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High level transactivation by a modified *Bombyx* ecdysone receptor in mammalian cells without exogenous retinoid X receptor

(transgene regulation/heterodimerization/hormone receptor)

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ABSTRACT **Our studies of the *Bombyx mori* ecdysone receptor (BE) revealed that, unlike the *Drosophila melanogaster* ecdysone receptor (DE), treatment of BE with the ecdysone agonist tebufenozide stimulated high level transactivation in mammalian cells without adding an exogenous heterodimer partner. Gel mobility shift and transfection assays with both the *ultraspiracle* gene product (Usp) and retinoid X receptor heterodimer partners indicated that this property of BE stems from significantly augmented heterodimer complex formation and concomitant DNA binding. We have mapped this "gain of function" to determinants within the D and E domains of BE and demonstrated that, although the D domain determinant is sufficient for high affinity heterodimerization with Usp, both determinants are necessary for high affinity interaction with retinoid X receptor. Modified BE receptors alone used as replication-defective retroviruses potently stimulated separate "reporter" viruses in all cell types examined, suggesting that BE has potentially broad utility in the modulation of transgene expression in mammalian cells.**

Of the nuclear receptor superfamily members cloned and characterized from insect species, ecdysone receptor (EcR) is the only member for which an activating ligand is known (1, 2). EcR is also distinctive as the only characterized nonmammalian nuclear hormone receptor with no apparent mammalian homolog. Consequently, EcR continues to be studied for potential application to the modulation of transgene expression in mammalian cell types and transgenic animals (3, 4). The first study in which the potential of *Drosophila melanogaster* EcR (DE) was examined as a modulator of transgene expression revealed that DE required N-terminal fusion of a heterologous transactivating domain for high level function in mammalian cells and that, whereas 20-OH ecdysone was not able to efficiently stimulate DE transactivation, the ecdysteroid muristerone A (murA) was a potent activator (3). Subsequent studies of EcR biology indicated that ligand specificity was in large part determined by the obligate heterodimer partner available for EcR interaction. In insect cells, the natural heterodimer partner of EcR is the *ultraspiracle* gene product (Usp) (5), which together with EcR and ligand forms the functional ecdysteroid response complex (6–8). In mammalian cells, the retinoid X receptors (RXRs) were found to be capable of substituting for Usp (6–8), although there were several limitations. One limitation already described was that the range of activating ligands was essentially limited exclusively to murA (3, 4, 6–8). A second limitation that took longer to fully define was that RXR (found in most, if not all, mammalian cell types) was a reluctant dimer partner of EcR, and very high endogenous levels were necessary for murA stimulation to occur (6–8). With the exception of 293 cells, DE could not support high level transactivation in the vast majority of mammalian cell types without superphysiological levels of RXR dimer partner supplied by cotransfection. The monkey cell line CV-1, widely used in studies of mammalian steroid hormone receptors, has been extensively characterized as nonpermissive for DE transactivation (6–8).

Recently, four full-length cloned EcRs, in addition to *Drosophila* EcR, were reported: *Bombyx mori* EcR (BE) (9), *Manduca sexta* EcR (10), *Chironomus tentans* EcR (11), and *Aedes aegypti* EcR (12). Unlike most previously characterized nuclear steroid receptors, these EcRs shared little sequence similarity with each other. Higgins and Sharp sequence alignments (13) performed in our laboratory, and comparisons performed by Fujiwara *et al.* (10), indicated that the EcR least similar to DE is from the silkmoth *Bombyx mori*, with less than 42% overall amino acid identity. Within the coding regions of both receptors, individual functional domains vary widely in both amino acid similarity and, to some extent, length. Overall, BE is 546 amino acids compared with 878 amino acids for DE.

BE and DE, with low levels of structural similarity, were chosen for side-by-side comparisons of both receptors in combination with either the insect heterodimer partner Usp or the mammalian homolog RXR to determine their degree of functional similarity in mammalian cell types. In preliminary transfection experiments, we found that BE, unlike DE, responded strongly to the ecdysone agonist tebufenozide. Surprisingly, tebufenozide-treated BE was also found to be capable of full transactivation even with no added exogenous RXR dimer partner. Gel mobility shift assays and the construction of chimeric BE–DE hybrid proteins revealed that this property stemmed from significant differences in the heterodimerization of DE and BE. Discrete determinants within the hormone-binding "E" domain and, unexpectedly, within the hinge or "D" domain of the BE protein were found to govern the high affinity ligand-stimulated RXR interaction. Three cell types stably cotransduced with a "reporter" virus and receptor-bearing recombinant retroviruses demonstrate the significance of these findings to the modulation of transgene expression in target mammalian cells.

MATERIALS AND METHODS

Expression Vectors and Transient Transfection Assays. All receptors were subcloned into vector LNCX (A. D. Miller, GenBank accession no. M28247; with an extended polylinker) for use in transient transfection assays. VBE (or CVBE when referring to the retrovirus) was produced by insertion of VP16

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Abbreviations: EcR, ecdysone receptor; DE, *Drosophila melanogaster* EcR; murA, muristerone A; RXR, retinoid X receptor; BE, *Bombyx mori* EcR; EcRE, ecdysone response element; LTR, long terminal repeat.

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(14) sequences with a synthetic ATG start codon into the amino-terminal region of BE up to, and in-frame with, the *Mlu*I site (corresponding to amino acid 26). VP16 primers: (5′) 5′-GAGAGAAGCTTATGGCGCGCCCGACCGATG and (3′) 5′-CACACACGCGTGTACTCGTCAATTCCAAG. VDE (or CVDE) was produced in a similar fashion by fusing VP16 sequences in-frame at a novel *Nco*I site corresponding to amino acid 68. The downstream primer was made *Nco*I compatible. Plasmid template (100 ng), each primer (500 ng), and reaction conditions outlined by the manufacturer for Pwo (Boehringer Mannheim) high fidelity polymerase, were used with a program of 1 min at 94°C, 1 min at 45°C, and 1 min at 72°C for 20 cycles for production of all PCR products used in this report. LNCX-Usp and RXR were produced from *Eco*RI fragments encoding the complete cDNAs, filling with the Klenow fragment of DNA polymerase, and inserting the fragments into the *Hpa*I site of LNCX. Orientation was determined by restriction endonuclease digestion. Transient transfection in CV-1 and 293 cells was performed by using standard methods (15) in triplicate in 24-well plates by calcium phosphate coprecipitation of 100-ng receptor(s), reporter plasmid E4-luc, and pCH110 as an internal control. Briefly, E4-luc is four tandem EcREs inserted upstream of a thymidine kinase gene minimal promoter directing luciferase expression. EcRE oligonucleotides were as described (6) with *Bam*HI–*Bgl*II compatible ends. Ligand (1 μM) was added at the time of transfection, and 40 h later cells were lysed and extracts were used for β-galactosidase assay and measurement of luciferase activity in an analytical bioluminescence photometer (ABL, Ann Arbor, MI).

CVDEiR and MS vectors used only as retroviruses in production of stable cell lines were produced as follows: The VDEiR expression cassette was constructed by modifying the ATG start codon of human RXRα to contain an overlapping *Bst*XI site for fusion into the ATG start codon of the 0.8-kb EMCV IRES (16, 17) sequence. The IRES-RXR cassette was then subcloned downstream of VDE in LNCX-ofVDE (CofVDE), a VDE variant vector with lower basal transactivation levels in superphysiological RXR environments, to produce CVDEiR. The MS retroviral reporter was produced by removing the Moloney murine leukemia virus enhancer core from the 3′ long terminal repeat (LTR) with *Nhe*I–*Xba*I digestion and replacing this sequence with four tandem EcREs, resulting in a reporter analogous to the E4-luc reporter plasmid (see below).

Gel Mobility Shift Assays. Gel mobility shift analysis was performed by *in vitro* translation of receptor ORFs subcloned into pBSK (Stratagene), pGEM-3 (Promega), and PSL301 (Invitrogen) in the presence of [^{35}S]Met by using T3/T7 TNT (Promega) transcription/translation and following the manufacturer's protocol. *In vitro* translated proteins were qualitatively examined by 5% SDS/PAGE with protocols as described elsewhere (15) and quantified by PhosphorImager (Molecular Dynamics) exposures of dried gels. Amounts of proteins used in gel shift assays were normalized by using quantitative data and correction for predicted Met residues in individual constructs. Double-stranded EcRE probes corresponding to response elements described above were labeled by filling of the Klenow fragment of DNA polymerase with [^{32}P]dCTP and unlabeled dGTP, dATP, and dTTP by standard methods. Reaction conditions for protein–probe interaction and gel electrophoresis were essentially as described by Yao *et al.* (8) except, to facilitate comparison between samples, reaction mixtures (including dimer partners and probe) were prepared as a mixture and distributed equally to individual tubes with receptor proteins. The reactions were allowed to proceed at 23°C for 5 min, at which time ligand or vehicle was added and the reaction allowed to continue for an additional 20 min.

Construction of BE–DE Chimeric Receptors. Chimeric receptors were produced by taking advantage of conserved DNA sequences and sites within the receptors and by introducing compatible sites by low cycle, high fidelity PCR (described above) of BE and DE templates to produce fusion proteins. DEBE-A/B was produced by PCR and replaced the BE A/B domain (amino acids 1–197) with amino acids 1–255 of DE introducing a novel *Apa*I restriction site. DEBE-C replaced the A/B and C domains (amino acids 1–273) of BE with amino acids 1–331 of the DE protein by introduction of a compatible *Eag*I site into BE at the fusion junction. DEBE-D replaced BE from amino acids 1–363 with amino acids 1–430 of DE by *Kpn*I digest. BEDE was the reverse of this construct and replaced amino acids 1–430 of DE with amino acids 1–363 of BE by using *Kpn*I partial digest. DEBH was produced from DEBE-C by removing all BE sequences except the D domain by *Kpn*I digestion and inserting sequences encoding the intact E and F domains (amino acids 430–878) of the original DE after partial *Kpn*I digestion. VEH was produced by excision of the central region of DEBH, including the heterologous hinge domain, and inserted into VDE, replacing the analogous region. Primer sequences for all PCR and PCR mutagenesis are available on request.

Construction of E Domain Chimeric Receptors. The BEDB chimera was produced by insertion of the 654-bp *Kpn*I–*Bgl*II fragment encoding precisely the DE hormone-binding domain into the identical analogous sites in BE. Two unique sites within the hormone-binding domain of BEDB were used in the production of the chimeras. A unique *Aat*II site lies approximately 120 bp downstream of the 5′ *Kpn*I site, and a unique *Eag*I sites lies approximately 440 bp downstream. PCR was used as described above, and corresponding fragments of BE with appropriate compatible ends were generated and subcloned into BEDB digested with *Kpn*I + *Aat*II (BKE), *Aat*II + *Eag*I (BAE), *Aat*II + *Bgl*II (BAB), and *Eag*I–*Bgl*II for BEB. The resulting constructs were translated, examined by SDS/PAGE, quantified, and normalized as described above. Conditions for gel mobility shift assay were also as described above.

Infection of Cells with Recombinant Retroviruses. Transient retroviral production in 293 cell types has been described (18). Forty-eight hours after transient retroviral production, the conditioned medium was removed, filtered through 0.45-μm filters, and frozen at −70°C until use. Ten-centimeter dishes of primary-cultured Fischer rat abdominal fibroblasts (19) and 293 and CV-1 cells at approximately 50% density were infected at a multiplicity of infection of approximately 0.05 with virus-containing media and 8 mg/ml Polybrene (Sigma) for 48 h. Infected cells were then selected with 4–6 mM L-histidinol until noninfected cells were cleared from the population: approximately 10 d for FF12s, 21 dafor 293s, and >30 d for CV-1s (CV-1 cell populations were resistant to rigorous selection with L-histidinol). Resistant colonies from multiple plates were trypsinized, pooled, and passaged. The resulting bulk population was then split and infected with receptor-bearing virus and reselected again in medium containing both L-histidinol and 400–800 μg/ml G418. The infection and selection process for the second round of viral infection proceeded as described for the first round. The final population of doubly resistant primary fibroblasts was then pooled and passaged in triplicate in 6- or 24-well Costar plates for β-galactosidase staining by standard methods or luciferase assay.

RESULTS

CV-1 Cells Support BE-Mediated Transactivation Without Exogenous Dimer Partner. Both receptors were tested to determine the effect that differential endogenous dimer partner availability had on transactivation of responsive promoters. Transient transfection analyses of N-terminal VDE and VBE cotransfected into either 293 cells (Fig. 1*A*) or CV-1 cells (Fig.

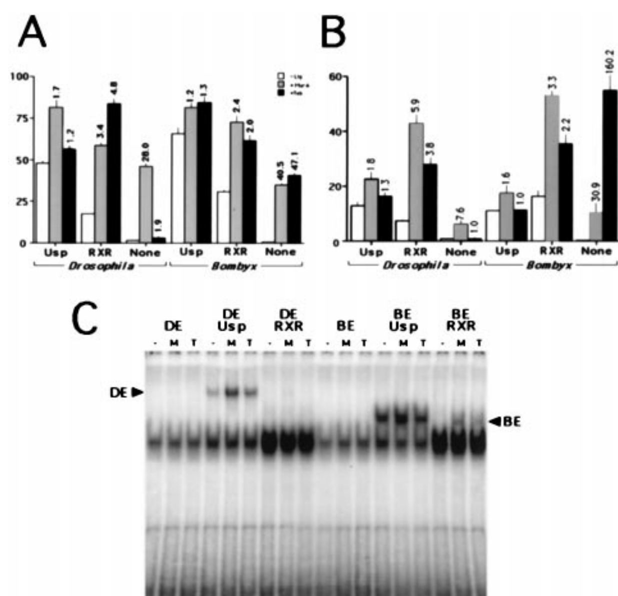

FIG. 1. A comparison of BE and DE by transient transfection and gel shift assays with both receptors and the dimer partners Usp or RXR. Relative luciferase activity from VDE and VBE transiently cotransfected into 293 (*A*) or CV-1 (*B*) cells with either Usp, RXR, or no added heterodimer partner and treated with either vehicle (open bar), 1 μM murA (shaded bar), or 1 μM tebufenozide (solid bar). Numbers at the top of bars represent fold induction relative to vehicle-treated samples. (*C*) Comparative gel mobility shift analysis of native EcRs in combination with Usp, RXR, or no added heterodimer partner. Symbols above lanes indicate treatment with vehicle (–), 1 μM murA (M), or 1 μM tebufenozide (T). Left arrowhead, DE + dimer partner shifted band; right arrowhead, BE + dimer partner shifted band. As in *A*, the band below the heterodimer shift bands is caused by ligand-independent binding of monomers to the probe (data not shown). The free probe is at the extreme lower edge of the autoradiogram.

1*B*) with a reporter plasmid (E4-luc) and Usp or RXR were performed.

In the presence of an equimolar exogenously added dimer partner, VDE and VBE transactivation was similar in both cell types (Figs. 1*A* and *B*). With Usp, both proteins were induced less than 2-fold by ligand and displayed a high level of basal transactivation. With RXR, VDE displayed an average relative 5.35-fold induction across both cell types, whereas VBE was induced only 2.35-fold, even though the absolute level of induction matched or exceeded the expression with VDE. The decreased relative induction of VBE + RXR resulted from approximately doubled basal activity levels compared with VDE + RXR. With no exogenous dimer partner, both receptors in both cell types exhibited dramatic 15- to 80-fold decreases in basal transactivation. With no exogenous dimer partner, VDE failed to respond to tebufenozide, whereas VBE continued to respond well to both murA and tebufenozide. The addition of RXR to VDE-transfected 293 cells increased the maximum murA-treated expression level by only 20%, indicating that the high level of endogenous RXR in 293 cells is near saturation for heterodimerization with DE. By comparison, CV-1 cells supported murA stimulation of VDE at only 13% of the expression level of VDE with added RXR (Fig. 1*B*). VBE was active at levels similar to VDE in the high RXR background of the 293 cells; however, in the CV-1 cells, tebufenozide-stimulated VBE exhibited both the highest absolute level of transactivation and the greatest relative induction (160.2-fold) of any of the other combinations of receptors and ligands tested (Fig. 1*B*). In the CV-1 cells, tebufenozide-stimulated VBE displayed 21-fold greater relative induction and an absolute expression level 9.25 times the level of VDE treated with murA.

BE Displays Facilitated Heterodimer Formation with Usp and RXR. The high level function of VBE in the low heterodimer partner environment of the CV-1 cells suggested that BE might be capable of efficient function with lower levels of dimer partner than DE. To explore this possibility, cell-free experiments with gel mobility shift analysis and equivalent amounts of *in vitro* translated BE and DE in combination with Usp or RXR dimer partners and both ligands were performed. Neither protein alone formed a dimeric complex with the EcRE probe unless either the Usp or RXR dimer partner proteins were added, as shown in Fig. 1*C*. In the presence of Usp, DE formed a comparatively weak interaction with the EcREs that was increased approximately 4-fold by murA and 2-fold by tebufenozide. In contrast, BE produced a strong shift with Usp that was only slightly influenced by either ligand. In the absence of any ligand, BE bound probe 5 times more efficiently than DE. With ligand, the BE probe shift was observed to still exceed the DE shift and was double the DE + Usp band volume. Both receptors combined with RXR displayed an absolute dependence on ligand for heterodimer formation; however, whereas BE + RXR with either ligand displayed a prominent shifted band, an equivalent amount of DE resulted in a barely detectable shifted band with murA and no detectable band shift with tebufenozide. The BE + RXR tebufenozide- and murA-induced shifts were determined to be >15 times the DE + RXR shift with murA.

BE–DE Chimeras Reveal That the D Domain Is Critical to High Affinity Heterodimerization. Gel mobility shift assays indicated that BE had higher affinity heterodimer formation than DE. To further define the subdomains and molecular determinants resulting in the BE high affinity phenotype, we used PCR mutagenesis and internal shared restriction endonuclease sites to produce BE–DE chimeric receptors that were assayed for their ability to bind the EcRE probe with either Usp or RXR and with or without ligand. Because previous results indicated that murA stimulated both EcRs, for purposes of direct comparison only murA was used for gel shift analysis of the chimeras. Fusion constructs began at the N terminus of BE and sequentially replaced the A/B (DEBE-A), C (DEBE-C), and D (DEBE-D) domains with DE sequences. BEDE was the reverse, encoding BE up to the D–E domain boundary with the DE E and F domains (Fig. 2*A*). In Fig. 2*B*, DEBE-A and DEBE-C with Usp shifted nearly as efficiently as native BE. The absence of shift for DEBE-D, however, and the substantial shift seen for BEDE + Usp suggested that the high affinity determinant for BE–Usp heterodimerization resided in the D, or hinge domain. The same constructs in combination with RXR revealed a different shift pattern (Fig. 2*C*). BE and DEBE-A both responded strongly to ligand to bind and shift the probe. DEBE-C with ligand was approximately 40% decreased relative to native BE; however, it still displayed 16-fold greater binding than native DE. DEBE-D had lost all high affinity DNA-binding complex formation and functioned indistinguishably from native DE with or without ligand. MurA-stimulated BEDE + RXR produced a band shift 4 times the intensity of the native DE + RXR stimulated with murA, but BEDE was clearly significantly impaired in heterodimerization with RXR compared with native BE and other higher affinity chimeras like DEBE-A and DEBE-C.

For further confirmation of the role of the BE D domain in dimer partner affinity, the D region of DE was replaced with the BE D domain to produce DEBH. Native DE and DEBH were compared side by side for binding to EcREs with both Usp and RXR dimer partners. As shown in Fig. 2*D*, compared with DE + Usp, DEBH + Usp averaged 5-fold greater probe binding with murA and 9-fold greater binding without ligand. DEBH + RXR exhibited both decreased probe shift in the absence of ligand and increased probe binding with murA

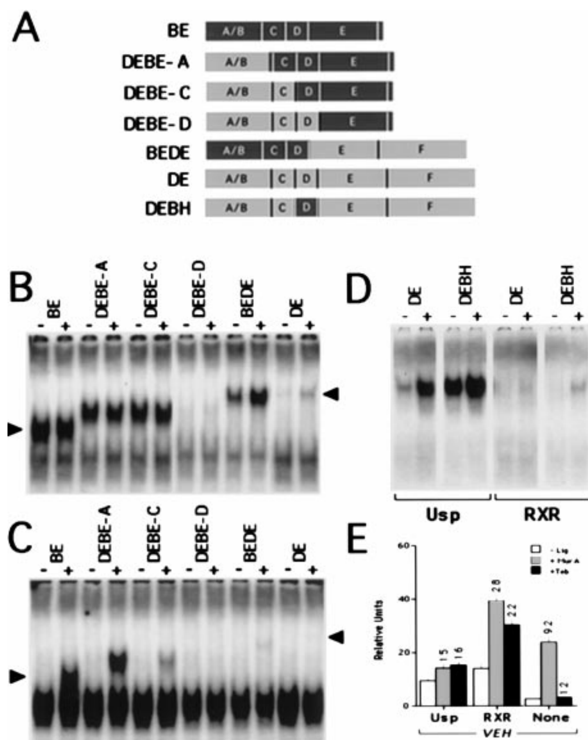

FIG. 2. Comparative gel mobility shift analysis of chimeric receptors in combination with Usp, RXR, or no added heterodimer partner. (*A*) Schematics of native and chimeric EcRs. BE sequences are depicted with dark shading and DE sequences are shown in light shading. The name of each chimera is to the left of the drawings. Vertical lines represent divides between the different labeled domains characteristic of nuclear hormone receptor family members. (*B*) Gel mobility shift of native and chimeric EcRs with Usp and vehicle (−) or 1 μM murA (+). The left arrow indicates the size of the native BE shift, and the right arrow indicates the location of the native DE band shift. The lower portions of all of the remaining autoradiograms were essentially identical with *A* and only the top half is depicted to allow for greater magnification of the area displaying the EcR heterodimer bands in this and subsequent figures. (*C*) Gel mobility shift of native and chimeric EcRs with RXR as in *B*. (*D*) Gel mobility shift of *in vitro* translated and normalized DE and hinge-substituted DEBH, with Usp or RXR as labeled. −, vehicle; +, 1 μM murA. (*E*) Transient transfection of VEH into CV-1 cells and directly comparable to Fig. 1*B*.

(approximately 3-fold over native DE) for a 10-fold relative induction by ligand compared with 2.5-fold for DE.

Transient transfection analysis of a VP16-DEBH construct (VEH) in CV-1 cells revealed that VEH shared characteristics of both DE and BE (Fig. 2*E*, directly comparable to Fig. 1*B*). VEH displayed the high basal transactivation level and low relative induction (average of 2.5-fold) characteristic of BE with superphysiological RXR. VEH displayed 5- to 8-fold greater basal and murA-activated expression than native DE in the absence of any exogenous dimer partner. MurA-stimulated VEH expression exceeded even the expression level of murA-stimulated BE by nearly 2-fold. As predicted, without the BE hormone-binding domain, DEBH did not to respond to tebufenozide. Additional chimeric proteins with a subset of BE D-region sequences did not function as well as DEBH with replacement of the entire D domain, suggesting that multiple discrete determinants presumably over much of the hinge region contributed to the high affinity phenotype (data not shown).

Discrete Functional Regions Within the E Domain Revealed by BE–DE Chimeras. Although these data reveal that the BE D domain alone was sufficient for high affinity heterodimerization with Usp, a comparison of the relative shift of the DEBE-D and BEDE chimeras with both partners suggested that additional determinants within the BE hormone-binding E domain contributed to a high affinity interaction with RXR. To further study the BE E domain, chimeric proteins were produced by using BEDE as a template. Five E-domain chimeras named BEDB, BKE, BAE, BAB, and BEB are shown in Fig. 3*A*. BEDB is identical with BE with the exception of complete replacement of the E domain with corresponding sequences from DE. BEDB was identical with BEDE with the exception of replacement of the large C-terminal DE F domain for the 20-amino acid F domain of BE. BKE, BAE, BAB, and BEB are derived from BEDB and are sequential, C-terminal moving replacements of three regions within the DE-derived E domain. Gel mobility shift assays with BE, BEDE, and BEDB revealed that BEDE and BEDB are essentially identical in their properties of complex formation (Fig. 3*B*), and this suggests that the F domain does not play a significant role in heterodimerization and DNA binding. In addition, BEDB recapitulates the high affinity USP binding and the low affinity RXR binding of BEDE.

The BEDB-derived chimeric receptors shown in Fig. 3 *C* and *D* reveal several functional subdomains over most of the BE E domain. By using unique internal sites, we subdivided the E domain into approximately thirds called E_1, E_2, and E_3. Chimera BKE with replacement of the DE $E_1$and E_2 regions and chimera BAE with replacement of only the E_2 region displayed very similar patterns of shift. Both chimeras were

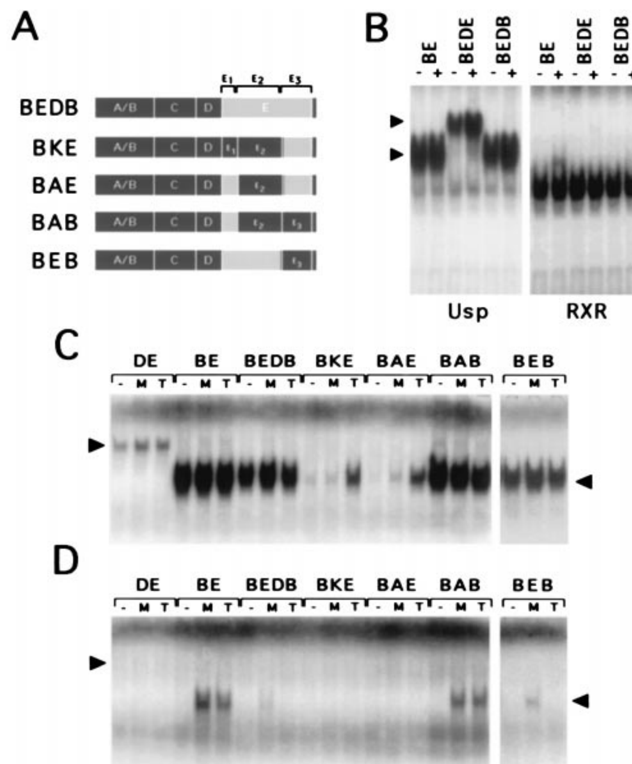

FIG. 3. Gel mobility shift analysis of EcR hormone-binding domain chimeras. (*A*) Schematics of chimeric receptors. Dark shading, BE sequences; light shading, DE sequences. E_1, E_2, and E_3 indicate subdivisions of the E domain. (*B*) Gel mobility shift of native BE and the BEDE and BEDB chimeras with Usp and RXR heterodimer partners. On the left is indicated the size of BE and BEDB band shifts (lower arrowhead) and BEDE band shifts (upper arrowhead). Gel mobility shift of native and E-domain chimeric EcRs with Usp (*C*) or with RXR (*D*). Treatments were with vehicle (−), 1 μM murA (M), or 1 μM tebufenozide (T). The left arrow indicates the size of the native DE shift, and the right arrow indicates BE-sized shifted bands. The BEB chimeras are slightly separated from the other lanes because they were run simultaneously on a separate gel.

significantly impaired in complex formation with Usp (Fig. 3*C*) and RXR (Fig. 3*D*) relative to the original BEDB chimera, suggesting that there were fundamental incompatibilities between subdomains of the *Bombyx* and *Drosophila* E domains. The similarity of these two constructs suggested that DE and BE E_1 regions were likely to be functionally similar, but that the E_2 regions had very different properties. Curiously, although both basal and murA-stimulated heterodimerization were inhibited, BKE/BAE-Usp heterodimerization was significantly stimulated by tebufenozide, indicating that the tebufenozide response determinant is within E_2 but that the high affinity RXR-binding determinant lies elsewhere. When E_2 was combined in tandem with E_3 in the chimeric receptor BAB, high affinity heterodimer formation indistinguishable from native BE heterodimerization was observed with both Usp and RXR. To determine whether the E_3 region alone could confer high affinity heterodimerization with RXR, the chimera BEB, with substitution of only the E_3 region, was produced and tested. BEB displayed a pattern of response essentially identical with the original BEDB with low-level interaction with RXR and only upon stimulation with murA. BEB, which lacked the E_2 region of native BE, was not responsive to tebufenozide. Taken together, these results indicate that the BE D domain and E_3 region, in combination with ligand-binding determinants within the E_2 region, function in concert to produce high affinity, ligand-dependent heterodimerization between BE and the RXR protein.

Comparison of VBE and VDE Function in Stably Transduced Cells. Building on transient transfection and gel shift data, we wanted to directly compare DE and BE variants for their ability to function at low or single copy in stably transduced cell types. In addition to direct comparison of CVDE and CVBE retroviruses, we also tested an RXR-bearing VDE variant called CVDEiR that previous studies had determined to provide the best combination of low basal expression and relatively high induction for VDE cotransferred with supplemental RXR (data not shown). These three receptor-bearing vectors and an empty LNCX control were used to infect 293, CV-1, and primary fibroblast cells harboring an EcRE-responsive retroviruses termed MS (Fig. 4*A*). MS encoded a 3′ LTR with the core enhancer elements replaced with four tandem EcREs and an internal simian virus 40 promoter directing resistance to the drug L-histidinol. Parallel MS vectors encoding either the LacZ gene (MS-Z) or a luciferase transgene (MS-luc) were used in this comparative study, the former as an indication of the number of responding cells and the latter for quantitative purposes.

Three cell types, 293s, CV-1s, and rat primary fibroblasts (line FF12), were selected for assay of reporter/receptor virus combinations based on their relative capacities to support high level DE-mediated transactivation. As shown in Fig. 1*A*, the human 293 cell line supports essentially full DE activation with no requirement for added RXR, whereas CV-1s require the cotransfection of exogenous dimer partner for DE function. FF12s were selected as the third cell type because they are refractory to chemical methods of transduction and reliant on the use of biological vectors such as recombinant retroviruses for efficient gene transfer. Furthermore, primary fibroblasts are frequently used as autologous donor cells for transplantation approaches to somatic gene therapy.

All three cell types infected with both the reporter and receptor-bearing vectors and treated for 72 h with either vehicle, 1 μM murA or 1 μM tebufenozide, are shown in Fig. 4*B*. Histochemical staining of MS-Z infected cells indicated that all three receptor types were capable of high level ligand-responsive transactivation in coinfected 293 cells. DE-derived constructs responded well to murA but were unable to respond strongly to tebufenozide. CVBE, on the other hand, responded moderately to murA but strongly to tebufenozide. MS-LacZ cell types infected with an empty LNCX vector

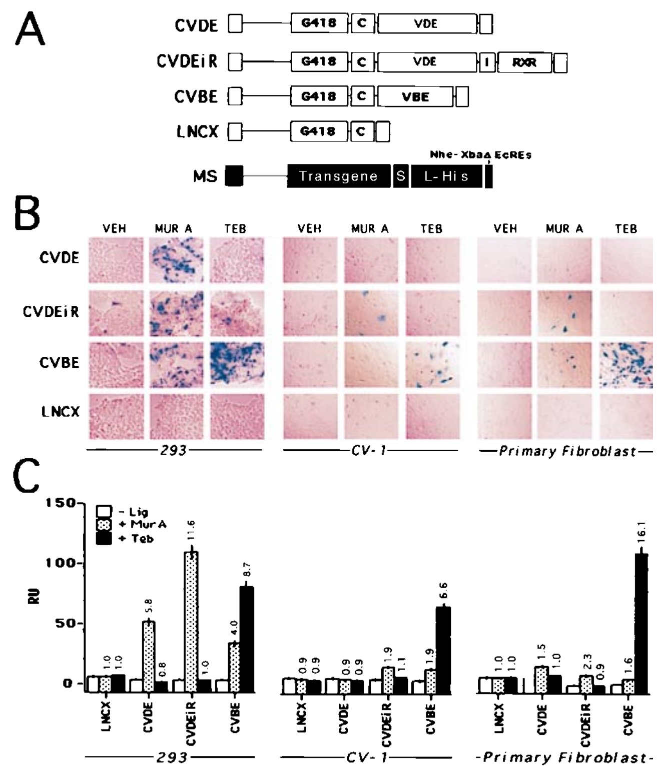

FIG. 4. Responsiveness of 293, CV-1, and FF12 cells coinfected with reporter and receptor-bearing retroviruses. (*A*) Schematic of receptor variant viral vectors (light shading) and the MS reporter virus (dark shading). Small, flanking empty boxes are LTRs, G418/L-histidinol are selectable markers, C/S is the cytomegalovirus or simian virus 40 promoter. Other nomenclature is described in the text. (*B*) β-Galactosidase histochemistry revealing positive MS-Z-infected cell types coinfected with receptor variants and treated with 1 μM ligand corresponding to the labels above (ligand), below (cell type), and to the left (receptor type) of the panels. Cell fields are taken from the approximate center of wells and are representative of staining. (*C*) Luciferase induction in MS-luc-infected cells analogous to the groups in *B*. Raw values are expressed as relative units normalized against LNCX values across cell types. Numbers at the end of bars represent fold induction relative to vehicle treatment.

displayed no staining when stimulated with any ligand and further confirmed observations from vehicle-treated cells that the MS vector permitted only relatively low basal levels of transgene expression. Approximately 30% of CVDE-, CVDEiR-, and CVBE-infected MS-Z 293 cells responded to murA. Tebufenozide stimulated <1% of CVDE-infected cells and <4% of CVDEiR-infected MS-Z 293s. More than 50% of CVBE-infected MS-Z 293s responded to tebufenozide. More dramatic differences were seen in CV-1 and FF12 MS-Z infected cells. CVDE alone was not inducible in either cell type, indicating that the endogenous level of RXR in either CV-1s or primary fibroblasts is not sufficient to support VDE-mediated transactivation. When the endogenous level of RXR is supplemented, as in CVDEiR, 3–10% of the infected population was observed to strongly respond to murA (Fig. 4*B*). CVBE, however, was clearly the most potent transactivator in the CV-1 and FF12 cell types with 20% of CV-1 and 55% of CVBE-infected FF12s cells histochemically positive.

Luciferase activity in MS-Luc cells infected in parallel with the same receptor viruses confirmed that CVBE was the most potent transactivator in the CV-1 and FF12 cell types and that all receptor types were functional in 293 cells (Fig. 4*C*). The MS-Z data suggest that the low absolute level of CVDEiR induction in CV-1 and FF12 cells with murA most likely reflects a small number of robustly responding cells in a largely

nonresponsive population as opposed to low level induction by most MS-luc-infected cells.

DISCUSSION

The results of these experiments reveal differences in the transactivation of EcRs derived from *Bombyx mori* and *Drosophila melanogaster* in mammalian cell types with low levels of the endogenous heterodimer partner RXR. These differences were found to stem, at least in part, from an increased capacity of the *Bombyx* receptor for heterodimerization with RXR. The determinants for high affinity dimerization with either RXR or Usp were found to lie within the BE D and E domains.

Substantial differences in the amino acid sequences of most cloned EcRs led us to speculate that these receptors might function very differently outside of their natural cellular environment. Functional differences in these receptors could be further implied by differences in ligand specificities. The development of "smart" insecticides like tebufenozide, targeted specifically at lepidopteran and coleopteran species, implied that unique molecular determinants within the individual hormone-binding domains of these EcRs existed.

Our interest in the EcR as a means of regulating transgene expression led to the study of tebufenozide as an alternative to murA because of the high cost and potential availability problems of murA for use in *in vitro* systems, transgenic animals, or animals with transplants of ecdysteroid-responsive cells. The requirement of tebufenozide for high level transactivation in the absence of superphysiological RXR originally led us to hypothesize that the ligand-binding E domain alone would determine the phenotype. We were surprised that the data indicated that the D, or hinge, domain was jointly responsible for high affinity RXR interaction and apparently solely responsible for high affinity BE interaction with Usp. We have not found a report implicating the D domain of mammalian class II nuclear hormone receptors in either dimer partner preference or affinity; therefore, it is possible that this D domain property is unique to the EcRs. BE D domain substitution alone was able to confer the high affinity phenotype to DE for its natural dimer partner Usp, and future experiments will determine whether the BE D domain can also alter the heterodimerization (or perhaps homodimerization) properties of mammalian nuclear receptors for their respective dimer partners.

E domain chimeras suggested that subdomains conferring high order response specifically to tebufenozide are likely to reside near the center of the E domain in E_2, between amino acids 402 and 508 of BE. Curiously, only BAB and BEB constructs (in addition to BEDB and in combination with RXR) responded well to murA, suggesting that a subdomain within the C-terminal 75 amino acid E_3 region might interact coordinately with a complementary region in E_2 to complete the muristerone-binding pocket. Crystallographic studies of the hormone-binding domains of retinoic acid receptors and thyroid hormone receptors (20, 21) support this supposition and describe multiple amino acids across the retinoic acid receptor and thyroid hormone receptor E domains brought into close functional proximity by protein folding.

Ligand specificities aside, these data also argue for multiple determinants over the E domain acting in a coordinated fashion for high affinity RXR interaction. Comparison of BKE BAE, and BAB suggests that the E_1 region of the BE E domain is unlikely to be involved in the high affinity phenotype because these amino acids are DE derived in the BAB chimera. Conversely, sequences within the C-terminal 75 amino acids are evidently necessary, but not sufficient, because BAB displays the high affinity phenotype ,whereas neither BAK or BEB is capable of full high affinity interaction. Only BAB, with both the E_2 and E_3 regions of BE, responded to both ligands to strongly interact with RXR. Production of additional E domain mutant receptors will shed further light on the association between EcR heterodimerization and their specificity for individual ligands.

Comparison of DE and BE variants by retroviral infection confirmed transient transfection data and further revealed that BE and, to a lesser extent, DE + RXR, were capable of high level transactivation with both the receptor and reporter constructs at low (and most likely single) copy. In an effort to accurately portray responsive cell populations we chose to present the responsive cells as "bulk" infected populations without subtraction of basal expression levels to calculate relative induction. Data in preparation indicates that readily isolated clonal cell populations of MS-CVBE cells and less common clones of MS-CVDEiR cells have inductive properties superior to the bulk population. Preliminary experiments also suggest that vectors currently in development with internal responsive promoters will have dramatically lower levels of basal expression than the EcRE-LTR of MS. The absence of required coinfected dimer partner and the smaller size of BE relative to DE make it particularly suited to use in single-plasmid retroviral vectors and other small viral vectors such as AAV.

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