Cryptocephal, the *Drosophila melanogaster* ATF4, Is a Specific Coactivator for Ecdysone Receptor Isoform B2

Sebastien A. Gauthier¹, Eric VanHaaften², Lucy Cherbas², Peter Cherbas², Randall S. Hewes¹*

¹ Department of Biology, University of Oklahoma, Norman, Oklahoma, United States of America, ²Department of Biology, Indiana University, Bloomington, Indiana, United States of America

**Abstract**

The ecdysone receptor is a heterodimer of two nuclear receptors, the Ecdysone receptor (EcR) and Ultraspiracle (USP). In *Drosophila melanogaster*, three EcR isoforms share common DNA and ligand-binding domains, but these proteins differ in their most N-terminal regions and, consequently, in the activation domains (AF1s) contained therein. The transcriptional coactivators for these domains, which impart unique transcriptional regulatory properties to the EcR isoforms, are unknown. Activating transcription factor 4 (ATF4) is a basic-leucine zipper transcription factor that plays a central role in the stress response of mammals. Here we show that Cryptocephal (CRC), the *Drosophila* homolog of ATF4, is an ecdysone receptor coactivator that is specific for isoform B2. CRC interacts with EcR-B2 to promote ecdysone-dependent expression of ecdysis-triggering hormone (ETH), an essential regulator of insect molting behavior. We propose that this interaction explains some of the differences in transcriptional properties that are displayed by the EcR isoforms, and similar interactions may underlie the differential activities of other nuclear receptors with distinct AF1-coactivators.

**Introduction**

Nuclear receptors are multifunctional transcription factors that mediate responses to steroids and other small hydrophobic signaling molecules. Most nuclear receptors have two transcriptional activation functions (AF1 and AF2). AF2 is formed by ligand-induced folding of the ligand-binding domain, and the structural basis of its interaction with coactivators is becoming known. AF1 designates a second, ligand-independent activation function often present in the N-terminal region of the receptor. AF1 sequences are not conserved, and the existence of an AF1 must be inferred from functional assays. Although the AF1s are of considerable interest, because they often differentiate receptor isoforms and because some have been shown to interact with general transcription factors, comparatively few AF1-coactivator interactions have been characterized. The relative contributions of AF1 and AF2 to transcriptional activation vary among receptors, and for any given receptor the relative contributions may depend upon the promoter context [1].

The three isoforms of EcR (FlyBase ID: FBgn0000546) have unrelated AF1 regions, each capable of mediating transcriptional activation in some contexts [2–6]. Although several coactivators and corepressors for the AF2 of EcR have been identified [7–13], the interacting factors for the unique AF1 domains remain unknown. The 17-residue AF1 region of isoform B2 is capable of the interacting factors for the unique AF1 domains remain unknown. The 17-residue AF1 region of isoform B2 is capable of...
bic interactions play roles in its dimerization, probably with the leucine zipper region of CRC. To test this idea further, we made several individual basic-to-acidic mutations within the CRC leucine zipper – at sites predicted to determine the dimerization specificity of the bZIP domain [16] – and tested the binding of the mutant CRC proteins to wild-type and E9K mutant EcR-B2 (Figure 2B). The binding properties of CRC-R347E and CRC-R353E were indistinguishable from those of wild-type CRC, but CRC-R361E bound EcR-B2-E9K. That an alteration in CRC reversed the effect of an EcR mutation strongly implies direct interaction.

Neither USP (FBgn0003964) nor the hormone ecdysone affected the CRC-EcR-B2 interaction as measured in our biochemical tests (Figure 2A). While AF1 activity is hormone-dependent in vivo, that is probably due to the effects of corepressors (e.g. SMRTER) that bind unliganded EcR/USP and suppress the activity of AF1 [4].

Loss of CRC Enhanced Phenotypes in Tissues Requiring EcR-B2

Both the crc mutant phenotype, which includes molting defects that result in supernumerary mouthparts in larvae and failure to evert the adult head at pupal ecysis, and the pattern of crc expression suggest a role for CRC in the ecdysone response [15]. We used a genetic interaction test to determine whether CRC functions as a modulator of EcR-B2 function in flies. We examined the effects of a single copy of crc [a spontaneous mutation, Q171R; FBal0001818] [15,17] on the phenotype produced by targeted expression of the dominant-negative mutant EcR-B1-F645A [2,4]. EcR-B1-F645A is normal in transcriptional repression (the effect of unliganded receptor), but it fails to mediate transcriptional activation. Because the EcR isoforms do not display isoform specificity in DNA binding [5], the EcR-B1-F645A mutant is thought to competitively inhibit all three endogenous EcR isoforms [18]. Hence, a reduction in EcR-B2 coactivator titer should selectively enhance the effects caused by EcR-F645A expression only in tissues requiring the EcR-B2 isoform.

Targeted expression of the dominant-negative receptor EcR-B1-F645A permits an examination of the properties of EcR function in specific tissues in the context of an otherwise normal animal [2]. crc is a recessive mutation, and crc/crc heterozygous cells are phenotypically normal. We generated sensitized tissues by targeting expression of EcR-B1-F645A to five different developmental domains, using specific GAL4 drivers [2]. As shown in Table 1, crc is a dominant enhancer of the EcR dominant negative phenotype in the Eip domain (largely larval epidermis) and in the slbo domain (specialized portions of the follicular epithelium of the egg chamber), but it had no significant effect in the GMR domain (primarily retinal epithelium), the dpp domain (primarily A/P disc boundaries), or the Lsp2 domain (fat body). We have previously described the EcR isoform requirements in each of these domains [2]. There was a remarkable
correlation: Where EcR-B2 is required for development, wild-type crc function was also required, and where normal development does not require EcR-B2, reduction of the CRC titer had little or no effect.

CRC Regulation of ETH Expression

In homozygous or hemizygous crc1 mutant larvae, expression of ETH is markedly reduced [19]. The loss of crc has similar effects on expression of an ETH-EGFP reporter gene, which contains the amino-terminal 17 amino acids of EcR-B2. In lanes labeled B2-E9K, E9K mutant EcR-B2 or E9K mutant GBT-B2-NS was substituted 1:1 for the corresponding wild-type protein. Full-length EcRs were precipitated with a mixture of two EcR-common region monoclonal antibodies, and GBT-B2-NS was precipitated with an antibody to the GAL4 DNA-binding domain. (B) A similar experiment, showing the binding of wild-type and mutant CRCs to full-length EcR-B2. Each incubation mixture contained the 35S-radiolabeled CRC protein listed above and the other components listed below, and precipitations were performed with the same mix of EcR common region antibodies as in (A). Wild-type CRC, CRC-R347E, and CRC-R353E bound to EcR-B2 but not EcR-B2-E9K. However, the basic-to-acidic mutation in CRC-R361E permitted binding to EcR-B2-E9K.

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CRC Regulation of ETH Expression

In homozygous or hemizygous crc1 mutant larvae, expression of ETH is markedly reduced [19]. The loss of crc has similar effects on expression of an ETH-EGFP reporter gene, which contains

Table 1. Dominant effects of crc1 on the phenotypes of EcR-B1-F645A expression.

| Driver (temperature) | crc+crc+ | crc/crc' | Ratio heterozygote/wild-type | Effect of crc' on EcR-F645A phenotype | EcR isoform requirement |
|----------------------|----------|----------|-------------------------------|---------------------------------------|-------------------------|
| GMR (20°)            | 4.7 (21) | 5.5 (40) | 1.2                          | none                                  | any                     |
| dpp (20°)            | 4.2 (33) | 8.5 (72) | 2.0                          | slight suppression                    | any                     |
| Lsp2 (25°)           | 3.7 (12) | 9.5 (25) | 2.6                          | slight suppression                    | any                     |
| Eip (16°)            | 5.1 (15) | 1.0 (13) | 0.27                         | enhancement                           | B2                     |
| slbo (25°)           |          |          |                              | enhancement                           | B2                     |

All flies contained, in addition to the indicated crc genotype, one copy of UAS-EcR-B1-F645A and one copy of the indicated driver. Quantitative data represent % viability to adult eclosion for the indicated genotypes; the nature of the lethality in each case is described elsewhere [5]. Effects of EcR-B1-F645A in the slbo domain were assessed qualitatively by observing the tendency of eggs laid by an affected female to collapse. Each datum was produced by crossing a driver stock to either UAS-EcR-B1-F645A/CyO (crc+/crc+) or UAS-EcR-B1-F645A crc'/crc' (hallucinate). Survival was determined by comparing the number of adults recovered which carry the UAS-EcR-B1-F645A-containing chromosome with the number of adults carrying the CyO balancer; the number in parentheses indicates the number of EcR-B1-F645A-expressing adults recovered. In a control experiment to determine the relative strength of transgene expression in each domain, the Gal4 drivers were crossed to UAS-nuclear-GFP (FBti0012492), the tissues were fixed in 4% paraformaldehyde and washed, and the intensity of direct GFP fluorescence in single confocal sections (signal - background) was quantified. The mean intensities of GFP fluorescence were: dpp (FBti0002123) 71.6+/−9.6, GMR (FBti0002994) 59.3+/−6.3, slbo (FBti0023076) 50.9+/−9.4, Eip (FBti0016770) 30.6+/−4.3, Lsp2 (FBti0018531) 25.8+/−4.8 (n=6). Thus, the intensity of driver expression was not correlated with the ability to enhance the EcR dominant negative phenotype.

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382 bp of the ETH promoter and precisely recapitulates the native pattern of ETH expression [14,19]. Thus, CRC up-regulates ETH expression.

CRC is expressed in many larval tissues, including the endocrine source of ecdysone [15]. Therefore, to test for cell-autonomous regulation of ETH expression by CRC, we used an ETH-GeneSwitch driver to drive transgenic crc RNAi (UAS-crc-RNAi) specifically in the Inka cells [Figure S1], the site of ETH synthesis [14]. GeneSwitch is a conditional GAL4 protein that is activated by addition of the progesterone antagonist RU486 to the food [20]. Compared to the control larvae, larvae with crc RNAi showed a 15-fold or greater reduction in ETH transcript levels (Figure 3A). Thus, CRC was cell-autonomously required in the Inka cells for full ETH expression.

**Regulation of ETH Expression by Ecdysone**

The *Drosophila melanogaster* ETH promoter contains a putative ecdysone response element [19,21], and ETH expression in the tobacco hawkmoth (*Manduca sexta*) fluctuates during the molts and is elevated in response to circulating ecdysteroids [22]. Therefore, we examined whether ETH expression is ecdysone-dependent. In larval and pupal *Drosophila*, expression of the ETH peptide hormone is restricted to 14 endocrine Inka cells located on the trachea [14]. We performed ETH in situ hybridization and found that ETH transcript levels increased gradually during the first few hours after metamorphosis was initiated (Figure 4A). The ETH transcript levels peaked 6–8 hr after the pulse of ecdysone that occurs at pupariation (Figure 4A), suggesting that ETH was transcribed in response to elevation of the circulating ecdysone titer.

We tested for direct ecdysone-dependence of ETH expression in young third instar stage larvae, when circulating ecdysone and ETH transcript and protein levels are low, by feeding them the major active form of ecdysone, 20-hydroxyecdysone (20E) [23]. These larvae carried the ETH-EGFP reporter gene. By 12 hr after the onset of the 20E treatment, the level of ETH-EGFP fluorescence was markedly elevated (Figure 4B). Thus, ETH expression was strongly up-regulated by circulating ecdysone.

To test for a direct, cell autonomous effect of ecdysone on ETH expression, we targeted EcR-B1-F645A dominant negative proteins specifically to the Inka cells with the ETH-GeneSwitch driver. Following Inka cell expression of the EcR dominant negative proteins, ETH transcripts were still present but at levels that were 2–6 fold lower than in wild-type larvae (Figure 3B). Thus, ETH expression was strongly stimulated by ecdysone and required EcR expression in the Inka cells.

**CRC and EcR-B2 Interacted to Boost ETH Expression In Vivo**

The EcR-B1-F645A mutant is effective as a dominant negative when it is expressed in excess of the wild-type isoforms (Table 1) [2,4]. However, the dominant negative EcR-B1-F645A protein competes poorly with wild-type EcR when both are expressed from identical promoters [2,4]. Therefore, to determine which EcR isoforms support up-regulation of ETH expression in the Inka cells, we performed competition experiments in which EcR-B1-F645A and individual wild-type isoforms were coexpressed under the control of the ETH-GeneSwitch driver. The ability of a wild-type EcR isoform to mitigate the effects of the dominant negative is indirect evidence in support of transcriptional activation of the ETH promoter by that isoform.

Supernumerary mouthparts result when larvae fail to complete ecdysis to either the second or the third larval instar, and they are a characteristic feature of the ETH and crc mutant phenotypes [14,15]. Over 95% of larvae with Inka cell-targeted EcR-B1-F645A expression had multiple mouthparts, and ~70% of these animals died as larvae (Figure 5A). Simultaneous expression of wild-type EcR-B2 or EcR-B2-E9K with EcR-B1-F645A fully
rescued lethality and ecdisys of the larval mouthparts, whereas EcR-B1 and EcR-A produced only partial rescue (Figure 5A). Within the Inka cells, ETH transcript levels were fully rescued by EcR-B2, but EcR-B1 and EcR-B2-E9K were ineffective at rescue (Figure 5B). Thus, of the three EcR isoforms, only wild-type EcR-B2 was capable of supporting full ETH expression and successful ecdisys. The E9K mutant of EcR-B2 failed to rescue ETH transcript levels, suggesting a model in which dimerization of EcR-B2 with CRC is required for ETH mRNA expression.

In *M. sexta*, 20E regulates ETH synthesis as well as the competency of the Inka cells to secrete ETH [24]. The ability of EcR-B2-E9K, and to a lesser extent EcR-B1 and EcR-A, to rescue ecdisys and lethality (Figure 5A) indicates that EcR likely regulates other Inka cell processes, such as ETH protein accumulation or secretory competence, that are necessary for signaling by ETH. We tested this hypothesis by performing ETH immuno-staining in EcR-B2-E9K rescue animals both before and after secretion at pupal ecdisys. Although EcR-B2-E9K did not stimulate ETH transcription (Figure 5B), it drove ETH protein accumulation in the Inka cells (Figure S2A). Consistent with the predicted role of EcR in the development of secretory competence, we also observed a marked decrease in accumulated ETH at pupal ecdisys (Figure S2B). These results show that EcR—likely through different sets of EcR isoforms and transcriptional coactivators—regulates ETH protein accumulation independently of ETH mRNA expression.

**Discussion**

Our experiments suggest that the 17-residue B2-specific N-terminus binds to the bZIP region of CRC, that an ionic interaction between EcR-B2-E9 and CRC-R361 plays some role in the binding, and that the interaction of the two proteins plays a crucial role in those tissues where EcR-B2 is essential. These tissues include the endocrine Inka cells, which display ecdisys-dependent upregulation of ETH transcripts and which require EcR-B2 and CRC for full ETH expression. Taken together, these findings implicate CRC as an isoform-specific transcriptional activator for EcR-B2.

In diverse systems, bZIP proteins interact with dyadic or palindromic promoter sequences as homodimers or heterodimers with other bZIP partners [25]. Dimerization involves regularly spaced hydrophobic amino acids that form a coiled-coil between two leucine zipper domains [26]. Other bZIP transcription factors are known to interact with nuclear receptors, modulating the activities of either AF1 or AF2 [27–29], but in the cases reported previously, bZIP proteins bind either to the DNA-binding domain or to the hinge domain of the nuclear receptor. By contrast, CRC (through its bZIP domain) appears to bind directly to the EcR-B2 AF1 region, and its interaction is specific to one EcR isoform.

ATF4, the mammalian homolog of CRC, plays a central role in stress responses [30]. The role of CRC in ecdisys signaling suggests the possibility of interesting and unexpected connections between stress responses and the control of developmental timing and metamorphosis.

The ETH promoter contains sequences matching the consensus half-sites for binding of ATF4 and EcR to DNA. These half-sites are separated by 4 nucleotides, and they are located within a highly conserved sequence (comparing *D. melanogaster* to several other *Drosophila* species) that is 138–171 nucleotides upstream of the ETH transcriptional start site [19]. Since bZIP proteins may bind first sequentially as monomers and then dimerize while bound to DNA [26,31], these observations suggest a model in which CRC participates in the stabilization of EcR-B2 binding to the ETH promoter. This interaction provides a basis for understanding some of the differences in transcriptional properties.
that are displayed by the EcR isoforms and perhaps other nuclear receptors with distinct AF1-coactivators.

Materials and Methods

Yeast Two-Hybrid Screening

Yeast two-hybrid assays were carried out using the Clontech Matchmaker yeast two-hybrid kit (Clontech, Mountain View, CA) and yeast strain H7C. The bait was a fusion of the GAL4 DNA-binding domain to either the 17-residue AF1 domain of EcR-B2, or the same fragment containing the mutation E9K. Binding was assayed as expression of β-galactosidase from a UAS-lacZ reporter.

In Vitro Protein Binding

Proteins were synthesized in vitro using the TNT reticulocyte lysate kit (Promega, Madison, WI); template plasmids were described previously or were generated by a similar procedure [4]. Binding reactions (50 μl) contained 3 μl of each indicated translation mix in buffer A (20 mM NaH2PO4, 150 mM NaCl, pH 8.0) were incubated at 4°C for 30 min. Then, 25 μl of a 50% slurry of Sepharose-protein A (Sigma) loaded with the indicated antibody was added and the incubation continued for 30 min. Beads were washed 3 times in buffer A and then boiled in SDS-PAGE sample buffer. The eluted proteins were separated by SDS-PAGE and radiolabeled proteins detected by autoradiography. For precipitation of full length EcRs, a mixture of the EcR-common region monoclonal antibodies, AG 10.2 and DDA 2.7 [6], was used with each at a 1:10,000 dilution of an ascites fluid. For precipitation of GBT-B2-NS, we used a commercial antibody to the GAL4 DNA-binding domain (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA).

Animals and Staging

Drosophila melanogaster were reared on standard cornmeal-yeast-agar media at 22–25°C unless otherwise noted. Oregon-R was used as the wild-type strain. Larvae at the onset of metamorphosis were scored based on the blue color intensity observed in the gut of third instar larvae fed with cornmeal-yeast-agar food supplemented with 0.1% bromophenol blue. We collected blue gut larvae (18 hours before pupariation) and clear gut larvae (4 hours before pupariation) [52]. Prepupae and pupae were selected based on the criteria reported by Bainbridge and Brownes [33] at the following stages: white puparium (P1 stage; at puparium formation), buoyant prepupa (P4 stage; 6.5–8 hours after puparium formation), and moving bubble prepupa (P4s stage; 12–13.5 hours after puparium formation).

The ETH-GeneSwitch (ETH-GSW) line was a kind gift from Michael Adams (University of California, Riverside) and Yoonsung Park (Kansas State University). It expresses a conditional, RU486-dependent GAL4 protein chimera [20] under the control of the 382 bp ETH promoter region [19,21]. First instar larvae carrying ETH-GSW and selected UAS constructs were transferred after hatching to cornmeal-yeast-agar media supplemented with 500 mM RU486 [34]. In larvae, the expression of a reporter gene under ETH-GSW/RU486 control was restricted to just the Inka cells (Figure S1).

The CRC and EcR loss-of-function transgenes included UAS-Crc-RNAi (Vienna Drosophila RNAi Center (VDRC) line #2935, FBa0084039) [35], UAS-Er-B1-W50A (FBii0029863), UAS-Er-B2-W56A, UAS-Er-B1-F645A (FBii002961), and UAS-Er-B2-E9K. The UAS-Er-B1 (FBii0023086), UAS-Er-B2 (FBii0023085), and UAS-Er-A (FBii002907) transgenes contain the three wild-type EcR isoforms [2].

20E Feeding

Freshly ecysed ETH-EGFP (FBa0136020) third instar larvae were transferred on cornmeal-yeast-agar media supplemented with 0.08 mg/ml 20E [36] and collected 12 hours later for analysis of EGFP fluorescence in the Inka cells.

Tissue Preparation and Image Analysis

Digoxigenin-labeled DNA probe preparation, whole-mount larval in situ hybridization, ETH in situ hybridization, anti-PETH
immunostaining, and ETH-EGFP imaging was performed as described [19]. In larvae, the Inka cells are identified by the tracheal metameremes (TMs) on which they are located, and the TMs are numbered 1 to 10, starting with the anterior end of the animal. To quantify the intensity of EGFP, immunostaining, and in situ hybridization signals, we measured the Intensity Index (AI) = S * I/B [B] where S is the surface area covered by the signal, I is the mean pixel intensity of the signal within this area, and B is the background signal intensity [19, 37]. This method takes into consideration the density of the signal distributed over the cell area, and it therefore normalizes for the angle at which the Inka cell is photographed and for heterogeneity in the spatial distribution of the signal. The measurements were taken using Adobe Photoshop (San Jose, CA, USA).

**Statistical Analysis**

Statistical tests were performed using the NCSS 2001 software package (Kaysville, UT). Bonferroni corrections were performed to minimize type I errors in multiple pair-wise comparisons (Rice, 1989). We used parametric statistics because the data generally followed a normal distribution. All values are means ± s.e.m., except as indicated.

**Supporting Information**

**Figure S1** The ETH-GeneSwitch driver directed transgene expression specifically to the Inka cells. The image is a 2D confocal z-series projection of a larva expressing a LVU-mCD8::GFP (FBti0012685) under the control of ETH-GeneSwitch. The larva was raised on food containing RU486. Expression of mCD8::GFP was limited to the Inka cells. The cells in tracheal metameremes (TM) 1 and 4–9 on one side are labeled with arrows. The additional signal in the gut was due to yellowish autofluorescence. Bar = 200 μM. (TIFF)

**Figure S2** EcR-B2-E9K rescued ETH protein expression and permitted ETH secretion. (A) In TM5, ETH protein expression in Inka cells expressing the dominant negative EcR-B1-F643A isoform was rescued by co-expression of EcR-B2-E9K but not wild-type EcR-B2. Means with the same lower case letters (TM5) were not significantly different (p>0.05). All larvae were fed RU486 and were dissected at ~12 hr after ecysis to the third instar. One-way ANOVA (TM5, p = 0.004955; TM6, p = 0.125) were performed with Bonferroni (all-pairwise) multiple comparison post-hoc tests (n = 4–8). (B) In Inka cells expressing the dominant negative EcR-B1-F643A isoform, a reduction in ETH immunostaining consistent with ETH secretion was observed following rescue by EcR-B2 and EcR-B2-E9K. All animals were fed RU486 as larvae and were dissected either 9 hr after puation formation ("pre-HE") or 30 min after head eversion ("post-HE"). Head eversion (pupal ecysis) occurs at approximately 12–13.5 hr after puation formation [33]. Control animals had the same genotype as the EcR-B2 rescue animals, but were not fed RU486. One-way ANOVA (TM5, p = 0.005607; TM6, p = 0.007636) were performed with Bonferroni (all-pairwise) multiple comparison post-hoc tests (n = 0). Note: Different developmental stages and confocal imaging settings were used for the experiments in panel (A) versus in (B), and the relative protein levels cannot be directly compared between these experiments. (TIFF)

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**Author Contributions**

Conceived and designed the experiments: SAG EV LC PC RSH. Performed the experiments: SAG EV LC RSH. Analyzed the data: SAG LC RSH. Contributed reagents/materials/analysis tools: LC PC. Performed the experiments: SAG EV LC RSH. Analyzed the data: SAG LC RSH. Contributed reagents/materials/analysis tools: LC PC. Wrote the paper: SAG LC RSH.

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