Introduction of estrogen-responsiveness into mammalian cell lines

Petra M.Druege, Ludger Klein-Hitpass, Stephen Green¹, Gary Stack¹, Pierre Chambon¹ and Gerhart U.Ryffel

Kernforschungszentrum Karlsruhe, Institut für Genetik und Toxikologie, Postfach 3640, D-7500 Karlsruhe 1, FRG and ¹Laboratoire de Génétique Moléculaire des Eucaryotes du CNRS, Unité 184 de Biologie Moléculaire et de Génie Génétique de l'INSERM, Faculté de Médecine, 11 rue Humann, F-67085 Strasbourg Cédex, France

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ABSTRACT

We introduced estrogen responsiveness as a new characteristic into rat hepatoma, mouse Ltk⁻ and human HeLa⁺ cells by transfecting the human estrogen receptor (ER) cDNA. To measure the estrogen response we used *Xenopus* vitellogenin gene A2 constructs linked to the bacterial CAT gene. Transient cotransfections of the ER cDNA and the vitellogenin gene-CAT constructs containing the estrogen responsive element (ERE) lead to a hormone dependent induction of CAT activity whereas cotransfected vitellogenin gene constructs lacking the ERE are not inducible. Stable transfections of ER cDNA into Ltk⁻ cells give rise to cell clones that are estrogen responsive as shown by transfection of various vitellogenin gene-CAT constructs. These results prove that the transfected ER is biologically active and is sufficient to make a cell estrogen responsive.

INTRODUCTION

Steroid hormone action is thought to involve at least two elements: a cis-acting DNA regulatory element and a trans-acting hormone receptor which interacts with this element (for reviews see refs. 1 and 2). By analysing the estrogen regulation of the *Xenopus* vitellogenin A2 gene, Klein-Hitpass et al. (3) have recently localised an estrogen responsive element (ERE). This ERE mediates the estrogen-dependent induction of a linked indicator gene after transfection into MCF-7 cells, a human breast cancer cell line containing estrogen receptors (ER). The MCF-7 ER cDNA has been cloned (4) and expressed in the ER-negative cell lines (5,6). The receptor produced in these cells, like the native MCF-7 ER, has a molecular weight of 66 kDa, binds estradiol with high affinity (K_d 0.5 nM) and associates tightly with the nucleus only in the presence of estradiol (6,7). To test the ability of the transiently expressed ER to stimulate gene expression, we introduced the ER expression vector together with a plasmid which contains the ERE into cell lines which normally do not express the estrogen receptor gene and are not estrogen responsive. We show here, that activation of the *Xenopus* vitellogenin ERE in various cell lines is dependent upon both the expression of the

ER and the addition of estradiol. Our results also demonstrate that estrogen responsiveness can be introduced as a new characteristic into mouse Ltk⁻ cells.

MATERIALS AND METHODS

Cell culture and transfections

The human HeLa tk⁻ cell line was routinely kept in Eagle's Medium, the rat hepatoma cell line FTO-2B in a 1:1 mixture of Dulbecco's Modified Eagle's Medium (DMEM) and Ham's F12 and the mouse Ltk⁻ cells in DMEM. All the standard media were supplemented with 10% fetal calf serum, 100 U/ml penicillin/streptomycin and 2 mM glutamine.

When cells were kept with defined amount of estrogen the following medium was used: Modified Eagle's Medium without phenol red supplemented with 2% Ultrosor SF (LKB), 2 mM glutamine, 15 µg/ml glycine and 100 U/ml penicillin/streptomycin (MEUS 2). Phenol red was omitted because it has recently been shown that it acts as a weak estrogen (8) and Ultrosor SF was used as a serum substitute lacking estrogen.

For transient transfections cells were seeded at a density of 1×10^6 /10 cm petri dish. Two days later they were rinsed twice with Tris buffered saline (TBS) (9) and then incubated with 1 ml of a mixture of 10 µg DNA, 0.5 µg DEAE dextran (Pharmacia 5×10^5 Mr) in TBS for 30 minutes at room temperature. The two cotransfected plasmids were mixed at a molar ratio of 1:1. After incubation the cells were washed with TBS and then incubated for exactly two minutes with a mixture of 10% DMSO in medium without serum. The DMSO was washed off and the cells were then incubated in the appropriate medium.

CAT assays

CAT activity was assayed from cell extracts two and four days after transfection as described (3). The activity is expressed as percentage chloramphenicol acetylated per amount of protein per two hours. For quantification the spots were cut out of the chromatogram and counted. 100% correspond to about 300.000 cpm. The background derived from cells transfected with control plasmids was subtracted.

RESULTS

Transient transfection of the estrogen receptor cDNA activates the cotransfected estrogen responsive element

To show that transient expression of the cloned ER can stimulate an ERE-dependent activity of an indicator plasmid, we used the ER expression vector HE0, which contains the MCF-7 ER cDNA (previously described as pKCR2-ER,

Figure 1: Expression of the vitellogenin gene indicator plasmid is dependent on the estrogen receptor

HEO (ER) is cotransfected either with pA2(-331/-87)tk-CAT8+ which contains the ERE (-331), or with pA2(-260/-87)tk-CAT8+ which lacks the ERE (-260) into FTO-2B cells. pBR322 (pBR) was used as control. Cells were kept in standard medium supplemented with 10% fetal calf serum. Cell extracts were assayed for CAT activity after two and four days.

in ref. 5). The cotransfected indicator plasmid, pA2(-331/-87)tk-CAT8+, contains the 5' flanking region of the vitellogenin A2 gene of *Xenopus laevis* from position -331 to position -87, linked to the tk promoter of Herpes simplex virus (-105 to +51) upstream from the bacterial chloramphenicol acetyl transferase (CAT) gene (3). This ERE-containing construct directs estrogen-dependent induction of CAT activity after transfection into human MCF-7 cells (3). As a control for the requirement of an ERE, we used the non-inducible construct pA2(-260/-87)tk-CAT8+ which lacks the ERE (3).

During the initial experiments the cells were maintained in culture medium supplemented with 10% fetal calf serum which provides an amount of estrogen sufficient to activate the estrogen receptor (10). Figure 1 shows the results obtained with the differentiated rat hepatoma cell line FTO-2B. When HEO was cotransfected with the indicator plasmid, pA2(-331/-87)tk-CAT8+, the CAT activity was strongly stimulated four days later. In contrast, no stimulation was observed when pBR322 was used in place of HEO. A response can be seen as soon as two days after transfection when using the calcium phosphate (data not shown) but not with the DEAE-dextran technique (see Fig. 1 and Table I).

Table I: Estrogen receptor stimulation of ERE-dependent CAT activity.

	plasmids transfected					
	pA2(-331/-87)tk-CAT8+			pA2(-260/-87)tk-CAT8+		
	pBR322	HEO	st.f.	pBR322	HEO	st.f.
cell lines						
FTO-2B						
2 days	6.6	9.3	1.4	2.8	3.5	1.3
4 days	3.6	53.2	14.8	4.0	4.0	1.0
Ltk ⁻						
2 days	0.1	0.6	6.0	0.2	0.1	0.5
4 days	1.0	33.0	33.0	0.3	0.05	0.2

CAT activity, measured two and four days after transfection, per 250 µg of protein. St.f. stands for stimulation factor.

When HEO was cotransfected with the non-inducible plasmid, pA2(-260/-87)tk-CAT8+, no stimulation of CAT activity was detected at either time point. The quantified results for the FTO-2B cells and the mouse fibroblastoid cell line

Figure 2: The activity of the transfected estrogen receptor depends on the presence of estrogen
HEO (ER) was cotransfected either with pA2(-331/-87)tk-CAT8+, which contains the ERE (-331), or with pA2(-260/-87)tk-CAT8+ which lacks the ERE (-260) into HeLa^{tk} cells. The vector pKCR2 was used as control (5). The cells were kept in MEUS 2 and 10⁻⁷ M estrogen was added when indicated (+). Cell extracts for CAT assays were prepared 4 days after transfection.

Table II: Estradiol induction of ERE-dependent CAT activity.

plasmids transfected						
pA2(-331/-87)tk-CAT8+			pA2(-331/-87)tk-CAT8+			
HEO			pKCR2			
10 ⁻⁷ M estrogen	-	+	ind.f.	-	+	ind.f.
cell lines						
HeLa tk ⁻	2.6	45.9	17.7	4.1	1.4	0.3
Ltk ⁻	3.5	76.9	22.1	2.7	5.0	1.9
pA2(-260/-87)tk-CAT8+						
HEO			pKCR2			
10 ⁻⁷ M estrogen	-	+	ind.f.	-	+	ind.f.
cell lines						
HeLa tk ⁻	1.4	1.9	1.4	1.7	1.5	0.9
Ltk ⁻	6.3	20.5	3.3	1.2	1.8	1.5

The CAT activity was measured using 20 µg of protein for the HeLa tk⁻ cells and 100 µg of protein for the Ltk⁻ cells. Cell extracts were prepared 4 days after transfection. Ind. f. stands for induction factor.

Ltk⁻ are listed in Table I. The stimulation factor ranges from about 15 to 33. These data demonstrate that the transiently expressed human ER is biologically active in both mouse and rat cell lines. In addition, activation of the CAT indicator gene is dependent upon the presence of the ERE since stimulation by the ER is only seen with pA2(-331/-87)tk-CAT8+ and not with pA2(-260/-87)tk-CAT8+.

The activation of the estrogen responsive element by the cotransfected estrogen receptor gene is hormone dependent

Figure 2 shows the data obtained after cotransfection of HEO with the inducible indicator plasmid into HeLa tk⁻ cells cultivated in the presence or absence of 10⁻⁷M estradiol. These results, and those of a similar experiment performed using Ltk⁻ cells, showed the induction by estradiol to be about 20-fold (Table II). The small induction of the plasmid lacking the ERE (pA2(-260/-87)tk-CAT8+) reflects the inducibility of the vector pBLCAT8+ (see below). Lower estrogen concentrations (10⁻⁹ or 10⁻⁸M) also stimulate CAT activity whereas progesterone (10⁻⁸M) or dexamethasone (10⁻⁷M) had no effect (data not shown).

Taken together, the data presented in Table I and II show that estrogen

Figure 3: Estrogen responsiveness is acquired as a stable characteristic by Ltk⁻ mouse cells after transfection with the human estrogen receptor cDNA
Four independent clones transfected with the human ER cDNA as well as the parental Ltk⁻ cells are transiently transfected with either the vitellogenin gene-CAT construct containing the ERE, pA2(-331/-87)tk-CAT8+ (-331), or the control plasmid pA2(-260/-87)tk-CAT8+ (-260). Cells were kept in standard medium containing 10% fetal calf serum and cell extracts were prepared four days after transfection.

regulation can be transiently introduced into various eukaryotic cell lines and that activation of the cotransfected *Xenopus* vitellogenin ERE is dependent upon both the receptor and estrogen.

Stable transfection of the estrogen receptor cDNA results in estrogen responsive cell clones

In order to see whether it is possible to create estrogen responsive cell lines, mouse Ltk⁻ cells were cotransfected with HEO and the neomycine resistance gene (pSV2neo) using standard techniques (3). After selection with G418 we obtained 12 independent clones. Transient transfection of these cloned cell lines with the inducible indicator plasmid pA2(-331/-87)tk-CAT8+ showed that all expressed high CAT activity in medium with estrogen (10% fetal calf serum), whereas the non-inducible plasmid pA2(-260/-87)tk-CAT8+ gave low CAT activity (Fig.3). In Ltk⁻ host cells the transfection of neither of the plasmids gave rise to a significant CAT activity.

Two of the cloned cell lines (Ltk⁻e33 and Ltk⁻e42) were further analysed for their response to estrogen. The results, summarised in Table III, show that the expression of two different indicator plasmids containing the ERE, pA2(-331/-87)tk-CAT8+ and pA2(-331/-297)tk-CAT8+ (see ref.3), can be induced to nearly 60 fold by estrogen in both of the cell lines. The results confirm

Table III: Characteristics of the acquired estrogen responsiveness of Ltk⁻ derived transfected cell clones.

cell clones 10 ⁻⁷ M estrogen	Ltk ⁻ e33			Ltk ⁻ e42		
	-	+	ind.f.	-	+	ind.f.
plasmids transfected						
pA2(-331/-87)tk-CAT8+	2.5	140.5	56.2	3.0	118.5	39.5
pA2(-331/-297)tk-CAT8+	2.0	76.5	38.2	3.0	175.0	58.3
pA2(-324/-87)tk-CAT8+	1.2	7.5	6.2	1.0	7.5	7.5
pA2(-260/-87)tk-CAT8+	0.8	2.0	2.5	0.8	3.1	3.9
pBL-CAT8+	0.9	5.5	6.1	1.4	7.9	5.6
pSV2-CAT	14.2	39.5	2.8	8.7	44.5	5.1

CAT activity was measured for the stable transfectants Ltk⁻e33 and Ltk⁻e42 two days after transient transfection of indicator or control plasmids. CAT assays were performed with 13.5 µg of protein for the indicator plasmids pA2(-331/-87)tk-CAT8+ and pA2(-331/-297)tk-CAT8+ and with 60 µg of protein for the control plasmids. All values given are calculated for 60 µg of protein. Ind.f. stands for induction factor.

that the 35 bp sequence is sufficient for optimal induction by estrogen (3). As controls we used two plasmids, one of them lacks the ERE (pA2(-260/-87)tk-CAT8+) and in the other one the ERE is partly deleted (pA2(-324/-87)tk-CAT8+, see ref. 3). The induction of both of these plasmids lies in the range of the values obtained with vectors without vitellogenin gene inserts (pBL-CAT8+, see ref.3, and pSV2-CAT, see ref.11). The inducibility of the vector pBL-CAT8+ which was used to test the vitellogenin gene inserts, is about 6 fold. A comparable inducibility is obtained with pSV2-CAT, which has a much higher basal activity. We interpret this relative weak induction as a general stimulatory effect of estrogen which is independent of the ERE. The data in Table III further document that the basal activity, without estrogen, of the various vitellogenin gene-tk-CAT-constructs is similar to the activity observed with the vector pBL-CAT8+. This is in contrast to our previous experiments with MCF-7 cells (3), where a hormone independent activator could be localised downstream of the ERE. Obviously this activator is not functional in Ltk⁻ cells.

DISCUSSION

The results presented here show that the receptor expressed from the cloned ER cDNA stimulates specific gene expression. Taken together with our previous

results concerning the ability of this protein to bind estradiol (5,7) we consider this to constitute the final proof that this cloned cDNA encodes the authentic estrogen receptor. Other steroid hormone receptors have been shown to bind directly to DNA at sites which are important for the transcriptional regulation of hormone-inducible genes (1,2). Our data suggests that the ER plays a key role as a transacting factor by stimulating the activity of a cis regulatory element, the ERE. These experiments also indicate that the addition of the ER alone into a non-target cell is sufficient to make it estrogen-responsive and that other factors which may be involved in the estrogen response are apparently not cell-specific. Our data confirm the previously defined (3) size and position of the ERE in the 5' flanking region of the vitellogenin A2 gene. In the Ltk⁻ cells which have acquired estrogen responsiveness as a stable characteristic after transfection with the human ER cDNA the inducibility by estrogen is dependent on the presence of the 35 bp sequence between -331 and -297 as originally observed in MCF-7 cells. The functional interaction of the human ER with an ERE derived from a frog gene in cell lines of different mammalian species further documents the high degree of conservation during evolution of the elements involved in estrogen control (3,12). Further experiments, of the type described here, will allow us to define the functional domains of the estrogen receptor important for the stimulation of gene transcription as well as to define in more detail the vitellogenin ERE.

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