Transcriptional activity of *Drosophila melanogaster* ecdysone receptor isoforms and ultraspiracle in *Saccharomyces cerevisiae*

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ABSTRACT

The *Drosophila melanogaster* ecdysone receptor (EcR) is produced in three isoforms, which mediate developmental processes such as metamorphosis. These isoforms were expressed in *Saccharomyces cerevisiae* to elucidate aspects of receptor transcription activity in a highly defined genetic model system. All three EcR isoforms showed ligand-independent transcriptional activation of an ecdysone reporter gene and the amount of activation correlated with the size of the N-terminal A/B (transactivation) domain present in the isoform: EcR-B1>EcR-A>>EcR-B2. Upon co-expression with ultraspiracle (Usp), transcriptional activation was further increased with EcR-B1 or EcR-A, but was unchanged with EcR-B2 or a truncated EcR lacking the A/B N-terminal domain (EcRΔA/B). Thus, the enhanced activity from Usp may depend on the presence of an N-terminal domain of EcR. Co-expression with Usp of several chimeric receptors of the EcR and the mouse androgen receptor

(mAR) identified one chimera, composed of the mAR N-terminus and the remainder from EcR (mAR\EcR-CDEF) that was transcriptionally silent and inducible by Usp. In contrast, the vertebrate homologue, human retinoic acid receptor (RXRα), showed ligand-independent transcription when co-expressed with EcRΔA/B but not mAR\EcR-CDEF. Therefore, RXRα does not require its partner to possess an N-terminal domain, yet is intolerant of a heterologous N-terminus. Similarly, the human vitamin D receptor, which has a short N-terminal region, showed greater ligand-independent transcription in the presence of RXRα than in the presence of Usp. These results reveal a mechanistic basis for the differential activities among the EcR isoforms, and between Usp and RXRα. Furthermore, they provided the foundation for a genetic screen to identify potential insecticides as well as accessory proteins for Usp and EcR. *Journal of Molecular Endocrinology* (2000) **24**, 183–191

INTRODUCTION

Insect metamorphosis is in part controlled by ecdysone, a steroid hormone. Ecdysone binds to the ecdysone receptor (EcR), inducing the transcription of a hierarchy of genes that harbor ecdysone-specific response elements (RE) upstream of their promoters. The *Drosophila melanogaster* EcR gene encodes for three isotypes (-A, -B1 or -B2) that are typical steroid/nuclear receptors and share common C-terminal DNA-binding domain and ligand-binding domain (LBD) sequences, but have different N-terminal A/B domain sequences

(Koeller 1992, Talbot *et al.* 1993). The EcRs also bind DNA in the absence of ecdysone, suggesting that unliganded EcRs also regulate target genes (Buszczak & Segraves 1998, Hall & Thummel 1998 and references therein). The EcRs are differentially expressed, with EcR-A being present in cells that differentiate to form adult tissues while EcR-B1 and EcR-B2 are predominantly expressed in larval cells fated to die. Such observations suggest that cell fate is, at least in part, controlled by EcR isoform expression (Talbot *et al.* 1993).

The EcR has an obligatory heterodimer partner called ultraspiracle (Usp). Usp is a homologue of

the vertebrate retinoid X receptor alpha (RXR α) (Oro *et al.* 1990), a steroid/nuclear receptor that is a heterodimeric partner to a growing list of nuclear receptors (reviewed in Mangelsdorf & Evans 1995). Usp and RXR α can substitute for one another in forming a heterodimeric receptor: RXR α can heterodimerize with EcR-B1 and Usp with the human vitamin D receptor (huVDR) (Yao *et al.* 1992). However, Usp is distinctly different from RXR α in at least two properties. First, it does not bind retinoic acid, and is probably an orphan receptor, although it has been reported that Usp binds juvenile hormone III (Jones & Sharp 1997). Secondly, like RXR α , Usp is a global regulator of genetic pathways, including some independent of ecdysone (Segraves 1994, Sutherland *et al.* 1995, Hall & Thummel 1998 and references therein). However, despite extensive searches for heterodimeric partners other than EcRs, only the *Drosophila* hormone receptor 38 (Sutherland *et al.* 1995) has been identified. The complex roles of Usp and EcRs in insect development may include more diverse mechanisms than those accommodated by simple models of ligand-activated gene transcription.

Saccharomyces cerevisiae lacks steroid/nuclear receptors and their specific co-regulators. As a result, yeast has been extensively employed by researchers in basic science and drug discovery to reconstitute steroid/nuclear receptor function (Garabedian 1993, Butt & Walfish 1996). Faithful ligand-activated target gene transcription is commonly observed in yeast cells expressing steroid/nuclear receptor(s) through the use of reporter plasmids with cognate REs upstream of synthetic promoters. Co-transfecting steroid-specific co-activator proteins and other transcription factor accessory proteins can optimize the activity of some receptors. Conversely, the absence of co-regulators in yeast provides a powerful *in vivo* system in which the intrinsic properties of receptors and subtle differences among receptors can be investigated. Using the yeast system, we identified in this study three novel aspects of the EcR and Usp: differences among EcR isoforms; a Usp specific activity; and a distinction between Usp and RXR α . Additionally, we constructed a yeast strain in which Usp activity is easily monitored. This strain can be used to screen for chemicals with potential insecticide activity, or for novel Usp and EcR accessory proteins.

MATERIALS AND METHODS

Construction of yeast expression plasmids

Standard methods for manipulating DNA and yeast were used (Sambrook *et al.* 1989, Guthrie & Fink

1991). Yeast expression plasmids (YEp) and the derivative containing a multiple cloning site (YEpc) were used to insert the open reading frames of the receptor used. Plasmids YEpcUSP and YEpEcR-B1 were constructed as described elsewhere (Dela Cruz & Mak 1997). Plasmids YEpEcR-A and YEpEcR-B2 were made from YEpEcR-B1, by excising an AflII-AscI fragment, and inserting polymerase chain reaction (PCR) fragments containing EcR-A and EcR-B2 specific sequences. The YEpEcR Δ A/B plasmid was made by excising from YEpEcR-B1 a BamHI-BspEI fragment and inserting two PCR fragments. Plasmids encoding chimeras were constructed using YEpEcR-B1 and YEpMAR5 (Mak *et al.* 1994b). Plasmid YEpEcR\MAR-DE was made by excising a SacI-BspEI fragment and replacing it with a PCR product derived from YEpMAR5 (MAR being mouse androgen receptor). Plasmid YEpMAR\EcR-DEF was made by inserting a DraIII-BssHII fragment from YEpMAR5 (Mak *et al.* 1994b) into the SacI-BspEI site of YEpEcR-B. Plasmids YEpMAR\EcR-EF and YEpMAR\EcR-E were made by cloning PCR fragments derived from EcR-B1 into YEpMAR6 (P Mak, unpublished observations). Plasmid YEpMAR-D\EcR-DEF was cloned similarly to YEpMAR\EcR-DEF except the DraIII-BssHII fragment was cloned into YEpMAR6. The plasmid YEpMAR\EcR-CDEF was constructed by inserting a PCR product from YEpMAR (Mak *et al.* 1994b) into YEpEcR Δ A/B. Plasmids YEpMAR5 and YEpRXR α are as described (McDonnell *et al.* 1989, Mak *et al.* 1994a), except the copper-responsive yeast metallothionein (CUP1) promoter drives expression of Usp and RXR α , and the constitutive yeast glyceraldehyde-3-phosphate dehydrogenase (TDH3) promoter drives expression of the EcRs, the chimeras, and huVDR.

Construction of reporter plasmids

The YEpEcRRE₂-LacZ reporter plasmid (Dela Cruz & Mak 1997) (RE=reporter element; subscript=in duplicate) was used to make YEp-UspRE₂-LacZ and YEp-VDRRE₂ (vitamin D reporter response element₂). A XhoI fragment from YEpEcRRE₂-LacZ was excised and replaced with tandem duplicate enhancer sequences separated by an arbitrary six bases. For YEp-UspRE₂-LacZ, the enhancer was the 21 base pair sequence corresponding to the -64 to -44 region of the chorion σ 15 promoter (Shea *et al.* 1990). For YEp-VDRRE₂-LacZ, the enhancer was a 25 base pair sequence corresponding to the -262 to -242 region of the rat 24-hydroxylase distal gene (r24-Hlase(d)) (Kephart *et al.* 1996). YRpA₂ has been described previously (Mak *et al.* 1994b).

S. cerevisiae host strains

Plasmid YEp*UspRE₂-LacZ* was used in the protease deficient strain BJ2168 which has the following genotype: MATa leu2 trp1 ura3–52 prb1–1122 pep4–3 prc1–407 gal2. Plasmid YEp*EcRRE₂-LacZ* and YRpA₂ were used in a canavanine permease (*can1*) mutant yeast strain, CGY44(DC45): MATa ste11-Δ1 his4–519 leu2 trp1 ura3 can1–101. Transformations of yeast cells were performed according to standard procedures and transformants were grown in the appropriate selective media for plasmid maintenance (Guthrie & Fink 1991).

Preparation of cytosolic extract

Yeast cells were grown at 30 °C overnight with constant agitation in minimal media supplemented with 2% glucose and 100 μM CuSO₄ as needed, and subcultured into fresh media in the absence or presence of steroid agonists, until the cell density reached mid-log phase (optical density (OD)₆₀₀=1.0). To prepare cytosolic extracts, cells maintained at 4 °C were harvested by centrifugation, washed twice, and lysed in 1/100 volume by five cycles of 1-min vortexing with glass beads (0.5 mm; B. Braun Instruments, Kronberg, Germany) followed by 1 min on ice and high speed centrifugation. The protein concentration of the supernatant or cytosolic extract, determined by the Bradford protein assay (Bio-Rad, Richmond, CA, USA), was typically between 5 and 10 mg/ml. For hormone-binding assays, the cells were resuspended in ice-cold TEDG (10 mM Tris, pH 8.0, 0.1 mM EDTA, 2 mM dithiothreitol, 10% glycerol) containing 0.4 M NaCl and the protease inhibitors (150 μg/ml phenylmethylsulfonyl fluoride, 4 μg/ml leupeptin, 3 μg/ml chymostatin and 1 μg/ml pepstatin). An equal volume of glass beads was added, the cells were lysed by vortexing and the suspension was centrifuged at 1000 *g* for 10 min to remove unbroken cells and then at 100 000 *g* for 30 min to pellet the particulate fraction and recover the cytosolic extract. For the β-galactosidase (β-Gal) assays, the cells were resuspended in 500 μl Z-buffer (60 mM Na₂HPO₄·7H₂O, 40 mM NaH₂PO₄·H₂O, 10 mM KCl, 1 mM MgSO₄·7H₂O, 50 mM β-mercaptoethanol, pH 7.0), lysed in a microfuge tube containing a one-third volume of glass beads, and microfuged for 10 min at 12 000 *g*.

Bioassays

The standard binding assay was performed in a total reaction volume of 200 μl TEDG, including 200–400 μg cytosolic extract and 2.5 nM [24,25,26,27-³H(N)]Ponasterone A (³H-Pon A),

195.2 Ci/mmol (New England Nuclear, Boston, MA, USA), in the absence or presence of 1000-fold excess of muristerone A (Mur A). After incubation for 1 h at 25 °C with constant agitation, the reaction was passed through an Inotech A21 glass fiber filter attached to the Inotech Cell Harvester (Inotech Biosystems Int., Rockville, MD, USA) maintained under vacuum, washed with three volumes of TEDG, and subjected to the vacuum for 1 additional min. The filters were placed in 3 ml scintillation cocktail (Ready Safe; Beckman Coulter Inc., Fullerton, CA, USA) and radioactivity was measured as c.p.m. in a Taurus Automated Liquid Scintillation Counter (ICN Biochemicals Inc., Irvine, CA, USA). The readings from assays performed in the absence and presence of Mur A represent total and non-specific c.p.m., and the difference is specific counts or specific binding of ³H-Pon A. Binding was done in triplicate and the data were analyzed using GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA).

The standard transcription assay consisted of preincubating 800 μl Z-buffer containing 5–20 μg cytosolic extract for 10 min at 28 °C and then adding 200 μl *O*-nitrophenyl β-D-galactopyranoside (4 mg/ml dissolved in water) for an additional 10 min. The reaction was stopped by adding 500 μl 1 M sodium carbonate and the OD₄₂₀ was measured. Each treatment was done in triplicate and the average values were converted to Miller units (Mu) mg protein (Miller 1972).

RESULTS

Behavior of EcR isoforms in yeast cells

Dela Cruz & Mak (1997) expressed the EcR-B1 isoform in yeast cells. Extracts from cells expressing the EcR-B1 and *Usp* showed high-affinity ligand binding in these studies. However, *in vitro* β-Gal assays showed that EcR-B1 activated *lacZ*, a reporter plasmid that contained an ecdysone RE. Neither co-expression of EcR-B1 with *Usp*, nor growth of these cells in the presence of ecdysone further increased expression of the reporter gene. This β-Gal assay was used here to compare EcR-B1, EcR-A and EcR-B2. In the absence of *Usp*, EcR-B1 showed significant ligand-independent transactivation (Table 1), while EcR-A displayed approximately 60% the activity of EcR-B1, and EcR-B2 showed about one-tenth the activity of EcR-B1. Co-expression with *Usp* increased by over fourfold the activation levels with EcR-B1, about threefold with EcR-A, and there was no detected increase with EcR-B2.

TABLE 1. The EcR isotypes and Usp show ligand-independent transcriptional activity. β-Gal activity in yeast strains containing the reporter plasmid YEp*EcRRE₂-LacZ*, and expression plasmids for YEp*EcR-B1*, YEp*EcR-A* or YEp*EcR-B2*, and YEp*c* (–) or YEp*USP* (+). The activity of untreated (–) and 10 μM ligand (Mur A)-treated cells are compared. Induction over the absence of nuclear receptor is calculated from a basal mean of 58. The results show the mean ± s.d. from four independent clones, for each condition, and are presented in standard Miller units (Mu/mg)

Nuclear receptors		β-Gal activity (Mu/mg)		Induction	
EcR isoform	Usp	(–)	Mur A	Activity/basal activity*	Fold activation by Usp
—	–	23 ± 52	57 ± 69	1	
—	+	88 ± 64	63 ± 72	1	1
EcR-B1	–	4103 ± 709	4135 ± 716	71 ± 9	
EcR-B1	+	18 007 ± 1967	15 691 ± 548	310 ± 34	4.4 ± 0.3
EcR-A	–	2523 ± 710	2890 ± 729	43 ± 13	
EcR-A	+	6694 ± 3187	6144 ± 2198	116 ± 37	3.0 ± 1.5
EcR-B2	–	480 ± 150	431 ± 107	8 ± 2	
EcR-B2	+	459 ± 120	517 ± 3	6 ± 2	0.9 ± 1.5

*Basal mean for all experiments is 58.

These experiments were repeated using cells that had been treated with Mur A, a high-affinity ecdysone receptor agonist. Mur A produced no significant increase in reporter activity in any of the ecdysone receptor expressing cells (Table 1). To eliminate the possibility that Mur A does not diffuse into the yeast cell, a number of structurally distinct ecdysone agonists, including RH5849 (a non-steroidal, diacylhydrazine ecdysone receptor ligand), were also tested and failed to induce transcription (data not shown). Radioligand binding studies with ³H-Pon A were performed with cells expressing Usp and each receptor isoform to determine whether the lack of responsiveness to Mur A was due to the inability of the ligand to bind the receptors. *In vitro* ligand competition curves (Fig. 1A–D) showed apparent high-affinity binding to ³H-Pon A, and an order of potency for ecdysone agonist competitors, Pon A>Mur A>ecdysone>RH5849, that was similar for the three isoforms. Since the three receptor isoforms showed similar binding kinetics, the differences in activity among them was not the result of differential ligand binding. In addition, the absence of transactivation in yeast cells expressing EcR-B2 cannot be explained by poor receptor expression since extracts from all three cells showed similar ligand binding (approximately 15 000 c.p.m./200 μg protein).

The enhanced reporter expression observed in cells co-expressing EcR-B1 or EcR-A suggested that Usp might have intrinsic ligand-independent transcription activation activity. To test this, a reporter plasmid containing a Usp RE was used to measure Usp-specific transactivation. However, this reporter was transcriptionally active in the absence of Usp, and was not further transactivated by Usp

(data not shown). Therefore, an approach employing receptor chimeras was used to assess for transactivation by Usp.

Behavior of EcR-mAR chimeras in yeast cells

Steroid/nuclear receptors are composed of structurally and functionally conserved domains. Transactivation is produced by the A/B domain, DNA binding is mediated by the C domain, the D domain is required for nuclear localization, the E domain determines ligand binding as well as heterodimerization, and the F domain (present in some and absent from other receptors) has a less defined function (reviewed in Mangelsdorf & Evans 1995). In yeast, the mAR displayed ligand-dependent transcription activation (Mak *et al.* 1994b). We therefore constructed receptor chimeras containing complementary portions of the EcR-B1 and the mAR (Fig. 2) to identify regions responsible for the observed ligand-independent transactivation of the EcR-B1.

As expected (Table 2), an EcR with a truncated A/B domain (EcRΔA/B) produced significantly less (44%) transcriptional activation compared with EcR-B1, and the activation of EcRΔA/B was not further increased with Usp. Since ligand binding is dependent on heterodimerization between Usp and EcR, radioligand-binding experiments were performed with extracts from cells expressing both Usp and EcRΔA/B. Saturation binding curves with these extracts containing both EcRΔA/B and Usp were similar to those with EcR-B1 and Usp (Fig. 1E and F). In addition, Scatchard analysis showed nearly identical high-affinity dissociation constants (*K_d*)

FIGURE 1. Competition with agonists of ^3H -Pon A binding to yeast cytosolic extracts containing Usp with EcR-B1, EcR-A and EcR-B2 or EcRΔA/B (A–D) and saturation binding and Scatchard analysis with Usp and either EcR-B1 or EcRΔA/B (E–F). The standard competition binding assay containing 2.5 nM ^3H -Pon A was done with increasing tenfold concentrations from 10 pM to 100 μM of the competitors, Pon A (●), Mur A (○), 20-hydroxyecdysone (Ec; ■) and RH5849 (Hydra; □). The results are graphs showing the percentage bound compared with the amount bound in the absence of a competitor. The standard saturation binding assay was done with concentrations of ^3H -Pon A of 0.1, 0.5, 1.0, 2.5, 5, 10, 20 and 40 nM and in the absence and presence of unlabeled Pon A. The results are shown in graph form for total binding (●), non-specific binding (■), and their difference, the specific binding (○). Scatchard plots were used to obtain apparent K_d values of 3 nM for the EcR and 2 nM for the EcRΔA/B respectively.

for both. Therefore Usp and EcRΔA/B associate together, and the inability of Usp to enhance EcRΔA/B transactivation is due to the absence of the EcR-B1 N-terminal domain.

To further characterize the contribution of the N-terminal domain of the EcR to the ligand-independent activity of Usp, Usp was co-expressed

with the EcR mAR chimeras. These chimeras were designed to substitute mAR domains throughout the EcR and included virtually all contiguous combinations of functional domains from the EcR and mAR. Usp increased reporter activity in all chimeras (Table 3) except one, mAR\EcR-DEF. Since no single EcR domain was required for this

FIGURE 2. Schematic comparison of the EcR (open boxes), mAR (hatched boxes), the N-terminal truncated EcRΔA/B, the chimeras between the EcR and mAR and Usp (checkered boxes). The recombinant proteins are depicted by the standard nomenclature designating the functional domains, A through F, of nuclear receptors and are aligned by their C domains. Protein N-termini are presented to the left and the C-termini to the right. The first amino acid and the number of the amino acid at the C-terminal of each domain are indicated. Also indicated are the addition of three amino acids (solid boxes) or two amino acids (horizontally lined box; in Usp, at the beginning on the left) to a number of the EcR forms and to Usp respectively. The names of the chimeras begin with the nuclear receptor which contributes the N-terminal domain(s), followed by a slash '/' indicating the junction between two nuclear receptors, and ends with the second nuclear receptor plus the domains which it contributes. The last chimera contains D domains from two nuclear receptors and this situation is indicated by a -D after the name of the first nuclear receptor (mAR-D).

activity, there is a large degree of flexibility in the ability of Usp to increase the activity of a receptor partner. This result suggested the possibility that the transactivation was due to the inherent activity of Usp. One of the receptor chimeras that responded to Usp, mAR\EcR-CDEF, produced no detectable ligand-independent reporter activation, indicating that Usp transcription is not dependent on a high basal level of reporter expression. The single non-responsive chimera, mAR\EcR-DEF, consisted of the A/B and C domains from mAR and the D, E and F domains from EcR. This result suggested incompatibility between the C domain of the mAR and the D domain of the EcR, because the extremely similar chimera mAR-D\EcR-DEF, which has an additional D domain from the mAR, and the 'reverse chimera' EcR\mAR-DE both showed increased reporter expression when co-expressed with Usp.

None of the chimeras containing the ecdysone LBD responded to Mur A treatment or bound ^3H -Pon A. In contrast, a receptor chimera containing the androgen LBD (EcR\mAR-DE) responded to testosterone, indicating that domain swapping was permissible in this system. Furthermore, reporter induction by EcR\mAR-DE with Usp (~ 2400 Mu/mg) and by EcR\mAR-DE with testosterone (3500 Mu/mg), when combined, approximated the induction by Usp and testosterone together (6489+144 Mu/mg). This apparent additivity suggested that testosterone and Usp induction of this receptor might occur by independent mechanisms.

RXR α and Usp are selectively interchangeable in yeast

Since RXR α can substitute for Usp in binding and transactivation in yeast (Dela Cruz & Mak 1997), we assayed RXR α for ligand-independent transcriptional activity. We compared RXR α and Usp activity with (1) mAR\EcR-CDEF, (2) the N-terminally deleted EcRΔA/B, and (3) huVDR, which has one of the shortest N-terminal domains of the known steroid receptors. RXR α , interestingly, produced responses opposite to those observed with Usp: RXR α did not activate mAR\EcR-CDEF but did activate EcRΔA/B (Table 3A). The transactivation of huVDR by RXR α was approximately tenfold higher than transactivation by Usp (Table 3B). Treatment with vitamin D (1,25-dihydroxyvitamin D $_3$) had no effect, in accord with previous observations (Kephart *et al.* 1996). These results demonstrated that Usp and RXR α shared the ability to induce ligand-independent transcription, but that Usp was dependent upon the presence of an A/B domain in its heterodimeric partner while RXR α was not.

DISCUSSION

In *S. cerevisiae*, expression of each of the three EcR isoforms resulted in a different level of transcription of a reporter gene containing an ecdysone RE. The level was proportional to the length of the N-terminal domain of the EcR isoform: EcR-B1 (226 amino acids) > EcR-A (197 amino acids) >> EcR-B2 (17 amino acids). A second correlation with the N-terminal domain was enhancement of transactivation by Usp, EcR-B1 > EcR-A and none with EcR-B2. Consistent with these properties, an N-terminal truncated EcR (EcRΔA/B) behaved similarly to EcR-B2. Since the receptors were expressed at similar levels (similar amount of ligand

TABLE 2. Different forms of the EcR show Usp-specific transcriptional activity. β -Gal activity in yeast strains containing a *LacZ* reporter gene, a form of the EcR, and lacking (–) or containing Usp (+). The RE-*LacZ* reporter plasmid contained either *EcRRE₂-LacZ* or *A₂-LacZ*, and was selected based on domain C of the EcR form listed in the first column. The yeast was grown in the absence (–), or the presence of either 10 μ M Mur A or 1 μ M testosterone. The results are presented in Miller units (Mu/mg) as a fold difference between the EcR in the absence as compared with the presence of Usp (Usp (fold induction)). The results are from two independent experiments

Nuclear receptors		β -Gal activity (Mu/mg)					
		No treatment		Mur A		Testosterone	
EcR chimera	Usp	Mu/mg	Usp (fold induction)	Mu/mg	Usp (fold induction)	Mu/mg	Usp (fold induction)
Tested with EcR response element							
—	–	49 $\pm$ 14		43 $\pm$ 11		52 $\pm$ 13	
—	+	70 $\pm$ 17	1.4 $\pm$ 0.4	66 $\pm$ 5	1.5 $\pm$ 0.5	73 $\pm$ 12	1.4 $\pm$ 0.3
EcR-B1	–	3199 $\pm$ 889		3172 $\pm$ 700		3234 $\pm$ 1063	
EcR-B1	+	10 558 $\pm$ 1246	3.3 $\pm$ 0.7	10 212 $\pm$ 913	3.2 $\pm$ 0.7	9846 $\pm$ 724	3.0 $\pm$ 1.0
EcRAA/B	–	1411 $\pm$ 395		1502 $\pm$ 389		1305 $\pm$ 250	
EcRAA/B	+	1435 $\pm$ 190	1.0 $\pm$ 0	1705 $\pm$ 167	1.1 $\pm$ 0.2	1521 $\pm$ 102	1.1 $\pm$ 0.2
EcR\mAR-DE	–	319 $\pm$ 77		320 $\pm$ 96		3548 $\pm$ 289	
EcR\mAR-DE	+	2349 $\pm$ 149	7.5 $\pm$ 1.5	2489 $\pm$ 289	7.7 $\pm$ 1.9	6489 $\pm$ 144	1.8 $\pm$ 0.3
mAR\EcR-CDEF	–	42 $\pm$ 12		51 $\pm$ 12		33 $\pm$ 11	
mAR\EcR-CDEF	+	1988 $\pm$ 378	47 $\pm$ 12	2536 $\pm$ 106	50 $\pm$ 12	3030 $\pm$ 173	92 $\pm$ 33
Tested with mAR response element							
—	–	101 $\pm$ 25		144 $\pm$ 24		89 $\pm$ 70	
—	+	96 $\pm$ 91	0.9 $\pm$ 0.7	191 $\pm$ 23	1.3 $\pm$ 0.1	37 $\pm$ 20	0.7 $\pm$ 0.8
mAR\EcR-DEF	–	315 $\pm$ 51		258 $\pm$ 38		ND	
mAR\EcR-DEF	+	183 $\pm$ 20	0.6 $\pm$ 0	229 $\pm$ 12	0.9 $\pm$ 0.1	ND	
mAR\EcR-EF	–	307 $\pm$ 52		304 $\pm$ 52		ND	
mAR\EcR-EF	+	2029 $\pm$ 122	6.7 $\pm$ 1.5	2054 $\pm$ 14	6.9 $\pm$ 1.1	ND	
mAR\EcR-E	–	304 $\pm$ 4		281 $\pm$ 71		ND	
mAR\EcR-E	+	2781 $\pm$ 214	9.2 $\pm$ 0.6	3751 $\pm$ 286	8.8 $\pm$ 3.2	ND	
mAR-D\EcR-DEF	–	322 $\pm$ 24		246 $\pm$ 62		ND	
mAR-D\EcR-DEF	+	2091 $\pm$ 247	6.5 $\pm$ 0.3	2022 $\pm$ 267	8.5 $\pm$ 2.0	ND	

ND, not determined.

TABLE 3. Usp and RXRα show differences in their ligand-independent transcriptional activity. β-Gal activity (Miller units; Mu) in yeast strains containing YEpRXRα or YEpUsp and (A) YEpEcRΔA/B or YEpEcR-CDEF with YEpEcRRE₂-LacZ, or (B) YEpV5 with YEpVDRRE₂-LacZ. Strains were grown in the absence (–) or presence (+) of vitamin D (Vit D)

		β-Gal activity (Mu/mg)		
		Nuclear receptor		
		Vector	Usp	RXRα
(A) EcR form (with YEpEcRRE ₂)				
—		93 ± 53	107 ± 53	5 ± 50
EcRΔA/B		2085 ± 509	1350 ± 291	4320 ± 1230
mAR\EcR-CDEF		83 ± 25	726 ± 246	128 ± 94
(B) VDR	Vit D (with VDRRE ₂)			
—	—	48 ± 22	59 ± 21	44 ± 23
—	+	63 ± 34	34 ± 14	47 ± 30
+	—	62 ± 34	279 ± 138	2752 ± 700
+	+	55 ± 21	600 ± 148	2891 ± 700

binding), the differences in transactivation potential was due to the intrinsic properties of the EcR isoforms and Usp. The ligand-independent activity of these receptors may be explained by the lack of *Drosophila* accessory proteins and steroid/nuclear receptor coregulators, i.e. co-repressors (reviewed in Mangelsdorf & Evans 1995). Evidence for co-repressor association with EcR, but not Usp or the Usp and EcR complex, was recently reported with the vertebrate co-repressors nuclear receptor co-repressor (N-CoR), silencing of mediator of retinoid and thyroid receptors (SMRT), and Alien, and the *Drosophila* homologue of Alien (Dressel *et al.* 1999, Thormeyer *et al.* 1999). Interestingly, in mammalian cells, Usp, but not RXRα, showed ligand-independent activity when co-expressed with EcR–B1 (Thomas *et al.* 1993). These results suggest that Usp ligand-independent activity might be physiologically relevant.

Two approaches were used to discriminate between the contribution of Usp and the EcR to the ligand-independent transcriptional activity. First, a reporter plasmid containing a Usp RE was not transactivated by Usp. In the second approach a receptor chimera that lacked constitutive activity, mAR\EcR-CDEF, yet responded to the presence of Usp was identified. Since mAR\EcR-CDEF failed to bind ³H-Pon A, it was possible that it was also transcriptionally uninducible by Usp, and merely provided the anchor for Usp, which was the source of the transcriptional activity (N-terminal A/B domain of Usp). This hypothesis was consistent with other observations. First, the EcR\mAR-CDE chimera was additively induced by Usp and testosterone; if the chimera was maximally induced

by testosterone, then Usp would have contributed the additional transcriptional activity. Secondly, Usp's vertebrate homologue, RXRα, produced transactivation when co-expressed with EcRΔA/B. Since the A/B domain is the major ligand-independent transcriptional activation domain, the observed activation was likely dependent upon RXRα. However, RXRα's ability to transactivate EcRΔA/B, as well as its greater ability than Usp to activate huVDR and inability to activate EcR\mAR-CDE, also emphasized a difference from Usp: that RXRα does not require the N-terminal domain of its partner and is less tolerant of a heterologous N-terminal on its partner. Interestingly, in mammalian cultured cells, ligand-independent activation by RXRα was reported to occur through the E domain of the orphan nuclear receptor 1 (Weibel & Gustafsson 1997).

A property of the yeast strain containing the mAR\EcR-CDEF and Usp is its potential ability to detect compounds that regulate Usp activity (Heinrich *et al.* 1997). Such compounds could be used as probes of Usp activity or could provide clues to the chemical structure of the Usp ligand. Furthermore, since Usp is essential for insect development and distinctive from RXRα receptors, such compounds would serve as leads to develop novel selective insecticides. Another potential use of this yeast strain, as well as others described in this report, is to identify nuclear receptor co-regulators and accessory proteins from insect cDNA libraries by expression cloning, since expression of such proteins should also alter Usp and EcR activity. The information obtained by using yeast to delineate the mechanism of function for EcR

isoforms and Usp should provide insights into their complex roles in insect biology and advance the understanding of the function of steroid/nuclear receptors in general.

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