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INTERACTION OF ESTROGEN RECEPTOR COMPLEXES WITH THE PROMOTER REGION OF GENES THAT ARE NEGATIVELY REGULATED BY ESTROGENS: THE α_{2u}-GLOBULINS

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Since estrogens strongly suppress the expression of α_{2u}-globulin genes in the rat liver, we studied the binding of estrogen-receptor complexes to fragments derived from α_{2u}-globulin gene RAO 01 using a DNA-cellulose competition assay. Rat uterus cytosol labelled with [^{3}H]estradiol was used as a source of the estrogen receptor. As a positive control in these experiments we used an oligonucleotide containing the estrogen response element (ERE) cloned into pUC18. Our experiments indicate that estrogen-receptor complexes bind specifically to the ERE and to a fragment of RAO 01 located in the 5'-upstream region (bp -606 up to -575). This fragment is conserved among other members of this gene family. This is the first time that in vitro estrogen receptor binding is observed to gene fragments derived from a gene that is repressed by this steroid in vivo. © 1992 Academic Press, Inc.

Steroid hormones regulate a variety of developmental and physiological responses in a wide variety of organisms. Their action is mediated by steroid-receptor complexes which interact with specific DNA sequences, termed hormone response elements (HREs), located mostly near or inside the regulated genes (1-8). Mostly these HRE sequences exert stimulatory effects on gene expression. In several cases, however, steroid hormones repress genes at the transcriptional level. Most of the systems for which data are available concern glucocorticoid-repressed genes (9,10).

Since the hepatic expression of the α_{2u}-globulin genes is strongly inhibited by estrogens, we have searched for a possible negative estrogen response element (nERE) in the 5'-upstream region of these genes.

The α_{2u}-globulins are closely related proteins encoded by a 20-odd gene family. They are secreted mainly by the male rat liver and in small amounts by other tissues (11-16).

Female rats do not produce α_{2u}-globulins in the liver and no α_{2u}-globulin mRNA could be detected in the livers of these animals. Androgen treatment of

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ovariectomized female rats induces the α_{2u}-globulins and the corresponding mRNA (17). Estrogens have a strong inhibitory effect on the expression of α_{2u}-globulin as can be seen in adult male rats where, after administration of oestradiol 17ß, the expression of the protein is fully repressed (18). It is unknown whether the effects of estrogens on the repression of this protein are the result of a direct interaction of estrogen-receptor complexes with the corresponding genes or whether other mechanisms are involved. Recently we isolated and characterized several members of the α_{2u}-globulin gene family and we demonstrated specific binding of glucocorticoid- and androgen-receptor complexes to 5'-upstream fragments of some of these genes (19-21). In the present paper we studied the binding of estrogen-receptor complexes to one member of the α_{2u}-globulin gene family.

MATERIALS AND METHODS

Materials

[^{3}H]17ß-estradiol (93 Ci/mmol) was obtained from NEN Dupont. Estradiol and calf thymus DNA from Serva and α-mono thioglycerol from Sigma. Cellulose (Cellex 410) was purschased from Bio-Rad. Enzymes were from Boehringer Mannheim. Norit A from ICN Pharmaceuticals Holland B.V. Dextran T70 from Pharmacia. Other chemicals were reagent grade. Oligonucleotides were prepared using a Cyclone DNA synthesizer (New Brunswick).

Preparation of estrogen receptor

Nine week old female Wistar rats were killed and their uteri were immediately frozen in liquid nitrogen. Routinely 500 mg of frozen tissue was pulverized in liquid nitrogen and homogenized in 2 ml PETG buffer (10 mM phosphate pH 7.5, 1.5 mM EDTA, 10 mM α-mono-thioglycerol and 10 % glycerol). The homogenate was centrifuged at 30,000 x g in a refrigerated Sorvall RC-2B centrifuge for 1 hour at 4 °C. The supernatant was recovered avoiding the lipid layer on the surface and [^{3}H]estradiol was added to a final concentration of 10 nM. After overnight incubation at 0 °C, free steroids were separated by treatment with dextran-coated charcoal (Norit A , 10 mg/ml; Dextran T70, 1 mg/ml PEG buffer). The cytosol was then used as such in the DNA-cellulose competition assay.

Preparation of DNA fragments

The source of the DNA fragments used in the DNA-cellulose competition assay has been reported previously (21).
A positive control for the assay was constructed by cloning an oligonucleotide, containing the estrogen responsive element, in the *Bam*HI site of pUC18. The oligonucleotde was constructed according to Klein-Hitpass *et al.* (22). The sequence of the oligonucleotide is:

5' G-A-T-C-T-A-G-G-T-C-A-C-A-G-T-G-A-C-C-T-A 3'
3' A-T-C-C-A-G-T-G-T-C-A-C-T-G-G-A-T-C-T-A-G 5'

A *Pvu*II digest of pUC18 resulted in a fragment of 322 bp which served as a negative control. The same digest of a clone containing the ERE resulted in a fragment of 343 bp which served as the positive control.
Other double stranded oligonucleotides used in the DNA-cellulose binding assay can be summarized as follows (only the coding strand is shown and lower case letters are parts of endonuclease cleavage sites):

A: (RAP 01) -511 tcgAGAAACTGACTCCTCC -496
B: (RAO 01) -502 tcgAGAATCTGACTCCTTC -487
C: (RAP 01) -499 gatCTCCTTTTGACCTCCTTCT -481
D: (RAO 01) -490 gatCTTCTTTCACATCCTTCT -473
E: (RAP 01 or RAO 01) -603 gatCCAAAAGAGGGTCATTTCCTGTGACTGGAG -574

DNA-cellulose competition assay

The assay was performed essentially as described previously (21), but all incubations were allowed to proceed at room temperature for 90 min. The incubation mixture contained : 1 μl DNA cellulose (corresponding to 3.4 μg DNA), 20-200 ng of competing DNA fragments and 20 μl [^{3}H]estradiol-receptor complex in a final volume of 300 μl incubation medium (PETG buffer + 50 mM NaCl). Washing and counting was performed as described previously.

RESULTS

Validation of the DNA-cellulose competition assay

A series of preliminary experiments were performed to delineate optimal conditions for the study of estrogen-receptor DNA interactions. The main results are summarized in Fig. 1. Panel A shows that calf thymus DNA displaces estrogen-receptor complexes from DNA-cellulose in a dose-dependent way. 50 % displacement is observed with 2.5 μg calf thymus DNA. 10 μg reduces the binding to approximately 20 % of the control value observed in the absence of added DNA. This concentration of calf thymus DNA was used to estimate 'non-specific' binding.

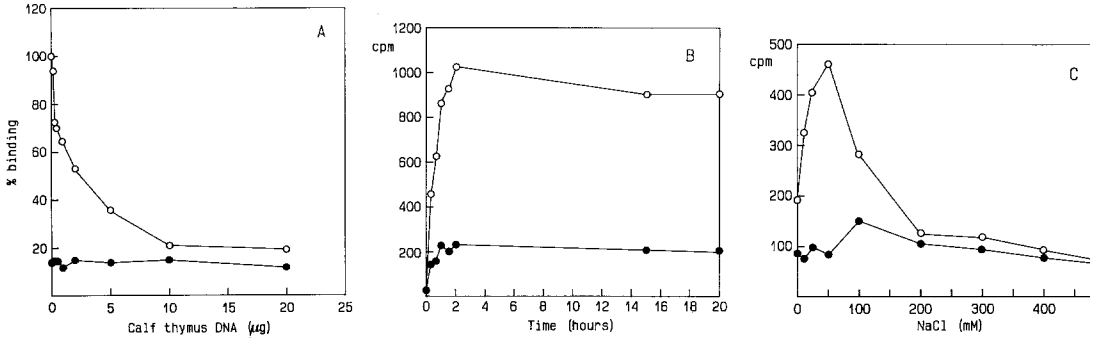

Figure 1. Validation of the DNA-cellulose competition assay. Panel A: Binding of [^{3}H]estradiol-receptor complex to cellulose (●) or DNA-cellulose (○) in the presence of increasing concentrations of competing sonicated calf thymus DNA. Samples were incubated for 90 min at room temperature in the incubation mixture described in Materials and Methods. Panel B: Binding of [^{3}H]estradiol-receptor complex to DNA-cellulose as a function of time. Samples were incubated as described in panel A but the incubation time was varied from 0 min up to 20 h. Measurements were performed in the presence (●) and in the absense (○) of 10 μg sonicated calf thymus DNA. Panel C: Influence of the concentration of NaCl in the incubation mixture on the binding of [^{3}H]estradiol-receptor complex to DNA-cellulose. Samples were incubated as described in panel A but the concentration of NaCl was varied from 0 up to 500 mM. Measurements were performed in the presence (●) and in the absence (○) of 10 μg sonicated calf thymus DNA. All points represent the mean of incubations performed in duplicate.

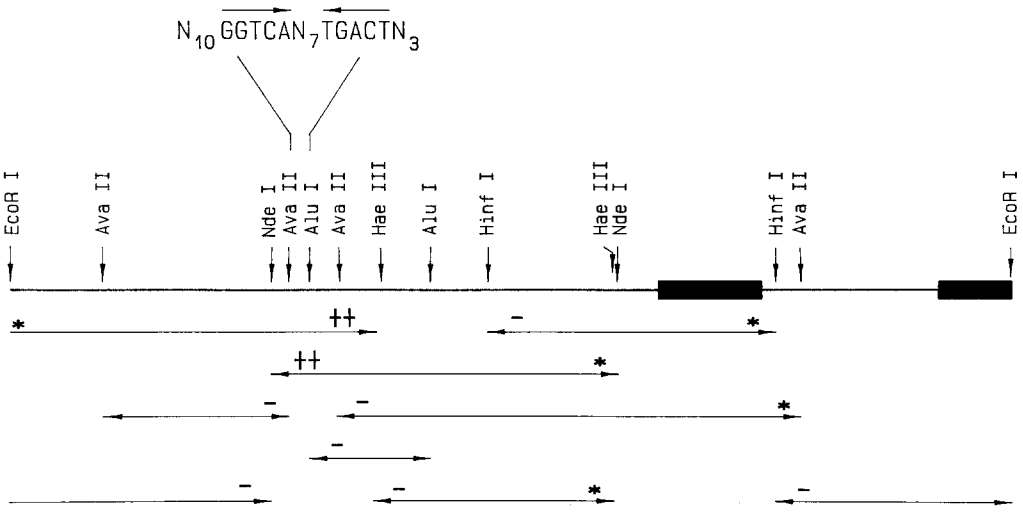

Figure 2. Localization of estrogen receptor binding along the RAO 01 gene. Black boxes correspond to exons and horizontal arrows to the restriction fragments studied. The fragments indicated by an * are presented in Fig. 3. The two fragments, which display higher affinity for the [^{3}H]estradiol-receptor complex, are indicated by ++. Two ERE-like sequences are indicated on top of the Figure.

A typical time-course experiment (panel B) illustrates that displaceable binding increases rapidly during the first hour of incubation and approaches a maximum after approximately 90 min. This was the incubation time used in all further experiments. Remarkably, after 20 hours of incubation at room temperature the estrogen-receptor DNA complex is still intact. A dose response curve with increasing amounts of cytosol showed that in our system 20 μl gave an optimal difference between total binding and non-specific binding (data not shown). Panel C shows the influence of the ionic strength of the incubation mixture. Total binding of estrogen-receptor complexes to DNA-cellulose increases up to 50 mM NaCl. From 100 mM NaCl on, the total binding decreases rapidly to reach the level of non-specific binding at 200 mM NaCl. Accordingly, 50 mM was chosen as the optimal ionic strength for all further experiments.

Comparison of the binding of estrogen-receptor complexes to gene fragments from α_{2u}-globulin and to a fragment containing the ERE of the vitellogenin A2 gene.

Our major objective was to study the binding of estrogen-receptor complexes to α_{2u}-globulin gene fragments derived from clone RAO 01 (19,21). The fragments studied covered the region between bp -1043 and bp +571. The exact localization of the studied fragments of clone RAO 01 are indicated in the lower part of Fig. 2. Some representative binding experiments are shown in Fig. 3.

In all experiments performed, the positive control, containing one copy of the ERE oligonucleotide, showed at least 10 times higher binding affinity for the estrogen receptor than the fragment of pGV451 (21) or the fragment of pUC18 (see

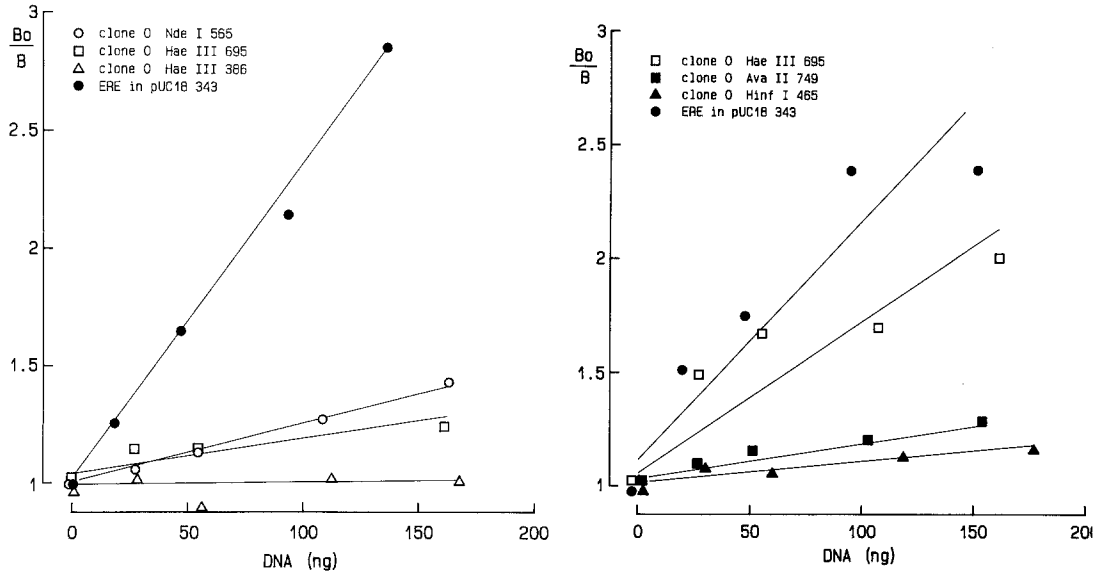

Figure 3. Binding of [^{3}H]estradiol-receptor complex to fragments from α_{2u}-globulin gene RAO 01. The value Bo/B was obtained by dividing the amount of [^{3}H]estradiol-receptor complex bound to DNA-cellulose in the absence of competing DNA (Bo) by the amount bound in the presence of competing DNA (B). Both Bo and B were corrected for 'non-specific' binding by subtracting the amount of radioactivity bound in the presence of 10 μg sonicated calf thymus DNA. Bo was calculated from incubations performed in quadruplicate. All other points represent the mean from incubations performed in duplicate. The exact localization of the fragments from RAO 01 is as follows. Left panel: (○) bp -73 to bp -638; (□) bp -463 to bp -1043 + 115 bp of the vector; (△) bp -77 to bp -463. Right panel: (□) bp -463 to bp -1043 + 115 bp of the vector; (■) bp +223 to bp -527; (▲) bp +184 to bp -282. The slope of each line is proportional to the affinity of the corresponding fragment for the estrogen receptor. All slopes are calculated by linear regression.

Materials and Methods) which were used as negative controls. Clone RAO 01 contains one region with an affinity for the estrogen receptor which is significantly higher than that of the fragments used as negative control (Fig. 2 and Fig. 3). Specific binding was observed with two overlapping fragments (*Nde*I, 565 bp and *Hae*III, 695 bp), whereas all the other fragments tested were negative. Taking into account that the *Ava*II (between bp -907 and -606) and the *Alu*I (between bp -575 and -376) fragments were negative, the region which displays binding can tentatively be localized between bp -606 and bp -575. To be sure that this 32 bp fragment was indeed the binding site for the estrogen receptor complex, we tested a double stranded oligonucleotide containing 30 bp of this fragment in our competition assay (oligonucleotide E). A double stranded oligonucleotide containing the ERE consensus sequence was used as a positive control in these experiments and several double stranded oligonucleotides of both clone RAO 01 and clone RAP 01 were used as negative controls (oligonucleotides A to D). Fig. 4 summarizes the results of DNA-cellulose competition assays with these double stranded oligonucleotides. Again, the ERE oligonucleotide displayed a high affinity for the estrogen receptor in comparison with oligonucleotide D (corresponding to bp

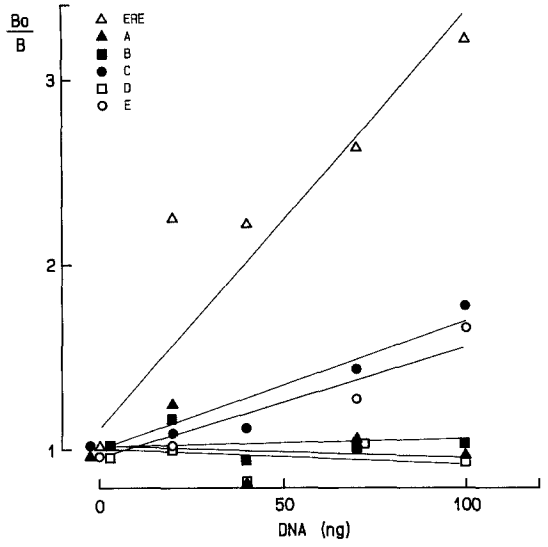

Figure 4. Binding of [^{3}H]estradiol-receptor complex to oligonucleotides derived from α_{2u}-globulin gene RAO 01 and RAP 01. Calculation was performed as described in Fig. 3. The exact localization of the oligonucleotides is as described in Materials and Methods.

-490 to -473 of clone RAO 01). Oligonucleotide E displayed an affinity similar to what we have seen for the two overlapping fragments (Fig. 3). This oligonucleotide contains a partially palindromic sequence which resembles the ERE but the two half sites are separtated by 7 instead of 3 nucleotides (see discussion). Interestingly, oligonucleotide C (RAP 01), which is the homologue of oligonucleotide D (RAO 01), also displays affinity for the estrogen receptor. As can be seen in Methods, this oligonucleotide contains a perfect half site (TGACC) of the ERE. In clone RAO 01, this sequence is mutated (C-ACA) and the *Alu*I fragment comprising this oligonucleotide does not bind the estrogen receptor. Two other oligonucleotides (A for RAP 01 and B for RAO 01; see Fig. 4 and Methods) also did not show any affinity for the estrogen receptor.

DISCUSSION

These data show that the estrogen-receptor binds specifically to RAO 01, a member of the α_{2u}-globulin gene family, *in vitro*. A crude receptor preparation was used in the present experiments and under these conditions the observed binding affinity is relatively low. Nonetheless, the validity of our observations is supported by a positive and several negative controls. A fragment of pUC18 which contains a classical ERE consistently displays high affinity for the estrogen-receptor whereas the same fragment without the ERE and several other fragments derived from plasmids or α_{2u}-globulin genes do not bind the estrogen receptor. Taking into account the overlaps observed between receptor binding fragments in the

competition assays, a 32 bp region could be delineated as the most likely site of interaction of RAO 01 with the estrogen receptor. This region contains an imperfectly palindromic sequence resembling the ERE but with 7 instead of 3 bp interspersed between the two half sites. Moreover, it is completely conserved between RAO 01 and RAP 01, another member of the α_{2u}-globulin gene family. An oligonucleotide (oligonucleotide E) containing this sequence also interacts with the estrogen receptor. The only other oligonucleotides which reacted positively in the DNA cellulose competition assay were the ERE itself and, remarkably, an oligonucleotide derived from RAP 01 which contains a perfect half site of the ERE (oligonucleotide C). The affinity of oligonucleotides C and E for the estrogen receptor is comparable but is again markedly lower than that of an oligonucleotide containing the classical ERE. It is conceivable that both oligonucleotides bind only receptor monomers whereas the perfect ERE binds a receptor dimer. Transfection expreriments will have to be performed to evaluate the functional significance of the observed estrogen receptor binding sites in the negative regulation of α_{2u}-globulin genes by estrogens. Up to now such studies for α_{2u}-globulin genes have only been successful for glucocorticoid induction (23) It may be mentioned that the GGTCA half palindromic element of the chicken ovalbumin gene promoter can bind the DNA binding domain of the human estrogen receptor *in vitro* (24). To be active in vivo, however, this ERE requires cell-specific factors since only chicken embryonic fibroblasts and not HeLa cells are functional. A similar need for cell-specific protein-protein interactions has been reported for the estrogen receptor mediated inhibition of CAT activity observed in A431 cells (25). Our data suggest that in the α_{2u}-globulin gene, estrogen receptor can interact at least in vitro with a non-canonical ERE. Whether this binding plays a role in the negative regulation of this gene and whether other cell specific factors are involved remains to be defined. Preliminary DNAseI protection experiments with rat liver nuclear extracts, however, show a clear footprint in the region overlapping this non-canonical ERE.

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REFERENCES

1. Bailly, A., Atger, M., Atger, P., Cerbon, M.A., Alizon, M., Vu Haï, M.T., Logeat, F. and Milgrom (1983) J. Biol. Chem. 258, 10384-10389.
2. Chandler, V.L., Maler, B.A. and Yamamoto, K.R. (1983) Cell 33, 489-499.
3. Scheidereit, C., Geisse, S., Westphal, H.M. and Beato, M. (1983) Nature 304, 749-752.

4. Dean, D.C., Gope, R., Knoll, B.J., Riser, M.E. and O'Malley, B.W. (1984) J. Biol. Chem. 259, 9967-9970.
5. Karin, M., Haslinger, A., Holtgreve, H., Richards, R.I., Krauter, P., Westphal, H.M. and Beato, M. (1984) Nature 308, 513-519.
6. Renkawitz, R., Schütz, G., von der Ahe, D. and Beato, M. (1984) Cell 37, 503-510.
7. Von der Ahe, D., Janich, S., Scheidereit, C., Renkawitz, R., Schütz, G. and Beato, M. (1985) Nature 313, 706-709.
8. Cato, A.C.B., Miksicek, R., Schütz, G., Arnemann, J. and Beato, M. (1986) EMBO J. 5, 2237-2240.
9. Beato, M. (1989) Cell 56, 335-344.
10. Levine, M. and Manley, J.L. (1989) Cell 59 405-408.
11. Roy, A.K., Chatterjee, B., Motwani, N.M., Demyan, W.F. and Nath, T.S. (1983) In Gene regulation by steroid hormones II (Eds Roy, A.K. and Clark, J.H.) Springer-Verlag New York Inc., NY, pp 299-310.
12. Kurtz, D.T. (1981) J. Mol. Appl. Genet. 1, 29-38.
13. Laperche, Y., Lynch, K.R., Dolan, K.P. and Feigelson, P. (1983) Cell 30, 453-460.
14. Vandoren, G., Mertens, B., Heyns, W., Van Baelen, H., Rombauts, W. and Verhoeven, G. (1983) Eur. J. Biochem. 134, 175-181.
15. Gubitz, R.M., Lynch, K.R., Kulkarni, A.B., Dolan, K.P., Gresik, E.W., Hollander, P. and Feigelson, P. (1984) J. Biol. Chem. 259, 12803-12809.
16. MacInnes, J.I., Nozik, E.S. and Kurtz, D.T. (1986) Mol. Cell. Biol. 6, 3563-3567.
17. Sippel, A.E., Feigelson, P. and Roy, A.K. (1975) Biochemistry 14, 825-829.
18. Roy, A.K. and Dowbenko, D.J. (1977) Biochemistry 16, 3918-3922.
19. Winderickx, J., Van Dijck, P., Dirckx, L., Volckaert, G. Rombauts, W., Heyns, W. and Verhoeven, G. (1987) Eur. J. Biochem. 165, 521-529.
20. Van Dijck, P., Lemaigre, F.P., Winderickx, J., Rousseau, G.G. and Verhoeven, G. (1987) Biochem. Biophys. Res. Commun. 149, 1099-1105.
21. Van Dijck, P., Winderickx, J., Heyns, W. and Verhoeven, G. (1989) Mol. Cell. Endocrinol. 64, 195-204.
22. Klein-Hitpass, L., Tsai, S.Y., Greene, G.L., Clark, J.H., Tsai, M.-J. and O'Malley, B.W. (1989) Mol. Cell. Biol. 9, 43-49.
23. Addison, W.R. and Kurtz, D.T. (1986) Mol. Cell. Biol. 6, 2334-2346.
24. Tora, L., Gronemeyer, H., Turcotte, B., Gaub, M.-P. and Chambon, P. (1988) Nature 333, 185-188.
25. Nag, A., Park, I., Krust, A. and Smith, R.G. (1990) Biochemistry 29, 2699-2702.