

# Effects of estrogen receptor and estrogen on the chromatin structure in estrogen receptor stable transfectants

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Abbreviations: ER, estrogen receptor; ERE, estrogen response elements; PR, progesterone receptor.

## Abstract

**The estrogen receptor (ER), a member of the nuclear hormone receptor superfamily recruits coactivators that modify local chromatin structure. Here we investigated the effect of the estrogen receptor and estrogen on the global chromatin structure and the local chromatin structure of the progesterone receptor gene during the process of transcriptional activation using Rat1+ER cells stably expressing the estrogen receptor. The total chromatin was more accessible to DNaseI in Rat1+ER cells than in the parental estrogen receptor-negative Rat1 cells. After 18 h of estrogen treatment, total chromatin was more dispersed in Rat1+ER cells than in Rat1 cells. The chromatin structure of the progesterone receptor gene was more sensitive to DNaseI in Rat1+ER cells than in Rat1 cells. However, the chromatin structure of the progesterone receptor gene did not change further on estrogen treatment. Our results suggest that under certain circumstances unoccupied estrogen receptors may play some role in reorganizing the repressive chromatin structure to induce gene activation.**

**Key Words:** Estrogen, Estrogen receptor, Progesterone receptor, DNaseI sensitivity, Chromatin

## Introduction

An estrogen-occupied estrogen receptor (ER) bound to estrogen response elements (ERE) can activate or repress transcription (Beato and Klug, 2000). The disruption of chromatin structure has been implicated in

estrogen-induced transcription activation (Burch and Weintraub, 1983; Burch and Fischer, 1990; DiRenzo *et al.*, 2000; Pham *et al.*, 1991; Seyfred and Gorski, 1990). Unoccupied ERs are associated with the chromatin as determined by *in vivo* crosslinking experiments (Wrenn and Katzenellenbogen, 1990). However, it is still unclear whether there is a direct relationship between estrogen-induced transcription and changes in chromatin structure (Mao and Shapiro, 2000). Transcription activation parallels chromatin structural changes in the promoter region of minichromosomes containing the 5' flanking region of the prolactin gene in rat pituitary-derived GH<sub>3</sub> cells (Seyfred *et al.*, 1989). SWI-SNF proteins, which facilitate transcription by remodeling chromatin (Peterson and Tamkun, 1995), are required for estrogen-induced transcription in yeast (Yoshinaga *et al.*, 1992). However, there are evidences to suggest that no direct correlation exists between transcription and chromatin structural remodeling: DNaseI hypersensitive sites of the prolactin gene appear early in development and do not change further with estrogen treatment (Durrin and Gorski, 1985); and transcription activation of the vitellogenin gene is poorly correlated with chromatin changes (Burch and Evans, 1986).

In this study, we addressed these issues by examining changes in chromatin structures and their sensitivity toward DNaseI in the stably transfected cells with ER (Rat1+ER). Rat1 cells that do not express endogenous ER were transfected with retroviral-vector construct containing human ER cDNA and stably ER expressing cells referred as Rat1+ER (Kaneko *et al.*, 1993). Rat1+ER cells expressed physiological levels of ER per cell, and activated the silent endogenous progesterone receptor (PR) gene on estrogen treatment (Kaneko *et al.*, 1993). The PR gene transcription rate gradually increased during a 24 h of estrogen treatment after an initial lag of 1-3 h (Lee and Gorski, 1996). It was suggested that the unoccupied ER may likely be bound to the chromatin and changes in chromatin structure was taken place for the gradual estrogen-induced PR gene transcription. To test this hypothesis, we examined DNaseI sensitivity of total chromatin and the chromatin of the 5' flanking regions and exon 1 of the PR gene. The presence of the unoccupied ER alone modified the chromatin structure of the PR gene as well as the total chromatin of Rat1+ER cells. Estrogen treatment of Rat1+ER cells caused an increase in DNaseI sensitivity at 18 h of the estrogen treatment; however, no changes in the chromatin of the PR gene were detected. The estrogen-induced gradual increase in the rate of

transcription of the PR gene was not due to the chromatin changes in the 5' flanking regions and exon 1 of the gene. These results suggest that the unoccupied ER is already bound to the chromatin, which leads to a structural change under some circumstances, such as in our clonal cell line.

## Materials and Methods

### Cell Culture

Rat1 and Rat1+ER cells were grown in phenol red-free Dulbecco's modified Eagles medium (DMEM; Sigma, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone, USA). Cells were grown at 37°C in humidified 95% air/5% CO<sub>2</sub> and fed every 1-2 day. Before treatment with 17-β-estradiol (Sigma), the cells were incubated for 24-48 h in DMEM, supplemented with 10% dextran-charcoal stripped FBS (ST-FBS). All estrogen treatments were done with medium containing ST-FBS. To maximize the response, 10 nM of estrogen was used.

### Chromatin Analysis

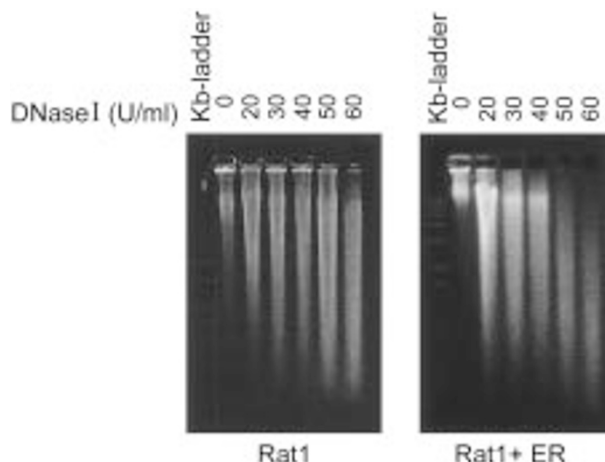
Cells were harvested by trypsinization, washed, and lysed in 0.25% Nonidet P-40 buffer [10 mM Tris-HCl, 3 mM CaCl<sub>2</sub>, 2 mM MgCl<sub>2</sub>, 1 mM dithiothreitol (DTT), and 10% glycerol]. Nuclei were suspended immediately in DNaseI digestion buffer (20 mM Tris-HCl, pH 7.5, 60 mM KCl, 15 mM NaCl, 0.5 mM DTT, 5 mM MgCl<sub>2</sub>, 1 mM CaCl<sub>2</sub>, and 10% glycerol). Increasing amounts of DNaseI were added and incubated at room temperature for 20 min, except that nuclei from GH<sub>3</sub> cells were treated for 6 min. Reaction was stopped by adding sodium dodecyl sulfate (SDS)-RNaseA-EDTA solution. Genomic DNA was isolated by proteinase K digestion overnight at 55°C, phenol/chloroform extraction, and ethanol precipitation (Shin *et al.*, 2000; Sun and John, 2000). Aliquots of isolated genomic DNA were visualized by agarose gel electrophoresis to examine global chromatin structural change. The rat PR sequences and clones (Kraus *et al.*, 1994) were a gift from Dr. Kelly E. Mayo at Northwestern University. The map of the 5' flanking regions of the PR gene examined is illustrated in Fig. 3. *Eco*RI was used to cleave the PR gene. *Eco*RI digestion of the genomic DNA results in a 3.1-kilobase (kb) parental PR fragment, which is from nucleotides -1378 to +1759. This 3.1-kb fragment includes both the distal and proximal promoters and two of the five EREs identified by Katzenellenbogen's group at -11059/-11037 and +615/+637, respectively (Kraus *et al.*, 1994). A DNaseI cut within the area, i.e., a DNaseI hypersensitive site, results in a sub-fragment. The length of the sub-band is the distance between the *Eco*RI cut and the DNaseI hypersensitive site. The PR probe that was used in this assay is a 987 bp *Pst*I

fragment, from +637 to +1624. Thirty micrograms of DNaseI-treated genomic DNA were digested with *Eco*RI and fractionated by electrophoresis in a 1% agarose gel in 1x TAE buffer (40 mM Tris-acetate, 1 mM EDTA). After electrophoresis, the DNA within the gel was depurinated (0.25 N HCl) for 10 min, denatured (1.5 M NaCl, 0.5 N NaOH) for 30 min, neutralized (0.5 M Tris-HCl pH 7.0, 1.5 M NaCl) for 30 min, treated in 20x SSC (3 M NaCl, 0.3 M sodium citrate, pH 7.0) for 30 min, and transferred downward to a nylon membrane (Schleicher & Schuell). The hybridization was done in 5x SSPE (0.75 M NaCl, 50 mM NaH<sub>2</sub>PO<sub>4</sub>, and 5 mM EDTA), 5x Denhardt's solution (15% Ficoll, 0.1% polyvinylpyrrolidone, and 0.1% bovine serum albumin), 0.5% SDS, and 100 µg/ml of denatured and sheared herring sperm DNA at 65°C for 36-48 h. Probes were labeled with <sup>32</sup>P-α-dATP by a random priming method. The membranes were washed with 0.2x SSC (0.3 M NaCl, 0.03 M sodium citrate, pH 7.0)/0.1% SDS at 65°C for 1 h, exposed, and visualized by either Phosphorimager (Molecular Dynamics) or autoradiography.

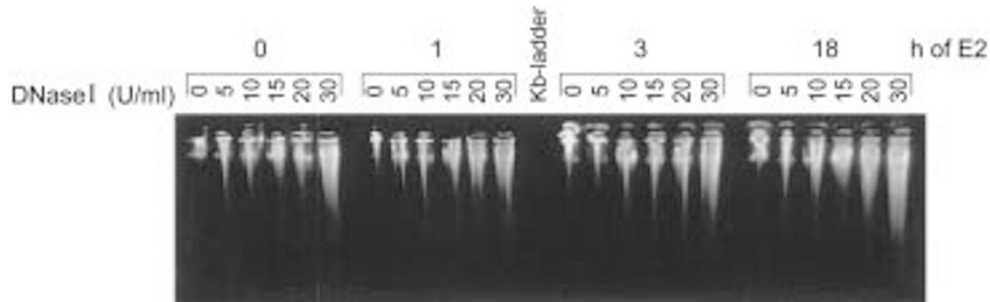
## Results

### Effects of unoccupied ER on the global chromatin structure

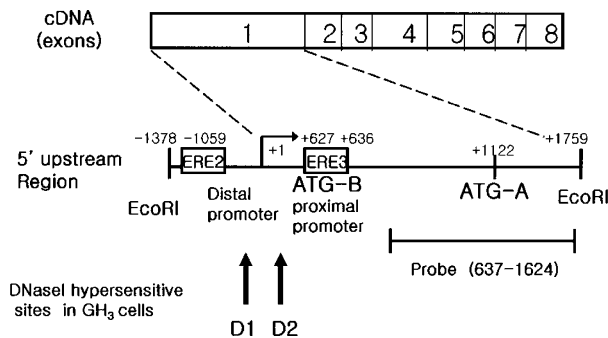
The chromatin organization of the Rat1 and Rat1+ER cells was compared. Nuclei from each cell type were isolated and treated with increasing amounts of DNaseI simultaneously. Genomic DNA isolated from DNaseI-treated nuclei was examined for the susceptibility of the total chromatin toward DNaseI by comparing the rates of



**Figure 1.** Chromatin structure in Rat1 and Rat1+ER Cells. Nuclei from Rat1 and Rat1+ER cells were simultaneously treated with increasing concentrations of DNaseI as indicated. Genomic DNA was purified and visualized by agarose gel electrophoresis. A 1-kb ladder was used as a size marker. The samples from Rat1 cells and Rat1+ER cells are indicated on the figure.



**Figure 2.** Time-course of chromatin changes in Rat1+ER Cells. Rat1+ER cells were treated with estrogen for the indicated times and harvested simultaneously. Nuclei were isolated and treated with increasing concentrations of DNaseI as indicated. A 1-kb ladder was used as a size marker. The estrogen treatment periods are indicated at the top of the figure.

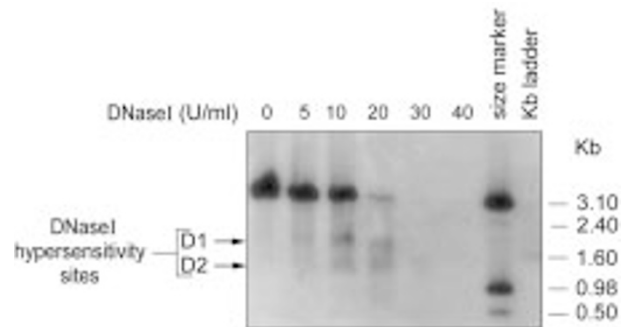


**Figure 3.** Schematic representation of the cDNA, the 5' flanking regions, and the locations of DNaseI hypersensitive sites of the rat PR gene. Positions of the exons (numbered 1-8) and map of the 5' flanking regions and exon 1 of the rat PR gene are shown. Positions of the *EcoRI* sites and the 987 bp *PstI* PR probe used in the DNaseI hypersensitive sites mapping are indicated. The 3.1-kb *EcoRI* fragment includes both the distal and proximal PR promoters, two translation start sites (ATG-B at +627; ATG-A at +1122) for PR A and B isoforms, and two EREs. Map is not drawn to scale. The positions of the two DNaseI hypersensitive sites are designated as D1 and D2. D1 is adjacent to the distal promoter and D2 is in the vicinity of the proximal promoter, which overlaps the translational start site of the PR B isoform and the internal ERE.

genomic DNA degradation (Figure 1). The chromatin of the Rat1+ER cells required less DNaseI to be degraded than did the chromatin of the Rat1 cells, suggesting that the chromatin was more dispersed by the presence of ER.

### Effects of estrogen on the global chromatin structure

The time-course of the estrogen-induced chromatin change in Rat1+ER cells was examined (Figure 2). Equal numbers of cells were seeded, treated with estrogen at different times, and harvested at the same time. Nuclei were treated with increasing concentrations of DNaseI and genomic DNA was purified. There was no significant global chromatin structure difference between 0, 1, and 3 h of estrogen treatment, respectively. However, the chromatin was more susceptible to DNaseI at 18 h of estrogen treatment, suggesting that estrogen slowly



**Figure 4.** Chromatin Structure of the PR Gene in GH<sub>3</sub> Cells. Nuclei from GH<sub>3</sub> cells were treated with increasing concentrations of DNaseI. Genomic DNA was digested with *EcoRI*. The 3.1-kb parental *EcoRI* bands are indicated. A size marker was prepared by cutting the PR plasmids with several restriction enzymes and loading approximately 10 µg aliquots to clearly designate the location of the DNaseI hypersensitive sites. The positions of the DNaseI hypersensitive sites are designated D1 and D2, as shown in Fig. 3.

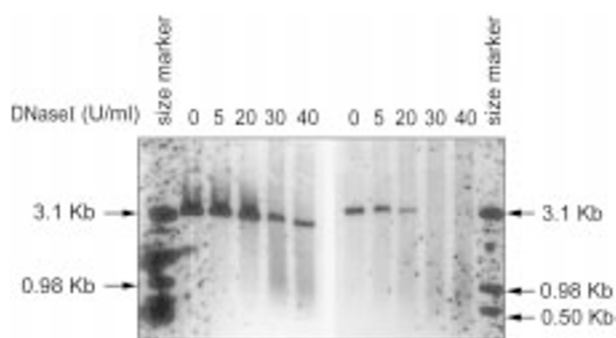
has been observed in several experiments and at other time points up to 36 h.

### Chromatin structure of the endogenous PR gene in GH<sub>3</sub> cells

Next, we examined specific chromatin structure changes of 5' flanking and exon 1 of the endogenous PR gene. Initially, DNaseI hypersensitive sites of the PR gene were mapped in GH<sub>3</sub> cells (Figure 3), where the PR gene is actively expressed. DNaseI hypersensitive sites are associated with important regulatory regions and are good criteria for determining chromatin structure disruption at specific local sites. Purified genomic DNA from DNaseI-treated nuclei was digested with *EcoRI* and probed by an indirect end-labeling. Two DNaseI hypersensitive sites were clearly detected (Figure 4). One is located near the distal promoter region and the other is at the proximal promoter, which overlaps one ERE. These locations are labeled D1 and D2 in Figure 3, respectively.

### Effects of unoccupied ER on the chromatin structure of the PR gene

The chromatin structures of the 5' flanking and exon 1 of the PR gene in Rat1 and Rat1+ER cells were compared. Nuclei from either cell type were isolated and treated with DNaseI simultaneously. Genomic DNA was purified from each sample, digested with *EcoRI*, and probed as shown in Figure 3. DNaseI degraded the PR gene in Rat1+ER cells faster than it degraded the gene in Rat1 cells (Figure 5). However, there was no DNaseI hypersensitive site on the PR gene in Rat1 or Rat1+ER cells. This indicated that the chromatin structures of the 5' flanking regions and exon 1 of the PR gene in Rat1+ER cells were more exposed than in Rat1 cells, although there were no specific changes in the local regions.



**Figure 5.** Chromatin structure of the PR gene in Rat1 and Rat1+ER cells. Nuclei from Rat1 and Rat1+ER cells were treated with increasing concentrations of DNaseI. Genomic DNA was purified and digested with *EcoRI*. The 3.1-kb parental *EcoRI* bands are indicated. The lane containing the size marker is labeled.

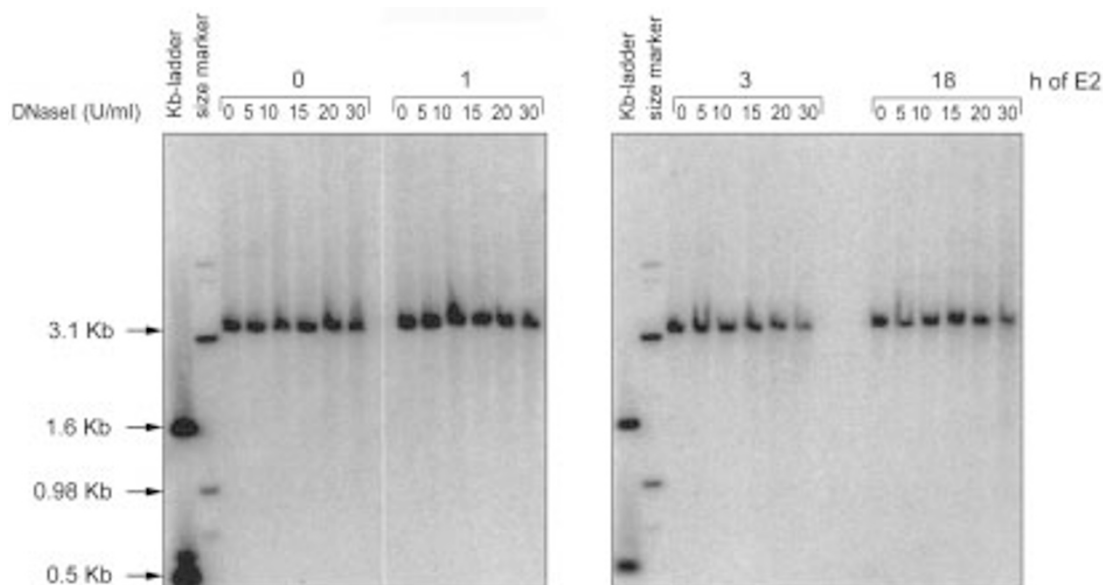
### Effects of estrogen on the chromatin structure of the PR gene

We hypothesized that the gradual increase in the rate transcription of the PR gene (Lee and Gorski, 1996) accompanies the disruption of the chromatin structure of the PR gene. The effect of estrogen treatment on the chromatin structure of the PR gene with time in Rat1+ER cells was examined (Figure 6). No DNaseI hypersensitive sites were detected and the DNaseI sensitivity of the PR gene did not change with estrogen treatment.

### Discussion

We examined the effects of unoccupied ER on chromatin structure by comparing the structures in parental ER-negative Rat1 and Rat1+ER cells. The global chromatin structure and the chromatin structure of the 5' flanking regions and exon 1 of the PR gene of the Rat1+ER cells were significantly dispersed as compared with the Rat1 cells. It appears that the presence of the ER itself caused this effect.

Interestingly, estrogen slowly caused the entire chromatin of Rat1+ER cells to be more dispersed. Vic *et al.* (1980) reported that estrogen induced decondensation of the entire chromatin in rat endometrium within 1 h. This effect appears to be related to transcription activity, because it is sensitive to actinomycin D but not to cycloheximide (Vic *et al.*, 1980). However, it is not known how these effects influence the transcription of specific estrogen-responsive genes.



**Figure 6.** Time-course of chromatin change of the PR gene in Rat1+ER cells. Rat1+ER cells were treated with estrogen for the indicated times. The DNaseI concentration for each lane is shown at the top. The 3.1-kb parental *EcoRI* bands are indicated. The lanes containing a 1-kb ladder and size marker are labeled.

We examined whether the chromatin structure of the PR gene remodeled with activation of transcription. However, the DNaseI hypersensitive sites in the 5' flanking regions and exon 1 of the PR gene present in GH<sub>3</sub> cells were not detected with transcription activation in Rat1+ER cells. It appears that mechanisms other than changes in chromatin structure enhance PR gene transcription in Rat1+ER cells, although it is possible that the EREs located outside the region examined are being regulated. There is evidence that chromatin structure does not always correlate with transcription. A truncated ER devoid of transcription activation function induces chromatin changes, indicating that some chromatin changes are due solely to DNA binding (Gilbert *et al.*, 1992). The administration of exogenous estrogen activates the silent vitellogenin gene (Burch and Weintraub, 1983). This is accompanied by an initial lag; however, subsequent induction of the gene by estrogen is much more efficient with no such a lag, indicating that the initial exposure to estrogen introduces modifications in the genome that facilitate transcription (Burch and Evans, 1986). This "memory effect" induced by estrogen is not due to chromatin structure changes or methylation states of the gene (Burch and Evans, 1986).

We did not find direct correlation between estrogen-induced transcription of the PR gene and DNaseI sensitivity changes of the 5' flanking regions and exon 1 of the PR gene. This was probably due to the detection limit of the methodology that we used. However, our results suggest that the unoccupied ER may already bound to the chromatin and may play a role in reorganizing chromatin that represses transcription activation in some circumstances, such as in our clonal cell line.

## Acknowledgements

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