Hormone- and DNA-Binding Mechanisms of the Recombinant Human Estrogen Receptor†

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ABSTRACT: We have investigated the hormone- and DNA-binding mechanisms of the wild-type human estrogen receptor (hER) overproduced in insect cells using a baculovirus expression system. The recombinant hER was indistinguishable in size (67 kDa) and immunogenically from the native human estrogen receptor in MCF-7 breast carcinoma cells. The recombinant hER was purified to 70–80% homogeniety with a two-step procedure that included ammonium sulfate precipitation and oligonucleotide affinity chromatography using a unique Teflon affinity matrix. The recombinant hER bound estradiol with a positively cooperative mechanism. At hER concentrations in excess of 13 nM the Hill cofficient reached a maximal value of 1.6, whereas, at lower hER concentrations, the Hill cofficient approached 1.0, suggesting that the hER was dissociated to the monomeric species and site–site interactions were diminished. The hER specifically bound an estrogen responsive element (ERE) from chicken vitellogenin II gene as measured by the gel mobility assay, ethylation, and thymine interference footprinting. Specific interference patterns suggest a two-fold symmetry of the hER binding to the ERE with each monomer of the hER bound in the major groove of the DNA. These data indicate that the recombinant hER is valuable to define the biochemical and structural properties of the native estrogen receptor.

The human estrogen receptor (hER)[1] is a member of a family of transcription factors which are regulated by the binding of their cognate ligands. The interaction of the hormone-bound hER with specific DNA sequences referred to as estrogen responsive elements (ERE) alters the transcription efficiency of ERE-containing genes, by as yet an unknown mechanism (Beato, 1989; Evans, 1988; Green & Chambon, 1988; Yamamoto, 1985). The hER cDNA has been cloned and sequenced and its complete amino acid sequence deduced (Green et al., 1986; Walter et al., 1985). The estrogen receptor consists of several conserved domains responsible for the various functions of the receptor. By functional analyses of point mutants, truncated mutants and domain-swapping among the steroid hormone receptors, it was determined that the carboxy terminus contains the ligand-binding domain and the amino terminus the transactivation domain, and that the DNA binding domain is centrally located (Krust et al., 1986; Kumar et al., 1986). While transient transfection assays of the mutated receptors have provided valuable functional information about the different structural domains of the receptors, detailed protein biochemical studies of the steroid receptors and their role in altering transcription require large quantities of receptor.

Recently, recombinant DNA technology has allowed expression of proteins in prokaryotic or eukaryotic host cells in amounts previously unattainable from conventional sources. Expression of small proteins or truncated receptor domains in bacterial hosts have successfully produced large quantities of protein for structural studies (Hard et al., 1990). However, overexpression of larger complex proteins such as the full-length steroid hormone receptors in bacterial systems has proven ineffective. The bacterial host lacks the enzymes which are required for proper posttranslational modifications of the overexpressed proteins resulting in improper folding and subsequent insolubility of the proteins (Schein, 1989). Expression of the hER (Metzger et al., 1988), chicken progesterone (Mak et al., 1989), glucocorticoid (Schena & Yamamoto, 1988), and vitamin D (McDonnell et al., 1989) receptors in yeast cells have produced more recombinant protein than mammalian cells however, like the bacterial systems, the yield of functional receptor is low. The yeast cells also appear unable to properly process the overexpressed proteins (Metzger et al., 1988; Vlak & Keus, 1990).

In contrast, it has been demonstrated that posttranslational processing of overexpressed proteins produced in Sf9 insect cells using the baculovirus expression system closely parallels processing events in mammalian cells, including signal sequence cleavage, phosphorylation, and glycosylation. Thus, the problem of protein solubility is limited while simutaneously providing large quantities of biologically active protein (Luckow, 1991; Luckow & Summers, 1988). The strong baculoviral polyhedrin gene promoter drives expression of the desired gene such that milligram quantities of recombinant protein are possible. A number of steroid hormone receptors have been expressed in the baculovirus–Sf9 cell system and are reported to be biologically active and structurally identical to the authentic proteins (Alnemri et al., 1991a,b; Binart et al., 1991; Chang et al., 1992; Christensen et al., 1991; Elliston

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[1] Abbreviations: AcNPV, *Autographa californica* nuclear polyhedrosis virus; bp, base pair(s); BSA, bovine serum albumin; cVit II ERE, chicken vitellogenin II estrogen responsive element; DTT, DL-dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; ERE, estrogen responsive element; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; hER, human estrogen receptor; HSC, high speed cytosol; LSC, low speed cytosol; n_H, Hill coeffecient; SDS, sodium dodecyl sulfate; Sf9, *Spodoptera frugiperda* cells; xVit A_2 ERE, *Xenopus* vitellogenin A2 estrogen responsive element.

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et al., 1990, 1992; Paul et al., 1990; Ross et al., 1991; Srinivasan & Thompson, 1990; Xie et al., 1992).

Brown and Sharp (1990) reported the expression of a recombinant hER in the baculovirus system using the hER cDNA sequence contained in the HEO plasmid (Green et al., 1986). The recombinant hER was found to be full-length, expressed in high quantity, and capable of specifically binding both hormone and ERE sequences. However, a functionally significant mutation in the HEO cDNA sequence was discovered resulting in the substitution of a valine residue for glycine at amino acid 400. The resulting mutant hER is temperature sensitive, resulting in a physiologically variant phenotype from that of the wild-type hER (Tora et al., 1989).

We report here that we have corrected the valine mutation to the wild-type glycine, overexpressed the wild-type hER in the baculovirus system, and demonstrated functional equivalence of the overexpressed hER to the classical ER. We describe a simple two-step purification scheme using an ERE-containing oligonucleotide Teflon affinity matrix which yields milligram quantities of highly purified hER. We also describe several biochemical aspects of the recombinant hER, including its DNA- and [^{3}H]estradiol-binding mechanisms.

EXPERIMENTAL PROCEDURES

Materials. The transfer vector pVL1393, the wild-type *Autographa californica* nuclear polyhedrosis virus (AcNPV), *Spodoptera frugiperda* (Sf9) cells, and the Grace's insect cell culture medium were obtained from Invitrogen (San Diego, CA). The 17β-[6,7-^{3}H(N)]estradiol (45.6 mCi/mmol) and [α-^{32}P]dATP (6000 Ci/mmol) were obtained from Du Pont/New England Nuclear. The Teflon affinity matrix was obtained from Glen Research Corp. (Sterling, VA) in 1 μM cartridges and the oligonucleotide synthesized on an Applied Biosystems 380A DNA synthesizer. Fetal bovine serum (heat inactivated), gentamicin, fungizone, salmon sperm DNA, and ethylnitrosourea were purchased from Sigma (St Louis, MO). The pUC19 plasmid was from Pharmacia.

Cell Culture. Sf9 insect cells were grown in TNM-FH medium (Summers & Smith, 1987) which consists of Grace's insect medium supplemented with 10% heat-inactivated fetal bovine serum, 3.3 g/L yeastolate, 3.3 g/L lactalbumin hydrosylate, 50 mg/mL gentamicin, and 2.5 mg/mL fungizone. Production of recombinant hER was accomplished in 25 cm^2 or 150 cm^2 flasks (Corning), seeded with log phase Sf9 cells [(1–2) $\times$ 10^6 cells/mL] from suspension cultures, and allowed to achieve a continuous monolayer at 27 °C. Cells were infected with 10–50 pfu/cell of hER recombinant or control viral stocks for 1 h and then incubated for 48–111 h.

Construction of the Recombinant Transfer Vector and Recombinant Bacluovirus. The HEO plasmid containing the human estrogen receptor (hER) cDNA sequence (Green et al., 1986) was digested with *Eco*R1. The 1806 bp hER cDNA fragment and the linearized pUC19 plasmid vector were combined 1:10 (vector:fragment) and incubated with 2 units of T4 ligase for 48 h at 20 °C. The resulting recombinant plasmid was transformed into competent DH5α cells and purified. The plasmid containing the correct orientation of the hER cDNA was identified by digestion with *Hin*dIII.

The hER cDNA in the HEO has three mutations: two mutations are silent and one is expressed resulting in a valine substitution for a glycine at position 400 (Tora et al., 1989). Two single-stranded complimentary oligonucleotides of 71 and 78 bases were synthesized to replace a fragment of the hER cDNA between the *Dra*III and *Bgl*II sites. This oligonucleotide contains a sequence that corrects the valine mutation at amino acid 400 to the wild-type glycine as well as destroying the *Dra*III site resulting in an effective assay for the repaired clones. The pUC19 hER plasmid was double digested with *Dra*III and *Bgl*II, gel purified, and combined with the annealed oligonucleotides at 1:100 (vector:oligonucleotide) in a 50-μL T4 ligation reaction for 20 h at 20 °C. The resulting recombinant plasmids were analyzed by digestion with *Bgl*II and *Dra*III in separate reactions. The repaired cDNA encoding the wild-type hER was then cloned into the baculovirus transfer vector pVL1393 at the *Eco*R1 site (pVL1393-hER). The cDNA orientation was checked by *Sma*I digestion, and the region around the insert was sequenced to confirm the wild-type correction. A large-scale plasmid preparation of pVL1393-hER was produced and purified by CsCl gradient centrifugation.

The recombinant transfer vector pVL1393-hER and the wild-type *A. californica* nuclear polyhedrosis virus (AcNPV) were then cotransfected using the calcium phosphate method into Sf9 insect cells, and 6 days later the resulting recombinant virus (AcNPV-hER) was harvested. The recombinant virus (AcNPV-hER) was purified using the plaque assay and Southern blot analysis with the 71 bp *Bgl*II to *Dra*III oligonucleotide insert of the hER cDNA described above as the probe. The recombinant virus was isolated by three successive plaque purifications (Summers & Smith, 1987).

Construction of the ERE-Containing Oligonucleotide Teflon Matrix. An ERE containing nucleotide 5′-GTCCAAAGTCAGGTCACAGTGACCTGATCAAAGTT-3′ was synthesized on a Teflon affinity matrix (Duncan & Cavlier 1988). The Teflon fibers were incubated for 22 h at 50 °C in fresh ammonium hydroxide and then washed with water. The Teflon matrix was washed with annealing buffer (20 mM Tris, pH 7.4, 1 mM EDTA, 1 M NaCl), degassed under vacuum, and combined with 1 μmol of soluble 5′-^{32}P-labeled complimentary oligonucleotide 5′-AACTTTGATCAGGTCACTGTGACCTGACTTTGGAC-3′ in a final volume of 0.7 mL of annealing buffer. The complimentary ERE-containing oligonucleotides were annealed at 95 °C for 5 min, 72 °C for 4 h, and 42 °C overnight. Annealing was approximately 23% producing 2.6 mg of double-stranded oligonucleotide attached to the Teflon support. The matrix was stored in 40 mM Tris, pH 7.4, 2 mM EDTA, 1 mM EGTA, 1 mM DTT, 200 mM KCl, and 0.01% phenol at 4 °C.

Purification of Recombinant hER. Infected cells were washed with phosphate-buffered saline, pH 7.5, and lysed by freeze thawing three times in hypotonic buffer 40 mM Tris, pH 7.4, 1 mM EDTA, 1 mM EGTA, and 1 mM DTT (TDEE) with 0.2 mM phenylmethanesulfonyl fluoride and 0.5 mM leupeptin. Low-speed cytosol (LSC) was prepared by centrifuging the lysed cells at 28000g for 10 min at 4 °C. The LSC was brought to a concentration of 400 mM KCl and 2 μM [^{3}H]estradiol and incubated for 2 h at 4 °C, followed by centrifugation for 45 min at 143000g to obtain the soluble high speed cytosol (HSC). Ammonium sulfate (40%) was added to the HSC and the precipitate centrifuged at 28000g for 10 min. The precipitate was dissolved in TDEE buffer (12 mL) containing 100 mM KCl, 50 μg/mL salmon sperm DNA, and protease inhibitors. The hER was recirculated for 15 h over a ERE-containing oligonucleotide Teflon matrix in a 1.5-mL column at 12 mL/h. The ERE-matrix was washed with 30 volumes of TDEE buffer containing 200 mM KCl. The hER was eluted with a 20-mL linear gradient of 200–800 mM KCl in TDEE buffer with 10% glycerol at 4 mL/h. Peak and shoulder fractions were pooled separately, and 100 μg/mL insulin with 0.1 mM leupeptin was added. The SDS gel electrophoresis was described by Laemmli (1970).

Antibody 5 and 6 Production. A 20 amino acid peptide that included residues 152–170 (peptide 5) and a 21 amino acid peptide that included residues 259–278 (peptide 6) of the human estrogen receptor each with a unique N-terminal cysteine was synthesized by American Peptide Co. (Santa Clara, CA) and purified by reverse-phase HPLC. The cysteine of the HPLC-purified peptide was conjugated to the ϵ-amino group of the lysine residues of *Limulus* hemacyanin with the bifunctional cross-linking reagent, succinimdyl 4-(*N*-maleimidomethyl)cyclohexane-1-carborylate (Pierce, Rockford, IL). The peptide–hemacyanin conjugates had substitution ratios of about 10. New Zealand female rabbits were injected subcutaneously at multiple sites with complete Freund's adjuvant containing 1 mg of peptide 5 or 6. After 1 month the rabbits received booster injections subcutaneously at multiple sites of 1 mg of peptide 5 or 6 in Fruend's incomplete adjuvant. The booster injections were repeated twice at 3 week intervals. Serum was collected 2 weeks after the last booster and at 2 week intervals thereafter. Antibody 5 and 6 showed a 50% immunoprecipitation of the hER complexed with [^{3}H]estradiol at a 1:1500 dilution of the sera. These antibodies did not show cross-reactivity with the glucocorticoid or progesterone receptor. The preparation and characteristics of the polyclonal antibody raised against the calf estrogen receptor and Western blot analysis have been previously described (Denton et al., 1992).

DNA Ethylation, Selective Cleavage, and Glycolization. Probe preparation and gel mobility shift assays performed as described in Koszewski and Notides (1991). Briefly, the hER in 20 mM HEPES, pH 7.5, 0.2 mM EDTA, 0.5 mM DTT, 100 mM KCl, 1 mM $MgSO_4$, 100 nM estradiol, 0.5 mg/mL BSA, 15% glycerol, and protease inhibitors was incubated with ^{32}P-labeled probe (20 fmol) and 2.5 μg of poly(dIdC) in 20 μL for 90 min at 4 °C. Samples were loaded onto a prerun 5% polyacrylamide gel followed by electrophoresis at 175 V for 3 h in 6.7 mM Tris, pH 7.5, 3.3 mM sodium acetate, and 1 mM EDTA with cooling and recirculation. Ethylation of ^{32}P-end-labeled 47 bp probe encoding the chicken vitellogenin II ERE (cVit ERE) (Burch et al., 1988) was performed with ethylnitrosourea as described (Siebenlist & Gilbert, 1980). Base hydrolysis of the ethylated phosphates was performed as described (Sakonju & Brown, 1982) with modifications according to the protocol of Koszewski and Notides (1991). Glycolization of thymidine residues was described by Howgate et al. (1968) and Rubin and Schmid (1980). Thymidine footprint of the hER was performed according to the method of Truss et al. (1990). Briefly, ^{32}P-labeled probe was heat denatured at 95 °C for 3 min in 30 mM Tris, pH 7.4, and treated with 0.20 mM $KMnO_4$ at 20 °C for 10 min. Stop solution (1.5 M NaOAc with 0.42 M β-mercaptoethanol) was added followed by 10 mM Tris, pH 8.0, and 1 mM EDTA. The $KMnO_4$-treated probe was then ethanol precipitated, washed with 80% ethanol, and dried under vacuum. The oxidized single strands were annealed by resuspension in 10 mM Tris, pH 8.0, 1 mM EDTA, and 30 mM NaCl, heating to 65 °C, and slowly cooling to room temperature. Reannealed probe was bound to hER in gel mobility binding reactions as described above. For interference experiments, the wet gel was exposed to X-ray film overnight to visualize the free and hER-bound DNA complexes. The DNA was recovered by electroelution. The DNA-containing eluent was made 300 mM with respect to sodium acetate and precipitated with ethanol in the presence of 4 μg of tRNA. Pellets were washed with 75% ethanol and dried under vacuum. Following cleavage of the modified thymidines by piperidine, the ^{32}P-labeled fragments were subjected to electrophoresis in a 8% sequencing gel and dried, followed by autoradiography.

Estradiol Binding by the Recombinant hER. Ammonium sulfate precipitates of the hER were dissolved in TDEE buffer containing 100 mM KCl, 0.5 mM leupeptin, and 10% glycerol. γ-Globulin was added to give a final protein concentration of 1.5 mg/mL. The hER was incubated with a varied concentration of [^{3}H]estradiol, and the nonspecific binding was measured by a parallel incubation with [^{3}H]estradiol plus a 300-fold excess of estradiol for 15 h at 4 °C. At the completion of the incubation, 50 μL of each mixture was removed and the total [^{3}H]estradiol concentration determined. Then 100 μL of a 50% slurry of hydroxylapatite (in 40 mM Tris, pH 7.4, 1 mM EDTA, 1 mM EGTA) was added and allowed to bind hER for 40 min. The hydroxylapatite was washed three times with 0.5 mL of 40 mM Tris, pH 7.4, 1 mM EDTA, 1 mM EGTA, and 100 mM KCl. The hydroxylapatite pellets were suspended in 1 mL of ethanol and counted in 5 mL of scintillation fluid, and the receptor-bound [^{3}H]estradiol was measured. Each value is the mean of two determinations.

RESULTS

Expression of Full-Length Recombinant Human Estrogen Receptor in Sf9 Cells. Expression of the wild-type human estrogen receptor (hER) was examined in insect Sf9 cells from 0 to 111 h following infection with the recombinant virus, AcNPV-hER. Surprisingly, the amount recombinant hER protein continued to increase up to 111 h (Figure 1A). This receptor expression was 1–2 days longer than that reported for other steroid hormone receptors (Alnemri et al., 1991a,b; Binart et al., 1991; Chang et al., 1992; Christensen et al., 1991; Elliston et al., 1990, 1992; Paul et al., 1990; Ross et al., 1991; Srinivasan & Thompson, 1990; Xie et al., 1992). The hER was full length (67 kDa) as shown by SDS gel electrophoresis and comprised approximately 4–6% of the soluble proteins. Western blot analysis showed that the 67-kDa recombinant hER was the only specific immunoreactive band present.

The gel mobility shift assay was used to assess the ability of the recombinant protein to interact with the cVit II perfect ERE as a function of time postinfection. There was a steady increase in the amount of hER–ERE complex formed, indicating that even at high levels of hER expression the hER was soluble and did not aggregate (Figure 1B). Antibody 6, directed against the hinge region of the hER, retarded the mobility of the protein–^{32}P-labeled ERE complex confirming the identity of the hER. Antibody 6 alone did not form a complex when incubated with the ^{32}P-labeled ERE (Figure 1B).

The Recombinant hER Binds Estradiol with a Positive Cooperative Binding Mechanism. Scatchard analyses were performed on [^{3}H]estradiol equilibrium binding data of partially purified recombinant hER, as a function of receptor concentration. At receptor concentrations of 0.3 nM and 1.2 nM, the Scatchard plots were linear and the Hill coefficients were 1.0, indicating the absence of cooperativity of ligand binding at these receptor concentrations (Figure 2). However, at concentrations of 7, 13, and 20 nM hER, the Scatchard plots clearly exhibited convex curves with corresponding Hill coefficients of 1.2, 1.5, and 1.6, respectively, indicating a highly positive cooperative ligand-binding mechanism (Figure 2). The observed concentration-dependent, cooperative binding mechanism of the recombinant hER is in agreement with our earlier studies of the bovine estrogen receptor. At a receptor concentration comparable to that found intracellularly, the estrogen receptor is in the dimeric form and displays the ligand-induced conformational changes in its hormone-binding

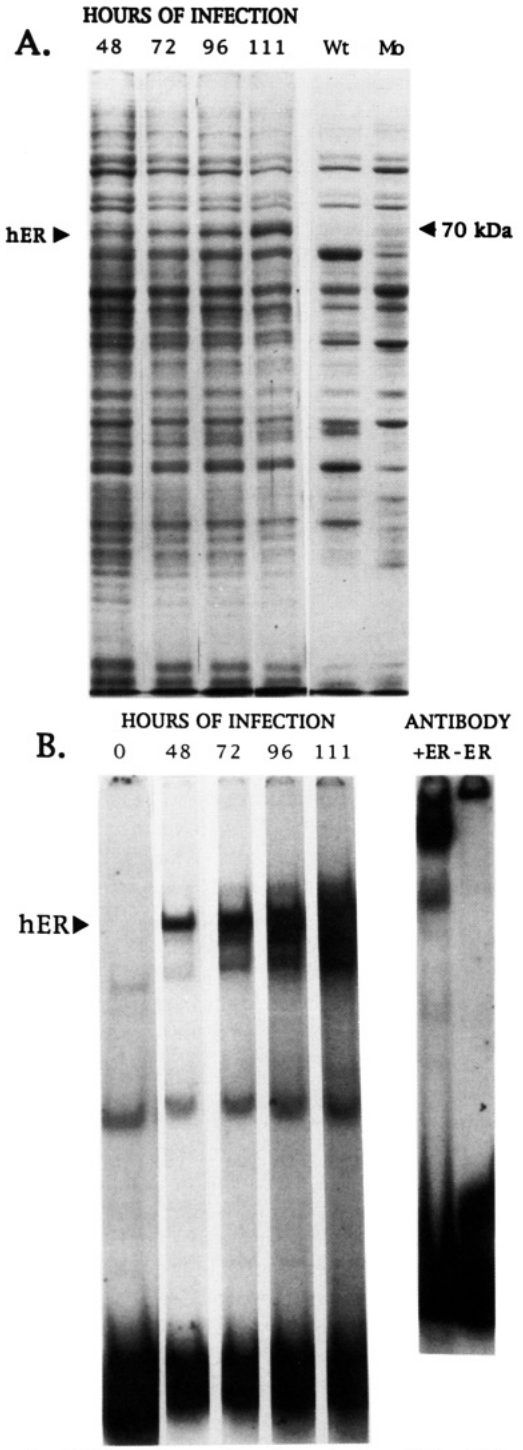

FIGURE 1: SDS gel electrophoresis and gel mobility shift analysis of the recombinant hER. (A) Cytosolic protein (100 μg) from Sf9 cells infected with the recombinant human estrogen receptor baculovirus (AcNPV-hER), for 48, 72, 96, and 111 h, or cells infected for 48 h with wild-type baculovirus (Wt) or mock infected (Mo) were subjected to SDS gel electrophoresis. The gel was then stained with Coomassie Blue. The position of the recombinant human estrogen receptor (hER) is noted. (B) Cytosolic protein (19 μg) from the AcNPV-hER infected Sf9 cells was incubated with 200 nM estradiol and a 47 bp ^{32}P-labeled ERE probe. The hER–ERE bound complexes were resolved on 5% nondenaturing polyacrylamide gels. Antibody 6 which is specific for the hER was included in the hER–ERE binding reaction (+ER) while (–ER) contains only antibody 6 without hER.

mechanism, whereas at lower receptor concentrations the receptor is dissociated into its monomeric form and site–site conformational changes are not present (Notides et al., 1981).

The estrogenic binding specificity of the hER is shown in Table I. Inhibition of [^{3}H]estradiol binding to the hER was achieved with potent estrogens and to a lesser degree with the

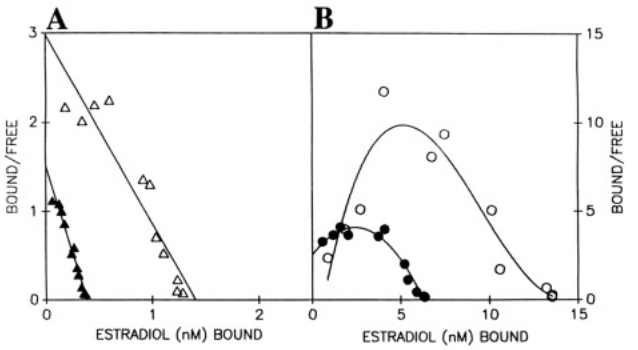

FIGURE 2: [^{3}H]Estradiol positive cooperative binding mechanism of the recombinant hER; Scatchard analysis of [^{3}H]estradiol binding using the recombinant hER at four concentrations. The receptor concentrations and Hill coefficients (n_H) are as follows: (A) 0.3 nM (▲), n_H = 1.0; 1.2 nM (△), n_H = 1.0; and (B) 7 nM (●), n_H = 1.2; and 13 nM (○), n_H = 1.5. The [^{3}H]estradiol binding with 20 nM recombinant hER is not illustrated. The final protein concentration was 1.5 mg/mL for all four concentrations of hER. Equilibrium binding was performed at 4 °C for 15 h, and the nonspecific binding was less than 10% for each point.

Table I: Steroid Specificity of Recombinant hER Expressed in Sf9 Insect Cells[a]

10-fold excess of unlabeled hormone	% inhibition
diethylstilbestrol	86
4-hydroxytamoxifen	84
17β-estradiol	83
estriol	55
17α-estradiol	51
estrone	47
ICI 164 384	40
dihydrotestosterone	1
progesterone	1
dexamethasone	0
testosterone	0

[a] A 40% ammonium sulfate fraction of the Sf9 cells containing the recombinant hER was used. The hER was equilibrated with 25 nM [^{3}H]estradiol for 1 h at 25 °C, without or with a 10-fold molar excess of unlabeled competitor. The 100% value was 1 pmol of specifically bound [^{3}H]estradiol per 0.2 mL. Each value is the mean of three determinations.

weaker estrogens and estrogenic antagonists but not by androgens, progesterone, or the synthetic glucocorticoid dexamethasone.

Purification of the Recombinant hER with an ERE-Containing Oligonucleotide Linked to a Teflon Matrix. The recombinant hER was purified by a two-step procedure (Figure 3). The cytosol from the Sf9 cells was precipitated with 40% ammonium sulfate resulting in a 70–80% recovery of the hER with a 2–3-fold purification of the receptor. The ammonium sulfate precipitate was dissolved in TDEE buffer containing 100 mM KCl and incubated with 50 μg/mL sonicated salmon sperm DNA. The protein–DNA mixture was passed over the ERE-containing oligonucleotide linked to a Teflon matrix. The affinity column was washed extensively and the recombinant hER eluted at approximately 450 mM KCl with a 20-mL linear gradient of 200–800 mM KCl. The hER is approximately 70% pure with one contaminant protein (30 kDa) accounting for the remaining protein. Thus, this two-step purification procedure allows us to purify approximately 1 mg of hER from four 150 cm^2 flasks of Sf9 cells.

The recombinant hER expressed in the Sf9 cells appears as a doublet following SDS gel electrophoresis (Figure 3), suggesting posttranslational modification. The doublet of the hER expressed in the Sf9 or MCF-7 cells have identical mobilities (data not shown). We and others (Washburn et

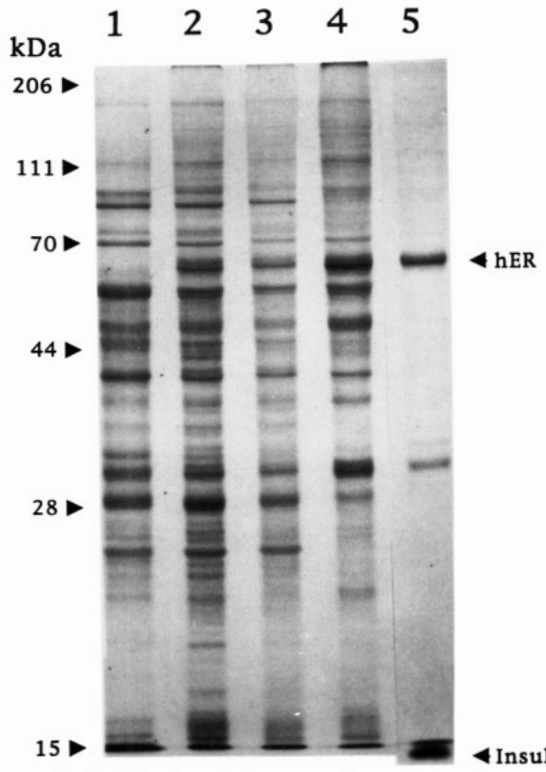

FIGURE 3: Purification of the recombinant hER using an ERE-containing oligonucleotide linked to a Teflon matrix; SDS gel electrophoresis of the recombinant hER purified from Sf9 cells. Lane 1 is a low speed cytosol of Sf9 cells infected for 48 h with wild-type baculovirus (AcNPV). Lane 2 is the low speed cytosol of Sf9 cells infected for 96 h with AcNPV-hER; lane 3 is the high speed cytosol, and lane 4 is the 40% ammonium sulfate fraction of the high speed cytosol. Each lane contains 150 μg of protein. Lane 5 contains 3.5 μg of hER eluted from the ERE-containing oligonucleotide Teflon column. One contaminant protein with a molecular mass of 30 kDa coelutes with the recombinant hER. The positions of the hER and insulin are noted.

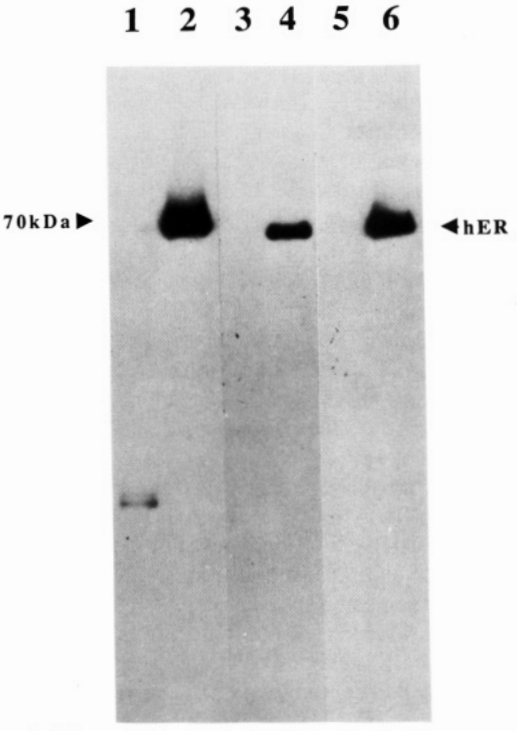

FIGURE 4: Western blot analysis of the purified recombinant hER and cytosolic proteins from Sf9 cells. Cytosolic proteins (50 μg) from Sf9 cells infected with the wild-type AcNPV baculovirus are in lanes 1, 3, and 5, and the purified hER (500 fmol) is in lanes 2, 4, and 6. The proteins were electrophoresed in a 10% SDS–polyacrylamide gel and then transferred to a poly(vinylidene difluoride) membrane. The membrane was blotted with a 1:500 dilution of a polyclonal antibody raised against the calf estrogen receptor (lanes 1 and 2), a 1:2000 dilution of antibody 5 (lanes 3 and 4), and a 1:2000 dilution of antibody 6 (lanes 5 and 6).

al., 1991) have observed that the upper band is produced by phosphorylation of the receptor. Phosphatase treatment of the receptor produced in Sf9 cells or MCF-7 cells results in dephosphorylation of the receptor, and the upper band collapses to the lower (data not shown) indicating that the Sf9 cells have posttranslationally phosphorylated the hER.

Western blot analysis with antibody 5 and antibody 6 directed against two different peptides of the hER and a polyclonal antibody against the bovine estrogen receptor, which cross-reacts with the hER (Denton et al., 1992), showed reactivity with the hER but not the 30-kDa protein (Figure 4) indicating it is not a fragment of the hER. The 30-kDa protein appears to comigrate with a protein found in the wild-type (AcNPV) baculovirus-infected Sf9 cells (Figure 3).

The Purified Recombinant hER Binds Specifically to an ERE. The crude or the ERE–oligonucleotide affinity-purified hER (30 nM) bound to a ^{32}P-labeled 47 bp oligonucleotide containing the chicken vitellogenin II (cVit II) ERE with the same relative intensity and mobility (Figure 5). The crude hER in the absence or presence of 1 mM $MgCl_2$ and/or 0.5 mg/mL of bovine serum albumin (BSA) did not affect the intensity or mobility of the specific hER–ERE complex that was formed. However, while the highly purified hER formed a comparable hER–ERE bound complex in the absence or presence of 1 mM $MgCl_2$, it was necessary to include BSA in the incubate. The BSA presumably prevents adsorption of the highly purified hER to nonspecific sites (Figure 5). BSA alone did not form a complex with the ^{32}P-labeled ERE (data not shown). The addition of insulin (2 mg/mL) resulted in the appearance of the purified hER–[^{32}P]ERE complex

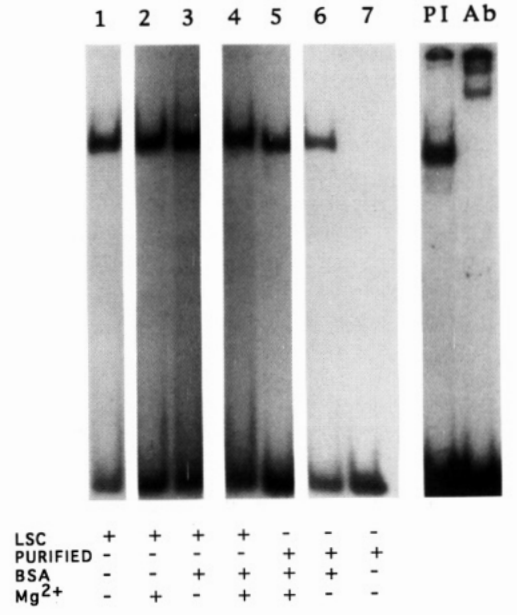

FIGURE 5: Purified recombinant hER binds specifically to an ERE; gel mobility shift assay of the hER using the ^{32}P-labeled cVit II ERE. Lanes 1–4 contain 30 nM unpurified hER in the low speed cytosol (LSC). Lanes 5–7 contain 30 nM hER purified by ERE–oligonucleotide affinity chromatography (PURIFIED). The hER was incubated with the 47 bp ^{32}P-labeled ERE at 4 °C for 45 min in the absence (lanes 1, 3, 6, and 7) or presence (lanes 2, 4, and 5) of 1 mM Mg^{2+}. Lanes 3–6 each contain 10 μg of bovine serum albumin (BSA). The purified hER–[^{32}P]ERE bound complex was incubated with preimmune serum (PI) or antibody 5 (Ab).

suggesting that inhibition of nonspecific adsorption of the purified hER was not unique to the BSA (data not shown). These data suggest that the purified estrogen receptor has an

intrinsic capacity to bind to its response element; nevertheless, it does not exclude the possibility that accessory proteins (Mukherjee & Chambon 1990) may facilitate estrogen receptor binding to its responsive elements. The intensity of the hER–ERE complex did not appear to be influenced by the absence or presence of estradiol; however, we consistently observed a slight difference in the mobilities of the hormone-bound and hormone-free hER–ERE complexes as reported by others (Fawell et al., 1990).

Ethylation and Thymine Interference with Recombinant hER Binding to the cVit II Perfect ERE. The individual contact points of the hER with the sugar–phosphate backbone of the coding strand of the cVit II perfect ERE demonstrate that the hER binds to its responsive element with a two-fold symmetry (Figure 6). The ^{32}P-end-labeled ERE was treated with ethylnitrosourea, which results in esterification of the sugar–phosphate backbone and alkylation of the purine residues (G ≫ A). After separation and recovery of hER-bound and free ERE on a nondenaturing gel, the phosphotriester positions were cleaved with base (NaOH), and the fragments were subjected to sequencing gel analysis (Figure 6A). The ethylation interference footprint indicated that four modified phosphate backbone positions on the coding strand of the perfect ERE contributed to receptor binding. The strongest interference was observed at phosphates pTpGACC located in the 3′ half of the ERE. Weaker interference was also seen in the 5′ half of the coding strand, pGpGTCA. Heating the ethylnitrosurea-treated DNA with piperidine resulted in the depurination of the N7-ethylated guanine to produce a guanine sequence ladder (Koszewski & Notides, 1991). Comparison of these footprint patterns allowed an estimate of the degree of interference caused by the N7-alkylated guanines at the corresponding phosphotriester position. The guanines in both halves of the palindrome 5′-GGTCA and TGACC-3′ strongly interfere with hER binding to the ERE. In contrast, the variant guanine located in the ERE three base spacer did not inhibit binding.

The contribution of the thymidine residues to specific hER binding following treatment of the ^{32}P-labeled ERE with $KMnO_4$ (Truss et al., 1990, 1991) indicates that each of the thymidines of the ERE are involved. Thymidine residues are attacked by $KMnO_4$ at the C5–C6 double bond of thymines in single-stranded DNA, resulting in a glycolization reaction that can be followed by oxidation to carbocyclic acid and/or ring opening (Howgate et al., 1968; Rubin & Schmid, 1990). After reannealing, the double-stranded ERE was bound to the recombinant hER. Free and hER-bound fractions of ^{32}P-labeled ERE were treated with piperidine, and fragments were subjected to denaturing gel analysis (Figure 6B). Both thymidine residues in the coding strand of the cVit II ERE strongly interfered with binding of recombinant hER. The modified thymidines in the adjacent flanking regions of the perfect ERE did not inhibit binding.

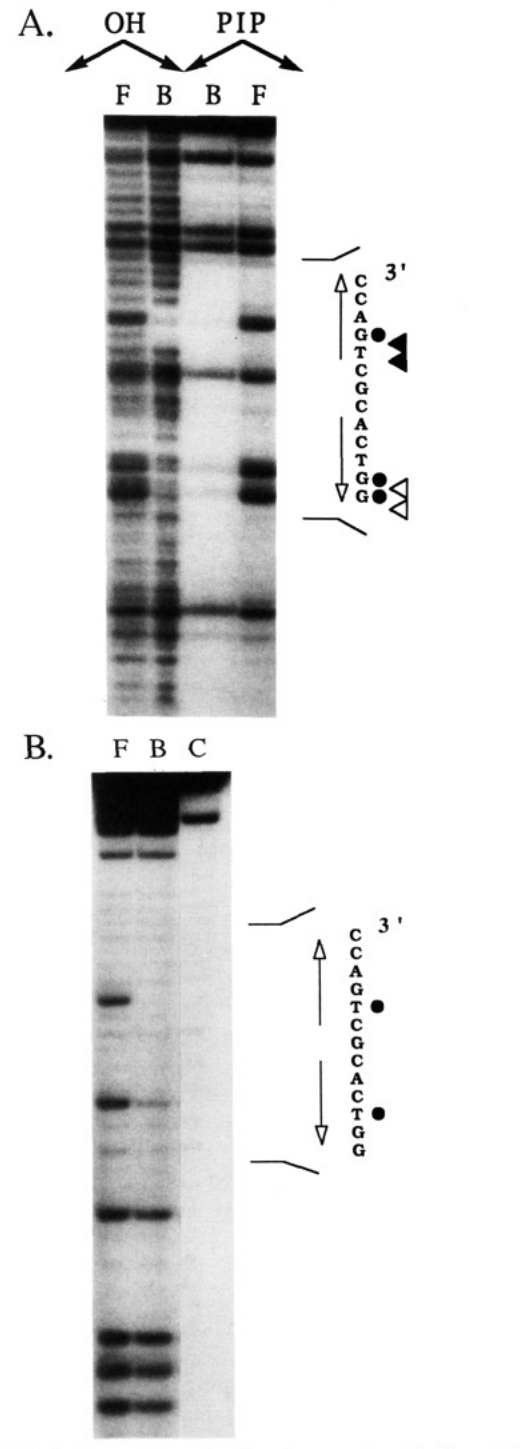

FIGURE 6: Ethylation and thymine interference footprinting of the recombinant hER. (A) The ethylated 47 bp cVit II ERE, selectively ^{32}P-labeled on the coding strand, was analyzed for hER contacts with the phosphates of the ERE. A gel mobility shift assay was used to separate the free (F) and hER-bound (B) ^{32}P-labeled DNA probe. Selective cleavage of the ethylated DNA from the hER bound complex was undertaken to determine the contribution of the phosphate backbone and alkylated G residues (●). The DNA recovered from the hER-bound complex and the free DNA was apportioned for cleavage reactions with 0.1 M sodium hydroxide (OH) or 1 M piperidine (PIP) for 30 min at 90 °C. The cleaved fragments were electrophoresed through 8% sequencing gels and dried, followed by autoradiography. Films were scanned with a laser densitometer; strong phosphate contacts (solid triangles) and weak phosphate contacts (open triangles) are noted. (B) In order to determine the contribution of the thymidine residues (●) to binding of the hER, the ^{32}P-labeled ERE was modified with $KMnO_4$ and incubated with the hER. After nondenaturing gel electrophoresis, the free (F) and hER-bound (B) oxidized DNA was isolated and cleaved with 1 M piperidine for 30 min at 90 °C. Unmodified control DNA was treated identically (C).

DISCUSSION

In the present work we have determined optimum conditions for the expression of the human estrogen receptor in Sf9 cells with a recombinant baculovirus vector. Previously the steroid hormone receptors were expressed in the baculovirus insect system for up to 2–3 days (Alnemri et al., 1991a,b; Xie et al., 1992; Ross et al., 1991; Binart et al., 1991; Christensen et al., 1991; Srinivasan & Thompson, 1990; Chang et al., 1992; Paul et al., 1990). Here we report that expression of the wild-type hER up to 5 days postinfection results in a substantial increase in receptor concentration and no evidence of receptor proteolysis or insolubility. From 2×10^8 infected Sf9 cells, we

typically recover 35 nmol (2.3 mg) of hER or approximately 230 pmol of hER per mg of protein, which is 1×10^8 receptors per Sf9 cell. This level of recombinant hER expression is approximately twice the level previously achieved for the hER (Brown & Sharp, 1990) and a 100-fold greater expression than reported for the human progesterone (Christensen et al., 1991) or human glucocorticoid (Alnemri et al., 1991; Srinivasan & Thompson, 1990) receptors with the baculoviral expression system. The addition of estradiol during hER expression did not increase the quantity of recoverable receptor (unpublished data). In contrast, the addition of 1,25-$(OH)_2D_3$ (Sone et al., 1990) or progesterone (Christensen et al., 1991) during expression of their respective receptors dramatically increased the quantity of recoverable receptors, presumably due to *in vivo* stabilization of the receptors. The recombinant hER expressed in the Sf9 cells is full length and biologically active with respect to hormone- and DNA-binding.

We found that affinity chromatography purification of the recombinant hER with an ERE oligonucleotide linked to a fibrous Teflon matrix (Duncan & Cavlier, 1988) was superior to the same ERE oligonucleotide coupled to agarose. The Teflon fiber is coated with copolymer containing a 25 atom spacer arm of mixed hydrophilic character to which the ERE oligonucleotide was linked. The concentration of the ERE oligonucleotide linked to the Teflon fiber was 600–1000-fold greater (250 nmol of the double-stranded ERE oligonucleotide on approximately 0.5 mL of Teflon fiber) than to the agarose (400 pmol/mL of gel). The Teflon-linked ERE affinity resin is both chemically and mechanically stable. An excess of 20 receptor purifications have been performed with no measurable loss of binding capacity. The very high capacity of specific receptor binding sites and very low nonspecific protein binding of the Teflon-linked ERE oligonucleotide makes it an ideal affinity chromatography matrix for purification of large quantities of receptor.

The 30-kDa protein that copurified with the hER during ERE oligonucleotide affinity chromatography was shown not to be a fragment of the hER (Figures 3 and 4). This protein was isolated by ERE oligonucleotide affinity chromatography from Sf9 cells infected with the wild-type baculovirus, indicating that it is from the Sf9-infected cells and binds independently of the hER (data not shown). It remains to be seen if changing oligonucleotide sequence flanking the ERE sequence or reducing the size of oligonucleotide linked to the Teflon matrix would eliminate the 30-kDa protein's binding.

The analysis of the equilibrium binding of estradiol by the recombinant hER reveals the mechanism by which estradiol regulates the conformation of the receptor. The Scatchard plot of the [^{3}H]estradiol binding is convex at receptor concentrations above 4 nM. The Hill coefficient, an index of cooperativity, reaches a maximum of approximately 1.6 at a receptor concentration between 7 and 20 nM, a value consistent with positive cooperative site–site interaction in a dimer. Thus, the hER exists in a reversible equilibrium between two conformational states with each state having a different affinity for estrogens. Estradiol binding therefore shifts the equilibrium toward the conformation of the receptor with the higher affinity for estradiol, the activated conformation of the receptor. The loss of cooperative estradiol binding at low hER concentrations indicates that the receptor is dissociated and site–site interaction of the two hormone binding sites is not present in the monomeric form of the receptor (Notides et al., 1981, 1985).

The equilibrium constant for hER dimerization is approximately 4 nM (data not shown), which is 10-fold higher than the equilibrium constant of dimerization (0.6 nM) observed

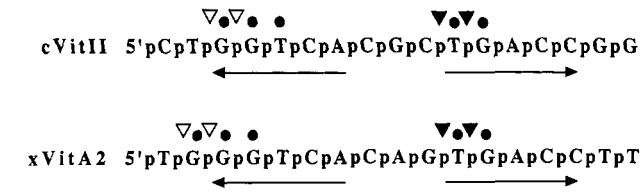

FIGURE 7: Comparison of the phosphate, guanine, and thymidine interference footprints of the cVit II ERE and the xVit A2 ERE. The interference footprint of the cVit II ERE using the wild-type hER reported here (Figure 5) is compared to the interference footprint of the xVit A2 ERE using the Val_{400} mutant hER reported by Truss et al. (1991).

for the bovine or rat estrogen receptor (Notides et al., 1981). Thus, the hER would be expected to show a qualitative difference to estrogens. Presumably, the estrogen responsiveness of the hER would be more dependent upon estrogen concentration and intracellular estrogen receptor concentration than the bovine or rat estrogen receptors.

The recombinant hER establishes four contact points with the sugar–phosphate backbone of the coding strand of the cVit II perfect ERE, two of which are strong and two weaker (Figure 6). The same footprint pattern was observed with the hER from nuclear extracts of MCF-7 cells (data not shown), further confirming the structural integrity of the recombinant protein. In contrast, the bovine estrogen receptor makes two strong and three weaker sugar–phosphate contacts with each strand of the cVit II perfect ERE (Koszewski & Notides, 1991). The contact points with the sugar–phosphates are identical with the hER and bovine ER except that the bovine ER establishes an additional weaker phosphate contact in the 3′ half of the ERE at TGpACC. Because of the high degree of conservation observed in the estrogen receptor's DNA-binding domain among species (Krust et al., 1986), this finding implies that regions outside this domain, which can vary substantially, may influence the receptor's points of contact with an ERE. It remains to be determined whether this additional phosphate contact established by the bovine ER results in a ER–ERE interaction with a higher binding affinity than the hER.

The interference footprint data for the guanine and thymidine residues indicates that modification of any of these residues within the two symmetrical halves of the palindromic cVit II ERE, but not the three nonconserved basepairs, interfered with hER–ERE binding. The bovine ER gave similar results (Koszewski & Notides, 1991). These observations, together with the sugar–phosphate backbone footprint discussed above, can be contrasted with the findings of Truss et al. (1991). Using transient transfection for expression of the HEO, they examined binding interactions to the *Xenopus* vitellogenin A2 (xVit A2) perfect ERE. While good agreement is seen in the phosphate, guanine, and thymidine interference footprints in the 3′ halves, TGACC, of both of these elements, striking differences are evident in the 5′ halves of these EREs (Figure 7). The two phosphate backbone contacts observed in the xVit A2 ERE are 5′-shifted by one phosphate position relative to that seen using the cVit II ERE. Strong interference is also seen with the guanine that is immediately 5′, or just outside, of the canonical ERE sequence of the xVit A2 gene. Finally, no thymidine interference is observed in the 5′ half, GGTCA, of the xVit A2 ERE, while modification of this residue results in strong interference to binding by either recombinant hER or bovine ER to the cVit II ERE. In short, the points of contact observed in the 5′ half of the xVit A2 ERE appear to be 5′-shifted by one position relative to the contacts seen with the cVit II ERE. We cannot rule out the possible influence of the point mutation that exists

in the HEO for generating dissimilar footprint patterns. However, the altered contacts may reflect sequence-dependent differences in local DNA structure for these particular ERE's concomitant with an inherent flexibility on the part of the ER dimer to maximize high-affinity binding interactions.

The expression of the hER in the baculovirus insect cell system provides a means of obtaining large quantities of human proteins for their biochemical and structural analyses. In addition, receptor–DNA interactions, the role of phosphorylation in transcriptional activation, and hormone-induced conformational changes of the hER can be investigated.

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