IN VITRO BINDING OF THE ESTROGEN RECEPTOR TO DNA: ABSENCE OF SATURATION AT EQUILIBRIUM

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1. Introduction

When estradiol (E_2) enters a target cell, it binds to its specific receptor (R) to form a complex (E_2R) which, more or less modified, is then translocated to the nucleus where it interacts with component(s) called nuclear 'acceptor', the biochemical nature of which has not yet been specified. DNA could be at least a part of this 'acceptor', since in vitro E_2R–DNA interactions have been shown by using ultracentrifugation in sucrose gradient [1], DNA cellulose chromatography [2] Sepharose [3], and DNA paper disk filtration [4]. However, the biological significance of these in vitro studies is questionable, since no straightforward DNA specificity has been found. Previous studies from this laboratory have shown that estrogens specifically favor the receptor DNA interaction [5] and that the partially proteolysed receptor obtained after Ca^{2+} or trypsin treatment has lost its ability to bind DNA [6]. These findings favor some relation with the in vivo estrogen induced translocation of the receptor to the nucleus. In addition, the saturation of DNA sites by the E_2R complex, has been reported [4], suggesting the presence of a limited number of high affinity DNA 'acceptor' sites for the receptor.

We have monitored a method allowing us to measure a sufficient number of DNA receptor complexes during the same experiment, in order to determine some binding characteristics of the estradiol receptor DNA interaction and particularly its reversibility and saturability*.

* Part of this work was presented at the IVth International Congress on Hormonal Steroids (September 1974, Mexico).

2. Material and methods

Calf uterine cytosol was prepared in Tris (10 mM) EDTA (1.5 mM) HCl pH 7.4 buffer as described previously [7] and labelled at 2°C for at least 90 min with 0.5 to 6 nM 6.7 ^{3}H estradiol (CEA, SA:48 to 60 Ci/mmole). It was thereafter incubated at 2°C or 25°C under agitation either with cellulose or with DNA cellulose powder prepared according to Alberts [8]. Generally 0.4 ml cytosol was incubated for 1 hr at 25°C with ≃ 10 μg of calf thymus DNA (Type I Sigma) adsorbed on 10 mg of cellulose Munktell No. 410. *E.coli* DNA prepared according to Marmur [9] was used in some experiments. The cellulose powder (± DNA) was washed with 10 ml of the homogenisation buffer, and then counted for radioactivity in an ethanol toluene scintillation mixture with a 25% efficiency according to external standardisation. The binding of E_2R to DNA was obtained by the difference between the radioactivity bound to DNA cellulose and to cellulose. Treatment of DNA cellulose by DNAse I (Worthington) abolished this difference. The receptor was also needed for the binding of ^{3}H estradiol to DNA which was 98% decreased after inactivation of the receptor protein by heating at 60°C for 20 min or its blockage by a 100-fold excess of non-radioactive E_2. Moreover when increasing concentrations of ^{3}H estradiol were used, the binding of the hormone to DNA increased in parallel to its binding to the cytosol receptor, and a plateau for DNA binding was reached when the estrogen receptor was saturated. The number of estradiol binding sites in the cytosol before (total E_2R) or after (free E_2R) incubation with cellulose

(± DNA) was evaluated by charcoal assay [7]. The free E_2R complex at the end of incubation was generally obtained by the difference between the total and the DNA bound E_2R complexes. The validity of this evaluation was based on control experiments where the free E_2R was directly measured in the supernatant at the end of the incubation, suggesting that the proportion of a weaker affinity binding of E_2R to DNA was low. The amount of adsorbed DNA, which decreased following the 25°C incubation, was also checked at the end of the interaction. DNA was hydrolysed at 90°C for 30 min in 1 ml of perchloric acid 0.5 M [10]. After centrifugation 2 min × 800 g of the powder, the supernatant was assayed at 260 nm in a Gilford spectrometer (1 A_{260} = 33 μg DNA). The protein concentration was assayed by UV absorption at 280 nm and 260 nm.

3. Results

3.1. *Equilibrium and reversibility of the E_2R-DNA interaction*

When the E_2R complex present in a salt free cytosol (2 or 8 mg/ml protein) was incubated with DNA cellulose, its binding to DNA increased with time and reached a maximum after 24 hr at 2°C and one to two hours at 25°C (not shown). At this plateau level, at most, 50% of the E_2R was bound to DNA. The remaining unbound E_2R was however still able to interact with DNA in the same proportion, indicating that under these conditions, the totality of the cytosol E_2R could bind DNA but that the affinity or the number of DNA 'acceptor' sites for E_2R would be very low. When incubation was further prolonged, a decrease in DNA binding was noticed. This biphasic curve observed at 25°C suggested a composite activation–inactivation process of the receptor induced by heat treatment. In the presence of 0.12 M KCl, the maximum of binding was attained faster both at 25°C and 2°C, but represented at most 10% of the total receptor. These results agreed with a thermo and ionic dependent 'activation' of the receptor [11] favoring its binding to DNA. The binding reversibility was further proved by using chase experiment (fig.1). After incubation at 25°C of a large excess of soluble DNA with the already formed E_2R DNA cellulose complexes, the remaining

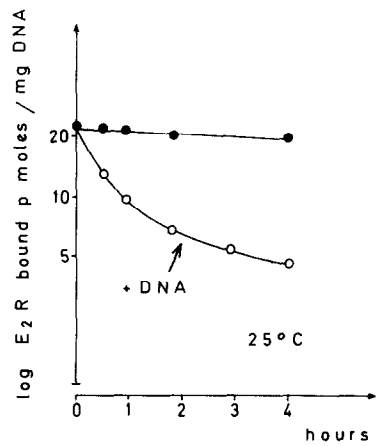

Fig.1. Chase experiment by soluble DNA at 25°C. Calf uterine cytosol (2 mg/ml protein) was incubated with ^3H E_2 5 nM for 90 mn at 0°C and then with the cellulose powder (± DNA) for 1 hr at 25°C. The particles of cellulose ± DNA were then washed as in method and then mixed under constant stirring at 25°C with 0.4 ml Tris–EDTA buffer containing ^3H E_2 0.5 nM in the presence (○) or absence (●) of a 50-fold excess of soluble DNA. After different periods of time, the powder was washed again by 10 ml of Tris–EDTA buffer and the E_2R complex still bound to adsorbed DNA was evaluated as in method.

E_2R bound to adsorbed DNA decreased in function of time. After the chase, the released estradiol was still bound to its receptor as tested by charcoal assay. This experiment indicated that E_2R was dissociated from the adsorbed DNA, its reassociation being prevented by the soluble DNA. The alternative according to which the adsorbed DNA would have been dissociated from the cellulose under these conditions was eliminated, since the soluble DNA did not modify the adsorbed DNA content and did not bind to cellulose under these conditions. The dissociation rate of the E_2R DNA complex did not obey a first order reaction suggesting that some other component (S) of the cytosol might interfere in the process of this dissociation.

3.2. *Absence of saturation of homologous and heterologous DNA by the E_2R complex*

Two kinds of experiments were performed:

(a) When the E_2R complex was raised in increasing concentrations of both E_2 and cytosol proteins, the binding of E_2R to DNA after 1 hr at 25°C seemed to

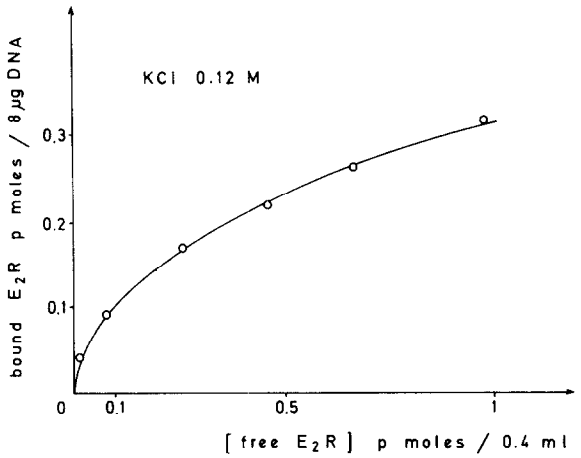

Fig.2. Apparent saturation of DNA by the E_2R complex (increasing E_2 and protein concentration). Calf uterine cytosol (2.5 mg/ml protein) containing 0.12 M KCl was incubated with ^3H E_2 6 nM for 3 hr at 2°C. Then it was diluted by Tris–EDTA KCl 0.12 M buffer to give 5 samples of increasing protein concentrations from 0.5 to 2.5 mg/ml. After incubation of these cytosol with the powder ± DNA for 1 hr at 25°C, the amount of the DNA bound and the free E_2R complexes were measured as in method.

be saturable (fig.2). From Scatchard plot, two kinds of binding sites were shown, one non-saturable and the other of limited binding capacity and of high affinity ($K_D \simeq 0.1$ nm). Similar results were also obtained by using sucrose gradient centrifugation in a 0.1 M KCl medium (J. André: unpublished), and DNA paper disk filtration [4].

(b) However, when the rise of E_2R complex was obtained by increasing only E_2 in a cytosol containing a constant protein concentration, the DNA binding was directly proportional to the unbound E_2R concentration, and no saturation could be observed. These results were obtained with or without 0.12 M KCl and by using calf thymus or *E.coli* DNA (fig.3a). The apparent better binding of E_2R to calf thymus than to *E.coli* DNA could not be ascertained, since the two kinds of DNA could be differently adsorbed on cellulose. Since the receptor was able to bind RNA in the absence of estrogen [5], it would have competed for DNA sites with the E_2R complex, mainly at low concentration of hormone. This would have given a sigmoid curve for the E_2R binding to DNA which was not observed. In addition, neither was the DNA saturation noticed when plotting the total R (filled and unfilled estrogen sites) bound to DNA versus the un-

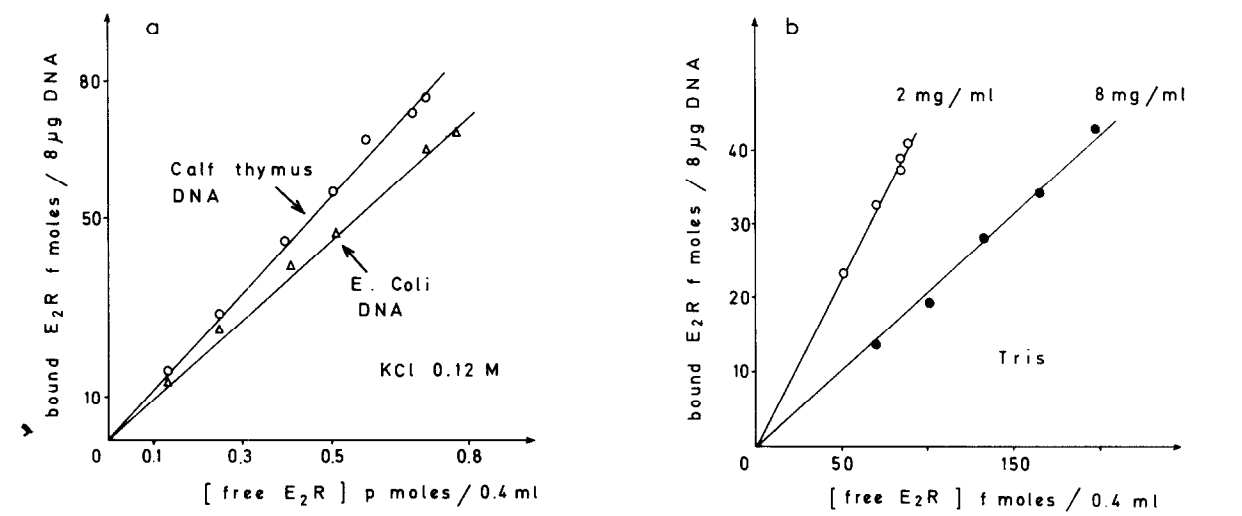

Fig.3. Absence of saturation of DNA by the E_2R complex (increasing only E_2 concentration). (a) Two kinds of DNA: Calf uterine cytosol (2.4 mg/ml protein) containing 0.12 M KCl was incubated for 4 hr at 2°C with increasing concentrations of ^3H estradiol (from 0.4 nM to 3.2 nM). Aliquots of each concentration were then stirred for one hour at 25°C with cellulose powder ± calf thymus (○) or *E.coli* (△) adsorbed DNA. (b) Two concentrations of proteins: A part of the calf uterine cytosol 8 mg/ml protein (●) was diluted with Tris–EDTA buffer to 2 mg/ml protein (○). Each cytosol was then incubated with 0.8 to 2.4 nM ^3H estradiol for 3 hr at 2°C. For each concentration, aliquots were thereafter stirred for 1 hr at 25°C with the powder (± DNA). In both cases the DNA bound and the free E_2R complexes were determined as in method.

bound E_2R complex, the unfilled estrogen receptor sites being labelled after the interaction with DNA. It was therefore concluded that the number of receptor binding sites on DNA was high enough to be non saturated under our in vitro conditions.

The pseudo saturation obtained by using increasing dilutions of cytosol was interpreted as secondary to an inhibitory effect of concentrated cytosol. In fact, when using two different dilutions of the same cytosol, which were then incubated with increasing amount of E_2 (fig.3b), the DNA binding observed under equilibrium conditions was proportional to the concentration of free E_2R in both cases, but was more effective in the diluted than in the concentrated cytosol. Whether cytosol component(s) decreased the R–DNA interaction in acting on DNA or on the receptor has not been determined.

4. Discussion

Using a technique which allowed us to measure rapidly and easily the interaction of estrogen receptor to DNA adsorbed on cellulose, the following observations have been made: (a) The E_2R–DNA interaction was reversible and reached an equilibrium more rapidly at 25°C than at 2°C. (b) Under equilibrium conditions, this interaction was not saturable when increasing the concentration of the E_2R complex, the ratio between the E_2R bound to DNA and the unbound E_2R being constant and at most equal to 1 for a 2 mg/ml cytosol protein. (c) An inhibitory effect of cytosol on the E_2R–DNA interaction was responsible for a pseudosaturation of DNA by the receptor when increasing concentrations of cytosol were used.

These results indicate that the interaction observed in vitro between the estrogen receptor and DNA, implies a great number of weak affinity DNA 'acceptor' sites. Recently, a similar conclusion was obtained for the cortisol receptor–DNA interaction [12]. Conversely, a limited number of high affinity DNA 'acceptor' sites have been reported for steroid receptor complexes on the basis of experiments performed in increasing the concentration of cytosol [4, 13, 14].

In order to assess the biological significance of the reported characteristics of the E_2R–DNA interaction, they can be compared with those described for systems more complex and closer to the in vivo conditions. In cell-free experiments, the binding of the E_2R complex to the nuclei or chromatin displayed similar time course, and nonsaturation of 'acceptor' sites [15–17]. Moreover, the degree of the nuclear transfer of the receptor induced by E_2 in the whole uterus [18] or by anti-estrogens in vivo [19] seems to be exclusively determined by the concentration of the cytosol receptor–ligand complex, and does not appear to be limited by any nuclear saturable process. In addition, the saturating level of DNA ($> 10^5$ 'acceptor' sites per cell nucleus), as indicated by our in vitro experiments, assuming one E_2 binding site per receptor and 6 pg DNA per cell, is superior to the maximum number of the estrogen receptor found in a living cell nucleus [20]. Thus the absence of a limited number of 'acceptor' sites under our in vitro conditions would agree with the in vivo situation. The characteristics of the in vitro interaction between steroid receptor and DNA, appear similar in many respects with the DNA binding of the cAMP–CAP protein complex observed in procaryotes [21]. However some very limited number of specific 'acceptor' sites on the DNA, which would be responsible for the regulation of a limited number of genes but would be unaccessible to our experimental assay, cannot be excluded.

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