Biochemical Pharmacology, Vol. 40, No. 12, pp. 2579–2585, 1990.
Printed in Great Britain.

0006–2952/90 $3.00 + 0.00

COMMENTARY

A NOVEL MECHANISM FOR EUKARYOTIC GENE EXPRESSION

THE INVOLVEMENT OF DNA TERTIARY STRUCTURE IN ESTROGEN RECEPTOR RECOGNITION OF ITS TARGET NUCLEOTIDE SEQUENCE

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A fundamental question in transcriptional regulation is how transcription factors accurately recognize their target nucleotide sequences from amongst the several billion base pairs in a typical eukaryotic genome. Is it the nucleotide sequence alone that controls the specificity of the transcription factor or are there additional transcriptional regulatory processes, such as differential expression of factors, limited access of transcription factors to regulatory sequences, or involvement of tertiary structure in the target nucleotide sequence? This commentary will address these issues by focusing on the binding mechanism of the estrogen receptor to its target nucleotide sequence. The estrogen receptor is an ideal model system to study transcriptional regulation because the main components of the system, the hormone effectors, the receptor protein and several estrogen responsive genes with their 5′ regulatory sequences have been identified and isolated. In this commentary, we propose a novel mechanism of estrogen receptor binding to DNA which involves tertiary structure of the DNA at the target nucleotide sequence. The implications of our proposed mechanism with regard to other transcription factors, and the possible importance of the mechanism for understanding eukaryotic transcription in general, will also be discussed.

Estrogen receptor—a hormone inducible transcription factor

The estrogen receptor is an important component in the regulation of development, differentiation, and cell growth. The estrogen receptor is a member of the steroid–hormone receptor superfamily (based on amino acid homology) which also consists of the glucocorticoid, progesterone, androgen, thyroid, erb A, vitamin D, retinoic acid, and mineralcorticoid receptors as well as functionally unidentified members [1]. These receptors have been divided into approximately four domains based on functional analysis of deletion mutants. The N-terminal domain, a weakly conserved region, is involved in interactions with other transcription factors [2, 3]. This region is followed by the DNA-binding domain which is the region of greatest homology, in both the nucleotide and the amino acid sequence, of the superfamily members. This domain contains two putative zinc fingers [4] and may be involved in dimerization [4, 5]. The DNA-binding region is separated from the hormone-binding domain by a hinge region. The hinge region, a low homology domain, may be involved in dimerization [5] as well as estrogen repression of transcription [6]. The hormone-binding domain controls transcriptional activation by binding estradiol and is involved in dimerization [2, 3, 5].

Estradiol, the most potent of the estrogens, binds reversibly and with high affinity to its receptor in a positively cooperative manner [7]. This cooperativity, together with other kinetic and molecular observations [8–11], indicates that the receptor exists as a homodimer. Importantly, the homodimer exists under physiologically relevant conditions, indicating that positive cooperativity of estradiol binding exists *in vivo* [7]. Positive cooperativity provides a more responsive regulatory mechanism for small changes in physiologically relevant hormone concentrations [7]. Our model of the estrogen receptor as a functional homodimer has been corroborated recently by observations from DNA-binding experiments with nuclear extracts from cells that had been cotransfected with wild type and truncated estrogen receptors [5]. Three different protein–DNA bands were observed in a gel-shift assay, which were interpreted as representing the two homodimer forms of receptor (wild type or truncated) plus a heterodimer of wild type and truncated receptor with an intermediate mobility.

The binding of estradiol by the receptor induces a conformational change that increases the affinity of the receptor for total DNA approximately 5- to 10-fold [12]. This conformational change can be detected by the alteration of the number of ionic bonds the receptor makes with DNA upon binding estradiol. In the absence of estradiol, the homodimer makes approximately 8 ionic bonds, whereas in the presence of estradiol the homodimer makes approximately 13 ionic bonds with nonspecific DNA [12]. The observed increase in affinity for total DNA may aid in target sequence location, because the binding of the receptor to the DNA would decrease

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Table 1. Estrogen responsive elements from various genes

Gene		ERE		Ref.
Xenopus vitellogenin A1	—336	GGGTCACAATGACCT	—322	[15]
Xenopus vitellogenin A2	—332 —312	AGGTCACAGTGACCT AAGTTAATGTAACCT	—318 —298	[16]
Xenopus vitellogenin B1	—335 —315	CAGTCACTGTGACCC AAGTTATCATGACCT	—321 —301	[17]
Xenopus vitellogenin B2	—336 —316	AAGTCACTTTGACCC AAGTTATCATGACCT	—322 —302	[18]
Chicken vitellogenin II	—621 —349	TGGTCAGCGTGACCG AGGTCAACATAACCT	—607 —335	[19]
Chicken ovalbumin	—78	AGGTCA	—73	[20]
Chicken apo very low density lipoprotein II	—222 —178	GGGGCTCAGTGACCC AGGTCAGACTGACCT	—208 —164	[21]
Rat prolactin	—1581	TTGTCACTATGTCCT	—1567	[22]
Rat luteinizing β	—1175	TGGACAGATGGTGTCCC	—1159	[23]
Human pS2	—406	AGGTCACGGTGGCCA	—392	[24]
Human uteroglobin	—265	AGGTCACCATGCCCT	—251	[25]
Consensus ERE		GGTCANNNTGACC		
Consensus GRE		GGTACANNNTGTTCT		
Consensus TRE		GGTCATGACC		

The location of the ERE from the start site is indicated. Highlighted nucleotides are deviations from the consensus ERE.

the diffusional mobility of the receptor from three dimensional to one dimensional as the receptor slides along the DNA searching for its specific target sequence [13, 14].

Binding mechanisms of estrogen receptor to its target DNA sequence

Orthodox model: The estrogen responsive element (ERE) dictates recognition and selectivity by the estrogen receptor. The ERE was identified by comparing sequences which confer estrogen responsiveness *in vivo* [15–25] as well as binding the estrogen receptor *in vitro* [22, 23, 25] (Table 1). A 13 base pair consensus sequence was derived which consists of the sequence GGTCANNNTGACC. This consensus ERE has a 5 base pair dyad symmetry separated by a 3 base pair spacer.

Current dogma suggests the following model for estrogen activation of transcription, which we have termed the orthodox model. The 13 base pair consensus ERE is thought to be sufficient to confer estrogen responsiveness *in vivo* [26–28]. Indeed, one base pair change in the consensus ERE destroys the ability of the sequence to confer estrogen responsiveness [26, 27]. Additionally, the consensus ERE has been demonstrated to provide selectivity for the estrogen response [26–30]. Thus, although the consensus glucocorticoid responsive element (GRE; GGTACANNNTGTTCT [1]) and the consensus thyroid responsive element (TRE; GGTCATGACC [1]) have either a relatively high homology or are identical to the ERE except for absence of the 3 base pair spacer, there is no overlap in hormone responsiveness. In fact, inversion of a single A/T base pair in each dyad of the ERE completely switched the steroid responsiveness of the ERE from estrogen to glucocorticoid [26, 28]. These observations led to the conclusions that the estrogen receptor requires only the 13 base pair ERE for recognition, selectivity, and the conferment of biological response.

In the orthodox model, the activated estrogen receptor locates and subsequently binds specifically to the ERE. Each monomer of the homodimer would interact with an identical sequence which is located on opposite sides of the double-stranded ERE [31]. Specific DNA binding is contributed by the first zinc finger of the receptor, and the second zinc finger contributes to nonspecific binding but may provide additional specific recognition [4]. The binding of the estrogen receptor alters the transcription rate through some unknown mechanism, which presumably involves the interaction with other factors, RNA polymeriase at a minimum, through the N-terminal region of the receptor [2, 3].

Paradoxes: Are there other considerations for a fully functional estrogen responsive element? The orthodox model proposes that estrogen responsiveness is conferred solely by the 13 base pair ERE; however, there are inconsistent observations which suggest that additional components besides the 13 base pair ERE are required. First, the estrogen response in some studies has been diminished compared to the response obtained with larger fragments containing the ERE [27]. These studies suggest that flanking nucleotide sequences play a role in estrogen responsiveness.

Second, the majority of EREs isolated thus far

appear to be imperfect rather than perfect palindromes (Table 1). Examination of these EREs shows that they have as much sequence homology to the GRE as they do to the ERE, yet no overlap in steroid responsiveness exists. Only four nucleotides are absolutely conserved among individual EREs (excluding the half-site ERE from the ovalbumin gene), which suggests that these are extremely important in receptor-ERE recognition (GGTCANNNTGACC). However, two of these are found in the same position in the GRE (GGTACANNNTGTTCT). Additionally, the TRE is identical to the ERE except for the absence of the three base pair spacer. This observation suggests that the spacing between the dyads is critical. Nevertheless, an ERE from the lutenizing β gene has been identified which has variant spacing (Table 1).

Third, when point mutations were made in the perfect ERE, the mutants did not retain estrogen responsiveness [27]. Disconcertingly, however, the mutant EREs, which did not function in this *in vivo* assay, have nonetheless been isolated and identified as conferring estrogen responsiveness on the pS2 and Xenopus vitellogenin B1 genes [24, 26].

Fourth, although perfect EREs may be able to operate independently, imperfect EREs usually have to operate in pairs [18, 26, 32]. Pairs of EREs will produce a synergistic estrogen response [18, 26, 32]. Cooperativity cannot be explained by the EREs each contributing a different perfect half of an ERE and thus forming a perfect ERE because imperfect EREs each bearing an imperfection in the same half of the palindrome will cooperate [26, 32]. Since the orthodox model implies that each monomer of the homodimer binds to an identical sequence on opposite sides of the double-stranded ERE, the formation of a complete binding site by two imperfect EREs is ruled out by this observation.

Fifth, one half-site consisting of a GGTCA sequence has been suggested to confer estrogen responsiveness in the chicken ovalbumin gene [20]. This half-site lies close to the TATA box, but this close proximity does not appear to account for the ability of the half-site to confer estrogen responsiveness since estrogen responsiveness was lost by single point mutations in the perfect ERE even though the ERE was close to the TATA box [26, 27]. This half-site was only operational in chick embryo fibroblasts, and the investigators suggest that stabilization by other factors allows this half-site to function as an ERE. It is unclear how the homodimer could bind with high affinity to a half-site, as the investigators suggest, because even if one monomer contacted the correct squence, the other monomer would be contracting nonspecific DNA, and one would expect that this would be thermodynamically unstable. Additionally, the probability of finding a GGTCA in a random sequence of bases is very high (1 out of every 1024 base pairs). The high incidence of the GGTCA sequence would suggest that in cells with the necessary auxillary transcription factors highly promiscuous estrogen responsiveness could occur. In summary, although the 13 base pair ERE is important in bestowing estrogen responsiveness, it remains unclear whether or not the 13 base pair ERE alone is sufficient.

What other regulatory processes could contribute to estrogen regulation of transcription? Plausible mechanisms for preventing an overlap in steroid responsiveness include differential expression of receptors, and inactivation of EREs, GREs or TREs. Control of estrogen responsiveness by differential receptor expression is not likely since estrogen, glucocorticoid and thyroid receptor have a broad, overlapping distribution *in vivo*. Inactivation could occur either by placing the appropriate DNA sequences in inaccessible chromatin or by the binding of inhibitory transcription factors. The thyroid hormone receptor appears to bind to EREs and act as an inhibitory transcription factor, that is it represses estrogen responsiveness [29]. The estrogen receptor appears to discriminate between TREs and EREs and will only weakly activate transcription from TREs [33]. These observations demonstrate that TREs and EREs are accessible for either interaction with thyroid or estrogen receptors. Simultaneous administration of glucocorticoids with estrogen produces an independent response to each hormone and thus the selective inaccessibility of glucocorticoid or estrogen responsive elements seems to be ruled out [34].

A possibility which we are exploring as an important determinant of receptor-ERE interaction is the tertiary structure of the DNA at the ERE. Thus, although ultimately the nucleotide sequence of the response element plays an important part in receptor recognition, the first phase of recognition and receptor interaction would be the structure of the target nucleotide site. This tertiary structure could be intrinsic to the response element or could be conferred onto the response element by flanking sequences. Differences in tertiary structure between the ERE, GRE and TRE could provide very dissimilar binding sites even though there is a high degree of nucleotide sequence homology.

Heterodox model: Evidence that the estrogen responsive element contains non-B DNA structure. Since we believed that the tertiary structure of the ERE might be important in receptor recognition, a DNA fragment containing the rat prolactin imperfect ERE was heat-treated followed by quick chilling in an attempt to trap inherent tertiary structure. The estrogen receptor preferentially bound with high affinity and specificity to the "coding strand" of the heat-quick chilled ERE [35]. The "noncoding strand" acted as an internal control showing that the estrogen receptor did not bind nonspecifically to single-stranded DNA [35]. The "coding strand" is believed to contain structure, i.e. non-B DNA, based on its anomalous mobility in a nondenaturing polyacrylamide gel [35].

Competitions (Fig. 1) with various DNAs demonstrated that the activated estrogen receptor preferentially binds with a 17-fold greater affinity to the double-stranded ERE than to nonspecific DNA. The higher affinity DNA site for the estrogen receptor is believed to form by transitory opening of the strands into a unique non-B DNA structure which is stabilized either by supercoiling or by proteins other than the estrogen receptor. The estrogen receptor binds with a 60-fold higher affinity to the unique structure of the ERE "coding strand" than the

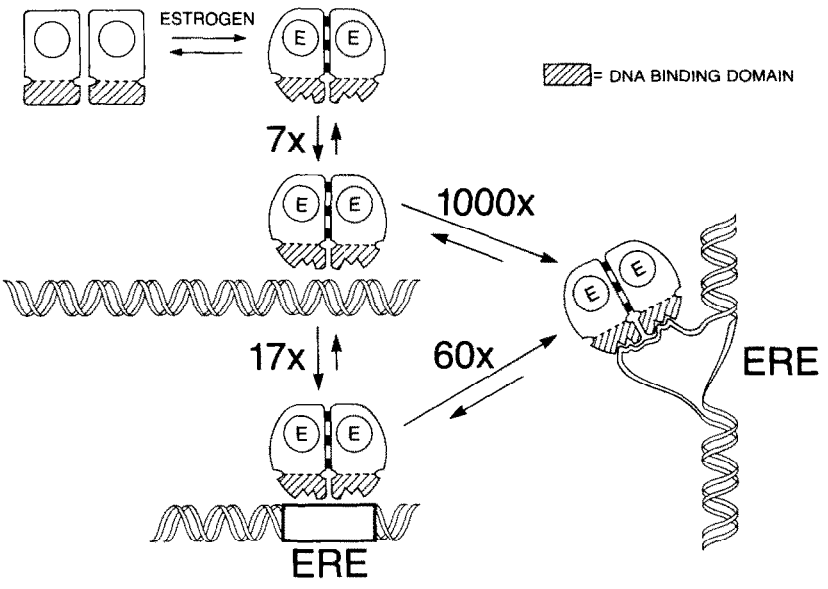

Fig. 1. Heterodox model of estrogen receptor interaction with DNA. Numbers indicate the increase in relative affinity.

double-stranded ERE. Presumably, there are single-stranded qualities to the structure since the receptor has a higher affinity for nonspecific single-stranded DNA than for specific double-stranded DNA. However, the probability of nonspecific DNA being in the single-stranded state is low due to the absence of stabilizing factors. The formation of the "receptor-coding strand" complex, which we term the heterodox model (Fig. 1 [35]), may be a crucial step in the mechanism of estrogen stimulation of transcription.

An important initial question was to determine whether or not the heterodox model operates for all EREs, just imperfect EREs, or only the rat prolactin ERE. We therefore analyzed the binding of receptor to both the perfect and imperfect ERE of the chicken vitellogen II gene. In each case the receptor preferentially bound the ERE when the ERE was in a non-B DNA form rather than the double-stranded form (Lannigan DA, Tomashek JJ and Notides AC, unpublished observations). Therefore, we believe that the heterodox model is a universal model for the mechanism of estrogen receptor recognition of the ERE.

Our model suggests that the estrogen receptor recognizes a structure which is unique to the ERE. The evidence of structure in the high-affinity site is based on the anomalous mobility in a nondenaturing polyacrylamide gel of the dissociated rat prolactin ERE [35]. Interestingly, both the perfect and imperfect dissociated EREs from the chicken vitellogenin showed the same anomalous gel mobility as the dissociated rat prolactin ERE (Lannigan DA, Tomashek JJ and Notides AC, unpublished observations). The anomalous gel mobilities suggest that the tertiary structure associated with the ERE had been recaptured successfully in each case. We think that the DNA structure is not intrinsic to the ERE but is most likely conferred by flanking sequences. This hypothesis is based on the observation that although the nucleotide sequences of the ERE, GRE and TRE are similar there is no evidence that the GRE or TRE contains non-B DNA.

Additional evidence for structure in the ERE is based on analyses for non-B DNA in a plasmid containing a 2 kilobase fragment from the upstream region of the rat prolactin gene. Enzymatic analysis by S1 nuclease and chemical analysis by potassium permanganate, osmium tetroxide, and diethylpyrocarbonate revealed that the ERE contains non-B DNA (Lannigan DA, Koszewski NJ and Notides AC, unpublished observations). Additionally, the enzymatic and chemical attack points were non-symmetrical with respect to the center of the palindrome (Lannigan DA, Koszewski NJ and Notides AC, unpublished observations). These results imply that the structures formed by the "coding and noncoding strands" of the rat prolactin ERE in "*in vivo*-like" DNA are different, and this difference is recognized by the estrogen receptor.

Returning to one of our original questions: how does the receptor distinguish the ERE from the GRE or TRE? If the ERE contains a unique tertiary structure, it would clearly be different from the GRE or TRE than indicated by inspection of their sequence alone.

Clearly, there are many questions to answer concerning the binding mechanism according to the heterodox model:

(1) How does the homodimer bind to one strand? It is easy to envision that two EREs could work in concert, each contributing a similar binding site to the monomers of the homodimer. This hypothesis suggests that the monomers sit head to tail. In the orthodox model, each monomer of the homodimer would interact with an identical sequence on opposite sides of the double-stranded ERE and thus the monomers would sit head to head [31]. Dimer orientation is different in the two models and could be used to distinguish between them.

(2) What is the structure of the ERE? Currently, we know that the ERE contains nucleotides in the non-B conformation. Presumably, however, there are single-stranded qualities to the structure since the receptor has a higher affinity for nonspecific single-stranded DNA than for specific double-stranded DNA.

(3) Does the structure exist *in vivo*? We have attempted to address this question by our analysis of non-B DNA in the 2 kilobase fragment from the upstream region of the rat prolactin gene. The observation of non-B DNA in our model system suggests that non-B DNA may exist *in vivo*, although verification of this will have to await analysis with agents that penetrate cells and modify non-B DNA which can then be detected.

(4) If the heterodox model is correct, why does an isolated ERE function in transient transfection assays [26–28]? One possibility, is that in these assays high concentrations of ERE were achieved, which would drive the formation of the receptor–ERE complex despite the low affinity of the receptor for a single unstructured ERE.

(5) How can a half-ERE function? This observation suggests the importance of flanking sequences which confer a unique structure to the ERE and thus prevent promiscuous estrogen responsiveness. The isolation of the flanking sequences which allow the ERE to adopt its unique conformation will help elucidate functional EREs from nonfunctional EREs.

(6) What is the function of the structure? The ERE structure could simplify the search the estrogen receptor has to make for its target nucleotide sequence in the nucleus. Instead of having to make individual contacts with specific bases to determine if the sequence contained an ERE, the receptor would initially simply scan for the unique structure of the ERE.

Synthesis of the two models and examples of other transcription factors with DNA-binding mechanisms similar to that of the estrogen receptor. Initially, the binding mechanism of the estrogen receptor for its target sequence appeared unique. This result seemed unusual since the estrogen receptor is a member of the steroid superfamily whose commonality includes a highly homologous DNA-binding region. In fact, an estrogen receptor can be converted from recognizing an ERE to recognizing a GRE by three amino acid changes in the region surrounding the first zinc finger [36]. This observation implies that the only distinction between the glucocorticoid and estrogen receptor binding mechanism is one of subtle differences in amino acid-nucleotide base contacts. We feel this observation can be explained by a two-stage model of estrogen receptor recognition for its ERE. In the first stage the receptor scans the DNA seeking the unique structure of the ERE. Upon locating this structure, specific amino acid-nucleotide base contacts are made. This first stage can be somewhat preempted if the equilibrium binding conditions are shifted from the *in vivo* conditions, i.e. if either the receptor or the DNA is in sufficient quantity (e.g. in transfection assays [26–28]) to drive the equilibrium so that the low affinity the estrogen receptor has for the unstructured ERE can be overcome to enable the receptor to bind. A crucial point of the heterodox model is that the receptor can bind to double-stranded ERE, but it does so with an affinity that is lower than that for the structured ERE which is the biologically relevant target site.

Examples of other transcription factors preferring to bind single-stranded versus double-stranded DNA are now coming to light. The TATA binding protein preferentially binds single-stranded nonspecific DNA over its double-stranded target sequence [37]. Unfortunately, the affinity for the single-stranded target sequence was not determined. Recently, a DNA binding protein which interacts with the sterol regulatory element (SRE) was isolated [38]. The SRE is responsible for the repression of gene transcription for the low density lipoprotein receptor and enzymes in the cholesterol biosynthetic pathway. This protein preferentially binds single-stranded versus double-stranded SRE [38]. Intriguingly, the SRE binding protein (which presumably mediates repression) binds with a higher affinity to the "noncoding strand" of the SRE [38]. Single-stranded sequence-specific binding proteins have been identified which bind to the adipsin promoter region [39]. Transcription from this gene increases on differentiation from preadipocytes to adipocytes. One of the identified proteins appears only in adipocytes and thus is differentiation specific [39]. These proteins bind to the "noncoding strand" of the DNA, but curiously enough some of them have no affinity for RNA of the same sequence [39].

Implications for eukaryotic transcription

Crick [40] postulated that single-stranded DNA would exist in introns and would be responsible for the control of transcription by providing binding sites for transcription factors. We would modify this model by suggesting that perhaps not just single-stranded DNA but non-B DNA, in general, constitutes the binding sites for various transcription factors. Crick's model may not be true for prokaryotes in which it is evident that prokaryote transcription factors prefer double-stranded DNA [41]. However, from our analysis of the binding mechanism of the estrogen receptor it appears that eukaryotes have adopted this system for transcriptional control. This method of control may be required because of the more complex eukaryotic genome. The simplest solution, to provide an additional layer of regulation, was for the DNA to adopt non-B conformations which could be identified by transcription factors.

Acknowledgement—D. A. L. would like to thank Ian G. Macara for useful discussions and critical reading of the manuscript.

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