Identification and characterization of a Drosophila nuclear receptor with the ability to inhibit the ecdysone response

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ABSTRACT In a search for retinoid X receptor-like molecules in Drosophila, we have identified an additional member of the nuclear receptor superfamily, XR78E/F. In the DNA-binding domain, XR78E/F is closely related to the mammalian receptor TR2, as well as to the nuclear receptors Coup-TF and Seven-up. We demonstrate that XR78E/F binds as a homodimer to direct repeats of the sequence AGGTCA. In transient transfection assays, XR78E/F represses ecdysone signaling in a DNA-binding-dependent fashion. XR78E/F has its highest expression in third-instar larvae and prepupae. These experiments suggest that XR78E/F may play a regulatory role in the transcriptional cascade triggered by the hormone ecdysone in Drosophila.

Both the retinoid X receptor (RXR) and ultraspiracle (Usp) are responsible for coordinating networks of gene expression. Proper signaling by the thyroid hormone receptor, the retinoic acid receptor, the vitamin D receptor, and the peroxisome proliferator-activated receptor is achieved through heterodimeric complexes with RXR (1–6). The ability of RXR-like molecules to form heterodimers is evolutionarily conserved as demonstrated by the fact that Usp, the Drosophila RXR, can replace RXR in heterodimers with the above mammalian nuclear receptors (7). In Drosophila, the functional ecdysone receptor complex is a heterodimer between the ecdysone receptor (EcR) and Usp (8, 9).

The EcR complex is responsible for mediating the transcriptional response to the steroid hormone 20-hydroxyecdysone leading to molting and metamorphosis. Analyses of the transcriptional cascade triggered by ecdysone, seen as puffing patterns in the Drosophila salivary gland chromosomes, led to proposal of a regulated cascade of transcription factors necessary for proper metamorphosis (10). Molecular characterization of the genes within the ecdysone-induced salivary gland puffs revealed that several of the associated genes encode orphan nuclear receptors. Orphan receptors have the characteristic DNA-binding domains (DBDs) and ligand-binding domains of the steroid/nuclear receptor superfamily but do not have identified ligands.

The biological role of many orphan receptors is not clear. In some cases, orphan receptors can modulate hormonal signaling of other nuclear receptors. Coup-TF and Seven-up can repress RXR/Usp-based signaling pathways (11–13, 29), whereas βFTZ-F1 can enhance transcription from gene targets of the EcR complex (14). Due to this potential of orphan receptors to modulate signaling pathways or possibly respond to specific hormones, we want to identify additional members of this family. This paper describes the orphan receptor XR78E/F and its possible role in modulating hormonal signaling pathways.†‡

MATERIALS AND METHODS

Genomic Southern Blotting and Isolation of cDNAs. Standard procedures were used for isolation of genomic DNA and Southern blotting (15, 16). The stringent and nonstringent hybridization conditions have been described (17), except that washes were performed with 2× SSC/0.1% SDS two times at room temperature and with 1× SSC/0.1% SDS at 65°C. DNA blots and libraries were screened with DNA encoding the mouse RXRβ BD. A size-selected genomic library was made by digesting Canton S genomic DNA with EcoRI, size-fractionating the fragments on an agarose gel, and isolating fragments of 2–5 kb. The DNA was ligated to λ ZAP II, EcoRI-digested phage arms and packaged according to the manufacturer’s instructions (Stratagene). Plasmids containing positive inserts were derived from phage according to the manufacturer’s instructions (Stratagene). An imaging disc cDNA library (provided by C. Zuker, University of California, San Diego) was screened with the cloned genomic fragment CSR 2.5.

Salivary Chromosome in Situ Hybridization. Chromosome in situ hybridizations were performed as described by McKewen et al. (18) except a digoxigenin-labeled DNA probe was used and detection was performed as recommended by the manufacturer (Boehringer Mannheim).

Developmental Northern Blot. Total RNA was isolated by homogenizing the animals in RNA extraction buffer (200 mM NaCl/20 mM Tris-HCl, pH 7.5/20 mM EDTA/2% SDS) that contained proteinase K (2.0 mg/ml) (Merck). The homogenate was incubated at 50°C for 1 hr and then extracted twice with phenol/chloroform (1:1) and once with chloroform/isoamyl alcohol (24:1). All nucleic acids were precipitated with ethanol. After resuspending the nucleic acid pellet in diethyl pyrocarbonate-treated water, an equal volume of 8 M LiCl was added and then the mixture was incubated for at least 1 hr at –20°C to precipitate the RNA. Standard procedures were used for Northern blotting (15).

Plasmids. CMX XR78E/F was constructed by placing the cDNA into CMXPLI, a derivative of the CMX expression vector (19). CMX GεEcR and CMX Usp have been described (7). The luciferase reporter constructs A MTV-hsEcRE3-LUC and A MTV-Eip28/29-LUC have been described (29).

Transfection Assays. Monkey kidney CV-1 cells were grown in Dulbecco’s modified Eagle’s medium containing 10% resin charcoal-stripped bovine calf serum. Twenty-four hours before transfection, cells were plated into 48-well plates. Cells were

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transfected using DOTAP according to the manufacturer's instructions (Boehringer Mannheim). Transfection assay mixtures contained (per well) 0.06 µg of reporter, 0.02 µg of each receptor, and 0.1 µg of CMX β-galactosidase to determine transfection efficiency. After 2 hr, the liposomes were removed and the cells were incubated for 36 hr in medium either without or with the ligand muristerone A (Zambon, Bresso, Italy) at a final concentration of 1 µM. Cell extracts were prepared and assayed for luciferase and β-galactosidase activity as described (19). All experimental points represent the average of three transfections.

Electrophoretic Mobility-Shift Assays (EMSAs). Protein for all EMSAs was generated by *in vitro* transcription/translation reactions using the TnT coupled reticulocyte lysate system (Promega) according to the manufacturer's instructions. The synthetic oligonucleotides were the same as described by Zelhof et al. (29) as well as the conditions for the EMSAs. The *Drosophila* embryo extract was a gift from J. Kadonaga (University of California, San Diego). The monoclonal antibody for EcR was a gift from David Hogness (Stanford University) and the Usp antiserum have been described elsewhere (7).

**Antisera Production.** Amino acids 1–160 of XR78E/F were cloned by PCR into the glutathion S-transferase fusion vector pGEX2T (Pharmacia). The subsequent glutathion S-transferase fusion protein was used to produce antisera as described by Yao et al. (7).

**RESULTS**

**Identification and Isolation of XR78E/F.** To examine whether genes for additional RXR-like molecules exist in *Drosophila*, we probed a blot of genomic DNA with DNA encoding the mouse RXRβ DBD. Under moderate stringency, two prominent signals were seen (Fig. 1A, lane 1). The 8-kb band corresponds to ultrasperacle (uSp) (20), and another band is detected at ~2.7 kb. To isolate this DNA, a size-selected genomic library was screened with the murine RXRβ DBD probe. Several positive clones were isolated and contained identical inserts. They were given the isolation name CSR 2.5. In *situ* hybridization maps CSR 2.5 to salivary chromosome region 78E/F (Fig. 1B). CSR 2.5 encodes a protein with a deduced amino acid sequence containing the zinc fingers characteristic of nuclear receptor DBDs (Fig. 2). Based on the position of CSR 2.5, we refer to this locus as XR78E/F (X receptor at 78E/F, where X indicates an unknown ligand). This is a separate locus from the E78 locus, encoding E78A and E78B, at salivary chromosome position 78C (21).

To further characterize this gene, we used CSR 2.5 to screen an imaginal disc cDNA library. Several clones were isolated and sequenced to obtain a complete, full-length cDNA se-

**Expression Pattern of XR78E/F.** To identify possible functions for XR78E/F, we examined its expression profile. Fig. 3 shows a blot of total RNA from various stages of *Drosophila* development. Although XR78E/F is not expressed strongly in embryos, its expression increases after the second- to third-instar molt. Quantification of the ratio of XR78E/F/RNA to *rp49* RNA shows a fairly constant expression level in the prepupal and pupal stages (data not shown). Based on studies of salivary glands cultured in ec dysone, XR78E/F expression does not appear to be strongly activated by ec dysone (data not shown). RNA in *situ* hybridization to embryos and imaginal discs show ubiquitous expression at low levels (data not shown).

**Binding Profile of XR78E/F.** Based on the homology with XR and Coup-TF/Seven-up, XR78E/F may share similar DNA-binding specificity. Using EMSAs and *in vitro* translated protein, we find that XR78E/F binds to hormone response elements consisting of direct repeats of the sequence AG-GTCA, in particular a direct repeat spaced by 1 nucleotide (DR1). To identify the relative affinity of XR78E/F for a DR1 compared to other direct repeats with different spacing, a
The addition of one response element to a nuclear embryo not only increases the affinity of XR78E/F for its target DNA, but also disrupts the binding of nuclear proteins to the XR78E/F complex. This effect is observed in several EMSA experiments using competition GST pull-down assays. In these assays, a radiolabeled XR78E/F was combined with a 10-fold molar excess of each unlabeled competitor oligonucleotide during the period of protein binding. XR78E/F has the highest affinity for a DR1 (27), but other direct repeats compete for binding (lanes 4-7). An oligonucleotide of equal size but containing only one of the two half sites does not compete (lane 3), suggesting that XR78E/F binds as a homodimer and not as a monomer. An inverted repeat, represented by the hs27 ecysone response element (EcRE) (27), also does not compete (lane 8). Interestingly, this binding profile is similar to that of Seven-up I and II. Heterodimerization with Usp and EcR has not been detected.

To check whether endogenous XR78E/F protein can bind to a DR1, we examined binding of proteins from Drosophila embryo nuclear extracts to a CRBPII element (28) (Fig. 4B). This element contains four AGGTCA half sites separated by 1 nucleotide. Several binding complexes are observed. Application of antisera against XR78E/F caused one complex to decrease or disappear (Fig. 4B, compare lanes 1 and 4, complex is indicated by arrow), while preimmune serum and antibodies to EcR and Usp have no effect on this complex (Fig. 4B, lanes 2 and 3). Control experiments with in vitro translated XR78E/F protein show that XR78E/F complexes comigrate with the putative XR78E/F CRBPII complex from embryos (data not shown) and that the XR78E/F complex is also disrupted upon addition of XR78E/F antisera (Fig. 4C).

XR78E/F Inhibits Ecdysone Signaling in a DNA-Dependent Fashion. Both Seven-up and XR78E/F appear to have the same DNA binding specificity and may possibly display overlapping functions in vivo. However, because the expression pattern of XR78E/F is different from seven-up, they are unlikely to perform redundant functions. XR78E/F RNA increases in third-instar larvae and is slightly influenced by ecdysone; thus, XR78E/F may contribute to regulation of the transcriptional cascade induced by ecdysone. We used transient transfection assays to test whether XR78E/F can alter ecdysone signaling. CV-1 cells respond to ecdysone only after

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**FIG. 3.** Developmental Northern blot of XR78E/F. Total RNA was isolated at the stages indicated and probed with a full-length CDNA clone. Only one band is detected and relative levels of expression were compared using rp49 levels as analyzed by Molecular Dynamics PhosphorImager (data not shown).

**FIG. 4.** XR78E/F DNA-binding competition assay. (A) EMSAs were used to determine the relative affinities of XR78E/F for different hormone response elements indicated. In vitro translated XR78E/F was incubated with 32P-labeled oligonucleotide representing a direct repeat spaced by one nucleotide (DR1) with or without (lane 1) a 10-fold excess of unlabeled competitor. EcRE represents an oligonucleotide based on the hs27 EcRE. Competitors are listed above each lane. (B) EMSA of a 32P-labeled CRBPII element (27) using Drosophila embryonic nuclear extract, with the addition of antibodies directed against specific nuclear receptors (Usp, lane 2; EcR, lane 3; XR78E/F, lane 4; preimmune serum, lane 5). The putative XR78E/F complex (arrows) is disrupted only in lane 4. (C) EMSA using in vitro translated XR78E/F to demonstrate specificity of the XR78E/F antisem. In A-C, arrows indicate position of the XR78E/F complex.
transfection of DNAs encoding both Usp and EcR (7). Using reporter constructs containing either five copies of the hs27 EcRE (ΔMTV-hsEcRE5-LUC) or three copies of the Eip 28/29 EcRE (ΔMTV-Eip28/29-LUC), we tested whether XR78E/F could alter ecdysone signaling.

As previously shown (7, 29), the transfection of DNA encoding Usp and GEcR (a modified EcR with the N-terminal transactivation domain of the glucocorticoid receptor) renders CV-1 cells competent for ecdysone-induced transcriptional activation (Fig. 5, compare columns 1 and 2 with columns 3). The difference in activation between the two reporters is probably due to the difference in copy number of the two response elements. Cotransfection of DNA encoding XR78E/F in addition to the DNAs encoding Usp and GEcR has different effects on hormone-induced transcriptional activity depending on the response elements. For the ΔMTV-Eip28/29-LUC reporter, the presence of XR78E/F substantially inhibited transcriptional activity. We see 29.8-fold induction of transcriptional activity in the absence of XR78E/F and only 4.1-fold induction in the presence of XR78E/F (Fig. 5B, columns 3 and 4). In the case of the ΔMTV-hsEcRE5-LUC reporter, the presence of XR78E/F did not affect transcriptional activity (Fig. 5A, columns 3 and 4). Thus, XR78E/F alters ecdysone signaling in a response element-dependent manner. With the ΔMTV-Eip28/29-LUC reporter, there is a slight increase in luciferase activity in response to XR78E/F and muristerone. This increase is slight and may overlap levels seen in controls (Fig. 5B, compare columns 5 and 6).

Why does XR78E/F inhibit ecdysone signaling on the Eip 28/29 EcRE but not the hs27 EcRE? One possibility is that XR78E/F competes with EcR/Usp for binding sites on the response elements and that XR78E/F binds to the Eip 28/29 EcRE and not the hs27 EcRE. Examination of the hs27 EcRE and the Eip 28/29 EcRE revealed that a degenerate direct repeat with 3-nucleotide spacing (DR3) is present in the Eip 28/29 EcRE but not in the hs27 EcRE. One half site of this DR3 is essential for EcR/Usp heterodimer binding to this element (29). From Fig. 4A, we know that a DR3 is a potential XR78E/F binding site, while the hs27 EcRE appears to be a poor binding site. To test whether XR78E/F has differential binding to the two EcREs, we used a competitive EMSA (Fig. 6) to determine the relative affinity of XR78E/F for the sequences present in both reporter constructs, as well as the importance of the direct repeat present in the Eip 28/29 EcRE. XR78E/F was bound to a radiolabeled DR1 element in the presence of a 25-fold molar excess of specific competitor DNAs. As expected, a DR1 competes for binding. Interest-

![Fig. 5.](image1)

![Fig. 6.](image2)
ingly, the wild-type Eip 28/29 element competes nearly as efficiently as the DR1 (Fig. 6, compare lanes 2 and 3). If either half site of the DR3 present in Eip 28/29 is mutated (represented by oligonucleotides Eip 28/29 1 and Eip 28/29 2), competition for binding is lost (lanes 4 and 5). The heterodimer of EcR and Usp will bind to the Eip 28/29 1 element, with lower affinity, but binding to the Eip 28/29 2 element is eliminated (29). The hs27 EcRE 2×-1 and 2×-2 oligonucleotides represent the two orientations of the individual hs27 EcREs existing in the reporter construct ΔMTV-hsEcRE-Luc. Neither of these oligonucleotides competes for DNA binding (lanes 6 and 7). These results suggest that XR78E/F inhibits ecdysone responsiveness from the Eip 28/29 element by direct competition for binding to the regulatory DNA sites and that XR78E/F fails to inhibit ecdysone responsiveness on the hs27 EcRE because it cannot bind to this element.

**DISCUSSION**

Here we have described the isolation and characterization of a *Drosophila* orphan nuclear receptor, XR78E/F. Even though XR78E/F was isolated with a RXR DBD-based probe, the receptor has higher homology to the DBD of the mammalian TR2 receptors. The mammalian TR2 receptors were isolated from human testis and prostate libraries and belong to the Coup subfamily of nuclear receptors (23–26). They have identical DBDs but are diverse in their ligand-binding domains. Their function is not known. Like TR2, XR78E/F shows homology with mammalian Coup-TF and *Drosophila* Seven-up. The ligand-binding domain of XR78E/F does not show any significant homology to other ligand-binding domains and does not have an identified ligand.

XR78E/F expression appears to be ubiquitous and low, with higher expression during the third-larval, prepupal, and pupal stages. The increase of expression during the third-larval instar is reminiscent of ecdysone-inducible genes, but little induction is seen in ecdysone organ cultures. Although mRNA levels in embryos are very low, functional protein appears to be present as judged by EMSAs and antibody shifting with embryonic nuclear extracts. The expression pattern of XR78E/F does not suggest a specific in vivo function, but biochemical data suggest that XR78E/F may modulate transcriptional activity induced by the ecdysone receptor complex or other hormone receptors via interactions with shared DNA-binding sites.

The close homology between the DBDs of XR78E/F and the *Drosophila* receptor Seven-up is reflected in their similar DNA-binding specificities. Like Svp, XR78E/F binds as a homodimer to variously spaced direct repeats, with the highest affinity for a DR1. Although XR78E/F shares similar DNA-binding specificities with Seven-up, XR78E/F is unlike Svp in that it represses ecdysone-induced transcription only on an EcRE to which it binds. Thus, XR78E/F regulates transcription via DNA binding, whereas Seven-up can regulate ecdysone-induced transcription independent of DNA binding (29). Based on the biochemical data and expression pattern of XR78E/F, it is possible that XR78E/F may modulate certain genes by raising the threshold level of the EcR complex necessary for transcriptional activation. Under this model, XR78E/F may act to dampen responses to any premature fluctuations in ecdysone titers to ensure proper spatial and temporal expression of ecdysone-inducible genes.

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