Differential control of gene activity by isoforms A, 131 and 132 of the *Drosophila* ecdysone receptor

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Abstract:

The steroid hormone ecdysone initiates molting and metamorphosis in *Drosophila* via a heterodimeric receptor consisting of EcR that binds hormone, and USP, a homolog of the vertebrate RXR receptor. EcR exists in three isoforms EcRA, EcRB1 and EcRB2 that are thought to direct specific physiological responses to ecdysone. These three isoforms differ only in their N-terminal A/B domain that implies that sequences responsible for the differential physiological effects lie within the A/B domains of the EcR isoforms. In the present study, we set out to determine the capability of the three isoforms and their A/B domains to control gene transcription. When full-length EcR plasmids were cotransfected into mammalian cells with a USP expressing and a cognate reporter plasmid, the three EcR isoforms showed striking differences in their ability to control gene transcription, both in the presence and in the absence of hormone. Furthermore, the A/B domains of EcRB1 and of EcRB2 when fused to the GAL4 DNA binding domain are sufficient to activate transcription of a reporter gene, in yeast as well as in mammalian cells. In contrast, a fusion construct containing the A/B domain of EcRA represses basal transcription of the reporter gene. All these findings emphasize the importance of the A/B domains of the three EcR isoforms for differentially controlling gene transcription. Furthermore, they provide evidence for the existence of an autonomous ligand-independent activation function (AF1) in the A/B domains of EcRB1 and EcRB2 and of an inhibitory function (IF) in the A/B domain of EcRA.

Abstract:

In *Drosophila melanogaster*, the steroid hormone 20-hydroxyecdysone (referred to here as ecdysone) is responsible for the complete reorganization of the body plan at the end of larval life that characterizes metamorphosis. At that time, several pulses of ecdysone activate a genetic cascade that leads to the destruction of larval tissues and their replacement by adult tissues and structures (reviewed in [1]).

The biological activity of ecdysone is mediated by a heterodimer of two members of the nuclear receptor superfamily: the ecdysone receptor, EcR [2] and Ultraspiracle (USP), a homolog of the vertebrate RXR receptors [3-5]. EcR exists in three isoforms, EcRA, EcRB1 and EcRB2, each being able to form heterodimers with USP [6-9]. Existence of three ecdysone receptor isoforms may explain partially how a single hormone drives such a variety of tissue- and stage-specific responses. Indeed, it has been shown that expression of these three EcR isoforms is differentially regulated according to the developmental fate of larval and adult structures [6,10,11]. Genetic analyses using isoform specific mutations have provided more direct evidence that EcR isoforms are functionally distinct [12,13]. However, the molecular basis for the distinct physiological roles of EcR isoforms is not yet understood. The three EcR isoforms contain a common 652 amino-acid C-terminal part that includes the DNA binding domain (C domain) and the ligand-binding domain (LBD or E domain). They

differ only in their N-terminal A/B domains suggesting that the amino-terminal region plays a crucial role in mediating the isoform-specific response to hormonal stimuli.

Numerous studies with vertebrate systems have shown that nuclear receptors generally stimulate transcription by means of two activation functions (AFs), AF1 located in the N-terminal A/B domain and AF2 located in the ligand-binding (D) domain. AF1 is ligand-independent in its transactivation properties while AF2 depends on hormone binding. Nevertheless, there is growing evidence indicating that these two independent AFs communicate via direct or indirect interactions leading to modulation of the overall transcriptional activity of a receptor [14-17]. Although there are several reports on the functional importance of specific binding by EcR and USP C domains to cognate ecdysone response elements (EcREs) [8,9,18], very little is known about their transactivating domains. We initiated studies to test the hypothesis that it is the A/B domain which determines the transactivation specificity of a given EcR isoform. In the present study, we demonstrate that the EcRA, EcRB1 and EcRB2 N-termini have distinct transcription controlling capacities. We also present evidence that each N-terminal A/B region of the EcRB isoforms exhibits an autonomous activation function, while that of EcRA appears to be repressive.

EXPERIMENTAL PROCEDURES
Plasmid construction

The DNA sequences coding for the various EcR and USP constructs were amplified using *Pfu* DNA polymerase (Stratagene). *EcRA*, *EcRB1* and *EcRB2* fragments were generated by PCR from the plasmids pWT57, pMK1 and pWT56, respectively. For *EcRA*, the reverse primer DEr (5'-ACCTCTCTAGACTATG-CAGTCGTCGAGTGGTC) was combined with the forward primer DEAf (5'CACCCGGATCCACCAT-GTTGACGACGAGTGGACAA), while for *EcRB1* and for *EcRB2*, DEr was combined with DEB 1f (5'CACC-CGGATCCACCATGAAGCGGCGCTGGTCGA-AC) or DEB2f (5'CACCCGGATCCACCATGGATACT-TGTGGATTAGTA), respectively. The underlined nucleotides show *Bam*HI, *Eco*RI and *Xba*I cloning sites. The boldface letters indicate the Kozak sequence placed adjacent to the start codon ATG for optimal translation. The expression plasmid encoding an A/B truncated *EcRB1* (252-878) was constructed using the forward primer DE-f (5'CACCCGGATCCACCATGA-AGAAGGGACCTGCGC-CA) and the reverse primer DEr. The expression plasmid encoding USP was constructed as follows: the cDNA for the *Drosophila* USP was generated using the forward primer DUf (TACCCGAATTCCACCATGGACAACTGCGACCAGGAC) and the reverse primer DUr (ACCTCTCTAGACTACTCCAGTTTCATCGCCAG). These fragments were digested with *Bam*HI and *Xba*I (EcR constructs) or *Eco*RI and *Xba*I (USP) and cloned in the pcDNA3 vector (Invitrogen).

The pPAL1CAT reporter plasmid contains four copies of a synthetic EcRE (PAL1) upstream of the *Drosophila melanogaster hsp70* promoter sequence (-50 to +200), in front of the CAT reporter gene [19].

All GAL4-derived expression plasmids for yeast cells are based on the pGBT9 expression vector (Clontech). pGBT9-EcRA(A/B), pGBT9-EcRB1(A/B), and pGBT9-EcRB2(A/B) were made by inserting PCR-amplified fragments coding for EcRA amino acids 1-234, EcRB1 amino acids 1-263 and EcRB2 amino acids 1-54, into *Bam*HI/*Sal*I, *Bam*HI/*Pst*I and *Bam*HI/*Pst*I sites, respectively.

The pGBT9-EcRB1(A/B) deletion mutants were generated by insertion of the corresponding PCR-amplified fragment into *Bam*HI/*Pst*I sites of the pGBT9 vector. All restriction sites were in frame with the template, ensuring an open reading frame from GAL4 through the entire PCR fragment.

The GAL4 expression plasmids for mammalian cells were derived from the pSCTEV-GAL4 plasmid containing the DNA-binding domain of GAL4 (residues 1-93) [20]. Briefly, the various *Bam*HI/*Pst*I EcR fragments were excised from the pGBT9 based constructs, blunted at the *Bam*HI site, and cloned into the pSCTEV-GAL4 vector which had been blunted at the Asp718 sites and then cut at the *Pst* I site. The pG$_5$E1bCAT reporter construct which contains five tandemly repeated GAL4 response elements has already been described [21]. All constructs were verified by sequencing.

Cell culture, transfection, and reporter gene assay

HeLa cells were maintained in Dulbecco's modified Eagle's medium supplemented with L-glutamine, 10% fetal bovine serum, and 100 U·mL^{-1} penicillin/streptomycin. For transient transfections HeLa cells were seeded in 10-cm dishes to give 60 - 80% confluence, and after growth for 6 h the cells were transfected by the standard calcium phosphate co-precipitation technique [22], using 3 µg of each expression plasmid, 5 µg of reporter plasmid (pG5E1b-CAT), 5 µg of plasmid pCH110 (Pharmacia) for transfection control, and calf thymus DNA to maintain a total of 20 µg DNA per plate. After 16 h, cells were washed with NaCl/Pi, and fresh medium containing muristerone A (Sigma) at 1 mm or solvent control (ethanol) was added. Cells were harvested 40 h after transfection, and CAT activity was assayed at 37 °C with 10-µg aliquots of total protein extract. The reaction period was varied between 1 and 6 h in order to optimize the production of monoacetylated [^{14}C]chloramphenicol. Substrate and acetylated products were separated by thin-layer chromatography, and the percentage conversion of [^{14}C]chloramphenicol to the acetylated forms was quantitated using a Molecular Dynamics PhosphorImager. CAT activity of each fusion protein was calculated by normalizing the percent conversion per unit reaction time, and it was standardized by division with control plasmid-derived β-galactosidase activity to yield `relative CAT activity'. Integrity, level of expression and GAL4 response element binding capacity of expressed fusion proteins were confirmed by an electrophoretic mobility shift assay using aliquots of the nuclear extracts (see below).

Yeast transformation and β-galactosidase assay

Yeast strain Y187, which has an integrated *LacZ* gene downstream of GAL4 binding sites, was transformed by the poly(ethylene glycol)/LiAc method [23] with pGBT9-based constructs (Clontech). Transformants were grown in selective medium, disrupted by three freeze/ thaw cycles and P-galactosidase activity was measured using 2-nitrophenyl β-D-galactopyranoside as substrate as specified by the manufacturer. All assays were performed in triplicates and repeated at least three times.

Electrophoretic mobility shift assays (EMSAs)

EcR[ΔA/B], EcRA, EcRB1 and EcRB2 were expressed *in vitro* using a reticulocyte lysate system as recommended by the supplier (TNT T7 Quick coupled Transcription/Translation kit, Promega). The probes used correspond to the double-stranded oligonucleotides *PAL1*/*A* and *hsp*27, a synthetic and a natural *EcRE* element, respectively [19]. For the binding reaction, 2 µL of programmed reticulocyte lysate or 6 µg of nuclear extract were preincubated for 15 min on ice in a buffer containing 20 mM Hepes/KOH (pH 7.4), 100 mM KCl, 5% glycerol, 2 mM dithiothreitol, 0.1% NP-40, 50 µg·mL^{-1} poly(dI-dC) in a total volume of 20 µL; muristerone A at a final concentration of 10 mM was included in the binding reaction mixture as indicated in the text. Approximately 1 ng (30 000 c.p.m.) of an end-filled probe was added to the binding reaction and further incubated at room temperature for 20 min. The complexes were separated on a 4% native polyacrylamide gel in 0.5 x Tris/borate/EDTA.

Nuclear extract from HeLa cells was prepared according to previous reports [24]. Five microliters of this extract were incubated for 15 min on ice with 20 µL binding buffer (50 mM KCl, 5 mM MgCl$_2$, 20 mM Hepes (pH 7.4), 20 µM ZnCl$_2$, 200 mg·mL^{-1} bovine serum albumin, 0.2 M spermidine, 5% glycerol, 100 µg·mL^{-1} poly(dI~dC), 75 µg·mL^{-1} salmon sperm (DNA). Double-stranded GAL4 response element DNA (made of the oligonucleotides 5'-GATCGCACAGTGCCGGAGGACAGTCCTCCGGTTCGAT and 5'-GATCATCGAAC-CGGAGGACTGTCCTCCGGCACTGTGC-3') was end-labeled by fill-in reaction with [α-^{32}P]dCTP to a specific activity of ≈ 10^7 c.p.m.·µg^{-1}, and it was added to the reaction.
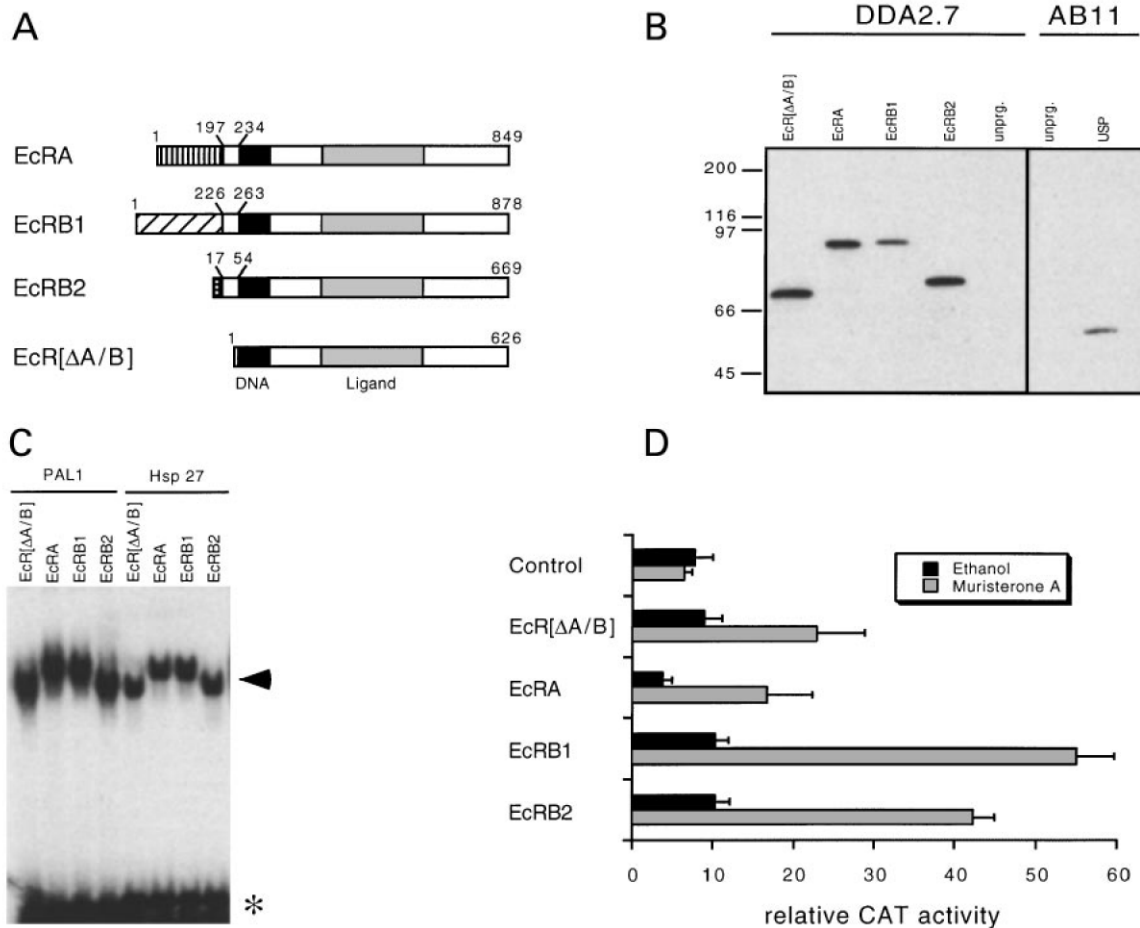
Fig. 1. Differential transcription control capabilities of EcRA, EcRB1, EcRB2 and EcR[ΔA/B]. (A) Schematic representation of the three EcR isoforms and of the A/B-truncated EcR used in the present studies. Black box designates C (DNA binding) domain. To the N-terminal side lies the A/B domain which contains a common region shown in white and an isoform-specific region marked by different patterns. (B) Western blot of *in vitro* translated receptor proteins. Expressed receptors and the monoclonal antibodies used for the detection reaction are indicated above each lane. The position and molecular mass of protein markers (in kDa) are indicated at the left. (C) EMSA of *in vitro* translated EcR isoforms and EcR[ΔA/B] in combination with USP on different EcREs after addition of 1 μM of muristerone A. The position of the various EcR/USP complexes are indicated by an arrow and the free probe by an asterisk. (D) Reporter CAT activity of HeLa cells transiently cotransfected by an expression plasmid containing the respective receptor sequence, a USP expression vector, and the pPAL1-CAT reporter construct. Relative CAT activity was determined in absence (black columns) or presence (gray columns) of 1 μM muristerone A, as described in the text. With each bar, average and standard deviation of three independent experiments are given.

Immunobloting

Equimolar expression of pcDNA3-based constructs was confirmed after *in vitro* transcription/translation (Promega) by Western blot analysis. Reaction mix (2 μL) was loaded onto a 10% SDS gel. After electrophoresis, proteins were transferred onto a nitrocellulose membrane (Schleicher & Schuell) and subsequently incubated with the monoclonal antibody DDA2.7 [2] directed against EcR, and AB 11 [25] against USP. HeLa nuclear extracts (20 μg) from transient transfection experiments were used in Western blot analyses conducted as described above. The blots were washed and developed by enhanced chemiluminescence.

RESULTS
DNA binding analysis of EcR isoforms

As the N-termini were shown to influence the DNA-binding properties of several nuclear receptors [26-28], we wanted to assess the DNA-binding capacity of the EcR isoforms and the truncated EcR (EcR[ΔA/B]), by utilizing an *in vitro* system (Fig. 1A). EMSAs were performed with labeled double-stranded oligonucleotides corresponding to ecdysone response elements (EcREs). One is a natural element of the *Drosophila hsp*27 gene [29], and the other is PAL1, the most potent EcRE, consisting of an idealized perfect palindrome with a single A/T base pair spacing [19]. *In vitro* translated EcR[ΔA/B], EcRA, EcRB1 or EcRB2 were combined with USP, and muristerone A was added to enhance dimerization and DNA binding. All proteins were expressed at similar

levels (Fig. 1B). As shown in Fig. 1C, all EcR isoforms as well as the truncated receptor exhibited a similar DNA-binding affinity. Under the given experimental conditions therefore the A/B region does not seem to differentially influence DNA binding of EcR isoforms and thereby the outcome of CAT activity assays.

Differential transcription control capabilities of EcR isoforms

To compare the transcriptional activity of the EcR isoforms, various EcR expression constructs were transiently transfected into HeLa cells along with an USP expression vector and pPAL1CAT which contains four copies of the synthetic EcRE PAL1. It has been shown previously that in addition to exhibiting maximal DNA-binding activity, PAL1 mediates the highest level of ligand-induced transactivation when compared to other EcREs [19]. This is potentially important because EcR is generally regarded a poor transactivator in mammalian cells [9,18,30]. Western blot analyses using DDA2.7 and AB11 antibodies against the EcR C domain and the USP D domain, respectively, revealed similar levels of expressed EcR forms and USP in all experiments (data not shown).
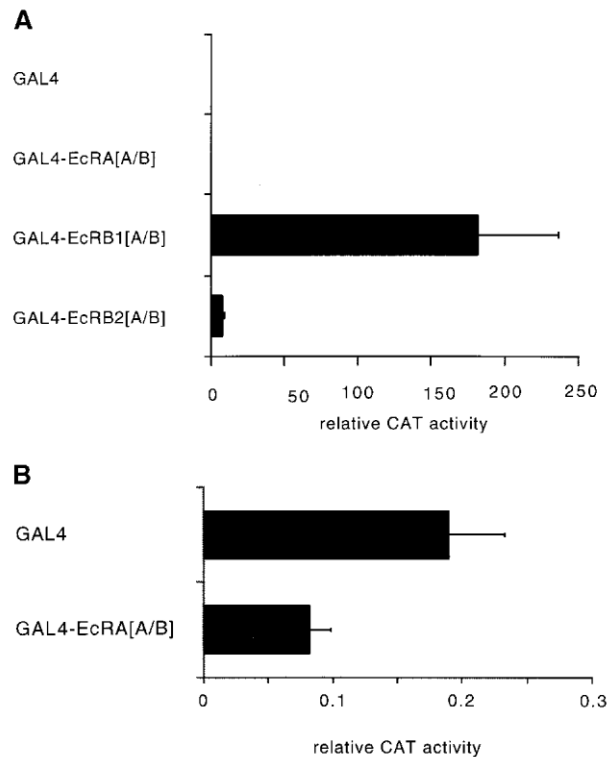
Fig. 3. Transcription controlling activity of the A/B region of EcR isoforms in HeLa cells. HeLa cells were transfected with constructs containing GAL4 DBD either alone or fused to the A/B domains of EcRA, EcRB1 or EcRB2. Reporter CAT activity was determined after 36 h. Cell lysates were incubated either for 1 h (A) of for 6 h (B), and the relative CAT activity was calculated as described in the text. Averages ($\pm$ SD) of three independent experiments are given, with the exception of the upper bar of Fig. 3B where the average ($\pm$ SD) of six independent experiments is shown. Note: the difference between the values of the upper and lower bar in Fig. 3B is statistically significant at $P = 0.95$.

Figure 1D shows the results of CAT assays performed using cell extracts from transfected HeLa cells. In this assay all EcR isoforms as well as the truncated receptor transactivated an EcRE-driven reporter gene in response to the ecdysone agonist muristerone A. However, there were significant differences between the transactivation potential of the EcR isoforms. EcR[AA/B] and EcRA have quite similar ligand-induced transcriptional activities suggesting that the A/B domain of EcRA does not contribute to its transcriptional activity. As a matter of fact, the basal activity of unliganded EcRA was even lower than that of EcR[AA/B]. With EcRB isoforms we could not detect any repressive effect, either in the absence or presence of ligand. In

fact, both EcRB1 and B2 showed an enhanced transcriptional activity when compared to the truncated EcR. It is interesting to note that EcRB1 and EcRB2 show nearly the same activation potentials, despite profound structural differences in their A/B domains, EcRB1 being just slightly more potent than EcRB2.
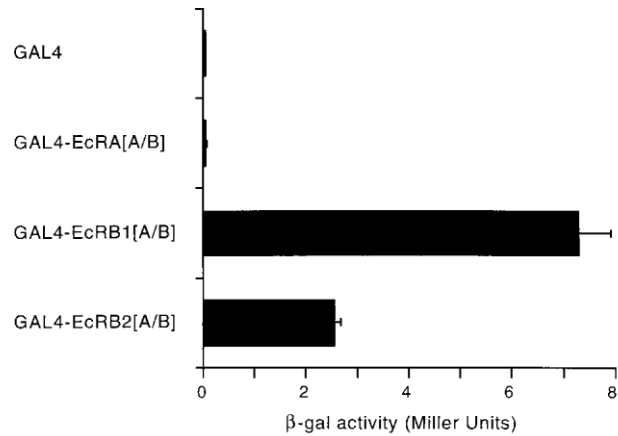
Fig. 2. Transcription controlling activity of the A/B region of the EcR isoforms in yeast. The A/B regions of EcRA (amino acids 1–234), EcR-B1 (1–263), and EcR-B2 (1–54) were fused to the GAL4 DNA-binding domain and transformed into yeast cells (strain Y187). Reporter gene activity evident as β-galactosidase activity was determined from crude lysates as described in the text and expressed as Miller units. Averages (± SD) of at least three independent experiments.

Fig. 4. Amino-acid sequence comparison of A/B domains of EcRB1 of different insect species. Dm, *Drosophila melanogaster* [2]; Aa, *Aedes aegypti* [42]; Ms, *Manduca sexta* [43]; Bm, *Bombyx mori* [44]. The numbers give amino-acid positions. Gaps in sequences are shown by dashes. Black boxes and gray shadings depict amino acids that are identical or conserved, respectively, among the four sequences.


Autonomous transcription control functions in the A/B domains of EcRA, EcRB1 and EcRB2

The fact that EcR isoforms deviate from each other in their capability to control transcription suggests that their A/B domains contain different transcription control regions which either act autonomously or in conjunction with the ligand-dependent activation function (AF2) thought to be located in the E domain. To investigate these possibilities, sequences encoding the A/B domain of the three EcR isoforms were cloned into the yeast expression vector pGBT9 to produce fusion proteins consisting of the respective A/B domain and the DNA binding domain (DBD) of GAL4. These constructs were transformed into the yeast strain Y187. As shown in Fig. 2, the empty plasmid carrying only the GAL4 DBD failed to induce P-galactosidase. Fusion of the A/B domain of EcRA to the GAL4 DBD did not stimulate transcription. By contrast, fusion of A/B domains of either EcRB1 or EcRB2 resulted in a strong stimulation of the reporter gene. The EcRB1 construct was about three times more efficient than the EcRB2 construct. These results indicate the presence of an autonomous activation function within the A/B domain of the EcRB isoforms.

To further characterize the activation potential of the various EcR A/B regions and to rule out the possibility that this phenomenon is restricted to yeast cells, we examined the transcriptional activity of the fusion proteins in HeLa cells containing no endogenous EcR. In this system we used the mammalian expression vector pSCTEV-GAL4 [20], and the constructs were cotransfected with the reporter plasmid pG5E1bCAT and with plasmid pCH110 for normalization of the CAT values. Correct expression and specific binding to the GAL4

response element were checked by EMSA using nuclear extract from transfected HeLa cells and a probe containing the GAL4 elements (data not shown).

The results obtained with the fusion proteins transfected into HeLa cells are shown in Fig. 3. As demonstrated with yeast cells, empty expression vector and the construct carrying the A/B domain of EcRA failed to induce reporter activity. Using longer incubation periods we were able to assess and to compare the transactivation potential of the empty plasmid and the EcRA[A/B] construct in the absence of ligand more precisely (Fig. 3B). Interestingly, the A/B domain of EcRA not only failed to activate transcription but actually repressed the basal transcription of the reporter gene as measured with the empty plamid. This repression of basal transcription was low but statistically significant (see Fig. 3B); it was consistently observed in nine independent experiments, each using a different plasmid preparation. This result indicates that the A/B domain of EcRA contains an autonomous inhibitory function. By EMSA we could rule out the alternative possibilities that the fusion protein with EcRA[A/B] would exhibit a reduced expression and/ or DNA binding ability compared to the fusion protein with EcRB1[A/B].
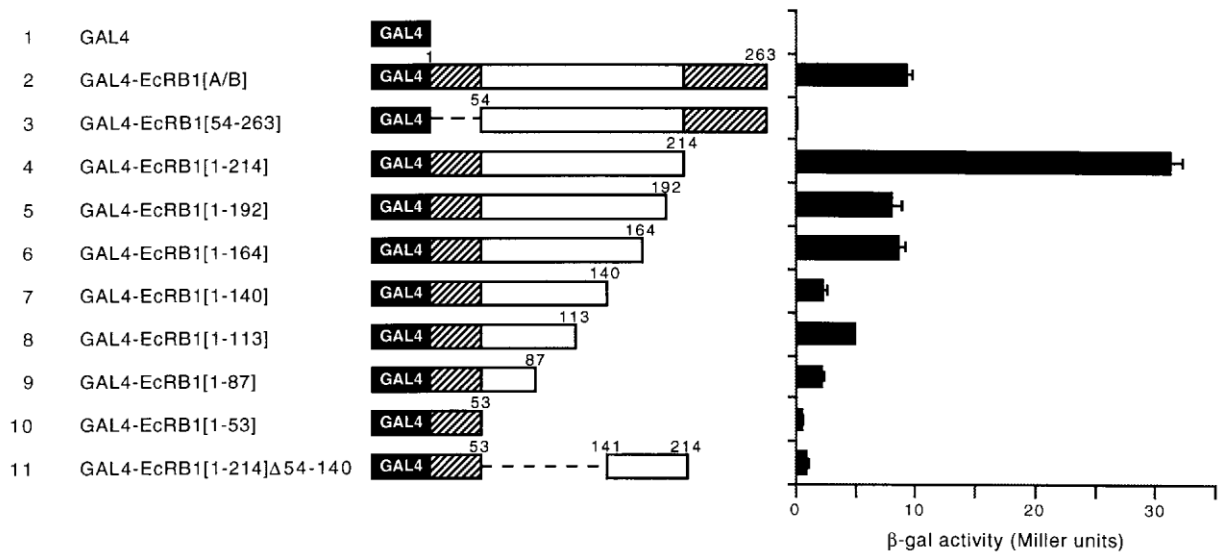
Fig. 5. Functional analysis in yeast of deletion mutants in EcRB1[A/B] fused to GAL4 DBD. The region which is conserved among EcRB1[A/B] from different insects (see Fig. 4) is shown as a hatched box. The DBD of GAL4 is indicated by a black box. The variable region of A/B is depicted as open box. Intervening deleted sequences are marked by dashed lines. Averages (± SD) of three independent experiments.
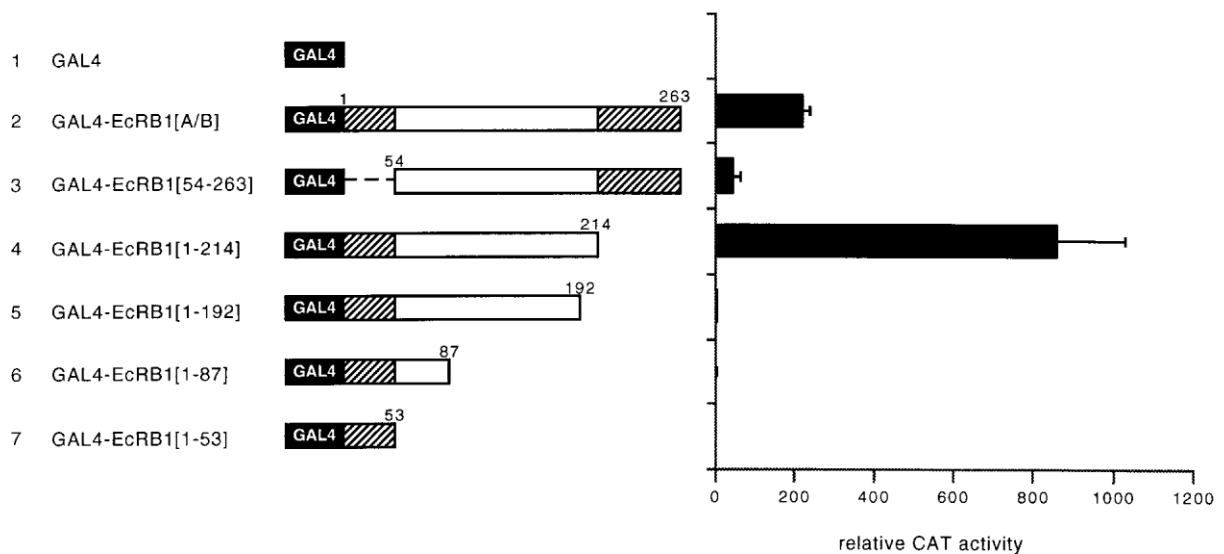
Fig. 6. Functional analysis in HeLa cells of deletion mutants in EcRB1[A/B] fused to GAL4 DBD. As Fig. 5 except CAT was used for monitoring reporter gene activity. For further explanations, see legend to Fig. 1D.

Similar to the experiments performed in yeast cells, fusion proteins containing an A/B domain of the B isoforms stimulated transcription, particularly when using EcRB 1 [A/B]. In contrast to the results obtained in yeast, a rather weak transcriptional activation was observed with EcRB2[A/B]. While these constructs contain only a portion of GAL4 DBD (94-147), they do not explain the low activity level of the EcRB2-GAL4 construct, because GAL4 constructs containing the entire GAL4 DBD gave similar results.

Mapping of the putative transactivation function (A~1) in the A/B domain of EcRB1

We wanted to determine the residues that are crucial for transcriptional activation in the A/B domain of the B1 isoform of *Drosophila* EcR. This isoform is not only the longest but also the most ubiquitous in insect species. Its A/B domain is usually highly variable among EcR proteins from different insects, although some subregions are conserved. A sequence alignment (see Fig. 4) with *Drosophila melanogaster* [2], *Aedes ageypti* [3 1], *Manduca sexta* [32], and *Bombyx mori* [33] shows that residues 1 – 53 and residues 214 – 263 are conserved whereas the intervening region is highly variable in length and amino-acid sequence. We therefore explored the possibility that one or the other of the two conserved regions contains the postulated AF1. We generated a series of deletion mutants in the A/B domain, fused them to the GAL4 DBD, and analyzed them for transcriptional activity in yeast and HeLa cells.

In yeast cells, truncation of the conserved amino-acid sequence 1 – 53 resulted in a complete loss of transcriptional activity suggesting that these amino acids are essential for transactivation (Fig. 5, line 3). Surprisingly, deletion of the conserved amino-acid sequence 215 – 263 resulted in a strong increase rather than a loss of transcriptional activity, revealing a possible inhibitory function in this stretch of amino acids (Fig. 5, line 4). Removal of residues 193 – 214 abolished this enhancement, and the remaining fragment exhibited almost the same activity as the full-length A/B domain (Fig. 5, lines 5 and 6). This points to the presence of a second transactivation focus in the short stretch of amino acids 193 – 214. However, this function appears to depend on amino-acid context as fusion of fragment 141 – 214 to sequence 1 – 53 resulted in only a small increase of transcriptional acivity compared to the activation observed with 1 – 53 alone (cf. Figure 5, line 11 with line 10). Extension of the C-terminal deletions generally caused a progressive reduction of the transcriptional activitation potential except for a construct that deleted region 114 – 140, suggesting the presence of another, weaker repressive function in the EcRB1 A/B domain (Fig. 5, line 7-10). The construct which contained only the conserved N-terminal amino acids stretch 1 – 53 exhibited a weak but significant activity (Fig. 5, line 10).

When tested in HeLa cells (Fig. 6) these mutant constructs yielded essentially the same results as the ones obtained with yeast. Removal of the conserved N-terminal amino acids 1-53 caused a substantial but incomplete loss of activity while deletion of the conserved C-terminal amino acids 215-262 strongly enhanced activity. In HeLa cells unlike yeast, the deleterious effect of removing the second activation focus i.e. amino acids 193 – 214, was so dramatic that possible modulatory functions of intervening amino-acids stretches in region 54 – 192 could not be studied. Likewise, a construct containing the putative N-terminal activation sequence 1 – 53 alone yielded no activity (Fig. 6, line 7). EMSAs performed with extracts from HeLa cells transfected with the various constructs showed that in all cases the corresponding fusion proteins were expressed at normal levels (data not shown).

DISCUSSION

The findings presented here show that the three isoforms of the ecdysone receptor of *Drosophila* not only differ qualitatively from each other as suggested by published developmental studies [6,10-13] but also quantitatively in terms of transcriptional control of reporter genes in transfected cells. In HeLa cells, the full length proteins, when coexpressed with USP, exhibited great differences in the transcriptional control of a CAT reporter gene being driven by an EcRE (Fig. 1D). In HeLa cells, as well as yeast cells, fusion proteins containing the A/B domain of one of the three isoforms fused to the DNA-binding peptide of GAL4 showed similar differences (Figs 2 and 3). In the three experimental regimes described here, the B1 isoform was strongest in transactivation whereas the A isoform was inactive or inhibitory. The effect of the B2 A/B domain was less consistent but always clearly positive. Dela Cruz and coworkers [34] found a different order of activity when expressing full-

length EcRA, EcRB1 or EcRB2 in yeast cells. However, this order (EcRB1 > EcRA > EcRB2) cannot really be compared with that observed by us and others (EcRB1 > EcRB2 > EcRA, see below) because of large deviations in the experimental parameters. Thus, we shall focus on the possible relevance of our results to the *Drosophila* system and *in vivo* situation.

Hu [35] introduced full-length EcRA, EcRB1 or EcRB2 or fusion proteins of their A/B domains with GAL4 DBD into an EcR-deficient/USP-containing *Drosophila* cell line by transfection. In both experimental series, EcRB1 or its fusion construct was most active in reporter gene activation whereas EcRA or its fusion construct was inactive. Salivary glands of larvae, which were mutant in EcRB1, and thus failed to respond to ecdysone by target gene activation as revealed by respective puff inductions, could be rescued by transgenic expression of EcRB1 or to a lesser degree by EcRB2. However, EcRA did not rescue the response [12]. Recent evidence suggests that ectopically expressed EcRA suppresses *DHR*3 gene activity (M. Schubiger, Department of Zoology, Seattle, WA, USA, personal communication). Likewise, ectopic expression of EcRA suppresses cuticular tanning in mutant animals (M. Schubiger, personal communication). It generally seems that among EcR isoforms, EcRA exhibits the weakest potential to rescue the development of EcR mutant animals (M. Schubiger, personal communication; P. Maroy, Department of Genetics, Szeged, Hungary, personal communication). In *Chironomus tentans* an EcR form (cEcR) was found whose A/B domain shares no sequence homology with any published EcR [36]. In transient transfection experiments, cEcR is inactive or slightly repressive. This repression is attributed to its A/B domain because a replacement of its A/B domain with that of *Drosophila* EcRB1 or with VP16 results in transcriptional activity (V. C. Henrich, M. Lezzi & M. Vögtli, unpublished observations). When fused to GAL4 DBD, cEcR[A/B] is inactive (K. Locher and J.-F. Mouillet, unpublished observations). Thus, the characterized EcR form of *Chironomus* functionally resembles *Drosophila* EcRA; inhibitory EcR forms may therefore exist in other insects. A situation in which one isoform is inducing transcription whereas another is repressing transcription provides a mechanism for fine tuning transcriptional levels, and has been observed for the α and β estrogen receptor isoforms [37,38].

It will be an interesting and challenging task to unravel the problem of whether and how the described quantitative differences are translated into the suggested qualitative differences, evident as tissue- and stage-specific effects of EcRB1, B2 and A during development. Here, we primarily asked how the activating or inhibitory effects of the three isoforms might be brought about. For example, do the A/B domains of the three isoforms function autonomously? The fact that any of these A/B domains could be separated from the rest of the EcR molecule and be attached to a foreign DNA-binding domain without losing their intrinsic property demonstrates that the inhibitory function of EcRA[A/B] and the activating function of EcRB1[A/B] and EcRB2[A/B] are largely autonomous.

In contrast to our observations with heterologous host systems, transfection of homologous host cells also reveals an inhibitory property of *Drosophila* EcRB1 and EcRB2 if assayed in the absence of ligand [39]. In fact, transferable, thus autonomous, silencing regions have previously been described for *Drosophila* EcR and shown to interact with corepressors like SMRTER and ALIEN [40-42]. However, these regions are located in the C-terminal part of EcR which is common to all three isoforms. Furthermore, corepressor binding to them is sensitive to ligand. They clearly differ from the inhibitory (or silencing) region described in the present paper which: (a) is specific for EcR isoform A, (b) is located in the N-terminus, i.e. outside the ligand binding domain, and (c) is able to function in the absence of the latter domain. We therefore propose the presence of an isoform-specific ligand-independent autonomous inhibitory function (IF) in the A/B domain of EcRA, which is able to interact with ubiquitous repressor molecules. Existence of an IF in the A/B domain of one of two isoforms has been reported for the human progesterone receptor [43,44].

Regarding the existence and location of autonomous activating functions (AFs) in nuclear hormone receptors, the general view is much clearer than with IFs [43]. There exists a ligand-dependent AF (AF2) in the E domain with a core motif in helix 12. Additionally there may occur ligand-independent AFs (AF1 and AF3) whose locations seem to vary. Most often, AF1 is found in the A/B domain of receptors. It is commonly accepted that an AF2 exists also in EcR and that it is located in helix 12 of the E domain [35]. The data presented in this

paper demonstrate that *Drosophila* EcRB1 and EcRB2 contain an AF1 which is located in the A/B domain. On the basis of sequence comparisons one may assume that the AF1 in the B1 isoform is widespread among arthropods (Fig. 4). Moreover, it seems to be ubiquitously active as it functions in yeast as well as in HeLa cells. The postulated AF1 of the B2 isoform is probably more specific both in terms of species distribution and tissue-dependent activity.

Having shown that the A/B domains of EcR isoforms are able to function autonomously does not preclude the possibility of their interaction with other domains within the EcR molecule. Seibel [45] showed that binding to EcREs is altered by the type of A/B domain present in a given EcR isoform when assayed in the absence of USP and ligand. In the presence of USP, though, such an effect of the A/B domain on binding to DNA could not be detected (Fig. 1C; [45]). This suggests that under special circumstances there might be intramolecular crosstalk between the A/B and the C (DNA binding) domain. Likewise, such a crosstalk must exist between the A/B and the E (ligand binding) domain. Muristerone A treatment of transfected HeLa cells switches EcRA from a repressive to an active state (Fig. 1D), probably by activating its AF2 in the E domain. A comparison with A/B-truncated EcR indicates, however, that this ligand effect is additive rather than cooperative as the ligand-induced activity is still lower with full-length than with A/B-truncated EcRA (Fig. 1D). This contrasts with the situation for EcRB1 and B2 where the effect of ligand is not simply additive but rather synergistic (Fig. 1D) indicating for the existence of cooperativity between the putative AF1 located in the A/B domain and the putative AF2 in the E domain. Cooperativity between AF1 and AF2 in nuclear receptors is a well known phenomenon and is thought to result from binding of multivalent coactivators (e.g. SRC-1, see [15,16,46]) to both AFs simultaneously. It will be interesting to identify such cofactors and to map their binding sites in the A/B domain of EcR isoforms.

In this study, we discovered an IF and AF1s in the A/B domains of EcRA, EcRB1 and EcRB2, respectively. A precise mapping of these functions was attempted only with EcRB1 because the IF in EcRA[A/B] is rather weak (HeLa cells) or even undetectable (yeast) while the short A/B domain of EcRB2 leaves little room for varying AF1 localization. With the A/B domain of EcRB1, experiments comprising a large series of deletions could be performed, both in HeLa as in yeast cells. The results obtained reveal a puzzling pattern of activating and inhibitory subregions. Unexpectedly but undoubtedly, the C-terminal region of EcRB1[A/B] (amino acids 215-263) must harbor a strong inhibitory function as its deletion results in a dramatic increase in the transactivation capacity of the respective A/B-GAL4 construct when assayed in either cell type. It remains to be shown whether this inhibitory function is autonomous and whether it shares properties with the described IF in the A/B domain of EcRA. The dominant AF1 function in EcRB1[A/B] seems to be spread over a large region as observed previously [35] with a different series of constructs containing EcRB1[A/B] deletions expressed in EcR-deficient *Drosophila* cells. However, within that region, the activation potential is unevenly distributed. Two `hot-spots' are envisaged there, one from amino acid 1 – 53 and another from amino acid 193-214. The autonomy of each region does not seem to be very pronounced. In addition, the relative strength of the two hotspots appears to vary, maybe in a cell-specific manner. Sequence searches for signature motifs or address sites for comodulators did not yield any clear result (not shown). Protein—protein interaction studies will probably provide insight into the mechanistic details of the postulated AFs and IFs in the A/B domains of EcR isoforms.

In conclusion it may be stated that the A/B domains of EcRB 1 and EcRB2 contain autonomous ligand-independent activation functions (AF1s) while that of EcRA rather exhibits an autonomous inhibitory function (IF). The molecular mode of action of these autonomous functions and their connection to the differential in vivo effects of EcR isoforms remains to be elucidated.

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