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Role of the two activating domains of the oestrogen receptor in the cell-type and promoter-context dependent agonistic activity of the anti-oestrogen 4-hydroxytamoxifen

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Various oestrogen responsive reporter genes and vectors expressing truncated or chimeric human oestrogen receptors (hER) containing either of the two independent hER transcriptional activation functions (TAF-1 and TAF-2) have been transfected into HeLa cells, chicken embryo fibroblast (CEF) or yeast cells to investigate the agonistic activity of the anti-oestrogen 4-hydroxytamoxifen (OHT). We demonstrate that the agonistic effect of OHT on the whole hER is due to the cell-type and promoter-context dependent activity of TAF-1. In similar experiments, we show that the anti-oestrogen, ICI 164,384, does not exhibit any oestrogenic activity and, therefore, acts always as a pure antagonist, even though it does not inhibit the activity of the isolated TAF-1. We also confirm that the wild type human oestrogen receptor has no ligand independent transcriptional activity. The implications of our results for the variable antagonist/agonist activity of anti-oestrogens *in vivo* are discussed.

Key words: antagonistic activity/agonistic anti-oestrogens/human oestrogen receptor/transcriptional activation function

Introduction

The oestrogen receptor (ER), a member of the nuclear receptor family, acts as ligand inducible transcriptional activator by interacting with specific *cis*-acting enhancer sequences, called oestrogen responsive elements (EREs) (for reviews see Green and Chambon, 1988, 1990; Beato, 1989; Ham and Parker, 1989). Alignment of the amino acid sequences of the human (hER) and chicken (cER) oestrogen receptors and functional dissection of the hER have shown that the ER has a molecular structure consisting of separable domains which can function independently. The DNA binding domain (DBD) (region C) and the hormone binding domain (HBD) (region E) are the most highly conserved regions (Krust *et al.*, 1986; Kumar *et al.*, 1987; Kumar and Chambon, 1988; see also Figure 1B). Two independent transcriptional activation functions, TAF-1 and TAF-2, have been characterized (Tora *et al.*, 1989a and refs therein). The activity of TAF-1 which is located within the N-terminal A/B region of the receptor is constitutive, whereas TAF-2 whose activity is hormone inducible is located within the HBD (Kumar *et al.*, 1987; Webster *et al.*, 1988, 1989; Lees *et al.*, 1989; Tora *et al.*, 1989a). Furthermore, the activity of both TAF-1 and TAF-2 is dependent on the target gene promoter context, and TAF-1 activity exhibits a marked cell specificity, being much more efficient in chicken embryo fibroblasts than in HeLa cells (Tora *et al.*, 1989a). Interestingly, hER also stimulates transcription in yeast in a strictly oestrogen dependent manner, indicating a conservation of the molecular mechanism underlying this activation across the range of eukaryotes (Metzger *et al.*, 1988). Moreover, it has been shown that a truncated receptor containing TAF-1 and the DBD (HE15, see Figure 1B), stimulates transcription in yeast cells very efficiently in a hormone independent manner (White *et al.*, 1988).

The effects of oestrogens can be antagonized by compounds such as 4-hydroxytamoxifen (OHT) and ICI 164,384 (ICI) (for refs see Jordan, 1984; Wakeling and Bowler, 1988a,b). Both OHT and ICI appear to antagonize oestrogen action by competing with oestrogens for receptor binding. OHT, however, can act as a partial oestrogen agonist depending upon the tissue and response examined, whereas ICI appears to be a pure antagonist (Wakeling and Bowler, 1988a,b; see Discussion for additional references). Previous studies *in vivo* using a chimeric receptor associating the DBD of the yeast transcriptional activator *GAL4* and the hER region E containing TAF-2 [GAL-ER(EF), see Figure 1B], have shown that OHT, in contrast to oestradiol (E2), cannot induce TAF-2 activity (Webster *et al.*, 1988). On the other hand, *in vitro* studies, using gel retardation and methylation interference assays, have demonstrated that hER bound to either E2 or OHT recognizes an ERE specifically (Metzger *et al.*, 1988; Kumar and Chambon, 1988; Lees *et al.*, 1989). Interestingly, in the *in vitro* gel retardation assay, the OHT-receptor−ERE complex migrates slightly more slowly than the corresponding complex assembled in the presence of E2 (Kumar and Chambon, 1988; Metzger *et al.*, 1988; Lees *et al.*, 1989).

From the above studies, it was concluded that the antagonist effect of OHT could result from its inability to induce the structural transition necessary to activate TAF-2, even though it may have the same effect as E2 on the binding of ER to ERE *in vivo* (Webster *et al.*, 1988). Since the activity of TAF-1 separated from the HBD does not depend on the presence of oestrogen (Kumar *et al.*, 1987; Tora *et al.*, 1989a), the possibility thus arises that the partial agonistic activity of OHT in some tissues may reflect the aforementioned cell-type and promoter-context specificity of TAF-1 activity (Green and Chambon, 1988). We have tested this hypothesis and report here that for a given oestrogen responsive target gene in a given cell, there is indeed a strong correlation between the activity of the isolated TAF-1 and the agonistic effect of OHT on the transcriptional activity of the whole hER. We show also that under similar conditions, ICI 164,384 has no agonistic effect, even though this compound does not inhibit the isolated TAF-1 activity. Finally, we confirm (see Tora *et al.*, 1989b) that the low level of 'constitutive' activity which can be observed in cells

grown in the presence of dextran-coated charcoal-treated serum with the wild type hER possessing a glycine at position 400 is most likely due to the imperfect removal of oestrogens from the treated serum.

Results

The agonistic effect of 4-hydroxytamoxifen on hER in HeLa cells and chicken embryo fibroblasts is correlated with the activity of TAF-1, whereas ICI 164,384 is never an agonist

Since the stimulatory activity of TAF-1 and TAF-2 is cell-type and promoter-context dependent (see above), the effect of the two anti-oestrogens 4-hydroxytamoxifen (OHT) and ICI 164,384 (ICI) was determined in both transiently transfected HeLa cells and chicken embryo fibroblasts (CEF), which are both devoid of any detectable levels of ER. Expression vectors encoding either the whole hER (HEO), an N-terminally truncated ER containing TAF-2 (HE19) or a C-terminally truncated ER containing TAF-1 (HE15) (see Figure 1B and Kumar *et al.*, 1986,1987) were tranfected together with the various oestrogen responsive reporter genes which have been used previously (Kumar *et al.*, 1987; Tora *et al.*, 1989a; Green *et al.*, 1988). ERE-tk-CAT, vit-tk-CAT, 17M/ERE-G.CAT and pS2-CAT have complex ER-inducible promoter regions containing several

Ⓐ ACTIVATION OF TRANSCRIPTION (FOLD STIMULATION)

	REPORTER GENE	ACTIVATOR GENE	HELA CELLS –	HELA CELLS E2	HELA CELLS OHT	HELA CELLS ICI	CHICKEN EMBRYO FIBROBLASTS –	CHICKEN EMBRYO FIBROBLASTS E2	CHICKEN EMBRYO FIBROBLASTS OHT	CHICKEN EMBRYO FIBROBLASTS ICI
		A HEO	1.0	70	1.5	0.8	1.0	10	6.0	0.8
①	vit-tk-CAT	B HE19	1.0	70	0.7	0.8	1.0	10	1.0	0.8
		C HE15	1.5	1.5	1.5	1.5	5.0	5.0	5.0	5.0
		A HEO	1.0	25	1.5	0.8	1.0	6.0	3.0	0.8
②	ERE-tk-CAT	B HE19	1.0	12	0.7	0.6	1.0	6.0	1.0	0.7
		C HE15	1.5	1.5	1.5	1.5	4.5	4.5	4.5	4.5
		A HEO	1.0	35	1.5	ND	1.0	30	15	ND
③	17M/ERE-G•CAT	B HE19	1.0	30	0.8	ND	1.0	20	1.0	0.8
		C HE15	1.7	1.7	1.7	1.7	18	18	18	18
		A HEO	1.0	20	5.0	1.0	1.0	20	10	1.0
④	pS2-CAT	B HE19	1.0	4.0	1.0	1.0	1.0	5.0	1.0	1.0
		C HE15	2.4	2.4	2.4	2.4	4.0	4.0	4.0	4.0
		A HEO	1.0	20	1.5	0.6	1.0	45	25	1.0
⑤	ERE-TATA-CAT	B HE19	1.0	1.5	0.5	0.5	1.0	1.7	1.0	1.0
		C HE15	1.2	1.2	1.2	1.2	25	25	25	25
⑥	17M2-G•CAT	A GAL-ER(EF)	1.0	100	1.0	1.0	1.0	35	1.0	1.0
		B GAL-ER(AB)	3.0	3.0	3.0	3.0	23	23	23	23

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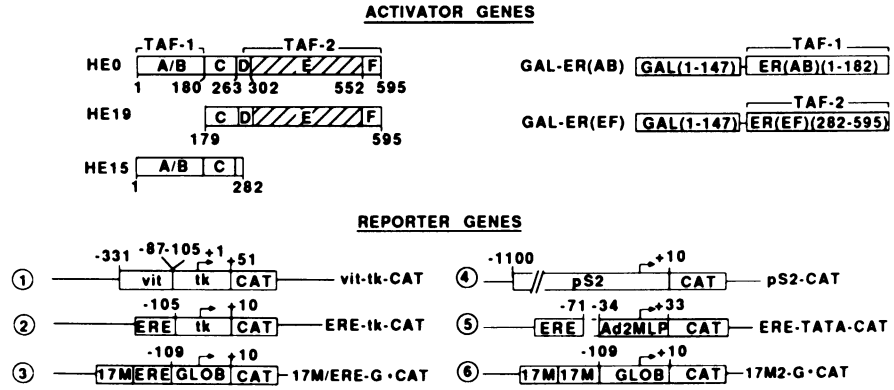

Fig. 1. Transcriptional stimulation by the human ER(HEO), the truncated ER mutants HE19 and HE15 and the chimeric receptors GAL-ER(EF) and GAL-ER(AB) of various reporter genes in HeLa cells and CEF in the presence and absence of oestradiol, 4-hydroxytamoxifen and ICI 164,384. (**A**) Compilation of the data of several experiments similar to those presented in Figures 2 and 3. Activation of transcription from vit-tk-CAT, ERE-tk-CAT, 17M/ERE-G.CAT, pS2-CAT, ERE-TATA-CAT by either HEO, HE19 or HE15, or from 17M2-G.CAT by either GAL-ER(EF) or GAL-ER(AB), in the presence of oestradiol (E2, 10 nM), 4-hydroxytamoxifen (OHT, 100 nM), ICI 164,384 (ICI, 100 nM) is depicted as fold stimulation over the control (−, ethanol vehicle alone) which has been taken as 1.0. Each value is an average (±20%) of at least three independent experiments. In the case of HE15 and GAL-ER(AB) the stimulation was expressed relative to the controls (no ligand addition) for HEO and GAL-ER(EF), respectively. HeLa cells and CEF were transfected as indicated with HEO, HE19, HE15, GAL-ER(EF) or GAL-ER(AB) together with vit-tk-CAT, ERE-tk-CAT, ERE-TATA-CAT, 17M/ERE-G.CAT, pS2-CAT and 17M2-G.CAT, and processed for measurement of CAT activity as described in Materials and methods. ND, not determined. (**B**) Schematic representation of the different activator and reporter genes used in this study.

upstream elements of either the thymidine kinase (tk), globin (G) or pS2 gene promoters, in addition to an ERE and a TATA box (see Figure 1B and Materials and methods). In contrast, ERE-TATA-CAT contains a minimal promoter composed of an ERE inserted upstream of the TATA region (−34 to +33) of the adenovirus-2 major late promoter (Ad2MLP). In all cases, a reference recombinant pCH110 expressing the bacterial β-galactosidase was co-transfected in order to correct CAT activity measurements for variations in transfection efficiencies. In each transfection series, the results (summarized in Figure 1A) were expressed as fold stimulation of transcription, relative to the values obtained with HEO in control cells not treated with oestradiol (E2) or anti-oestrogens.

In agreement with our previous reports (Kumar *et al.*, 1987; Tora *et al.*, 1989a) and depending on the complex promoter reporter gene used, HE19, which contains TAF-2, was 20−100% as efficient as E2-inducible activator as HEO (compare lanes 1−4, A and B, in Figure 1A; Figure 2 and data not shown), whereas it was very inefficient at stimulating transcription from the minimal promoter of the ERE-TATA-CAT reporter gene (lane 5A and B in Figure 1A; Figure 2) in both cell types. As expected (Kumar *et al.*, 1987; Tora *et al.*, 1989a), in HeLa cells, HE15, which contains TAF-1, stimulated transcription poorly from the various complex promoters (note however, its relatively higher activity with pS2-CAT) and the minimal promoter ERE-TATA-CAT (Figure 1A, lanes 1−5, A and C; Figure 2 and data not shown). In contrast in CEF, depending on the promoter context, the constitutive activity of HE15 was 20−75% that of HEO in the presence of oestradiol. These differential stimulation activities of HE19 and HE15 in HeLa cells and CEF reflect intrinsic properties of TAF-2 and TAF-1, respectively, as shown by using the chimeric activators GAL-ER(EF) and GAL-ER(AB) (Figure 1B) in which the hER regions containing TAF-2 and TAF-1 are attached to the DNA binding domain of the yeast *trans*-activator GAL4 (Figure 1A, lanes 6A and B; Figure 3; see also Webster *et al.*, 1988 and Tora *et al.*, 1989a).

In no case was the stimulation of transcription by either HE15 or GAL-ER(AB) affected by the presence of oestradiol or the anti-oestrogens OHT and ICI, confirming that their effect on the transcriptional activity of the hER is mediated by region E which contains the HBD and TAF-2 (Figure 1A; Figures 2 and 3 and data not shown). In agreement with our previous report (Webster *et al.*, 1988), both OHT and ICI did not induce the activity of TAF-2, whether it was present in HE19 or GAL-ER(EF) (Figure 1A; Figures 2 and 3 and data not shown), and in both cases induction by E2 could be antagonized by either OHT or ICI (data not shown).

In contrast, the effects of these two anti-oestrogens on the activity of the whole hER was markedly different (HEO in Figure 1A; Figure 2 and data not shown). In all cases, no stimulation of transcription was observed in the presence of ICI, and this compound antagonized the effect of E2 (data not shown), in keeping with previous studies characterizing it as a pure anti-oestrogen (refs in Introduction and Discussion). In all cases, however, OHT was an efficient inducer of HEO stimulatory activity in CEF, whereas it had very little if any effect on HeLa cells except on pS2-CAT transcription in agreement with our previous report (Berry *et al.*, 1989). Note, that in keeping with these observations, OHT antagonized only weakly the effect of E2 on stimulation of transcription by hER in CEF, irrespective of which reporter gene was used (data not shown).

Interestingly, there was in general a good correlation between the ability of OHT to induce transcription of the various reporter genes by the whole hER and the efficiency with which HE15 could stimulate transcription (see Figure 1A). Taken together with the inability of OHT to induce

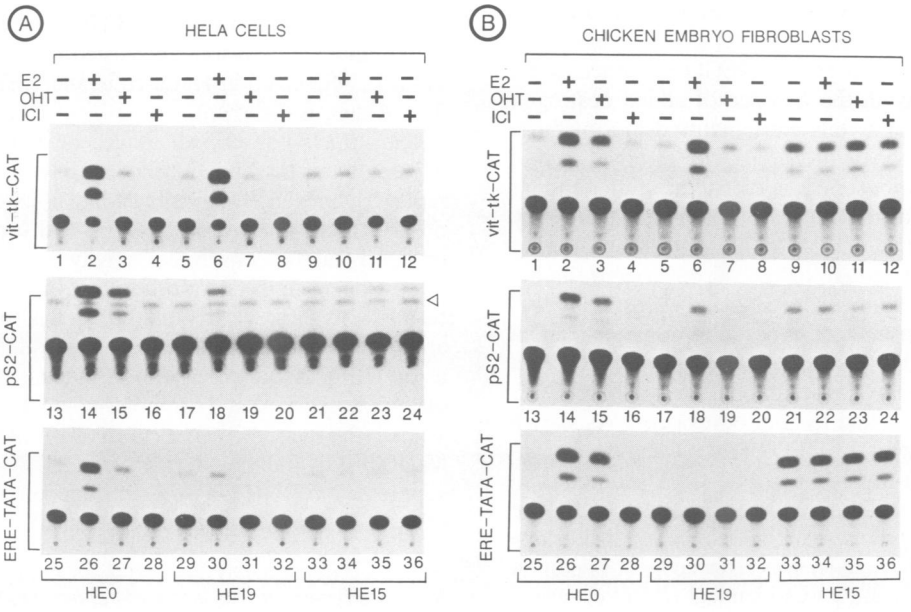

Fig. 2. Transcriptional activation of various reporter genes by HEO, HE19 and HE15 in HeLa cells and CEF. A representative CAT assay experiment using cell extracts from HeLa cells (**A**) and CEF (**B**) transfected (Materials and methods) with vit-tk-CAT, pS2-CAT or ERE-TATA-CAT together with HEO (lanes 1−4; 13−16; 25−28). HE19 (lanes 5−8; 17−20; 29−32) or HE15 (lanes 9−12; 21−24; 33−36), is shown. Cell cultures were maintained in the absence (−) or in the presence (+) of oestradiol (E2, 10 nM), 4-hydroxytamoxifen (OHT, 100 nM) and ICI 164,384 (ICI 100 nM) as indicated. CAT activity was assayed after normalization for β-galactosidase activity from the reference plasmid pCH110, as described in Materials and methods. The open triangle points to an unknown contaminating derivative.

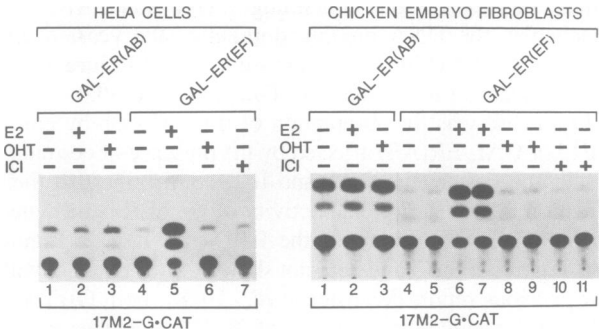

Fig. 3. Transcriptional stimulation of 17M2-G.CAT by the chimeric activators GAL-ER(EF) and GAL-ER(AB) in HeLa cells and CEF. HeLa cells and CEF were transfected with the 17M2-G.CAT reporter gene in the presence of GAL-ER(AB) or GAL-ER(EF), and CAT assays were performed as described in Materials and methods. E2 (10 nM), OHT (100 nM) and ICI (100 nM) were added as indicated.

TAF-2 activity (see above), these results indicate that OHT can act as an agonist on hER when transcription from the responsive promoter can be stimulated by TAF-1.

Similar effects of anti-oestrogens on hER bearing a valine or a glycine at position 400

We have recently demonstrated that the hER cDNA which was initially cloned (Walter *et al.*, 1985; Green *et al.*, 1986) and is contained in the expression vector HEO, contains an artefactual point mutation which results in the substitution of a valine for a glycine at amino acid position 400 (Gly400→Val400) (Tora *et al.*, 1989b). Using the expression vector HEGO encoding a glycine instead of a valine at position 400, we have shown that the Val400 mutation in the hER HBD destabilized its structure in such a manner that the hER encoded in HEO, but not in HEGO, irreversibly lost its oestrogen binding capacity when exposed to 25°C for 1 h in the absence of oestradiol (Tora *et al.*, 1989b). Using vit-tk-CAT as a reporter gene we have also shown that, in contrast to HEO, HEGO exhibited some transcriptional activity in the absence of added oestrogen when the recipient HeLa cells were cultured in a medium treated with dextran-coated charcoal to remove steroid hormones. This apparently oestrogen independent 'constitutive' activity of HEGO in HeLa cells was drastically decreased by the addition of OHT or ICI 164,384, indicating that it was due to residual levels of oestrogens in the 'stripped' culture medium (Tora *et al.*, 1989b).

In view of the present cell-type and promoter-context dependent oestrogenic effect of OHT on HEO, we investigated whether the transcriptional activity of HEGO would be similarly affected by anti-oestrogens. As shown in Figure 4, in contrast to HEO, HEGO activated transcription from the vit-tk-CAT and pS2-CAT promoters to some extent, in either HeLa cells or CEF, in the absence of E2 addition (−, in panels A and B). However, in all cases this constitutive HEGO activity was reduced by ICI addition to a background level which was identical to that observed for HEO in the absence of E2 or in the presence of ICI. On the other hand, the effect of OHT on HEGO constitutive activity was variable, but clearly related to its oestrogenic effect on HEO activity (see Figures 1 and 2). In the case of vit-tk-CAT in HeLa cells where OHT has very little if

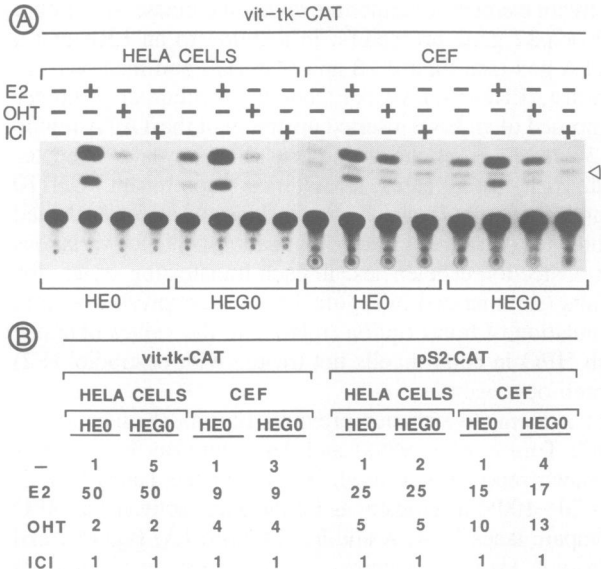

Fig. 4. Transcriptional activation of vit-tk-CAT and pS2-CAT by HEO and HEGO in the presence of oestradiol, 4-hydroxytamoxifen and ICI 164,384. (**A**) HeLa cells and CEF were transfected with 400 ng and 200 ng, respectively, of either HEO (expressing the Val400 hER) or HEGO (expressing the Gly400 hER), together with the vit-tk-CAT reporter gene (Materials and methods). Ethanol (−), E2 (10 nM), OHT (100 nM) or ICI (100 nM) were added as indicated. The open triangle points to an unknown contaminating derivative. (**B**) Quantification of the data from the experiment shown in panel A and from several similar experiments (see e.g, Figure 5). The transcriptional activation of vit-tk-CAT or pS2-CAT by either HEO or HEGO in the presence of the ligands (as indicated) is expressed taking the activation by HEO in the absence of any ligand (−) as 1.0. The average values (±30%) from the different experiments are given.

any agonistic effect on HEO, HEGO constitutive activity was decreased by OHT addition (see also Tora *et al.*, 1989b). In all other cases where OHT exerted an oestrogenic effect on HEO, its addition stimulated HEGO constitutive activity to the same levels as those observed with HEO (vit-tk-CAT in CEF, pS2-CAT in HeLa cells and CEF, Figures 4 and 5, and data not shown).

The effects, described above, of ICI 164,384 on the apparently oestrogen independent activity of HEGO on two reporter genes in HeLa cells and CEF extend our previous observations (Tora *et al.*, 1989b), and further support our proposal that this constitutive activity is due to residual levels of oestrogens in the steroid-hormone-stripped medium. Our results also show clearly that OHT has the same cell-type and promoter-context dependent agonistic effect on HEGO as on HEO. As in the case of HEO, this effect is certainly due to the cell-type and promoter-context activity of TAF-1, since the constitutive activity of HE19G (the Val400→Gly400 counterpart of HE19) is as efficiently suppressed by OHT as the E2-induced activity of HE19 (Figure 5; see also Figures 1A and 2).

4-Hydroxytamoxifen is an efficient oestrogenic agonist in yeast

We have previously shown that the Val400 hER (HEO) can efficiently stimulate transcription in yeast from a minimal promoter containing an ERE inserted upstream from the *GAL*1 TATA box region (pYERE1, Figure 6B, see Metzger

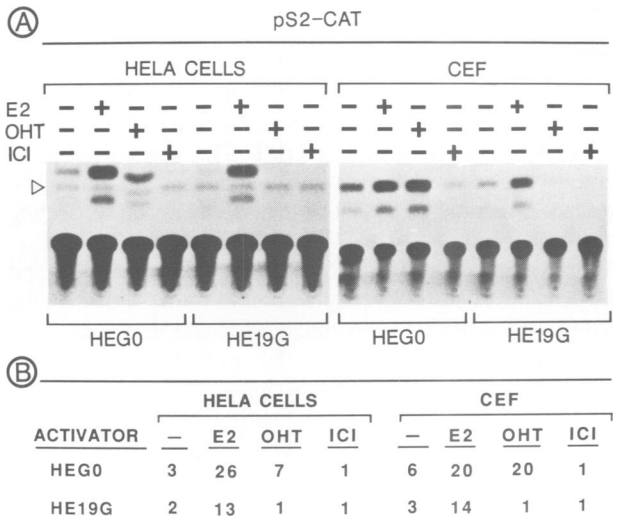

Fig. 5. Transcriptional activation of pS2-CAT by HEGO and HE19G in HeLa cells and CEF. (**A**) CAT assays of extracts of HeLa cells and CEF transfected with the pS2-CAT reporter gene along with HEGO and HE19G as activators (Materials and methods). E2 (10 nM), OHT (100 nM) and ICI (100 nM) were added as indicated. The open triangle points to an unknown contaminating derivative in the case of HeLa cells only. (**B**) Quantification of the data from the experiment shown in panel A and from several similar experiments. The transcriptional activation of pS2-CAT by either HEGO or HE19G is expressed taking in each case the activation by HEGO or HE19G in the presence of ICI 164,384 as 1.0. The average values (±30%) from the different experiments are given.

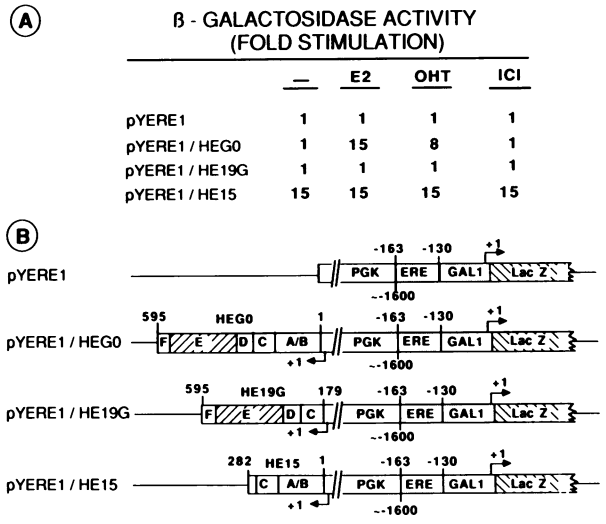

Fig. 6. Transcriptional stimulation in yeast by human Gly400 ER (HEGO) and its truncated derivative HE19G in the presence and absence of oestradiol, 4-hydroxytamoxifen and ICI 164,384. (**A**) yeast cells transformed with pYERE1, pYERE1/HEGO, pYERE1/HE19G and pYERE1/HE15 (panel B) were grown as described in Materials and methods in the presence or absence of E2 (5 nM), OHT (100 nM) and ICI (1 μM) as indicated. β-galactosidase activity was assayed and expressed as fold stimulation (±10%), taking the control value (−, ethanol alone, ~60 β-galactosidase units) as 1. (**B**) Schematic representation of the chimeric vectors used in yeast. The chimeric vectors express wild type Gly400 hER (HEGO) or truncated hER mutants (HE19G, HE15) from the *PGK* promoter. The *lacZ* gene coding for β-galactosidase is under the control of an ER dependent promoter which contains an ERE located upstream of the TATA box sequence of the *GAL1* promoter (see Metzger *et al.*, 1988 and White *et al.*, 1988).

et al., 1988). Using yeast cells tranformed with pYERE1/HEGO we have reported that Gly400 hER stimulates transcription to a similar extent in a fully E2 dependent manner (Tora *et al.*, 1989b; see also Figure 6), which strongly supports the conclusion that the constitutive activity of HEGO in cultured animal cells is due to the presence of residual oestrogens in the steroid-hormone-stripped medium. We have also reported that HE15 is almost as efficient as the whole hER at stimulating transcription in yeast (White *et al.*, 1988; see also Figure 6).

The results obtained here with yeast cells transformed with pYERE1/HE19G, indicate that TAF-2, in contrast to TAF-1, has very little if any transcriptional activity in yeast [Figure 6, compare pYERE1/HEGO, pYERE1/HE19G and pYERE1/HE15; HE19G and HEGO protein levels were similar as judged from Western blots (data not shown)]. Note that the transcriptional activity for HE19G was not increased by using the recombinant pYERE3/HE19G which contains three consensus EREs in tandem, in place of a single ERE (D.Metzger, unpublished data). In analogy with the situation in CEF, the strong activity of TAF-1 in yeast cells suggested that OHT could be an efficient oestrogen agonist in yeast. The transcriptional activity of HEGO was indeed efficiently stimulated by OHT in pYERE1/HEGO transformed yeast cells [Figure 6A, similar results were obtained with 5 μM OHT (data not shown)]. On the other hand, no stimulation of transcription was observed in the presence of ICI 164,384 (Figure 6A). However, the stimulatory effect of E2 (5 nM) or OHT (100 nM) was not blocked by its addition to the growth medium at 10 μM (data not shown), raising the possibility that ICI 164,384 cannot penetrate into yeast cells.

Discussion

4-Hydroxytamoxifen, but not ICI 164,384, acts as an oestrogenic agonist when the hER transcriptional activation function TAF-1 is active on its own

The present results show clearly that the oestrogenic effect of OHT on stimulation of transcription by the hER is both cell-type and promoter-context dependent. In CEF, OHT acts as an efficient agonist irrespective of the promoter context, whereas in HeLa cells it has very little agonistic effect except in the case of the reporter gene which contains the pS2 gene promoter. Since in all cases, and in agreement with our previous results (Webster *et al.*, 1988), the hER transcriptional activation function TAF-2 cannot be induced by OHT [HE19 or GAL-ER(EF), see Figure 1], the agonistic effect of OHT must be ascribed to TAF-1 whose activity is insensitive to OHT [HE15 or GAL-ER(AB), see Figure 1]. Accordingly, TAF-1 (HE15) stimulates transcription efficiently from all oestrogen responsive promoters in CEF, whereas only pS2-CAT is significantly stimulated in HeLa cells [the particular role of TAF-1 in stimulation of transcription from pS2-CAT in HeLa cells is also indicated by the lower efficiency of HE19 (versus HEO) when compared with other complex promoters, see Figure 1]. The tight correlation between the activity of TAF-1 and the oestrogenic effect of OHT is especially striking in the case of yeast cells in which TAF-2 has no activity, whereas HE15 is as active as the whole hER (see Figure 6). It has been shown that hER produced either in animal cells (Kumar and Chambon,

1988), in yeast cells (Metzger *et al.*, 1988) or *in vitro* (Lees *et al.*, 1989) binds as efficiently and specifically to an ERE *in vitro* when either oestradiol or OHT is present. We propose therefore that OHT acts as an oestrogen agonist whenever it promotes binding of the hER to a target gene promoter from which transcription can be activated by TAF-1 on its own. Conversely, it will act as a pure antagonist, whenever the activation of a given promoter is fully dependent on TAF-2 activity. On the other hand ICI 164,384 would be a pure antagonist in all cases, because it may in some way prevent binding of the hER to DNA. Whether this is in fact the case should be further investigated, since apparently conflicting results have been published concerning this point. In one study, ER synthesized *in vivo* and extracted in the presence of molybdate appears unable to bind to DNA *in vitro* in the presence of ICI 164,384 (Weatherill *et al.*, 1988; Wilson *et al.*, 1990), whereas in another study the binding of *in vivo* synthesized ER extracted in the absence of molybdate could be stimulated by the addition of ICI 164, 384, albeit with a lower efficiency than by addition of oestradiol or hydroxytamoxifen (Martinez and Wahli, 1989). In addition, binding of ER synthesized *in vitro* to an ERE was not prevented by ICI 164, 384 (Lees *et al.*, 1989. Our unpublished results).

No ligand independent transcriptional activity of the wild type human oestrogen receptor

Using the oestrogen responsive reporter gene vit-tk-CAT transfected in HeLa cells cultured in a phenol red-free and hormone-stripped medium, we have previously observed a significant activation of transcription by the wild type hER (HEGO) which possesses a glycine at position 400 instead of the artefactual valine which is present in the hER cDNA (HEO) which was originally cloned from MCF-7 cells (Tora *et al.*, 1989b). A similar seemingly 'oestrogen independent' activation of transcription was also observed by us using the chicken ER (CEO) and by others (White *et al.*, 1987; Lees *et al.*, 1989) using the mouse ER (mER), both of which contain a glycine at position 400. Because the oestrogen independent activation of vit-tk-CAT by HEGO in HeLa cells could be fully inhibited by OHT (Tora *et al.*, 1989b), and because activation of transcription by HEGO in yeast was fully dependent on oestrogen addition, we concluded that these 'oestrogen independent' activations were due both to a higher stability of Gly400 hER (when compared with Val400 hER) in the absence of oestrogen and to the presence of residual levels of oestrogens in the hormone-stripped culture medium (Tora *et al.*, 1989b). On the other hand, Lees *et al.* (1989), who found that some oestrogen responsive reporter genes were activated by mER in NIH 3T3 cells cultured in the presence of tamoxifen, concluded from their study that mER can bind an ERE *in vivo* in the absence of ligand, and activate transcription through the activation function located in the N-terminal A/B region (TAF-1).

Our present results in animal cells show clearly that the lack of inhibition by OHT of the 'ligand independent' HEGO activation of transcription cannot be taken as evidence for constitutive binding of the wild type Gly400 hER to an ERE. In fact, addition of OHT to the culture medium may even result in some cases in an increase in the 'constitutive' activity (see pS2-CAT with HEGO in Figure 4). On the other hand, in all cases the presence of the pure anti-oestrogen ICI 164,384 resulted in a complete suppression of HEGO 'constitutive' activity.

The inability of the Gly400 hER to activate constitutively an oestrogen responsive element in the absence of ligand was best illustrated by its complete inactivity in yeast cells, in the absence of added E2 or OHT. The yeast results also demonstrate clearly that activation of transcription in the presence of tamoxifen cannot be taken as evidence supporting constitutive binding of the ER to DNA.

We conclude therefore that the so-called 'ligand independent' 'constitutive' activation of transcription by the Gly400 hER (HEGO), the chicken ER and the mouse ER, results most probably from the presence of residual oestrogens in the hormone-stripped culture medium. Moreover, our results indicate clearly that OHT cannot be used to probe for a possible ligand independent activation of transcription by the ER.

Implications for agonist/antagonist activity of anti-oestrogens

The partial oestrogenic activity of tamoxifen and its active metabolite 4-hydroxytamoxifen is well established both *in vitro* and *in vivo*. The degree of agonism varies, however, with the species, tissues, cells and response being studied (for reviews see Furr and Jordan, 1984; Pasqualini *et al.*, 1988). Proliferation of the oestrogen dependent human MCF-7 breast carcinoma cells *in vitro* is stimulated by OHT (Sonnenschein *et al.*, 1985; Katzenellenbogen *et al.*, 1987; Bignon *et al.*, 1988; Wakeling, 1989; and refs therein). Tamoxifen and OHT have variable activity, ranging from fully oestrogenic to totally anti-oestrogenic, for the induction of a number of RNA species (including pS2 and progesterone receptor mRNAs) and proteins in cultured MCF-7 and other oestrogen dependent breast carcinoma cells (Horwitz *et al.*, 1978; May and Westley, 1987; Cavaillès *et al.*, 1988; May *et al.*, 1989; and refs therein). Interestingly, the magnitude of these inductions was different in another oestrogen dependent breast cancer cell line (Westley *et al.*, 1989). On the other hand in all cases studied, ICI 164,384 acted as a pure anti-oestrogen in cultured breast carcinoma cells and also antagonized the tamoxifen agonistic effects (Wiseman *et al.*, 1989; Thompson *et al.*, 1989; May *et al.*, 1989; Wakeling, 1989). The partial oestrogenic activity of tamoxifen and OHT *in vivo*, particularly on rodent uterus, vagina and mammary gland is well established and its variability with the animal species, the tissue and the parameter studied is well recognized (Furr and Jordan, 1984; Wakeling and Bowler, 1988a; Pasqualini *et al.*, 1988; and refs therein). On the other hand, ICI 164, 384 was found to be devoid of oestrogenic activity in all cases and blocked the partial agonist activity of tamoxifen (Wakeling and Bowler, 1988a,b; Pasqualini *et al.*, 1988; and refs therein). Tamoxifen has also been shown to have oestrogenic properties in human (Ferrazzi *et al.*, 1977; Boccardo *et al.*, 1981) and it was reported that OHT stimulates the proliferation of human endometrial adenocarcinoma cells (Satyaswaroop *et al.*, 1984; Gottardis *et al.*, 1988; Anzai *et al.*, 1989; Friedl *et al.*, 1989). Interestingly, adjuvant tamoxifen therapy of breast cancers may increase the incidence of endometrial carcinoma (Fornander *et al.*, 1989). Tamoxifen may also have an oestrogenic effect on bone cells, since it appears to prevent osteoporosis (Jordan *et al.*, 1987; Love *et al.*, 1988).

Our results raise the interesting possibility that the cell-type and promoter-context dependent agonistic activity of 4-hydroxytamoxifen observed here with various transfected oestrogen responsive genes, could account for most of the tamoxifen agonistic effects which are seen *in vivo* and are dependent on the tissue, cell type and parameters studied. The variations in tamoxifen agonism *in vivo* would depend on the ability of the hER activation function TAF-1 to stimulate transcription in particular tissues or cells from specific oestrogen responsive promoters. In contrast, the pure anti-oestrogenic activity of ICI 164,384 would result from its complete inability to 'induce' the transcriptional activity of the hER, even under cell-type and promoter-context conditions in which TAF-1 is active, as is the case here in chicken embryo fibroblasts. In this respect, these cells together with the oestrogen responsive reporter genes used in this study may be useful in providing a convenient *in vitro* assay for the identification of additional pure anti-oestrogens which may be valuable for the treatment of non-malignant oestrogen responsive diseases as well as for adjuvant therapy in breast cancers.

Materials and methods

ER expression vectors and reporter plasmids

HEO, HE19 and HE15 (Kumar *et al.*, 1986, 1987), HEGO (Tora *et al.*, 1989b), GAL-ER(AB) and GAL-ER(EF) (Webster *et al.*, 1989), have been described (see also Figure 1B). HE19G was constructed by replacing the region of HE19 containing glycine 400 with the corresponding region of HEGO. The reporter plasmids (Figure 1B), vit-tk-CAT, 17M/ERE-G.CAT, ERE-TATA-CAT, 17M2-G.CAT, pS2-CAT (see Tora *et al.*, 1989a), and ERE-tk-CAT (Green *et al.*, 1988) have been described. pYERE1 (Metzger *et al.*, 1988), pYERE1/HE15 (White *et al.*, 1988) and pYERE1/HEGO (Tora *et al.*, 1989b) have also been described. pYERE1/HE19G was constructed by inserting the *Bam*HI fragment of the hER cDNA sequence of HE19G into the *Bgl*II site of pYERE1.

Cell transfection and CAT assays

Chicken embryo fibroblast primary cell cultures (CEF) were prepared from 9–11 day old embryos according to Solomon (1975). HeLa cells and CEF were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% dextran-coated charcoal-treated fetal calf serum (Horwitz and McGuire, 1978) without phenol red (Berthois *et al.*, 1986). Insulin was added in the case of CEF (1 μg/ml). HeLa cells at 50% confluence in 9 cm Petri dishes were tranfected using the calcium phosphate coprecipitation technique (Tora *et al.*, 1989b). HeLa cells were transfected with either 1 μg of vit-tk-CAT, ERE-tk-CAT, ERE-TATA-CAT, 17M/ERE-G.CAT, 10 μg of pS2-CAT or 200 ng of 17M2-G.CAT as reporter genes, together with either 400 ng of HEO, HEGO, HE19, HE19G, HE15, or 200 ng of GAL-ER(AB) or GAL-ER(EF) as activator genes. HeLa cell transfections included 2 μg of the reference plasmid pCH110 (Pharmacia) and Bluescribe M13+ DNA (BSM+, Stratagene) as carrier DNA to make a total of 15 μg of DNA. CEF were transfected with either 1 μg vit-tk-CAT, ERE-tk-CAT, ERE-TATA-CAT, 17M/ERE-G.CAT, 5 μg pS2-CAT or 200 ng of 17M2-G.CAT together with either 200 ng of HEO, HEGO, HE19, HE19G, HE15 or 100 ng of GAL-ER(AB) or GAL-ER(EF) as activator genes. CEF transfections contained also 1 μg of pCH110 and BSM+ DNA to a total of 10 μg DNA. Ethanol (as a control), or oestradiol (E2, 10 nM), 4-hydroxytamoxifen (OHT, 100 nM), ICI 164,384 (ICI, 100 nM) prepared in ethanol were added 1 h post-transfection, where appropriate. The calcium phosphate precipitate was removed after 18–20 h, rinsed with DMEM, and replaced with fresh medium containing appropriate ligands or ethanol.

After an additional 18–24 h, the cells were rinsed and harvested in chilled phosphate-buffered saline, lysed with a single cycle of freeze–thaw (−80°C/20°C) in 200 mM Tris–HCl pH 7.5. The extracts were centrifuged at 10 000 *g* for 15 min at 4°C to remove cell debris. One fifth of each extract was assayed for β-galactosidase activity (Tora *et al.*, 1989b). HeLa cell extracts corresponding to 0.25 U of β-galactosidase for vit-tk-CAT and 17M2-G.CAT, 1 U for ERE-tk-CAT, 17M/ERE-G.CAT and ERE-TATA-CAT, and 5 U for pS2-CAT were used for CAT assays. For CEF cells, all the assays contained cell extracts equivalent to 5–10 U of β-galactosidase. CAT assays were performed in a total volume of 200 μl for 1 h at 37°C in the presence of 0.1 μCi of [^{14}C]chloramphenicol (50 μCi/mmol, Amersham) and 40 μl of 4 mM acetylcoenzyme A (Sigma). The acetylated and non-acetylated forms of labelled chloramphenicol were separated by TLC, autoradiographed and then excised and quantitated by liquid scintillation counting.

Growth of yeast culture and β-galactosidase assay

The yeast strain TGY14.1 was transformed as described (Metzger *et al.*, 1988; White *et al.*, 1988) to yield the pYERE1 series of transformants. Cells were grown to an OD_{600} of ~0.6 units in a medium containing 0.67% yeast nitrogen base without amino acids, 0.5% casamino acids and 1% glucose. Ethanol, E2 (5 nM or 100 nM), OHT (100 nM or 5 μM) or ICI (1 μM or 10 μM) was then added. The yeast cells were harvested 2 h later. About 10^7 cells (equivalent to 0.2 OD units) were taken for measuring β-galactosidase activity (Metzger *et al.*, 1988; Tora *et al.*, 1989b).

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