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Mechanisms involved in regulation of the estrogen-responsive pS2 gene

Review Article

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Abbreviations: **ER**, estrogen receptor; **EREs**, estrogen response elements; **DMS**, dimethylsulfate; **LMPCR**, ligation mediated polymerase chain reaction

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Summary

Estrogen is a hormone of critical importance in the development and maintenance of normal reproductive tissues and has been implicated in initiation of mammary carcinogenesis. Estrogen's actions are mediated through an intracellular estrogen receptor (ER), which interacts with estrogen response elements (EREs) to bring about changes in transcription of estrogen-responsive genes. Although it is clear that the ER-ERE interaction is a critical link in the chain of events that lead to transcription activation, the mechanisms by which transcriptional changes occur remain unclear. We present evidence that ERE sequence and ligand act as allosteric modulators of ER conformation and that these conformational changes most likely play a role in regulating transcription of estrogen-responsive genes.

I. Introduction

The importance of estrogen action in the development and maintenance of normal reproductive function in the female has been well established. Estrogen has been extensively used in birth control pills to regulate ovulation and avoid pregnancy. More recently, it has become clear that estrogen's effects extend well beyond reproductive tissues. Estrogen plays an important role in maintenance of cardiovascular health (Mendelsohn and Karas 1994; Stevenson et al., 1994; Subbiah 1998), bone mineral density (Smith et al., 1994; Yang et al., 1996), and neural function (Toran-Allerand 1996; Wickelgren 1997). Estrogen's ability to confer these very positive effects has led to the widespread use of estrogen replacement therapy in women with postmenopausal symptoms (Stanford and Colditz 1996). Surprisingly, it is now clear that estrogen plays a crucial role in the male reproductive tract and is required for production of viable sperm (Hess, Bunick, and Bahr 1995; Hess et al., 1997). Studies in ER-deficient mice indicate that estrogen may also influence behavior in both males and females (Ogawa et al., 1996a,b, 1997, 1998).

Antiestrogens have also been used in a number of clinical situations. Tamoxifen has been used in breast cancer therapy in women with ER-positive tumors (Tormey et al., 1976; Wasterberg 1980) and has resulted in a 40% reduction in breast cancer recurrence (Early Breast Cancer Trialists' Collaborative Group 1992) as well as favorable effects on lipid profiles and bone mineral density (Love et al., 1991, 1992). Tamoxifen is also being tested for its ability to prevent breast cancer in women who are at risk for developing this disease (Davidson 1992; Powles 1998). Raloxifene has positive effects on bone mineral density and cardiovascular health without stimulating proliferation in the endometrium and may be useful in breast cancer prevention (Delmas et al., 1997; Gustafsson 1998). Clinical trials have been instituted to test the ability of ICI 182,780 to prevent breast cancer recurrence and treat tamoxifen-resistant tumors (DeFriend et al., 1994; Howell et al., 1995; Wakeling et al., 1991). Although the importance of estrogens and antiestrogens in human health is indisputable, the mechanisms by which these compounds bring about their effects are unclear.

The actions of estrogen and antiestrogens are mediated by the intracellular estrogen receptor (ER). Upon binding to ligand, the ER interacts with estrogen response elements (EREs) present in target genes and initiates changes in transcription. To better understand how hormone and antihormone regulate gene expression, we have examined

the ER-ERE interaction in *in vitro* and *in vivo* assays and considered the ability of different ERE sequences to activate transcription.

II. Different estrogen response elements activate transcription to different extents

Although the consensus ERE (Klein-Hitpass et al., 1988) found in the *Xenopus laevis* vitellogenin A2 gene is a high affinity binding site for the ER and the most thoroughly characterized ERE, the overwhelming majority of ERE sequences identified in endogenous genes differ from the consensus sequence by one or more nucleotides. To understand how different EREs regulate gene expression, we examined the ability of the ER to activate transcription of simple promoters containing either the *Xenopus laevis* vitellogenin A2 ERE (GGTCAnnnTGACC, Ref. Klein-Hitpass et al., 1988), which contains a palindromic, consensus ERE sequence, the *Xenopus laevis* vitellogenin B1 ERE2, which contains a one base pair change in the 5' end of the half palindrome (AGTCAnnnTGACC, Ref. Walker et al., 1984), or the human pS2 ERE (GGTCAnnnTGGCC, Ref. Nunez et al., 1987), which contains a one base pair mismatch in the 3' ERE half site. When Chinese Hamster Ovary cells were transfected with reporter plasmids containing either the A2, B1, or pS2 ERE separated from the TATA sequence by 2.6 helical turns, exposure to 10 nM 17ß-estradiol increased transcription 12.7-, 2.4-, and 3.8-fold, respectively (**Fig. 1**). Increasing the spacing between the ERE and TATA sequence to 3 helical turns decreased the ability of the A2 ERE to activate transcription by 55%, increased the ability of the pS2 ERE to activate transcription by 35%, but had no significant effect on B1 ERE activity. Further increasing the distance between the ERE and TATA sequence to 3.6 helical turns restored the activity of promoters containing the A2 and pS2 EREs to the original levels, but decreased the activity of the promoter containing the relatively weak B1 ERE. Thus, in spite of the fact that the promoters were identical, except for the ERE sequence, these three promoters had very different abilities to activate transcription. Furthermore, their abilities to effectively activate transcription were dependent upon the phasing of the ERE and TATA sequences.

From these findings we hypothesized that individual EREs might serve as allosteric modulators of ER conformation and that these DNA-induced changes in ER conformation may in turn influence ER-protein interactions and lead to changes in transcription activation. Thus it seemed possible that the A2 and the pS2 ERE sequences might elicit unique changes in ER conforma-tion, which fostered the interaction of the receptor with different sets of transcription factors and resulted in different levels of transcription activation.

The ability of DNA to induce changes in protein conformation has been reported for a number of different transcription factors (Alber, 1993; Frankel and Kim, 1991; Pabo and Sauer, 1992; Spolar and Record, 1994; Steitz, 1990; von Hippel, 1994). More importantly, NMR and crystal structure studies carried out with the ER and glucocorticoid receptor DBDs have demonstrated that a dimerization interface is induced upon binding to DNA (Hard et al., 1990; Luisi et al., 1991; Schwabe et al., 1993a,b). Work with mutant GRs provide further support for this hypothesis (Lefstin et al., 1994; Starr et al., 1996).

III. Differential interaction of ER-specific antibodies with the A2 and pS2 ERE-bound ER suggest differences in receptor conformation

To determine if there were differences in epitope availability when the intact receptor was bound to the A2 or pS2 ERE, antibody supershift experiments were carried out.

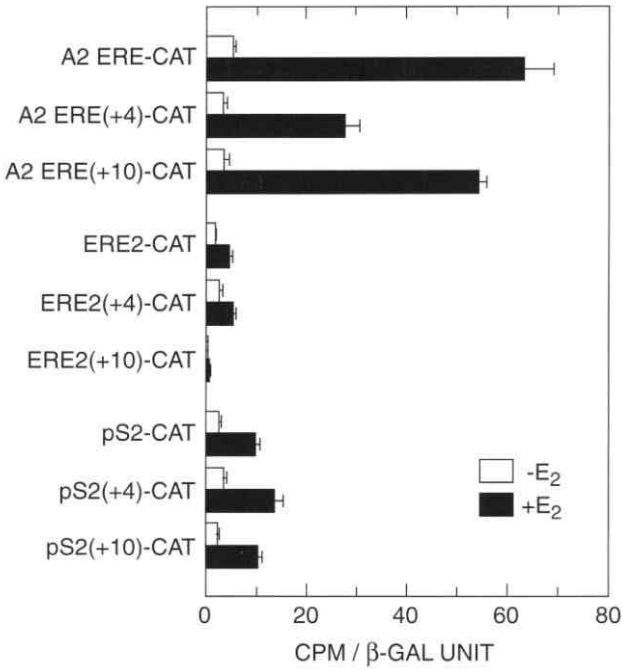

Fig. 1 The A2, B1, and pS2 ERE activate transcription to different extents. CHO cells were transfected with CAT reporter plasmid, human ER expression plasmid, β-galactosidase expression plasmid, and pTZ nonspecific DNA using the calcium phosphate coprecipitation method. Cells were treated with ethanol vehicle (-E_2, open bars) or 10 nM 17ß-estradiol (+E_2, solid bars). Each reporter plasmid was included in at least 6 independent experiments. Values are presented as the mean ± S.E. (From Nardulli *et al*, 1996).

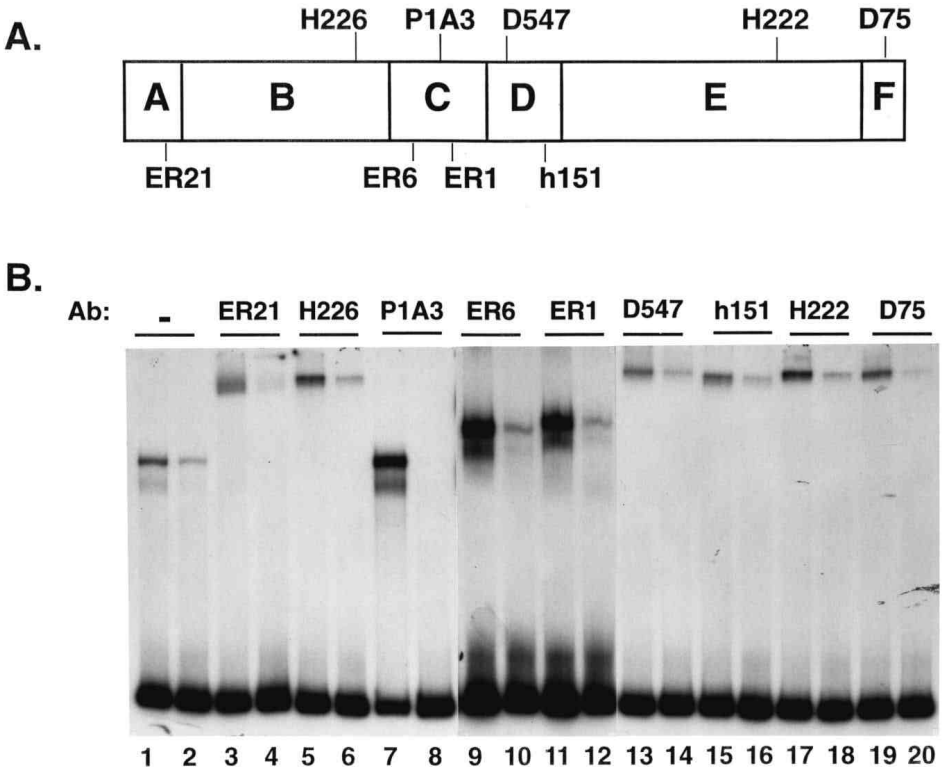

Fig. 2. Antibodies to various ER epitopes can detect differences in conformation of the A2 and pS2 ERE-bound receptor.

A. Schematic representation of the epitopes for ER-specific antibodies utilized.

B. Partially purified, yeast-expressed ER was combined with A2 ERE-containing DNA fragments (odd lanes) or pS2 ERE-containing DNA fragments (even lanes). After a short incubation, antibodies (Ab) were added to the binding reactions as indicated and the complexes were fractionated on a non-denaturing acrylamide gel. The complexed DNA and free probe were visualized by autoradiography. (From Wood *et al*, 1998).

A series of monoclonal and polyclonal antibodies, which had been made against several different ER regions (**Fig. 2A**), were utilized with partially purified yeast-expressed ER and ^{32}P-labeled A2 or pS2 ERE-containing DNA fragments. Partially purified ER and DNA fragments containing either the A2 or pS2 ERE were incubated with or without antibodies made against different ER epitopes. The protein-DNA complexes were fractionated on non-denaturing polyacrylamide gels. The most striking difference in epitope availability was observed with the monoclonal antibody P1A3, which was made against purified *Xenopus laevis* DBD. P1A3 enhanced the ER-A2 ERE interaction approximately six fold (**Fig. 2B**, compare lanes 1 and 7) and strongly inhibited the ER-pS2 ERE interaction (compare lanes 2 and 8), but failed to supershift either the A2 or pS2 ERE-ER complex. Two other antibodies also discriminated between the A2 and pS2 ERE-bound ER. ER21, which binds to the amino terminal region, and D75, which binds to the carboxy terminal region, both enhanced the supershifted ER-A2 ERE complex formation approximately two-fold (compare lanes 1 with 3 and 19), but did not alter (ER21) or decreased (D75) formation of the ER-pS2 ERE complex (compare lane 2 with 4 and 20). The other antibodies tested (H226, ER6, ER1, D547, h151 and H222) supershifted both the A2 and pS2 ERE-containing complexes in a similar manner. The differential interaction of three ER-specific antibodies with A2 and pS2 ERE-bound ER implied that there were differences in ER epitope availability not only in the DBD, but also in the amino and carboxy termini of the receptor.

IV. Protease sensitivity assays detect differences in the A2 and pS2 ERE-bound ER

To more directly assess possible differences in receptor conformation, protease sensitivity assays of A2 and pS2 ERE-bound ER were carried out. These assays utilize limited proteolysis of a DNA-bound protein to produce a pattern of digestion based upon amino acid accessibility and provides information about native protein conformation (Schreiber et al., 1988; Tan and Richmond, 1990). ^{32}P-labeled DNA fragments containing the A2 or pS2 ERE were combined with partially purified ER.

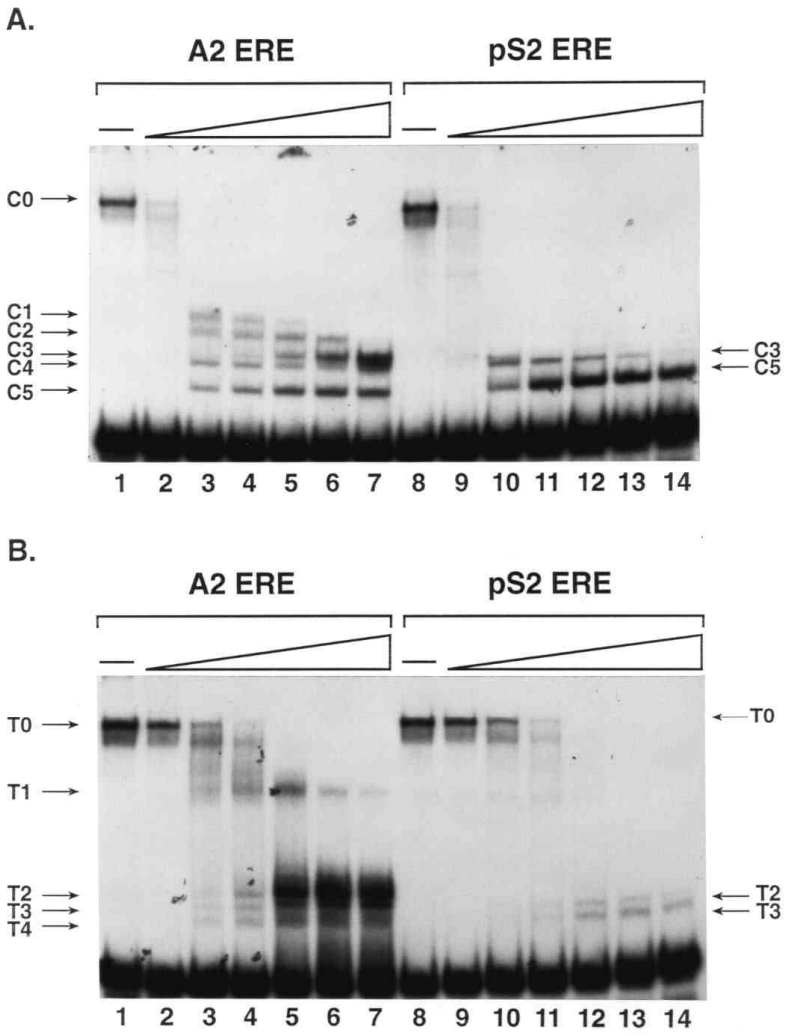

Fig.3. Distinct protease digestion patterns of A2 and pS2 ERE-bound ER provide evidence for ERE-mediated differences in receptor conformation.

· Partially purified, estrogen-occupied ER was combined with A2 or pS2 ERE-containing DNA fragments. After a short incubation, 0, 0.05, 0.5, 1.25, 2.5, 3.75, or 5 ng chymotrypsin was added to the binding reaction. ER-DNA complexes and free DNA were fractionated on a nondenaturing acrylamide gel and the gel was dried and subjected to autoradiography. The undigested ER-DNA complex (C0) and ER-DNA complexes formed with chymotrypsin-proteolyzed receptor (C1 - C5) are indicated.

· Partially purified ER and A2 or pS2 ERE-containing DNA fragments were combined as in panel A except that 0, 0.05, 0.5, 1.25, 2.5, 3.75, or 5 ng trypsin was added to the binding reactions. The undigested ER-DNA complex (T0) and ER-DNA complexes formed with trypsin-proteolyzed receptor (T1 - T4) are indicated. (From Wood *et al*, 1998).

Increasing amounts of chymotrypsin were added to the reactions and the resulting protein-DNA complexes were fractionated on a non-denaturing, acrylamide gel. The differences in the digestion patterns observed with the A2 ERE-bound ER and the pS2 ERE-bound ER were striking (**Fig**. **3A**). Limited chymotrypsin digestion of the A2 ERE-bound ER produced a larger stable ER-DNA complex (**Fig**. **3A**, C3) than that observed with the pS2 ERE-bound ER (C5). The number of intermediate ER-DNA complexes observed with A2 and pS2 ERE-bound ER was also quite distinct. While chymotrypsin digestion of the A2 ERE-bound receptor produced several ER-DNA complexes of intermediate size, digestion of the pS2 ERE-bound ER produced few intermediate sized ER-DNA complexes. The difference in digestion patterns observed with these two EREs was not due to differences in ER or DNA concentrations, chymotrypsin concentrations, association of different proteins with the ER, or increased dissociation of the ER from the pS2 ERE as discussed previously (Wood, Greene, and Nardulli 1998). Proteolysis of the A2 and pS2 ERE-bound ER with trypsin also produced very distinct digestion patterns (**Fig**. **3B**). The pS2 ERE-bound receptor appeared to be particularly susceptible to trypsin cleavage as evidenced by the loss of ER-DNA complex at higher trypsin concentration. Therefore, we believe that the different digestion patterns we observed with the A2 and pS2 ERE-bound ER resulted from differences in receptor conformation and that the conformation was dictated by the ERE sequence.

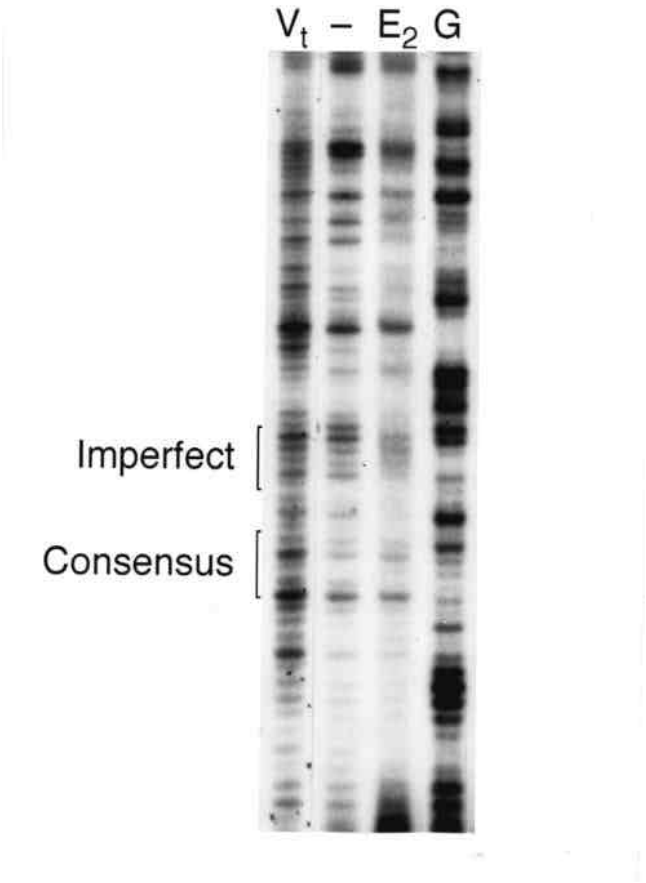

Fig. 4. DNase I *in vivo* footprinting of the pS2 ERE. MCF-7 cells, which had been maintained on serum-free medium for six days, were exposed to either control vehicle (-) or 10 nM E_2 (E_2) for 24 hours and then treated with lysolecithin and DNase I. Genomic DNA was isolated and used in *in vivo* footprinting. Naked genomic DNA samples, which had been treated *in vitro* with either DNase I (V_t) or DMS (G), were included as references. Consensus and imperfect ERE half site locations are indicated.

V. Both the unoccupied and estrogen-occupied receptor may be involved in regulating estrogen-responsive genes

Our long term goal is not to understand how the ER interacts with supercoiled plasmids in transfection assays or linear DNA fragments in acrylamide gels, but rather to understand how estrogen-responsive genes are regulated in target cells. To define how the ERE is involved in regulating endogenous genes in living cells, we have used *in vivo* footprinting to examine the endogenous pS2 ERE in intact MCF-7 breast cancer cells.

MCF-7 cells were treated with either control vehicle or 10 nM E_2 and then exposed to DNase I. DNase I-treated genomic DNA was used in ligation mediated polymerase chain reaction (LMPCR) footprinting analysis (Mueller and Wold, 1992) to examine the pS2 ERE, which is located from -393 to -405 relative to the transcription start site of the pS2 gene (Nunez et al., 1989). When *in vitro*-treated DNA was compared to *in vivo*-treated DNA, nucleotides within and adjacent to the consensus ERE half site appeared to be occupied in the absence of hormone (**Fig**. **4**, Compare V_t and -). Exposure of MCF-7 cells to E_2 resulted in a more extensive pattern of protection, which included both the imperfect and consensus ERE half sites (**Fig**. **4**, E_2). The occupation of the pS2 ERE in the presence and in the absence of hormone suggests that the ERE may be involved in silencing as well as activation of the pS2 gene. It should also be noted that other DNA regions 3' of the ERE were protected by proteins suggesting that the ER-occupied receptor may recruit other proteins to the ERE.

VI. *In vivo* footprinting suggests that unoccupied, estrogen-occupied and antiestrogen-occupied ER recruit different sets of transcription factors to the pS2 5' flanking region

It has become clear from various studies that ligand binding alters receptor conformation (Beekman et al., 1993; Bourguet et al., 1995; Hansen and Gorski, 1986; Renaud et al., 1995; Wagner et al., 1995). Recent crystal structure studies demonstrate that E_2 and raloxifene induce distinct differences in the conformation of the ligand binding domain (Brzozowski et al., 1997). To determine whether ligand-induced changes in ER conformation are involved in recruitment of different proteins to the ERE, *in vivo* footprinting experiments were carried out to examine the endogenous pS2 gene in MCF-7 cells that had or had not been exposed to hormone and subjected to DMS treatment. LMPCR footprinting analysis (Mueller and Wold, 1992) indicates that distinct differences were apparent in the footprinting patterns when MCF-7 cells had or had not been exposed to E_2. When MCF-7 cells were maintained in an estrogen-free environment, the footprinting pattern observed was very similar to that of *in vitro* DMS-treated naked, genomic DNA, except that three adenine residues (**Fig**. **5**, Compare G and -), one of which was located in the consensus ERE half site, displayed an increased sensitivity to DMS methylation. When cells were exposed to E_2, one guanine residue in the imperfect ERE half site was protected and the adenine residue in the consensus ERE half site again displayed increased sensitivity to DMS methylation (**Fig**. **5**, E_2). Even more striking was that the pattern of protection extended to include sequences flanking both sides of the ERE and multiple regions 3' of the ERE. These findings sup-

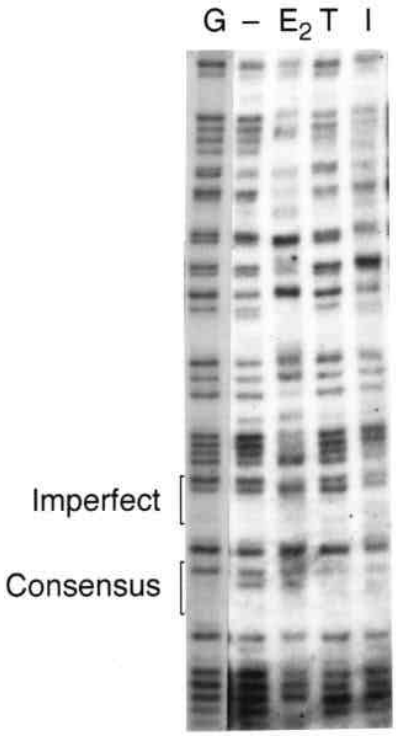

Fig. 5. DMS *in vivo* footprinting of the pS2 ERE. MCF-7 cells were exposed to either control vehicle (-), 10 nM E_2 (E_2), 100 nM 4-hydroxytamoxifen (T), or 100 nM ICI 182,780 (I) for 2 hours and treated with DMS. Genomic DNA was isolated and used in *in vivo* footprinting. Naked genomic DNA that had been treated *in vitro* with DMS (G) was included for reference. Consensus and imperfect ERE half site locations are indicated.

port the idea that the E_2-occupied, ERE-bound receptor was involved in recruiting numerous other proteins to the pS2 5' flanking region.

When MCF-7 cells were treated with 4-hydroxytamoxifen, the footprinting pattern observed was strikingly similar to that of *in vitro* DMS-treated naked DNA, except that a guanine residue in the consensus ERE half site (**Fig**. **5**, T) and two guanines in more distant regions 3' of the ERE were protected. Thus, 4-hydroxytamoxifen treatment of MCF-7 cells resulted in minimal changes in the protection of this region of the pS2 5' flanking region. When MCF-7 cells were treated with ICI 182,780, a very different and distinct footprinting pattern was observed. Guanine residues in the consensus (**Fig**. **5**, I) and imperfect ERE half sites and adjacent nucleotide sequences were protected. Numerous changes in protein-DNA interactions were also observed at multiple sites 3' of the ERE. Thus, the two antiestrogens tested, one a partial agonist/antagonist and the other a pure antagonist, produced very different footprinting patterns.

The divergent footprinting patterns observed with control-, estrogen-, and antiestrogen-treated MCF-7 cells suggest that unoccupied, estrogen-occupied, and antiestrogen-occupied ER associate with different sets of coactivator and/or corepressor proteins and that these proteins in turn form an interconnected protein-DNA complex, which serves to modulate gene expression.

The extensive pattern of protection flanking the ERE suggests that this region is intimately associated with a number of proteins. Numerous coactivators and corepressors associated with the steroid receptors have been identified (Horwitz et al., 1996). Recent studies have also identified coactivator and corepressor proteins with histone acetylase and deactylase activities, respectively (Heinzel et al., 1997; Nagy et al., 1997; Pazin and Kadonaga, 1997). Association of ER with these coregulators could be important in modulating chromatin structure and the accessibility of transcription factor binding sites in native chromatin. Estrogen treatment could release corepressor proteins and promote interaction of the receptor with coactivators resulting in changes in local chromatin structure.

VII. The basal promoter is poised for transcription even in the absence of hormone

TATA and CAAT boxes are often present in the proximal promoters of inducible genes and are involved in formation of the basal transcription complex. The footprinting patterns in the TATA and CAAT regions of the pS2 gene (Jeltsch et al., 1987) were quite similar when MCF-7 cells had been treated with either control vehicle or E_2 and then exposed to DNase I (**Fig**. **6**). Interestingly, DNase I hypersensitive sites (*) were observed flanking the TATA and CAAT sequences in both control vehicle- and E_2-treated cells. The presence of hypersensitive sites in these regions suggests that protein-induced conformational changes in DNA structure brought about by binding of transcription factors to these regions may enhance the susceptibility of specific nucleotides to DNase I cleavage (Suck, 1994). The ability of the TATA binding protein to induce DNA bending has been demonstrated (Kim et al., 1993) and is consistent with this hypothesis. Since the TATA and CAAT sequences were flanked by hypersensitive sites before and after hormone treatment, it appears that the basal promoter is accessible and poised for transcription even in the absence of hormone.

Fig. 6. DNase I *in vivo* footprinting of the pS2 TATA and CAAT sequences. MCF-7 cells were treated with control vehicle (-) or 10 nM E_2 (E_2) and then exposed to DNase I. Genomic DNA was isolated and used in *in vivo* footprinting. Naked genomic DNA samples, which had been treated *in vitro* with either DNase I (V_t) or DMS (G), were included as references. Regions of DNase I hypersensitivity are indicated (*). TATA and CAAT sequence positions are noted.

VIII. Changes in ER conformation induced by ligand and ERE sequence may play a role in regulating estrogen-responsive genes

A number of studies have demonstrated that the activity of many ERE-containing promoters is cell-type specific (Berry et al., 1990; Metzger et al., 1995; Montano et al., 1996; Tora et al., 1989; Tzukerman et al., 1994). It is generally thought that these tissue-specific effects are brought about by restricting the expression of required regulatory cofactors to target cells. A more versatile way of differentially regulating gene expression would be to provide the receptor with a large repertoire of functional surfaces that can be formed and serve as contact points for other cellular proteins. The presentation of these functional surfaces and the selection of ER-associated proteins would provide tremendous regulatory versatility to a single cell harboring multiple estrogen-responsive genes. Thus, it appears that the estrogen receptor may be subject to two ligands: hormone and DNA. Binding of either ligand could induce changes in receptor conformation that could lead to recruitment of different sets of proteins to the 5' flanking region of estrogen-responsive genes in target cells and ultimately result in differential gene expression.

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