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ESTROGEN RECEPTOR ALTERS THE TOPOLOGY OF PLASMID DNA CONTAINING ESTROGEN RESPONSIVE ELEMENTS

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Summary: We have recently used DNA containing estrogen responsive element (ERE) sequences for affinity purification to prepare calf uterine estrogen receptor (ER) at near homogeneity. The capacity of this purified ER to alter DNA topology upon binding was examined. Although the ER is not a topoisomerase, the presence of ER changes the distribution of topoisomers generated by incubation of plasmid DNA with excess wheat germ topoisomerase I. This effect is larger in plasmids containing a consensus ERE sequence. Two dimensional gel electrophoretic analysis suggested that interaction of ER and ERE causes negative supercoiling in regions of the plasmid accessible to topoisomerase I, resulting from overwinding of DNA contacting the ER. The extent of topological alteration was dependent on ER concentration. We suggest that the observed conformational changes in the DNA could have a role in regulation of transcription.

Interaction with estrogen transforms the estrogen receptor (ER) protein into a form capable of binding with high affinity to estrogen responsive element (ERE) sequences in the 5' regulatory regions of target genes (1). This binding interaction regulates the level of transcription (2-4). We have previously demonstrated high affinity binding of partially purified ER to plasmids containing the ERE consensus sequence (5). However, the mechanism by which the ER carries out its functions as a transcriptional regulatory factor have not been clarified. It is known that some DNA-binding proteins locally melt DNA (6,7), and change the topological conformation of DNA (8). Such an effect, if exhibited by ER, could be biologically relevant.

Investigation of topological effects of ER on DNA requires preparation of pure ER that retains the capacity to bind specifically to both hormone and ERE. The ERE affinity chromatography method developed in this laboratory (9) yields ER with these properties. Using this purified ER , we report here results demonstrating that the ER-ERE interaction introduces a conformational change into the plasmid. Although interpretation of these results must be tempered by the understanding that ER carries out its functions in association with other transcription factors (10,11), we suggest that changes in DNA conformation induced by ER binding could contribute to the regulatory function of the receptor.

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Abbreviations: ER,estrogen receptor; ERE, estrogen responsive element.

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Materials and Methods

Chemicals and enzymes - [2,4,6,7,16,17-[^{3}H]]Estradiol-17ß (141 Ci/mmol) was purchased from Amersham (Arlington Heights. IL). Wheat germ topoisomerase I was from Promega (Madison, WI). Polydeoxy(inosinate-cytidilate) (poly (dI·dC)) was from The Midland Certified Reagent Company (Midland, TX). Agarose NA was from Pharmacia (Uppsala, Sweden). *Hind*III digested lambda DNA (fragment sizes shown in the results are 23, 9.4, 6.4, 4.4, 2.3 and 2.0 kb pairs) was from New England Biolabs (Beverly,MA). Dithiothreitol and Tris (ultrapure) were from Boeringer Mannheim Biochemicals (Indianapolis,IN). Phenylmethylsulfonyl fluoride (PMSF), Nonidet P-40 (NP-40), estradiol-17ß, and chloroquine diphosphate were from Sigma Chemical Company (St Louis, MO). Carboxymethyl bovine serum albumin (BSA) was prepared as described (9). All other chemicals were reagent grade.

Plasmids - The pGEM-7Zf(+) plasmid (3000-bp) was purchased from Promega. The 38-bp ERE consensus sequence segment, containing the core consensus sequence 5'-GGTCAGAGTGACC-3', was prepared as described previously (5). The *Sma* I site of the pGEM-7Zf(+) plasmid was cut. Into this site was inserted either the 38-bp consensus ERE (producing plasmid Z16.F17 (3071-bp)), or two tandemly linked consensus EREs (plasmid Z16.F10 (3109-bp)), or eight tandemly linked consensus EREs (plasmid Z16.0121 (3337-bp)).

Purification of the estrogen receptor - The ER was purified from 40 g of calf uteri using the sequence specific DNA affinity column method described previously (9). Briefly, ER was partially purified from calf uteri by ammonium sulfate precipitation followed by heparin-agarose column chromatography (heparin-agarose purified ER). Further purification was carried out by DNA-affinity chromatography (DNA-affinity purified ER). Purity of heparin-agarose purified ER and DNA-affinity purified ER were 2% and 95%, respectively, based on densitometry of silver stained gels after sodium dodesyl sulfate (SDS) polyacrylamide gel electrophoresis. The concentrations of purified ER fractions were adjusted to approximately 1.1 nM ER in 40 mM Tris-HCl (pH 7.5), 0.5 mM PMSF, 1 mM dithiothreitol,100 mM KCl, 0.1 mM EDTA, 0.1% NP-40, 1 µg/ml poly(dI·dC), 100 µg/ml carboxymethyl BSA and 33% glycerol. The ER could be stored at -80 °C for 3 days without loss of topological activity.

Topoisomerase I relaxation assay - Zero to 278 µl of purified ER were preincubated with 10 µl of 100 nM plasmid DNA for 150 min at 4 °C . After preincubation, 0.5 U of wheat germ topoisomerase I and 5 mM NaCl were added, and the mixture was incubated for 30 min at 37 °C. The final volume was 300 µl and final concentrations of dimeric ER and plasmid DNA were 0-1.0 nM and 0.33 nM in polymer molecules, respectively. The reaction was stopped by adding EDTA and SDS to 16 mM and 0.16% final concentrations, respectively. DNA was precipitated with ethanol and resuspended in 80 mM Tris-phosphate (pH7.7) and 0.8 mM EDTA (TPE buffer) containing 10% sucrose. DNA samples were analyzed by agarose gel electrophoresis . The gels were subsequently stained with 2.5 µg/ml ethidium bromide, and then photographed using Polaroid 55 film.

Results

ER Binding Induces Topological Changes in DNA

The capacity of ER to induce topological changes in DNA was measured using a topoisomerase I relaxation assay. Negatively supercoiled plasmid DNA, when exposed to excess wheat germ topoisomerase I, is converted to a distribution of topoisomers that are centered near fully relaxed helical density. The basis of the assay is that if ER-ERE interaction shifts the superhelical density in the region of binding, a corresponding opposite shift will occur in the rest of the plasmid, assuming no single or double stranded DNA breakage occurs. When the ER-DNA complex is subsequently exposed to topoisomerase I, the topoisomerase will generate a relaxed population of DNA molecules still bound to ER. When the ER/DNA complex is subsequently dissociated by SDS, any local helical change stabilized by the ER will propagate to the rest of the plasmid. Comparison of the topoisomer distributions generated in the presence *versus* absence of ER, allows a quantitative estimation of the topological effects of ER binding.

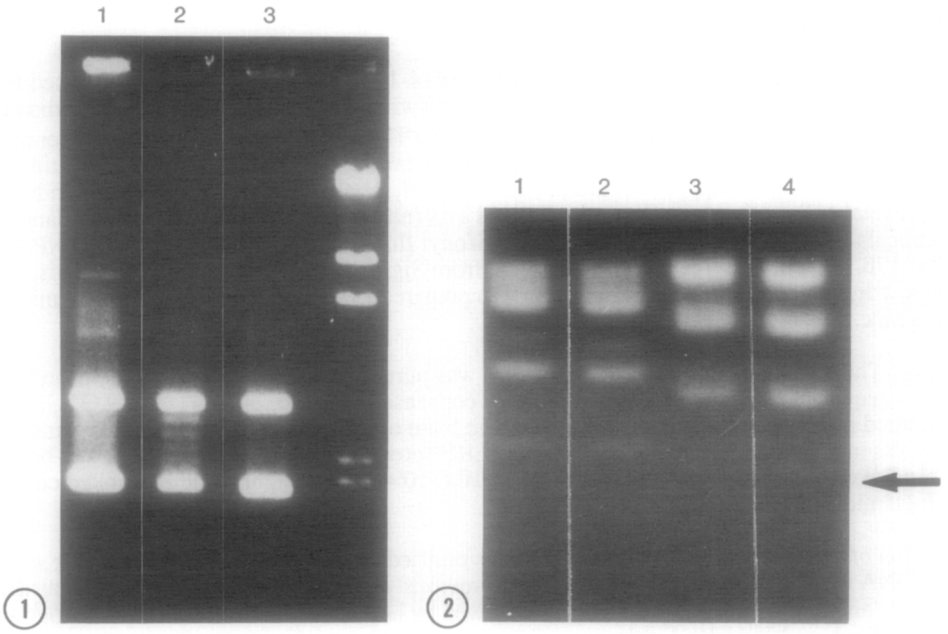

Figure 1. Topoisomerase activity of partially purified ER.
Plasmid Z16.0121 was incubated without ER (lane 1), with heparin-agarose purified ER (lane 2), or with DNA-affinity purified ER (lane 3), in the topoisomerase I relaxation assay. The samples were loaded onto a 0.8% agarose minigel. Electrophoresis was carried out at 75 mA for 2 h in TPE buffer. The gel was stained as described in the Materials and Methods section. *Hind* III digested lambda DNA fragments were used as markers.

Figure 2. Change in DNA conformation resulting from binding of ER.
Plasmids pGEM-7Zf(+) (lanes 1,2) and Z16.F17(lanes 3,4) were incubated with no ER (lanes 1 and 3) or 1.0 nM ER (lanes 2 and 4) in the topoisomerase I relaxation assay, and then subjected to electrophoresis in a 1% agarose gel. Electrophoresis was carried out at 50V for 20h in TPE buffer.The gel was stained as described in the Materials and Methods section.

Important considerations are that the ER preparation be free of any contaminating DNA binding proteins that could affect the topology of the plasmid DNA, or any topoisomerases that could shift the final distribution of topoisomers. The final ER preparation (95% pure), tested at concentrations used in subsequent assays, displayed no topoisomerase activity on plasmids containing EREs (Fig. 1 lane 3). Evidently, the ER has no inherent activity as a topoisomerase, and the preparation is free of detectable topoisomerase contamination. On the other hand, partially purified ER tested after the heparin-agarose chromatography step (2% pure) contained demonstrable topoisomerase activity, presumably as a contaminant (Fig. 1 lane 2). Therefore, to investigate whether ER caused topological effects, we employed the nearly homogeneous ER obtained after DNA affinity purification.

The presence of ER resulted in a shift in the topoisomer pattern of plasmids containing an ERE (Fig. 2 lane 4 arrow). However, ER had little effect on the topoisomer pattern of plasmids lacking EREs (Fig. 2 lane 2). ER that had been incubated at 70 °C for 30 min, caused no topological change in plasmids having or lacking EREs (data not shown). The change in topological state in the presence *versus* absence of EREs supports the conclusion that these effects are mediated by the ER and not by a contaminating DNA binding protein.

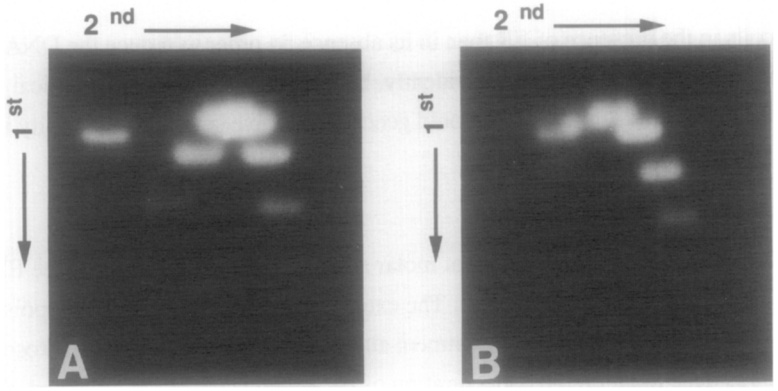

Figure 3. Two-dimensional gel electrophoresis of plasmid topoisomers.
Plasmid Z16.F17 was subjected to the topoisomerase I relaxation assay (in quadruplicate) in the absence or presence of ER. The 4 samples were then combined in one well to have sufficient DNA for two-dimensional gel electrophoresis. Electrophoresis in the first dimension was carried out in 1% agarose in TPE buffer at 50 V for 20 h at room temperature, and then the gel was soaked in TPE buffer containing 2.5 μg/ml of chloroquine diphosphate for 12 hr. Then electrophoresis in the second dimension was carried out in the latter medium at 50 V for 10 hr at room temperature. Results in the absence (A) and presence (B) of ER are shown. Relaxed DNA migrates slower than supercoiled DNA in the first dimension, whereas originally negatively-supercoiled, relaxed and positively-supercoiled DNA migrate at slow, intermediate and fast rates, respectively, in the second dimension.

The Direction of the Topological Change

The direction of the change in superhelicity induced by ER binding was determined by two dimensional electrophoresis of the products generated by topoisomerase I activity in the presence *versus* absence of ER (Fig. 3). ERE containing plasmids exposed to topoisomerase I in the absence of ER, displayed an array of topoisomers centered around the relaxed circular form, with some topoisomers having plus 1 or 2 and minus 1 or 2 supercoils visible (Fig. 3A). The presence of ER resulted in a shift of topoisomer distribution, to more positively supercoiled topoisomers (Fig. 3B). The topoisomerase removed more

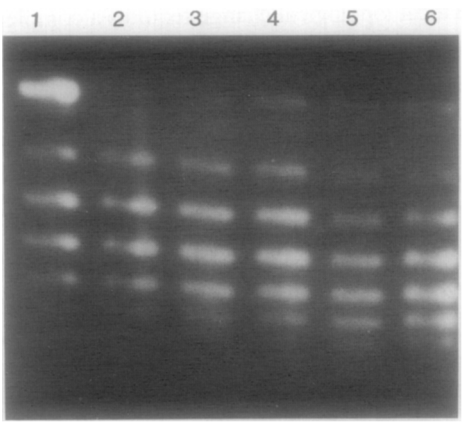

Figure 4. Dependence of conformational change on ER concentration.
Plasmid Z16.F10 was incubated with various concentration of ER in the topoisomerase I relaxation assay. Reactions contained either no ER (lane 1), 0.13 nM ER (lanes 2), 0.25 nM ER (lane 3), 0.38 nM ER (lane 4), 0.5 nM ER (lane 5) or 1.0 nM ER (lane 6), and were analyzed by electrophoresis in a 1% agarose gel containing 2.5 μg/ml chloroquine diphosphate. Chloroquine diphosphate was used to maximize the separation of each band.

negative supercoils in the presence of ER than in its absence, in order to reduce the DNA to a distribution around the fully relaxed state. Evidently, by increasing positive superhelical density in the region where it contacted the DNA, the ER could generate negative superhelical density in areas of the plasmid accessible to topoisomerase I.

Effects of Varying ER Concentration

ER was preincubated with plasmid Z16.F10 at molar ratios ranging from zero to three ER/DNA polymer prior to the topoisomerase I reaction. The extent of change induced in the topoisomer distribution observed after topoisomerase treatment and chloroquine diphosphate agarose gel electrophoresis was dependent on the concentration of ER added (Fig. 4). As expected, the increase in supercoiling approached a plateau value as the amount of ER was increased. This maximum effect of ER represents the sum of specific interaction with EREs and nonspecific interaction with other regions of the plasmid DNA.

Discussion

We have demonstrated that the binding of purified calf uterine ER to DNA causes a change in the topology of the DNA. ER purified to near homogeneity was previously shown to bind with high affinity to plasmid DNAs containing a 38-nucleotide long ERE consensus sequence (9). ER binding affinity and capacity was reduced for plasmids containing EREs with nucleotide changes in the inverted repeat region of the consensus sequence (12). Even lower ER binding was observed to plasmids lacking an ERE (5). Similarly, the extent of observed topological change in the presence of ER was greater for plasmids having *versus* lacking a consensus ERE. This relationship between topological change and ER binding affinity verifies that the observed topological effects are caused by the ER and not by an unknown DNA binding protein contaminant. The topological change was ER concentration-dependent (Fig.4).

Our results indicate that the binding of ER to an ERE site induces a local conformational change in the positively supercoiled direction. Because the ER does not appear to induce single or double strand DNA breaks (Fig.1), the local topological change requires a complementary change in the negative supercoiled direction throughout the remainder of the plasmid. When topoisomerase I reacts with the complex, this increased negative supercoiling is removed, but the local positive supercoiling, stabilized by the ER, is retained. When the ER/DNA complex is then denatured prior to gel analysis, the local positive supercoiling is distributed throughout the plasmid (Fig. 3).

Other transcription factors, DNA binding proteins, and RNA polymerases have been reported to unwind DNA (13,14). The binding of ER also was reported to result in transitory separation of the strands of the ERE (15). However, DNA strand separation would not show the same directional change as the results shown here. Our results are consistent with the hypothesis that the DNA wraps around the ER in a toroidal coil, a process that could generate positive supercoil stress (13,14). This type of binding mode, although with a winding direction that generates negative supercoil stress, has been reported for the transcription factor TFIIIA (16-18). One alternative possibility is that the ER binds double stranded DNA in two separate locations forming a loop. The appropriate loop conformation could also generate positive supercoil stress between the points of protein DNA contact. The known ERE core consensus binding site (13-bp) is probably too short to sustain the observed conformational

changes as a direct helix distortion resulting from forced addition of coils. Furthermore, for binding of the glucocorticoid receptor to the glucocorticoid responsive element, recent models do not appear to require significant local distortion of the DNA helix (19).

In this paper we demonstrate alterations in the conformation of DNA induced by the binding of ER. Although other proteins and complex mechanisms are involved in the initiation and regulation of transcription (20), the topological changes observed here could be a significant part of this process.

Acknowledgments

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