A baculovirus expression system was used to over-produce the human estrogen receptor in insect cells. The estrogen receptor made in this system is full-length, binds estrogen specifically, and is recognized by a monoclonal antibody to the human estrogen receptor. The recombinant estrogen receptor binds the estrogen response element (ERE) in both the absence and presence of estrogen if the binding is carried out in the absence of Mg²⁺. In the presence of Mg²⁺, the estrogen receptor binds the ERE in a hormone-dependent fashion. This effect is more pronounced at higher temperatures. Tamoxifen, a nonsteroidal anti-estrogen, is able to stimulate ERE binding to the same extent and under the same conditions as estradiol. Estriadiol stimulates formation of an estrogen receptor-ERE complex with an increased mobility in native gels as compared with the complex formed without hormone or with tamoxifen. These results demonstrate that specific DNA binding of the estrogen receptor is not absolutely dependent on the presence of hormone and that estradiol but not tamoxifen is able to induce a change in the estrogen receptor. This differential effect of estradiol and tamoxifen may be important in understanding the role of the receptor to activate target genes differentially.

Estrogen plays an important role in the growth and differentiation of a number of normal tissues in mammals as well as a pivotal part in the regulation of reproduction. In addition, the activity of the estrogen receptor significantly influences the behavior and treatment of greater than a third of human breast cancers (1). The human estrogen receptor is a member of a superfamily of nuclear receptors for small hydrophobic ligands including the steroid hormones, thyroid hormone, and retinoic acid (2). As a class, these receptors are transcription factors whose activity is regulated allosterically by hormone binding. A dissection of the molecular mechanism by which estrogen activates its receptor is central to our understanding of the regulation of gene expression by the estrogen receptor. The mechanism by which the estrogen receptor activates transcription is unknown. An important step in the activation is the recognition by the estrogen receptor of a specific DNA element termed the estrogen response element (ERE). This element has been identified in the promoters of a number of estrogen-responsive genes. The ERE from the vitellogenin promoter consists of a 13-base pair palindromic sequence and represents a consensus binding site (15–18). An analysis of the specific estrogen receptor-ERE complexes will allow a greater understanding of the requirements for early steps in the regulation of gene expression by the estrogen receptor.

Tamoxifen, a triphenylethylene anti-estrogen, is widely used in the treatment of breast cancer, yet the mechanism by which tamoxifen antagonizes the action of estradiol is unknown. Tamoxifen competes with estradiol for binding of the estrogen receptor (19). In addition, a derivative of tamoxifen, 4-hydroxytamoxifen, has been shown to stimulate dimerization and sequence-specific DNA binding of the estrogen receptor (20). Thus, tamoxifen differs from estradiol in activity at a step distal to the induction of estrogen receptor-ERE complexes. An understanding of this difference will provide significant insight into the functioning of the estrogen receptor.

We have used recombinant human estrogen receptor made in a baculovirus expression system to study the role of estrogen in the stimulation of sequence-specific DNA binding in vitro. A model is proposed in which the estrogen receptor-ERE complex can exist in at least three distinct states and in which these are responsible for the differences in biological activity of estrogen, estrogen withdrawal, and tamoxifen.

**Human Estrogen Receptor Forms Multiple Protein-DNA Complexes**

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1 The abbreviations used are: ERE, estrogen response element; SDS, sodium dodecyl sulfate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; ER, estrogen receptor.
containing the human estrogen receptor cDNA was a gift of P. Chambon (CNRS, Strasbourg, France). The EcoRI fragment from the EcoRI containing the entire estrogen receptor-coding region was incubated with the Klenow fragment of DNA polymerase I to produce blunt termini and then cloned into the BamHI site of the baculovirus expression vector pVL841 (gift of M. Sumner, Texas A&M University) using BamHI linkers. The resulting plasmid, pVLER, was co-transfected with wild-type AcNPV baculovirus DNA into Sf9 cells, and 4 days later a recombinant viral supernatant was harvested. This was used to infect fresh Sf9 cells, and recombinant viruses containing the estrogen receptor coding region were identified using plaque hybridization and visual screening (21). Positive plaques were purified through three further rounds of plaque purification.

**Gel Electrophoresis and Western Blotting**—Total protein extracts for analysis by gel electrophoresis and Western blotting were prepared from virus-infected or mock infected Sf9 cells by boiling the cells in SDS-polyacrylamide gel electrophoresis sample buffer. The extracts were separated on discontinuous 10% SDS-polyacrylamide gels (22) and either stained with Coomassie Brilliant Blue or transferred to nitrocellulose for Western blotting. The D75 anti-estrogen receptor monoclonal antibody used for Western blotting was the gift of G. Greene, University of Chicago (23).

After transfer for Western blotting, the filters were blocked, incubated with the primary antibody, and then developed with an anti-rabbit secondary antibody linked to alkaline phosphatase (Bio-Rad) as described (24).

**Preparation of Protein Extracts from Infected Insect Cells**—For production of recombinant estrogen receptor extract, 6000-cm² cell face dishes (Nunc) were seeded with 2 * 10⁶ Sf9 cells in Grace's insect medium supplemented with 10% charcoal-treated serum (19). Twenty-four hours later, the cells were infected with 20–50 plaque-forming units/cell of estrogen receptor recombinant or control viral stocks. S100 extracts were prepared 72 h postinfection as described (24). Typical protein concentrations of the S100 extracts were 30–40 mg/ml. The level of receptor measured by [3H]estradiol binding was 125 pmol/ml. This represented approximately 10% of the level of receptor estimated by Coomassie Brilliant Blue staining of SDS-polyacrylamide electrophoresis gels. A similar difference was observed by Metzger et al. (14) when the receptor was expressed in yeast.

**Gel Mobility Shift Assay**—Plasmids containing the probe or competitor sequences were constructed using synthetic oligonucleotides. Oligonucleotides ERE, EREM1, and ERCOUP were cloned into the BamHI site, and XERE9 was cloned into the SalI site of the pUC18 polylinker. The sequences of the oligonucleotides were: ERE, 5'-AGGTCACAGTGACCT-3'; EREM1, 5'-AGGACACAGTGTCCT-3'; ERCOUP, 5'-TGGTATGGATCGCCTTTT-3'; XERE9, 5'-GAGCATTTGGTGCC-3'. End-labeled probe was prepared from plasmid DNA following digestion with EcoRI and HindIII and incubation with the Klenow fragment of DNA polymerase I in the presence of 100 μCi of [α-32P]dATP (6000 Ci/mmol, Du Pont-New England Nuclear) and electrophoresis in a 2% agarose mini-gel. Probes had a specific activity of 10⁶ cpm/μg. The level of receptor measured by [3H]estradiol binding was 125 pmol/ml. This represented approximately 10% of the level of receptor estimated by Coomassie Brilliant Blue staining of SDS-polyacrylamide electrophoresis gels. A similar difference was observed by Metzger et al. (14) when the receptor was expressed in yeast.

**RESULTS**

**Expression of the Human Estrogen Receptor in Insect Cells**—A baculovirus expression system (21) was used to overexpress the human estrogen receptor (ER). The plasmid HE0 (7) was used as the source of the human ER cDNA. It has been shown recently that this plasmid contains a point mutation in the hormone-binding domain of the ER (Gly100 for Val100), which decreases its affinity for estradiol (26). The coding region for the ER was cloned into the transfer vector pVL941 under the control of the polyhedron promoter for expression in insect cells (21). To obtain the recombinant viral stock, the resulting plasmid pVLER was co-transfected with wild-type baculovirus AcNPV DNA into Sf9 cells. The ER recombinant virus (AcNPV-ER) was identified using plaque hybridization, and the positive plaques were purified through three further rounds of plaque purification.

The approximate expression level of the human ER in this system was determined by an SDS-polyacrylamide gel analysis of total protein extracts made from infected cells (Fig. 1). A prominent band at M, 67,000 was present in extracts from the ER recombinant virus-infected cells (lane 3) but was not evident in extracts from the mock infected or wild-type virus-infected cells (lanes 1 and 2, respectively). We estimate that the ER represents 3% of the total protein in infected cells.

In order to confirm that this protein being overexpressed in the system was in fact the human ER, Western blotting was performed using the D75 monoclonal antibody made against human ER (Fig. 2). A prominent band of 67 kDa was observed in the ER recombinant extract (lane 4) whereas no cross-reacting material was seen in either mock infected or wild-type infected cell extracts (lanes 2 and 3, respectively). Several species that migrated as a series of bands slower than that of the full-length ER reacted with the D75 antibody (lane 4). These may represent post-translational modifications of the ER protein.

Extracts from Sf9 cells infected with the ER recombinant or control virus were assayed for estrogen binding using the dextran-coated charcoal method (19). There was no specific estrogen binding in either mock infected or control virus-infected cell extracts. However, extracts made from cells infected with the ER recombinant virus contained 125 pmol/ml [3H]estradiol binding activity (data not shown).

**Requirements for Specific DNA Binding**—Extracts from Sf9 cells infected with the ER recombinant or control baculoviruses were assayed by native gel electrophoresis for binding to estradiol.

**FIG. 1. SDS-polyacrylamide gel analysis of infected Sf9 cells.** Total protein extracts from mock infected (MO, lane 1), wild-type baculovirus-infected (WT, lane 2), or recombinant human estrogen receptor virus-infected (ER, lane 3) were analyzed on a 10% SDS-polyacrylamide gel and stained with Coomassie Brilliant Blue as described under "Materials and Methods." Molecular weights correspond to the positions of prestained standards (Bio-Rad). The positions of the recombinant estrogen receptor (ER) and baculovirus polyhedron (P) are noted.
Multiple Estrogen Receptor-DNA Complexes

FIG. 2. Western blot of infected Sf9 cells with a monoclonal antibody against the human estrogen receptor. Total protein extracts from mock infected (MO, lane 2), wild-type baculovirus-infected (WT, lane 3), or recombinant human estrogen receptor virus-infected (ER, lane 4) were analyzed on a 10% SDS-polyacrylamide gel and transferred to nitrocellulose for Western blotting as described under “Materials and Methods.” Molecular weights correspond to the positions of prestained standards (M, lane 1).

FIG. 3. Recombinant human estrogen receptor binds specifically to the ERE. Gel mobility shift assay of the estrogen receptor-ERE complex using the 32P-labeled ERE probe either in the absence ((−) lanes 1 and 3) or presence ((+) lanes 2 and 4) of 0.1 μM estradiol (E2) as assayed by gel mobility. Binding was performed without competitor DNA (lane 1) or in the presence of unlabeled competitors ERE (lane 2), EREmt1 (lane 3), or XERE9 (lane 4), ERCOUP (lane 5).

FIG. 4. Estrogen receptor binds the ERE in the absence of hormone. Formation of the estrogen receptor-ERE complex using the 32P-labeled ERE probe either in the absence ((−) lanes 1 and 3) or presence ((+) lanes 2 and 4) of 0.1 μM estradiol (E2) was assayed by gel mobility. Binding was carried out at 20 °C for 20 min followed by either 15 min of additional incubation at 20 °C in the absence of MgCl₂ (lanes 1 and 2) or 15 min of additional incubation at 37 °C in the presence of 5 mM MgCl₂.

of a specific estrogen response element. Extracts of the ER recombinant virus-infected cells generated a specific DNA-protein complex (Fig. 3, lane 1). Control extracts from uninfected Sf9 cells or Sf9 cells infected with a recombinant baculovirus encoding the herpes simplex virus αTIF protein (24) did not bind the ERE (data not shown). The DNA sequence requirements for specific ER binding were investigated using variants of the ERE as competitors in the gel retardation assay. These included a double point mutant (20)

of the ERE, EREmt1; an unrelated sequence from the promoter of the Xenopus estrogen receptor, XERE9; and a sequence from the human ER promoter, ERCOUP, which contains a sequence homologous to the COUP sequence found in the ovalbumin promoter (27). These sequences contain a perfect half-site of the palindromic ERE. The specific complex formed between the ER and the 32P-labeled ERE probe (Fig. 3, lane 1) was effectively competed by a 20-fold molar excess of unlabeled ERE (Fig. 3, lane 2). This complex was not significantly competed by the other oligonucleotides tested (Fig. 3, lanes 3-5). These results indicate that the human ER overexpressed in this system recognizes the ERE specifically. It is important to note that a fragment containing an ERE half-site, ERCOUP, did not bind the ER and must have an affinity at least 20-fold lower than the palindromic ERE.

The initial experiments testing the specific binding of the ER were carried out at 20 °C in the absence of MgCl₂. A specific ER-ERE complex was observed in both the presence and absence of estradiol (Fig. 4, lanes 1 and 2). In addition, there was no significant difference in the total amount of specific complex in the presence or absence of estradiol over a wide range of DNA and protein concentrations (data not shown). The most visible effect of addition of estradiol in these experiments was a small but reproducible increase in the mobility of the complex (Fig. 4, lanes 1 and 2). This increase in mobility was observed over the entire range of probe and protein concentrations tested. The increase in mobility was not due to the chemical presence of the steroid, as the nonsteroidal

D. Shapiro, personal communication.

V. Kumar and P. Chambon, personal communication.
estrogen diethylstilbestrol had the same effect (data not shown).

In order to determine the conditions for hormone-dependent binding of the ER, the effects of incubation temperature and ionic environment were investigated. When complexes preformed by incubation at 20 °C and in the absence of Mg²⁺ were incubated further in the presence of 5 mM Mg²⁺ and at 37 °C, a significant estradiol requirement for binding was observed (Fig. 4, lanes 3 and 4). In the absence of hormone, the ER-ERE complex was not stable under these conditions. In the presence of estradiol, these changes in incubation conditions did not significantly alter the total amount of complex formed or its increased mobility in native gels (Fig. 4, lanes 2 and 4).

The effects of temperature and ionic environment on the hormonal dependence of the binding of the ER to the ERE were next assayed independently. In the absence of Mg²⁺, DNA binding was observed in both the presence and absence of estradiol at all of the temperatures tested (Fig. 5). At 4 °C (lanes 1 and 2) and 20 °C (lanes 3 and 4) the level of binding was almost equivalent with or without estradiol, but at 30 °C (lanes 5 and 6) and 37 °C (lanes 7 and 8) a significant dependence of binding upon estradiol was observed. The previously noted shift in the mobility of the complex upon addition of estradiol was reproduced at all temperatures tested. The hormonal dependence of binding was tested when reactions were incubated at different ionic strengths (Fig. 6). At 20 °C, an increased Mg²⁺ concentration restored the estradiol dependence of ER binding. This effect was observed at 5 mM Mg²⁺ and was more significant at 10 mM Mg²⁺ (Fig. 6, lanes 7–10). In general, high KCl concentrations decreased the level of binding in both the presence and absence of estradiol although a differential effect was observed at 400 mM KCl (Fig. 6, lanes 15 and 16).

Full hormone dependence for binding of the ER required incubation at both elevated temperature and Mg²⁺ concentrations. The effects of temperature and Mg²⁺ concentration on the hormonal dependence of ER binding were apparently additive. The effects of increasing the temperature from 20 to 30 °C and the Mg²⁺ concentration from 0 to 5 mM summed to account for the total binding at the higher temperature and ionic conditions.

Tamoxifen Induces a Novel ER-ERE Complex—The ability of tamoxifen, a nonsteroidal anti-estrogen, to stimulate the binding of ER to the ERE was also analyzed using the gel mobility assay (Fig. 7). Significantly, tamoxifen was able to stimulate ERE binding to the same extent as estradiol even in the presence of Mg²⁺ and at elevated temperature (Fig. 7B, lanes 2–5). However, tamoxifen did not induce the increase in the mobility of the ER-ERE complex which was observed with estradiol (Fig. 7, A and B, lanes 2–5). When increasing concentrations of tamoxifen were added to a constant satu-
rating amount of estradiol in the binding reaction, the mobility of the complex shifted to the slower moving form (Fig. 7, A and B, lanes 6–8). The requirement for a 100-fold higher concentration of tamoxifen than estradiol is due to its approximately 100-fold lower affinity for the ER (19). Thus, tamoxifen induces a specific ER-ERE complex with a mobility in native gels which is similar to that formed in the absence of hormone at low temperature and ionic strengths. It differs from this complex in that it is stable at high temperatures and ionic strengths. This novel complex with properties of both the complex formed in the absence of hormone and the estradiol-induced complex may contribute to the weak estrogenic properties of tamoxifen reported in some systems (28).

**DISCUSSION**

Unraveling of the complex biology governed by estrogen requires an understanding at a molecular level of the mechanism by which estrogen regulates the ER as a transcription factor. The receptor must be modified by the binding of the steroid, and it is important to determine its various states. Antagonists of estrogen action such as tamoxifen offer the ability to probe the activity of the ER under alternate conditions and may offer clues to the mechanism of ER action. We have produced the human ER in a baculovirus expression system and have studied the conditions influencing the formation of the ER-ERE complex.

At least three distinct ER-ERE complexes exist. In the absence of hormone, a complex forms between the ER and the ERE if Mg"+ is omitted from the binding reaction. Either raising the temperature of the binding reaction or addition of Mg"+ suppresses the formation of this ER-ERE complex. Binding of the ER requires dimerization, and the physical interactions being affected by these changes could either be responsible for dimerization or DNA binding. The formation of this ER-ERE complex implies that there may be some level of ERE occupancy even in the absence of estradiol in vitro. This may function in some genes to control the basal level of transcription and may allow these genes to be transcriptionally poised for the addition of an estrogenic signal. Recent work with the thyroid hormone receptor has shown that it too can bind its response element in the absence of hormone (29, 30). In view of this, it is noteworthy that in certain cell types, ER-dependent transcriptional activation is seen in the absence of exogenously added estrogen (26). Thus, the ER-ERE complex may form in vitro in the absence of estrogen and provide an additional level of gene regulation.

The ER-ERE complex formed in the presence of estradiol can be distinguished from the no hormone complex on the basis of two criteria. These are a differential sensitivity of the complexes to Mg"+ and an increased mobility of the estradiol-induced complex in native gels. The complex formed in the presence of estradiol was shown to be more stable than that formed without hormone. Only the former complex was stable at 37 °C and in the presence of 5 mM Mg"+ . It should be noted that under these latter conditions, binding of ER to the ERE is estrogen dependent. The basis for this increased stability is unclear, but it may involve stabilization of ER dimers by hormone under these conditions. Alternately, the effect may be on the stability of the ER monomer. The increased mobility of the estradiol-induced complex is independent of its stability, as tamoxifen is able to induce a complex that is stable to Mg"+ but shows a mobility that is similar to the no hormone complex. Thus, this change in mobility signals an estradiol-induced change in the ER and may reflect the activation of the hormone-inducible transactivation domain (9).

The ER-ERE complex formed in the presence of tamoxifen is distinct from either the no hormone complex or the estradiol complex. The tamoxifen complex displayed the same stability to temperature and Mg"+ as did the estradiol complex. Interestingly, the mobility of the tamoxifen complex was more similar to the no hormone complex. This would imply that the effect of tamoxifen differs from the effect of estradiol in a way distinct from the induction of specific DNA binding by the ER. This difference is reflected by the increased mobility of the estradiol-induced complex in native gels and may signal differential activation of the hormone-inducible transactivation domain (9). For example, the steroid may induce a conformation that strongly activates transcription whereas tamoxifen may stabilize binding but not activate transcription in the same fashion and may even inhibit transcription. Thus, tamoxifen could exhibit its anti-estrogenic effects on promoters that require the estrogen-inducible change in the transactivation domain for activity. In these cases, tamoxifen would be a competitive inhibitor of estrogen action or may bind and suppress transcription initiation. In cases in which both estrogen-dependent DNA binding and induction of the hormone-responsive transactivation domain are essential, tamoxifen would be expected to be a transdominant suppressor.

Under other conditions and in certain cell types, tamoxifen is known to have estrogenic activity (28). This may be due to the ability of tamoxifen to induce stable ERE binding under conditions that do not favor estrogen-independent binding of the ER. In these cases a second non-estrogen-dependent transactivation domain contained in the amino-terminal portion of the ER may be utilized (10). Thus, the apparent paradox of tamoxifen having both antagonistic and agonistic properties would be explained on the basis of its ability to stimulate the formation of a complex that has properties of both the no hormone and estradiol-induced complexes.

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**Note Added in Proof**—In support of our findings, Lees et al. (Lees, J. A., Fawell, S. F., and Parker, M. G. (1989) Nucleic Acids Res. 17, 5477–5488) have reported binding of the mouse ER to the ERE in the absence of hormone.

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