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Estrogen Receptor and Aromatic Hydrocarbon Receptor in the Primate Ovary

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We have previously shown by immunocytochemistry and autoradiography the presence of estrogen receptors (ER) in rhesus monkey ovary. Intense chromogen staining showed specific binding for ER in nuclei of germinal epithelium and granulosa cells of antral follicles; and radiolabeled ligand bound specifically to functional corpora lutea (CL). Although it is accepted that the germinal epithelium of the primate ovary contains ER, some controversy still persists regarding the intraovarian localization of this molecule. In addition, no data exist that localize the aromatic hydrocarbon (dioxin) receptor (AHR), which is known to modulate ER, to the primate ovary. In the present study, we show the presence of ER using Western blot analysis, and ER capable of binding DNA within intraovarian compartments in two species of the genus *Macaca* (rhesus macaque, *Macaca mulatta* and stumptail macaque, *Macaca arctoides*); extend these findings to human ovarian granulosa cells (GC) using Western blot, reverse transcription-polymerase chain reaction (RT-PCR), and gel mobility-shift analysis; and localize the AHR to intraovarian compartments of the macaque ovary by Western blots and gel-shift assays. These experiments strongly suggest that estrogens can exert effects on follicle development directly at the ovary, and provide the first direct evidence that AHR-mediated toxicity may be manifested at the ovary to induce possible antifertility effects.

Key Words: Estrogen receptor; aromatic hydrocarbon receptor; ovary; corpus luteum; macaque; human.

Introduction

The role of estrogens in follicular development and function has been reviewed recently (Dierschke et al., 1994). Mounting evidence suggests that estrogen (E) can exert an inhibitory influence on the development of follicles (depending on stimulus, dose, and time of administration), both via a centrally mediated mechanism (Dierschke et al., 1985), and directly at the ovary (Hutz, 1989). However, conflicting evidence exists regarding the presence of ER in the rhesus macaque *(Macaca mulatta)* ovary. Hild-Petito et al. (1988) and Chandrasekher et al. (1994) suggested that estrogen receptors (ERs) are present only in the germinal epithelium of the rhesus ovary, and absent from corpus luteum (Stouffer and Duffy, 1995). However, ER is present in ovary from several taxa, including rodent (Kim and Greenwald, 1987) and baboon (Billiar et al., 1992). Additionally, we have shown using both immunocytochemistry and autoradiography the presence of ER in the rhesus monkey ovary (Hutz et al., 1993). With regard to the human ovary, the situation of ER localization is more equivocal.

Previous reports describing immunocytochemical localization have shown intense staining for ER in human granulosa cells (GC) from dominant preovulatory follicles, just prior to the luteinizing hormone (LH) surge, and weak staining in GC from antral follicles in the midfollicular phase of the menstrual cycle (Iwai et al., 1990). Sections of corpus luteum taken just after ovulation showed no staining for ER in luteal cells (Iwai et al., 1990). Additionally, the surrounding stromal cells and theca interna showed no staining for ER throughout the menstrual cycle (Iwai et al., 1990; Horie et al., 1992). In a more recent report, Wu et al., (1993) demonstrated the presence of ER mRNA by reverse transcription-polymerase chain reaction (RT-PCR) in cumulus–oocyte complexes from women participating in an assisted-reproduction program; message for ER was observed only in the cumulus–oocyte complex, not in cumulus cells alone or in mural GC. On the other hand,

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Hurst et al. (1995), using RT-PCR, were able to amplify ER cDNA from GC of women undergoing ovarian stimulation for in vitro fertilization (IVF) protocols. The functionality of human granulosa cell ER was verified by transfecting a human GC line with an ERE-linked chloramphenicol acetyltransferase (CAT) gene containing plasmid, showing increased CAT activity in the presence of estrogen (Hurst et al., 1995). The discrepancies in the human studies may be owing to various factors, including the ovarian stimulation protocol, cell-isolation protocol, and methods of detection used.

Although the presence or absence of ER in the ovary has been much studied, little is known regarding molecules that modulate or perturb ovarian E action. We have initiated experiments aimed at perturbing the normal expression of ER in the mammalian ovary. The halogenated aromatic hydrocarbons (HAH) are known modulators of ER in many tissues, e.g., rat liver and uterus (Romkes et al., 1987), and rat ovary (Chaffin et al., 1996). The most biologically potent of the HAH is the environmental pollutant 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). TCDD-induced toxicity is mediated via a soluble receptor, termed the aromatic hydrocarbon receptor (AHR; Poland and Knutson, 1982). TCDD is a known antifertility agent in several species, and is implicated in developmental defects in female rats and primates (Barsotti et al., 1979; Chaffin et al., 1994; Gray and Ostby, 1995). To date, AHR has not been localized to the primate ovary, nor has TCDD been shown to regulate ovarian ER in primates.

In the current study, we seek to extend the findings of Hutz et al. (1993) using data from Western immunoblots, gel-shift assays, and RT-PCR to include rhesus and stumptail *(M. arctoides)* monkey, and human ovarian granulosa cells so as to demonstrate the presence of ER in vitro. In addition, the localization of AHR in the primate ovary will be established.

Results

Using Western blotting, a strong signal for ER was observed in early, middle, and late corpora lutea of rhesus macaque (Fig. 1). Whole rhesus and stumptail ovaries, devoid of the germinal epithelium, were also positive for ER by Western blot (Fig. 1). Strong signal was detected from rhesus endometrium, whereas a signal of much lower intensity was observed in spleen. CL from the early luteal phase showed a very low signal for AHR, whereas mid- and late CL showed a stronger signal (Fig. 2); signal from monkey endometrium was similar in intensity to that of early CL.

ER message was present in purified human GC by RT-PCR (data not presented). The results, however, were not consistent from one subject to the next, with approx 40% of women being ER mRNA positive, suggesting that there is variability among subjects undergoing similar ovarian stimulation protocols. When the purified GC were pooled from at least three subjects, gel-shift analysis showed functional ER in the human cells (Fig. 3).

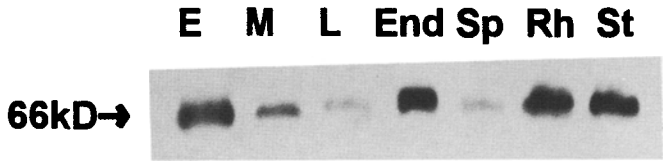

Fig. 1. Photograph of an autoradiogram of Western blot for ER in rhesus macaque corpus luteum (CL). Protein extracts are from early (E), mid (M), and late (L) CL, endometrium (End), spleen (Sp), and whole rhesus (Rh) and stumptail (St) ovary devoid of germinal epithelium.

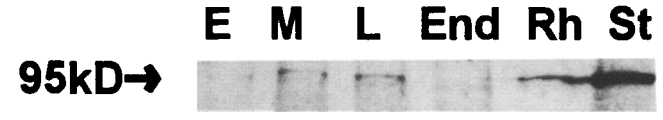

Fig. 2. Photograph of an autoradiogram of Western blot for AHR in rhesus macaque corpus luteum (CL). Protein extracts are from early (E), mid (M), and late (L) CL, endometrium (End), and whole rhesus (Rh) and stumptail (St) ovary devoid of germinal epithelium.

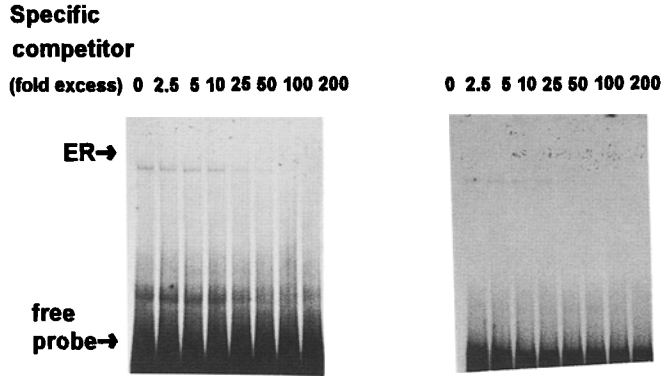

Fig. 3. Photographs of autoradiograms from gel-shift assays using 20 μg of human granulosa cell protein. Specific competitor refers to the presence of nonradioactive, specific competitor (numbers above each figure indicate the amount [molar-fold excess] of competitor). ER, estrogen receptor/ERE complex; AHR, Ah receptor/DRE complex; free probe, unbound, labeled response element DNA.

A strong signal was obtained from both rhesus and stumptail macaque for ER DNA binding from whole ovaries devoid of germinal epithelium (Fig. 4). A distinct signal was also obtained from mid- and late luteal phase CL, whereas a much weaker signal was present in CL from early luteal phase extracts (Fig. 5). Both CL and whole ovaries stripped of germinal epithelium were positive for AHR DNA binding (Figs. 6 and 7). In whole ovarian protein extracts, multiple shifted bands were observed (Fig. 6), whereas only a single band was observed in extracts from the corpus luteum (Fig. 7). The results for gel shift were

Fig. 4. Gel-shift assay using rhesus macaque ovarian (devoid of germinal epithelium) whole-cell protein extracts with the ERE. Bound ER is labeled "ER/ERE." +, 200-fold molar excess of nonradioactive ERE; –, no competitor.

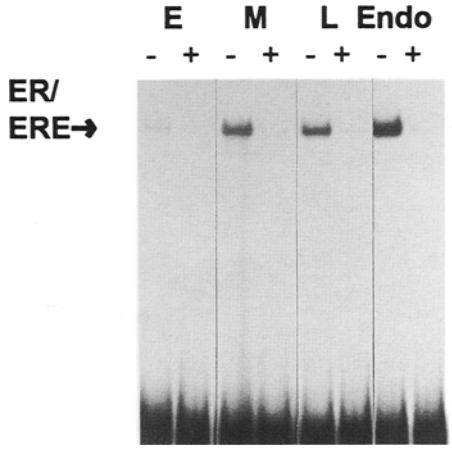

Fig. 5. Gel-shift assay using rhesus macaque CL protein extracts and ERE DNA. Bound ER is "ER/ERE." E, early CL; M, mid-CL; L, late CL; +, 200-fold molar excess of nonradioactive ERE; –, no competitor.

both specific based on the ability to reduce signal-to-background levels with unlabeled oligonucleotide response elements, and also based on the fact that the binding of labeled ERE was not reduced in the presence of 10 μg of poly d*T*-dC (data not presented). No signal was obtained using labeled ERE and protein extracts from stumptail macaque lung; however, specific binding of DRE to stumptail lung protein extracts was observed (data not presented).

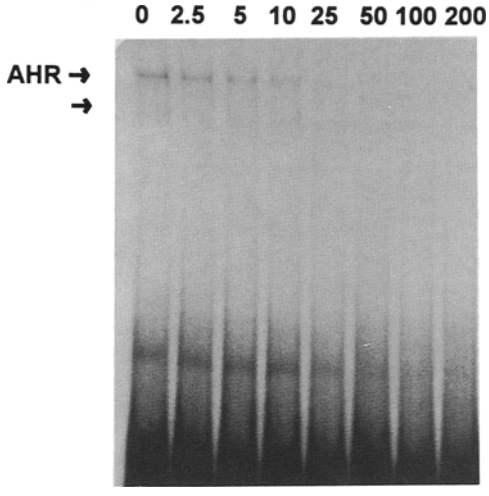

Fig. 6. Photograph of an autoradiogram from a gel-shift assay using whole-cell protein extracts from rhesus macaque ovary devoid of germinal epithelium. Numbers above each figure indicate the amount (molar-fold excess) of competitor. AHR and arrows, Ah receptor/DRE complexes. Note that multiple shifted bands were observed.

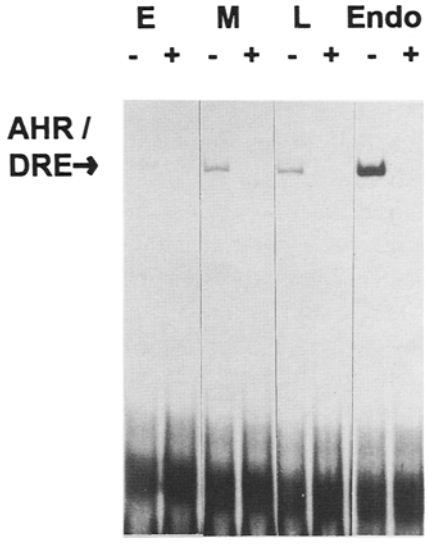

Fig. 7. Gel-shift assay using rhesus macaque CL protein extracts and dioxin-response element DNA. Bound AHR is "AHR/DRE." E, early CL; M, mid-CL; L, late CL; +, 200-fold molar excess of nonradioactive DRE; –, no competitor.

Discussion

Previous studies (Hild-Petito et al., 1988; Chandrasekher et al., 1994; reviewed in Stouffer and Duffy, 1995; Zelinski-Wooten and Stouffer, in press) have shown that intraovarian ERs are not expressed in the primate ovary other than in the external epithelial layer; however, Hutz et al. (1993) have suggested that ER are localized not only to germinal epithelium, but also to granulosa cells of antral follicles, to cells of the CL, and to interstitium. In the present study, we extend the findings of Hutz et al. (1993), using Western

immunoblots and RT-PCR data, by demonstrating the presence of ER message and protein, and AHR protein in the primate ovary; also, by gel shift, we show ER and AHR capable of binding DNA in vitro in ovarian cells other than in epithelium.

Estrogen Receptor

Both rhesus and stumptail macaque appear to express ER in whole ovarian homogenates devoid of the ER-rich germinal epithelium. Although the exact intraovarian site of ER expression is not currently known, the results from the present study substantiate earlier findings demonstrating the presence of ^{3}H-E_2 and I-125-E_2 binding sites in ovarian sections containing antral follicles and corpora lutea (Hutz et al., 1993). ER was also localized by Western blot and gel-shift assay to early, mid-, and late CL. Using RT-PCR and immunocytochemistry, the presence of ER has been shown to be absent from macaque luteal tissue (Hild-Petito et al., 1988; Chandrasekher et al., 1994; Stouffer and Duffy, 1995). Therefore, the role of E as a regulator of luteal function and demise remains controversial. It is unlikely that estrogen acts as a luteolytic agent in the macaque via direct actions at the CL, but rather via feedback inhibition of pituitary LH (e.g., Schoonmaker et al., 1982; reviewed in Stouffer, 1988). The data from the gel-shift assay indicate that ER DNA binding activity in CL is lowest in the early luteal phase and equivalent in mid- and late CL.

The discrepancy between the Western blot and gel-shift assays may be attributable to bioactive isoforms of ER not detectable by the H222 antibody, although this hypothesis remains untested. The lack of ER mRNA as assayed by RT-PCR in cells of the corpus luteum (Chandrasekher et al., 1994), and the declining expression of ER in the Western blot across the luteal phase may indicate protein remaining from the preovulatory follicle, rather than owing to new synthesis. The increase in ER DNA binding activity across the luteal phase may be indicative of a "nonclassical" ER isoform in the CL. This hypothesis is supported by the recent discovery of a novel form of ER in the rat ovary (Kuiper et al., 1996).

RT-PCR data from human GC suggest that ER message is present in these cells, but further investigation is required to determine why the message was not seen in all of the samples tested. The discrepancy may be owing to several possibilities, including the nature of the fertility disorder and the ovarian stimulation protocol used. Although granulosa cells pooled from multiple patients did contain ER capable of binding DNA in vitro, we did not observe immunoprecipitable ER. However, in our hands, the gel-shift assay is much more sensitive than our Western blots, and we had more protein available for the gel shifts. Whether the difference is additionally owing to isoform variation not detected by our monoclonal antibodies (MAbs) is unknown at present. We are currently investigating the apparent technique-based discrepancies.

These data add to the growing body of evidence that suggests that E may play a direct role in ovarian function in primates, including the induction of follicular atresia (Dierschke et al., 1995). This study also extends the observations of ER in stumptail macaque and human ovary, suggesting that ovarian ER is common to other primate species (Billiar et al., 1992). One feature of ovarian ER that remains unresolved, but salient, in the role of E-induced atresia (and other E-mediated activities) is the regulation of the ER gene. ER gene expression is very likely to be both menstrual cycle- and even cell-cycle dependent (Milan Tomanek, personal communication), making studies of in vivo regulation difficult.

Ah Receptor

Several reports have focused on the effects of TCDD as an E-modulatory and antifertility agent. TCDD is thought to modulate its effects by binding to AHR. In rats, TCDD has been shown to interfere with the maintenance of pregnancy, fetal growth, and development (Murray et al., 1979; Giavani et al., 1983). In macaques, TCDD exposure resulted in significant hormonal alterations, failure to conceive and in endometriosis (Barsotti et al., 1979; Rier et al., 1993). TCDD's effects on reproductive functions may occur via modulating E and/or ER (Umbreit and Gallo, 1988). Depending on the species, tissue, and hormonal status of an animal, TCDD has been shown to modulate ER is different ways (DeVito et al., 1992; White et al., 1995; Chaffin et al., 1996).

The present study shows the presence of AHR within the macaque ovary. The mRNA for AHR has been shown to exist in human pancreas, kidney, muscle, liver, brain, heart, and at very high levels in lung and placenta (Dolwick et al., 1993). We now show for the first time its presence in the primate ovary by Western blot and gel-shift assay. Because it is difficult to procure sufficient purified GC material from human ovaries, we are currently developing PCR primers that will allow us to elucidate the presence of AHR message in this tissue. Evidence of this kind may suggest a direct role of TCDD at the level of the GC, and may thus further explain TCDD's antifertility effects (Rier et al., 1993; Gray and Ostby, 1995).

Monkey endometrial tissue has very low levels of immunoreactive AHR, suggesting that expression of AHR is low or that AHR is rapidly degraded in this type of tissue. Smaller fragments, <95 kDa, were observed in protein extracts from whole ovaries (devoid of germinal epithelium) from rhesus macaque (Fig. 6), but not in protein extracts from macaque CL (Fig. 7). The multiple shifted bands observed in the gel-shift assay from whole ovarian protein suggest the presence of single-stranded binding proteins, although this is not substantiated. AHR is observed in a wide variety of tissues, notably placenta in humans (Dolwick et al., 1993) and uterus in rat (Chafffin and Hutz, 1995). We detected AHR by gel shift and Western blot in whole ovaries and isolated CL. Levels of AHR appear to be

lowest in early CL, and increased in mid- and late luteal phase tissues. As with the ER, the role of ovarian AHR is currently not understood. AHR has been shown to be a critical factor in mouse blastocyst differentiation (Blankenship et al., 1993; Peters and Wiley, 1995), although the wide tissue distribution of this receptor argues for a broader or more multifaceted role. It is clear, however, that AHR modulates the E signal, at least in rats (Chaffin et al., 1996).

There appears to be an exquisite, yet arcane, interplay between the E and HAH signaling systems. Although little is known regarding the mechanism of TCDD regulation of ER, it has become clear that the E-modulatory effects of this environmental xenobiotic are profound. In the current study, we have localized some apparently key molecules in this potential regulation and have demonstrated their functionality via their ability to bind DNA in vitro.

Materials and Methods

Nonhuman Primate Tissues

Three cycling female rhesus macaques and one stumptail macaque were used in this study. Ovaries were removed either during necropsy (stumptail) or ovariectomy (rhesus). Ovaries were taken without regard to cycle. CL were obtained from two different rhesus monkeys at early (day 2–4), middle (day 6–8), and late (day 13–15) stages of luteal development (courtesy R. Stouffer, Oregon Regional Primate Research Center, Beaverton, OR). Tissues were frozen until used for protein isolation.

Since previous studies have suggested that ER are localized only to the germinal epithelium of the rhesus macaque ovary (Hild-Petito et al., 1988; Chandrasekher et al., 1994), this layer of epithelial cells was removed in the following manner while the ovaries were frozen on dry ice. Ovaries were first cleaned of connective tissue and oviduct, if present, using a scalpel, and the outer 1–3 mm of cells were cut off the periphery of the entire tissue. The efficacy of the procedure was determined by fracturing the frozen ovary, cryosectioning, and staining a small portion of the tissue with Giemsa. The removal of germinal epithelium was nearly complete (data not presented).

Human Granulosa Cell Isolation and Purification

Human granulosa cells were obtained from 16 women participating in an IVF protocol at Rush Presbyterian St. Luke's Medical Center, Chicago, IL. All patients signed approved consent forms prior to the start of the study. Granulosa cells were collected from follicular fluid aspirates from women treated with the GnRH agonist luprolide acetate (Lupron; TAP Pharmaceuticals, Abbott Park, IL) and with human menotropins, hMG and FSH (Pergonal and Metrodin; Serono Laboratories Inc., Randolph, MA). Human chorionic gonadotropin (hCG, 10,000 IU im; Serono Laboratories, Randolph, MA), was administered to induce oocyte maturation and follicular luteinization. Transvaginal aspiration of oocytes and follicular fluid was performed 34–36 h after hCG injection. Cellular material was collected from the relatively clear follicular fluid aspirates (with little blood cell contamination) and centrifuged for 10 min at 300*g* to pellet the cells. The follicular fluid was removed, and the pellet was resuspended in DMEM/F12 (without phenol red), supplemented with 0.01% BSA. The GC were separated from red blood cells over a 50% Percoll gradient. Because ER were previously found in a specific subset of T-lymphocytes (Cohen et al., 1983), white blood cells were removed from the GC suspension using anti-CD45 magnetic immunobeads (AMAC Inc., Westbrook, ME) as previously described by Best et al. (1994). The GC were washed twice in DMEM/F12, pelleted, and immediately snap-frozen over dry ice. The cells were maintained at –80°C until used for ER analysis by RT-PCR, Western blot, and gel-shift assays.

Western Immunoblotting

Whole-cell protein was extracted from rhesus and stumptail macaque ovaries devoid of germinal epithelium, and from rhesus macaque CL. Protein extraction for Western blots was prepared as previously described by Bettini et al. (1992). Proteins (10 μg) were separated on 9% SDS-PAGE and transferred onto PVDF membrane (Immobilon, Millipore, Inc., Bedford, MA). The blots were incubated for 1 h at room temperature and then overnight at 4°C with H222 or H226 ER antibody (courtesy G. Greene, University of Chicago, Ben May Laboratories). Following a series of washes, blots were incubated with a peroxidase-conjugated goat antirat secondary antibody (Sigma, St. Louis, MO) for 2 h at room temperature. For AHR, the primary polyclonal antibody PA3-513 (ABR Inc., Golden, CO) was used. A 2-h incubation at room temperature with a peroxidase-conjugated goat-antimouse secondary antibody (Sigma) was performed. This last incubation (for both ER and AHR procedures) also contained a 1:2000 dilution of the blotting grade avidin-horseradish peroxidase conjugate (Bio-Rad Laboratories, Richmond, CA) for visualization of the mol-wt markers. For ER analysis, rhesus macaque endometrium was used as a positive control; spleen, known to contain very little ER (Westerlind et al., 1995), was used as a "low-concentration tissue." Protein extracts from gram-negative bacterial cells were also used as a negative control for both ER and AHR detection. An additional negative control for both ER and AHR was the omission of the primary antibody from the Western blot procedure. All washes were performed with TTBS (pH 7.5); 10% normal goat serum was used as a blocker with the secondary antibody/TTBS solution. The blot was exposed to X-ray film for 30 s after immersion in an enhanced chemiluminescence (ECL) solution (Pierce, Rockford, IL).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

The RT-PCR method was used to amplify ER cDNA in small quantities of purified human GC. Cellular material

was obtained from 10 women and individually analyzed for each PCR experiment. Approximately 3000 cells were lysed at 90°C in a 10-μL vol of 0.05*N* NaOH. The cellular material was pelleted, and the supernatant was transferred to a fresh tube with 10 μg proteinase K. The tube was incubated at 55°C for 30 min and then at 95°C for 5 min to inactivate the enzyme. The cellular debris was pelleted by centrifugation, and the supernatant was used immediately for cDNA synthesis. RT-PCR was carried out with the GeneAmp EZ r*Tth* RNA PCR kit (Perkin Elmer, Branchburg, NJ) and 1 μL of RNA. The PCR annealing reaction was carried out at 55°C using ER and β-actin primers per Wu et al. (1993; primers were from Biosynthesis Inc., Denton, TX). The β-actin primers were used in a control reaction. To ensure that the amplification was not owing to any contamination, a negative control was used whereby RNA was omitted from the PCR mixture. To demonstrate that cDNA rather than genomic DNA was used as a template in the reaction, a parallel reaction was performed for each set of primers in which RNase was added for 15 min at 37°C prior to the addition of any RT-PCR components. Following PCR, the amplified products and DNA size markers were electroporesed on a 1% agarose gel. The authenticity of the RT-PCR products was verified by Southern blot using a human ER cDNA probe (courtesy P. Chambon, Strasbourg, France).

Gel Mobility-Shift Assay

Oligonucleotides used for the gel-shift assays were commercially synthesized by Bio-Synthesis, Inc. (Demon, TX.). The estrogen-response element (ERE) was from the *Xenopus* vitellogenin promoter (5'-CAAAGTCAGGTCA CAGTGACCTGATCAAA-3') and the dioxin-response element (DRE) was from the murine cytochrome P4501A1 (cyp1AI) promoter (5'-GGGCCTGAGCACGCGTGTCA GGCGGGAC-3'). In each case, the cognate response element is underlined. Single-stranded oligonucleotides were end-labeled with ^{32}P-dATP using T4 polynucleotide kinase and annealed to form double-stranded response elements.

Whole-cell protein extracts were prepared as described in Bettini et al. (1992). All manipulations were done at 4°C. For the gel-shift assay, 20 μg of protein were incubated with 80 fmol of ^{32}P-labeled response element and 0.7 μg poly d*T*-dC, with or without 1000-fold molar excess nonradioactive, specific competitor for 30 min at 24°C. Protein–DNA complexes were resolved on an 8% nondenaturing acrylamide gel followed by autoradiography. For ER binding assays, whole-cell lung protein was used as a negative control (lung is AHR$^+$ in primates; Dolwick et al., 1993). Pooled human GC from follicular fluid aspirates of women undergoing ovarian stimulation for assisted reproduction were also used for gel-shift assays using the same oligonucleotides as with the nonhuman primate tissues. Each GC pool contained purified cells from at least three women. Each assay was replicated twice.

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