 bp mismatch to the AAE FTZ-F1 binding site (14) fails to compete (Fig. 6, lanes 7−9). These data demonstrate that DHR39 binds to two FTZ-F1 binding sites and does not bind to a mutated FTZ-F1 site that also fails to bind FTZ-F1 protein (14, 16).

DHR39 represses and FTZ-F1 activates distal *Adh* transcription

To determine if there is a functional role for DHR39 in the control of *Adh* transcription we over expressed the protein in *Drosophila* tissue culture cells together with *Adh* reporter genes (Fig. 7). The first reporter gene was pWT (Fig. 1B) containing an intact *Adh* gene and a wild type AAE. The second was pΔ (Fig. 1B) an *Adh* plasmid with the 12 bp linker replacement deletion of the DHR39 and FTZ-F1 binding site in the AAE. The recipient cells were the *Drosophila* cell line 1006−2 (24) in which the AAE is active (13, 14). In the absence of the *DHR39*-expressing plasmid (pMT-DHR39) there was approximately 5 fold more ADH activity detected with pWT than with pΔ, indicating that pΔ lacks an important positive cis-acting regulatory element for distal transcription as seen previously (14). Increasing amounts of pMT-DHR39 led to gradual decreases in ADH expression (Fig. 7A. shaded bars), which derives from distal transcription (13, 14). Transfection with 5000 ng of pMT-DHR39 consistently

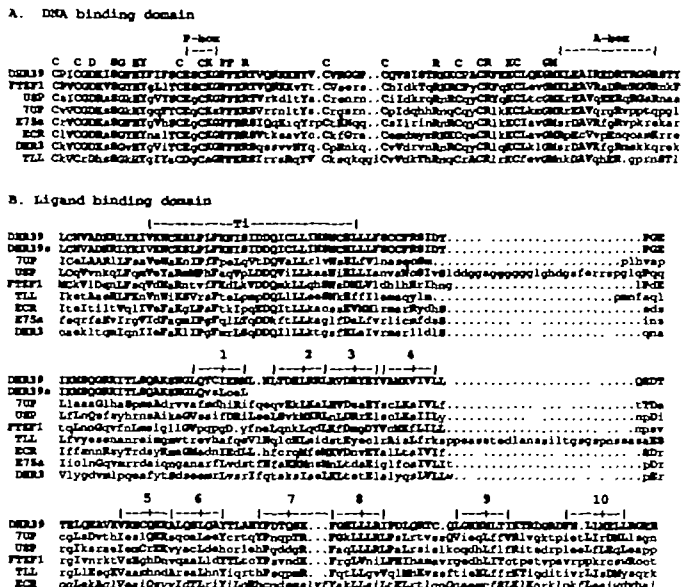

Figure 8. Amino acid sequence comparison of the DNA and putative ligand binding domains of DHR39 and other *Drosophila* steroid hormone receptor superfamily members. The DNA binding domains (A) and ligand binding domains (B) were aligned using the sequence analysis program PILEUP (29). DHR39 and the short DHR39 isoform (DHR39s) are compared to ultraspiracle (USP; 30, 31, 32), seven-up (7UP; 2), FTZ-F1 (16), tailless (TLL; 3), ecdysone receptor (ECR; 4), E75a (33), *Drosophila* hormone receptor 3 (DHR3; 34). **A.** Invariant amino acids in the DNA binding domain are indicated above the alignment. The location of the P-box and A-box (35) are also indicated. Similarity to DHR39 in the other receptor sequences is indicated by bold uppercase single letter amino acid codes (D-E, I-V-L, L-M, R-H, R-K, S-T,F-W-Y). **B.** The conserved Ti domain and heptad repeat dimerization domain (36, 37) are indicated above the alignment.

resulted in a 2 fold reduction in ADH activity. In control experiments in which the metallothionein promoter was not induced with copper (data not shown) there was no significant change in levels of ADH activity. No change in ADH activity was seen when pΔ was used as the reporter (Fig. 7A, open bars). These results suggest that DHR39 is a DNA binding site-dependent repressor of distal *Adh* expression.

We identified several cDNA clones, represented by C12 (Fig. 2 and 3), that encode a shortened version of the DHR39 protein lacking the putative dimerization region of the LBD, therefore we wanted to determine if this protein differs functionally from the full length receptor. Similar co-transfection experiments (pMT-DHR39-short, Fig. 7B) indicated that over expression of the short DHR39 protein had no effect on the level of ADH activity. We conclude that the C-terminal portion of the LBD is required to repress distal *Adh* transcription in this tissue culture cell line.

To test the functional role of FTZ-F1 we performed similar co-transfection experiments by over expressing the late isoform of FTZ-F1 (Fig. 7C). In contrast to the DHR39 over expression, FTZ-F1 activates distal *Adh* transcription in a dosage-dependent manner. Transfection with 5000 ng of pMT-FTZF1 consistently resulted in greater than 2 fold increases in ADH activity.

DISCUSSION

In this report we describe the cloning of a new member of the steroid-thyroid-retinoic acid receptor superfamily. DHR39 contains the characteristic DNA binding and ligand binding domains. Figure 7 shows the comparison of DHR39 protein in these regions to other *Drosophila* steroid receptor superfamily members. DHR39 has a high degree of identity in the DNA binding domain to FTZ-F1 (Fig. 8A). These two orphan receptors bind to a site in the AAE as well as to a regulatory element upstream of the segmentation gene *fushi tarazu* (16, 38). DHR39 and FTZ-F1 have identical P-boxes (Fig. 8A). The P-box has been demonstrated to specify DNA sequence recognition (39, 40, 41, reviewed in ref. 42). DHR39, FTZ-F1, the murine homolog of FTZ-F1, embryonal long terminal repeat-binding protein (ELP, 43) and murine liver receptor homolog-1 (LRH-1, 44) share a common P-box and represent a new subfamily within the steroid receptor superfamily. In addition, DHR39, FTZ-F1, ELP, and LRH-1 also share a region immediately C-terminal to the conserved DNA binding domain termed the A-box (Fig. 8A). The A-box of another superfamily member, H-2RIIBP (RXRβ), has been shown to be critical for binding to a specific response element in the promoter of the gene encoding cellular retinol binding protein II (35). Likewise, the common A-box in DHR39 and FTZ-F1 may play a similar role, in addition to the P-box, in specifying the overlapping binding sites of these two proteins. This domain has recently been shown to specify FTZ-F1 binding (45).

Although the DNA binding domains are highly conserved between FTZ-F1 and DHR39, less similarity is seen in the putative ligand binding domains (LBD, Fig. 8B). The LBDs of the superfamily members consists of two regions, the Ti domain and a dimerization domain (36, 37). In other steroid receptor superfamily members the Ti domain has been suggested to play a role in transcriptional inactivation in the absence of ligand (37). Of the *Drosophila* steroid receptor proteins, the Ti domain of DHR39 is most similar to the RXRα homolog ultraspiracle (59%) (30, 31, 32). This region is even more closely related (71% similar) to the human progesterone receptor (46). The Ti domain of FTZ-F1 is 55% similar to DHR39 and is 65% similar to ultraspiracle.

The dimerization domains within the LBD are characterized by a series of heptad repeats (36, 37). These repeats contain hydrophobic amino acids in positions 1 and 8, and either hydrophobic or charged amino acids with hydrophobic side chains in position 5. In the more conserved region between repeats 1–8 DHR39 is most similar to seven-up (45%) and ultraspiracle (43%). FTZ-F1 is most similar to ultraspiracle (48%) and only 39% similar to DHR39 in this region. Interestingly, the short open reading frame of cDNA C12 lacks these heptad repeats (Fig. 8B).

A model for activation and repression of the AAE

The similar protein structural domains but opposing activities of DHR39 and FTZ-F1 highlight an additional complexity of regulation. Two transcription factors with different functions may bind to the same cis-acting element and elicit opposite transcriptional responses. We have shown that DHR39 binds to two different FTZ-F1 sites, one from the *Adh* adult enhancer and a second from the zebra element of the fushi tarazu gene. These two proteins may bind to common sites at other loci as well. Although we have demonstrated that FTZ-F1 and DHR39 can activate and repress *Adh* expression in cultured cells, the temporal patterns of *FTZ-F1* and *DHR39* RNA suggest that FTZ-F1 may activate distal *Adh* transcription during development. It is unclear whether DHR39 plays any role in the normal regulation of ADH. More detailed immunocytochemical or in situ hybridization data are needed to determine whether these two

orphan receptors are expressed in ADH-expressing or non-expressing cells of the developing fly.

Several possibilities exist for mechanisms of DHR39 repression of the distal *Adh* promoter in the co-transfection assay. It may simply prevent FTZ-F1, which is present in these cells (14), from binding to the positive cis-element in the AAE. DHR39 might compete with FTZ-F1 for binding to the AAE or prevent FTZ-F1 from binding by direct protein-protein interaction. Alternatively DHR39 may act as a repressor by titrating ancillary factors which interact with both proteins such as components of the transcription apparatus or other proteins that bind to the AAE (14, 47, 48, 49) to prevent transcription initiation. The short ORF of DHR39, when expressed in the co-transfection assay, did not affect distal *Adh* expression. This suggests competition for DNA binding alone is not the mechanism of repression and that the LBD sequence participates in the process of transcriptional repression by the DHR39 protein. This alternative ORF has an intact DNA binding domain but lacks much of the putative ligand binding and dimerization domain. This truncated protein presumably can still bind the DNA; the original *DHR39* clone that was isolated from the expression library lacked the ligand binding domain, however it still bound specifically to the *Adh* adult enhancer. These observations suggest that if the DHR39 short ORF can bind the DNA, it is still not sufficient to prevent endogenous FTZ-F1 from binding. The repressor function of DHR39 might require the binding of a ligand or dimerization with itself or other members of the steroid receptor superfamily. Further experiments are planned to address these possibilities.

Recently it has been shown that ultraspiracle can form heterodimers with the ecdysone receptor (9) and is required to confer ecdysone responsiveness to cultured mammalian cells. DHR39 and ultraspiracle share similar organization for 5 of the 10 heptad repeats (Fig. 8, repeats 1, 2, 3, 5, and 8) in the putative dimerization domain. DHR39 is most closely related to seven-up (45% similarity) and ultraspiracle (43% similarity) within this domain. Ultraspiracle and seven-up are the *Drosophila* homologs of RXRα and COUP-TF respectively (2, 5). RXRα has been shown to form heterodimers with the retinoic acid, thyroid hormone, vitamin D and peroxisome proliferator-activated receptors (50, 51, 52, 53, 54, 55, 56). COUP-TF has recently been shown to form heterodimers with thyroid hormone receptor (57). It is possible that like ultraspiracle, DHR39 acts by forming heterodimers with other steroid receptor superfamily members. In addition the presence of the short form of DHR39 protein represented by the C12 class of cDNA would add another level of complexity to regulatory pathways involving DHR39 and FTZ-F1.

Drosophila steroid receptor superfamily members appear to function at the molecular level in ways analogous to vertebrate receptors. The potential to form heterodimers is conserved in the *Drosophila* RXRα homolog ultraspiracle (9). Here we present the first evidence that two different receptor-like proteins can bind to a common element and either activate or repress transcription. This may be analogous to the ability of COUP-TF to down regulate hormonal induction by vitamin D, thyroid hormone, and retinoic acid receptors which have similar binding sites to COUP-TF (57, 58). The apparent similar mechanisms between vertebrate and invertebrate nuclear receptors, combined with the powerful genetic system offered by *Drosophila* provides exciting avenues toward the mechanistic understanding of developmental and physiological roles of this important family of transcription factors.

ACKNOWLEDGEMENTS

We thank Elaine Reynolds for the in situ localization of DHR39 to polytene chromosomes, G. Lavorgna and C. Wu for sharing the *FTZ-F1* late cDNA sequence prior to publication. M. M. was supported by a De Kiewiet fellowship. This work was supported by grants from the NIH to C. B. and B. S.

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