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OESTROGEN ENHANCES THE RESPONSIVENESS OF THE MMTV-LTR TO GLUCOCORTICOID IN ZR-75-1 HUMAN BREAST CANCER CELLS

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Summary—Treatment of ZR-75-1 human breast cancer cells with oestrogen has no direct effect on the expression of a transfected MMTV-LTR but enhances its inducibility in response to glucocorticoid treatment. This effect which can be produced with both oestradiol and diethylstilbestrol is specific to induction of the MMTV-LTR, no effect of the treatment on expression driven by the RSV-LTR being observed. The effect can be observed in cells pre-treated with oestrogen prior to removal of DNA and glucocorticoid addition but not in cells where oestrogen is added after removal of the DNA. The possible mechanisms of these effects and their relationship to the induction of oestrogen-responsive genes by this hormone are discussed.

INTRODUCTION

The promoter within the long terminal repeat (LTR) of mouse mammary tumour virus (MMTV) has been widely used as a model of a steroid responsive promoter. The induction of this promoter in response to glucocorticoid for example has been known for some time (for reviews see Refs [1, 2]) and DNA sequences capable of binding the complex of glucocorticoid and its receptor have been identified within the promoter (for review see Ref. [3]). These glucocorticoid response elements (GRE) are capable also of binding the complexes of other steroid hormones and their receptors including both progesterone and androgens [4, 5]. Such binding results in the MMTV-LTR being inducible by these hormones in cells which contain the appropriate receptors [6, 7].

In contrast, the induction of oestrogen responsive genes by treatment with this hormone is mediated by oestrogen response elements (ERE) in the DNA which though related to the GRE sequence are distinct from it [8, 9]. In agreement with this distinction, the MMTV-LTR which does not contain any ERE sequences was not inducible by oestrogen in the T-47D human breast cancer cell line [10], even though this line contains a functional oestrogen receptor and responds to oestrogen by changes in growth rate [11] and changes in cellular gene expression [12]. Similarly no modulatory effect of oestrogen on the response of the MMTV-LTR to glucocorticoid or other steroids was observed in these cells [10]. Despite this lack of effect however, it has been shown that the oestrogen receptor can recognise glucocorticoid response elements in the MMTV-LTR and bind to them with high affinity *in vitro* leading to the suggestion that under some circumstances oestrogen might modulate the expression of the MMTV-LTR or its response to other steroids [13].

To investigate this possibility further and determine whether the lack of an oestrogen effect on the MMTV-LTR was generally observed in other oestrogen-responsive cells, we have studied the effect of oestrogen treatment on the MMTV-LTR in the oestrogen responsive human breast cancer cell line ZR-75-1 [14]. We report here that, although as in T-47D cells, oestrogen has no direct effect on the MMTV-LTR it does enhance the responsiveness of the LTR to treatment with glucocorticoid.

MATERIALS AND METHODS

Culture of ZR-75-1 cells

ZR-75-1 cells [14] (a kind gift of Dr P. Darbre) were grown as described by Darbre *et al.*[15] in DMEM supplemented with 10% foetal calf serum. Cells were routinely maintained and passaged in a 10^{-8} M concentration of 17 beta-oestradiol which was removed for experiments on the effect of oestrogen withdrawal.

Steroids

17 beta-oestradiol, dexamethasone, diethylstilbestrol and tamoxifen were all purchased from Sigma Chemical Company and were dissolved in ethanol and added to culture media at the indicated concentrations.

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Plasmid DNA

The MMTV CAT plasmid (a kind gift of Dr M. Parker) contains a 1.5 kb Pst I fragment spanning the entire MMTV-LTR linked to a promoterless chloramphenicol acetyl transferase (CAT) gene whilst the pA2-tk-CAT construct (a kind gift of Professor G. Ryffel) contains the sequences of the oestrogen responsive vitellogenin promoter from −331 to −87, cloned upstream of the herpes simplex virus thymidine kinase promoter linked to the CAT gene. The RSV-CAT construct is as described by Gorman *et al.*[16].

Transfection of ZR-75-1 cells

Introduction of DNA into ZR-75-1 cells was carried out using the basic calcium phosphate procedure as described by Gorman[17] which had been optimized to obtain maximal expression of the transfected DNA in the ZR cells. The cells were plated out at a density of 2.5×10^5 cells per 90 mm plate 24 h before transfection. After this time, the calcium phosphate precipitate of DNA was added directly to the medium and allowed to remain in contact with the cells for 18 h. The DNA-containing medium was then removed and replaced with fresh medium; cells were harvested 72 h later. The protein content of the cellular extracts was assayed by the method of Bradford[18] and samples which had been equalized for protein content were assayed for CAT activity as described by Gorman[17].

RESULTS

In initial experiments we compared the effect of different doses of dexamethasone on the expression of the MMTV-LTR introduced into ZR cells cultured in the presence of 17 beta-oestradiol at a concentration of 10^{-8} M or in its absence. As shown in Fig. 1, the presence of oestradiol resulted in a dramatic increase in the glucocorticoid responsiveness of the MMTV-LTR allowing detectable levels of CAT activity to be observed even in cells incubated with a 10^{-8} M concentration of dexamethasone whereas CAT activity was barely detectable in the 10^{-6} M sample in the absence of oestrogen. This difference was not produced by a direct induction of the MMTV-LTR by oestrogen since in the absence of dexamethasone, no expression of the LTR was detectable in the presence or absence of oestrogen (Fig. 1). Similarly the effect does not appear to be produced by a non-specific increase in the ability of oestrogen-treated cells to take up or express any exogenously added DNA since the levels of expression of a transfected construct in which the non steroid-inducible long terminal repeat of Rous Sarcoma virus drives the expression of the CAT gene were similar in both cell types (Fig. 1). Similar results were obtained in three replicate experiments.

It is clear therefore that in ZR cells, oestrogen can modulate the glucocorticoid responsiveness of the MMTV-LTR. In order to further investigate the relationship of this effect to other oestrogen mediated responses, we tested whether it could be reproduced with another oestrogen, diethyl-stilbestrol. As shown in Fig. 2 [compare parts (a) and (b)] this compound when added at a concentration of 10^{-6} M produced the same enhancement of glucocorticoid responsiveness as was seen for oestradiol. In contrast, the effect of oestradiol was not abolished by the addition of the anti-oestrogen, tamoxifen at a concentration of 10^{-6} M together with the 17 beta-oestradiol [Fig. 2 (b) and (c)].

Having established that the effects we observe could be produced with two different oestrogens, we investigated the time at which the oestrogen must be

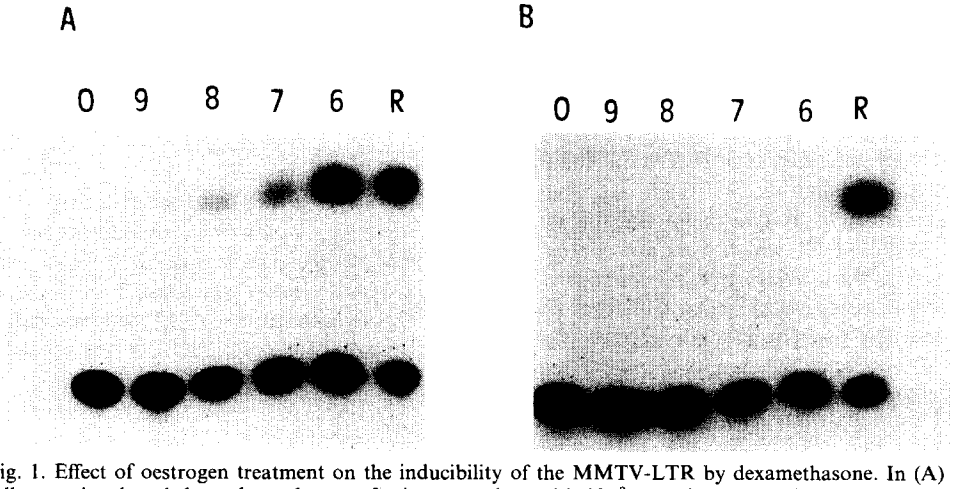

Fig. 1. Effect of oestrogen treatment on the inducibility of the MMTV-LTR by dexamethasone. In (A) cells were incubated throughout the transfection procedure with 10^{-8} M 17 beta-oestradiol whilst in (B) cells were incubated in the absence of the hormone. Tracks labelled 0, 8 (10^{-8} M) etc., indicate the expression of the MMTV-LTR in the absence (0) or the indicated molar concentration of dexamethasone. R indicates the expression of an RSV-CAT construct.

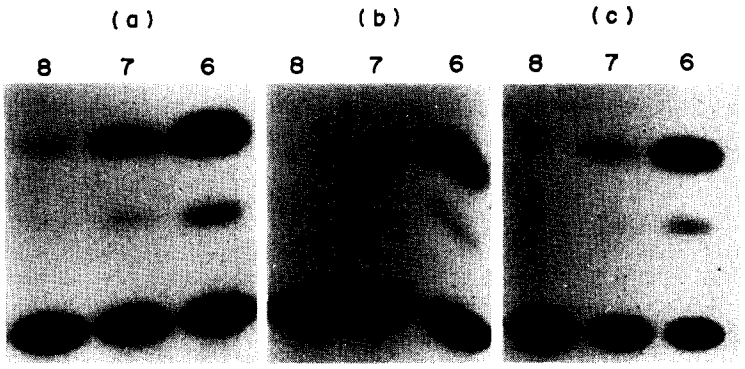

Fig. 2. Effect of another oestrogen and an anti-oestrogen on inducibility of the MMTV-LTR by glucocorticoid. In (a) cells were exposed to 10^{-6} M diethylstilbestrol, in (b) to 10^{-8} M 17 beta-oestradiol and in (c) to 10^{-8} M 17 beta-oestradiol in the presence of 10^{-6} M of the anti-oestrogen tamoxifen. Tracks are labelled as in Fig. 1, indicating the molar concentration of dexamethasone to which each sample was exposed.

present for the effect to occur. Thus in the experiments in Fig. 1, the oestradiol-treated sample was maintained in the presence of the hormone for the 24 h prior to addition of DNA, for the 18 h during which the DNA was present and for a further 72 h after removal of the untransfected DNA prior to harvesting (see Fig. 3, treatment a). It is during this latter period that the glucocorticoid is added and the transfected DNA is actually expressed [19]. We therefore investigated whether removal of the oestradiol at the time the calcium phosphate precipitate of DNA was removed would abolish the effect (treatment c in Fig. 3). As shown in Fig. 4 (b) this is not the case, cells from which oestradiol has been removed at this point, still showing strong responsiveness of the MMTV promoter to glucocorticoid comparable to that obtained when oestradiol is present throughout. In contrast cells treated in the reciprocal manner and exposed to oestrogen only after the untransfected DNA had been removed (treatment b in Fig. 3) showed only a very small enhancement of the responsiveness to glucocorticoid [Fig. 4(a)]. Similar results were obtained in three replicate experiments.

Hence the effect of oestrogen on the glucocorticoid responsiveness of the MMTV-LTR, can be observed if the hormone is present prior to the addition of the DNA and at the time of DNA addition but it need not be present when the glucocorticoid is actually added. To investigate this effect further we studied whether this enhancement of glucocorticoid responsiveness could be obtained if the oestrogen was only present prior to the addition of the MMTV-LTR. To do this two samples of cells were maintained in oestrogen containing medium prior to addition of MMTV DNA. One sample was removed from oestrogen immediately prior to DNA addition (treatment d in Fig. 3) whilst the other was, as before, removed from oestrogen when the calcium phosphate

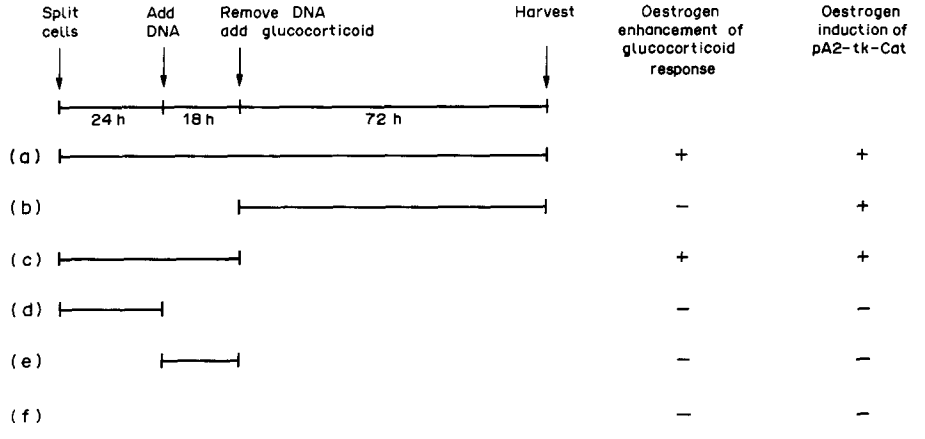

Fig. 3. Summary of the various oestrogen treatments employed and their effects on gene expression. The solid lines in (a)–(f) indicate the period when oestrogen was present whilst the blank spaces indicate when the hormone was withdrawn. A plus sign indicates that glucocorticoid responsiveness of the MMTV-LTR was enhanced by the treatment or that expression of pA2-tk-CAT was increased whereas a minus indicates that no effect was observed. The actual results of these experiments are shown in Figs 1 and 4–6.

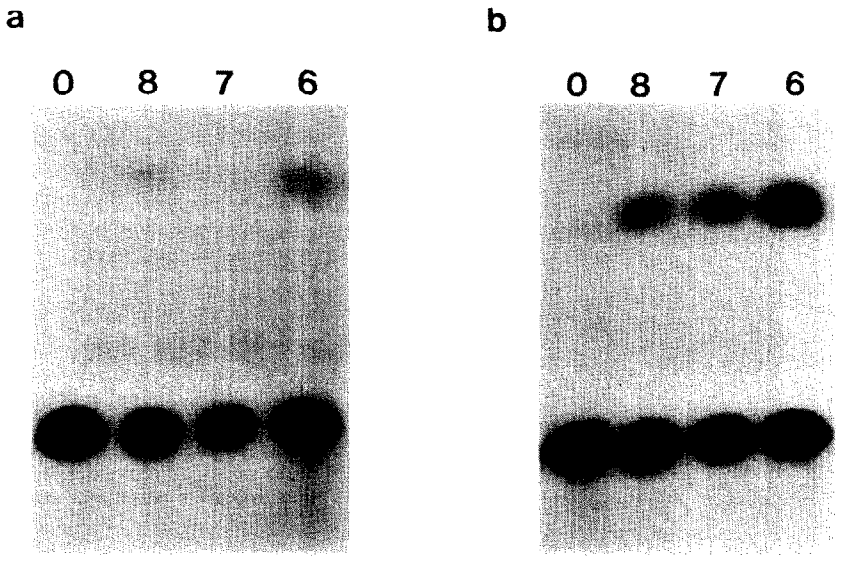

Fig. 4. Effect on glucocorticoid inducibility of the MMTV-LTR when oestrogen is added only following removal of the calcium phosphate DNA precipitate (part (a) treatment b in Fig. 3) or when it is present prior to and during DNA addition and is removed along with the DNA precipitate (part (b) treatment c in Fig. 3). Tracks are labelled as in Fig. 1, indicating the molar concentration of dexamethasone to which each sample was exposed.

precipitate was removed 18 h later (treatment c in Fig. 3). As shown in Fig. 5, removal of the oestrogen prior to removal of the DNA abolished its effect on glucocorticoid responsiveness of the MMTV-LTR indicating that the presence of oestrogen solely prior to DNA addition is insufficient for this effect [Fig. 5(b)]. In contrast, the presence of oestrogen both prior to addition of the DNA and when the DNA was added (treatment c in Fig. 3) was, as before, sufficient to produce the effect [Fig. 5(a)]. We therefore investigated whether the presence of oestrogen only for the 18 h in which the DNA was actually present would be sufficient for the enhancement of glucocorticoid responsiveness (treatment e in Fig. 3). As shown in Fig. 5 (c) however, this was not the case, no enhancement of glucocorticoid responsiveness being observed when the oestrogen was present only for the period of DNA addition.

These results (summarized in Fig. 3) indicate therefore that to obtain the oestrogen-mediated enhancement of glucocorticoid responsiveness, the oestrogen must be present both for the period immediately prior to addition of the DNA and whilst the DNA is present but that either period alone or addition of oestrogen following removal of DNA is insufficient. To investigate the relationship of this

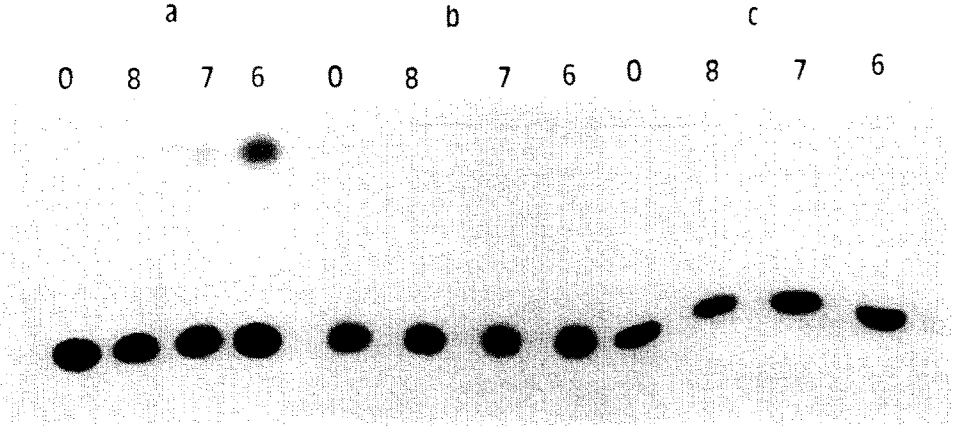

Fig. 5. Effect on glucocorticoid inducibility of the MMTV-LTR when oestrogen is present both prior to addition of the DNA and when the DNA is present (part (a) treatment c in Fig. 3), only prior to addition of the DNA (part (b) treatment d in Fig. 3) or only when DNA is present (part (c) treatment e in Fig. 3). Tracks are labelled as in Fig. 1, indicating the molar concentration of dexamethasone to which each sample was exposed.

effect to the induction of oestrogen responsive genes by the hormone we determined the effect of exposing ZR cells to oestrogen for various periods, on the induction of an oestrogen responsive gene. To do this cells were transfected using the normal procedure with a plasmid pA2-tk-CAT in which the oestrogen response elements of the vitellogenin gene drive the expression of the herpes simplex virus thymidine kinase promoter linked to the CAT gene [20]. During the transfection procedure cells were exposed to oestrogen for each of the periods indicated in Fig. 3 and the effect on the expression of pA2-tk-CAT assessed by a CAT assay following harvesting of the transfected cells.

The results of these experiments (Fig. 6, summarized in Fig. 3) revealed two distinct effects of the different periods of oestrogen exposure. Firstly, high level expression of pA2-tk-CAT was induced by any treatment which included exposure to oestrogen following DNA removal (treatments a and b in Fig. 3, data not shown). This was as expected since the normal means of stimulating a hormone responsive promoter is to add the hormone following DNA removal, as we have done throughout this study to study the effect of glucocorticoid on the MMTV-LTR. This effect of oestrogen is therefore distinct from the oestrogen-mediated enhancement of glucocorticoid responsiveness which does not occur when the oestrogen is added only after removal of the DNA. Interestingly however, a second effect was also observed in that some induction of the pA2-tk-CAT construct by oestrogen could be produced by oestrogen pre-treatment of the cells both prior to DNA addition and whilst the DNA was present (treatment c in Fig. 3, see Fig. 6), although this induction was less than that observed when oestrogen was present throughout. In contrast no effect was observed when the oestrogen was present only prior to DNA addition or while the DNA was present (treatment d and e in Fig. 3, see Fig. 6). This second effect is therefore exactly identical with the requirement for oestrogen-mediated enhancement of glucocorticoid responsiveness suggesting that the two effects are related to one another (see Fig. 3 for summary of these data).

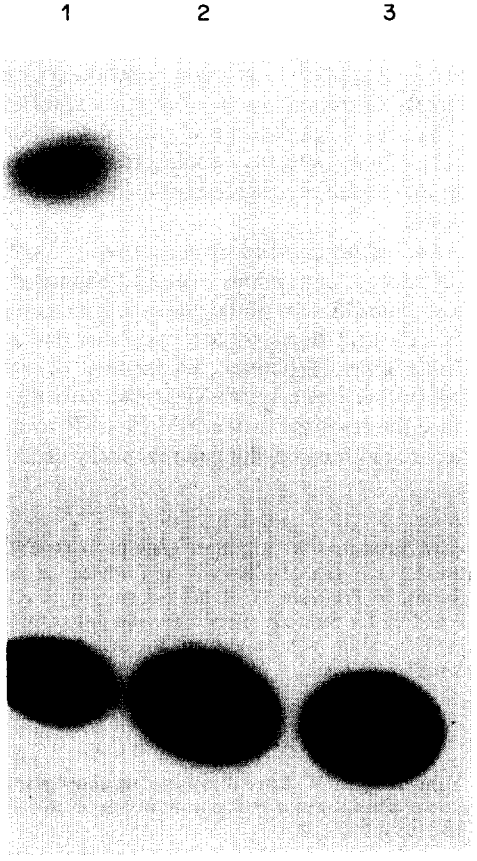

Fig. 6. Effect of various periods of treatment with oestrogen on the expression of the oestrogen-inducible pA2-tk-CAT plasmid. Track 1 indicates the expression of the plasmid in cells exposed to 10^{-8} M oestradiol both prior to and during DNA addition (treatment c in Fig. 3), track 2 the expression in cells exposed to hormone only prior to DNA addition (treatment d in Fig. 3) and track 3 the expression in cells exposed to hormone only during DNA addition (treatment e in Fig. 3).

DISCUSSION

It has been known for some time that the MMTV-LTR can be activated by a range of steroid hormones such as glucocorticoid, progesterone and androgen [1, 2, 5–8] and the sequences in the LTR involved in mediating these effects have been precisely defined [3, 4, 21]. In contrast the MMTV-LTR lacks DNA sequences mediating response to oestrogen [8, 9, 22] and in agreement with this no effect of physiological concentrations of oestrogen on the expression of the MMTV-LTR has previously been reported [6, 10, 13]. Here we show however, that oestrogen treatment of ZR-75-1 cells can enhance the response of the MMTV-LTR to glucocorticoid although oestrogen does not have any independent effect on the expression of the LTR.

It is known that oestradiol can bind weakly to the androgen receptor and hence, when present at very high concentrations beyond the physiological range, can act as a weak androgen and activate the MMTV-LTR [10, 23]. In contrast the effects we observe here appear to be mediated via binding of the hormone to the oestrogen receptor itself since they are only observed at physiological doses of oestradiol, 100-fold below those which are necessary for androgen like effects. Moreover these effects are also reproducible with high doses of another oestrogen, diethylstilbestrol which has no affinity for the androgen receptor. In contrast no antagonism of the effect of oestradiol was observed when the anti-oestrogen tamoxifen was added. This is consistent however, with the effects we observe being mediated via the oestrogen receptor; a similar lack of effect of an anti-oestrogen on the induction of gene expression

mediated by a complex of oestrogen and its receptor having also been observed for the oestrogen mediated induction of the pNR-1 gene in human breast cancer cells [24] whilst complexes of anti-glucocorticoid and its corresponding receptor fail to interact productively with the MMTV-LTR [25]. Further studies with more potent anti-oestrogens such as 4-hydroxy tamoxifen will be necessary to confirm that the effects we observe occur via the oestrogen receptor however.

The effect of oestrogen on the glucocorticoid-responsiveness of the MMTV-LTR appears to be a specific one rather than being mediated via an effect on the growth ability of the cells or their ability to take up DNA. Thus the effect was specific to the MMTV-LTR, no enhancement of the expression of the RSV-LTR being observed in any of the oestrogen treatments whilst only the glucocorticoid inducibility of the LTR rather than its basal level of expression in the absence of dexamethasone was affected. Similarly, the presence of oestrogen over the 18 h period when cells actually take up the DNA was insufficient for the effect to be observed suggesting that the hormone is not acting by promoting uptake of transfected DNA. This finding is consistent with several previous studies which have shown that virtually all cells in a monolayer culture can take up DNA and that initial uptake of DNA is not the factor limiting expression in the transfection of most cell types [26, 27].

Hence the effects we observe appear to be mediated via a specific effect of the oestrogen receptor-oestrogen complex on glucocorticoid inducibility of the MMTV-LTR. This process does exhibit differences however, from the action of the oestrogen receptor-oestrogen complex on constructs such as pA2-tk-CAT which are directly inducible by oestrogen. Thus the induction of pA2-tk-CAT expression by oestrogen was observed when oestrogen was added only after the DNA had been removed to the same extent as when the hormone was present throughout. In contrast, only a very small effect on the glucocorticoid inducibility of MMTV-CAT was observed under these conditions. This indicates that pre-treatment of the cells with oestrogen is required for maximal inducibility of MMTV-CAT but not for the enhanced expression of pA2-tk-CAT. Interestingly however, the expression of pA2-tk-CAT could also be induced by oestrogen when the hormone was present both prior to and during DNA addition but not when it was present for either of these periods alone exactly paralleling the requirement for oestrogen-mediated enhancement of MMTV-CAT inducibility.

Taken together these data are not consistent with the effects we observe being mediated via the direct binding of the oestrogen receptor to the GRE sequences in the MMTV-LTR observed by others [13]. Rather it seems likely that during the pre-treatment oestrogen acts by inducing the expression of another protein which in turn enhances the effect of glucocorticoid on the MMTV-LTR. The minimal enhancement of the effect of glucocorticoid if oestrogen is added only at the same time as dexamethasone could then be explained either on the basis that insufficient time was available for maximal *de novo* synthesis of the oestrogen-induced protein or that it must be present prior to glucocorticoid addition to facilitate the assembly of a stable transcriptional complex immediately upon glucocorticoid addition. Similarly the lack of effect of oestrogen on the glucocorticoid-inducibility of the MMTV-LTR observed by Glover and Darbre in T-47D breast cancer cells [10] could be explained by differences in the basal level or inducibility of this protein in these cells compared to ZR-75-1 cells possibly caused by a difference in the chromatin structure of the corresponding gene.

It remains unclear however, what the identity of the oestrogen-inducible protein might be. A number of reports have shown that the progesterone receptor is inducible by oestrogen in human breast cancer cells including ZR cells [28, 29] but no induction of the glucocorticoid receptor itself by oestrogen treatment has been reported in such cells. In contrast it has been shown that the glucocorticoid receptor-associated 90 kDa heat shock protein is inducible by oestrogen treatment in rat uterus [30]. However, since this protein is suggested to act as an anchorage protein preventing the receptor activating gene expression in the absence of glucocorticoid (for discussion see Refs [31, 32]) its induction would presumably reduce rather than enhance the glucocorticoid inducibility of the LTR.

To resolve this question we are currently investigating the effect of our various treatment regimes on the expression in ZR cells of the glucocorticoid receptor, the 90 kDa heat shock protein and other oestrogen-inducible genes. Whatever the identity of the inducible protein however, it is clear that oestrogen can enhance the response of the MMTV-LTR to glucocorticoid in at least one human mammary cancer cell line.

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