Functional transfer of an elementary ecdysone gene regulatory system to mammalian cells: Transient transfections and stable cell lines

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Abstract. A 3.1 Kb fragment of a *Drosophila melanogaster* ecdysone receptor (EcR) cDNA (splice product, *EcR B1*) comprising the 2.6 Kb coding region with 218 base pairs of 5' and 258 base pairs of 3'-untranslated sequence, was cloned into the mammalian expression vectors pHβAPr-1 and pSG5 (which place *EcR* under the control of a human β-actin and a SV40 early promoter, respectively). Chinese hamster ovary cells have been stably transfected with the β-actin promoter construct. Antiserum, prepared against an EcR-fusion protein, has been used to demonstrate the synthesis of an apparently complete ecdysone receptor in a stable cell line produced in this way. Nuclear extracts from this line exhibit specific binding activity for the *D. melanogaster* hsp 27 ecdysone response element in mobility shift analyses.

Ecdysteroid induction of reporter gene activity has been demonstrated in Chinese hamster ovary cells both by transient transfection analysis and in stably transfected cell lines which constitutively produce the *D. melanogaster* ecdysone receptor.

INTRODUCTION

Diptera and mammals have been phylogenetically separated for at least 500 million years (Morris, 1993). Nonetheless, there is clear evidence that elements within the gene regulatory machinery have been conserved between these groups during their long separation. For example, the human transcription factor Sp1, when expressed in *Drosophila melanogaster* tissue culture cells, can activate gene expression (Courey et al., 1989) and mammalian homeobox genes can function very similarly to their *Drosophila* homologues when inserted into transgenic flies (Zhao et al., 1993). Conversely, the product of the *Drosophila* gene *Kruppel,* expressed in human and monkey cells, can act as a specific repressor of transcription (Licht et al., 1990).

In *Drosophila* a whole host of gene systems are under the control of the steroid hormone 20-hydroxyecdysone (see, e.g., Richards, 1981; Thummel, 1990; Hill et al., 1993). It was postulated by Ashburner et al. (1974) that binding of 20-hydroxyecdysone to a receptor protein converted it to a form capable of activating genes. More recently, a gene encoding this postulated receptor, *EcR,* has been cloned (Koelle et al., 1991) and sequences in promoter sites capable of binding to it (ecdysone response elements, *EcRE's*) have been characterised (Riddihough & Pelham, 1987; Cherbas et al., 1991). The ecdysone gene regulatory system is not found in mammalian cells (see, e.g., Burdette, 1962). The transfer

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of this system to mammalian cells thus offers the possibility of specific external control of transgenes linked to *EcRE's* by administration of ecdysteroids. However, for the system to prove useful for the control of genes for gene therapy or for optimisation of expression of transgenes for economic advantage in the production of transgenic animals, it must function in the context of a situation in which both *EcR* genes and the genes coming under ecdysteroid regulation are stably incorporated into mammalian cell genomes.

Several laboratories have shown, by transient cotransfection analyses, that the ecdysone gene regulatory system of *D. melanogaster* can function in mammalian cells (Christopherson et al., 1992; Yao et al., 1992; Thomas et al., 1993). Evidence has been adduced for the notion that the EcR protein binds functionally to *EcRE's* as a heterodimer with another member of the steroid receptor superfamily, the product of the *ultraspiracle* gene in *D. melanogaster* or the retinoid X receptor in mammalian cells (Yao et al., 1992; Thomas et al., 1993). In this communication we examine some of the properties of Chinese hamster ovary (CHO) cells, stably transfected with a gene encoding the *D. melanogaster* EcR under the control of a human β-actin promoter. We show that they acquire the ability to produce a new protein ᴄᵒ apparent molecular weight 105 KD, which reacts with antibodies raised against a segment of the ecdysone receptor expressed in *E. coli*, and a binding activity, in mobility shift assays, for the hsp27 *EcRE*. We demonstrate the ecdysteroid-inducibility, in CHO cells, of a chloramphenicol acetyl transferase (CAT) reporter gene linked to a promoter containing *EcRE's* when transiently cotransfected with a plasmid expressing EcR. We also show reporter gene inducibility in cells constitutively producing the receptor from stably incorporated *EcR* genes when the *EcRE-CAT* reporter is introduced transiently, and when it too is stably incorporated into the genome.

MATERIAL AND METHODS

Plasmids

pSV40-EcR was constructed by excising a 3110 base pair Fsp1-HindIII fragment, containing the complete 2634 base pair coding region of a *D. melanogaster* ecdysone receptor, with 218 base pairs of 5'-untranslated sequence and 258 base pairs from the 3'-untranslated region, from a plasmid pMKI bearing a EcR-cDNA (Koelle et al., 1991; kind gift of the Hogness laboratory). This fragment was end-filled and ligated into the end-filled BamHI site of the expression vector pSG5 (Greene et al., 1988).

pHβA-EcR was constructed by cloning the FsP1-HindIII fragment described above into the HindIII site of the human β-actin expression vector pHβAPr-1 (Gunning et al., 1987).

The reporter plasmid p(EcRE)₅-CAT, in which five copies of a sequence containing the *D. melanogaster* hsp27 *EcRE* (Riddihough & Pelham, 1987) were inserted into the HindIII site of the plasmid pMMTV-CAT (Hollenberg & Evans, 1988) 93 base pairs upstream of the transcription start site for the MMTV promoter, was a kind gift from Dr W. Segraves.

The 1.8 kb KpnI fragment of *D. melanogaster* EcR cDNA, which encompasses the region encoding the hormone binding domain, was generated from EcR cDNA plasmid pMK1 (Koelle et al., 1991) and cloned into the Smal I site of plasmid pGEX-3 (Smith & Johnson, 1988) to give pGEX-EcR.

Cell culture

Chinese hamster ovary (CHO) cells and Swiss 3T3 mouse cells were maintained in 50% (v/v) Dulbecco's modified Eagle's medium (DMEM) and 50% (v/v) Ham's F12 nutrient mixture (GIBCO) supplemented with 10% fetal bovine serum. CV1 monkey kidney cells were maintained in modified Eagle's medium (MEM) (GIBCO) supplemented with 10% fetal bovine serum.

Transient transfections

DNA-calcium phosphate mediated transfection was carried out by the coprecipitation method (Ausubel et al., 1992). One day before transfection, CHO cells were plated out at $5-8 \times 10^5$ cells per 6 cm diameter

culture dish in DMEM/F12 medium supplemented with 10% fetal bovine serum. Three hours before the addition of the DNA-calcium phosphate coprecipitate, the cells were washed with phosphate buffered saline (PBS, Sambrook et al., 1989) and cultured in fresh DMEM plus 10% fetal bovine serum. The cells were incubated in the presence of the coprecipitate for 18 hours before excess DNA was washed away with PBS. The cells were then cultured for another day in DMEM/F12 supplemented with 10% (v/v) fetal bovine serum with or without added ponasterone A before harvesting. Transfections included a β-galactosidase expression plasmid pPGK-LacZ (McBurney et al., 1991) as an internal control for transfection efficiency, and pUC18 DNA to bring the total amount of DNA added per dish up to 10 μg. Cells were washed with PBS, harvested by mechanical scraping in 0.25 M Tris-HCl (pH 7.8) and disrupted for enzyme extraction by three freeze-thaw cycles. CAT and β-galactosidase enzyme activities were assayed on duplicate or triplicate plates by the organic solvent procedure as described in Sambrook et al. (1989), experiments repeated two or more times and mean values reported.

Lipofection was carried out using DOTAP (Boehringer-Mannheim) at 15 μg/ml. Duplicate dishes of subconfluent CHO cells were cotransfected with p(EcRE)$_5$-CAT (1μg/ml), pPGKNeo (1μg/ml) was added as an independent reporter gene to allow monitoring of transfection efficiency and either pSV40-EcR (1μg/ml) or pUC19 (1μg/ml) to act as a control carrier DNA for transfections. Commercially available ELISA kits were used to quantify the synthesis of CAT (Boehringer-Mannheim, Aust.) and Neomycin phosphotransferase II (NPTII) (5 Prime-3 Prime, Inc., USA) in extracts of cells 48 h after transfection (Hannan et al., 1993). Variation between experiments was minimised by normalising the level of CAT to NPTII in the same extract.

Preparation of stably transfected cell lines

To prepare cells containing a stably integrated EcR gene, CHO cells were transfected with 2.5 μg pHβA-EcR DNA and 0.5 μg neomycin resistance expression vector, pPGKNeo. (McBurney et al., 1991). Geneticin (G418) (600 μg/ml) was added to the culture medium for selection. Colonies resistant to G418 were isolated and further purified by limit dilution cloning. These colonies were assayed for EcR activity by transfection with reporter gene construct p(EcRE)$_5$-CAT.

Cell lines in which both an ecdysone receptor expression plasmid and reporter plasmid were stably integrated were similarly prepared except that 2.5 μg of p(EcRE)$_5$-CAT, 2.5 μg pHβA-EcR and 0.5 μg pPGKNeo were used in the transfection.

Hormones

20-hydroxyecdysone was purchased from Sigma Chemical Company. Ponasterone A was a kind gift from Denis Horn.

Preparation of antiserum

pGEX-EcR was employed for production of a gluthathione S-transferase-fusion protein in E. coli. An overnight culture was diluted 1 : 10 in fresh medium and grown until A$_{600}$ reached 1-2. Isopropylthiogalactoside was added to 0.1 mM and incubation continued for another 2-6 hours. Cells were pelleted and resuspended in 1/10 volume of 50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 100 mM NaCl containing 5 mg/ml lysozyme and incubated for 20 min on ice. Following sodium deoxycholate treatment (0.5 μg/ml) at 37°C for 5 min, the lysate was subjected to DNase 1 (5 μg/ml) digestion at 37°C for 20 min and pelleted at 12,000 g for 15 min. As the expressed fusion protein is highly insoluble, the pellet was washed twice with 5 M urea in 0.1 M Tris-HCl (pH 8.3), solubilised in sodium dodecyl sulphate (SDS) and size fractionated by SDS-polyacrylamide gel electrophoresis. Protein bands were visualized in ice-cold 0.2 M KCl and the fusion protein band was cut out. For immunogen preparation the gel slice was cut into small pieces, mixed with sterile 0.9% NaCl and further fragmented by passage several times through narrow gauge syringe needles. Approximately 30 μg of the fusion protein was taken for each injection of female BAlB/c mice.

Immunoblot analysis

Cells at subconfluence on 10 cm dishes were washed with 10 ml TBS, scraped into 2 ml TBS and pelleted by centrifugation for 15 sec in a microfuge. Protein extraction was performed essentially as described by Schreiber et al. (1989). The cell pellet was resuspended in 100 μl buffer containing 10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM ditiothreitol (DTT), 0.1 mM phenylmetylsulphonyl fluoride (PMSF) for 15 min after which 12 μl of a 10% solution of Nonidet P40 was

added. The tube was vigorously vortexed for 5 sec and centrifuged for 30 sec in a microfuge. The nuclear pellet was resuspended in 29 µl of ice cold buffer containing 20 mM HEPES (pH 7.9), 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF. The tube was rotated for 15–20 minutes at 4°C. The extract was centrifuged for 5 min at 4°C in a microfuge, the supernatant aliquoted and stored at –70°C. 5 µg of protein for each sample was subjected to SDS polyacrylamide electrophoresis in a 6% gel. The protein on the gel was electrophoretically transferred to a sheet of nitrocellulose membrane (Schleicher & Schuell). The membrane was blocked by overnight incubation in "blotto" containing 5% skim milk and 0.5% Tween-20 after which it was transferred to a similar buffer but containing 0.1% Tween-20. The blot was probed with appropriately diluted polyclonal antiserum to EcR protein in buffer containing 0.1% Tween 20. Bands were visualised utilising a horse radish peroxidase-linked second antibody and the enhanced chemiluminescence kit (ECL, Amersham) according to the procedure described by the manufacturer.

Nuclear extracts and gel mobility shift assays

Nuclear extracts were prepared as described above for immunoblot analysis. The pellets were resuspended in buffer containing 0.4 M NaCl for extraction of protein. The binding reaction mixture (20 µl) contained 5–10 µg crude nuclear protein with 2 µg poly(dI-dC) in 15 mM Hepes (pH 7.9), 60 mM KCl, 0.5mM DTT, 0.1 mM EDTA, 0.5 mM PMSF and 10% glycerol.

^{32}P-labelled double-stranded oligonucleotide encompassing the EcRE from D. melanogaster hsp 27 EcRE was generated by anealing oligo 5'ATGCGAGAGACAAGGGTTCAATGCACTTGTCCAATG3' and oligo 5'GCATCATTGGACAAGTGCATTGAACCCTTGTCTCTC3' and filling in with Klenow polymerase. The binding reaction was performed by preincubation on ice for 10 min, followed by incubation at room temperature for 10 min with 20 fmol probe (approximately 1–2 × 10^4 cpm) and other oligonucleotide competitors as indicated in the text. The reaction mixture was then loaded onto a 10% nondenaturing polyacrylamide gel (acrylamide:bis 60:1) in TGE buffer (25 mM Tris-HCl, pH8.3, 192 mM glycine, 0.5 mM EGTA) with 5% added glycerol and run in TGE buffer. After electrophoresis the gels were dried down for autoradiography.

Southern blotting

For DNA preparation, CHO cells were released by trypsinisation, pelleted by centrifugation, resuspended in PBS and pelleted once more. The cells were resuspended in 1 ml of 100 mM Tris-HCl (pH 8.0), 5 mM EDTA, 5 mM NaCl and lysed by the addition of SDS (0.5%). Proteinase K was added to a final concentration of 100 µg/ml and digestion carried out overnight at 37°C. The viscous solution was extracted once with phenol : chloroform (1:1) and again with chloroform alone. The aqueous phase was collected, made 0.3 M with respect to sodium acetate and nucleic acid was recovered by ethanol precipitation. Nucleic acids were re-dissolved in 1 ml TE and ethanol precipitated a second time. The DNA pellet was dried and redissolved in TE to a concentration of 250 µg/ml).

DNA (2 µg) was digested overnight with 8 units of SacI restriction endonuclease at 37°C. The resultant DNA fragments were separated by electrophoresis in a 0.8% agarose gel in TBE buffer. DNA was transferred to Hybond N by capillary blotting overnight in 0.4 M NaOH.

After blotting, filters were rinsed in 2 × SSC and pre-hybridized for 30 min in 5 × SSPE, 5 × Denhard's solution, 0.2% SDS, 42% formamide at 42°C. Filters were then hybridized overnight in the same buffer containing a ^{32}P-labelled probe (10^6 cpm/ml) specific for the DNA binding domain of the D. melanogaster EcR gene. The DNA encoding the binding domain of the EcR was isolated within a 420 bp fragment from plasmid pMK1 by digestion with SacI and EcoRI. 50 ng of this DNA was denatured at 95°C for 5 min. Radiolabelled probe was prepared using the GIGAprime DNA Labelling Kit (Bresatec Ltd., Adelaide, Australia) essentially according to the manufacturer's instructions except that random primers were replaced with the specific primers ecdna1 (5'TGCCTGGTTTGCGGCGACAGG3') and ecdna2 (5'CATACCCACGGCCAGGCACTT3'). Unincorporated label was removed by size exclusion chromatography over Biogel-P60 (Biorad Ltd., Sydney, Australia). After hybridisation, filters were washed for 2 × 15 min in 2 × SSC, 0.2% SDS and for 2 × 15 min in 0.2 × SSC, 0.2% SDS at room temperature. Filters were dried and radioactive profiles detected using a Molecular Dynamics PhosphorImager.
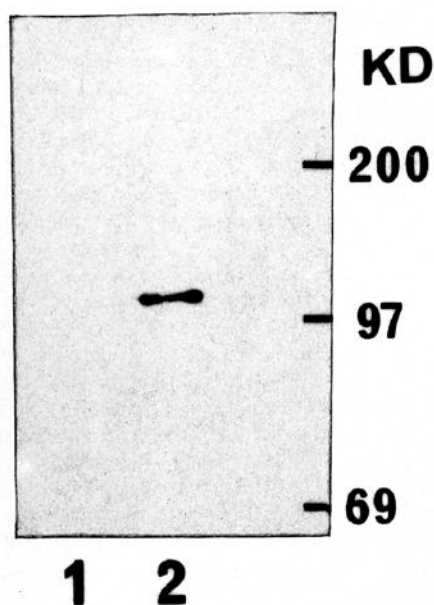
KD

— 200

— 97

— 69

1 2

Fig. 1. Immunoblot analysis of the synthesis of ecdysone receptor protein in control CHO cells (lane 1) and CHO cell line CR2, stably transformed with the EcR expression vector pHβA-EcR (lane 2).

RESULTS

Expression of functional ecdysone receptor in stably transfected CHO cells

A plasmid, pHβA-EcR, containing the complete coding sequence of a *D. melanogaster* ecdysone receptor (form EcR B1) under the control of a human β-actin promoter (Gunning et al., 1987), was constructed for expression in mammalian cells. Following cotransfection of CHO cells with pHβA-EcR and a neomycin phosphotransferase II expressing plasmid, pPGKNeo, several neomycin resistant clones were obtained. An extract from one of these, CR2, was assayed by immunoblotting for the presence of ecdysone receptor protein employing a specific antiserum prepared as described in Material and methods. A single band, absent from untransfected cells, was detected by the antiserum (Fig. 1). Its electrophoretic mobility corresponded to 105 KD, consistent with that expected for the conceptually translated *D. melanogaster EcR* gene (Koelle et al., 1991).

The ecdysone receptor protein synthesised in *D. melanogaster* cells has the ability to bind to ecdysone response elements in vitro. In order to examine whether the ecdysone receptor protein synthesised in stably transfected CHO cells is biologically active by this criterion, mobility shift assays were carried out using a ^{32}P-labelled oligonucleotide containing an *EcRE* from the *D. melanogaster* hsp27 promoter (see Materias and methods). The result (Fig. 2) shows binding activity for the *EcRE* in extracts from stably transfected cell line CR2 but not in extracts from control CHO cells. Competition for binding was exhibited by unlabelled *EcRE* but not by a nonspecific sequence.

To confirm the presence of stably integrated *EcR* genes, and to obtain a semiquantitative estimate of copy number, DNA was prepared from the cell line CR2 twelve passages after clonal isolation and from the parental CHO cell line. After cleavage with SacI, DNA samples were analysed by Southern blotting using as a probe a ^{32}P-labelled 198 base pair fragment encompassing the region of the gene encoding the DNA binding domain. The
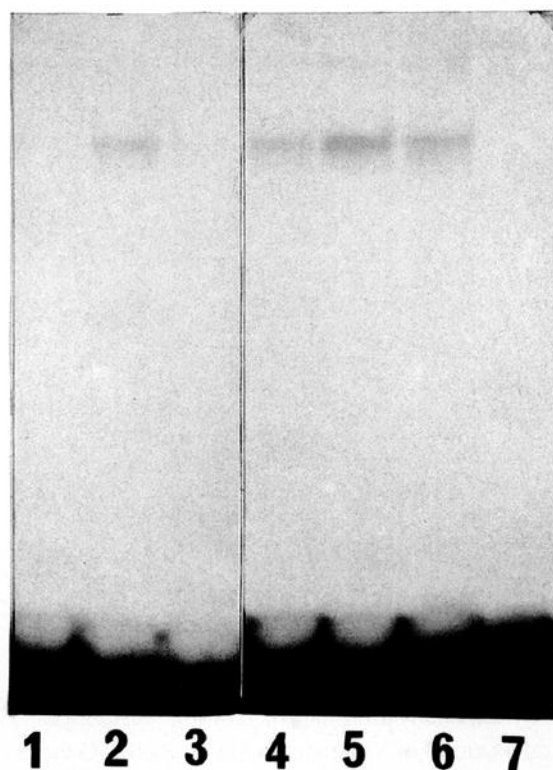
383

Fig. 2. Band-shift analysis showing the ability of the ecdysone receptor protein synthesised in the transformed CHO cell line, CR2, to bind to the *EcRE* sequence in vitro. Lanes 1–6 depict incubations of labelled *EcRE* and nuclear extracts from the cells. Lane 1, 10 µg extract from control CHO cells; lane 2, 10 µg of extract from cell line, CR2, binding in the presence of a 50-fold excess of non-specific competitor sequence (5'TGCTTTCTCCAGTTTTCTGTTAATG GCGAAA3'); lane 3, 10 µg extract from cell line CR2 binding in the presence of a 50-fold excess of unlabelled *EcRE*; lane 4, 10 µg cell line CR2 binding in the presence of a 5-fold excess of unlabelled *EcRE*; lane 5, 10 µg extract from cell line CR2, no competitor; lane 6, 5 µg extract from cell line CR2, no competitor; lane 7, *EcRE* in the absence of cell extract.

result (Fig. 3) clearly showed the presence of *EcR* in CR2 but not in the parental CHO cells. Comparison with the signals from measured amounts of plasmid containing the gene indicated that there were of the order of 25 copies of *EcR* per cell.

Ecdysteroid mediated control of gene expression in transient assays and stable CHO cell lines

For an initial assessment of the ability of the ecdysone receptor synthesised in mammalian cells to regulate gene activity, cells were cotransfected with plasmid, pSV40-EcR (in which *EcR* is under the control of the SV40 early promoter) and a series of reporter plasmids containing varying numbers of *EcRE's* inserted into different sites within a MMTV promoter linked to a CAT gene. A number of different cell types were tested employing different ecdysteroids as potential inducers.

The first positive results were obtained when CHO cells, after transient transfection with pSV40-EcR and p(EcRE)$_5$-CAT, were exposed to the ecdysone analogue, ponasterone A, which displays a higher affinity than 20-hydroxyecdysone for the native *D. melanogaster* receptor. A strong induction was observed either for calcium phosphate-transfected cells monitored for CAT enzymatic activity (Fig. 4A1,2) or for DOTAP-transfected cells monitored for CAT by immunological assay (Fig. 4B1,3). A number of controls were performed. Fig. 4A3 depicts the low level of background in cells transfected with the parental expression vector, pSG5 (not containing the receptor gene) and p(EcRE)$_5$CAT. Fig. 4A4,5 indicate the low induction of gene activity with a receptor
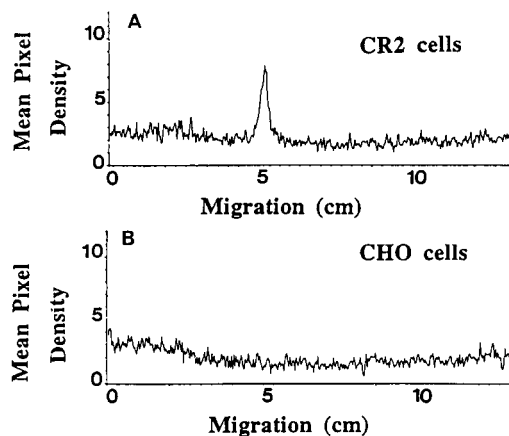
Fig. 3. Southern blot detection of *EcR* in the stably transfected cell line CR2. DNA from CR2 cells (trace A) and from the parental CHO cells (trace B). DNA purified from the two cell lines was cut by SacI, subjected to Southern blotting and probed with a segment of the *EcR* gene encompassing the DNA binding domain as described in Material and methods. Comparison with plasmid standards containing *EcR* run on the same gel indicated 25 copies of *EcR* per cell.

producing plasmid and a reporter (pMMTV-CAT) lacking *EcRE's*. Fig. 4A6 shows the low level of expression when cells are cotransfected with pSG5 and pMMTV-CAT. When pSV40-EcR and p(EcRE)$_5$-CAT are introduced into CHO cells by lipofection, the level of CAT induction is even more striking (Fig. 4B1,3); in this system reporter gene expression remains at a basal level if hormone is added but there is no ecdysone receptor in the cells (Fig. 4B2). Hormone inducibility was also observed in Swiss 3T3 mouse cells but not in monkey CV1 cells (data not shown).
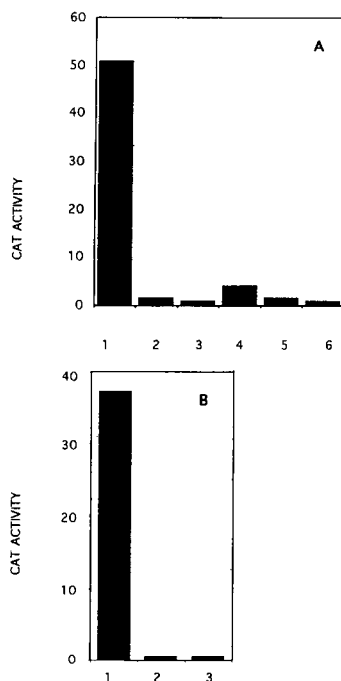

Fig. 4. The control of a chloramphenicol acetyl transferase (CAT) reporter gene in CHO cells by the ecdysone regulatory system in transient transfection assays. A: Transfection by the calcium phosphate procedure and CAT assayed by enzymatic activity. Cells were cotransfected with: 1 and 2 plasmids p(EcRE)$_5$-CAT and pSV40-EcR; 3 p(EcRE)$_5$-CAT and pSG5; 4 and 5 pMMTV-CAT and pSV40-EcR; and 6 pMMTV-CAT and pSG5. CAT activity was normalised with respect to β-galactosidase activity expressed from the plasmid pPGK-LacZ which served as an internal control on the transient transfections. The ecdysone analogue, ponasterone A was present at a concentration of 50 μM in 1 and 4 and absent from 2, 3, 5 and 6. B: Liposome-mediated transfection and CAT assayed by immunoreactivity. Cells were cotransfected with the plasmids p(EcRE)$_5$-CAT, pPGKNeo and either 1 and 3 pSV40-ECR or 2 pUC19. 1 and 2 were incubated with 50 μm ponasterone A and 3 without the hormone. CAT activity was normalised with respect to neomycin phosphotransferase II expression from pPGKNeo, which served as an internal control for transfection efficiency.

385

40

30

CAT ACTIVITY
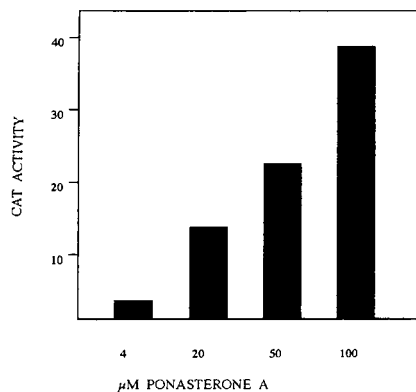
20

10

4   20   50   100

μM PONASTERONE A

Fig. 5. The effect of ponasterone A concentration on reporter gene induction in CHO cell line CR2, stably transfected with the ecdysone receptor expression plasmid, pHβA-EcR and transiently transfected with the reporter plasmid p(EcRE)₅-CAT.

For the ecdysone system to be usefully employed for the control of gene expression in gene therapy or in transgenic animals, it must also function from *EcR* genes stably integrated into the host cell genome. We have shown above that a stable cell line, CR2, in which the construct pHβA-EcR has been stably incorporated into the CHO cell chromosomes, constitutively produces a new protein exhibiting the size, immunological reactivity and in vitro DNA binding properties expected for a full-length *D. melanogaster* ecdysone receptor. Fig. 5 records the finding that, when this cell line is transiently transfected with p(EcRE)₅-CAT, ponasterone A addition leads to induction of reporter gene activity and that this induction increases monotonically with increasing ponasterone A concentration.

Of course, for use in gene therapy or in transgenic animals, the receptor producing gene and the effector gene under ecdysteroid-regulation will normally both need to be incorporated into host cell chromosomes. Cell lines, stably transfected for *EcR* and *EcRE-CAT*, were produced by selection for neomycin resistance following cotransfection with pHβA-EcR, p(EcRE)₅-CAT and pPGKNeo. The induction of reporter gene activity in one of these stable cell lines, 2CR72, is recorded in Fig. 6. Again significant induction is seen, increasing with increasing ponasterone A concentration.

20

15

CAT ACTIVITY

10

5

1

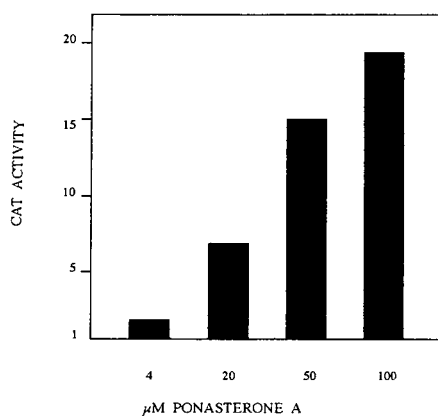4   20   50   100

μM PONASTERONE A

Fig. 6. Ponasterone A-dependent reporter gene regulation in CHO cell line, 2CR72, stably transfected with both the ecdysone receptor expression plasmid, pHβA-EcR, and the reporter plasmid, p(EcRE)₅-CAT.

DISCUSSION

During the early phases of this work, different cell types, different reporter constructs with varying numbers of *EcRE's* inserted into different positions in the MMTV promoter, and different ecdysone analogues were tested in transient transfection assays. The most active form of molting hormone in *Drosophila*, 20-hydroxyecdysone, gave little or no induction of reporter gene activity in mammalian cells. However, when the tighter-binding analogue, ponasterone A, was employed highly significant, specific induction was observed. It is not known whether the greater inductive effect of ponasterone A simply reflects its well-established greater affinity for the *D. melanogaster* ecdysone receptor (see, e.g., Yund et al., 1978), greater uptake by mammalian cells, differences in metabolic fate or some other cause. However, the small or insignificant effects of 20-hydroxyecdysone in our assays are consistent with the data presented for mammalian cells by Christopherson et al. (1992). The relatively large ponasterone A inductive effects observed in our work are no doubt influenced by the range of concentrations of the analogue employed (0 to 100 μM), the number of *EcRE's* in the modified MMTV promoter (smaller effects were seen with smaller numbers of *EcRE's*, data not shown), the strength of the promoter driving transcription of the *EcR* gene (the SV40 early and human β-actin promoters in the present study) and other proteins interacting with the EcR protein.

Cell-type specificity was also observed during our studies. CV1 monkey kidney cells exhibited little or no response while mouse 3T3 cells (data not shown) and CHO cells gave significant ecdysteroid induction of reporter gene activity when appropriately transfected. This cell-type specificity is consistent with the notion that the EcR protein functions as a heterodimer with the product of the *ultraspiracle* gene in *Drosophila* and, in mammalian cells, with the related retinoid X receptor which is present at different levels in different cell types (Yao et al., 1992, 1993; Thomas et al., 1993). Presumably our CHO and 3T3 cells are endogenously expressing retinoid X receptor or another functionally related protein. Clearly the level of expression of retinoid X receptor, and possibly other specific transcription factors, will influence the ability of introduced EcR to regulate the expression of transgenes in mammalian cells. It should be recalled that the very high levels of ecdysteroid induction of reporter gene activity observed in *Drosophila* cells (up to some three orders of magnitude, see, e.g., Koelle et al., 1991) may reflect secondary interactions with other specific transcription factors. This notion is consistent with long-standing cytogenetic data in *D. melanogaster*, e.g., the fact that mutations in the *Broad Complex* residing in the early ecdysteroid puff 2B5, a long way from the *EcR* gene at 42A (Segraves, 1988), significantly inhibit the ecdysone-induced puffing response at other loci (Belyaeva et al., 1981). The *Broad Complex* is now known to encode a set of proteins whose structures are consistent with a role as transcription factors (DiBello et al., 1991).

In addition to transient transfection assays, we have produced stable cell lines in which *EcR*, under the control of a human β-actin promoter, has been incorporated into the chromosomes of CHO cells. The stably transfected cell line CR2 constitutively produces a new protein with the size, immunoreactivity and DNA binding properties expected for the EcR protein of *D. melanogaster*. Expression of EcR protein has no apparent toxic effects on CHO cells. CR2 cells exhibit no significant change in cell size, morphology or doubling time from the parental CHO cells and provide a convenient, reproducible way of studying the effects of EcR protein in mammalian cells. We have also shown that the EcR

protein produced by the cell line, CR2, is sufficient to impart ecdysteroid inducibility on a reporter gene construct introduced into the cells by transient transfection. Furthermore, the cell line, 2CR72, in which both *EcR* and reporter genes have been integrated into the CHO host cell's chromosomes, exhibits direct ponasterone A-induced gene activity, providing a model in which both functional receptor genes and reporter genes should be stably inherited as would be required for applications in gene therapy and transgenic animals.

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