HMGA1 Enhances the Transcriptional Activity and Binding of the Estrogen Receptor to Its Responsive Element†

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ABSTRACT: The estrogen receptor (ER) plays a critical role in the development of hormone-dependent cancer. Since HMGA1, a member of the "high mobility group" proteins, is overexpressed in certain malignant cells, we investigated the interaction between these nuclear proteins. Transfection of the HMGA1 expression vector increased 2-fold the transcriptional activation of ERE containing promoter by E_2. Furthermore, the HMGA1 protein stimulated severalfold the binding of purified ER to the consensus ERE oligonucleotides in gel mobility shift assays and saturation assays. However, HMGA1 could not bind alone either to consensus or to modified EREs, and the minor groove binding drug distamycin A failed to prevent the synergism between ER and HMGA1. This could suggest that the binding of HMGA1 to DNA was not required for its stimulatory effect. Antibody supershift assays showed that HMGA1 was required for increased binding and suggest a protein−protein interaction between those factors. This was confirmed by pull down assay. These data show that HMGA1 acts in concert with the ER to regulate the expression of estrogen responsive genes through a mechanism that does not require direct binding to DNA. These observations may be relevant in malignant cells expressing both proteins.

The estrogen receptor (ER)[1] is a member of a superfamily of nuclear receptors (NR3A) (*1*) that have common structural and functional domains with two highly conserved regions: the central DNA-binding domain and the C-terminal hormone-binding domain (*2*, *3*). The most highly conserved region among the members of this superfamily of receptors is the DNA-binding domain (DBD). This domain is responsible for the specific interaction of ER with the estrogen response element (ERE), a palindrome consisting of GGTCA half-sites separated by a linker sequence of 3 bp (*4*−*6*). Following estrogen stimulation, the ER binds to ERE and induces transcriptional activation of estrogen target genes. These transcriptional effects of the ER probably account for its well-documented implication in the development of hormone-dependent cancers (*7*).

The action of ER is tripartite involving the receptor, its ligands, and its co-regulatory proteins. The association of nuclear receptors with co-activators (*8*) and accessory proteins (*9*) augments receptor−DNA interaction. Co-repressors involved in modulating the hormone-responsive gene transcription have been described (*10*, *11*). Therefore, transcription of steroid hormone-responsive genes may be subject to the combined effects of activators and repressors that modulate the cellular response to the ER-estrogen complex.

Co-activators such as CBP/p300 may act in concert with architectural proteins involved in transcriptional regulation of genes (*12*). The high-mobility-group (HMG) protein family constitutes a class of important proteins involved in transcriptional regulation. HMGB1 (previously HMG1) (*13*), a ubiquitous intracellular protein, enhances the binding of ER-DBD to ERE and could act in synergy with co-activators to regulate expression of estrogen-responsive genes (*14*). It is not known whether HMGA1 proteins, which are other members of the HMG protein superfamily, also display similar properties and effects.

The "high mobility group" A1 proteins (HMGA1) (previously designated HMG I(Y)) (*13*) are founding members of a class of non-histone nuclear proteins known as architectural transcriptional factors (*15*−*17*). HMGA1a and HMGA1b, the two most abundant HMGA1 proteins found in mammalian cells that differ from each other by only 11 amino acids (*18*, *19*), are produced by translation of alternatively spliced transcripts encoded by the HMGA1 gene (aka, *HMGIY*) on human chromosome 6p21 (*18*, *19*). Because of their many shared biochemical and biological characteristics (*17*), we will, for convenience, refer to these two proteins

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[1] Abbreviations: ER, estrogen receptor; HMGA1, high mobility group AT-hook, HMGB, high mobility group Box; NR, nuclear receptor; DNRE, distal negative regulatory element, ERE, estrogen responsive element; DBD, DNA-binding domain; CAT, choramphenicol acethyltransferase.

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as simply HMGA1. The structural feature that distinguishes HMGA1 from other HMG proteins is the presence of multiple DNA-binding domains called AT-hooks that preferentially bind to the minor groove of AT-rich regions of DNA (*20*−*22*). The HMGA1 proteins recognize their substrate's structure rather than nucleotide sequence and, therefore, also specifically recognize altered DNA structures such as bent DNA, four-way junctions, and the distorted regions of DNA on nucleosome core particles (reviewed in ref *17*). They also have the ability to bend, unwind, and introduce supercoils into DNA substrates and to specifically interact with other proteins, many of which are transcription factors (*17*). Because of this unusual combination of characteristics, the HMGA1 proteins are thought to play an important role in regulating the expression of many different genes in vivo (*17*, *23*−*32*).

The HMGA1 proteins are barely expressed in normal adult tissues in both rodents and humans (*33*, *34*). However, it has been suggested that alterations in the HMGA1 gene could play an important role in the generation of benign or malignant tumors (reviewed in refs *17*, *26*, *35*, and *36*). Indeed, a high expression of HMGA1 has been described in several solid tumors including thyroid (*37*−*39*), prostate (*40*), uterus (*41*), colon (*42*−*44*), pancreas (*45*), and breast tumors (*26*, *46*). A correlation was also found between neoplastic transformation and the expression of HMGA1 in mammalian cells (*47*, *48*). These and other reports suggest that HMGA1 is an oncogene whose overexpression is highly correlated with both cancerous transformation and increased malignancy.

Since both ER and HMGA1 proteins are suspected to be involved in tumor development and progression, it was of interest to test whether they could functionally interact. This possibility is supported by the fact that HMGB1 displays a synergistic effect with the ER and regulates ER responsive genes (*49*, *50*). However, HMGB1 and HMGA1 differ in their structure and in their mode of interaction with DNA (*16*, *17*). The aim of the present study was to investigate the possible interaction between the ER and HMGA1. Such an interaction might be relevant in estrogen responsive tumors. The ability of HMGA1 to enhance transcription of ERE and binding of ER to consensus and imperfect ERE sequences that were modified in their half-site or in their structure (*51*) was especially examined. Our results showed that HMGA1 enhances transcriptional activity of promoters containing ERE and increases binding of ER to ERE.

MATERIALS AND METHODS

Materials. All reagents were of molecular biology grade and were purchased from Life-Technologies. Oligonucleotides were purchased from Genset SA (Paris, France). Purified human recombinant estrogen receptor was purchased from PanVera Corporation (Madison). The human recombinant HMGA1 protein used in these studies (specifically the HMGA1a isoform) was produced and purified by HPLC (>90%) as previously described (*52*). For the reasons noted above, in this report, for convenience, the HMGA1a recombinant protein is referred to as simply HMGA1. Antibodies [anti-HMGA1 and anti-ER(α)] were purchased from Santa Cruz. ^{32}P-Radiolabeled nucleotides were from Amersham Pharmacia Biotechnology (France). Dac-30 was purchased from Eurogentec.

Cell Culture. The human breast carcinoma cell line MCF-7 was maintained in Dulbecco's Modified Eagle Medium (DMEM, Life-Technologies) with phenol red and supplemented with 10% foetal calf serum, 100 units/mL penicillin and 100 μg/mL streptomycin (Life-Technologies, Diamant, Puteaux, France), and 0.5 μg/mL fungizone (Squibb, Princeton, NJ). Forty-eight hours before the experiments, the medium was removed and replaced by DMEM without phenol red (Life-Technologies) supplemented with 10% charcoal-treated calf serum (desteroidized), and the same concentrations of penicillin, streptomycin, and fungizone.

Plasmids. The Firefly luciferase expression plasmid (pGL3 basic vector) was purchased from Promega. The *p*-α glob-RLuc was already described by Morel et al. (1999) (*53*) and used as a control in transfection experiments because it displays a stable activity under the treatments used in this study. The ERE-*tk*-CAT reporter plasmid was cloned as previously described (*54*) into the *Hin*dIII site of the *tk*-CAT plasmid (gift from Dr. C. Forest, Meudon, France). The pRc-CMV-HMGA1 expression vector was created by subcloning of the full-length human HMGA1a cDNA (*19*) into the pRc-CMV eukaryotic plasmid expression vector (In vitrogen). As with the recombinant protein, the HMGA1a protein produced in cells transfected with this vector is simply referred to as HMGA1.

Transfection Experiments. Transfection experiments in MCF-7 cells were performed with Dac-30 according to the manufacturer's instructions: 1 day prior to the transfection, cells (2×10^5 cells/6 cm dish) were seeded into DMEM without phenol red, 10% charcoal-treated foetal calf serum, penicillin, streptomycin, and fungizone. Each dish was transfected with 1 mL of medium containing 12 μg of DAC-30 solution mixed with 1 mL of medium containing 1 μg of the reporter *tk*-CAT or ERE-*tk*-CAT plasmid, 1 μg of the control α glob-RLuc plasmid, and increasing concentrations of the expression pRc-CMV-HMGA1 plasmid (0, 1, and 2 μg). This was completed with various amounts of pRc-CMV plasmid to obtain 4 μg of total DNA. Four hours after the transfection, the medium was removed and replaced by fresh complete medium containing 10% charcoal-treated foetal calf serum. Sixteen hours later, cells were treated with estrogen (10^{-7} M). After an additional 24-h incubation, cells were homogenized for chloramphenicol acetyltransferase (CAT) and luciferase assays. For each condition, the experiment was run in triplicate.

Luciferase Assay. Luciferase activity was used to determine and to normalize the transfection efficiency in all culture dishes (*55*). It was assayed using a kit from Promega according to the manufacturer's instructions. Briefly, the transfected cells were washed twice with 5 mL of calcium and magnesium free PBS, and lysed in 500 μL of Reporter Lysis Buffer 1X (Promega) for 15 min. After a 5 min centrifugation, 20 μL of the supernatants was mixed with 100 μL of luciferase assay reagent (Promega) at room temperature. The luciferase activity was measured using a luminometer 30 s after addition of the assay reagent.

CAT Assay. The CAT activity was determined using the two-phase assay described by Massaad et al. (*54*). Briefly, 60 μL of cellular extract, heated at 65 °C for 10 min, was incubated with 1 mM chloramphenicol, 30 μM acetyl CoA, and 0.5 μCi [^{3}H]acetyl CoA (NEN product no. NET-290 L) at 37 °C for 30 min. The solution was then transferred to a

Table 1: Nucleotides Sequences of the Probes[a]

Element	Sequence	
ERE (1)	5′-gaaAGGTCA **TGG** TGACCTac-3′	(strand I)
Consensus	3′-cttTCCAGT **ACC** ACTGGAtggcgg-5′	(strand II)
ERE (2)	5′-gaaATGTCA **TGG** TGACCTac-3′	(strand I)
	3′-cttTACAGT **ACC** ACTGGAtggcgg-5′	(strand II)
DNRE	5′-cagATTTAAGTCTAATTTAAAGTcgt-3′	(strand I)
	3′-gtcTAAATTCAGATTAAATTTCAgca-5′	(strand II)

[a] ERE, estrogen responsive element; DNRE, distal negative regulatory element. Unrelated nucleotides are shown in lower case. Underlined bases are those that differ from the consensus sequence. The linker sequence is in bold letters.

minivial and layered with 4 mL of Econofluor (NEN product no. NEF 969). After vigorous mixing, the two phases were allowed to separate for at least 15 min, and the radioactivity was then counted in a scintillation counter. Under these conditions, the product of the reaction, acetylated chloramphenicol, but not unreacted acetyl-CoA, can diffuse into the Econofluor phase. For these experiments, blanks were obtained by assaying CAT activity in cells that have undergone the same treatment in the absence of a CAT plasmid. Results were expressed in transcriptional activity (%) that represents CAT/luciferase ratios; 100% is the maximal activation elicited by the ERE-*tk*-CAT plasmid. The Mann and Whitney *U*-test was used to compare the mean of transcriptional activity between the different treatments. All calculations were carried out using Sigma Stat Software. The level accepted as significant was $p < 0.05$.

Oligonucleotides Used, Platination, and Radiolabeled Probes. Two-estrogen response elements [ERE(1), ERE(2) sequences] and the minimal distal negative regulatory element (DNRE) were synthesized and used as probes for this study (Table 1). The ERE sequences and the cisplatin adduct synthesis were previously described (*51*). DNRE sequence was already described by Lopez et al. (*27*). Probes were obtained by annealing the coding and noncoding strands. The duplex molecules were labeled by the Klenow fragment of DNA polymerase I in the presence of [α-^{32}P] dCTP (>3000 Ci/mmol; 1 Ci = 37 GBq).

Electrophoretic Mobility Shift Assay (EMSA). Binding reactions were carried out in 20 μL of reaction mix [10 mM HEPES (pH 7.8), 0.1 mM EDTA, 50 mM NaCl, 50 mM KCl, 5 mM $MgCl_2$, 4 mM spermidine, 2 mM dithiothreitol, 10% glycerol, 1 mg/mL poly(dG-dC).poly(dG-dC)., 0.1 mg/mL of bovine serum albumin]. Ninety nanograms of human recombinant ER-alpha and/or 30 ng of recombinant human HMGA1 protein were mixed with the binding reaction then preincubated with 0.1 ng of each probe for 30 min at room temperature. A 6% polyacrylamide gel (acrylamide/bisacrylamide, 29:1) containing 0.25× Tris-borate-EDTA was run at 300 V/12 cm for 30 min, before loading the reaction mixture for 45 min (300 V/12 cm). Then, the gels were dried and autoradiographed, the complexes were quantified using a phosphoImager (Image Quant Software), and the results were expressed as percent of control.

Saturation Assays. To compare the binding of the ER to ERE(1) in the presence or absence of HMGA1, "Saturation assays" were performed. ER (90 ng) or ER (90 ng) + HMGA1 (30 ng) was incubated with the reaction mix and increasing amounts of labeled ERE(1) (0.1, 0.2, 0.5, 1, 2, 4, 6, 8, 10 ng). Incubations and electrophoresis were carried out as described above.

Detection of Nucleoprotein Complexes. Electrophoretic mobility shift assay was performed using nonradioactive blunt ends ERE (24 bp) at 2 ng, and the same concentration of ER and HMGA1 proteins. After electrophoretic separation, nucleoprotein complexes were electrotransferred to nitrocellulose sheets, which were probed with a rabbit polyclonal antibody ERα diluted at 1:10000. The detection was carried out using a peroxidase-conjugated anti-rabbit antibody at 1:5000 dilution. Immunoreactive bands were visualized using the enhanced chemiluminescence detection system according to the manufacturer's instructions (Amersham Corp.).

Competition Assays with Distamycin A. Distamycin A, a minor groove-binding drug, which blocks DNA binding of HMGA1 proteins (*56*), was used as competitor in our experiments. Proteins were incubated with 1 μL of the drug at 10 nM, 100 nM, 500 nM, and 1 μM for 10 min, then the ERE(1) and DNRE probes were added and incubated for 30 min at room temperature. Complexes were resolved by electrophoresis, bound forms were quantified by the phosphoImager (Image Quant Software) and results were expressed as percent of control.

Supershift Experiments. Supershift experiments were performed according to two different approaches. The first involved preincubation of ER (90 ng) and/or HMGA1 (30 ng) for 30 min with either 0.1 ng of ERE(1) or DNRE probe at room temperature; the purified polyclonal rabbit anti-ERα antibody and/or purified polyclonal rabbit anti-HMGA1 antibody were then added for 4 h on ice. The following concentrations were used for the ER antibody: 0.1, 0.2, and 0.4 μg which correspond to 1:40, 1:20, and 1:10 dilutions. For the HMGA1 antibody, the following concentrations were used: 0.1, 0.2, 0.4, and 0.8 μg which correspond to 1:40, 1:20, 1:10 and 1:5 dilutions. The second approach involved preincubation of proteins and antibodies for 30 min on ice before adding the probe for 30 min at room temperature. For both procedures, purified polyclonal rabbit IgG was used as negative control and was treated similarly. Complexes were resolved by electrophoresis as described above, then dried and autoradiographed by phosphoImager.

Pull-Down Assays. Each pull down assay was carried out in 200 μL of Tris 50 mM, pH 7.5, 75 mM NaCl, 10% glycerol containing protease inhibitors/Pefabloc. One microgram of ER and 10 μg of HMGA1 were incubated for 30 min on ice with or without the ERE oligonucleotide (100 ng). Then 1 μg of purified polyclonal ER antibody (Santa Cruz Biotechnology) or polyclonal rabbit IgG (used as control) was added. After 4 h incubation at 4 °C, 20 μL of protein A-Sepharose (Pharmacia) was added for 1 h on a wheel at 4 °C. The mixture was then centrifuged and 20 μL of the supernatant were removed (input). The pellets were washed 4 times in the same buffer without protease inhibitors/Pefabloc at room temperature. For each sample, Laemmli buffer was added and then boiled for 2 min. After separation of bound proteins on 12% SDS−PAGE gels and "Western" blotting, proteins were detected with anti-HMGA1 antibody.

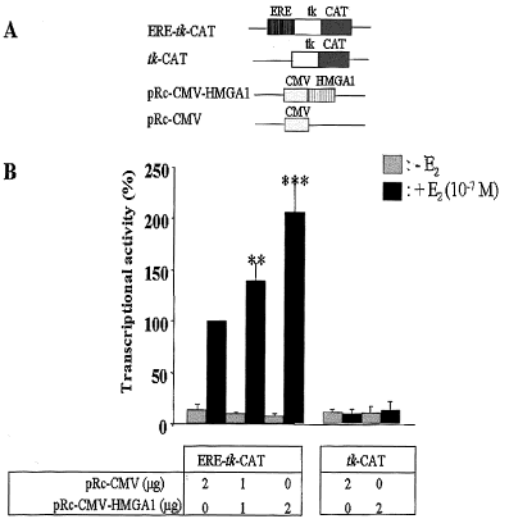

FIGURE 1: HMGA1 overexpression in MCF-7 cells. (A) Structure of vectors. (B) MCF-7 cells were transfected with 1 μg of the ERE-*tk*-CAT or *tk*-CAT plasmid, 1 μg of the control α glob-Rluc, and increasing concentrations of the expression HMGA1 vector (0, 1, and 2 μg). E_2 was added at the final concentration of 10^{-7} M. Total transfected DNA amount was kept constant with the pRc-CMV plasmid. The transcriptional activity represents CAT activity/Luc activity. The results are the mean SD of four independent experiments with three replicates for each point. The Mann and Whitney *U*-test was used for statistical analysis, (*) $p < 0.001$, (**) $p < 0.0001$.

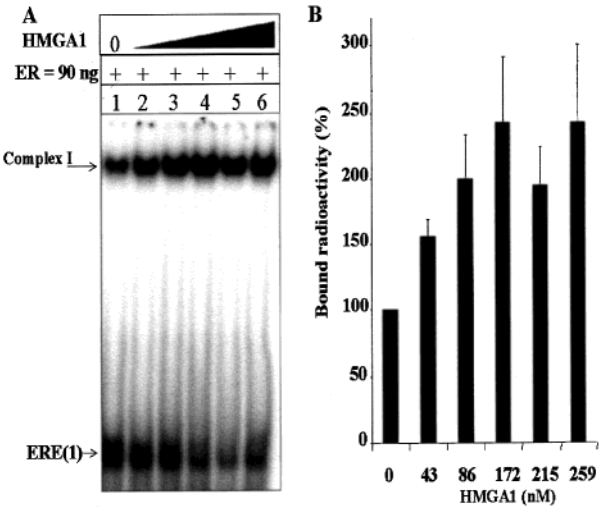

FIGURE 2: HMGA1 increases ER binding to ERE. (A) EMSA was performed with 90 ng of purified human recombinant estrogen receptor (Panvera) and the ERE(1) probe (0.1 ng) in the absence (lane 1) or the presence (lanes 2−6) of increasing amounts of purified human recombinant HMGA1 protein (10, 20, 30, 40, and 50 ng). (B) The % of bound radioactivity in the complex I (I) [ERE-(1)-ER] was calculated after quantitation with a PhosphoImager and ImageQuant software. The results are the mean SD of three independent experiments.

RESULTS

Overexpression of HMGA1 in MCF-7. To determine whether HMGA1 could modulate ERE regulated genes, we cotransfected the mammary tumor MCF-7 cells, which contain a functional ER, with the ERE-*tk*-CAT construct (which includes one copy of ERE) and increasing amounts of pRc-CMV-HMGA1 expression plasmid (Figure 1A). 17-β-Estradiol (E_2) (10^{-7} M) was added to the medium 24 h after transfection.

Preliminary experiments showed that the pRc-CMV plasmid did not affect the basal activity or the induced activity of both *tk*-CAT and ERE-*tk*-CAT. However, CAT activity was significantly increased in E_2 induced cells cotransfected with the pRc-CMV-HMGA1 expression vector. Increasing concentrations of the expression vector caused an enhancement of the reporter gene activity (Figure 1B). The expression vector (up to 2 μg) did not affect the basal activity (-E_2) of the ERE-*tk*-CAT reporter gene in transfected cells. Reporter gene activity was also unaffected in the *tk*-CAT transfected cells treated or not by E_2 (Figure 1B). These results indicate that HMGA1 could enhance the activity of promoters containing ERE in the presence of ER and E_2.

HMGA1 Increases ER Binding to ERE. The results obtained by the transfection assays led us to study the molecular mechanism involved in the increase of the transcriptional activity.

First, we evaluated the effect of HMGA1 on the binding of ER to an ERE(1), using electrophoretic mobility shift assays (EMSA). When ERE(1) was incubated with increasing concentrations of the recombinant HMGA1 protein, no band shift was observed (data not shown). However, when both recombinant ER and HMGA1 proteins were combined, an increase of the band shift intensity was observed (Figure 2A). This effect was increased as a function of the HMGA1 concentration up to 30 ng (Figure 2B). Therefore, in the following experiments, only 30 ng of HMGA1 was used.

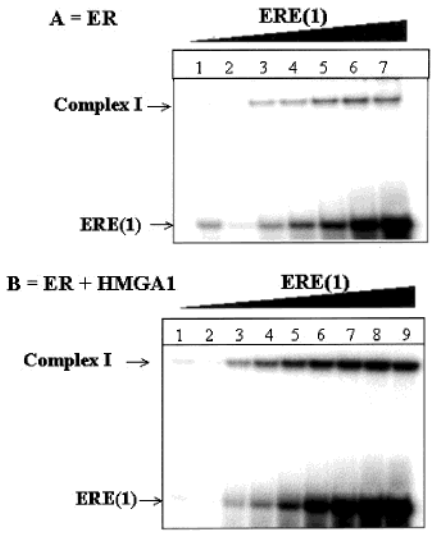

FIGURE 3: Saturation assays. (A) EMSA was performed using recombinant human ER (90 ng) and increasing amounts of radiolabeled ERE(1) (0.1, 0.2, 0.5, 1, 2, 4, and 6 ng). (B) HMGA1 (30 ng) and ER (90 ng) were incubated with increasing amounts of radiollabeled ERE(1) (0.1, 0.2, 0.5, 1, 2, 4, 6, 8, and 10 ng).

HMGA1 Increases the Amount of ER Bound to ERE. To characterize the effect of HMGA1 on the ER, saturation assays were performed. ER or a combination of ER + HMGA1 was incubated with increasing concentrations of labeled ERE(1). As shown in Figure 3, panels A and B, when both proteins were combined, the complex was more abundant than when ER alone was added. Blotting of the EMSA confirmed this observation. Using a specific ER antibody, results obtained after transferring the nucleoprotein complex showed an increase of the band intensity when ER and HMGA1 were combined compared to ER alone (Figure 4A). These results indicate that the enhanced transcriptional

activation of ER by HMGA1 detected ex vivo was also observed in vitro.

Effect of Distamycin A on the Nucleoprotein Complex. To determine how HMGA1 is associated with the nucleoprotein complex I, distamycin A, a minor groove drug that prevents the binding of HMGA1 proteins to DNA (*56*) was tested in EMSA.

DNRE, a specific binding site of HMGA1, was used as positive control in our experiments. As expected, the formation of complex II (consisting of two closely migrating complexes) that corresponds to the binding of DNRE to HMGA1 was directly inhibited by distamycin in a dose-dependent manner (Figure 5A, lanes 2−5, and Figure 5B) (*27*). In contrast HMGA1 could not bind to ERE(1) either in the presence (Figure 5C, lanes 2−4) or in the absence of distamycin A (Figure 5C, lane 1). In the presence of ER, the mean of several experiments did not show any significant difference between untreated and treated samples. At the same concentrations, distamycin A did not display any inhibitory effect on complex I in the presence of HMGA1 (Figure 5C, lanes 11−14, Figure 5D). Thus, complex I was not directly inhibited by distamycin A, which suggests that the effect of HMGA1 on the binding of ER to ERE(1) was not dependent on its binding to the minor groove.

Evidence for the Presence of HMGA1 in Complex I. We next hypothesized that HMGA1 could interact directly with ER without binding to ERE(1). To test this hypothesis, supershift experiments were performed using purified polyclonal rabbit anti-HMGA1 and purified polyclonal rabbit anti-ER antibodies. As described in the Materials and Methods , two EMSA conditions were used (antibodies was added before or after the probes); both of them gave the same result. When DNRE, our positive control for HMGA1, was used as a probe, the anti-HMGA1 antibody (applied at dilutions of 1:40, 1:20, 1:10, 1:5) was able to inhibit the formation of the complex II but did not supershift it (Figure 6A, lanes 2−5), probably because the binding of the antibody to the HMGA1 protein prevented it from binding to DNA.

The polyclonal rabbit anti-ER was able to supershift the ER-ERE complex (complex I) formed in the absence (Figure 6B, lanes 2 and 3) and in the presence of HMGA1 (Figure 6B, lanes 8 and 9). Both polyclonal rabbit IgG (Figure 6B, lane 6) and the HMGA1 antibody (Figure 6B, lane 5) used at 1:5 dilution did not affect complex I formation.

When ER was combined with HMGA1, the HMGA1 antibody was able to inhibit the formation of complex I (Figure 6B, lanes 11−13) (inhibition of 5%, 31% and 72% respectively for 1:20, 1:10, 1:5 dilutions of anti-HMGA1 antibody). The polyclonal rabbit IgG was inefficient (Figure 6B, lane 14). Therefore, the inhibition of the complex I formation in the presence of the anti-HMGA1 antibody suggests the presence of its target protein in the complex probably because the binding of the antibody to the HMGA1 protein prevented it from binding to ER.

To determine whether ER interacts directly with HMGA1, pull-down assays were carried out. ER was combined with HMGA1 in the absence or presence of 100 ng of ERE oligonucleotide. Then, the anti-ER antibody was added, and the immunocomplex was pulled down with protein A sepharose and analyzed by "western blots". In the absence of DNA, the anti-ER antibody precipitates HMGA1 (Figure 7A, line 3) as well as in the presence of 100 ng of ERE oligonucleotide (Figure 7B, line 3). We did not detect any precipitate when the control rabbit immunoglobulins were used for immunoprecipitation (Figure 7, panels A and B, line 4). The precipitation of ER was confirmed by western blotting (data not shown). Taken together these results indicate that HMGA1 interacts with ER.

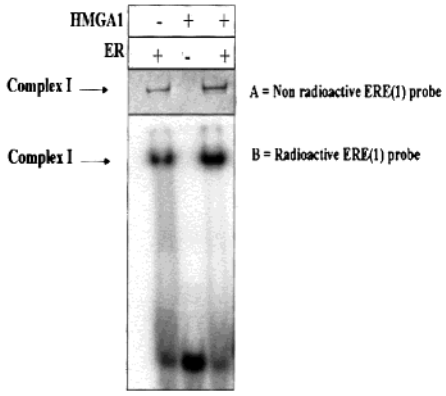

FIGURE 4: Detection of nucleoprotein complexes. (A) EMSA was performed using 2 ng of nonradioactive blunt end ERE(1). After incubation with ER (lane 1), HMGA1 (lane 2) or ER + HMGA1 (lane 3) the nucleoprotein complexes were electrotransferred and probed with rabbit polyclonal antibody anti-ERα. Complex I (I) was visualized using a chemiluminescence detection system. (B) EMSA was carried out under the same conditions except that labeled ERE(1) was used as a probe at a concentration of 0.1 ng, then the gel was dried and autoradiographed.

HMGA1 Increases ER Binding to Mutated and Modified EREs. To evaluate whether HMGA1 could enhance the ability of ER to interact with modified ERE sequences, EMSAs were performed. Sequences listed in Table 1, which are modified in their half-site [ERE(2)] or in their linker sequence by a single cisplatin adduct [ERE(2)-Pt], were used. E_2 was added at 10^{-7} M. As seen in Figure 8, the combination of ER and HMGA1 increased the binding of ER to ERE(2) (lane 3) and ERE(2)-Pt (lane 7). Addition of E_2 did not significantly increase the binding of ER to the ERE sequences (lanes 4 and 8). These results suggest that HMGA1 could increase the binding of ER to various EREs that are modified in their sequence or in their DNA conformation.

DISCUSSION

A number of studies reported that purified steroid hormone receptors bind poorly to their recognition sequences when compared to receptors associated with other cellular proteins. The addition of crude cellular extracts or other proteins restores the ability of purified receptors to bind to DNA. This suggests that nuclear receptors do not act alone but require the participation of other cellular proteins to bind to DNA (*9*, *49*, *50*, *57*). In this work, we demonstrated that HMGA1 could increase ex vivo the transcriptional activity of ERE and in vitro the binding of ER to ERE.

In MCF-7 cells, which express endogenously ER, an ERE-containing promoter was activated 7-fold by treatment with E_2. Under the same condition, overexpression of HMGA1 significantly enhanced this transcriptional activity (14-fold induction) without affecting the basal activity of the reporter gene. This is the first demonstration showing that HMGA1 could activate the transcriptional activity of the estrogen receptor, a member of the nuclear receptor superfamily. It

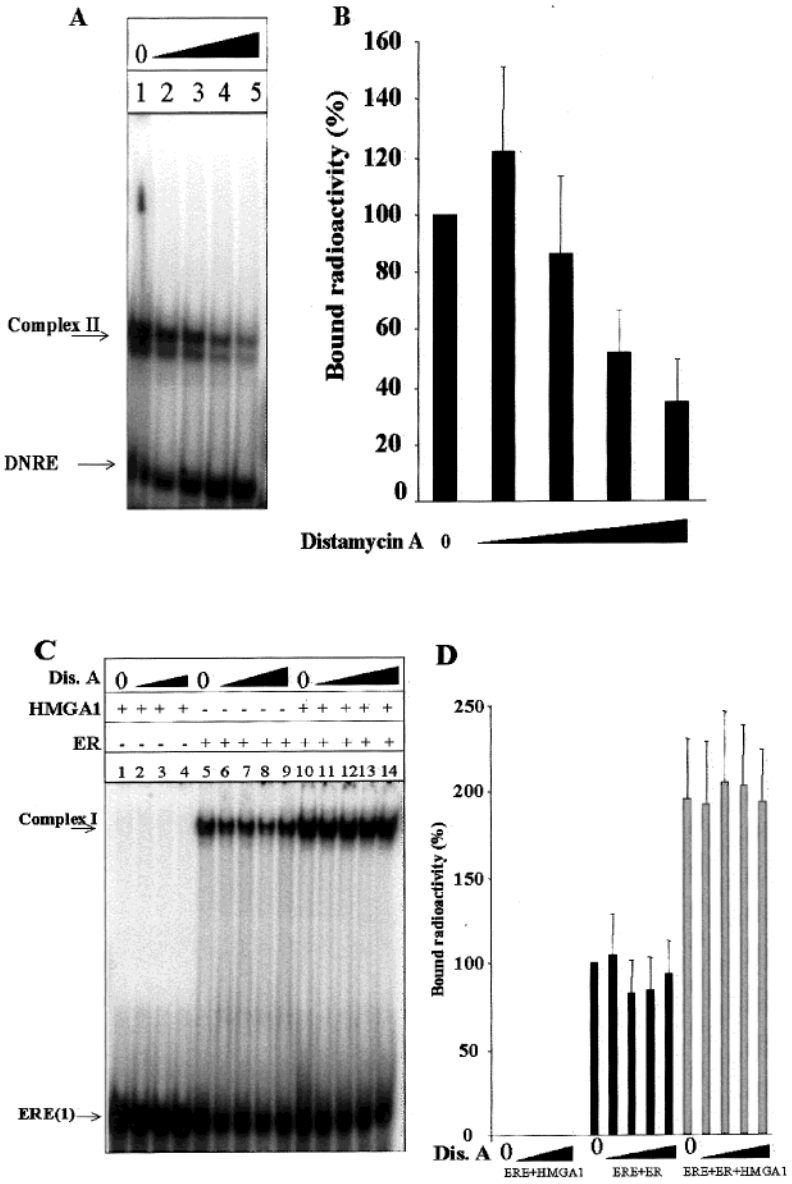

FIGURE 5: Effect of distamycin A on protein−DNA complex formation. (A) The DNRE probe used as control was incubated with HMGA1 in the absence (lane 1) or in the presence of increasing concentrations of distamycin A [1 nM, 100 nM, 500 nM and 1 μM (lanes 2−5)]. The band produced corresponds to the complex II. (B) The fraction of the radioactivity included in the complex II was calculated and the results were expressed as percent of control (0 nM of distamycin A). (C) The ERE(1) probe was incubated with HMGA1 (lanes 1−4), ER (lanes 5−9), and ER + HMGA1 (lanes 11−14). Lanes 1, 5, and 10 did not receive any additional treatment. Distamycin A was added at the final concentration of 1 nM (lanes 6, 11), 100 nM (lanes 2, 7, 12), 500 nM (lanes 3, 8, 13), and 1 μM (lanes 4, 9, 14). (D) Radioactivity in complex I was counted and the results were expressed as percent of control (ER without distamycin A). The results are the mean SD of four independent experiments.

was already known that HMGA1 stimulates the transcription of several genes including IFN-beta, interleukin-2 receptor-alpha, and E-selectin (*23*, *28*−*30*), represses the expression of other genes such as interferon-A and interleukin-4 (*27*, *31*, *32*), and modulates nucleosome and DNA−protein interaction (*48*). A common feature of these processes is the assembly of higher order nucleoprotein complexes. Since endogenous hormone-responsive genes contain multiple transcription factor-binding sites that are separated by varying distances, HMGA1 could display a similar effect on the higher order nucleoprotein complexes formed on these genes in vivo.

We investigated whether HMGA1 could directly bind to ERE in the absence of AT-rich sequences. As expected, our data show that HMGA1 alone could not bind to ERE. Nevertheless, in the presence of ER, addition of HMGA1 increased the binding of ER to the ERE in a dose dependent manner. Moreover, when we tested the effect of HMGA1 on a modified ERE [ERE(2)] which displays a 2-fold lower binding affinity for ER (*51*), the same phenomenon was observed. Therefore, a good relationship was found between the ability of HMGA1 to enhance ER binding to ERE in vitro and its ability to increase transcriptional activity ex vivo.

An important question raised by these findings was how HMGA1 could increase binding of ER to its responsive element without binding to DNA. Saturation assays showed that HMGA1 increases the binding of ER to ERE(1). A more detailed kinetic analysis is required to better characterize this effect. Moreover, anti-ER antibodies proved that there was an increase of the amount of ER in the complex in the

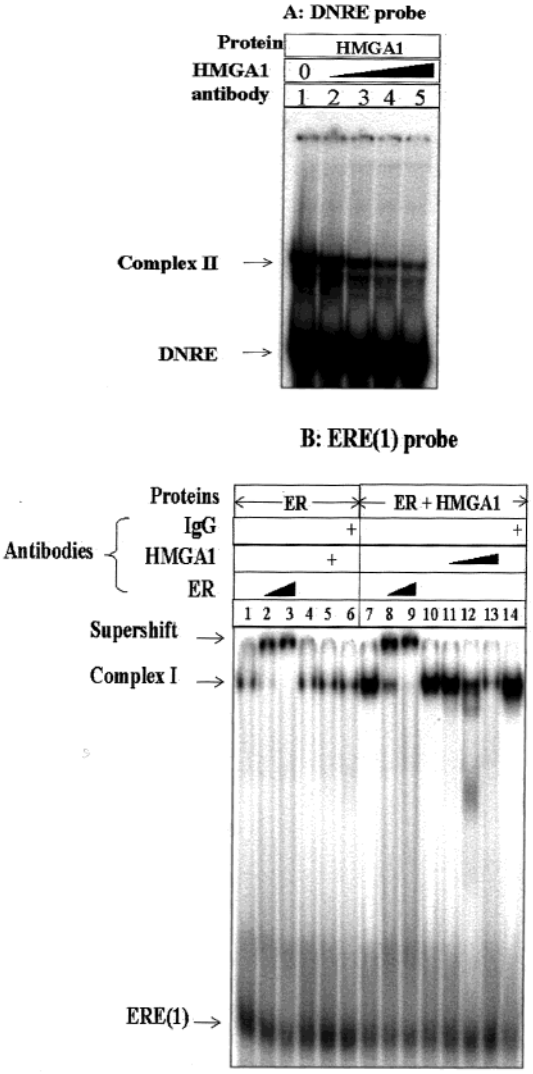

FIGURE 6: Supershift assays: HMGA1 is included in complex I. (A) The DNRE labeled probe (0.1 ng) was incubated with 30 ng of HMGA1 in the absence (lane 1) or in the presence of 0.1, 0.2, 0.4, and 0.8 μg of anti-HMGA1 antibody (1:40, 1:20, 1:10, and 1:5) (lanes 2−5). (B) The ERE(1) labeled probe was used at 0.1 ng; ER (90 ng) was added alone (lanes 1 and 4) or in the presence of 0.2 and 0.4 ng of anti-ER antibody (1:20 and 1:10 dilutions) (lanes 2 and 3) or with 0.8 μg of anti HMGA1 antibody (1:5 dilution) (lane 5) or with 0.8 μg of purified polyclonal rabbit IgG (1:5) (lane 6). Lanes 7−14 correspond to the combination of ER (90 ng) with HMGA1 at 30 ng concentration. Lanes 7 and 10 did not receive any serum. Lanes 8 and 9 were related to the preincubation with 0.2 and 0.4 μg of anti-ER antibody (1:20 and 1:10 dilutions). Lanes 11−13 correspond to the preincubation with 0.2, 0.4, and 0.8 μg of anti-HMGA1 antibody (1:20, 1:10, and 1:5). Lane 14 corresponds to the preincubation with 0.8 μg (1:5) of the purified polyclonal rabbit IgG. This experiment was repeated three times with essentially the same results.

presence of HMGA1 (Figure 4). Thus, we hypothesized that the HMGA1 protein participates in the formation of a stable or transient ternary complex by interacting directly with ER without binding to ERE. Indeed, a HMGA1-specific antibody inhibited the formation of complex I formation in the presence of ER and HMGA1. This suggests that HMGA1 is present in the complex through a protein−protein interaction with the ER. However, we have failed to observe a supershifted complex with the HMGA1 antibody. Nevertheless, we were able to pull down HMGA1 by the ER antibody. This is a good argument in favor of an interaction between

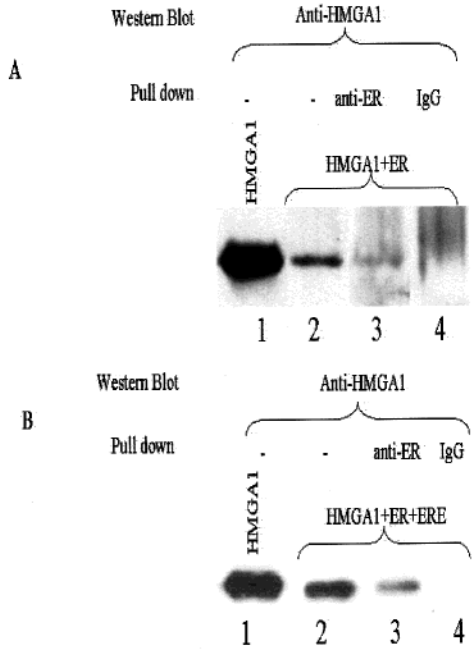

FIGURE 7: Evidence of ER and HMGA1 interaction by pull down assay. One microgram of ER and 10 μg of HMGA1 was incubated alone (A) or with 100 ng of ERE sequence (B) and then 1 μg of the purified polyclonal anti-ER antibody (lane 3) or polyclonal rabbit IgG (lane 4) was added. Pull down assays were performed using protein A sepharose. Bound proteins were separated by SDS−PAGE followed by western blotting using the rabbit anti-HMGA1 antibody. Lane 1 represents 1 μg of recombinant HMGA1 protein, and lane 2 corresponds to an aliquot which represents 10% of proteins used in the pull down assay. Precipitation of ER was confirmed by western blotting (not shown).

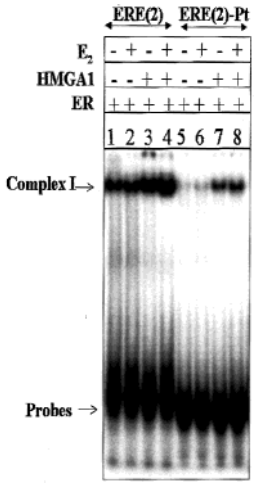

FIGURE 8: HMGA1 enhances binding of ER to modified ERE(2). EMSA was performed with 90 ng of purified human recombinant estrogen receptor and 0.1 ng of ERE(2) (lanes 1−4) or the ERE-(2)-Pt (lanes 5−8) probe in the absence (lanes 1, 2 and 5, 6) or in the presence of 30 ng of HMGA1 (lanes 3, 4 and 7, 8). E_2 was added at 10^{-7} M in lanes 2, 4, 7, and 8.

ER and HMGA1 proteins which could be increased in the presence of DNA.

The interaction of HMGA1 with DNA was assessed by the use of distamycin A, an inhibitor of HMGA1 binding to the minor groove. Indeed, distamycin A did not modify either the formation of the complex containing ER-ERE or the ER-HMGA1-ERE complex.

Several authors have reported a protein−protein interaction involving HMG proteins. John et al. (*29*) showed that Elf-1

was physically associated with HMGA1 and with NF−Kappa B p50 and c-Rel. More recently, Chin et al. (*58*) have likewise demonstrated that HMGA1 can stimulate serum response factor mediated transcription by binding to DNA, as have Himes et al. (*59*) for the gene activation by the AP-1, NF-AT and NF-κB transcription factors, and Lewis et al. (*60*) for the PU.1 transcription factor. Similarly, Boonyaratanakornkit et al. (*61*) and Romine et al. (*49*) described an interaction between HMGB1 and HMGB2 with steroid hormone receptors to enhanced their DNA binding in vitro and transcriptional activity in mammalian cells. They demonstrated that these HMG-box proteins did not bind to the steroid response element but enhanced binding of the receptors to their specific sites without altering the mobility of the DNA−protein complex in gel mobility shift experiments. These investigators therefore concluded that HMG-box proteins could act in concert with steroid hormones to provide the geometry and sequence specificity required for transcription of hormone-responsive genes. A similar mechanism could apply to HMGA1.

Finally, we tested the effect of HMGA1 on EREs containing a single d(GpG)cisPt adduct which display a 8-fold lower affinity for ER (*51*). The platination of EREs in the linker sequence between the ERE half-sites decreases the interaction of ER to these EREs (*51*). Interestingly, when HMGA1 was combined with ER, enhanced binding was observed but the initial affinity was not restored. In contrast to HMGB1 which binds selectively to the cisplatin adduct (*62*), HMGA1 alone cannot bind to platinated sequences. Despite the fact that HMGA1 belongs to the superfamily of the HMG proteins, its DNA binding characteristics and its expression are different from that of HMG box proteins. The ability of HMGA1 to increase ER binding to platinated DNA is probably not due to direct binding of HMGA1 to DNA. Therefore, this result is in good agreement with the conclusion that the interaction is due to protein−protein binding.

In this study, we demonstrated that HMGA1 acts in synergy with the ER to enhance its DNA binding and transcriptional activity. It would be interesting to determine whether the expression of HMGA1 in hormonally dependent cancers would explain the particular sensitivity of tumor cells to estradiol. Furthermore, given the high degree of conservation among the steroid receptor superfamily members, it could be predicted that the binding of other steroid hormone receptors would also be enhanced in the presence of HMGA1, which may be involved in regulating transcription of a number of hormone-responsive genes.

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