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ANALYSIS OF ESTROGEN RECEPTOR INTERACTION WITH TERTIARY-STRUCTURED ESTROGEN RESPONSIVE ELEMENTS

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Abstract—An initial crucial step in estrogen activation of gene expression is the interaction of the estrogen receptor with a specific nucleotide sequence [estrogen responsive element (ERE)]. Previously, we found that the estrogen receptor binds preferentially and with high affinity to the lower strand of the rat prolactin imperfect ERE which contains tertiary structure (Lannigan DA and Notides AC, *Proc Natl Acad Sci USA* **86**: 863–867, 1989). Using perfect and imperfect EREs from the upstream region of the chicken vitellogenin II gene, we have now extended our findings and have determined that the estrogen receptor preferentially interacts with either perfect or imperfect EREs which contain tertiary structure. A similar structure is present in a synthetic 42 bp oligonucleotide corresponding to the lower strand of a perfect ERE with flanking sequences from the rat prolactin ERE. Moreover, deviations from the ERE consensus sequence decrease the binding of the estrogen receptor to the tertiary-structured ERE. We also have determined that ERE flanking sequences contribute to the affinity of the receptor for the tertiary-structured ERE. Furthermore, ERE flanking sequences can influence the types of interactions that the estrogen receptor makes with the tertiary-structured ERE.

The estrogen receptor is an inducible transcription factor which depends on steroid hormone for its activation. The estrogen receptor binds to a specific nucleotide sequence termed the estrogen responsive element (ERE)§. A consensus ERE has been derived which is a perfect palindromic sequence consisting of a five base pair (bp) dyad separated by a three bp spacer [1–16]. This 13 bp sequence has been shown to confer estrogen responsiveness as well as specificity in a heterologous system [17–19]. A single base pair change destroys the ability of the 13 bp sequence to confer estrogen responsiveness [17–19]. However, perfect palindromic EREs appear to be the exception *in vivo* [1–5, 7–9, 11–13, 15]. Indeed, some DNA fragments which do not contain sequences that are highly homologous to the consensus ERE have been shown to confer estrogen responsiveness [1, 8]. These observations suggest that there appears to be a requirement for additional components besides homology to the ERE consensus sequence in order for imperfect palindromic EREs to confer estrogen responsiveness.

An attractive possibility as an additional component is that the tertiary structure of the ERE may play a role in the recognition of the estrogen receptor for its specific sequence. Tertiary structure is defined as any DNA conformation which is not B-form. Indeed, we have found that the estrogen receptor binds preferentially and with high affinity to the lower strand of the rat prolactin imperfect ERE which contains tertiary structure [20]. This high affinity site was obtained by heating the double-stranded ERE so that the strands separated, immediately followed by quick chilling, under dilute conditions, to favor intrastrand bonding. No binding could be detected to the heat/quick-chilled upper strand of the ERE, indicating the specificity of estrogen receptor binding. The estrogen receptor was able to bind double-stranded ERE but with a 60-fold lower affinity than that to the heat/quick-chilled ERE. The heat/quick-chill treatment of the ERE is thought to restore the native tertiary structure to the lower strand. We postulate that this native structure may either contain single-stranded regions or possess properties in common with single-stranded DNA, since the estrogen receptor has a higher affinity for single-stranded nonspecific DNA than for the double-stranded ERE [20].

However, there are still many questions concerning the involvement of ERE tertiary structure in forming the high affinity binding site for the estrogen receptor which we address in this paper. First, can an isolated synthetic oligonucleotide containing an ERE form a high affinity binding site for the estrogen receptor? Second, is the high affinity site for the estrogen receptor formed solely by the ERE or are flanking sequences involved? Third, is the involvement of tertiary structure in forming the high affinity binding site for the estrogen receptor unique to the rat prolactin imperfect ERE or a general mechanism for estrogen receptor recognition of perfect as well as other imperfect EREs?

We have determined that a synthetic oligonucleotide containing an ERE can form a high affinity binding site for the estrogen receptor. The

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§ Abbreviations: ERE, estrogen responsive element and cVTGII, chicken vitellogenin II.

deletion of sequences greater than 14 bp away from the ERE decreased the affinity of the estrogen receptor for the tertiary-structured ERE. The estrogen receptor preferentially interacted with both perfect and imperfect EREs which contain tertiary structure. Additionally, estrogen receptor binding to the tertiary-structured ERE was affected by deviations from the ERE consensus sequence. Moreover, flanking sequences can influence the types of interactions that the estrogen receptor makes with the tertiary-structured ERE.

MATERIALS AND METHODS

Purification of the calf uterine estrogen receptor

The estrogen receptor from calf uterus was purified to homogeneity as described [7, 21].

Isolation and ^{32}P-end-labeling of the ERE-containing fragments

The fragments containing the rat prolactin ERE were isolated by various restriction digestions of either pUC13 containing the upstream region between nucleotide −1956 to −1370 or pGEM3 containing the upstream region between nucleotide −1784 to −1530 of the rat prolactin gene [22, 23] (provided by Richard A. Maurer, University of Iowa). The chicken vitellogenin II (cVTGII) perfect ERE was isolated on a fragment obtained from a *Hgi* A1/*Mbo* II digestion of pGEM3 which contained a fragment obtained from a *Pst* I digestion of pTK.CAT containing the upstream region between nucleotide −1133 to −69 of the cVTGII gene [3] (provided by John B. E. Burch, Fox Chase Cancer Center). The cVTGII imperfect ERE was isolated on a fragment obtained from a *Dra* I/*Mbo* II digestion of pGEM3 which contained a fragment obtained by *Pst* I/*Bam* HI digestion of pTK.CAT containing the upstream region between nucleotide −1133 to −69 of the cVTGII gene.

The fragments were radiolabeled on the lower or upper strand by digestion with the appropriate restriction enzyme followed by dephosphorylation and subsequent rephosphorylation by [^{32}P]ATP and polynucleotide kinase. Subsequent digestion with a second appropriate restriction enzyme yielded the desired fragment. The desired radiolabeled fragment was then gel purified and electroeluted from the gel.

Competition experiments

The competition experiments were performed as previously described [20]. Briefly, competitors were added to the labeled fragments containing the rat prolactin ERE (1 fmol) and a 100-fold excess by weight of poly dIdC added either before (structured competitors) or after (double-stranded competitors) the heat/quick-chill procedure. Aliquots were then added to the estrogen receptor (final concentration 8.3 nM) and incubated for 90 min at 5°. The samples were then electrophoresed on either a 4% (for the *Dra* I to *Sau* 3A fragment) or a 5% gel (30:1) (for the *Nsi* I to *Sau* 3A fragment).

Oligonucleotide experiments

Two oligonucleotides containing either the sequence 5′AATTCTCTTCTGACCCCAAAGCACTCTAGGTCATAGTGACCAAAAT3′ or 5′ATTTTGGTCACTATGACCTAGAGTGCTTTGGGGTCAGAAGAG3′ were synthesized. (The ERE is underlined.) These oligonucleotides correspond to either the lower or upper strand of a perfect ERE with rat prolactin ERE flanking sequences. Four additional nucleotides were added onto the 5′ end of the lower oligonucleotide to facilitate subcloning. The oligonucleotides were either annealed and purified or used separately in the gel mobility assay [20] and run on a 5% gel (30:1).

Estrogen receptor titration

Purified estrogen receptor [106 nM estrogen receptor in TDK (Tris–HCl, pH 7.4, 1 mM dithiothreitol, 445 mM KCl, 10% (v/v) glycerol)] was initially diluted with TDK to 7.44 the final concentration desired. The receptor was then diluted to 1.11 the final concentration by addition of 60% Dignams' solution [24] without KCl containing 100 nM estradiol, insulin-chain A (0.03 ng/μL) and incubated for 30 min at 5°. The labeled DNA (2 fmole) was heat/quick-chilled with a 12,000-fold excess by weight of poly dIdC, added to the receptor and incubated for 90 min at 5°. The samples were then run on a pre-run gel as previously described [20] except that the *Nsi* I to *Sau* 3A fragment containing the rat prolactin ERE was run on a 5% gel.

RESULTS

Can an isolated synthetic oligonucleotide containing an ERE form a high affinity binding site for the estrogen receptor?

To rule out the possibility that the ERE high affinity site for the estrogen receptor was an artifact caused by either the heat/quick-chill procedure or the presence of the complementary strand, the gel mobility assay was performed with synthetic oligonucleotides. Two complementary oligonucleotides were synthesized which contained a perfect ERE with flanking sequences from the rat prolactin ERE. The oligonucleotides were radiolabeled and either annealed or used separately in the gel mobility assay. No retarded bands were observed upon incubation of the annealed oligonucleotides with purified estrogen receptor (14.3 nM) (Fig. 1). However, a retarded band was observed upon incubation of the oligonucleotide corresponding to the lower strand (Fig. 1) with purified estrogen receptor (14.3 nM). Heat/quick-chilling of the oligonucleotide did not increase the amount of receptor binding (Fig. 1). Thus, the estrogen receptor preferentially binds the ERE oligonucleotide rather than the double-stranded ERE. This preferential binding by the estrogen receptor to the ERE oligonucleotide occurs without either the heat/quick-chill treatment of the oligonucleotide or the presence of the complementary strand.

Is the high affinity site for the estrogen receptor solely formed by the ERE or are flanking sequences involved?

The minimum size of DNA containing an ERE

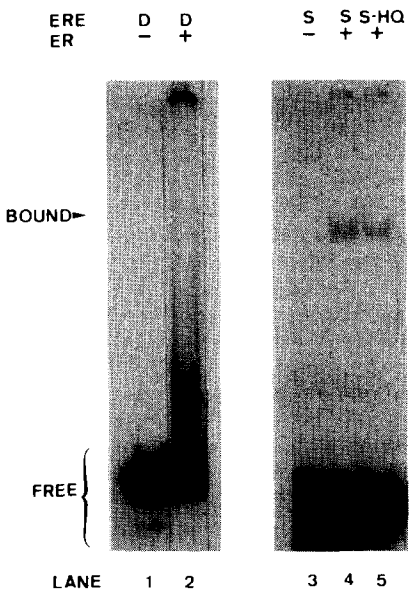

Fig. 1. Estrogen receptor (ER) binding to an ERE contained either on double-stranded DNA or on a synthetic oligonucleotide. Oligonucleotides containing a perfect ERE with ERE flanking sequences from the rat prolactin gene were synthesized. The oligonucleotides were either annealed and purified (D) (lanes 1 and 2) or the lower strand was used separately (S) with (lane 5) or without (lanes 3 and 4) the heat/quick-chill treatment. Estrogen receptor (14.3 nM) was then added (lanes 2, 4 and 5) or not (lanes 1 and 3).

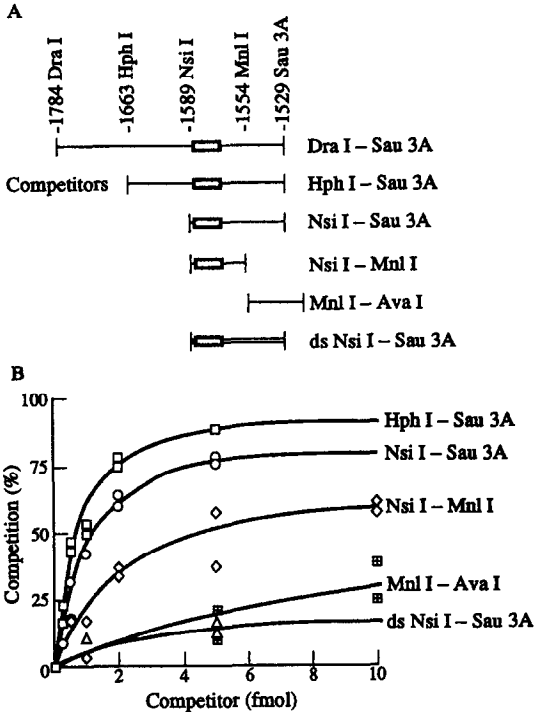

Fig. 2. Competition experiments. (A) Illustration of the restriction fragments from the upstream region of the rat prolaction gene which were used in the competition experiments. The open boxes indicate the location of the ERE on the restriction fragments. (B) Percent competition using the various restriction fragments to compete against the binding of the estrogen receptor to the heat/quick-chilled rat prolactin ERE contained either on the *Dra* I to *Sau* 3A (−1784 to −1529 or the *Nsi* I to *Sau* 3A (−1589 to −1529) restricting fragments. The heat/quick-chilled *Hph* I to *Sau* 3A (−1663 to −1529) and *Nsi* I to *Sau* 3A (−1589 to −1529) restriction fragments were used to compete against the binding of the estrogen receptor to the ERE contained on the heat/quick-chilled *Dra* I to *Sau* 3A (−1784 to −1529) restriction fragment. The heat/quick-chilled *Mnl* I to *Ava* I and the double-stranded (ds) and heat/quick-chilled *Nsi* I to *Mnl* I (−1589 to −1554) restriction fragments were used to compete against the binding of the estrogen receptor to the ERE contained on the heat/quick-chilled *Nsi* I to *Sau* 3A (−1589 to −1529) restriction fragment.

that could form the high affinity site of the receptor was determined by the relative affinity of the estrogen receptor for various sized DNA competitors (Fig. 2). Relative affinity is defined as the concentration of competitor that caused a 50% decrease in the intensity of radiolabeled ERE binding to the receptor. Estrogen receptor (14.3 nM) was incubated with labeled heat/quick-chilled restriction fragments from either −1784 to −1529 (*Dra* I to *Sau* 3A) or from −1589 to −1529 (*Nsi* I to *Sau* 3A) from the rat prolactin upstream region. These restriction fragments contained the ERE which is located from −1581 to −1569. The heat/quick-chilled restriction fragments from −1663 to −1529 (*Hph* I to *Sau* 3A) and from −1589 to −1529 (*Nsi* I to *Sau* 3A) were used to compete for binding to the −1784 to −1529 (*Dra* I to *Sau* 3A) fragment. The *Dra* I to *Sau* 3A restriction fragment contains two binding sites for the estrogen receptor [20]. However, only the relative affinity for the various competitors against the ERE which is located from −1581 to −1569 is shown since only this estrogen receptor binding site has been demonstrated to confer estrogen responsiveness *in vivo* [7]. The relative affinity of the estrogen receptor for the ERE contained on the *Nsi* I to *Sau* 3A fragment was only slightly lower compared to that obtained for the restriction fragment from *Hph* I to *Sau* 3A (Fig. 2B). The *Nsi* I to *Sau* 3A restriction fragment was subsequently used in the competition experiments with the heat/quick-chilled *Nsi* I to *Mnl* I (−1589 to −1554), the *Mnl* I to *Ava* I (−1554 to −1529) and the double-stranded *Nsi* I to *Sau* 3A restriction fragments. The estrogen receptor had a 6-fold lower affinity for the −1589 to −1554 (*Nsi* I to *Mnl* I) restriction fragment than for the −1589 to −1529 (*Nsi* I to *Sau* 3A) restriction fragment (Fig. 2B). This loss in affinity of the estrogen receptor for the ERE occurred by removal of sequences 14 bp away from the ERE. This result is in contrast to the observations with the fragments from −1663 to −1529 (*Hph* I to *Sau* 3A) and −1589 to −1554 (*Nsi* I to *Mnl* I) in which the affinity of the estrogen receptor for the ERE contained on these fragments was similar even

though in the −1589 to −1554 (*Nsi* I to *Mnl* I) fragment the ERE was only 9 nucleotides away from the 5′ end of the ERE on the lower strand. This result suggests that removal of sequences 14 bp away from the 3′ end of the ERE contributes to the affinity of the estrogen receptor for the structured ERE rather than simply being due to the closer position of the ERE to the end of the DNA fragment. The estrogen receptor had a much lower affinity for the −1554 to −1529 (*Mnl* I to *Ava* I) restriction fragment than for the −1589 to −1554 (*Nsi* I to *Mnl* I) restriction fragment. The *Mnl* I to *Ava* I fragment consisted of the rat prolactin upstream region from −1554 to −1529 plus an additional 5 bases from the pGEM 3 vector which makes this fragment 30 bases long. Thus although the −1589 to −1554 (*Nsi* I to *Mnl* I) and −1554 to −1529 (*Mnl* I to *Ava* I) fragments were similar in length, their abilities to compete for estrogen receptor binding to the *Nsi* I to *Sau* 3A fragment were very different. Since the *Mnl* I to *Ava* I fragment does not contain an ERE, this result indicates that the ERE is important for the estrogen receptor competition by the various heat/quick-chilled fragments. As previously observed [20], double-stranded EREs were weaker competitors than heat/quick-chilled EREs (Fig. 2B).

Additional evidence that flanking sequences play a role in forming the high affinity binding site for the estrogen receptor is indicated by observations that the preferential binding of the estrogen receptor for the lower strand of the rat prolactin ERE versus the upper strand diminished as the ERE containing fragment decreased in size (data not shown). However, the preference of the receptor for the heat/quick-chilled ERE versus a heat/quick-chilled nonspecific DNA was maintained (Fig. 2).

Is the binding mechanism of the estrogen receptor to the rat prolactin imperfect ERE anomalous or a general mechanism for estrogen receptor recognition of perfect as well as other imperfect EREs?

Estrogen receptor binding to perfect and imperfect EREs containing tertiary structure. We have shown previously that the estrogen receptor binds preferentially to the lower strand of the rat prolactin ERE which contains tertiary structure [20]. Therefore, we first wanted to determine if this binding preference is idiosyncratic to the rat prolactin ERE or represents a general mechanism for the interaction of the receptor with EREs. The upstream region of the cVTGII gene contains a perfect, GGTCANNNTGACC (−626 to −613), and an imperfect GGTCANNNTAACC (−348 to −335) ERE, either of which can independently confer estrogen responsiveness [3]. The binding preference of the estrogen receptor to restriction fragments containing either the cVTGII perfect or imperfect EREs was tested in gel mobility experiments analogous to those performed with the rat prolactin imperfect ERE [20]. When purified estrogen receptor (14.3 nM) was incubated with either the double-stranded cVTGII perfect or imperfect ERE, no retarded bands were detected under these conditions (Fig. 3). However, the purified estrogen receptor is capable of binding to double-stranded ERE [20]. The various restriction fragments were heated to allow strand separation followed by quick-chilling in conditions which favored intrastrand bonding, to allow the formation of tertiary structure at the ERE. The lower and upper strands for the perfect (Fig. 3) and imperfect (Fig. 3 and data not shown) cVTGII strands had separated since they had different mobilities in the gel system and, therefore, both ERE strands must contain tertiary structure (i.e. be in a non-B DNA conformation). Most importantly, when either the heat/quick-chilled perfect or imperfect EREs were incubated with purified estrogen receptor (14.3 nM), retarded bands appeared (Fig. 3). Since the estrogen receptor used in these studies was purified to homogeneity [7, 21] and the binding assays were performed with high concentrations of nonspecific DNA (see Materials and Methods), the retarded bands represent specific high affinity binding of the estrogen receptor to the various EREs. Additional evidence that the multiple bands all represent estrogen receptor interaction with the ERE is discussed below. These results indicate that the estrogen receptor preferentially binds EREs containing tertiary structure as opposed to the double-stranded ERE. Thus, the purified estrogen receptor interacts with the cVTGII perfect and imperfect ERE in a manner analogous to that with the rat prolactin ERE.

Analysis of the ratio of bound ERE over free ERE demonstrates that the estrogen receptor preferentially bound the perfect cVTGII ERE approximately 2-fold greater than it bound the imperfect cVTGII ERE (Fig. 3). This result

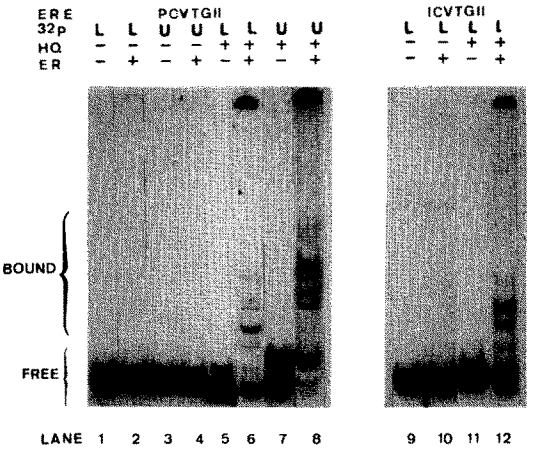

Fig. 3. Gel mobility pattern of estrogen receptor binding to either the double-stranded or heat/quick-chilled perfect and imperfect cVTGII ERE. The perfect (lanes 1–8) and imperfect (lanes 9–12) EREs from the cVTGII were ^{32}P-labeled on the indicated strand (lower L, upper U) and then were either kept double-stranded (lanes 1–4, 9 and 10) or were heat/quick-chilled (HQ) (lanes 5–8, 11 and 12). Estrogen receptor (14.3 nM) was either absent (lanes 1, 3, 5, 7, 9 and 11) or present (lanes 2, 4, 6, 8, 10 and 12). This figure comes from a collection of experiments. However, these experiments contained the appropriate double-stranded ERE to allow for normalization of the data.

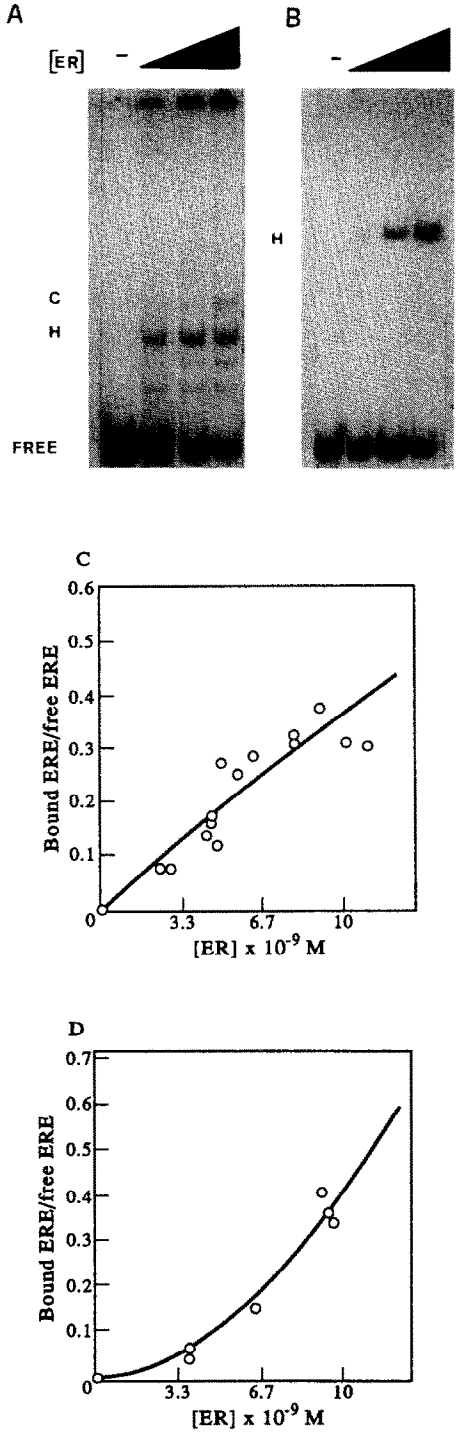

Fig. 4. Estrogen receptor titration of the heat/quick-chilled rat prolactin ERE contained on either the *Hph* I to *Sau* 3A or *Nsi* I to *Sau* 3A fragments. Increasing concentrations of estrogen receptor were added to the heat/quick-chilled rat prolactin ERE contained either on a (A) *Hph* I to *Sau* 3A or a (B) *Nsi* I to *Sau* 3A restriction fragment labeled on the lower strand and the gel mobility assay was performed. (C) The ratio of the bound ERE in an individual band over the total free ERE was plotted versus the [ER] from the bands marked H in panels A and B. These bands demonstrate the hyperbolic curve and are superimposable. The retarded bands just below H in panel A also demonstrated hyperbolic binding (data not shown). (D) The ratio of the bound ERE in an individual band over the total free ERE was plotted versus the [ER] from the band marked C in panel A. This band demonstrates the concave up curve.

demonstrates that deviations from the ERE consensus sequence influence the affinity of estrogen receptor binding to the structured ERE. Intuitively, this result is expected. Importantly, however, this result demonstrates the specificity of estrogen receptor interaction with tertiary-structured EREs.

Note that retarded bands are observed when either the radiolabeled lower or upper strand of the perfect cVTGII ERE was incubated with the estrogen receptor (Fig. 3). Curiously, the estrogen receptor demonstrated a 2-fold binding preference for the upper strand compared to the lower strand (data not shown). Since this ERE is a perfect palindrome, one would expect that the estrogen receptor would bind both strands with similar affinities. This observation supports the role of ERE flanking sequences in influencing estrogen receptor binding to the ERE as discussed above.

Estrogen receptor interactions with the rat prolactin and the cVTGII EREs. The rat prolactin ERE was isolated either on a restriction fragment from −1663 to −1529 (*Hph* I to *Sau* 3A) or a restriction fragment from −1589 to −1529 (*Nsi* I to *Sau* 3A). Each fragment was radiolabeled on the lower strand, heat/quick-chilled and titrated with purified receptor. Receptor titration with the *Hph* I to *Sau* 3A fragment resulted in multiple retarded bands on the autoradiogram (Fig. 4A) whereas receptor titration with the *Nsi* I to *Sau* 3A fragment resulted in only a single band on the autoradiogram (Fig. 4B). Confirmation that the retarded bands contained estrogen receptor was shown by western blot analysis which demonstrated that the band generated from the *Nsi* I to *Sau* 3A fragment contained estrogen receptor (data not shown). Additionally, competition experiments with the *Nsi* I to *Sau* 3A fragment could compete out the multiple bands which appeared with the *Hph* I to *Sau* 3A fragment (data not shown).

Increasing amounts of estrogen receptor were incubated with either the cVTGII perfect or imperfect ERE which were radiolabeled only on the lower strand. Multiple retarded bands appeared on the autoradiograms from the titration of the receptor with each of the heat/quick-chilled cVTGII EREs (Figs. 5 and 6).

Estrogen receptor titration to the various EREs produced similar patterns in the gel mobility assays (with the exclusion of the rat prolactin ERE contained on the *Nsi* I to *Sau* 3A restriction fragment) except that a broad band appeared in the pattern for the imperfect EREs which corresponded to a doublet for the perfect ERE (Figs. 4–6). The broad bands were assumed to consist of an unresolved doublet. The autoradiograms from the estrogen receptor titrations were scanned by laser densitometry and the ratios of the bound ERE over free ERE were plotted versus the total estrogen exceptor concentration for each of the bound bands (Figs. 4–6 and data not shown). Analysis of the resultant graphs revealed two types of binding. Each ERE (with the exclusion of the rat prolactin ERE

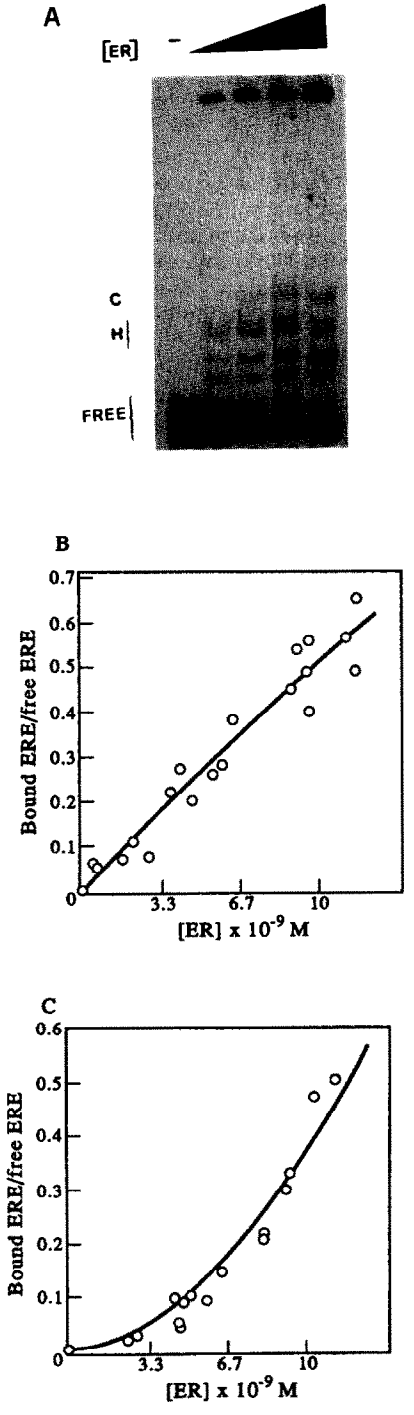

Fig. 5. Estrogen receptor titration of the heat/quick-chilled perfect cVTGII ERE. (A) Increasing concentrations of estrogen receptor were added to the heat/quick-chilled cVTGII perfect ERE labeled on the lower strand and the gel mobility assay was performed. (B) The ratio of the bound ERE in an individual band over the total free ERE was plotted versus the [ER] from the bands marked H in panel A. This band demonstrates the hyperbolic curve. The retarded bands just below H in panel A also demonstrated hyperbolic binding (data not shown). (C) The ratio of the bound ERE in an individual band over the total free ERE was plotted versus the [ER] (open circles) from the band marked C in panel A. This band demonstrates the concave up curve.

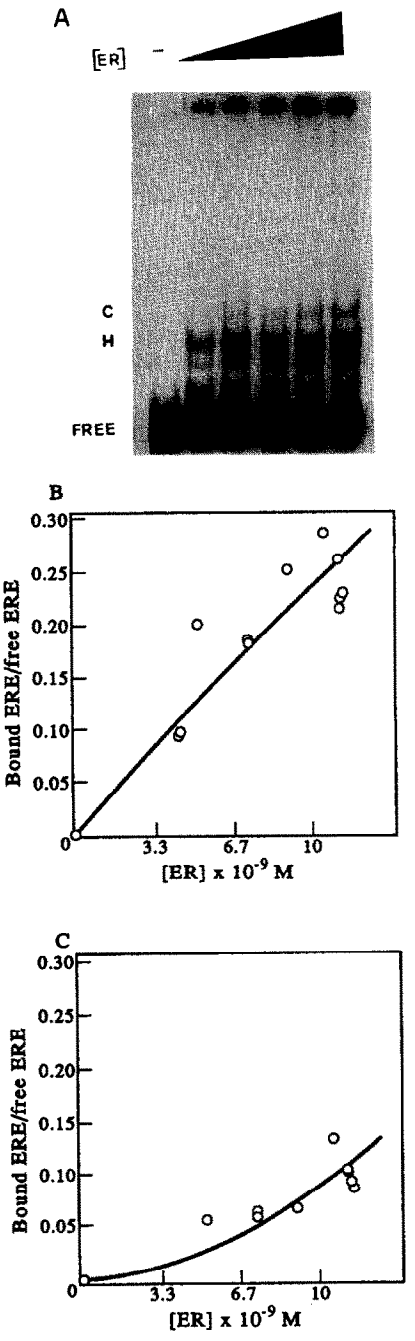

Fig. 6. Estrogen receptor titration of the heat/quick-chilled imperfect cVTGII ERE. (A) Increasing concentrations of estrogen receptor were added to the heat/quick-chilled cVTGII imperfect ERE labeled on the lower strand and the gel mobility assay was performed. See legend of Fig. 5 for explanation of panels (B) and (C).

contained on the *Nsi* I to *Sau* 3A fragment) produced bands which demonstrated hyperbolic binding (Figs. 4C, 5B and 6B) and a band which demonstrated concave up binding (Figs. 4D, 5C and 6C). Estrogen receptor binding to the rat prolactin ERE on the

Nsi I to *Sau* 3A fragment demonstrated only a hyperbolic curve (Fig. 4C). The reason for obtaining multiple bands corresponding to hyperbolic binding is unclear. However, these multiple bands do represent estrogen receptor binding to the ERE since competition experiments using the rat prolactin ERE contained on the *Nsi* I to *Sau* 3A fragment could compete out the multiple bands (data not shown). The hyperbolic curves from the rat prolactin ERE contained either on the *Hph* I to *Sau* 3A or the *Nsi* I to *Sau* 3A fragments were superimposable. Thus, the flanking sequences removed from the *Hph* I to *Sau* 3A fragment can affect the type of interaction the estrogen receptor makes with the ERE.

DISCUSSION

The results presented here illustrate the preferential interaction of the estrogen receptor with either imperfect or perfect EREs which contain tertiary structure. Since the strands have different mobilities in the gel system, the DNA is obviously not in the B-form. The specific nature of estrogen receptor interaction with tertiary-structured EREs was demonstrated by first, showing that the estrogen receptor preferentially bound to an ERE oligonucleotide compared to a double-stranded ERE. This result ruled out artifacts caused either by the heat/quick-chill procedure or the presence of the complementary strand. Second, estrogen receptor binding to tertiary-structured EREs was decreased by deviations from the ERE consensus sequence.

Multiple retarded bands were observed when estrogen receptor bound to the EREs (with the exclusion of the rat prolactin ERE contained on the *Nsi* I to *Sau* 3A fragment). These bands have been shown to contain estrogen receptor by competition experiments using the rat prolactin ERE contained on the *Nsi* I to *Sau* 3A fragment. Upon incubation with estrogen receptor, the *Nsi* I to *Sau* 3A fragment produced only one retarded band. This retarded band was shown to contain estrogen receptor by western blot analysis. Additionally, since the binding conditions were performed using estrogen receptor purified to homogeneity and with high concentrations of nonspecific DNA, the bands represent specific high affinity binding of the estrogen receptor to the ERE. These multiple retarded bands reflect different types of estrogen receptor interactions with the ERE. These interactions result in different types of binding curves which are observed upon titration of the estrogen receptor with the various EREs. Preliminary analysis suggests that the data are consistent with a model in which positive cooperative binding occurs. Additional experiments will be necessary to determine if the different binding curves are the result of monomer, dimer or tetramer interactions with the ERE. The ERE contained on the *Nsi* I to *Sau* 3A fragment did not demonstrate concave up binding whereas the ERE contained on the *Hph* I to *Sau* 3A fragment did. These data suggest that flanking sequences can influence the types of interactions the estrogen receptor makes with the ERE.

In the gel mobility assay, deletion of sequences 14 bp away from the ERE decreased the affinity of the estrogen receptor for the tertiary-structured ERE. Cato *et al.* [25] demonstrated that sequences outside of the steroid responsive element modulated steroid responsiveness. However, these flanking sequences provide sites for DNA-binding proteins. In the experiments presented here flanking sequences contribute to the formation of the tertiary-structured ERE.

We have proposed a model for estrogen receptor interaction with DNA which involves the formation of a tertiary-structured ERE. The estrogen receptor does bind to B-form ERE but with a lower affinity than to the tertiary-structured ERE. We suggest that it is the interaction of the estrogen receptor with the tertiary-structured ERE which is important *in vivo*. Curtis and Korach [26] have reported that specific estrogen receptor–ERE complexes are obtained only with B-form ERE. However, the conditions of their binding assay appear to favor the formation of the B-form ERE and, therefore, their results are not directly applicable to our experiments. In support of our model, Mukherjee and Chambon [27] have also demonstrated that purified estrogen receptor has a low affinity for B-form DNA. Additionally, we have observed that the ERE exists in a non-B DNA conformation in the absence of heat/quick-chilling or any protein (unpublished observation). This non-B DNA conformation appears to be labile (unpublished observation). Our current hypothesis is that *in vivo* either the estrogen receptor or other proteins stabilize the non-B DNA conformation of the ERE. Using various non-B DNA probes we have observed differences in the reactivity of ERE sequences on the upper and lower strands (unpublished observation). This result suggests that the two strands of the ERE exist in different conformations. This observation may explain the preferential binding of the estrogen receptor for a particular strand of the ERE. There are now a number of transcription factors which have been shown to preferentially bind a non-B form versus a B-form of their responsive elements [28–32]. This result suggests that responsive elements for other transcription factors contain tertiary structure *in vivo*. The involvement of tertiary-structured DNA in regulating transcription by providing high affinity binding sites for transcription factors is an alluring possibility.

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