Receptor Interconversion Model of Hormone Action. 3. Estrogen Receptor Mediated Repression of Reporter Gene Activity in A431 Cells†

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ABSTRACT: The chicken estrogen receptor exists in three interconvertible forms, two of which bind estradiol with high affinity and one which lacks the capacity to bind estradiol. Interconversion is regulated by reactions involving ATP/Mg^{2+}. By cotransfecting into A431 cells estrogen receptor cDNA in an expression vector together with the pA2 (−821/−87) tk-CAT vitellogenin construct, we demonstrate that constitutive expression of chloramphenicol acetyltransferase (CAT) activity can be regulated either by selection of ligand or by modifying phosphorylation reactions in the recipient cells. In the presence of estrogen receptors, constitutive expression of CAT activity is inhibited in three situations: (i) in the absence of an estrogenic ligand; (ii) in the presence of an anti-estrogen; and (iii) in the presence of an estrogenic ligand together with 12-*O*-tetradecanoylphorbol 13-acetate (TPA). Estrogen receptor mediated repression of constitutive CAT activity is not observed with the pA2 (−331/−87) tk-CAT construct, indicating that DNA sequences required for repression are located between −821 and −331 base pairs upstream of the transcription initiation site.

Our focus has been to provide a model of hormone action which would explain receptor-mediated agonist versus antagonist function. An early observation was that estrogen receptors existed in two ligand binding states of differing affinity (Smith et al., 1979). It was also noted from detailed studies of other receptor–ligand interactions that membrane-bound receptors also shared this common feature of dual affinity binding. It was therefore speculated that control of receptor conversion between different binding states or conformations might provide a general mechanism to explain agonist/antagonist action. The estrogen receptor system in the chick oviduct has been selected as a model to test the receptor interconversion hypothesis (McNaught & Smith, 1986; McNaught et al., 1986; Raymoure et al., 1986).

Interconversion between various forms or conformations of the receptor has been shown to be regulated by ATP/Mg^{2+} (McNaught et al., 1986). It has also been shown that the anti-estrogen hydroxytamoxifen stabilizes the R_x form of the receptor and inhibits the ATP/Mg^{2+}-dependent conversion to R_y (Raymoure et al., 1986). Since it has been shown by many investigators that in general the anti-estrogen receptor complex is transcriptionally inactive, it is hypothesized that estrogen-mediated transcription of estrogen-responsive genes is modified, either directly or indirectly, by intracellular phosphorylation/dephosphorylation reactions (McNaught et al., 1986; Raymoure et al., 1986; Dayani et al., 1990). Experiments were therefore designed to determine whether the transcriptional activity of the estrogen receptor can be controlled by modifying intracellular phosphorylation events.

The appropriate cell for the transfections should not contain estrogen receptors and should be responsive to factors which could modify ATP/Mg^{2+}-dependent intracellular phosphorylation in alternative ways. The A431 cell line provided such a system since it has high tyrosine kinase activity and responds to epidermal growth factor (EGF)[1] by activating the tyrosine kinase activity of the EGF receptor (Hunter et al., 1984). It also responds to the phorbol ester TPA by stimulating the kinase C system which counteracts the tyrosine kinase activity as demonstrated by conversion of the high-affinity EGF receptor to its lower affinity form. Kinase C activation results in increases in threonine and serine kinase activities (Hunter et al., 1984; Lin et al., 1986). In common with estrogen receptor interconversion (McNaught et al., 1986; Raymoure et al., 1986), both types of kinase activity in A431 cells directly involve ATP rather than cAMP. It was surmised, therefore, that if the receptor interconversions so clearly regulated by ATP/Mg^{2+} in vitro (McNaught et al., 1986; Raymoure et al., 1986) were important in vivo, then differing modes of utilizing ATP/Mg^{2+} in A431 cells would result in differential regulation of estrogen receptor mediated events.

The 5′-flanking region of the vitellogenin gene was selected as a regulatable unit since vitellogenin gene transcription is regulated in vivo by estrogen and the DNA sequence containing an estrogen response element (ERE) has been well-defined (Klein-Hitpass et al., 1986; Druege et al., 1986). The CAT reporter gene was chosen since it is readily assayable. A CAT construct containing the thymidine kinase promoter was selected since increased activation of CAT activity can readily be monitored through this promoter (Klein-Hitpass et al., 1986), and because it is constitutively active, it is possible to assay for repression and activation with the same construct.

In this report, we describe the effects of agents, which are known to alter phosphorylation reactions in A431 cells, on

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[1] Abbreviations: CAT, chloramphenicol acetyltransferase; DES, diethylstilbestrol; DME, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; TPA, 12-*O*-tetradecanoylphorbol 13-acetate.

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estrogen receptor mediated expression of a CAT reporter gene containing the estrogen response element from the *Xenopus* vitellogenin 2A gene linked to a thymidine kinase promoter.

Experimental Procedures

Cell Culture. The A431 cells were routinely grown in Dulbecco's modified Eagle's medium (high glucose variety) supplemented with 5% fetal bovine serum, penicillin (100 units/mL), and streptomycin (100 units/mL) in a humidified, 5% CO_2-gassed incubator at 37 °C.

DNA Constructs. The *Xenopus* vitellogenin gene has an estrogen regulatory DNA element located in the 5′-flanking region. The construct used in these experiments was designated as follows: pA2 (−821/87) tk-CAT, consisting of the vitellogenin A2 sequence from position −87 to −821 cloned into pBL-CAT, a plasmid containing the thymidine kinase promoter in front of the CAT structural gene. A similar plasmid containing a truncated 5′-flanking region pA2 (−331/−87) tk-CAT was also used (Klein-Hitpass et al., 1986). These sequences have been shown to encompass the estrogen response element (ERE).

The chicken estrogen receptor cDNA cloned into the *Eco*RI site of the expression vector pKCR2 (O'Hare et al., 1981) was kindly provided by Drs. Green and Chambon (Strasbourg, France) and is designated CERO (Krust et al., 1986). The *Xenopus* pA2 constructs were a generous gift from Dr. Ryffel (Karlsruhe, Federal Republic of Germany).

Transfection Protocol. The day before transfection, 2.2×10^6 A431 cells were plated per 100-mm petri dish in DME without phenol red but supplemented with 5% fetal bovine serum. The next day, by use of the calcium phosphate method (Gorman, 1985), cells were transfected with 5 μg each of vit-tk-CAT (or vit-CAT) and CERO DNA per dish. Following transfection, the cells were glycerol-shocked for 2 min, rinsed in phenol red free DME, and grown in DME without phenol red supplemented with 5% charcoal-stripped fetal bovine serum. At this stage, the cells were treated with hormones and/or growth factors as described under Results. These agents were added at the following concentrations: DES, 10 nM; hydroxytamoxifen, 10 nM; EGF, 20 ng/mL; TPA, 10 nM. Twenty-four hours later, the treatments were repeated without any media change, and cells were harvested for CAT assay after another 18 h.

The CAT assay was performed according to the method described by Gorman (1985). The reaction was carried out for 1 h at 37 °C with 150 μg of cytosol protein.

Estrogen Receptor Assays. Estrogen receptors in the transfected or nontransfected cell were measured either in cytosol preparations or by whole cell uptake 42 h after transfection, according to the procedure of Smith et al. (1985) which was modified for estrogen receptors. Isolation of receptors from whole cells in preparation for sucrose gradient analysis has also been described previously (Syms et al., 1985).

Results

Cotransfection of A431 Cells with the pA2 (−821/−87) tk-CAT and Expression Vector Containing Estrogen Receptor cDNA (CERO). The calcium phosphate protocol (Gorman, 1985) was used for transfection of A431 cells. The pA2 (−821/−87) tk-CAT construct consists of the vitellogenin A2 sequence containing the well-characterized ERE (Klein-Hitpass et al., 1986). CERO represents the chicken estrogen receptor cDNA cloned into the pKCR2 expression vector (Krust et al., 1986). After transfection and glycerol shock, the cells were washed and maintained in phenol red free DME containing 5% charcoal-stripped fetal bovine serum. The

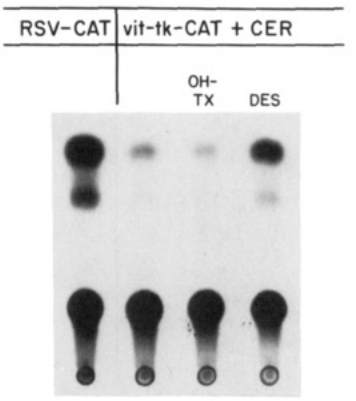

FIGURE 1: Chloramphenicol acetyltransferase (CAT) activity in the cytosol of A431 cells cotransfected with pA2 (−821/−87) tk-CAT and the chicken estrogen receptor (CER). After transfection, no ligand was added to one group, the anti-estrogen hydroxytamoxifen (10 nM) was added to a second group, and the third was treated with DES (10 nM). RSV-CAT was used as a control. Cytosol was assayed 42 h after treatment. The two upper spots are the acetylated forms of chloramphenicol.

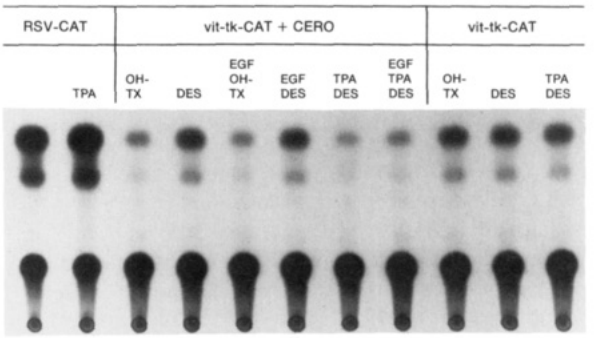

FIGURE 2: CAT activity in the cytosol of A431 cells transfected with RSV-CAT (control), pA2 (−821/−87) tk-CAT and CERO (CER), or pA2 (−821/−87) tk-CAT alone. The cells were treated as shown for 42 h before assaying.

culture dishes were taken randomly for treatment with hormones. Twenty-four hours later, additional hormones were added, and after 42 h, the cells were harvested, cytosol was isolated, and equal amounts of protein were used to measure CAT activity. It is clear from the results shown in Figure 1 that CAT activity is expressed at high levels in the presence of DES. In the absence of ligand or with hydroxytamoxifen, CAT is expressed only at low levels.

When the pA2 (−821/+14) CAT construct, which lacks the thymidine kinase promoter, was cotransfected with CERO, very low levels of CAT activity were induced in the presence of diethylstilbestrol. This induction was never observed in the absence of ligand or in the presene of hydroxytamoxifen.

Effects of TPA and EGF on Estrogen Receptor Mediated Regulation of pA2 (−821/−87) tk-CAT in A431 Cells. An attempt was made to modulate estrogen receptor mediated CAT activation using pA2 (−821/−87) tk-CAT in A431 cells by adding TPA and EGF. Following transfection with DNA, culture dishes were picked randomly for treatment with factors. Figure 2 demonstrates, in agreement with Figure 1, that diethylstilbestrol activates CAT activity in the presence of the estrogen receptor; hydroxytamoxifen has no effect. Figure 2 also provides an example that the estrogen receptor is capable of repressing CAT activity. Furthermore, Figure 2 shows that while EGF is ineffective in modulating estrogen induction, addition of TPA blocks DES induction of CAT activity. This attenuation by TPA also occurs in the presence of EGF. It can also be seen that in the absence of estrogen receptors, CAT

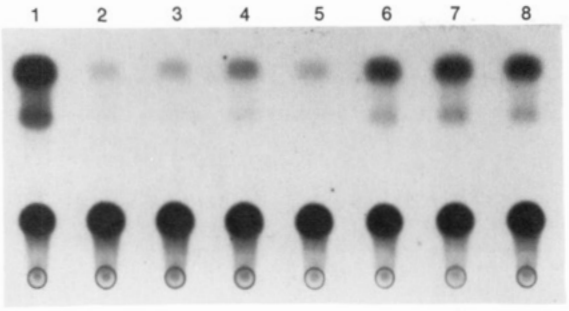

FIGURE 3: CAT activity in cytosol of A431 cells cotransfected with RSV-CAT, pA2 (871/−87) tk-CAT and CERO, or pA2 (−871/−87) tk-CAT with pKCR2. The cells were treated for 42 h after transfection and prior to cytosol preparation. Lane 1, RSV-CAT. Lanes 2–5, CERO + pA2: Nil, TX-OH, estradiol, estradiol + TPA. Lanes 6–8, pKCR2 + pA2: TX-OH, estradiol, estradiol + TPA.

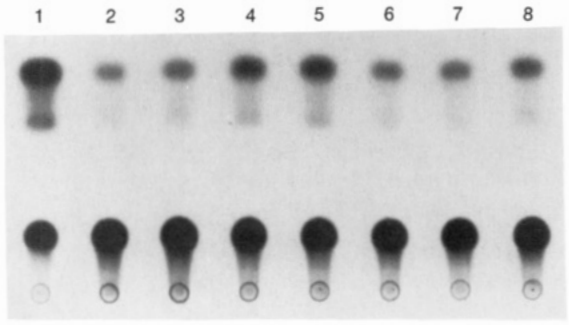

FIGURE 4: CAT activity in the cytosol of A431 cells transfected with RSV-CAT (control), pA2 (−331/−87) tk-CAT and CERO, or pA2 (−331/−87) tk-CAT with pKCR2. The cells were treated for 42 h after transfection and prior to cytosol preparation. Lane 1, RSV-CAT. Lanes 2–5, CERO + pA2: Nil, TX-OH, estradiol, estradiol + TPA. Lanes 6–8, pKCR2 + pA2: TX-OH, estradiol, estradiol + TPA.

activity is equivalent to that observed in the presence of receptor + DES and that TPA alone has no effect on constitutive CAT activity. These results suggest that the estrogen receptor is capable of repressing constitutive CAT activity and that this repression is relieved upon addition of DES in the absence of TPA. This conclusion is supported by data from five experiments.

Additional control experiments were performed. When the expression vector pKCR2, that is CERO lacking the estrogen receptor cDNA insert, was cotransfected into the cells together with pA2 (−821/−87), repression of CAT activity is not observed. Moreover, TPA has no inhibitory effect on CAT activity in the absence of the estrogen receptor (Figure 3). That estrogen-induced derepression is not as marked as that shown in Figure 2 probably reflects increased metabolism of the estrogenic ligand; estradiol was used in place of DES. Figure 4 shows that when CERO was cotransfected with pA2 (−331/−87), TPA had no inhibitory effect on estradiol-induced CAT activity and the estrogen receptor in the absence of estrogen also had no inhibitory effect on CAT activity. These data indicate that estrogen receptor mediated repressive effects are associated with sequences between −877 and −331. This region is upstream of the ERE.

In Figure 5, the relationship between incubation time with ligand and increase in CAT activity is shown. The effect of TPA was also monitored. Maximal activity is induced following 46 h of exposure to ligand. At times of exposure of less than 24 h, no effect of TPA and diethylstilbestrol on CAT activity is evident. However, after 24 and 46 h, in agreement with the representative experiments shown in Figures 2 and 3, TPA represses any estrogen-mediated increase in CAT activity.

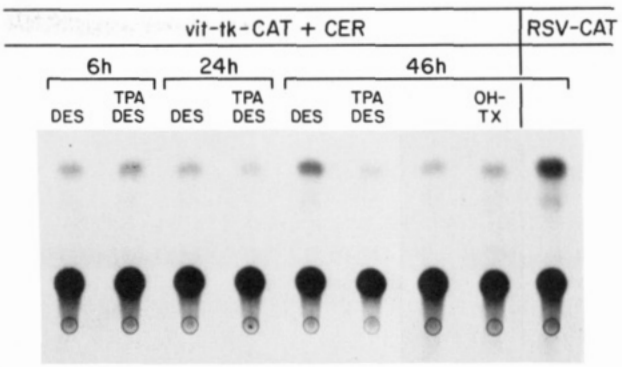

FIGURE 5: Time-dependent effect of DES and TPA on cells cotransfected with pA2 (−821/−87) tk-CAT and CERO (CER). The CAT assay was performed 46 h after transfection in all the groups, but they were treated with DES and with DES + TPA for various lengths of time (6–46 h), or with no ligand or hydroxytamoxifen (OH-TX) for 46 h.

Analysis of Estrogen Receptors in Cotransfected Cells. The measurement of estrogen receptors either by whole cell uptake or in cytosol showed the complete absence of receptors in A431 cells. When the cells were transfected with CERO and assayed 42–46 h later for estrogen receptors by whole cell uptake methods (Smith et al., 1985), and by sucrose density gradient analysis following receptor isolation from nuclei, cells treated with TPA contained similar quantitaties of receptors to those found (2 pmol of receptor/mg of protein) in the untreated cells. These results show that phorbol ester treatment had no inhibitory effect on estrogen receptor synthesis.

Discussion

We had shown previously that estradiol binding affinity is modified by ATP/Mg^{2+}-mediated reactions (McNaught et al., 1986; Raymoure et al., 1986) and we speculated that the transcriptional mode of the estrogen receptor is modulated by phosphorylation/dephosphorylation reactions (Dayani et al., 1990). Experiments were designed to test this speculation. The chicken estrogen cDNA cloned into an expression vector (CERO) was particularly appropriate for these studies because of extensive characterization of the chicken estrogen receptor system (Smith et al., 1979; McNaught & Smith, 1986; McNaught et al., 1986; Raymoure et al., 1986; Taylor & Smith, 1982; Smith & Schwartz, 1979; Smith & Taylor, 1981).

The results of experiments described herein suggest that under specific conditions the nonliganded estrogen receptor can act as a repressor. Furthermore, in A431 cells, the estrogen receptor is capable of acting as a repressor even in the presence of estrogen. TPA, a known activator of kinase C in A431 cells, prevented both DES- and estradiol-induced activation of CAT activity in pA2 (−821/−87) tk-CAT. However, these events appear to involve sequences far upstream of the vitellogenin ERE since repression was not observed with pA2 (−331/−87) tk-CAT.

The observed regulation in CAT activity cannot be explained by differences in transfection efficiencies since transfected dishes of cells were randomly selected for hormonal treatment following transfection and in each experiment the estrogen receptor mediated repression of CAT activity was reproducible. Moreover, the construct lacking 490 bp of 5′-flanking sequence acted as an additional control since receptor-mediated repression was never observed using this construct. Interestingly, the effect was also cell specific as receptor-mediated inhibition of CAT activity was not observed in T47D cells (data not shown), implying that cell-specific protein–protein interactions

are involved. This mechanism was proposed to explain estrogen receptor mediated repression of prolactin gene transcription (Adler et al., 1988).

It is not necessarily surprising that estrogen-mediated repression/derepression can be observed. For example, it has been shown for glucocorticoid receptors that certain mutants which are unable to bind hormone fail to transcriptionally activate the MTV promoter whereas other non-steroid binding mutants can cause constitutive activation (Hollenberg et al., 1987). Hence, it has been proposed that binding of the steroid hormone to its wild-type receptor induces a conformational change in the protein which allows activation of transcription. While the observations could be explained on the basis of differential DNA binding, DNA binding alone is insufficient to explain activation of transcription since it is well established that anti-estrogen–receptor complexes bind to DNA but are not transcriptionally active. Different conformations of receptor–transcriptional protein complexes are probably stabilized according to binding of hormone agonists or antagonists or by altered phosphorylation. We speculate that transcription of steroid-responsive genes is controlled by the conformation of the receptor complex.

Our studies also provide the first indication that TPA can negatively affect transcription to result in repression rather than activation of a gene product. Previously, phorbol ester tumor promoters were shown to induce the transcription of a variety of genes including c-myc and c-fos (Lau & Nathan, 1985; Greenberg & Ziff, 1984). The studies indicate that these processes are mediated via protein kinase C. Recently, it has been reported that TPA can activate the SV40 transcriptional enhancer element to result in as much as a 45-fold stimulation of an adjacent CAT reporter gene; the event appears to be directly mediated by a phosphorylation process which does not require intermediate protein synthesis (Greenberg & Ziff, 1984). In the presence of CERO, the negative effect of TPA on CAT activity might have been explained by the possible preferential recruitment of transcription factors to the SV40 enhancer in the CERO construct. If the concentration of transcription factors in these cells was rate limiting, it would result in decreased endogenous CAT activity. However, this explanation is unlikely since no major decrease in CAT activity occurs in response to TPA when pKCR2 (CERO in which the estrgen receptor cDNA has been deleted) is cotransfected with vit-tk-CAT into A431 cells. Furthermore, when pA2 (−331/−87) tk-CAT is used in place of pA2 (−821/−87) tk-CAT, no repression is observed.

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Registry No. DES, 56-53-1; estradiol, 50-28-2.

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