Juvenile hormone potentiates ecdysone receptor-dependent transcription in a mammalian cell culture system

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Abstract:
Insect development is guided by the combined actions of ecdysteroids and juvenile hormones (JHs). The transcriptional effects of ecdysteroids are mediated by a protein complex consisting of the ecdysone receptor (EcR) and its heterodimeric partner, Ultraspiracle (USP), but a corresponding JH receptor has not been defined conclusively. Given that the EcR ligand binding domain (LBD) is similar to that of the JH-responsive rat farnesoid-X-activated receptor (FXR), we sought to define experimental conditions under which EcR-dependent transcription could be promoted by JH. Chinese hamster ovary (CHO) cells were transfected with a plasmid carrying an ecdysteroid-inducible reporter gene, a second plasmid expressing one of the three amino-terminal variants of *Drosophila* EcR or an EcR chimera, and a third plasmid expressing either the mouse retinoid X receptor (RXR), or its insect orthologue, USP. Each of the EcR variants responded to the synthetic ecdysteroid, muristerone A (murA), but a maximal response to 20-hydroxyecdysone (20E) was achieved only for specific EcR combinations with its heterodimeric partner. Notably, the *Drosophila* EcR isoforms were responsive to 20E only when paired with USP, and only EcRB2 activity was further potentiated by JHIII in the presence of 20E. EcR chimeras that fuse the activator domains from VP16 or the glucocorticoid receptor to the *Drosophila* EcR DNA-binding and ligand-binding domains were responsive to ecdysteroids. Again, the effects of JHIII and 20E were associated with specific partners of the chimeric EcRs. In all experiments, the LBD of EcR proved to be the prerequisite component for potentiation by JHIII, and in this conformation may resemble the FXR LBD. Our results indicate that EcR responsiveness is influenced by the heterodimeric partner and that both the N-terminal domain of EcR and the particular ecdysteroid affect JHIII potentiation.

Keywords: Nuclear receptor; FXR; RXR; Ecdysteroid; Juvenile hormone

1. Introduction

Insect development is driven by the action of two hormone classes, the ecdysteroids and the juvenoids ([Riddiford, 1994, Gilbert et al., 2000 and Thummel, 2002]). Simplistically, the simultaneous presence of ecdysteroids and juvenile hormone (JH) leads to larval–larval molting, while an ecdysteroid peak alone is responsible for initiating metamorphosis. JH is also necessary for reproductive processes such as adult female vitellogenesis ([Wyatt and Davey, 1996]). Despite its central importance for insect processes, a single receptor for JH has not been definitively demonstrated in any insect or developmental period, although there has been an abundance of evidence that JH acts upon transcriptional activity ([Wyatt and Davey, 1996 and Dubrovsky et al., 2002]) and also modulates the transcriptional regulation of ecdysteroids ([Cherbas et al., 1989, Farkas and Knopp, 1997, Hiruma et al., 1999 and Zhou and Riddiford, 2002]).

In the fruit fly, *Drosophila melanogaster*, JH influences a variety of processes throughout development ([Riddiford and Ashburner, 1991 and Dai and Gilbert, 1991]) and JHs also modify the influence of ecdysteroid-inducible effects on development ([Chihara and Fristrom, 1973, Restifo and Wilson, 1998 and Richard et al., 2001]). Further, the ability of JH analogues to modulate ecdysteroid-induced chromosomal puffing ([Richards,

1978]) and induce ectopic expression of *Broad*, an early ecdysteroid-responsive gene ([Zhou and Riddiford, 2002]) implies that JH acts upon the ecdysteroid receptor itself.

Ultraspiracle (USP), the nuclear receptor that dimerizes with the ecdysone receptor (EcR) to form the functional ecdysteroid receptor complex ([Thomas et al., 1993, Yao et al., 1993 and Riddiford et al., 2000]) has been implicated as the JH receptor based on its ability to mediate a transcriptional response to methyl epoxyfarnesoate (JHIII) via a direct repeat (DR12) response element in insect cells ([Jones et al., 2001 and Xu et al., 2002]). USP is the insect orthologue of the vertebrate retinoid X receptor (RXR; [Oro et al., 1990]), which itself is responsive to the JH agonist, methoprene ([Harmon et al., 1995]).

Evidence for other mediators of JH action in *Drosophila* have also been presented. These include MET, defined genetically by a methoprene-resistant mutation and encoding a member of the bHLH-PAS transcription family ([Ashok et al., 1998]), which in turn belongs to a class of nuclear receptor-interacting proteins. Other possible mechanisms for JH action involve other nuclear receptors ([Dubrovsky et al., 2002]) and second messenger-mediated phosphorylation events ([Yamamato et al., 1988]). In fact, while the distinctions between these mechanisms have been emphasized, it is yet conceivable that JH regulation in *Drosophila* affects many activities involving the ecdysteroid receptor dimer, phosphorylation of USP during development ([Henrich et al., 1994 and Song et al., 2003]), contributing cofactors, and/or other heterodimeric partners. In other words, JH action may influence the dynamic interplay of several components in the ecdysone receptor complex ([Lezzi et al., 1999]).

Whereas USP has garnered considerable attention for its possible mediation of JH effects, little attention has been paid to the similarity of USP's partner, EcR ([Koelle et al., 1991]), with the vertebrate farnesoid-activated X receptor (FXR; [Forman et al., 1995]). FXR is a nuclear receptor that is strongly activated by JHIII when assayed in a mammalian cell culture system ([Forman et al., 1995]) and induces transcription from a promoter containing the *hsp27* ecdysone response element (EcRE; [Riddihough and Pelham, 1987]). The ligand-binding domain (LBD) of rat FXR shows about 40% amino acid identity with *Drosophila* EcR, primarily among islands of residues amid more diverse peptide stretches. While it is unknown whether the JHIII–FXR interaction involves direct binding, the possibility that EcR functionally resembles FXR bears investigation.

The ability to reconstitute ecdysteroid-responsive transcriptional effects in an otherwise nonresponsive mammalian cell culture by transfection with EcR offers a strategy for testing such features as ecdysteroid-induced transcriptional activity, as well as any possible effects of juvenoids ([Christopherson et al., 1992]). In order to explore the functional capacity of EcR, and its possible similarity to FXR, we have devised a series of experiments to test the ability of various EcR isoforms ([Talbot et al., 1993]) and chimeras ([No et al., 1996]) to mediate a transcriptional response to ecdysteroids and JHIII in mammalian cells which normally lack endogenous FXR and EcR activity. These studies demonstrate that JHIII potentiates the transcriptional inducibility of ecdysteroids acting via EcR and its heterodimeric partner at submaximal dosages.

2. Materials and methods

2.1. *Cell growth and transfection conditions*

Chinese hamster ovary (CHO K1) cells were grown in Dulbecco's modified Eagle medium: nutrient mixture F-12 (1:1) containing 5% fetal bovine serum and supplemented with 50 μg/ml penicillin, and 50 μg/ml streptomycin (Life Technologies) in a water-jacketed incubator held at 37 °C and maintained with a 5% CO_2 atmosphere.

The transcriptional assay utilized in the study is based upon those applied to earlier studies ([Forman et al., 1995]) with specific modifications for the current study. Cells were seeded in six-well polypropylene culture plates (Falcon) with 10^5 cells per well on the day prior to transfection. Transfection was subsequently performed using either calcium phosphate ([Kitareewan et al., 1996]) or a GenePorter reagent (Gene Therapy Systems, Inc, San Diego, CA) following the manufacturer's protocols. Each well received 1.25 μg of $(EcRE)_5$-ΔMTV-CAT (five copies of the *hsp27* EcRE inserted into a mouse mammary tumor virus (MTV) promoter

upstream of the chloramphenicol acetyltransferase (CAT) gene) or $(EcRE)_5$-ΔMTV-LUC (the same promoter attached to firefly luciferase), 1.25 μg of pCH111(SV40 early promoter linked to the β-galactosidase gene), and 0.25 μg of each expression plasmid (EcR, FXR, RXR, USP) that was tested. The cells were incubated with plasmid DNA for 7 h and then washed with 1×PBS. Muristerone A (murA; Alexis Biochemicals) or 20-hydroxyecdysone (20E; Sigma) were dissolved in ethanol to a concentration of 10 mM and diluted as necessary to the final concentration in 2 ml of fresh incubation medium that was then applied to the cells. Similarly, JHIII (Sigma) was dissolved in dimethyl sulfoxide (DMSO) to a concentration of 80 mM and diluted into the incubation medium to its final concentration (20, 40, 80, 160 μM). For experiments to test the effects of chenodeoxycholate (CDCA, Sigma) on FXR and EcR, CDCA was dissolved in DMSO at 20 mM and diluted in the culture medium to a final concentration of 20 μM. A corresponding volume of ethanol and DMSO were added to control cells for all experiments. For all experiments, the cells were allowed to incubate with the medium for 24 h before collection and cell lysates were prepared by described methods ([Kitareewan et al., 1996]). Both β-galactosidase and CAT reporter activity were measured based on previously used methods ([Kitareewan et al., 1996]). Luciferase assays using luciferin followed the specifications of the manufacturer.

From ^{3}H-chloramphenicol counts (for measuring CAT activity) or relative luciferase activity (RLU) measurements, transcriptional activity was measured for each cell lysate. The counts were then normalized by adjusting for differences in β-galactosidase activity, since its expression is controlled by a constitutive promoter and provides an estimate of cell mass. All presented data are normalized fold-inductions based on differences in reporter gene activity between hormonally treated and control cells. In all the cases reported here, the level of β-galactosidase activity generated by a constitutive promoter in treatment groups was at least 75% of the level found in an accompanying control group, and therefore, effects of hormone treatments on cell growth rates do not account for fold-induction differences observed.

2.2. *Description of plasmid vectors*

The GrdEcR vector used in this study has been described previously ([Yao et al., 1992]), as was the VP16dEcR ([No et al., 1996]); the VP16CfUSP vector was kindly provided by Reddi Palli ([Palli et al., 2003]). The USP vector contains the USP LBD of *Choristoneura fumeriferana*, and both VP16 chimeras contain an N-terminal domain that is active in mammalian cells ([Louvion et al., 1993]). The three *Drosophila* EcR isoform vectors (EcRA, EcRB1, EcRB2) were kindly provided by Dr. Markus Lezzi; their construction is described ([Mouillet et al., 2001]). The FXR (rat), mRXRα (mouse), pCHIII and $(EcRE)_5$-ΔMTV-CAT vectors were also described previously ([Forman et al., 1995 and Kitareewan et al., 1996]). The $(EcRE)_5$-ΔMTV-LUC construct was produced by subcloning the promoter region of MTV into the multiple cloning site of the p-LUC plasmid (Promega) and resembles the $(EcRE)_5$-ΔMTV-LUC described previously ([No et al., 1996]).

3. Results

3.1. *JHIII potentiates ecdysteroid-induced transcriptional activity in a mammalian cell cell line transfected with EcR*

In an initial series of studies, a GRdEcR chimera that consists of the rat glucocorticoid receptor (GR) activation domain attached to the EcR DBD and LBD was cotransfected along with mRXRα into CHO cells. The response of transfected cells to murA was measured via the $(EcRE)_5$-ΔMTV-CAT reporter plasmid that carries tandem repeats of the *hsp27* EcRE. The GRdEcR/RXR combination evoked a detectable response at dosages as low as 0.1 μM murA. Furthermore, JHIII potentiated the response of murA in a dose-dependent manner (using 20, 40, 80, and 160 μM JHIII) at submaximal murA dosages (0.1 and 1 μM murA; Fig. 1). JHIII did not display the ability to evoke a response that was greater than the maximal level induced by 10 μM murA, nor did JHIII by itself show an effect on transcription mediated by the GRdEcR chimera, despite the structural resemblance between the LBDs of EcR and the vertebrate FXR, which is highly responsive to JHIII alone ([Forman et al., 1995]).

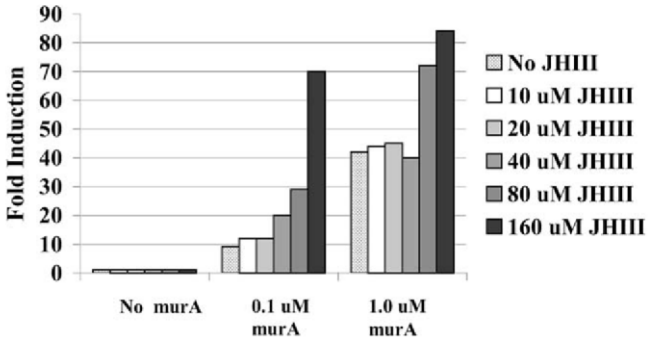

Fig. 1. Potentiating effects of JHIII dosage on murA-induced response mediated by GRdEcR chimera and mRXR using an $(EcRE)_5$ΔMTV-CAT reporter gene in CHO cells. All treatments carried out as described in Materials and methods. Results of one experiment that typified the responses seen in numerous replicates at these dosages.

3.2. *Ecdysteroid responsiveness and JHIII potentiation in EcR chimeras depends upon activating ligand and heterodimeric partner*

The potentiation experiments were repeated with a chimera encoding the VP16 activation domain connected to the DBD and LBD of *Drosophila* EcR, using luciferase as a reporter gene, $(EcRE)_5$-ΔMTV-LUC. The VP16dEcR, partnered with RXR, showed a response to muristerone A (as low as 0.01 μM) that was further potentiated by JHIII (Fig. 2a,b). In fact, VP16dEcR tested with muristerone A generated the most sensitive and robust ecdysteroid response (based on normalized fold-induction) in all the experiments reported here. This chimera also displayed a discernible response to 20E at 10 μM (over 20-fold) using RXR, but this activity was minimally affected by the additional presence of JHIII.

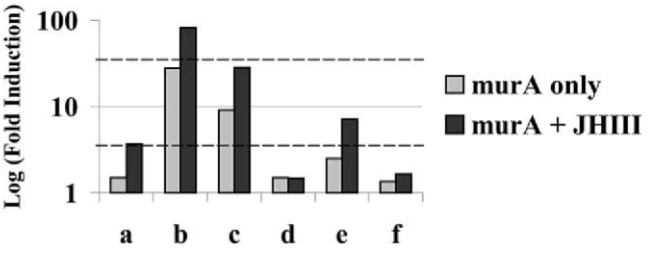

Fig. 2. Ecdysteroid response and potentiation effects of JHIII (log fold induction based on normalized activity in RLUs) measured for various EcR combinations and mouse RXR. Hatched lines indicate five- and 50-fold induction levels. For all combinations, 80 μM JHIII was used. Dosage of murA was based upon preliminary experiments to determine a submaximal dosage wherein JHIII effect, if any, was detectable. (a) VP16dEcR/mRXR at 0.01 μM murA; (b) VP16dEcR/mRXR at 0.1 μM murA; (c) VP16dEcR/VP16CfUSP at 0.1 μM murA, (d) EcRA/mRXR at 1 μM murA; (e) EcRB1/mRXR at 0.1 μM; (f) EcRB2/mRXR at 0.1 μM murA. All data are based on the mean normalized fold-inductions from at least three replicates, except for EcRA (two replicates). Range of fold-inductions was less than 15% among replicates.

The VP16dEcR was also tested with VP16CfUSP (Fig. 2c). The same degree of potentiation was observed using this EcR/USP combination as with the VP16dEcR/RXR dimer when using murA, except that a higher murA dose was required to achieve the same efficacy. The normalized level of JHIII-mediated potentiation of the murA response was similar with either RXR or VP16CfUSP substituted as VP16dEcR's heterodimeric partner. However, the combination of VP16dEcR with VP16CfUSP combination showed no response to 10 μM 20E and was not affected by the additional presence of JHIII.

3.3. *Drosophila EcR isoforms display different capabilities in mammalian cells that depend upon ligand and heterodimeric partner*

In order to evaluate the activity of the natural *Drosophila melanogaster* EcR isoforms, each (A, B1, or B2) was cotransfected into CHO cells with the aforementioned VP16CfUSP fusion protein and transcriptional response evaluated. All three isoforms showed some transcriptional capability in the mammalian cell culture system, though none showed the same dosage sensitivity seen earlier with VP16dEcR/RXR. When tested in the absence of hormone, the EcRB1/VP16CfUSP combination showed a relatively high level of (ligand-independent) transcription, between 10 and 20-fold higher than the basal levels found in any other experiment involving EcR. The EcRA isoform also showed an elevated basal level of transcription (two- to three-fold higher when tested with VP16CfUSP), whereas the basal activity of the B2/VP16CfUSP dimer was about the same as those produced by VP16dEcR/RXR and GRdEcR/RXR. With VP16CfUSP, all three *Drosophila* isoforms were induced by about 30–40-fold at 1 μM murA.

When tested with VP16CfUSP at 0.1 μM murA, the response of all isoforms was further potentiated by the additional presence of JHIII (80 μM; Fig. 3) and the effect proved to be dose-dependent, as noted earlier for GRdEcR (data not shown). Moreover, the range of the normalized fold inductions was small for all the experiments reported in Fig. 3, varying by less than 15%.

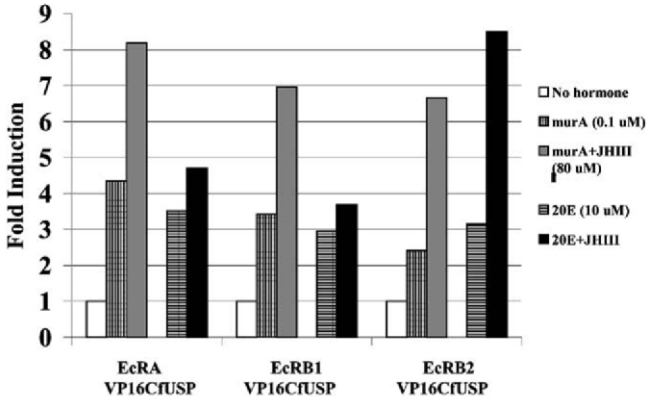

Fig. 3. Effects of murA, 20E and JHIII on RLU activity induced by $(EcRE)_5$ΔMTV-LUC in CHO cells cotransfected with a *Drosophila* EcR isoform and VP16CfUSP. All activities are normalized fold-inductions from cells incubated with hormone for 20 h compared to luciferase activity levels in cells incubated with solvents only. Each fold induction represents an average based on three or four (B1) replicates, and range was less than 15% mean fold-inductions for each data point. All combinations were also tested with JHIII alone (80 μM), which registered no significant effect on normalized RLU activity.

The *Drosophila* EcR isoforms and VP16CfUSP were also tested with 20E (Fig. 3). At a dosage of 10 μM 20E, all three generated a consistent and discernible transcriptional response, but only the EcRB2/VP16CfUSP dimer was potentiated significantly by the additional presence of JHIII. The singular ability of B2 to be potentiated by JHIII in the presence of 20E, whereas all the isoforms were potentiated in the presence of murA, reveals that the effect of JHIII depends upon both the N-terminal domain of EcR and the activating ecdysteroid.

The dimeric partner also affected the activity observed among the three EcR isoforms. In contrast to the robust response observed when RXR was tested with VP16dEcR, RXR did not mediate a response to murA as a dimer with either EcRA or B2. Only EcRB1/RXR displayed a response to murA among the three isoforms (Fig. 2d–f), but the levels of transcription were relatively low and dramatically reduced from those noted for the B1/USP combination described earlier. The murA response was further potentiated by JHIII with the B1/RXR combination, though JHIII by itself failed to evoke any response. Unlike the 20E response noted for all the isoforms with USP as a partner, none of them showed a 20E response with RXR as a heterodimeric partner and absolute transcription levels were also relatively low with RXR.

When contrasting the activity evoked by RXR and USP with the EcR isoforms and chimeras, it is evident that ligand-independent and ligand-dependent transcription, as well as JHIII potentiation depend upon an interplay of the EcR N-terminal domain, the activating ecdysteroid, and the heterodimeric partner. The most relevant observations include: (1) responsiveness of the natural EcR isoforms to 20E requires USP as a dimeric partner; (2) among these isoforms, further JHIII potentiation in the presence of 20E occurs only with the B2 isoform and USP; (3) specific combinations of the EcR N-terminal domain and the heterodimeric partner (e.g. VP16 and RXR, B2 and USP) result in a functional receptor that is capable of showing an ecdysteroid response and/or JHIII potentiation; and (4) ligand-independent levels transcriptional activity depend upon both the EcR N-terminal domain and the heterodimeric partner, as noted for B1. The potentiation observed in the experiments cannot be attributed to the activation of RXR by either JHIII or a JHIII metabolite, since JHIII showed no activity by itself on the assays ([Harmon et al., 1995 and Saez et al., 2000]).

3.4. *FXR interacts with RXR, but not USP, to respond to JHIII*

Because the FXR/RXR heterodimer unmistakably responds to JHIII, a series of experiments was carried out to determine the ability of insect USP to mediate an ecdysteroid and/or JHIII response in conjunction with FXR. The combination of FXR and USP evoked little response in CHO cells to JHIII (Fig. 4), and that is attributable to a low endogenous expression level of RXR characterized in preliminary experiments. The additional presence of ecdysteroids (murA or 20E) with JHIII induced no elevation of FXR-mediated activity. On the other hand, USP was unable to potentiate a response to the strongest activator of FXR, the bile acid CDCA at 20 μM ([Chiang et al., 2000]), and EcR was unresponsive to CDCA alone or as a potentiator of murA response (data not shown). Therefore, the pharmacological relationship that can be deduced is that a specific FXR activator, JHIII, can potentiate the transcriptional response of EcR induced by ecdysteroids. From these experiments, it was also concluded that the JHIII response exhibited by FXR requires RXR as a heterodimeric partner, and that USP cannot be substituted to mediate this response and probably dimerizes weakly, if at all, with FXR.

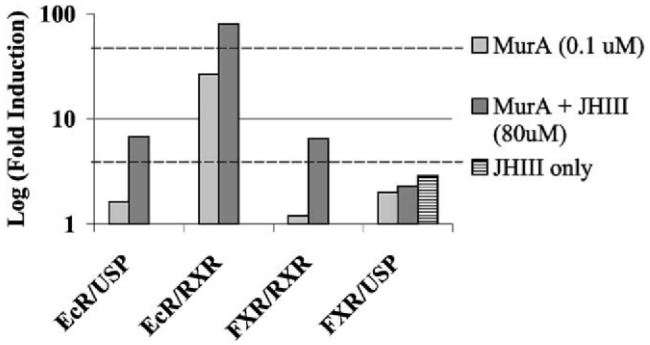

Fig. 4. Response of nuclear receptors to muristerone A and JHIII in CHO cells. All activities are normalized log (fold inductions) based on RLU activity from a simultaneous run that was repeated twice and generated similar trends, though dosage levels varied. EcR refers to VP16dEcR, USP refers to VP16CfUSP. FXR and RXR refer to natural mammalian forms. Hatched lines indicate five-fold and 50-fold induction levels.

4. Discussion

In these cell culture experiments, the presence of a ligand-bound EcR proved to be a prerequisite for observing the potentiative effects of JHIII. Among various EcR forms, the effects of ecdysteroids and JHIII were neither global nor uniform, revealing underlying patterns that implicate multiple factors which cooperatively influence receptor activity, including the activating ecdysteroid, the N-terminal domain of EcR, and the heterodimeric partner.

4.1. *Ligand-dependent and independent activity among EcR constructs*

A collective evaluation of the results (Table 1) shows that murA exhibits activity with a wider range of ecdysteroid receptor heterodimers and is more potent than 20E. Further, the EcR combination influences responsiveness. For instance, only EcRB1 among the *Drosophila* isoforms worked with RXR, and only when murA was the activating ligand. The three natural *Drosophila* isoforms showed a response to 20E only in conjunction with their natural partner, USP. From these observations, it is apparent that 20E responsiveness involves a compatibility between the N-terminal domain of EcR and the heterodimeric partner. This is further indicated by the ability of the VP16dEcR/VP16CfUSP combination to respond only to murA, and not 20E.

Table 1
A summary of ecdysteroid responsiveness and JHIII potentiation among various combinations of EcR with either RXR or USP proteins

Ecdysone receptor	MurA		MurA + JHIII		20E		20E + JHIII	
Description	RXR	USP	RXR	USP	RXR	USP	RXR	USP
GrdEcR	+	n.d	+	n.d.	−	n.d.	−	n.d.
VP16dEcR	++	+	+	+	+	−	−	−
EcRA	−	+	−	+	−	+	−	−
EcRB1	+	+	+	+	−	+	−	−
EcRB2	−	+	−	+	−	+	−	+

Proteins are described in Materials and methods. + designates a change in transcriptional level that exceeds 2.5-fold, ++ indicates a response at a lower dosage than other EcR forms. Responses are based on dosages of 0.1 μM murA, 10 μM 20E, and 80 μM JHIII. For murA and 20E, + indicates inducibility; for columns involving JHIII, + indicates observed potentiation that exceeds two-fold above the levels observed with ecdysteroid alone.

The B1/USP combination displayed the highest levels of basal transcriptional activity, confirming the recognized role of the N-terminal domain for ligand-independent activity. While the inducibility seen in CHO cells with the three isoforms generally resembled the pattern seen earlier in HeLa cells, the high level of B1 basal activity relates to a cell-specific aspect of CHO cultures, since this effect is not seen in HeLa cells. When amino acids 214–263 are deleted from the B1 isoform, it becomes much more active in HeLa cells ([Mouillet et al., 2001]). Possibly, this portion of the N-terminal domain normally interacts with a repressive factor in HeLa cells that is not present in CHO cells.

4.2. *JHIII potentiation among EcR constructs*

The N-terminal domain of EcR and its heterodimeric partner also affect the ability of JHIII to potentiate the response to murA or 20E in CHO cells, though it is unclear whether this involves an activity or the absence of an inhibiting, structural constraint in some N-terminal sequences. The most notable example of specificity is provided by the three *Drosophila* isoforms with USP. All were potentiated by JHIII when murA was the activating ligand, but only the 20E response of B2/USP was substantially potentiated by the simultaneous presence of JHIII. Just as the only isoform/RXR combination that was responsive to hormone was B1/RXR (in the presence of murA), only the B1/RXR isoform was further potentiated by JHIII, and only when murA was the activating ecdysteroid. The specificity imposed by 20E will require further examination, though the tentative conclusion is that structural constraints are imposed by 20E binding which limit the receptor complex's subsequent capacity for potentiation.

The differential ecdysteroid- and JHIII-dependent transcriptional activities noted among the EcR isoforms may offer insights concerning the lack of correspondence between cellular isoform titers and developmental effects in *Drosophila* tissues ([Cherbas et al., 2003]). The B isoforms have been functionally distinguished from the EcRA isoform ([Bender et al., 1997 and Hu et al., 2003]), and recent studies have further separated biological roles for B1 and B2 ([Cherbas et al., 2003]). The B2 N-terminal domain is shorter than B1 and capped with an amphipathic helix ([Talbot et al., 1993 and Hu et al., 2003]). Alternative isoforms in the rat FXR differ greatly in their ability to mediate ligand-dependent transcriptional activity, providing a precedent for this possibility ([Zhang et al., 2003]).

As already noted for the *Drosophila* isoforms, the activating ecdysteroid also determined the ability of JHIII to potentiate a response in other EcR dimers. For instance, the VP16dEcR/RXR combination was responsive to both 20E and murA, but JHIII was incapable of potentiating the response to 20E in this combination, whereas JHIII strongly potentiated the murA response. The compatibility of ligand, EcR, and heterodimeric partner is further exemplified by the VP16dEcR/VP16CfUSP combination. This dimer responded to murA and was further potentiated by JHIII, but did not respond to 20E at all.

4.3. *FXR and EcR*

The comparative studies of FXR and EcR indicate that these two nuclear receptors are not seamlessly interchangeable. Nevertheless, there appears to be considerable functional similarity between the two receptors, because a variety of JH agonists and synthetic analogues, including methoprene and pyriproxyfen, strongly potentiate ecdysteroid responsiveness (Henrich and Weinberger, unpublished). In other words, some activators are also EcR potentiators. FXR activators include phytochemicals known to possess ecdysteroid agonist and insecticidal activity ([Forman et al., 1995]; [Oberdorster et al., 2001]; [Baker et al., 2000]; Weinberger, unpublished). When ingested by insects, such compounds conceivably induce an overstimulated ecdysteroid response and ultimately, an abnormal or premature larval molt. The effect is specific since CDCA is the most potent FXR activator known, but does not modulate EcR-dependent activity, presumably because this bile acid is not biologically relevant for insect receptors.

4.4. *Mechanism for JHIII potentiation*

The mechanistic explanation for JHIII potentiation is that EcR, bound to its cognate ligand, acquires a conformation that allows further activation by JHIII either directly or via an indirect interaction. As noted earlier, it is unclear whether the N-terminal domain plays a passive or facilitative role in the process. Significantly, while fold-potentiation caused by JHIII remains constant over a range of submaximal murA doses, the absolute transcriptional activity attributable to a fixed JHIII dose increases as ecdysteroid molarity increases, indicating that the number of ligand-activated EcR proteins is the critical and rate-limiting factor. In other words, the effect is not a general cellular response that would be the same for a given dose of JHIII, regardless of ecdysteroid molarity. Apparently, as the maximal level of promoter responsiveness is attained in the cell culture system, the weak effect of JHIII is overwhelmed by a saturating number of activated ecdysteroid receptors.

The mechanism of JHIII potentiation is unambiguously different from effects of JH analogues reported previously with RXR. RXR is activated through its LBD by methoprene acid, a metabolite of methoprene, and with the retinoic acid receptor (RAR) generates a response to the ligand through a direct repeat element ([Harmon et al., 1995]). Known RXR ligands also increase the responsiveness of VP16dEcR to murA via the *hsp27* response element ([Saez et al., 2000]) to supra-maximal levels, whereas potentiation by JHIII occurs at a submaximal response through already activated EcR molecules. Another distinction is that RXR ligands activate the VP16EcR/RXR complex even when ecdysteroids are not bound to the EcR partner ([Saez et al., 2000]). By contrast, the effects of JHIII in this study required the simultaneous presence of ecdysteroids for any response to be observed.

This mechanism does not actually address or contradict the results involving USP and the effects of JHIII. Foremost, this study utilizes an *hsp27* EcRE to investigate the responsiveness of the EcR/USP heterodimer, whereas a DR12 element has been used in other experiments to investigate the ability of USP to mediate response to JHIII alone in an insect cell line that endogenously expresses EcR. Interestingly, the DR12 element also evokes a synergistic responsiveness when tested with both 20E and JHIII ([Jones et al., 2001 and Xu et al., 2002]). It is conceivable that USP behaves differently vis-à-vis JHIII in different receptor complexes and on different promoter elements. In fact, the functional distinctions implied by these studies may relate to the separable larval and metamorphic roles of USP ([Hall and Thummel, 1998 and Henrich et al., 2000]).

These experiments require a dosage of 40–80 μM JHIII to see a clearcut potentiation, and potentiation is specific to certain receptor combinations. Other nuclear receptors display transcriptional responsiveness in the

10–100 μM range with activators known to bind to the LBD ([Schmidt et al., 1992, Forman et al., 1995, Kitareewan et al., 1996 and Staudinger et al., 2001]). The situation may reflect the limitations of working in a heterologous and reconstituted transcriptional system in which critical receptor and cellular transport interactions are suboptimal or missing. In other words, ecdysteroids and JHIII might be taken up inefficiently, excluded actively ([Hock et al., 2000]), and/or metabolized rapidly by CHO cells ([Harmon et al., 1995]). Finally, temporal effects upon transcriptional activity may also prove to be an important consideration. The working assumption of these experiments, which rely upon 24-h incubations with hormone, is that inducible transcriptional activity results in the constant accumulation of the reporter protein, a pattern that differs markedly from the dynamic ecdysteroid response observed in *Drosophila* salivary glands ([Henrich et al., 1999 and Thummel, 2002]).

The normalized fold-induction caused by JHIII above the murA response seen in these experiments is also relatively modest, albeit highly repeatable and constant within a given experimental regime. As cell culture and gene microarray experiments have illustrated, replicable and consistent changes in gene expression as low as 1.2-fold can have relevance for understanding biological processes ([Mouillet et al., 2001, Staudinger et al., 2001 and Jin et al., 2001]). Given the difficulty that has surrounded the effort to identify the mode of JH action in insects, therefore, it is plausible to conjecture that the tools for recognizing and assessing the effects of JH action have only recently become available.

As with any cell culture system, however, these effects indicate what the functional ecdysteroid receptor can do in vitro, and it remains to be seen whether it actually behaves similarly in vivo. The notion that ecdysteroid receptor activity is altered by the simultaneous presence of ecdysteroids and juvenoids is consistent with recent molecular and biochemical evidence ([Zhou and Riddiford, 2002]). The tools available in *Drosophila* provide a means to test the relevance of these observations directly.

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