HMG-1 Stimulates Estrogen Response Element Binding by Estrogen Receptor from Stably Transfected HeLa Cells

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Estrogen receptor (ER) toxicity has hampered the development of vertebrate cell lines stably expressing substantial levels of recombinant wild-type ER. To isolate clonal lines of HeLa cells stably expressing epitope-tagged ER, we used a construction encoding a single bicistronic mRNA, in which FLAG-epitope-tagged human ERα (fER) was translated from a 5′-translation initiation site and fused to the neomycin resistance gene, which was translated from an internal ribosome entry site. One stable HeLa-ER-positive cell line (HeLa-ER1) produces 1,300,000 molecules of fER/cell ($\sim$20-fold more ER than MCF-7 cells). The HeLa fER is biologically active *in vivo*, as judged by rapid death of the cells in the presence of either 17β-estradiol or *trans*-hydroxytamoxifen and the ability of the cell line to activate a transfected estrogen response element (ERE)-containing reporter gene. The FLAG-tagged ER was purified to near homogeneity in a single step by immunoaffinity chromatography with anti-FLAG monoclonal antibody. Purified fER exhibited a distribution constant (K_D) for 17β-estradiol of 0.45 nM. Purified HeLa fER and HeLa fER in crude nuclear extracts exhibit similar K_D values for the ERE (0.8 nM and 1 nM, respectively), which are approximately 10 times lower than the K_D of 10 nM we determined for purified ER expressed using the baculovirus system. HMG-1 strongly stimulated binding of both crude and purified HeLa fER to the ERE (K_D of 0.25 nM). In transfected HeLa cells, HMG-1 exhibited a dose-dependent stimulation of 17β-estradiol-dependent transactivation. At high levels of transfected HMG-1 expression plasmid, transactivation by ER became partially ligand-independent, and transactivation by *trans*-hydroxytamoxifen was increased by more than 25-fold. These data describe a system in which ER, stably expressed in HeLa cells and easily purified, exhibits extremely high affinity for the ERE, and suggest that intracellular levels of HMG-1 may be limiting for ER action. (Molecular Endocrinology 13: 632–643, 1999)

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INTRODUCTION

As a heavily studied member of the steroid/nuclear receptor superfamily of ligand-regulated transcription factors (1–6), substantial interest has centered on the mechanism by which estrogen receptor (ER) regulates gene expression. The importance of ER in the growth and functioning of the mammalian reproductive system, in osteoporosis, in breast and uterine cancer, and in heart disease has intensified interest in ER action. Despite the availability of a great deal of information on ER action, fundamental questions remain in areas such as the role of phosphorylation in ER action, the process by which ligand binding and/or phosphorylation transforms the receptor into a transcription activator, and the mechanism(s) by which ER activates gene transcription and regulates mRNA stability.

The toxicity of ER in mammalian cells has made expression of substantial levels of wild-type ER quite difficult, hindering both structural and mechanistic studies of ER action. Although the ER has been expressed in *Escherichia coli*, yeast, and in the baculovirus system (7–10), these systems suffer from several problems. While ER fusion proteins have been expressed in *E. coli*, the level of expression was relatively low (7), and appropriate posttranslational modification of the ER was unlikely to have occurred. ER production in yeast has been hampered by proteolysis and by low levels of functional expression (9). ER expressed and purified in insect cells using the baculovirus system (10–12) binds to DNA containing the estrogen response element (ERE) poorly (see below), or not at all, unless additional proteins such as HMG-1 are present (11, 12). When ER is expressed in mammalian cell lines, the cells stop growing and lyse after exposure to low concentrations of estrogen (13, 14). Consequently, ER-expressing cell lines have expressed relatively low levels of wild-type ER (15). One approach to circumvent the toxicity of wild-type ER has been to utilize the less toxic ER_{val400} mutant (16, 17). Using this ER mutant, stable mammalian cell lines have been developed (13, 14, 18–26), some of which express very high levels of this less toxic mutant ER. While

these cell lines will continue to be extremely useful, the ER expressed in these cell lines lacks an epitope tag to facilitate isolation of ER-protein complexes and ER purification.

To prevent overgrowth of our culture by cells that had either lost the ER gene through recombination, or inactivated the promoter driving ER transcription, we used a bicistronic expression system (27), which tightly couples expression of the ER and the antibiotic resistance genes by transcribing them as a single bicistronic mRNA. Translation of the bicistronic mRNA occurs from two different translation initiation sites. HeLa-ER1, one of the stable HeLa-ER cell lines we developed using the bicistronic mRNA system, expresses high levels of biologically active epitope-tagged human ERα.

Several proteins have been reported to enhance the binding of steroid receptors to their DNA response elements (28, 29). Recent studies have focused on high-mobility group protein 1, HMG-1. HMG-1 is a highly conserved nonhistone chromosomal protein that binds to DNA without exhibiting sequence specificity, but exhibits a strong binding preference for DNAs in nonlinear conformations (30). HMG-1 enhances the binding of purified progesterone receptor (PR) (31, 32), ER expressed in nonmammalian cells (11, 12), and ER DNA-binding domain (33) to their respective response elements. Recently, Edwards and co-workers (12) extended their earlier work on HMG-1 action and demonstrated that HMG-1 enhances transactivation in intact cells and DNA binding by purified estrogen, androgen, and glucocorticoid receptors. However, HMG-1 did not stimulate DNA binding by nonsteroid nuclear receptors. We used both crude nuclear extracts and purified FLAG epitope-tagged ER (fER) from the HeLa-ER1 cells to analyze the effect of HMG-1 on binding of the fER to the ERE. Since fER in crude HeLa-ER1 nuclear extracts and purified fER exhibited similar high-affinity binding to the ERE, it was surprising that addition of HMG-1 strongly stimulated ERE binding by both purified fER and by fER in crude HeLa-ER1 nuclear extracts. In transient transfections of HeLa cells and of MDA-MB-231 human breast cancer cells, HMG-1 elicited a dose-dependent stimulation of both 17β-estradiol (E_2)-dependent and E_2-independent transactivation of an ERE-containing reporter gene. A high level of transfected HMG-1 expression plasmid increased transactivation by E_2 by approximately 5-fold while transactivation by *trans*-hydroxytamoxifen was increased by 27-fold.

RESULTS

Epitope-Tagged Flag-ER and Wild-Type ER Exhibit Similar Transactivation Potential in HeLa Cells

To facilitate ER purification, the FLAG epitope (34) was added in-frame at the N terminus of the ER. In transient transfections of HeLa cells expressing saturating and subsaturating levels of the receptors, fER and wild-type ER exhibited similar ability to activate transcription of an ERE-containing reporter gene (Fig. 1A). Recently, Kraus and Kadonaga (35) also concluded that adding an N-terminal FLAG epitope does not alter the properties of the ER.

Production of Clonal HeLa Cell Lines Stably Expressing ER

Using both constitutive and regulated promoters, our previous efforts to isolate stable cell lines expressing substantial levels of epitope-tagged ER were unsuccessful. We concluded that the toxicity of wild-type ER leads to progressive overgrowth of the culture by cells that have lost the ability to synthesize ER. We therefore elected to use the bicistronic mRNA expression system (Ref. 27; Fig. 1B), in which expression of both the fER and the neomycin phosphotransferase gene are under the control of a single promoter. The plasmid containing this bicistronic construct was introduced into HeLa cells by electroporation, and the cells were

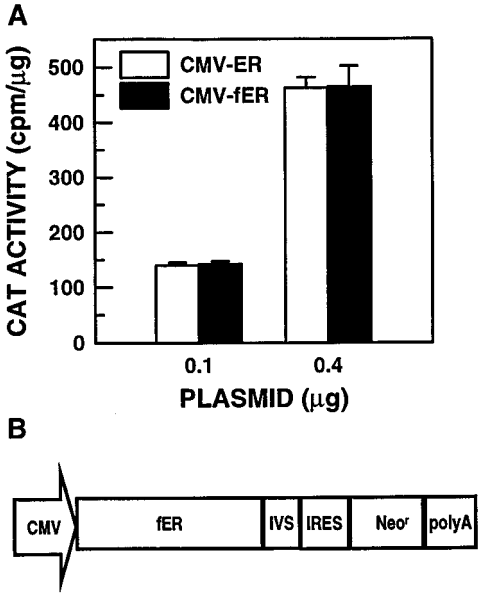

Fig. 1. Epitope-Tagged ER and Wild-Type ER Elicit Similar Activation of an ERE-Containing Reporter Gene

A, HeLa cells were cotransfected by electroporation with the indicated quantities of pCMV-ER (*open bars*) or pCMV-fER (*solid bars*) expression plasmid, 20 μg of pATC4 reporter gene (36), and 4.2 μg of pCMV-luciferase internal standard. The cells were maintained in 10^{-8} M E_2 for 48 h before harvesting. Cell extracts were prepared and CAT assays were performed as described in *Materials and Methods*. The data represent the mean ± SEM for three separate transfections. B, A schematic representation of the bicistronic ER expression cassette. The fER is followed by an intron (IVS) and an internal ribosomal entry site (IRES). This results in transcription of the fER and the neomycin resistance gene (Neo^r) as a single bicistronic mRNA.

maintained under continuous G418 selection. Seven clones exhibiting reasonable growth rates were isolated and analyzed for fER content. All seven of the G418-resistant clones expressed significant levels of fER, with levels ranging from 20,000–1,300,000 molecules of fER per cell (Table 1). The cloned cell line with the highest level of fER expression, designated HeLa-ER1, was chosen for more detailed study. The HeLa-ER1 cell line shows no sign of the progressive loss of fER expression we previously observed and has maintained its high level of fER expression for more than 1 yr. This cell line expresses about 20 times more ER than standard lines of MCF-7 human breast cancer cells (Ref. 13; Table 1).

The fER in HeLa-ER1 Cells Is Biologically Active *in Vivo*

To determine whether the FLAG-hERα expressed in HeLa-ER1 cells is functional *in vivo*, we determined whether it retained the characteristics of biologically active ER. Functional liganded ER should 1) show nuclear localization when ligand is present, 2) be toxic to the cells at high expression levels, and 3) activate transcription of an ERE-containing reporter gene. To ascertain the subcellular location of fER in the presence and absence of E_2, we prepared nuclear and cytosol extracts and analyzed their ER content by Western blotting. In the absence of E_2, approximately half of the fER was in the cytosol. Addition of E_2 resulted in complete nuclear localization of the fER (data not shown). Although the level of fER in the HeLa-ER1 cells is quite high, most of the fER is in a soluble and salt-extractable form, with only a small fraction in the insoluble nuclear pellet.

To assess fER toxicity, cell growth was monitored in either the presence or absence of 17β-estradiol, or of the antiestrogens, *trans*-hydroxytamoxifen (TOT) and ICI 182,780 (Fig. 2). 17β-Estradiol had no effect on the growth of wild-type HeLa cells, which lack ER (Table 1). TOT or ICI 182,870 also showed no effect on HeLa cell growth (data not shown). Addition of 17β-estradiol, or of the antiestrogen TOT, resulted in rapid killing of the HeLa ER-1 cells. Estradiol and TOT were also toxic to HeLa-ER2 cells (data not shown), which contain a much lower level of ER than the HeLa-ER1 cells (see Table 1). In contrast, the pure antiestrogen ICI 182,870 was not toxic to the HeLa-ER1 cells and actually enhanced their growth (Fig. 2).

Table 1. fER Levels in Stably Transfected HeLa Cells

Cell Line	fER Level (receptors/cell)[a]
HeLa-ER1	1,300,000
HeLa-ER2	48,000
HeLa-ER3	92,000
HeLa-ER4	20,000
HeLa-ER5	177,000
HeLa-ER6	148,000
HeLa-ER7	72,000
HeLa	0
MCF-7	55,000

[a] ER and fER determination was by whole-cell ER assay as described in *Materials and Methods.*

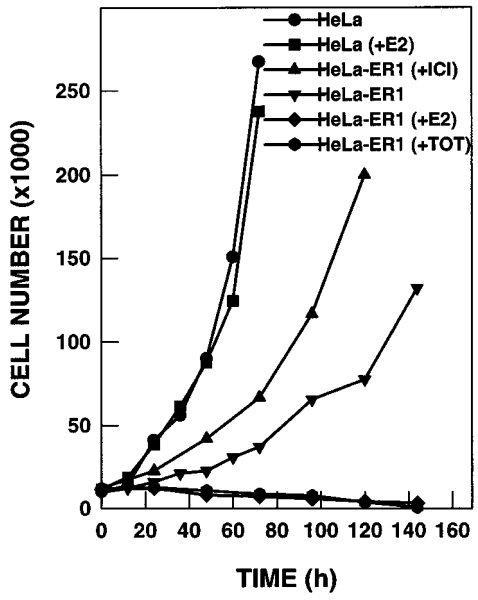

Fig. 2. Estradiol and TOT Are Toxic to Hela-ER1 Cells

Initially, HeLa-ER1 cells were plated at 10,000 cells per well in 24-well plates before the addition of 17β-estradiol, TOT, or ICI 182,780. In all cases hormone was dissolved in ethanol and added to medium to a final concentration of 5 nM. An equivalent volume of the ethanol vehicle was added to control media.

Since transcription activation requires the fER to be competent in ligand binding, DNA binding, and interaction with coactivators, transactivation is a useful test of the biological activity of the fER. The fER effectively transactivated a transiently transfected 2ERE-thymidine kinase (TK)-chloramphenicol acetyl transferase (CAT) reporter gene over a range of E_2 concentrations (Fig. 3). The estradiol dose-response curve exhibits saturation at 10^{-9} M, which is in good agreement with the K_D of the ER for E_2 (see below). Even at the very high level of fER in the HeLa-ER1 cells, there was no transcription of the reporter gene in the absence of added E_2. This finding is in agreement with our recent report that under standard cell culture conditions transcription activation by ER requires the presence of estrogen (36). TOT did not activate transcription of the 2ERE-TK-CAT reporter gene and antagonized E_2-mediated transactivation.

Purification of the fER

To purify the fER, we prepared nuclear extracts from HeLa-ER1 cells and purified the FLAG-tagged ER by immunoaffinity chromatography with an immobilized monoclonal antibody against the FLAG epitope. To isolate pure fER, the column was washed exhaus-

tively, and the fER was eluted using the FLAG peptide. SDS-PAGE of the eluted fER showed only a single band at the position expected for ER, suggesting the fER was nearly homogeneous (Fig. 4A, fER). Western blotting with the ER-specific monoclonal antibody, H222 (37), which recognizes an epitope near the C terminus of the ER and with the anti-FLAG M2 antibody, which recognizes the N-terminal FLAG epitope, revealed a single band comigrating with the mol wt standard at about 66 kDa (Fig. 4, B and C). The presence of a single band in the silver-stained gel of pure fER and in Western blotting with antibodies specific for N-terminal and C-terminal epitopes demonstrates that the fER did not undergo significant proteolysis during purification.

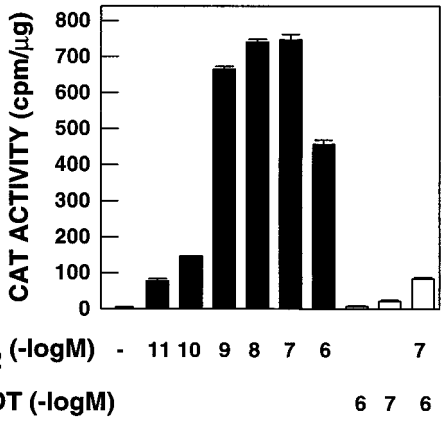

Fig. 3. Hormone-Dependent Transactivation by fER in HeLa-ER1 Cells

HeLa-ER1 cells were transfected with the ERE-containing reporter plasmid 2ERE-TK-CAT and maintained in the indicated concentrations of 17β-estradiol, TOT, or estradiol + TOT. Submaximal CAT activity at 10^{-6} M estradiol could be due to estradiol toxicity (see Fig. 3). Transfections and CAT assays were as described in *Materials and Methods*. The data represent the mean ± SEM for three separate transfections.

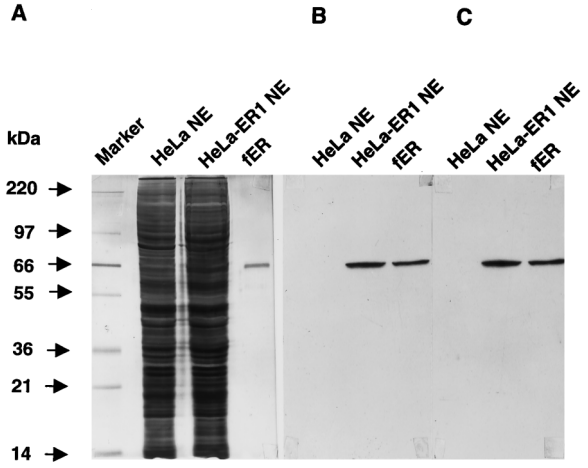

Fig. 4. Electrophoretic Analysis of Purified fER

A, Protein (10 μg) from a nuclear extract of wild-type HeLa cells (HeLa NE), or from a HeLa-ER1 nuclear extract (HeLa-ER1 NE), and about 10 ng of purified fER protein were fractioned by SDS-PAGE, and proteins were visualized by silver staining. The same amount of HeLa and HeLa-ER1 extracts and about 4 ng of purified fER protein were analyzed by Western blotting and visualized using the ECL system with either anti-FLAG M2 antibody (panel B) or the ER-specific monoclonal antibody H222 (panel C) as the primary antibody.

Purified fER and Crude ER Exhibit the Same Affinity for 17β-Estradiol

We analyzed binding of 17β-estradiol to the fER in crude nuclear extracts and after purification of the fER to near homogeneity. The concentrations of the crude and purified fER used in the binding assays were approximately 1 nM. Analysis of ligand binding using Scatchard plots resulted in linear plots with both crude and purified ER (Fig. 5). The crude and purified ER exhibited K_D values for 17β-estradiol of 0.45–0.5 nM, which is in the range of affinities determined for wild-type ER in various cell lines (38, 39). These data indicated that other cellular proteins, which are removed on purification of the fER to near homogeneity, do not influence its ability to bind ligand.

ERE Binding by fER Purified from HeLa-ER1 Cells

In preliminary studies we used electrophoretic mobility shift assays to examine the interaction of purified fER with a labeled ERE. The upshifted band was supershifted with the ER-specific monoclonal antibody, H222, but not with a control anti-BSA antibody (Fig. 6A, H222, Anti-BSA), indicating it was an ER-ERE complex. In addition, binding of ER to the ERE was competed by increasing amounts of unlabeled ERE, but not by a 100- and 1000-fold molar excess of

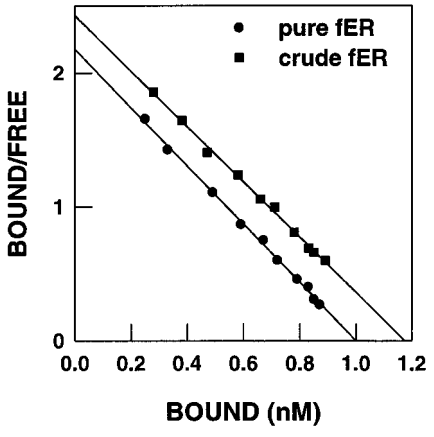

Fig. 5. Crude fER and Purified fER Exhibit the Same Affinity for 17β-Estradiol

Extracts containing the same amount of fER were incubated with increasing amounts of [^{3}H]estradiol in the presence or absence of unlabeled estradiol. Bound [^{3}H]estradiol was assayed by adsorption onto hydroxylapatite and quantitated by scintillation counting.

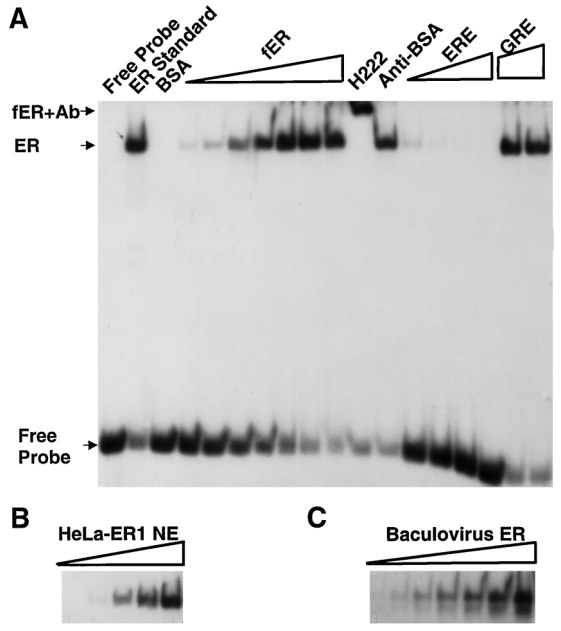

Fig. 6. Analysis of ER Binding to the ERE Using Electrophoretic Mobility Shift Assays

A, Binding of purified fER to the ERE was analyzed in electrophoretic mobility shift assays. The ER standard is 20 μg of protein from a whole-cell extract of COS cells transiently transfected with an ER expression plasmid. BSA carrier (10 μg) did not alter the mobility of the labeled probe. fER concentrations were 0.08 nM, 0.15 nM, 0.4 nM, 0.7 nM, 1.5 nM, 2.2 nM, and 3.0 nM. anti-ER monoclonal antibody H222 (0.46 ng) and 1.5 nM ER were used to show that ER antibody supershifts the complex. The same amount of rabbit anti-BSA was used as a control in the antibody supershift assay. The specificity of fER binding to the ERE was shown by competition with increasing amounts of unlabeled ERE, but not by a 100-fold or 1000-fold excess of unlabeled GRE/PRE. B, Increasing amounts of fER in crude HeLa-ER1 nuclear extracts were analyzed for ERE binding in electrophoretic shift assays. The fER concentrations were 0.08, 0.15, 0.4, 0.7, 1.5, and 3.0 nM. C, Purified ER, expressed in the baculovirus system, binds to the ERE. The ER concentrations were 0.2, 0.4, 0.8, 2, 4, 8, and 20 nM.

unlabeled GRE/PRE (Fig. 6A, ERE and GRE). In protein titrations, the purified fER exhibited a concentration-dependent increase in binding to the ERE (Fig. 6A, fER). ERE binding by fER in crude nuclear extracts and by purified fER was similar (Fig. 6B). Both crude fER and purified fER exhibited high-affinity binding to the ERE.

Our finding that fER purified to near homogeneity bound with a high affinity to the ERE was surprising in light of recent reports that ER expressed in insect cells using the baculovirus expression system bound to the ERE very poorly or not at all, unless HMG-1 was added (11, 12). Under our binding and electrophoresis conditions, commercially available ER, purified to more than 80% homogeneity from insect cells infected with recombinant baculovirus, exhibited readily detectable binding to the ERE (Fig. 6C), with an affinity for the ERE

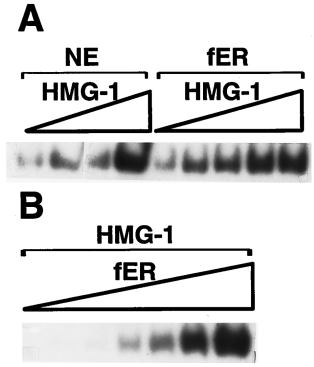

Fig. 7. HMG-1 Enhances Crude and Purified fER Binding to ERE in EMSA

A, A subsaturating concentration of 0.1 nM (2 fmol) of purified fER (fER) or of fER in HeLa-ER1 nuclear extract (NE) was incubated with increasing amounts (0–1,000 ng) of purified recombinant HMG-1, and binding to the ERE was analyzed by EMSA. B, To determine the K_D of the ER in the presence of HMG-1, 200 ng of HMG-1 were incubated with purified fER at 0.007, 0.014, 0.03, 0.07, 0.14, 0.28, and 1.4 nM.

approximately 10-fold lower than that of purified fER from HeLa-ER1 cells (see below).

HMG-1 Enhances Binding of fER to the ERE

While our data showed that purified fER was capable of high-affinity binding to the ERE, it did not address the question of whether HMG-1 could enhance binding of fER to the ERE. To more directly examine the role of HMG-1 in binding of fER to the ERE, we used the method recently described by Jayaraman *et al.* (40) to express and purify His_6-tagged HMG-1 in *E. coli.* The HMG-1 was more than 90% pure as judged by SDS-PAGE. To minimize oxidation, the HMG-1 was stored in a buffer containing 1 mM dithiothreitol (12, 40). Addition of HMG-1 resulted in a strong stimulation of binding of purified fER to the ERE (Fig. 7A, fER). To quantitatively assess the effect of HMG-1 on fER binding to the ERE, we titrated a constant concentration of HMG-1 with increasing amounts of fER (Fig. 7B). In the presence of purified HMG-1, purified fER exhibited a K_D of 0.25 nM for binding to the ERE (Figs. 7B and 8), 3- to 4 times lower than the 0.8 nM K_D we observed for purified fER in the absence of HMG-1 (Fig. 8). Surprisingly, a clear stimulation of binding was also observed when HMG-1 was added to crude HeLa-ER1 nuclear extracts (Fig. 7A, NE). These data suggest that even in crude HeLa cell nuclear extracts, in which HMG-1 has been shown to be present at high levels (40), HMG-1 can be limiting for ER binding to the ERE. HMG-1 also enhanced binding of the commercially obtained purified baculovirus-expressed ER to the ERE (data not shown), eliciting a similar 3- to 4-fold increase in binding for both the purified fER and the baculovirus-ER.

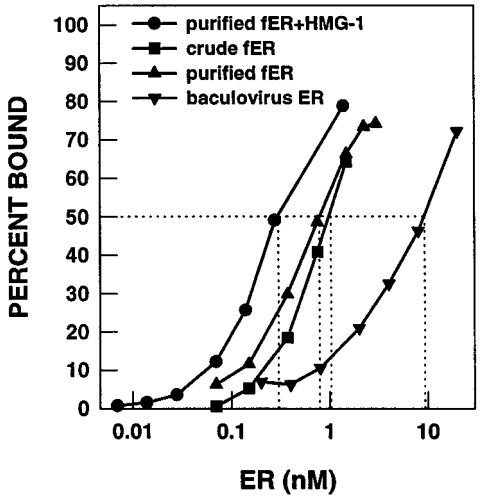

Fig. 8. Affinity of ER Preparations for the ERE and Effect of HMG-1 on Binding to the ERE

The data are taken from the results of Figs. 7–8. Gel-shifted bands were quantitated by PhosphorImager scanning.

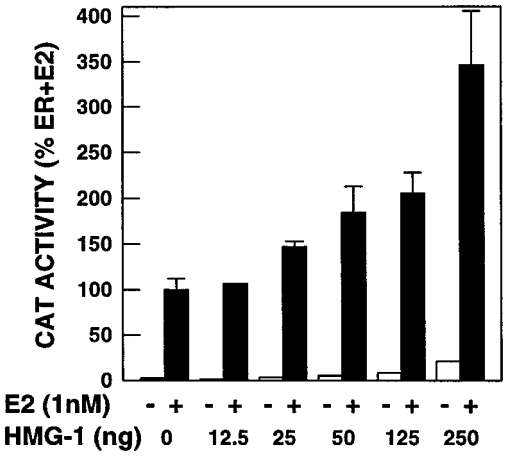

Fig. 9. HMG-1 Stimulates Transactivation by ER in Transiently Transfected HeLa Cells

HeLa cells were transiently transfected with CMV-hERα expression plasmid, the 2ERE-TK-CAT reporter gene, and with increasing amounts of an HMG-1 expression plasmid as described in *Materials and Methods*. Carrier pTZ18U DNA was used to ensure that the total amount of transfected DNA was the same in each transfection. CAT activity represents a normalized value with ER+E_2 with no transfected HMG-1 taken as 100%. When error bars are shown, the data represent the mean ± SEM for three separate transfections.

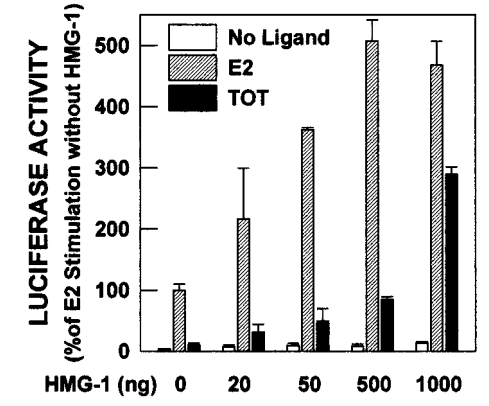

Fig. 10. HMG-1 Differentially Enhances E_2- and TOT-Induced Transactivation by ER

MDA-MB-231 cells were transiently transfected with CMV-hER expression plasmid, a 4ERE-LUC reporter gene, and with increasing amounts of an HMG-1 expression plasmid as described in *Materials and Methods*. Carrier pTZ18U DNA was used to ensure that the total amount of transfected DNA was the same in each transfection. When ligand was present, the cells were maintained in either 10 nM E_2 or 10 nM TOT for 48 h before harvesting. Luciferase activity represents a normalized value with ER+E_2 with no transfected HMG-1 taken as 100%. The data represent the mean ± SEM for three separate transfections.

HMG-1 Stimulates Transactivation by ER

If levels of HMG-1 are truly limiting *in vivo,* synthesis of additional HMG-1 should stimulate transactivation by subsaturating levels of ER. To test the effect of HMG-1 on ER action *in vivo*, we transfected increasing amounts of an HMG-1 expression plasmid (31, 41) into HeLa cells. HMG-1 alone did not activate transcription of the 2ERE-TK-CAT reporter gene (data not shown). When ER and E_2 were present, there was an increase in transactivation of the cotransfected 2ERE-TK-CAT reporter gene with increasing levels of transfected HMG-1 expression plasmid (Fig. 9). These data provide the evidence that HMG-1 levels are limiting for ER action in intact cells. These data also show that transfected HMG-1 elicits a modest, but readily detectable, level of ligand-independent transactivation by ER. Our observation that HMG-1 elicited a low level of ligand-independent transactivation by ER raised the possibility that HMG-1 might also increase the agonist activity of an antiestrogen. To evaluate this possibility, we examined the effect of HMG-1 on transactivation by TOT in MDA-MB-231 cells, which have been used previously to study antiestrogen action (42).

HMG-1 Strongly Enhances the Agonist Activity of TOT

We transfected increasing amounts of HMG-1 expression plasmid into ER- negative MDA-MB-231 cells. In agreement with the data from HeLa cells, increasing levels of transfected HMG-1 elicited a 4- to 5-fold increase in E_2-dependent and E_2-independent transactivation by ER. In the absence of transfected HMG-1, TOT activated transcription to about 10% of the level exhibited by E_2. In contrast to the modest 5-fold activation of E_2-dependent transcription, at 1 μg of transfected HMG-1 expression vector, transactivation by TOT increased 27-fold (Fig. 10) and was almost 3 times greater than transactivation by E_2 in the absence of HMG-1 (Fig. 10). At 1 μg of transfected HMG-1, transactivation by TOT was approximately

60% of the level of transactivation observed with E_2 at the same level of HMG-1.

DISCUSSION

High-Level Expression of ER Is Toxic

Stable cell lines expressing PR or glucocorticoid receptor from constitutive promoters have been reported (43–45). However, high-level expression of ER is extremely toxic to most mammalian cells, and attempts to express high levels of wild-type ER in standard mammalian cell lines using strong constitutive and regulated promoters have typically resulted in low levels of expression followed by progressive loss of ER expression (14). Although we carefully eliminated known sources of estrogen from the culture medium, using several variants of the regulated tetracycline expression system or constitutive promoters, we were unable to isolate clonal cell lines stably expressing levels of ER in the range we report here (data not shown). Only the bicistronic system has enabled us to successfully isolate stable cell lines expressing high levels of wild-type ER. In the bicistronic system, expression of the ER and the neomycin resistance gene are tightly linked, so that ER production is not lost without concomitant loss of production of neomycin phosphotransferase, the enzyme that confers neomycin resistance. An added advantage of the bicistronic system is that the internal ribosome entry site used to initiate translation of the neomycin resistance gene is attenuated (27). This means that substantial levels of the bicistronic mRNA must be present to produce sufficient neomycin phosphotransferase for the cells to grow in the neomycin analog, G418. This may partially account for the high level of ER production, 1,300,000 molecules per cell, seen in the stable HeLa-ER1 cell line. The recently described internal ribosome entry site system (IRES) (27) has so far not found wide application. However, a closely related approach, which predates the IRES system, in which a constitutive promoter expresses a single bicistronic mRNA without an internal ribosome entry site, was used successfully to express high levels of glucocorticoid receptor in stably transfected cells (43).

The 1,300,000 molecules of ER per cell expressed by the HeLa-ER1 cells is significantly lower than the level of ER_{val400} expressed using a regulated promoter system in CHO cells (13, 14). The CHO cell system expressing ER_{val400} will continue to find applications when the very highest levels of ER expression are required, while the HeLa-ER1 system should find applications in situations where an epitope-tagged ER is useful, or when a large-scale suspension culture of the ER-expressing cell line is required.

While our cell culture medium lacks known sources of estrogens, the HeLa-ER1 cells still exhibit significantly slower growth than wild-type HeLa cells (Fig. 2). That this results, at least in large part, from expression of fER, and not from continuous selection for resistance to G418, is shown by the increased growth rate of HeLa-ER1 cells when the pure antiestrogen ICI 182,780 is added to the culture medium. However, the mechanism by which the pure antiestrogen increases the growth rate of the cells is unclear. ICI 182,780 and a closely related member of this series of antiestrogens, ICI 164,384, have been reported to both stimulate the degradation of ER and block its uptake into the cell nucleus (46, 47). This raises the possibility that nuclear localization of high levels of unliganded ER is somewhat toxic, even in the absence of estrogen. Alternatively, the antiestrogen could be antagonizing the effect of traces of estrogen remaining in our culture medium. However, in the absence of added E_2, there is no detectable transcription of an ERE-containing reporter gene transfected into the HeLa-ER1 cells (Fig. 3), indicating that there is not a significant concentration of estrogen in the culture medium.

Interestingly, the antiestrogen TOT, which does not activate transcription of a transfected ERE-containing reporter gene (Fig. 3), kills the HeLa-ER1 cells even more efficiently than 17β-estradiol. Efficient killing of cells expressing ER by both 17β-estradiol and tamoxifen has been reported previously in CHO and mammary epithelial cell lines (13, 14), suggesting that this is a general phenomenon. Recent studies suggest that estrogens and TOT may kill breast cancer cells by different mechanisms (48).

ER Expressed in HeLa-ER1 Cells Is Functional *in Vivo* and *in Vitro*

While the high level of fER in the HeLa-ER1 cells (1,300,000 molecules ER/cell) contributes enormously to their utility as a source of functional ER, the superphysiological level of ER in these cells raises the related questions of whether the properties of this recombinant fER are similar to those of native ER, and whether all or most of the fER is actually functional. *In vivo* and *in vitro* characterization of the fER demonstrates that its properties are similar to those of naturally occurring ER. The liganded fER displays nuclear localization as does ER in naturally occurring cells (49). The fER efficiently activates transcription of an ERE-containing reporter gene in response to nanomolar concentrations of 17β-estradiol. Transactivation is completely dependent on the presence of added 17β-estradiol and is antagonized by the antiestrogen TOT. This pattern of antiestrogen activity is similar to that seen in both ER-positive cell lines and in cells transiently transfected with an ER expression plasmid (36). *In vitro*, the crude and purified fER exhibit an affinity for 17β-estradiol typical of ER from a variety of sources (38, 39).

It is difficult to unequivocally demonstrate that all of the fER is functional *in vitro*. However, comparison of the intensity of the bands seen in Western blots of fER, and of wild-type MCF-7 cell ER, with the amount of ligand bound by the two ER preparations suggests

that most and perhaps all of the fER is competent to bind hormone. Comparison of the amount of nearly homogeneous fER with the amount of labeled 17β-estradiol bound supports the view that most of the fER is competent to bind hormone. Analysis of the interaction of the purified fER with the ERE also supports the view that most of the fER is functional. Perhaps because different measurement techniques were used, there is considerable variation in reported K_D values for binding of ER to the ERE (50, 51). The 0.8 nM K_D we determined for purified fER is within the range of published values. If a large majority of the fER molecules were unable to bind DNA, we would be unlikely to observe such a low K_D.

Recent reports indicated that purification of ER expressed in insect cells using the baculovirus system resulted in loss of the ability to bind to the ERE (11, 12). In agreement with other studies (10, 35), we find that commercially obtained purified ER expressed in insect Sf9 cells binds with reasonably high affinity to the ERE. Using electrophoretic mobility shift assays we determined a K_D of 10 nM for ERE binding, which agrees with the 10 nM K_D for the ERE determined by the commercial supplier using a fluorescence assay. Our finding that ER expressed in insect cells exhibits a 12-fold reduction in affinity relative to the human ER expressed in human cells (Fig. 8) suggests that protein folding or posttranslational processes, such as receptor phosphorylation, may be different in the two systems. Since the purified ER expressed in insect cells was obtained commercially and was purified using a different method than we employed, it is also possible that the difference in affinity seen with these receptor preparations reflects differences in the preparation and purification of these ERs.

HMG-1 Potentiates Binding of ER from HeLa-ER1 Cells to the ERE and Is Limiting in HeLa Cells

To facilitate comparison to data obtained in crude nuclear extracts, and to provide an environment that more nearly simulates nuclear DNA, in which EREs are located in a large excess of nonspecific DNA binding sites, our binding assays were done in 3 μg of nonspecific DNA. Even in the presence of this large approximately 30,000-fold excess of nonspecific DNA, HMG-1 clearly stimulated ERE binding by both purified fER and by fER in crude HeLa-ER1 cell nuclear extracts. The K_D of 0.25 nM for purified fER binding to the ERE in the presence of HMG-1 is much lower than other values obtained using gel mobility shift assays (50).

Our observation that adding HMG-1 to fER-containing nuclear extracts elicits a several fold increase in fER binding to the ERE explains our finding that purifying the fER does not reduce its affinity for the ERE. Because the conditions used for gel mobility shift assays differ from those in intact nuclei, we examined the ability of HMG-1 to stimulate transactivation by fER *in vivo*. In both HeLa cells and MDA-MB-231 cells, HMG-1 elicited a dose-dependent increase of several fold in transactivation by ER. Taken together, the ability of HMG-1 to stimulate binding of ER to the ERE in crude nuclear extracts and the ability of transfected HMG-1 to stimulate ER-dependent transactivation strongly suggest that, despite the presence of high levels of HMG-1 in HeLa cell nuclei (40), HMG-1 levels may be limiting for ER action. Our data support the proposal of Edwards and co-workers (12) that under at least some conditions HMG-1 levels might be limiting for steroid hormone action (12) and suggests that modulating either the intracellular level of HMG-1 or the number of free nuclear HMG-1 binding sites could alter ER binding to the ERE.

HMG-1 Strongly Enhances the Agonist Activity of TOT

In both HeLa cells and MDA-MB-231 cells, transfected HMG-1 elicited a dose-dependent increase of 3- to 5-fold in transactivation by unliganded ER. These data are in agreement with our earlier observation that an ER mutant exhibiting enhanced binding to the ERE *in vivo* exhibited significant ability to activate transcription in the absence of estrogen (52, 53). Because the fold stimulation of transactivation by HMG-1 was similar for both unliganded ER and for ER when E_2 was added to the culture medium, it remained possible that the activation of unliganded ER was actually due to the presence of traces of estrogen in the culture medium. To evaluate this possibility we examined the effect of HMG-1 on transactivation by TOT. HMG-1 enhanced transactivation by TOT far more effectively than it enhanced transactivation by E_2. At 1 μg of transfected HMG-1 expression plasmid, transactivation by E_2 increased 5-fold while transactivation by TOT increased 27-fold, and transactivation by TOT was 62% of the level seen with E_2. Interestingly, an ER mutant exhibiting enhanced affinity for the ERE also exhibited enhanced transactivation by TOT (52, 53).

Studies from several laboratories have led to the concept that DNA binding can be one of several factors modulating the activity of steroid receptors (52–56). The ability of HMG-1 to enhance ligand-independent transactivation by ER and to increase the agonist activity of TOT supports earlier proposals that the ER gains the ability to activate transcription through an activation pathway. In this model, ligand binding, phosphorylation through signal transduction pathways, differential interaction with coactivators and corepressors, and DNA binding jointly contribute to the receptor's transactivation potential. Thus, binding to the ERE without added HMG-1 can be thought of as moving the ER part way down its activation pathway, while enhanced binding of the ER to the ERE in the presence of added HMG-1 moves the receptor further down its activation pathway, decreasing the requirement for ligand and allowing TOT, normally a weak agonist, to show strong agonist activity. While our data are consistent with this provocative idea, the role

of ERE binding in the changes that render the ER competent to activate transcription remains largely obscure.

Possible Mechanisms of HMG-1 Stimulation of Transactivation by ER

Although HMG-1 has been reported to enhance sequence-specific binding by P53 (40), PR (12, 31, 32), ER (11, 12), ER DNA binding domain (33), and other steroid receptors including GR and AR (12), and HOX family members (57), the mechanism by which HMG-1 enhances sequence-specific DNA binding has not been clearly established. We find that the electrophoretic mobility of fER-ERE complexes formed in the presence or absence of HMG-1 is identical, suggesting a weak interaction between HMG-1 and DNA, an interaction that is not sufficiently stable to persist during electrophoresis. These data are in agreement with earlier work in which HMG-1 did not alter the electrophoretic mobility of protein-DNA complexes (11, 12, 31–33).

HMG-1 exhibits a strong preference for binding to nonlinear DNA. At limiting nuclear HMG-1 concentrations, HMG-1 would therefore be expected to preferentially bind to curved or bent DNAs. Some EREs are located in regions of the DNA likely to exhibit preferential binding by HMG-1. For example, the EREs in the *Xenopus* vitellogenin genes are located directly adjacent to a region of curved DNA (58, 59), and it is possible that binding of HMG-1 to this region of DNA facilitates binding of ER to the nearby vitellogenin EREs.

The most plausible explanation for the mechanism by which HMG-1 stimulates binding of ER to the ERE relates to its ability to induce or stabilize bending in DNA and its preferential binding to nonlinear or bent DNA. ER induces DNA bending upon binding to the ERE (60). Recently, we demonstrated that ER DNA binding domain preferentially binds to DNA bent in the same direction as the ER-induced DNA bend (61). It is possible that HMG-1 either distorts the DNA conformation to facilitate ER binding or, by its preferential binding to the bent DNA around the ER-ERE binding site, helps to stabilize the ER-induced bent DNA conformation.

Our data demonstrate that transfected HMG-1 can enhance transactivation by unliganded ER and by E_2-ER and TOT-ER complexes in intact cells. While it is most likely that the ability of HMG-1 to facilitate binding to the ERE is responsible for these increases in transactivation by ER, other explanations have not been excluded. HMG-1 proteins appear to play a role in mediating assembly of nucleoprotein complexes (62), in chromatin decondensation (63), and in transcription by RNA polymerase II *in vitro* (64). Although effects of HMG-1 on chromatin assembly and disassembly seem less likely with the transiently transfected genes we used than for chromosomal genes, we cannot formally exclude the possibility that effects on chromatin structure and the basal transcription apparatus also contribute to the stimulation of ER-mediated transactivation by HMG-1.

In this work we describe the isolation of HeLa-ER cell lines using a generally applicable method for isolation of stable cell lines expressing a toxic protein. With the ability of HeLa cells to grow in large-scale suspension cultures, fER expression levels more than 10-fold higher than are seen in naturally occurring mammalian cells, and a simple one-step purification of the epitope-tagged fER, the HeLa-ER1 cells provide a useful complement to previously described ER-expressing cell lines (13, 14). The HeLa-ER1 system should find application in studies of ER-associated proteins and in biochemical and structural studies that require substantial quantities of highly purified, biologically active, mammalian ER. Our studies with fER from HeLa-ER1 cells reveal that nuclear HMG-1 levels appear to be limiting for ER action *in vitro* and demonstrate that in intact cells, HMG-1 strongly enhances the agonist potential of TOT.

MATERIALS AND METHODS

Construction of Plasmid pIE

Plasmid pIE was constructed by subcloning the entire protein-coding region of the human ER cDNA with the FLAG tag on its N terminus (65) into the *Eco*RI/*Bam*HI site of pIRESIneo (CLONTECH, Palo Alto, CA).

Establishment of the Hela-ER Cell Lines

HeLa cells adapted for growth in DME/10% charcoal-dextran-treated FBS were grown to 60–70% confluence in 100-mm culture plates. The cells were transfected with 20 μg of *Ssp*I-linearized pIE DNA by electroporation (200 V, 1180 μFarad, low resistance using a Cell-Porter from GIBCO-BRL, Gaithersburg, MD). The medium was replaced with selection media (50% DME/10% charcoal-dextran-FBS, 50% conditioned medium + 1 mg/ml G418) approximately 24 h post transfection. After 10 days, colonies were isolated and reseeded into 24-well culture plates in selection media. Cell lines were further expanded to confluence in standard growth medium (without conditioned medium) in T175 flasks. For long-term growth, the cells were maintained under selection in medium containing 0–200 μg/ml G418.

Transient Transfections and CAT Assays

Transient transfections in HeLa-ER1 cells were performed using electroporation as described above for production of the stable cell lines, with the exception that 20 μg of 2ERE-TK-CAT DNA and 4 μg of cytomegalovirus (CMV)-luciferase as internal standard were used and the media contained different concentrations of 17β-estradiol (10^{-11} M to 10^{-6} M) and/or TOT (10^{-6} M, 10^{-7} M). After 48 h, the cells were harvested, and mixed-phase CAT assays were performed as we have described (66). Transient transfections of HeLa cells to compare transactivation by fER and by wild-type ER were done by electroporation as described above using the indicated quantities of CMV-ER or CMV-fER expression plasmids, 20 μg of ATC4 (36), and 4.2 μg CMV-luciferase as internal standard. The cells were maintained in 10^{-8} M E_2 for

48 h before harvesting. Transient transfections in HeLa cells using HMG-1 were performed using Tfx-20 reagent (Promega, Madison, WI), using the manufacturer's protocol. Transfections were done in six-well plates, containing 50 ng of CMV-ER, 50 ng of CMV-luciferase as internal standard, 900 ng of 2ERE-TK-CAT, the indicated amount of an HMG-1 expression plasmid (31, 41), and PTZ18 U to bring the total amount of DNA to 1250 ng. The indicated concentrations of hormone were added immediately after transfection. After 48 h, the cells were harvested for CAT assays (66). Transient transfection in MDA-MB-231 cells was performed using calcium phosphate (42). Transfections were done in six-well plates containing 10 ng of CMV-ER, 50 ng of PRL-SV40 (from Promega) as internal standard, 1 μg of 4ERE-LUC (constructed by G. de Haan in this laboratory), the indicated amounts of an HMG-1 expression plasmid, and PTZ 18U to bring the total amount of DNA to 2.67 μg. Forty eight hours after the cells had been shocked, the dual luciferase assay (Promega) was performed using the manufacturer's protocol.

Preparation of Cell Extracts and Immunopurification of fER

Cells used for making nuclear and cytosol extracts were grown in medium without added hormone. Extracts made in the presence of estradiol were pretreated by resuspending the harvested cells (without serum), adding E_2 to 10^{-7} M, and then incubating at 37 C for 30 min. Cell extracts were prepared as we have described (67) except that after centrifugation to remove the nuclear pellet we retained the cytosol. The nuclear resuspension buffer was TEG 500 (50 mM Tris, pH 7.9, 0.1 mM EDTA, 0.5 mM EGTA, 0.5 M KCl, 1 mM dithiothreitol, 50 μM $ZnCl_2$, 10% glycerol, 50 ng/μl leupeptin, 5 ng/μl phenylmethylsulfonyl fluoride, 5 ng/μl pepstatin A, 0.5 ng/μl aprotinin), and the ammonium sulfate precipitation and dialysis steps were omitted. For fER purification, estradiol-treated HeLa-ER1 nuclear extracts were adjusted to 300 mM KCl and applied to an anti-FLAG epitope immunoaffinity column (Anti-FLAG M2 affinity Gel, Eastman Kodak, Rochester, NY), at 50 μl of packed resin per ml of nuclear extract. The column was subsequently washed 10 times with a total of 100 volumes of TEG300 containing 8 mM (3-[(3-chloramidopropyl)dimethylammonio]-1-propane-sulfonate, and the fER was eluted with FLAG peptide (N-DYKDDDDK-C, 0.2 mg/ml) in TEG100.

Western Blots

Approximately 4 ng of purified fER and 20 μg of nuclear extract containing or lacking fER were analyzed by electrophoresis on a 10% Glycine-SDS polyacrylamide gel, and the proteins were electroblotted onto a nitrocellulose membrane. The membrane was probed with the anti-FLAG M2 monoclonal antibody (at 1:2000 dilution) or ER-specific primary antibody H222 at 0.12 μg/ml, incubated with horseradish peroxidase-conjugated secondary antibodies (at 1:2000 dilution) and detected by chemiluminescence with the ECL kit (Amersham, Arlington Heights, IL).

ER Ligand-Binding Assays

Whole-cell ER assays were carried out as described by Zhuang *et al.* (66). *In vitro* estrogen-binding assays were modified from the method of Carlson *et al.* (39). The ER was diluted into binding buffer (50 mM Tris, pH 7.5, 10% glycerol, 10 mM mercaptoethanol, 500 μg/ml BSA). The bound ligand was assayed by adsorption onto hydroxyapatite for 15 min at 4 C, followed by three washes with 1 ml 0.05 M Tris, pH 7.3. After the last wash, the pellet was resuspended in 0.5 ml of ethanol and counted in 5 ml of scintillation fluid.

Electrophoretic Mobility Shift Assays

Electrophoretic mobility shift assays were carried out as described previously (61) with some modifications. Briefly, end-labeled ERE-containing probes (10,000 cpm/reaction) were combined with the indicated amounts of purified ER, HeLa-ER1 nuclear extract or HMG-1, and 500 ng/μl BSA (or the amount of BSA required to reach 10 μg of total protein when crude nuclear extracts were used); 3 μg of poly-dIdC (Sigma) were present as nonspecific carrier DNA, 10% glycerol, 75 mM KCl, 15 mM Tris-HCl, pH 7.9, 0.2 mM EDTA, and 0.4 mM dithiothreitol in a volume of 20 μl and incubated at 25 C for 15 min. For antibody supershift experiments and competition electrophoretic mobility shift assays, conditions were the same, except for a preincubation for 10 min on ice with either antibody (rabbit anti-BSA was a gift from S. Miklasz, University of Illinois) or DNA competitor before addition of the labeled ERE-containing probes. After probe addition, the reaction mixtures were incubated at 25 C for 15 min and subjected to low ionic strength 8% PAGE using a water jacket to maintain the gel at 4 C with buffer recirculation. Gels were dried before autoradiography, and free and bound forms of ERE and ER-ERE complex were quantitated by PhosphorImager analysis (Molecular Dynamics, Sunnyvale, CA).

Determination of ER Concentrations and Calculation of K_D Values

The amount of ER in the ER preparations from HeLa cells and baculovirus was determined by whole-cell ER assay as described above. We also determined the actual amount of protein in purified fER preparations (which migrate as a single band on SDS gels). To confirm that the baculovirus and crude ER preparations contained the amount of ER determined in the whole-cell ER assays, we used side-by-side Western blots using purified fER as a standard and the ER-specific H222 monoclonal antibody to detect the ER.

We calculated K_D values for binding of the ER preparations to the ERE essentially as described by Kim *et al.* (61). Briefly, the K_D is the amount of ER required to up-shift 50% of the labeled ERE probe in a gel mobility shift assay. Under our gel shift conditions, essentially all of the labeled probe remained in the probe band, or was in a discrete up-shifted band.

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