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Genetics

Ecdysteroid-dependent regulation of genes in mammalian cells by a *Drosophila* ecdysone receptor and chimeric transactivators

(steroid hormone receptor/gene regulation/inducible transcription)

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ABSTRACT **Steroid receptors are members of a large family of transcription factors whose activity is tightly regulated by the binding of their cognate steroid ligand. Mammalian steroid hormone receptors have been exploited to obtain the regulated expression of heterologous genes in mammalian cells. However, the utility of these systems in cultured cells and transgenic animals is limited by the presence of endogenous steroids and their receptors. We show that a *Drosophila* ecdysone receptor can function in cultured mammalian cells as an ecdysteroid-dependent transcription factor. The activity of the ecdysone receptor was not induced by any of the mammalian steroid hormones tested. The DNA-binding and transactivation activities of viral, mammalian, or bacterial proteins were rendered ecdysteroid-dependent when fused to the ligand-binding domain of the ecdysone receptor. The ecdysone receptor may prove useful in selectively regulating the expression of endogenous or heterologous genes in mammalian cells.**

The steroid hormone receptor superfamily represents an evolutionarily conserved group of proteins that influence developmental and metabolic processes primarily by functioning as ligand-dependent transcription factors (1–3). Structural analysis of receptor proteins has identified domains of these proteins that function to bind DNA or ligand and to enhance transcription (4–7). Ligand binding is required for the interaction of some receptors with their cognate response elements *in vivo* (8) and may also control dimerization and transcriptional activation properties (9, 10).

The ability of transcription factors to function in heterologous species has provided a system to analyze the functional domains of these factors and a novel mechanism to control the expression of heterologous genes (11, 12). Mammalian steroid hormone receptors have been shown to function when expressed in yeast (13–15) and *Drosophila* cells (16).

We wished to develop a system to specifically regulate heterologous genes in mammalian cells in tissue culture and in transgenic animals. Such a system could provide a powerful approach to understanding the functions of the product of that gene. Systems that exploit the ability of mammalian steroid hormone receptors to function as ligand-dependent transcription factors have proved useful in regulating the expression of heterologous genes in mammalian cells (17, 18). However, the applications of these systems in cultured cells or transgenic animals are limited by the presence of endogenous steroid receptors and their ligands. In this report we show that the *Drosophila* ecdysone receptor (EcR) protein, a member of the steroid hormone receptor superfamily (19), can function as a ligand-dependent transcription factor in mammalian cells. The activity of the EcR is induced upon administration of certain ecdysteroids but not by any of the mammalian hormones tested. Novel target-gene specificity was obtained by using chimeric receptors containing the EcR ligand-binding domains fused to heterologous DNA-binding domains. Finally, the activity of these chimeric proteins could be increased by inclusion of a potent viral transactivation domain.

MATERIALS AND METHODS

Hormones. All hormones were purchased from Sigma with the exception of muristerone A, which was obtained from Simes (Milan), and ponasterone A, a gift from David King (University of California, Berkeley). Hormones were dissolved in ethanol at a final concentration of 1 mM.

Construction of Plasmids. Reporter plasmids $pEc_4M_{-77}CO$, $pG_4M_{-77}CO$, and $pE_4M_{-77}CO$ contain four copies of a 28- or 29-base-pair DNA sequence containing an ecdysone response element (EcRE) (5′-GATCCGACAAGGGTTCAATGCACTTGTCA-3′), a glucocorticoid response element (GRE) (5′-GATCCGTAGCTAGAAACAGACTGTTCTGA-3′), or an estrogen response element (ERE) (5′-GATCCGTAGCTAGGTCAGACTGACCTGA-3′), respectively, at position −77 of a mouse mammary tumor virus (MMTV) promoter–chloramphenicol acetyltransferase (CAT) gene fusion. The MMTV promoter in this reporter gene was constructed by using a synthetic oligonucleotide and extends from position −77 to position −16 relative to the site of transcription initiation within the MMTV long terminal repeat (LTR). Reporter plasmid $pG_4M_{-77}GO$ was derived from $pG_4M_{-77}CO$ by replacing the CAT gene with a human growth hormone cDNA. To construct the reporter plasmid $pOC_{-33}CO$, the GREs and MMTV promoter of $pG_4M_{-77}CO$ were replaced with an oligonucleotide encoding the minimal cytomegalovirus (CMV) immediate early (IE) promoter (−33 to −19). $pX_4C_{-33}CO$ was derived from $pOC_{-33}CO$ by addition of four copies of a Lex operator (5′-TCGACGTACTGTATGTACATACAGTACC-3′) immediately upstream of the minimal CMV IE promoter.

Receptor construct pRSV.GGG contains a Rous sarcoma virus (RSV) LTR driving the expression of a cDNA encoding the rat glucocorticoid receptor (GR) (20). The receptor plasmid pRSV.GEG was derived from pRSV.GGG by replacing a *Hin*dIII–*Aha* III fragment of the rat GR cDNA with a double-stranded oligonucleotide (5′-AAGCTTCAGGATGTCATTACGGAGTACTGACATGTGAAGGCTGCAAAGTATTCTTT-3′) incorporating two amino acid substitutions (G458E, S459G) that alter the DNA-binding specificity to allow ERE recognition (21, 22). In constructs denoted "V,"

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Abbreviations: EcR, ecdysone receptor; GR, glucocorticoid receptor; EcRE, ecdysone response element; GRE, glucocorticoid response element; ERE, estrogen response element; MMTV, mouse mammary tumor virus; RSV, Rous sarcoma virus; LTR, long terminal repeat; CMV, cytomegalovirus; IE, immediate early; CAT, chloramphenicol acetyltransferase.

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the sequence encoding amino acids 153–406 of the rat GR is replaced by the sequence encoding the transcriptional activation domain of the herpes simplex virus VP16 protein (amino acids 411–490). Receptor expression plasmid pRSV.GGEc was constructed by replacing the sequence encoding the GR ligand-binding domain in pRSV.GGG (amino acids 528–795) with a sequence coding for the ligand-binding domain of the EcR (amino acids 329–878). Similarly, to construct GXEc, the cDNA encoding the ligand-binding domain of GXG (referred to as NLxC in ref. 5) was replaced with DNA encoding the EcR ligand-binding domain. The expression of the RSV LTR used in the receptor expression plasmids was not affected by muristerone A. Sequence files of all reporter and receptor genes in this study are available upon request.

Cell Culture, Transfection, and Analysis of Reporter Gene Activity. Human 293 cells (embryonic kidney cell line) were cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's nutrient mixture F12, supplemented with 10% fetal bovine serum. Subconfluent cultures grown in 60-mm culture dishes were transfected (5) with 2.0 μg of the receptor expression plasmid, or the parental expression plasmid pRSV as a control, and 0.5 μg of the reporter plasmid. As an internal control for transfection efficiency, 0.5 μg of the control plasmid pRSV.hGH was included in the transfection mixture. After transfection, cells were treated with hormone as indicated. Cells were harvested 36–40 hr after transfection, and extracts were prepared by freezing three times in dry ice and thawing at 37°C. CAT assays were performed as described (23). The values were normalized to the expression of human growth hormone secreted into the medium, and the average values of three independent experiments are shown. "Fold induction" represents the level of expression of the reporter gene in cells incubated with hormone divided by the expression of that reporter gene in extracts from cells transfected with the RSV expression vector lacking a receptor insert and incubated in the absence of added ligand.

Total cellular RNA was prepared by the RNAzol method (Cinna/Biotecx Laboratories, Friendswood, TX). Briefly, 10^7 cells were homogenized in 1 ml of RNAzol, and 0.1 ml of chloroform was added. After incubation for 15 min on ice, the samples were centrifuged at 12,000 × *g* for 15 min. The aqueous phase was collected, and RNA was precipitated with 0.5 ml of 2-propanol, pelleted at 12,000 × *g* for 15 min, washed in 80% ethanol, and suspended in water. For RNase protection experiments, 25 μg of total cellular RNA was hybridized for 16 hr with ^{32}P-labeled antisense probes. The probes were prepared and the RNase digestion was carried out as described (24). Protected fragments were fractionated in a 6% polyacrylamide sequencing gel and visualized by autoradiography.

RESULTS

Only Specific Ecdysteroids Are Agonists for the EcR in Mammalian Cells. A cDNA clone for the *Drosophila* EcR was obtained by screening a cDNA library prepared from *Drosophila* early pupal larvae with oligonucleotide probes based on the partial EcR sequence (referred to as DHR23 in ref. 25). The deduced amino acid sequence of the clone used here matches the published sequence of the EcR (19). The highest region of homology between the EcR and other members of the steroid receptor superfamily is found in a cysteine-rich region (residues 264–329) that corresponds to the DNA-binding domain. The putative ligand-binding domain shares limited homology with retinoic acid and thyroid hormone receptors and the vitamin D receptor. The N-terminal domain (residues 1–263) does not share significant homology with any steroid receptor superfamily member (19).

The EcR has been reported to regulate transcription of genes containing EcREs in *Drosophila* tissue culture cells treated with 20-hydroxyecdysone (19). We determined whether the EcR could function in mammalian cells treated with ecdysteroids to enhance the transcription of a reporter gene containing EcREs linked to the MMTV promoter and CAT gene. Human 293 cells were cotransfected with a RSV-based expression vector that encodes the EcR and the reporter gene $Ec_4M_{-77}CO$, which contains four copies of an EcRE from the *Drosophila Hsp27* promoter (26), linked to a murine tumor virus promoter–CAT construct. CAT activity was determined in extracts from cells incubated with or without the ecdysteroids α-ecdysone, 20-hydroxyecdysone, polypodine B, ponasterone A, or muristerone A. Neither α-ecdysone, 20-hydroxyecdysone, nor polypodine B acted as agonists for the EcR in mammalian cells. In contrast, expression of the reporter gene was increased markedly in cells treated with muristerone A and to a lesser extent in cells treated with ponasterone A (Fig. 1). The EcR did not regulate expression of the control reporter genes $G_4M_{-77}CO$ and $E_4M_{-77}CO$, which contain binding sites for the GR (GRE, Fig. 1) or for the estrogen receptor (ERE; see Fig. 4), respectively. Half-maximal stimulation of reporter gene activity by the EcR was observed at 0.5 μM muristerone A and ponasterone A. Thus, in mammalian cells the EcR acts in an

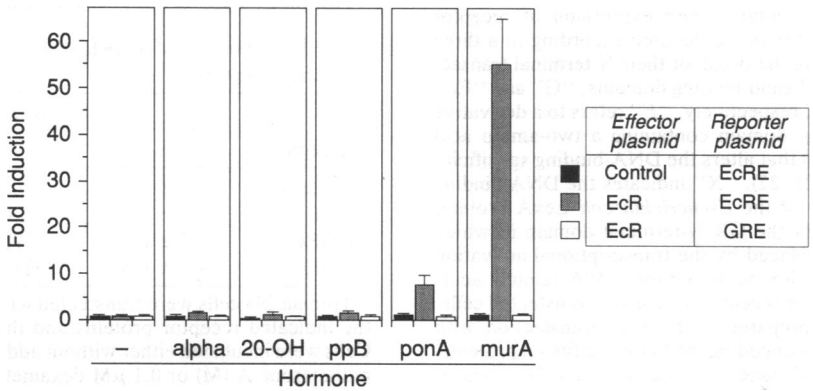

FIG. 1. Specific ecdysteroids are agonists for the EcR in mammalian cells. Human 293 cells were cotransfected with 2.5 μg of the expression plasmid pRSV.EcR or the parental expression plasmid pRSV (Control) and 0.5 μg of the reporter plasmid $pEc_4M_{-77}CO$ (EcRE) or $pG_4M_{-77}CO$ (GRE). As a control for transfection efficiency, 0.5 μg of the control plasmid pRSV.hGH was included in the transfection mixture. After transfection, cells were treated without (–) or with 1 μM α-ecdysone (alpha), 20-hydroxyecdysone (20-OH), polypodine B (ppB), ponasterone A (ponA), or muristerone A (murA). Values were normalized to the expression of human growth hormone, and the average values of three independent experiments are shown.

ecdysteroid-dependent fashion to selectively stimulate expression from an EcRE-containing reporter gene.

It is unclear why 20-hydroxyecdysone and polypodine B, which are agonists in *Drosophila* cell lines, fail to activate the EcR in mammalian cells. The only structural difference between 20-hydroxyecdysone and the weak agonist ponasterone A is that the latter lacks a hydroxyl group at position 25; the strong agonist muristerone differs from ponasterone A solely by the addition of hydroxyl groups at positions 5 and 11 (27). It will be interesting to determine whether there are differences in the modification or metabolism of the EcR or ecdysteroids in mammalian and insect cells. The activities of certain glucocorticoid ligands for their receptor have also been shown to differ significantly in yeast and mammalian cells (M. Garabedian, personal communication).

To determine whether mammalian steroid hormones could act as agonists for the EcR, we tested representative members of classes of hormones known to activate mammalian steroid receptors. Cells were transfected with the EcR expression vector and $Ec_4M_{-77}CO$ reporter gene and treated without hormone or with 1 μM dexamethasone, 17β-estradiol, aldosterone, corticosterone, hydroxycorticosterone, testosterone, dihydrotestosterone, 3,3',5-triiodo-L-thyronine, promegestone, retinol acetate, or 1,25-dihydroxyvitamin D_3. Reporter gene activity was determined as described in Fig. 1. None of these hormones stimulated expression of the reporter gene above background levels (results not shown). These results support the idea that EcR activity is selectively regulated in mammalian cells by ecdysteroids.

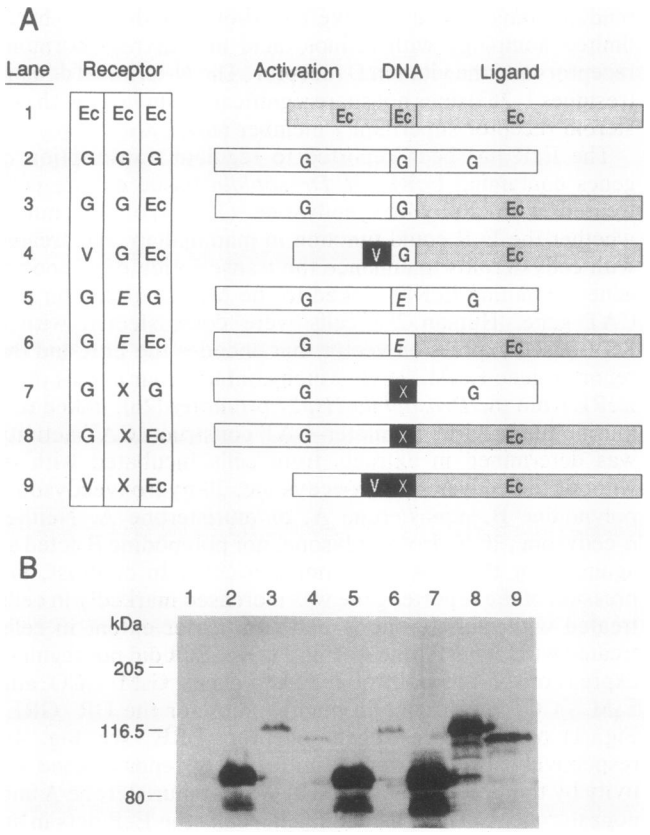

FIG. 2. Schematic representation and expression of receptor proteins. (*A*) Receptor constructs are denoted according to a three part nomenclature describing the origin of their N-terminal transactivation, DNA-binding, and ligand-binding domains. "G" and "Ec" refer to the GR and the EcR, respectively. "*E*" refers to a derivative of the rat GR DNA-binding domain containing a two-amino acid substitution (G458E, S459G) that alters the DNA-binding specificity to allow ERE recognition (21, 22). "X" indicates the DNA-binding domain (amino acids 1–87) of the *Escherichia coli* LexA protein. "V" denotes a derivative of the GR N-terminal domain in which amino acids 153–406 are replaced by the transcriptional activation domain of the herpes simplex viral protein VP16 (amino acids 411–490). (*B*) Accumulation of receptor proteins in transfected cells. Whole cell extracts were prepared 48 hr after transfection with receptor expression plasmids encoding the following fusion proteins: lane 1, EcEcEc; lane 2, GGG; lane 3, GGEc; lane 4, VGEc; lane 5, G*E*G; lane 6, G*E*Ec; lane 7, GXG; lane 8, GXEc; lane 9, VXEc. The blots were reacted with monoclonal antibody BuGR2, which recognizes an epitope in the N-terminal domain of the rat GR (33). This epitope is contained within all of the receptor derivatives used in this study, with the exception of the EcR; thus lane 1 serves as a control for background reaction with the antibody. Positions of molecular size markers are indicated.

Chimeric EcRs Regulate the Expression of Genes Containing GRE or EREs. A remarkable feature of steroid hormone receptors is the degree to which individual domains can function when combined with domains of heterologous proteins. The DNA-binding specificity of a receptor can be altered by replacing its DNA-binding domain with one from another steroid receptor (28) or from bacterial (5) or yeast (6, 7) DNA-binding proteins. In some cases, the activity of a heterologous protein becomes hormone-regulated when that protein is fused to a steroid receptor ligand-binding domain. For example, the transactivation or transformation activities of the adenovirus E1A protein or c-Myc, C/EBP, or c-Fos protein can be brought under hormonal control by fusion of a steroid receptor ligand-binding domain (29–32).

We determined whether the ligand-binding domain of the EcR could be used to regulate the DNA-binding and transactivation domains of the mammalian GR. We constructed a chimeric receptor gene, GGEc, in which the sequence encoding the ligand-binding domain of the GR was replaced with that of the EcR (Fig. 2*A*). Western blot analysis indicated that GGEc was expressed in transfected cells, although at considerably lower levels than the intact GR (Fig. 2*B*). As expected, the EcR failed to induce the expression of the GRE-containing reporter gene $G_4M_{-77}CO$ (Table 1). However, expression of the GRE-containing reporter gene was induced more than 700-fold by GGEc in muristerone-treated cells. The relative efficacies of ecdysteroids as agonists for the chimeric receptor and the EcR are identical; neither α-ecdysone, 20-hydroxyecdysone, nor polypodine B acted as agonists, whereas maximal induction in response to ponasterone A was about 15% of that observed with muristerone A.

Results of the CAT assays were confirmed and extended by direct analysis of transcripts initiated at the regulated promoter. RNA isolated from transfected cells was assayed by RNase mapping experiments using probes complementary

Table 1. Induction of a GRE-responsive gene by chimeric receptors

Receptor	Fold induction (mean ± SD)		
	−	M	D*
EcEcEc	1.0 ± 0.2	0.9 ± 0.2	0.8 ± 0.2
GGG	1.0 ± 0.3	1.0 ± 0.3	347 ± 28
GGEc	1.7 ± 0.3	798 ± 57	1.2 ± 0.1
VGEc	4.7 ± 0.8	3117 ± 240	1.4 ± 0.3

Human 293 cells were transfected with effector plasmids encoding the indicated receptor proteins and the reporter gene $G_4M_{-77}CO$. Cells were incubated either without added hormone (−) or with 1 μM muristerone A (M) or 0.1 μM dexamethasone (D).

*Human 293 cells express low levels of endogenous GR, which results in a small induction (<6-fold) of reporter gene $G_4M_{-77}CO$ in dexamethasone-treated cells. This background was subtracted from the results shown. Transfection of 293 cells with expression plasmids encoding the intact GR, but not the EcR derivatives, resulted in substantial increases in dexamethasone-dependent induction of $G_4M_{-77}CO$ compared with control transfections.

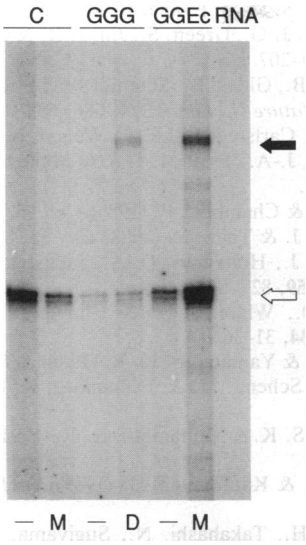

FIG. 3. RNase protection analysis of transcripts induced by receptor proteins. Total RNA was prepared from cells 48 hr after transfection with the RSV expression vector lacking a receptor gene insert (control) or with expression plasmids encoding either GGG or GGEc receptor (see Fig. 2A) and the reporter gene $G_4M_{-77}GO$. Assays were performed as described (24) with 25 μg of total cellular RNA. The lane marked "RNA" received only tRNA and serves as a control. The 377-base protected band for $G_4M_{-77}CO$ and the 294-base protected band from the internal control gene (expressed from a CMV IE enhancer/promoter construct) are indicated by the filled arrow and open arrow, respectively. The small (2- to 3-fold) apparent increase in the expression of the internal control gene seen in GGEc-expressing cells treated with muristerone A was not reproducibly observed. −, No hormone; M, muristerone A; D, dexamethasone.

to the GRE-containing reporter gene and a cotransfected control gene expressed from the CMV enhancer and promoter. Fig. 3 shows that GGEc acts in a hormone-dependent fashion to stimulate expression from a GRE–MMTV promoter construct.

We then determined whether an EcR fusion protein could regulate genes normally responsive to estrogens. We constructed a derivative of GGEc that incorporated a two-amino acid change in the DNA-binding domain of the rat glucocorticoid receptor (G458E, S459G) that has been shown to alter the DNA-binding specificity of the GR such that it will bind to a consensus ERE (21, 22). This fusion protein, GEEc (Fig. 2A), regulated the ERE-containing reporter gene $E_4M_{-77}CO$ in a muristerone-dependent fashion (Fig. 4).

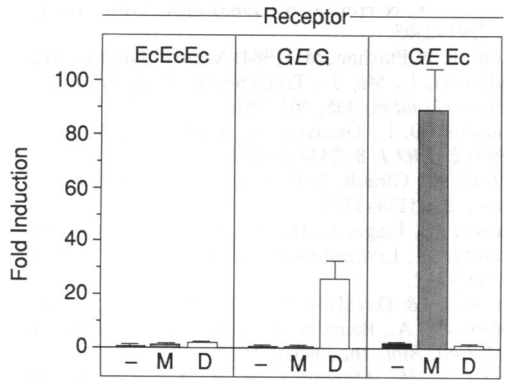

FIG. 4. Induction of EREs by chimeric receptors. Cells were transfected with expression plasmids encoding either EcR (EcEcEc), GEG, or GEEc and the reporter plasmid $pE_4M_{-77}CO$. Cells were incubated for 48 hr without (−) or with muristerone A (M) or dexamethasone (D). Reporter gene activity was determined as described in *Materials and Methods*.

Table 2. Transcriptional activation by receptor–LexA fusion proteins

	Fold induction (mean ± SD)			
	$X_4C_{-33}CO$		$OC_{-33}CO$	
Receptor	−	+	−	+
EcEcEc	1.2 ± 0.2	1.4 ± 0.1	NT	NT
GXG	1.0 ± 0.1	1.1 ± 0.2	NT	NT
GXEc	1.0 ± 0.2	44.3 ± 8.3	0.7 ± 0.2	0.9 ± 0.3
VXEc	4.7 ± 1.3	563 ± 61	0.8 ± 0.4	1.0 ± 0.2

Effector plasmids encoding the indicated receptor proteins were transfected with a reporter gene either containing ($X_4C_{-33}CO$) or lacking ($OC_{-33}CO$) Lex operators. Cells were incubated either with (+) or without (−) muristerone A.

Ecdysteroid Regulation of Bacterial DNA-Binding and Viral Transactivation Domains. We next determined whether the EcR ligand-binding domain could be used to regulate the activity of a DNA-binding domain not normally expressed in mammalian cells. Such fusion proteins might prove useful in regulating the expression of exogenous genes introduced into mammalian cells. We constructed the chimeric gene GLxEc by replacing the sequence coding for the GR DNA-binding domain in GGEc with the sequence encoding the DNA-binding domain of the *Escherichia coli* LexA repressor (34) (Fig. 2A). A reporter gene, $X_4C_{-33}CO$, was constructed that contained four copies of a 26-base-pair Lex operator (35) at position −33 of the CMV promoter. GLxEc had no effect on the expression of a control reporter gene that lacked the Lex operator (Table 2). However, transcription of $X_4C_{-33}CO$ was strongly induced by GLxEc, and this induction was fully hormone-dependent. As controls we showed that $X_4C_{-33}CO$ was not induced in cells treated with muristerone and cotransfected with expression plasmids encoding either the EcR, which lacks the LexA DNA-binding domain, or GLxG, which contains the GR ligand-binding domain.

Finally, we determined whether the activity of EcR fusion proteins could be further enhanced by inclusion of a potent viral transactivation domain. We constructed VGEc and VLxEc fusion genes by replacing a portion of the GR N-terminal activation domain in GGEc and GLxEc, respectively, with the acidic activation domain of the herpes simplex viral protein VP16 (36, 37) (Fig. 2A). In transfected cells, these proteins accumulated to similar levels as derivatives lacking the VP16 activation domain (Fig. 2B). Both VGEc and VLxEc acted in an ecdysteroid-dependent fashion to induce activity of the appropriate reporter gene (Tables 1 and 2). However, the activity of VGEc and VLxEc was 5- and 10-fold greater than GGEc and GLxEc, respectively. We note that the basal level of expression of the reporter gene increased in cells transfected with vectors expressing VGEc or VXEc. This presumably reflects some ligand-independent activity of these proteins. However, the EcR ligand-binding domain can be used to regulate the activities of viral, mammalian, and bacterial DNA-binding or transactivation domains.

DISCUSSION

Our results demonstrate the feasibility of using nonmammalian steroid hormone receptors to regulate genes in mammalian cells. In addition to the human 293 cells used in this study, transactivators containing the EcR ligand domain also act in a ligand-dependent fashion in CHO cells (P.J.G., data not shown) and in CV-1 monkey cells and F9 murine embryonal carcinoma cells (J. N. Miner, personal communication). There are several important features of this system. The EcR

acts as a potent and selective regulator of the transcription of genes containing EcREs, and the activity of the EcR can be further modified by replacing its DNA-binding or transactivation domain with the corresponding domain from a heterologous protein. Importantly, EcR activity is regulated by ecdysteroids, which are not normally expressed in mammalian cells (38). Although we did not survey all of the mammalian steroids, none of those representing the most abundant of the natural murine steroids acted as agonists for the EcR. Thus, it is conceivable that the transcriptional regulatory activities of the EcR or EcR fusion proteins will be completely dependent on the administration of exogenous ligand. Of course, it will be important to determine the effects of ecdysteroids and of the expression of the EcR on the physiology of mammalian cells. In preliminary experiments we have not detected any gross alterations on the growth or metabolism of 293 or CHO cells incubated with muristerone A and expressing the EcR.

Several reports have demonstrated the feasibility of using the *E. coli lac* repressor to regulate gene expression in mammalian cells. Both the *lac* repressor- and the steroid receptor-based systems can induce or repress (39–41) transcription. An attractive feature of a steroid receptor-based regulatory system is that the amount of induction can be varied by altering the concentration and time of exposure to the ligand. Second, transcriptional regulation of engineered genes by the EcR, or by derivatives that contain different activation or DNA-binding domains, may be achieved without perturbing the expression of any other cellular genes. Finally, in theory, EcR fusion genes can be constructed to regulate the expression of virtually any gene for which a cis-acting regulatory sequence and its cognate DNA-binding domain have been identified.

Ornitz *et al.* (42) have described a binary system for regulating expression of heterologous genes in transgenic mice. In this system, a "transactivator" strain expressing the yeast GAL4 protein is crossed with "target" strains containing a transcriptionally silent transgene controlled by upstream activation sequences. The bigenic progeny of this cross express both transgenes in the same tissue. We suggest that bigenic systems incorporating the EcR or EcR gene fusions may provide a general method to control the abundance, time course, and/or tissue-specific expression of endogenous or exogenous genes. For example, tissue-specific and developmentally regulated expression of transgenes controlled by EcREs could be achieved by targeting the expression of the EcR with appropriate tissue-specific enhancers. However, in contrast to the GAL4 system, the expression of that transgene could then be induced at appropriate times by "switching on" its activator with ecdysteroids. The development of a system for regulated expression of endogenous and exogenous genes in eukaryotic cells should provide an important method to study the function of those gene products.

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