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CO-OPERATION OF PROGESTATIONAL STEROIDS WITH EPIDERMAL GROWTH FACTOR IN ACTIVATION OF GENE EXPRESSION IN MAMMARY TUMOR CELLS

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Summary—The progesterone receptor belongs to a class of ligand binding transcription factors that regulate transcription by interacting with specific DNA sequences on hormone regulated genes. In human mammary tumor T47D cells that contain both progesterone and epidermal growth factor (EGF) receptors, the progestin-induced transactivation at various hormone regulated promoters is enhanced by EGF. The effect of EGF is rapid and does not require new protein synthesis. EGF treatment does not alter the DNA binding activity of the progesterone receptor nor does it affect the total ligand-dependent phosphorylation of this receptor. These results suggest that EGF enhances the transactivation property of the progesterone receptor through mechanisms other than those involving a direct interaction of this receptor with its cognate binding sites.

INTRODUCTION

Steroid hormones regulate gene expression by binding to their corresponding receptors which in turn interact with discrete DNA sequences on regulatable genes to modulate their activity [1]. Although details of the receptor-DNA binding have been extensively worked-out [1], little information is available on the molecular events that follow this binding or processes that regulate the transactivating property of steroid receptors.

The action of steroid hormones on growth of human mammary tumor cells has been shown to be modulated by polypeptide growth factors [2, 3] but so far there are no reports describing a direct involvement of these growth factors in the regulation of gene expression by steroid hormones. In this communication we show that epidermal growth factor (EGF) and transforming growth factor α (TGFα) enhance the progestin response at various hormone regulated promoters in human mammary tumor T47D cells. The effect of these growth factors is mediated by activation of the EGF receptor but does not appear to require alteration of the DNA binding activity of the progesterone receptor. These results suggest that factors other than those controlling the binding of the receptor to DNA are responsible for the enhanced progestin response by EGF and TGFα in the human mammary T47D cells.

RESULTS AND DISCUSSION

To determine whether growth factors influence progestin regulation of gene expression, we studied the effect of EGF on progestin response in the human mammary tumor cell line T47D that contains functional EGF and progesterone receptors [4, 5]. These cells were stably co-transfected with the progestin-inducible MMTV CAT [6] or PRE-tk-CAT [7] and pSV$_2$neo [8] constructs and three clones were isolated for further analyses. The selected clones showed variable levels of inducibility by R5020 as determined by CAT assay [Fig. 1(A)] EGF alone had no effect on CAT activity in all the clones studied but EGF and R5020 increased CAT activity 2–4-fold higher than the level induced by R5020 alone [Fig. 1(A)]. The EGF effect on R5020 response was observed independent of whether the hormone response element at the MMTV promoter or a synthetic progesterone

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response element (PRE) linked to the thymidine kinase promoter was used as the indicator gene [Fig. 1(A)]. The EGF effect on progestin action was also evident when progestins such as norethisterone or medroxyprogesterone acetate were used instead of R5020 (results not shown).

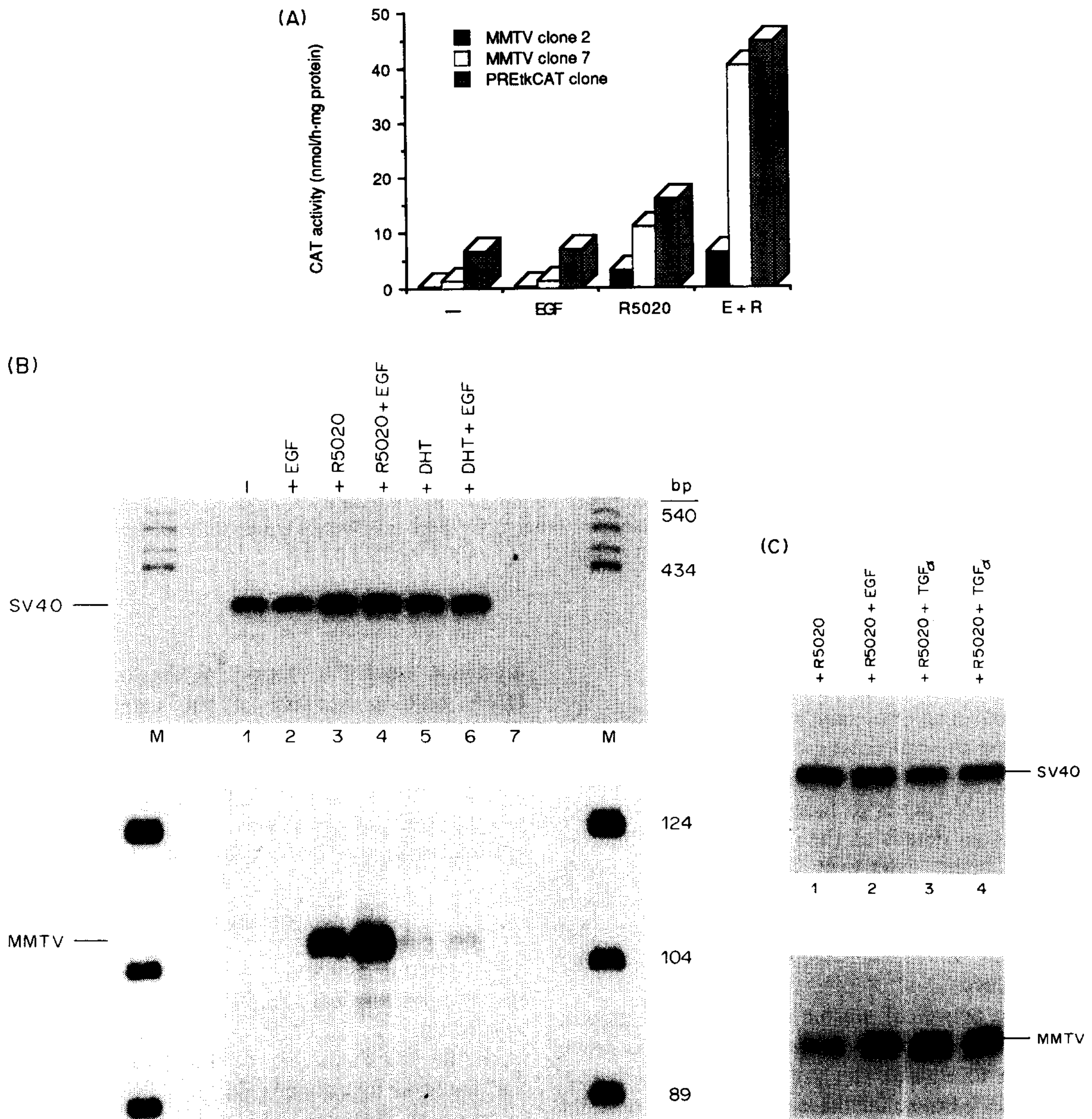

Fig. 1. Effect of EGF and TGFα on progestin response. T47D cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) and 0.6 μg/ml insulin. Stable transfections were carried out with pMMTV-CAT [6] or PRE-tk-CAT [7] and pSV$_2$neo [8] constructs using the calcium phosphate precipitation method of Wigler *et al.*, 1979 [20]. Transfected clones were selected in medium supplemented with 500 μg/ml G418. Selected clones were cultured in RPMI medium supplemented with 25 μg/ml bovine serum albumin (BSA) and treated for 40 h without, with 10^{-8} M R5020, 20 ng/ml EGF (Sigma), 10^{-7} M DHT, (50 ng/ml or 100 ng/ml) TGFα (Bissendorf Biochemicals) or a combination of steroids and growth factors. (A) CAT activity of the transfected T47D clones after treatment with the indicated hormone and growth factor was determined as described by Gorman *et al.*, 1982 [21]. E + R stands for EGF and R5020; (B) S1 nuclease mapping of the SV40 and MMTV LTR transcripts in T47D clone 7 cells after treatment of the cells with the indicated combination of steroids and EGF. Total cellular RNA (30 μg) were used for the assay. The SV40 and MMTV LTR probes used have been described previously [22]. Lane 7 contains S1 nuclease mapping products carried out with 30 μg yeast RNA and M (Marker) stands for lane with labeled HaeIII fragments of plasmid pBR322. The SV40 and MMTV LTR transcripts are indicated; and (C) S1 nuclease mapping of the SV40 and MMTV LTR transcripts in T47D clone 7 cells after treating the cells with the indicated steroid and growth factors. Lane 3 contains RNA from cells treated with 50 ng/ml TGFα and lane 4 with 100 ng/ml TGFα.

To investigate whether the effect of EGF on R5020-induced CAT activity results from changes at the transcriptional level, we analyzed the accumulation of transcripts at the MMTV LTR promoter in the T47D clone 7 cells after growth factor and steroid hormone treatment using S1 nuclease mapping technique. As in the case of the CAT assay, we found that EGF had no detectable effect on transcription at the MMTV promoter [Fig. 1(B), lane 2] but significantly increased the progestin response at this promoter [Fig. 1(B), compare lanes 3 and 4]. No enhancement of androgen response by EGF was observed when the cells were treated with the androgen dihydrotestosterone (DHT) instead of R5020 [Fig. 1(B), lanes 5 and 6]. In addition, neither DHT nor R5020 with or without EGF had any effect on transcription at the promoter of the control pSV_2neo construct [Fig. 1(B), see the SV40 band]. These results demonstrate that the EGF effect is restricted to constructs bearing a PRE. The PRE must therefore be the target for the effect of EGF on progestin response.

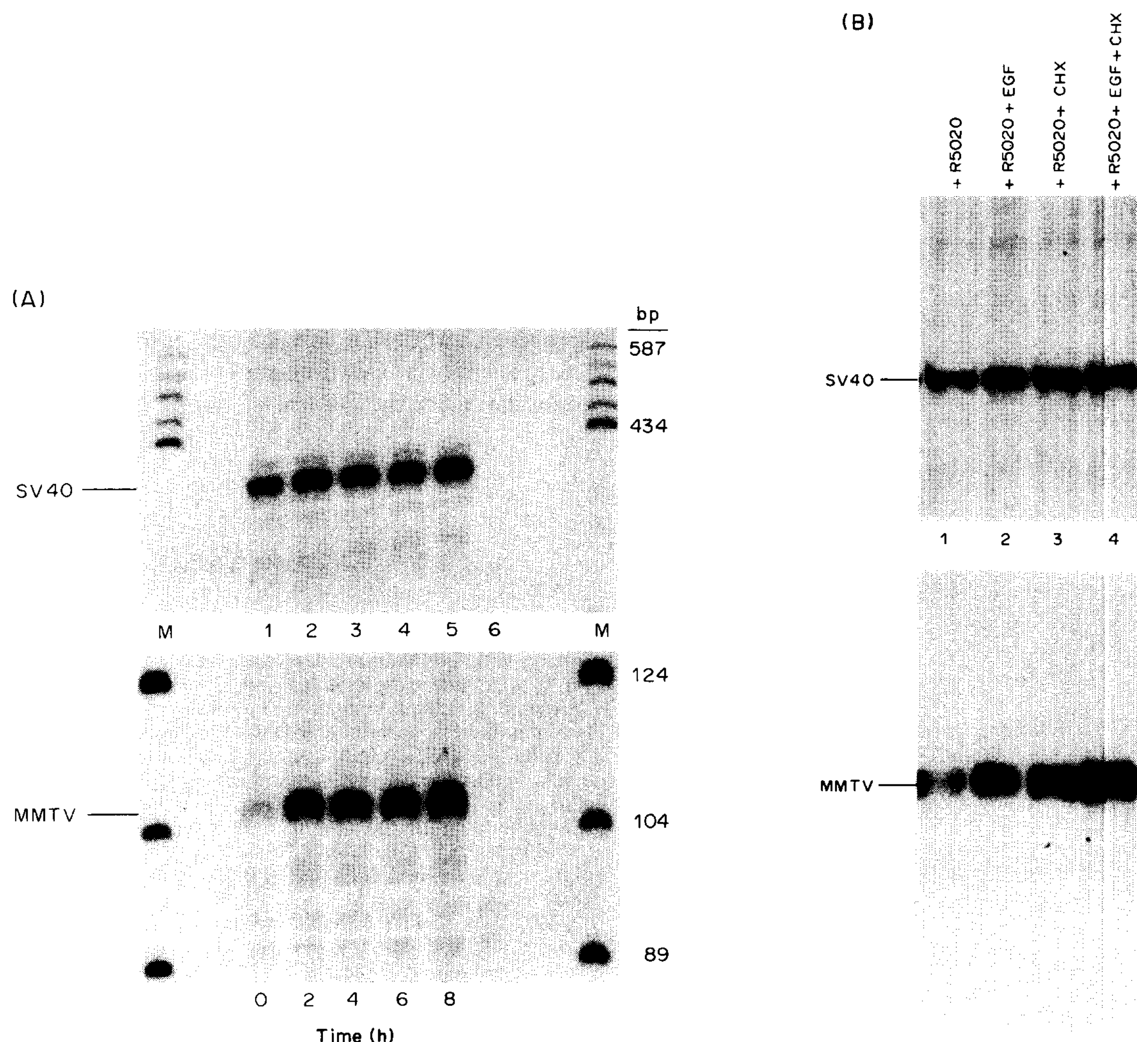

Fig. 2. The EGF effect on progestin response does not require new protein synthesis. (A) The effect of EGF on R5020 response is rapid. T47D clone 7 cells cultured in RPMI medium supplemented with 25 μg/ml BSA and treated for 40 h with 10^{-8} M R5020 were further treated with EGF (20 ng/ml) for the indicated lengths of time. The cells were harvested, total cellular RNA isolated and S1 nuclease mapping was carried out as described previously [22] with 10 μg RNA. Lane 6 contains products of S1 nuclease mapping carried out with 10 μg yeast RNA. M stands for lane with labeled HaeIII fragments of pBR322; and (B) Inhibition of protein synthesis does not abolish the EGF effect on R5020 response. T47D clone 7 cells cultured in the absence of FCS were treated with 10^{-8} M R5020 for 16 h. Thereafter, as indicated, the cells were treated with cycloheximide (20 μg/ml) and/or EGF (20 ng/ml) for 2 h. When cycloheximide and EGF were administered together the cells were treated first with cycloheximide for 5 min before the addition of EGF and further incubation for 2 h with both substances. S1 nuclease mapping experiments with 20 μg total RNA were carried out as described in Fig. 1.

Experiments to determine the time it takes for EGF to influence progestin action at the MMTV promoter showed a response that is already maximal by 2 h after addition of this growth factor [Fig. 2(A)]. The EGF effect was not abolished by cycloheximide, an inhibitor of protein synthesis [Fig. 2(B), lanes 3 and 4]. Cycloheximide rather enhanced the level of expression at the MMTV promoter [Fig. 2(B), compare lanes 1 and 2 with 3 and 4]. Taken together these results indicate that the effect of EGF does not require *de novo* protein synthesis.

When EGF binds its receptor, it activates a tyrosine protein kinase that mediates the activity of this growth factor [9]. This effect is mimicked by TGFα that also binds to the EGF receptor (for review see Ref. [10]). That EGF receptor activation is involved in the stimulation of progestin response is shown by experiments in Fig. 1(C) in which TGFα at two different concentrations 50 ng/ml [Fig. 1(C), lane 3] and 100 ng/ml [Fig. 1(C), lane 4] enhanced R5020 response. A further hint that EGF receptor activation is involved in the EGF effect comes from inhibition studies with tyrphostin (RG50863), a specific inhibitor of EGF receptor phosphotyrosine kinase activity [11, 12]. Tyrphostin inhibits dose-dependently the effect of EGF on R5020 response without showing any inhibitory effect on R5020 response alone [Fig. 3(A)]. This demonstrates that the kinase activity of the EGF receptor is involved in the increased progestin response to EGF.

The progesterone receptor increases transcription at hormone regulated promoters after binding progestins. Ligand binding is associated with increased phosphorylation of the progesterone receptor [13, 14] and binding of the receptor to discrete DNA sequences [1]. To find out whether the increased transcriptional activity by the progesterone receptor in response to progestin and EGF involves a further increase in receptor phosphorylation, we studied the phosphorylation of the B-form of the progesterone

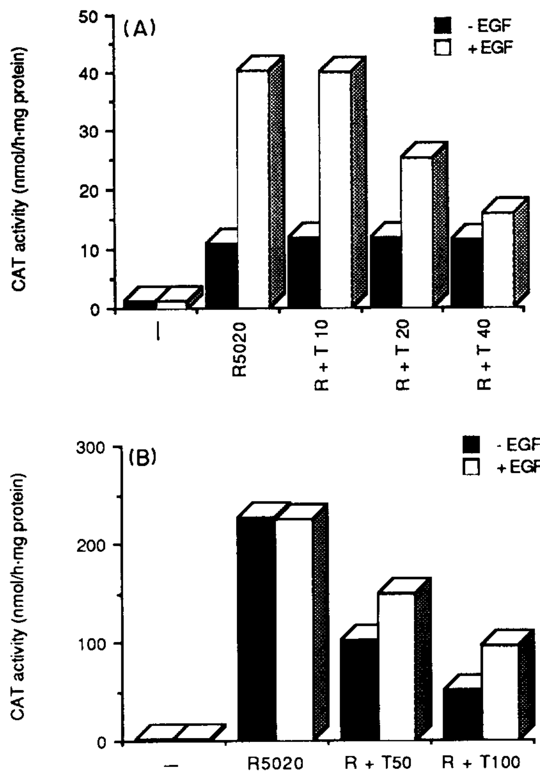

Fig. 3. Inhibition of EGF effect on R5020 response by tyrphostin. T47D clone 7 cells were cultured for 40 h in RPMI medium supplemented with 25 μg/ml BSA and 10^{-8} M R5020 with or without EGF in the presence of different concentrations of tyrphostin RG50863. The cells were then harvested and CAT activity was determined as described by Gorman *et al.*, 1982 [21]. Presented as bar diagrams are the responses of R5020 or R5020 + EGF in the presence of tyrphostin in cells that respond to EGF (A) and altered T47D cells that no longer respond to EGF treatment (B). (–) = untreated cells, (R) = cells treated with 10^{-8} M R5020, and (T10, 20, 40, 50 and 100) = various concentrations of tyrphostin in (μM). EGF concentrations used were 20 ng/ml for (A) and 60 ng/ml for (B).

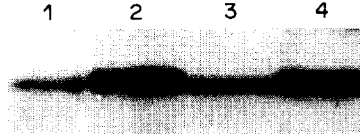

Fig. 4. The effect of EGF on progesterone receptor phosphorylation. T47D clone 7 cells were cultured to confluency in RPMI medium supplemented with 10% FCS stripped of hormone by charcoal treatment. At confluency this medium was replaced by phosphate-free Dulbecco's modified Eagles medium (DMEM) (Gibco) supplemented with 25 μg/ml BSA for 2 h. Thereafter [^{32}P]orthophosphate was added to 250 μCi/ml. The cells were labeled for 4 h in the absence of hormone, with 10^{-8} M R5020, EGF (20 ng/ml) or with R5020 and EGF. After labeling, the cells were washed twice with phosphate buffered saline (PBS) containing 10 mM NaF and lysed in 50 mM Tris, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate (DOC), 0.1% sodium dodecyl sulphate (SDS), 50 mM NaF and protease inhibitors (pepstatin 0.625 μg/ml, PMSF 1 mM, leupeptin 0.05 mM, bacitracin 0.1 mg/ml, aprotinin 0.07 mg/ml) for 45 min at 4°C. The lysates were centrifuged 100,000 *g* at 4°C for 30 min and the resulting supernatants were incubated with anti-progesterone monoclonal antibody Let 126 [23] for 16 h at 4°C. The complexes were incubated with anti-mouse IgG for 4 h at 4°C and then purified by centrifugation for 30 min at 6900 *g* on 1 M sucrose cushion in PBS containing 1% Triton X-100, 0.5% DOC, 0.1% SDS. The pellet was washed twice with PBS containing detergents and once with PBS containing 10 mM NaF. The resulting pellet was boiled for 10 min in Laemmli Sample buffer and subjected to electrophoresis on a 9% polyacrylamide Laemmli gel. Untreated cells (lane 1), cells treated with 10^{-8} M R5020 (lane 2), with 20 ng/ml EGF (lane 3) and with R5020 and EGF (lane 4).

receptor in the T47D clone 7 cells after treatment with EGF in the absence and presence of R5020. EGF on its own did not influence the phosphorylation of the progesterone receptor [Fig. 4(A), compare lane 1 with 3], neither did this growth factor in the presence of R5020 alter the level of the ligand-dependent phosphorylation of the receptor (Fig. 4, compare lane 2 with 4). Thus a total increase in the steroid-induced phosphorylation of the progesterone receptor does not appear to be correlated with the enhancement of progestin response by EGF. This finding is consistent with the data of Rao *et al.*, 1987 [15], in which it was shown that EGF had no influence on the total ligand-dependent phosphorylation of the progesterone receptor. However these results together with our results do not rule out the possibility of minor changes in phosphorylation of the progesterone receptor which could only be observed by analyses of the phosphoamino acid or phosphopeptide composition of the progesterone receptor. Further experiments are therefore required to clarify this point.

To determine whether progestin-induced DNA binding activity of the progesterone receptor is altered by EGF treatment, we carried out gel-retardation experiments with extracts from T47D cells treated with EGF, R5020 or R5020 and EGF using a PRE oligonucleotide [7]. Treatment of the cells without hormone or with EGF alone did not confer DNA-binding activity to the receptor (Fig. 5, lanes 1 and 2). In agreement with published results, the receptor acquired the ability to bind DNA upon R5020 treatment (Ref. [1] and Fig. 5, lane 3). This DNA-binding activity was however not significantly altered by simultaneous treatment of the cells with R5020 and EGF (Fig. 5, compare lane 3 with 4). These results demonstrate that the effect of EGF on progestin response is not associated with a significant increase in binding of the receptor to DNA. That the receptor binding is specific for the PRE is demonstrated by the ability of 200-fold molar excess of specific but not non-specific oligonucleotides to compete for binding of the receptor to the labeled PRE (Fig. 5, lanes 11, 12 and 7,

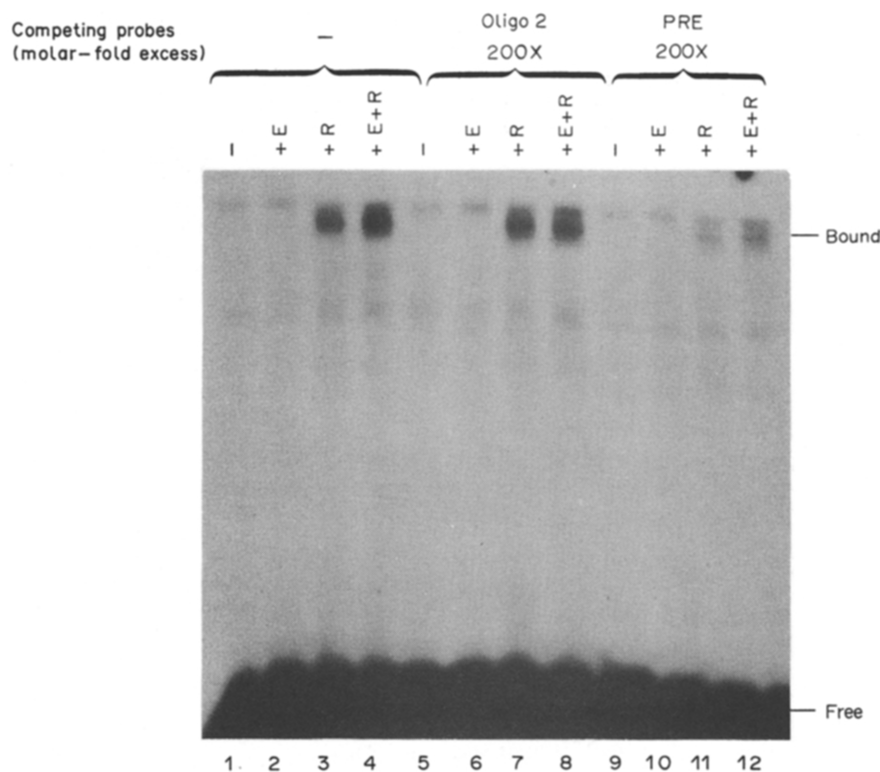

Fig. 5. The effect of EGF on progestin-response is not mediated by increased binding of the liganded progesterone receptor to DNA. Extracts from T47D clone 7 cells cultured in RPMI medium in the absence of FCS but treated with 10^{-8} M R5020 (+R), EGF (20 ng/ml) (+E) or EGF + R5020 (+E + R) for 2 h were prepared as described by Eul *et al.*, 1989 [24]. Gel-retardation experiments were carried out with the PRE sequence 5′AGCTTAGAACACAGTGTTCTCTAGAG3′ using the procedure of Eul *et al.*, 1989 [24].Competition experiments were carried out with a 200-fold molar excess of unlabeled PRE sequence or oligo 2 sequence (5′GATCCAGGGTTTAAATA3′).

8). Taken together, our results suggest that the EGF effect on progestin response may be due to alteration in function of factors other than the progesterone receptor itself.

In the course of our experiments we observed a loss of EGF effect after prolonged culturing of our T47D cells [Fig. 3(B)]. In such cells, the progestin response was much higher than the response in cells used in our initial studies [compare R5020 response of T47D clone 7 cells in Figs 3(A) and (B)] and was partially inhibited by tyrphostin even in the absence of added EGF [Fig. 3(B)]. Note that higher concentrations of tyrphostin are now required for the inhibition [Fig. 3(B)]. An EGF response could only be observed in these cells after a prior repression of the R5020 response by tyrphostin followed by treatment with higher concentrations of EGF (60 ng/ml) [Fig. 3(B)]. These results suggest that in such cell lines either the EGF receptor had already been activated even in the absence of added EGF or other pathways distal to the EGF receptor activation are already functional. Another possible explanation of this phenomenon is that mammary tumor cells secrete growth substances into their culture media [16, 17], some of which are related to EGF and TGFα, which could activate the EGF receptor even in the absence of added EGF. Alternatively, certain batches of fetal calf serum used in culturing these cells may have varying levels of EGF that could constitutively activate the EGF receptor. Our results that the progestin response in the altered cells is much higher than in the original cells treated with EGF indicate that constitutive production of EGF or TGFα may not be the only factors that influence the progestin response in the T47D cells. Further experiments are in progress to determine what other factors affect the response of this steroid hormone.

Taken together, our results indicate that the progestin response determined in some mammary tumor cells may already have been influenced by activation of the EGF receptor. It however remains to be established how the activation of this receptor leads mechanistically to increased progestin response. Further elucidation of this effect will greatly increase our understanding of the biologic behavior and/or pathogenesis of human breast cancers because of the prognostic value of the progesterone receptor and the EGF receptor homologue (HER2/neu) in human mammary cancers [18,19].

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