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# Regulation of the estrogen-responsive pS2 gene in MCF-7 human breast cancer cells

Jongsook Kim [a], Larry N. Petz [a], Yvonne S. Ziegler [a], Jennifer R. Wood [a], Sara J. Potthoff [b], Ann M. Nardulli [a,*]

[a] *Department of Molecular and Integrative Physiology, University of Illinois at Urbana-Champaign, 524 Burrill Hall, 407 South Goodwin Ave., Urbana, IL 61801, USA*

[b] *Department of Biochemistry, University of Illinois, Urbana, IL 61801, USA*

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**Abstract**

To understand how hormones and antihormones regulate transcription of estrogen-responsive genes, in vivo footprinting was used to examine the endogenous pS2 gene in MCF-7 cells. While the consensus pS2 estrogen response element (ERE) half site was protected in the absence of hormone, both the consensus and imperfect ERE half sites were protected in the presence of estrogen. 4-Hydroxytamoxifen and ICI 182,780 elicited distinct footprinting patterns, which differed from those observed with vehicle- or with estrogen-treated cells suggesting that the partial agonist/antagonist and antagonist properties of 4-hydroxytamoxifen or ICI 182,780, respectively, may be partially explained by modulation of protein-DNA interactions. Footprinting patterns in and around the TATA and CAAT sequences were identical in the presence and in the absence of estrogen suggesting that the basal promoter is accessible and poised for transcription even in the absence of hormone. In vitro DNase I footprinting experiments demonstrated that the estrogen receptor bound to the pS2 ERE and that adjacent nucleotides were protected by MCF-7 nuclear proteins. These findings indicate that transcription of the pS2 gene is modulated by alterations in protein binding to multiple sites upstream of the basal promoter, but not by changes in protein-DNA interactions in the basal promoter. 

*Keywords:* Estrogen; Antiestrogen; Transcription; Chromatin

## 1. Introduction

Estrogen is a hormone of crucial importance in the development and maintenance of normal reproductive function and is also involved in initiation and proliferation of mammary tumors. Estrogen's actions are mediated through the intracellular estrogen receptor (ER), which interacts with estrogen response elements (EREs) in target genes to initiate changes in gene transcription [1]. In addition to the previously identified ERα, a second receptor, ERβ, has been identified and has demonstrated the ability to induce transcription of ERE-containing reporter plasmids [2,3].

The pS2 gene is an estrogen-responsive gene, which is expressed in breast cancer cells, but not in normal mammary cells [4,5]. Exposure of MCF-7 human breast cancer cells to 17β-estradiol ($E_2$) activates transcription of the single-copy pS2 gene [6] and results in increased levels of pS2 mRNA and secreted protein [5,7–9]. Transient transfection assays have demonstrated that a single imperfect ERE confers estrogen-responsiveness to this gene [10].

The pS2 gene provides an ideal model system to study estrogen-regulated gene expression. The pS2 5′ flanking region contains the elements of a classic estrogen-responsive gene including a TATA box, a CAAT box, and a single imperfect ERE [6,10]. In addition, expression of the pS2 gene has been used as a marker of estrogen responsiveness in ER-containing breast cancer cells, an indicator of disease progression, and a predic-

* Corresponding author. Tel.: +1-217-244-5679; fax: +1-217-333-1133.

*E-mail address:* anardull@life.uiuc.edu (A.M. Nardulli).

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tor of the success of antiestrogen therapy in breast cancer patients [9–13]. Thus, by examining the pS2 gene in MCF-7 cells, we can not only learn about general mechanisms involved in regulation of estrogen-responsive genes, but also gain specific insight into how the pS2 gene is regulated in human breast cancer cells.

Numerous studies aimed at delineating how estrogen-responsive genes are regulated have included the use of transient transfection assays. While these studies have provided us with a great wealth of information, transfection experiments have significant limitations. Cells transfected with receptor expression vectors typically contain much higher receptor levels than are found in normal target cells. This overexpression can lead to increased basal transcription and anomalous expression of reporter plasmids. Cells used in transfection assays may not contain accessory proteins needed for proper regulation and if present, these proteins may be present in limiting quantities. Multicopy reporter plasmids often contain synthetic promoters and sequences that bear little resemblance to native genes. Finally, the supercoiled plasmids used in transfection studies fail to take into account the role of native chromatin structure in regulating gene transcription.

To gain a better understanding of how endogenous, naturally occurring estrogen-responsive genes are regulated, we have used in vivo ligation mediated polymerase chain reaction (LMPCR) footprinting analysis to examine the 5′ flanking region of the endogenous estrogen-responsive pS2 gene in MCF-7 human breast cancer cells. By complementing these in vivo studies with in vitro footprinting and DNA binding assays, we have examined the role the ERE and the basal promoter in regulating pS2 gene expression and begun to define mechanisms by which estrogen and antiestrogens mediate their effects in target cells.

## 2. Materials and methods

### 2.1. Cell culture and ER preparations

MCF-7 (K1) cells [14] were maintained in Eagle's Minimum Essential Medium (MEM) supplemented with 5% fetal calf serum and then transferred to MEM with 5% charcoal stripped [15] calf serum for 5 days and serum-free Improved MEM with 6 μg/ml transferrin, 0.25 mg/ml bovine serum albumin, 6 ng/ml insulin and 3.75 ng/ml hydrocortisone [16] 6 days before experiments were initiated. MCF-7 (K3) cells [14] were maintained in MEM with 5% charcoal stripped calf serum, harvested, and incubated with 10 nM $E_2$ for 20 min. Nuclear extracts were prepared as described [17], except that nuclei were extracted with buffer containing 0.5 M KCl. Viral stock for the production of ERα was kindly provided by J. Kadonaga and L. Kraus, University of California, San Diego, CA. ERα was expressed and purified as described by Kraus and Kadonaga [18].

### 2.2. Northern blot analysis

MCF-7 cells were exposed to control vehicle or 10 nM $E_2$ for 0.25–24 h. Total RNA was isolated using the Ultraspec RNA isolation system (Biotecx, Houston, TX) according to the manufacturer's instructions. A total of 10 μg of total RNA was fractionated on a 1.5% agarose gel and transferred to a nylon membrane. Hexamer primed $^{32}$P-labeled pS2 and 36B4 [5,9] DNA fragments were used to probe the nylon membrane. Bands were visualized by autoradiography and quantitated using a phosphorimager and Imagequant software (Molecular Dynamics, Sunnyvale, CA).

### 2.3. In vivo footprinting

For DNase I footprinting, MCF-7 cells, which had been treated with vehicle control or $E_2$ for 24 h, were permeabilized with 0.5% NP-40 for 3 min at 4°C or 0.2 mg/ml lysolecithin for 1 min at 25°C and treated with 750 U DNase I/ml. For dimethylsulfate (DMS) footprinting, MCF-7 cells, which had been treated with vehicle control or hormone for 2 h, were treated with 0.1% DMS for 2 min at 25°C. Isolation of genomic DNA and LMPCR footprinting was carried out essentially as described by Mueller and Wold [19]. A total of 1–2 μg genomic DNA was subjected to LMPCR using nested primers, which annealed to DNA sequences in the pS2 gene regions of interest. The linker primer oligos LMPCR1 and LMPCR2 described by Mueller and Wold [19] were also used. However, the first two 5′ nucleotides of LMPCR1 were omitted to limit secondary structure formation. The primers used to examine the pS2 ERE on the non-coding strand were: primer 1, 5′GGGATTACAGCGTGAGCCACTGC3′; primer 2, 5′AAAGAATTAGCTTAGGCCTAGACGGAATGG3′; and primer 3, 5′CTTAGGCCTAGACGGAATGGGCTTCAT3′. The annealing temperatures used for the primers were 60, 62, and 64°C, respectively. Excess primer 2 was removed using biotinlylated LMPCR1 and the Linker Tag Selection method [20]. Nested primers used to examine the basal promoter on the coding strand were: primer 4, 5′GGGCGCAGATCACCTTGTTC3′; primer 5, 5′GCCATTGCCTCCTCTCTGCTCC3′; and primer 6, 5′CCATTGCCTCCTCTCTGCTCCAAAGG3′. The annealing temperatures for these oligos were 56, 61, and 67°C, respectively.

### 2.4. Plasmid construction

To create pTZpS2(−666/+75), a 741-bp DNA fragment containing sequence from the 5′ flanking re-

gion of the pS2 gene was synthesized by PCR amplification of −666 to +75 of the pS2 gene using sequence specific primers. The amplified, blunt-ended DNA fragment was inserted into Sma I-cut, dephosphorylated pTZ18U. Insert junctions were checked using DNA sequencing and the plasmids were purified on cesium chloride gradients.

### 2.5. In vitro DNase I footprinting

To examine the pS2 ERE, primers, which annealed 71 bp upstream (pS2for3 5′GCGCCAGG CCTACAATTTCATTATTAAAACCAA3′) and 87 bp downstream (pS2rev3 5′CAGGTCCTACTCATATCTGAGAGGCCCTCCC3′) of the pS2 ERE were subjected to 30 rounds of PCR amplification with 30 ng of pTZpS2(−666/+75) to produce 235-bp fragments. To examine the basal promoter elements, primers, which annealed 90 bp upstream (UpS2TATA.3 5′ATGTAGCTTGACCATGTCTAGGAAACACCTTTGAT3′) and 45 bp downstream (primer 6) of the TATA sequence were subjected to 30 rounds of PCR amplification with 30 ng of pTZpS2(−666/+75) to produce 203-bp DNA fragments. Labeling of the ERE- or TATA-containing DNA fragments was carried out with $^{32}$P-labeled pS2rev3 or primer 6, respectively. The singly end-labeled amplified fragments were fractionated on an acrylamide gel and isolated. End-labeled DNA fragments (100 000 cpm) containing the pS2 ERE or basal promoter elements were incubated for 15 min at room temperature in a buffer containing 10% glycerol, 50 mM KCl, 15 mM Tris, pH 7.9, 0.2 mM EDTA, 1 mM $MgCl_2$, 50 ng of poly dI/dC and 4 mM DTT in a final volume of 50 μl with either 0–60 μg of nuclear extract from $E_2$-treated MCF-7 cells or 0.6–2.4 pmol of purified flag-tagged, $E_2$-occupied ER. Bovine serum albumin or ovalbumin was included in the binding reaction so that the total protein concentration in each reaction was 2.5 μg for purified ER or 60 μg for nuclear extracts. Poly dI/dC was increased to 1 μg per reaction when nuclear extracts were used. A total of 1–2 U of RQ1 ribonuclease-free DNase I (Promega, Madison, WI) was added to each sample and incubated at room temperature for 0.75–8 min. The DNase I digestion was terminated by addition of stop solution (200 mM NaCl, 1% SDS, 30 mM EDTA and 100 ng/μl tRNA). DNA was phenol/chloroform extracted, precipitated, fractionated on an 8% denaturing acrylamide gel. Radioactive bands were visualized by autoradiography.

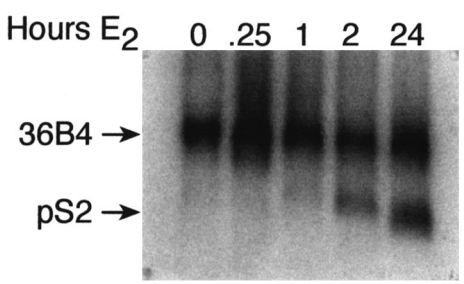


Fig. 1. Effects of $E_2$ on pS2 mRNA production in MCF-7 breast cancer cells assessed by Northern blot analysis. Total RNA was isolated from MCF-7 cells that had been treated with $E_2$ for varying periods of time, fractionated on an agarose gel, and transferred to a nylon membrane. $^{32}$P-labeled pS2 and 36B4 probes were used to detect the mRNAs. Equivalency of mRNA loading was monitored by normalization to levels of constitutively-expressed 36B4.

### 2.6. Gel mobility shift assays

Gel mobility shift assays were carried out essentially as described [21,22]. $^{32}$P-labeled (10 000 cpm) 235-bp DNA fragments containing the pS2 ERE were incubated for 15 min at room temperature in a buffer containing 10% glycerol, 50 mM KCl, 15 mM Tris, pH 7.9, 0.2 mM EDTA, 50 ng of poly dI/dC and 4 mM DTT in a final volume of 20 μl with either 10 μg of MCF-7 nuclear extract or 90 fmol of purified ER. BSA was included when purified ER was used so that the total protein concentration in each reaction was 2.5 μg. When MCF-7 nuclear extracts were used, the non-specific DNA for each reaction included 1 μg of salmon sperm DNA and 2 μg poly dI/dC. For antibody supershift experiments, the ER-specific monoclonal antibody, h151 (kindly provided by Dean Edwards, University of Colorado, Denver, CO) was added to the protein-DNA mixture and incubated for 5 min at room temperature. Low ionic strength gels and buffers were prepared as described [23]. Radioactive bands were visualized by autoradiography.

## 3. Results

### 3.1. Estrogen treatment of MCF-7 cells increases pS2 mRNA levels

In order to limit basal expression of the pS2 gene, MCF-7 breast cancer cells were maintained on serum free medium for 6 days and then exposed to either control vehicle or $E_2$ for 0.25–24 h. The 10-nM $E_2$ concentration used in these studies has been shown to fully occupy the receptor and maximally stimulate pS2 gene expression [24]. Basal and $E_2$-induced pS2 mRNA levels present in MCF-7 cells were determined using Northern blot analysis. Increased levels of pS2 mRNA were detected after a 1-h exposure of cells to $E_2$ and continued to increase up to 24 h (Fig. 1). In contrast, the level of constitutively expressed 36B4 mRNA [9], which was used as an internal control, remained constant. Quantitation from four independent determinations demonstrated that pS2 mRNA transcripts increased 16-fold after 24 h of $E_2$ treatment. These

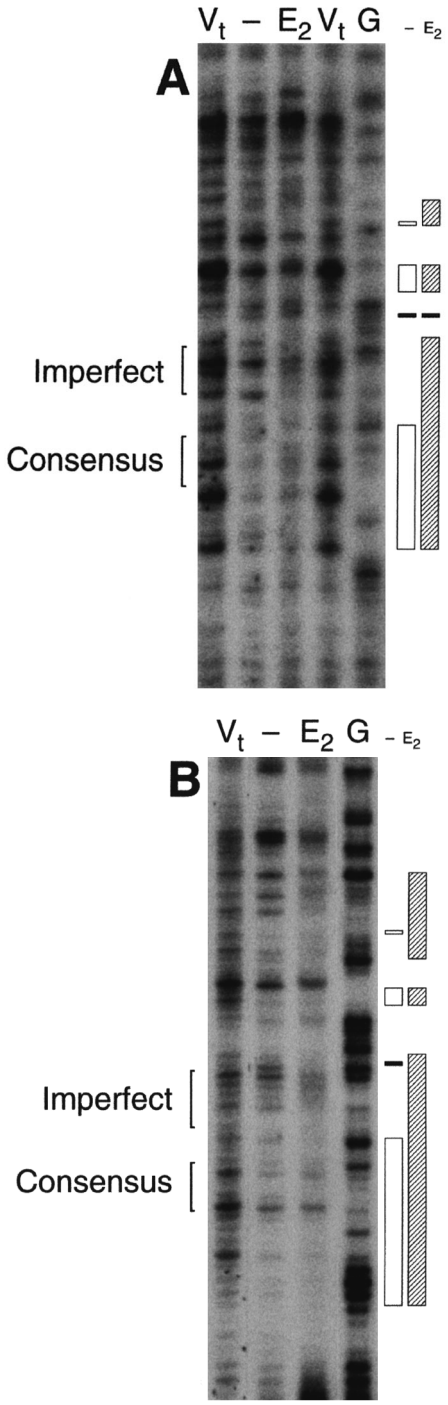


Fig. 2. In vivo DNase I footprinting of the pS2 ERE. MCF-7 cells were exposed to control vehicle (–) or $E_2$ and then treated with either NP-40/DNase I (panel A) or lysolecithin/DNase I (panel B). Genomic DNA was isolated and used for in vivo LMPCR footprinting. Naked genomic DNA samples, which had been treated in vitro with either DNase I ($V_t$) or DMS (G), were included as references. Nucleotides protected in control (open bars) or $E_2$ (hatched bars) treated cells are indicated to the right of the autoradiogram. Solid bars indicate regions of DNase I hypersensitivity.

findings are in good agreement with previous determinations [8–10,24] and indicate that the pS2 gene was essentially quiescent in the absence of exogenously added $E_2$ but responded robustly to $E_2$ treatment. Thus, we were assured that the serum-free conditions utilized in our studies fully supported pS2 gene expression.

### *3.2. In vivo DNase I footprinting reveals protection of the endogenous pS2 ERE*

To define how the ERE is involved in regulating endogenous target genes in living cells, we used in vivo DNase I footprinting to examine the endogenous pS2 ERE residing in native chromatin in MCF-7 cells. This technique utilizes the non-specific cleavage properties of DNase I to identify DNA regions that are protected by proteins. The pS2 ERE is located from −393 to −405 relative to the transcription initiation site and is comprised of a 5′ consensus ERE half site and a 3′ imperfect ERE half site (5′GGTCAnnnTGGCC3′; [10]).

MCF-7 cells were treated with ethanol vehicle or $E_2$, harvested, and then exposed to an NP40/DNase I mixture to cleave accessible DNA sequences that were not protected by proteins. Cells were lysed, DNA was isolated, and LMPCR procedures were carried out. Isolated, naked genomic DNA was treated with DNase I in vitro and used as a reference to identify DNA sequences that were susceptible to cleavage in the absence of proteins. A comparison of in vitro- and in vivo-cleaved genomic DNA revealed that the consensus ERE half site and adjacent sequences were protected when MCF-7 cells were maintained in a hormone-free environment (Fig. 2A, compare $V_t$ and –). Regions of protection (open bars) and DNase I hypersensitivity (solid bar) were also observed in the absence of hormone. When MCF-7 cells were treated with $E_2$ and subjected to in vivo DNase I footprinting procedures, regions of protection (Fig. 2A, $E_2$, hatched bars) and enhanced cleavage (solid bar) were similar to those observed in the absence of hormone. However, the protection at the ERE was extended to include the imperfect ERE half site and adjacent nucleotide sequence.

As a comparison, in vivo DNase I footprinting experiments were also carried out using MCF-7 cells that had been treated with a lysolecithin/DNase I mixture. When in vitro-treated DNA was compared to in vivo-treated DNA, nucleotides within and adjacent to the consensus ERE half site were protected in the absence of hormone (Fig. 2B, compare $V_t$ and –, open bar). DNase I hypersensitivity and regions of protection were also observed adjacent to the imperfect ERE half site. Exposure of MCF-7 cells to $E_2$ resulted in a more extensive pattern of protection than was observed in the absence of hormone. Both the imperfect and consensus ERE half sites were protected and nucleotides adjacent to the imperfect ERE half site were more extensively protected when cells were exposed to $E_2$ (Fig. 2B, $E_2$, hatched bars).

Similar footprinting patterns were observed in the region of the pS2 ERE in four to five independent LMPCR experiments using two sets of MCF-7 cells that had been permeabilized with either NP40 or lysolecithin. Although some variation in individual nucleotides protected was detected, which probably resulted from slightly different amounts of DNase I entry into the cells, it was clear that the overall observations were quite similar. The consensus ERE half site was protected in the absence of hormone, both ERE half sites were occupied in the presence of $E_2$, and sites adjacent to the imperfect ERE half site were more extensively protected after $E_2$ treatment.

### 3.3. DMS footprinting delineates nucleotides involved in modulating pS2 expression

In order to obtain additional information about proteins interacting with the pS2 5′ flanking region, MCF-7 cells were treated with control vehicle or $E_2$ and then exposed to DMS in order to methylate individual guanine residues that were not intimately associated with proteins. Cells were lysed, DNA was isolated, methylated guanines were cleaved with piperidine, and LMPCR procedures were carried out. 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 cultured in a hormone-free environment, the footprinting pattern observed was very similar to that of in vitro DMS-treated naked, genomic DNA, except that three adenine residues, one of which was located in the consensus ERE half site, displayed an increased sensitivity to DMS methylation (Fig. 3, compare G and –). Enhanced DMS cleavage of adenine residues can result from binding of a protein to the major groove of the DNA helix and/or distortion of the DNA helix causing adenine residues in the minor groove to become more accessible to DMS treatment. Only one guanine residue in this region appeared to be protected in the absence of hormone.

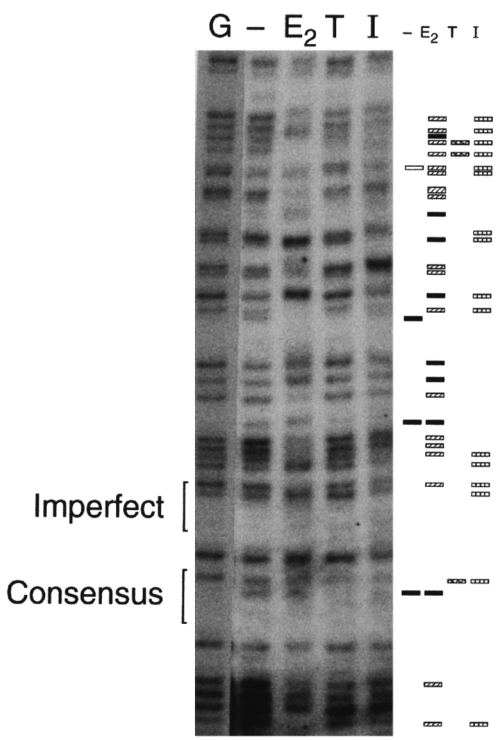


Fig. 3. In vivo DMS footprinting of the pS2 ERE. MCF-7 cells were exposed to control vehicle ( – ), 10 nM $E_2$ ($E_2$), 100 nM 4-hydroxytamoxifen (T), or 100 nM ICI 182,780 (I) and then treated with DMS. Genomic DNA was isolated and used in in vivo LMPCR footprinting. A naked genomic DNA sample, which had been treated in vitro with DMS (G), was included for reference. Nucleotides protected in control (open bars), $E_2$ (hatched bars), 4-hydroxytamoxifen (crosshatched) or ICI 182,780 (striped bars) treated cells are indicated to the right of the autoradiogram. Solid bars indicate regions of enhanced cleavage.

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 displayed increased sensitivity to DMS methylation (Fig. 3, $E_2$), as was observed in the absence of hormone. Even more striking was that the pattern of protection extended to include sequences flanking both sides of the ERE and multiple regions adjacent to the imperfect ERE half site. Enhanced adenine and guanine cleavage was also observed. These findings reinforced the idea that $E_2$ was not only affecting the interaction of protein(s) with the ERE, but multiple other protein-DNA interactions as well, particularly in regions adjacent to the imperfect ERE.

### 3.4. Treatment of MCF-7 cells with antiestrogens elicits unique footprinting patterns

A subject of great clinical interest has been to delineate how tamoxifen, an antiestrogen with agonistic and antagonistic properties, and ICI 182,780, a pure antiestrogen, inhibit disease recurrence in breast cancer patients [25–27]. Although 4-hydroxytamoxifen has a weak agonistic effect on pS2 mRNA levels, ICI 182,780 does not increase pS2 RNA levels [24,28,29]. The effects of these compounds have been studied using in vitro DNA binding assays and transient transfection assays, but their effects on protein-DNA interactions at the molecular level in a native gene have not been addressed.

When MCF-7 cells were treated with 4-hydroxytamoxifen, the footprinting pattern observed was strikingly similar to that of in vitro DMS-treated naked genomic DNA, except that a guanine residue in the consensus ERE half site (Fig. 3, T, cross hatched bars) and two more distant regions 3′ of the ERE were strongly protected. Thus, treatment of MCF-7 cells with 4-hydroxytamoxifen resulted in minimal changes

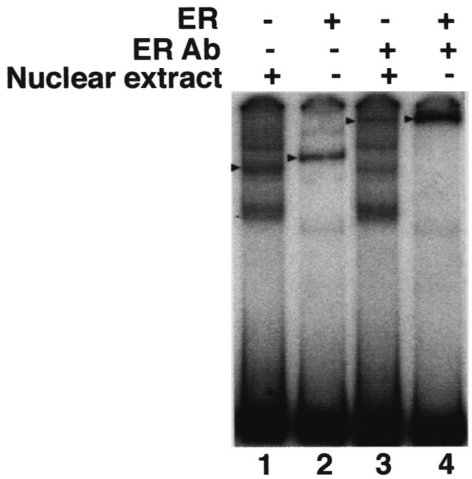


Fig. 4. Binding of MCF-7 and purified ER to the ERE-containing DNA fragments. The 235-bp $^{32}$P-labeled DNA fragments containing pS2 5′ flanking region from −505 to −270 were incubated with nuclear extracts from $E_2$-treated MCF-7 cells (lanes 1 and 3) or purified ER (lanes 2 and 4). The ER-specific antibody h151 (ER Ab) was added to the binding reaction as indicated. The $^{32}$P-labeled oligos were fractionated on a non-denaturing gel and visualized by autoradiography. Arrowheads (►) indicate ER-containing DNA complexes.

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. 3, I, striped bars) and imperfect ERE half sites and adjacent nucleotide sequence were protected. Numerous changes in protein-DNA interactions were also observed at multiple sites adjacent to the imperfect ERE half site. Thus, the two antiestrogens tested, one a partial agonist/antagonist and the other a pure antagonist, produced very different footprinting patterns.

### *3.5. The ER and other proteins interact with the pS2 promoter in vitro*

In vivo footprinting is a powerful technique which can identify cis elements involved in mediating changes in transcription. However, it cannot be used to identify factors bound to these elements. Thus, a series of in vitro experiments was carried out to characterize the interaction of proteins with the pS2 promoter. Since ERα is present at high levels in MCF-7 cells [14], but ERβ is not expressed [30], it seemed likely that ERα was responsible for the protection of the pS2 ERE seen in our in vivo footprinting experiments. To determine if this was the case, gel mobility shift experiments were carried out. When $^{32}$P-labeled DNA fragments, each comprised of 235 bp of pS2 of 5′ flanking region (−505 to −270), were incubated with MCF-7 nuclear extracts, several gel-shifted bands were observed (Fig. 4, lane 1). In contrast, when purified, flag-tagged ERα (hereafter referred to as ER) was used, a single gel-shifted band was present (lane 2). Addition of the ER-specific antibody h151 to the binding reaction containing nuclear extract resulted in the disappearance of a single band (lane 1, ►), which migrated slightly faster than the purified ER-DNA complex (lane 2, ►), and the appearance of a more slowly migrating, supershifted band (lane 3, ►). As expected, the ER-specific antibody efficiently supershifted the receptor-DNA complex (lane 4, ►). These findings indicate that the protein-DNA complex, which migrated slightly faster than the purified ER-DNA complex, contained the MCF-7 ER. The difference in migration of the MCF-7 ER-DNA complex and the purified ER-DNA complexes most likely resulted from the slightly larger size of the flag-tagged, purified ER or minimal proteolytic cleavage of the MCF-7 ER. These experiments demonstrate that the ER from MCF-7 nuclear extracts and purified ER bind to the region of the pS2 promoter containing the ERE.

In vitro DNase I footprinting was carried out to determine if the pS2 ERE was protected by protein present in nuclear extracts of $E_2$-treated MCF-7 cells. When $^{32}$P-labeled DNA fragments containing 235 bp from −505 to −270 of the pS2 5′ flanking sequence were combined with increasing amounts of nuclear extract, the consensus ERE half site was more extensively protected than the imperfect ERE half site and regions adjacent to the imperfect ERE half site were protected (Fig. 5). Several regions of hypersensitivity were also observed. Experiments carried out with nuclear extracts from MCF-7 cells that had been treated with ethanol vehicle produced identical footprints (data not shown). Kraus and Kadonaga [18] have demonstrated that when an ERE-containing DNA template, which does not respond to hormone in vitro, is assembled into nucleosomes, it regains estrogen responsiveness suggesting that appropriately structured DNA is required for hormone-regulated gene expression in vivo and in vitro.

To determine whether the ER was able to bind to the ERE in the absence of other MCF-7 nuclear proteins, in vitro DNase I footprinting experiments were also carried out with $E_2$-occupied, purified ER. The 235- bp $^{32}$P-labeled DNA fragments containing the pS2 ERE and flanking sequences were incubated with increasing concentrations of baculovirus-expressed, purified ER. As increasing amounts of purified ER were added to the binding reaction, an incremental increase in protection of the pS2 ERE was observed (Fig. 6). The consensus ERE half site was protected at lower ER concentrations than the imperfect ERE half site demonstrating the receptor's preference for the consensus ERE half site. Regions flanking both sides of the ERE displayed increased hypersensitivity to DNase I cleavage. The region adjacent to the consensus ERE half site was particularly sensitive to DNase I cleavage.

### 3.6. The pS2 basal promoter is poised for transcription even in the absence of hormone

All our in vivo and in vitro assays supported the idea that the pS2 ERE is instrumental in regulation of the pS2 gene. However, the ERE does not function in isolation, but requires the participation of other cis elements for regulated gene expression. Since previous studies have suggested that proteins bound to the ER interact directly or through adapter proteins with transcription factors bound to the basal promoter in order for transcription to occur [31–34], we were particularly interested in examining whether hormone treatment affected the interaction of proteins with the basal promoter. Surprisingly, the footprinting patterns in the TATA and CAAT regions of the pS2 gene were nearly identical when MCF-7 cells were treated with either control vehicle or $E_2$ and then exposed to DNase I (Fig. 7). Two small regions of protection and two extended regions of protection were observed before and after $E_2$ treatment (hatched bars). Interestingly, multiple DNase I hypersensitive sites were observed flanking the TATA

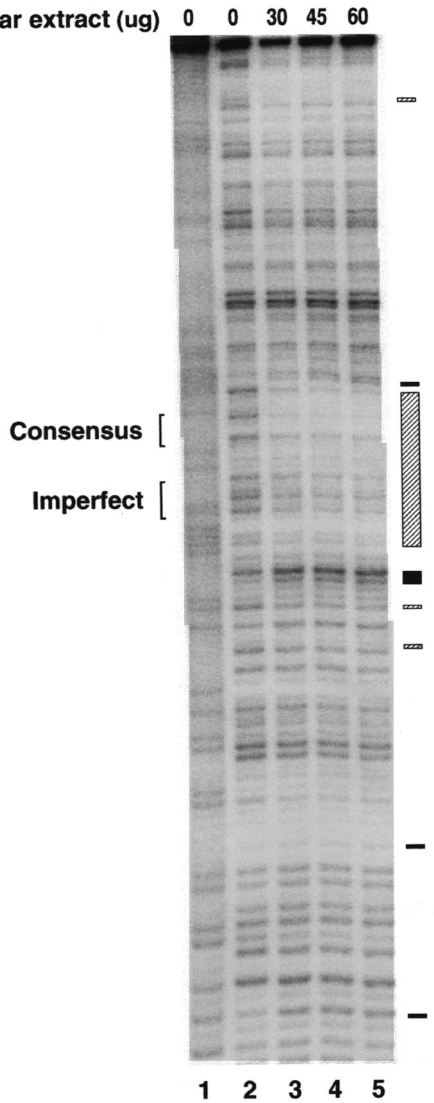


Fig. 5. In vitro DNase I footprinting of the pS2 ERE with MCF-7 nuclear extracts. The 235-bp $^{32}$P-labeled DNA fragments containing pS2 5′ flanking region from −505 to −270 were incubated with increasing concentrations of nuclear extract from $E_2$-treated MCF-7 cells (lanes 3–5). The binding reactions were subjected to limited DNase I digestion and the cleaved DNA fragments were fractionated on a denaturing gel. DNA fragments cleaved in vitro with DMS (lane 1) or DNase I (lanes 2) in the absence of proteins were included as references. The locations of the consensus and imperfect ERE half sites are indicated. Protected (hatched bars) and hypersensitive (solid bars) nucleotides are indicated to the right of the autoradiogram. Because of the labeling procedures used, in vivo and in vitro footprinting patterns are in opposite orientations.

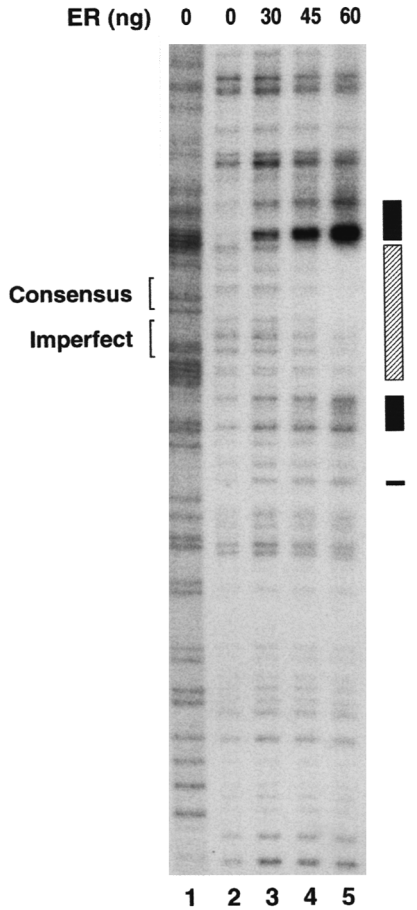


Fig. 6. In vitro DNase I footprinting of the pS2 ERE with purified ER. The 235-bp $^{32}$P-labeled DNA fragments containing pS2 5′ flanking region from −505 to −270 were incubated with increasing concentrations of purified $E_2$-occupied ER (lanes 3–5). The binding reactions were subjected to limited DNase I digestion and the cleaved DNA fragments were fractionated on a denaturing gel. DNA fragments cleaved in vitro with DMS (lane 1) or DNase I (lanes 2) in the absence of proteins were included as references. The locations of the consensus and imperfect ERE half sites are indicated. Protected (hatched bar) and hypersensitive (solid bars) regions are indicated to the right of the autoradiogram. Because of the labeling procedures used, in vivo and in vitro footprinting patterns are in opposite orientations.

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 brought about by binding of transcription factors to these regions may enhance the susceptibility of specific nucleotides to DNase I cleavage [35].

This basal promoter region was also examined using in vitro DNase I footprinting. A 203-bp $^{32}$P-labeled DNA fragment containing the pS2 promoter from $-154$ to $+49$ was incubated with nuclear extracts from $E_2$-treated MCF-7 cells. As seen in the in vivo footprinting experiments, numerous hypersensitive regions flanked both sides of the TATA and CAAT sequences (Fig. 8). Regions of protection were also observed and were particularly evident in the region between the TATA and CAAT sequences.

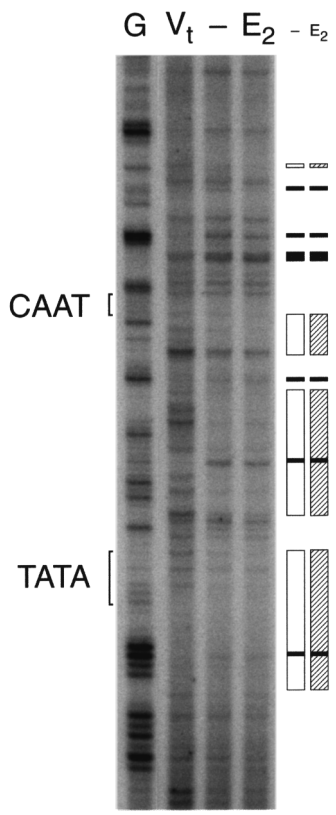


Fig. 7. In vivo DNase I footprinting of the basal promoter. MCF-7 cells were treated with control vehicle (−) or 10 nM $E_2$ ($E_2$) and then exposed to NP40/DNase I. Genomic DNA was isolated and used in in vivo LMPCR footprinting. Naked genomic DNA samples, which had been treated in vitro with either DNase I ($V_t$) or DMS (G), were included as references. Nucleotides protected in control- (open bars) or $E_2$- (hatched bars) treated cells are indicated to the right of the autoradiogram. Solid bars indicate regions of DNase I hypersensitivity.

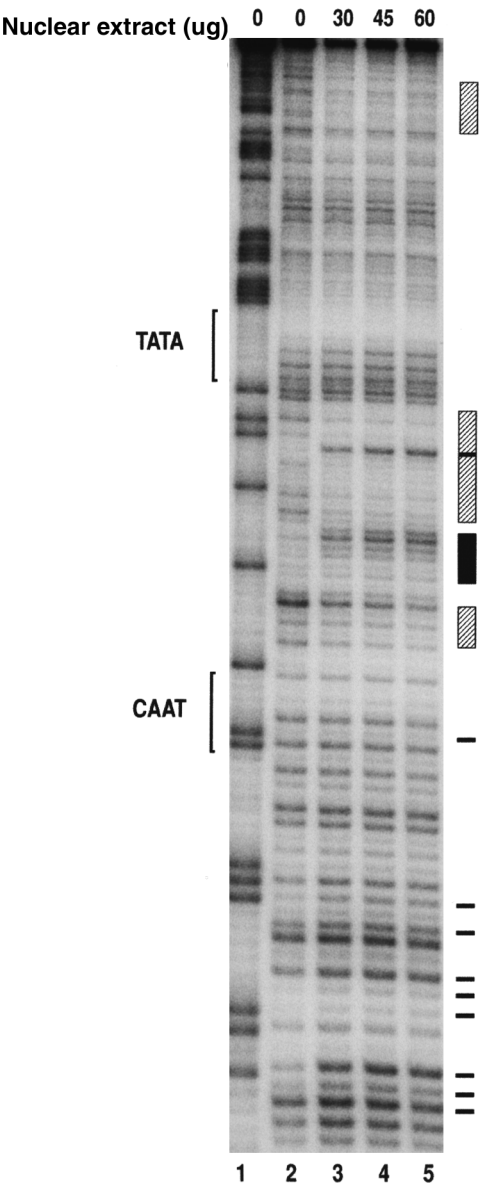


Fig. 8. In vitro DNase I footprinting of the pS2 basal promoter with MCF-7 nuclear extracts. The 203-bp $^{32}$P-labeled DNA fragments containing pS2 5′ flanking region from $-154$ to $+49$ were incubated with increasing concentrations of nuclear extract from $E_2$-treated MCF-7 cells (lanes 3–5). The binding reactions were subjected to limited DNase I digestion and the cleaved DNA fragments were fractionated on a denaturing gel. DNA fragments cleaved in vitro with DMS (lane 1) or DNase I (lanes 2) in the absence of proteins were included as references. The locations of the TATA and CAAT sequences are indicated. Protected (hatched bars) and hypersensitive (solid bars) nucleotides are indicated to the right of the autoradiogram. Because of the labeling procedures used, in vivo and in vitro footprinting patterns are in opposite orientations.

## 4. Discussion

A number of methods have been employed to examine regulation of the estrogen-responsive pS2 gene. Extensive studies carried out by Chambon and coworkers have defined the effects of estrogen treatment on the

synthesis of pS2 mRNA and delineated specific sequences in the pS2 5′ flanking region involved in regulating gene expression [5,7–10,28]. These studies have been extremely informative and have formed the basis of our current understanding of how the pS2 gene is regulated in breast cancer cells. However, in a study comparing the expression of the endogenous pS2 gene and a transiently transfected reporter plasmid containing 1.1 kb of the pS2 5′ flanking region, the endogenous pS2 gene residing in native chromatin responded differently to antiestrogen treatment than the transiently transfected pS2 promoter residing in a supercoiled reporter plasmid [24]. These findings indicate that DNA context may be important for the appropriate regulation of the pS2 gene and that chromatin structure may exert an additional level of control on regulation of this gene.

### 4.1. Role of the ERE in estrogen-regulated pS2 gene expression

To more fully understand how the endogenous estrogen-responsive pS2 gene is regulated, we have used high resolution in vivo footprinting to examine the interaction of proteins with the endogenous pS2 5′ flanking region residing in native chromatin. We found that when MCF-7 cells were treated with $E_2$, the ERE and regions flanking this sequence were extensively protected using both DNase I and DMS in vivo footprinting analysis. DNase I footprinting revealed that the consensus ERE half site was protected in the absence of hormone and that both ERE half sites and adjacent flanking sequences were protected after hormone treatment. The enhanced DMS sensitivity of an adenine residue in the consensus ERE half in the absence and in the presence of $E_2$ and the protection of a guanine residue in the imperfect ERE half site in the presence of $E_2$ further support the DNase I footprinting results.

Our in vivo footprinting analysis complemented by our in vitro binding studies, supports the idea that unoccupied ER is bound to the consensus ERE half site in the absence of hormone and that an $E_2$-occupied ER is bound to both ERE half sites in the presence of hormone. Since we do observe protection of nucleotide sequence adjacent to the consensus ERE half site in the absence of hormone in our in vivo footprints, it is possible that ER binding to the consensus ERE half site could be stabilized by interaction with a protein bound to adjacent nucleotide sequence. The ability of the receptor to bind to the consensus ERE half site in the absence of hormone could also be fostered by a rather loose association of the ERE-containing DNA with histones [36]. Taken together, our findings imply that differential occupation of the ERE may be involved in silencing, activation, and maintenance of pS2 gene expression.

It is interesting to note that hypersensitive sites are present in our in vivo and in our in vitro footprints. Such hypersensitivity may result from distortion or bending of the DNA helix by protein binding. We and others have demonstrated that the ER induces distortion and directed bending in ERE-containing DNA fragments [37–40]. Binding of ER to the ERE could feasibly explain the hypersensitivity observed in this region.

Our findings contrast with those of the apo very low density lipoprotein 5′ flanking region in which the EREs were occupied only after estrogen treatment [41,42]. However, studies of a number of hormone-responsive genes indicate that there is significant variation in the protection of hormone response elements. While some response elements are occupied only in the presence of hormone [43–47], others appear to be unaffected by hormone treatment [46,48–50]. These apparent differences in the occupation of various hormone response elements may be due to the presence of tissue-specific accessory factors, the inaccessibility of protein binding sites due to promoter organization, or the transient nature of protein-DNA interactions.

### 4.2. Mechanisms regulating antiestrogen action

Tamoxifen, a non-steroidal antiestrogen, has been extensively used in breast cancer therapy and is also being tested for its ability to decrease the onset of breast cancer [51]. ICI 182,780, an estradiol analogue, has also been used in breast cancer treatment and may prove to be useful in limiting disease recurrence in tamoxifen-resistant tumors [52]. While tamoxifen has both agonistic and antagonistic actions [26,53,54] and moderately enhances pS2 mRNA levels in MCF-7 cells [24,28], ICI 182,780 is classified as a pure antiestrogen [55] and does not induce pS2 mRNA levels in MCF-7 cells [29].

Antiestrogens have had a tremendous impact on breast cancer treatment and yet, the mechanisms by which they bring about their effects are largely unknown. Our in vivo footprinting experiments provide us with a first glimpse of how these compounds function at the level of the gene and demonstrate that 4-hydroxytamoxifen and ICI 182,780 have very different effects on the interaction of proteins with the pS2 5′ flanking region. Although few proteins were recruited to the pS2 5′ flanking region after 4-hydroxytamoxifen treatment of MCF-7 cells, multiple proteins were recruited after treatment of MCF-7 cells with ICI 182,780. The different patterns of protein-DNA interaction observed after treatment of cells with 4-hydroxytamoxifen and ICI 182,780 may underlie the partial agonist/antagonist and pure antagonist properties, respectively, of these two drugs.

4-Hydroxytamoxifen and ICI 182,780 produced very different footprints, which were distinct from the footprints observed with no hormone or $E_2$ treatment. What is unclear at this point is how such differences in footprinting patterns are brought about. Part of the answer may lie in the changes in ER conformation that accompany binding of hormone or antihormone. A number of studies have provided evidence that individual ligands may induce specific changes in ER conformation [56–58]. Recent X-ray crystallographic studies of the estrogen and antiestrogen-bound ER ligand binding domain demonstrate that there are dramatic differences in the orientation of helix 12 when the ligand binding domain is occupied by $E_2$ or raloxifene [59]. Such changes in receptor conformation could result in the presentation of different functional ER surfaces and form the basis for the recruitment of specific sets of transcription factors to the promoter.

### *4.3. Role of the basal promoter in transcription activation*

A previous in vitro transcription study suggested that ER binding to the ERE enhanced transcription by stabilizing binding of proteins to the basal promoter [60]. Surprisingly, however, elements in the endogenous pS2 basal promoter appeared to be largely unaffected by $E_2$ treatment in our in vivo footprinting studies. There was no evidence of enhanced protection or altered hypersensitivity in this region after $E_2$ treatment suggesting that the basal promoter is accessible and poised for transcription even in the absence of hormone. In support of this idea, Sewack and Hansen [36] have reported that the nucleosome containing the TATA sequence is not altered by $E_2$ treatment and that the loose association of this nucleosome with the histone octamer may allow protein complex formation in the presence and in the absence of hormone. Thus, it appears that recruitment and binding of transcription factors to the basal promoter does not play a role in modulating transcription of the pS2 gene.

The pS2 TATA and CAAT sequences were flanked by hypersensitive sites before and after hormone treatment. Since the TATA binding protein binds to the minor groove of the DNA helix and induces DNA to bend [61], this increased sensitivity to DNase I may result from distortion of the DNA helix brought about by binding of the TATA binding protein and other factors associated with the basal transcription complex.

### *4.4. Role of other proteins in estrogen-regulated transcription activation*

Although estrogen treatment did not result in recruitment of proteins to the basal promoter, it did play a substantial role in recruitment and binding of proteins to other regions of the pS2 5′ flanking region, in particular, those regions within the ERE and adjacent to the imperfect ERE half site. Our in vivo and in vitro footprinting experiments with MCF-7 nuclear extracts demonstrated that several sites flanking the ERE were protected by proteins after $E_2$ treatment providing evidence that a number of proteins intimately associated with the pS2 promoter play an integral role in regulating gene expression.

Numerous groups have reported the association of steroid hormone receptors with coactivators and corepressors (reviewed in [62] and references therein). Recent studies have also identified coactivator and corepressor proteins with histone acetylase and deacetylase activities, respectively [63–66]. Association of ER with these coregulators may be important in modulating the accessibility of transcription factor binding sites in native chromatin. The divergent footprinting patterns observed with 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 may in turn form an interconnected protein-DNA complex. Estrogen treatment could release corepressor proteins and promote interaction of the receptor with coactivators. Taken together, our in vivo and in vitro experiments provide us with a more physiologically relevant view of how estrogens and antiestrogens regulate the expression of estrogen-responsive genes in target cells.

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