Pergamon

J. Steroid Biochem. Molec. Biol Vol 48, No. 1, pp. 1–13, 1994

0960-0760/94 $24.00 + 0.00

0960-0760(93)E0019-4

General Review

Nuclear Accessory Factors Enhance the Binding of Progesterone Receptor to Specific Target DNA

Paul Prendergast, Sergio A. Oñate,* Kurt Christensen and Dean P. Edwards†

Department of Pathology, University of Colorado Cancer Center, Denver, CO 80262-0216, U.S.A.

The human progesterone receptor (PR) is dependent upon hormone and a nuclear accessory factor(s) for maximal binding to progesterone response elements (PRES) *in vitro*. Recombinant full-length PR, expressed in a baculovirus system and purified to apparent homogeneity, was used as a substrate to isolate and identify the accessory factor(s). The major PRE binding enhancement activity present in nuclear extracts was shown to be associated with the high mobility group chromatin protein HMG-1. Moreover, HMG-1 was equally effective in enhancing the DNA binding of both the A and B isoforms of PR. Enhancement of PRE binding was highly selective for HMG-1 as a single purified protein and was not mimicked by a general protein stabilization effect. In gel mobility shift assays, it appeared that HMG-1 enhanced PRE binding without stably participating as a component of the final DNA–PR complex, suggesting that HMG-1 acts indirectly by modifying the PR protein or the target DNA. HMG-1 is a sequence-independent DNA binding protein that recognizes distorted DNA structures and is also able to promote further distortions by bending DNA. Enhancement of PRE binding was found to be intrinsic to the conserved DNA binding domain of HMG-1 suggesting that HMG-1 acts by promoting a structural alteration in the target PRE-DNA.

J. Steroid Biochem. Molec. Biol., Vol. 48, No. 1, pp. 1–13, 1994

INTRODUCTION

Steroid hormone receptors are a family of ligand dependent transcriptional activators that function by binding to *cis*-acting hormone response elements (HREs) of target genes [1]. Major unanswered questions in our understanding of steroid hormone action are how the ligand converts receptors from an inactive to an active transcriptional regulator and how receptor binding to HREs increases transcription of target genes. Although consensus nucleotide sequences for functional HREs and structural features in the receptor that are important for binding have been defined, much remains to be learned about receptor–DNA recognition mechanisms. A recurring theme in the literature is the existence of nuclear auxiliary proteins that modulate the *in vitro* DNA binding activity of sequence specific transcription activators. In some cases, these auxiliary proteins have also been shown to effect transcriptional activity *in vivo*. Accessory proteins that facilitate DNA binding have been described for the activated T-cell transcription factor NF-AT [2], serum response factor SRF [3], *fos–jun* [4], NF-kB [5–7], CREB/ATF [7] and several members of the steroid receptor family [8–22]. It appears that there may be common auxiliary proteins for distinct subgroups of transcription activators. For example, the DNA binding activities of *fos–jun*, NF-kB, Myb, and CREB/ATF, are sensitive to oxidation–reduction and a protein termed redox factor ref-1 has been cloned and isolated that is capable of mediating both redox regulation and enhancement of the DNA binding of these transcription factors [7]. The retinoic acid X receptor (RXR), which is itself a member of the steroid receptor family, appears to serve

*Present address: Cell Biology Department, Baylor College of Medicine, Houston, TX 77030, U.S.A.
†Correspondence to D. P. Edwards at the Department of Pathology (B216), University of Colorado Health Sciences Center, 4200 East Ninth Avenue, Denver, CO 80262-0216, U.S.A.
Received 18 Oct. 1993; accepted 5 Nov. 1993.

in this capacity for a subgroup of intracellular receptors that includes thyroid hormone receptor (TR), vitamin D_3 receptor (VDR), retinoic acid receptor (RAR) and two orphan receptors, the peroxisome proliferator activator receptor (PPAR) and COUP-TF [12–15, 23, 24]. RXR is a former orphan receptor since a stereoisomer of all-*trans* retinoic acid, 9-*cis* retinoic acid, has now been identified as the endogenous ligand for RXR [25]. It appears that RXR enhances DNA binding by heterodimerization where both RXR and the other receptor partner are both components of the DNA complex. Whether proteins other than RXRs can also heterodimerize with and enhance DNA binding of this subgroup of receptors remains to be resolved [8–11].

Nuclear proteins that enhance the DNA binding of the classical steroid hormone receptors have also been described. Two non-histone chromosomal proteins of 60–70 kDa that bind to the spacer nucleotides of palindromic estrogen response elements (EREs) were shown to enhance estrogen receptor (ER) binding [18]. It has also been shown that efficient binding of highly purified human ER to an ERE *in vitro* requires the addition of a 45 kDa single stranded DNA binding protein [19]. A small heat stable 3–7 kDa polypeptide was reported to increase the binding of glucocorticoid receptor (GR) to DNA–cellulose [16] and a larger protein of 93 kDa was described to increase binding of GR to chromatin [17]. More recently, a receptor accessory factor (RAF) associated with a protein of 130 kDa, was demonstrated to enhance the binding of recombinant GR and androgen receptor (AR) to their cognate target DNA sequences [20]. It appeared that enhancement of DNA binding was associated with RAF forming a ternary complex with receptor and DNA.

We have shown in earlier studies that the human progesterone receptor (PR) also requires an auxiliary nuclear protein for maximal binding to progesterone response elements (PREs) *in vitro* [21]. The major PRE enhancement activity present in nuclear extracts was identified to be associated with the high mobility group chromatin protein HMG-1 [22]. The present paper provides an overview of our studies of auxiliary proteins that enhance the DNA binding of PR and includes both published [21, 22], and unpublished results. A mechanism is proposed by which accessory factors enhance receptor binding to specific DNA sites by promoting structural alterations in the target DNA.

EXPERIMENTAL

Cell culture, baculovirus expression and receptor preparation

PR-rich T47D human breast cancer cells and PR-negative MDA231 cells were cultured as described previously [26, 27]. *S. frugiperda* (Sf9) insect cells were grown at 27°C in Grace's insect medium (GIBCO) supplemented with 3.3 g/l of yeastolate (GIBCO), 3.3 g/l of lactalbumin hydrolysate (GIBCO), 25 μg/ml Gentamicin (Irvine) and 10% fetal bovine serum (Hyclone). For infection with recombinant baculovirus vectors, Sf9 cells were plated at a density of 1×10^6 cells/ml in spinner vessels. Cells in suspension culture were infected with recombinant viruses at a multiplicity of infection of 2.0 for 48 h at 27°C. Construction of recombinant baculoviruses expressing either the intact full length A or B isoforms of human PR and their functional properties have been detailed previously [28].

Recombinant receptors expressed from baculovirus were prepared as whole cell extracts (WCE) of infected Sf9 cells by lysing cells in TEDG [10 mM Tris-base, pH 7.4, 1 mM EDTA, 1 mM dithiothreitol (DTT), 10% glycerol] supplemented with a cocktail of protease inhibitor [26] and 0.5 M NaCl. Samples were clarified by centrifugation at 105,000 *g* for 30 min. Receptor binding to hormone *in vivo* was accomplished by incubating Sf9 insect cells at 27°C for the last 4 h of infection with 100 nM of the synthetic progestin, R5020. WCEs were dialyzed at 4°C against TEDG to reduce salt concentrations prior to immunoprecipitation and DNA binding assays. Receptor concentrations in cell extracts were estimated by steroid binding and by immunodot blot assay as described previously [29], and protein concentrations were measured by the method of Bradford [30].

Purification of recombinant human PR

Separately expressed PR-A and PR-B from baculovirus vectors in Sf9 insect cells were purified by immunoaffinity chromatography. Monoclonal antibody (MAb) affinity resins were constructed by chemically crosslinking the B-30 or the AB-52 MAb to protein G Sepharose (Pharmacia-LKB) using 10 mM dimethylpimelimidate by methods described previously [26]. MAbs were purified from ascites fluids and coupled to protein G-Sepharose at a concentration of a 4–6 mg/ml. The B-30 MAb which recognizes the B isoform of PR was used to purify PR-B. AB-52 MAb which recognizes both the A and B isoforms was used to purify PR-A [26]. Approximately 300×10^6 Sf9 insect cells were used for each receptor purification. To follow receptor during purification, WCEs were incubated for 8 h at 4°C with [^{3}H]R5020 to allow some exchange with unlabeled R5020 bound to PR. Excess free [^{3}H]R5020 and NaCl concentrations in WCEs were reduced by dialysis against TEDG. Dialyzed WCEs were then incubated for 4 h at 4°C on an end-over-end rotator with a 1.0 ml suspension of MAb-coupled protein G-Sepharose. The beads were then collected by centrifugation at 1500 rpm for 5 min and washed by resuspension and centrifugation in 15 ml of TEG (TEDG minus DTT). This was followed by 3 more washes with 15 ml of TEG containing 0.5 M NaCl and one additional wash with 15 ml of TEG. Resins were then transferred to a new tube and washed twice more with 15 ml of TEG. For elution of PR, resins were transferred to a 2.0 ml microfuge tube and exposed to alkaline pH. Briefly, beads were suspended in 600 μl of 50 mM Tris-base, 1 mM EDTA containing 20% glycerol that was adjusted to pH 11.3 with NaOH. The

beads were immediately centrifuged and the supernatant was removed and mixed with 200 μl of a neutralization/renaturation buffer comprised of 400 mM Tris–HCl (pH 7.4), 40 mM $MgCl_2$, 40 mM DTT, 4 mM EDTA, 0.4 mM EGTA, 100 mM NaCl, 0.2 mM $ZnCl_2$ and 50% glycerol. Alkaline pH elution steps were repeated 4–6 times and the separate eluates were pooled. The majority of hormone (60%) originally bound to PR remains bound during alkaline pH elution and neutralization (data not shown). Purified PR can be frozen at −70°C in aliquots and will retain biological activity for approx. 1–2 months, if not repeatedly freeze–thawed.

Gel electrophoresis and Western blotting

SDS polyacrylamide gel electrophoresis (SDS–PAGE) and Western blot analyses of PR were carried out as described previously [26, 27, 29] except that peroxidase conjugated anti-mouse IgG and ELISA was used as the detection method instead of radioactive protein A and autoradiography. Silver staining of SDS–PAGE was performed by the method of Heukeshoven and Dernick [31].

Purification of HMG-1

HMG-1 was purified from calf thymus by a slight modification of the method of Adachi *et al.* [32]. Briefly, acid soluble chromosomal proteins were extracted with 5% perchloric acid and precipitated with cold 25% trichloroacetic acid. The precipitate was pelleted by centrifugation at 10,000 rpm for 20 min. The pellet was dissolved in water and dialyzed against 10 mM Tris–HCl, pH 7.8. The dialysate was applied directly to a PBE94 polybuffer-exchange resin (Pharmacia-LKB) used under anion exchange conditions. The PBE94 column was washed in 10 mM Tris–HCl, pH 7.8 to remove weakly bound proteins and then eluted with a linear 0–1.5 M NaCl gradient prepared in 10 mM Tris–HCl, pH 7.8. Eluted fractions were analyzed by SDS–PAGE and silver staining and by Western blotting with a rabbit anti-sera to HMG-1. Western blot of HMG-1 was performed as for PR except that the detection method was by autoradiography using an ^{125}I-donkey anti-rabbit IgG (11 μCi/μg, Amersham). The rabbit anti-sera to HMG-1 [33] was diluted 1:150 in Western blot dilution buffer and the ^{125}I-secondary antibody was diluted 1:2000 ($\approx$100,000 dpm/ml).

Quantitative immunodot blot assay

To determine the quantity of PR protein present in WCEs and after purification, an immunodot blot assay was used as described previously with some minor modifications [29]. As a standard for known amounts of PR, cytosols from T47D breast cancer cells were measured for the number of PR sites by steroid binding assay and then serially diluted (2-fold) in TEDG to generate a series of PR concentrations ranging from 2500 to 1.2 fmol/300 μl. An assumption in this assay is that all PR in T47D extracts is capable of binding steroid. Samples of purified PR were initially diluted 1:10 and WCEs were diluted 1:100. Whatman filter paper (3 mm) and nitrocellulose were equilibrated for 30 min at 25°C (or room temperature) in TEDG and then placed in a Biorad E6-well dot blot manifold. Samples in 300 μl volumes were applied under vacuum to each well of the manifold and washed once under vacuum with 300 μl TEDG. The nitrocellulose was then removed, blocked with 1% bovine serum albumin (BSA)/0.1% Tween-20 and incubated with 10 μg/ml of AB-52 MAb and processed in the same manner as for Western blot assays. Nitrocellulose filters were incubated with [^{35}S]protein A (Amersham, at 50,000 cpm/ml), dried and exposed to X-ray film. Each spot was quantitated by direct scanning of the radioactivity of the nitrocellulose filter with a Phosphorimager (Molecular Dynamics).

Gel-mobility shift assay

DNA-binding assays by gel-mobility shift were performed as described previously with minor modifications [27, 34]. PR (in fmol or ng as indicated in the figure legends) in whole cell extracts or in purified form were incubated for 1 h at 4°C with 0.3 ng of end-labeled [^{32}P]PRE oligonucleotide (sp. act. of 20,000–30,000 cpm/0.1 ng of DNA). The DNA binding reaction also contained poly dA-dT:poly dA-dT as non-specific competitor DNA and 2 μg of gelatin in a DNA binding buffer consisting of 10 mM Tris-base, pH 7.5, 50 mM NaCl, 5 mM DTT, 2 mM $MgCl_2$, and 10% glycerol. The DNA binding reactions (25 μl) were subjected to electrophoresis and autoradiography as described previously [27, 34]. In experiments in which other proteins were added to purified PR, the receptor and protein fractions were mixed and preincubated for 30 min at 4°C prior to addition of DNA. Quantitation of free [^{32}P]PRE and PRE complexes was carried out by direct scanning of dried gels for radioactivity using the Series 400 Molecular Dynamics PhosphorImager.

RESULTS

PR requires both hormone and a nuclear accessory protein(s) for efficient binding to specific DNA

In human cells, PR is expressed from the same gene as two amino terminal variants termed PR-A (90–94 kDa) and PR-B (118–120 kDa). PR-A is truncated, missing 164 amino terminal residues, and arises from alternative use of a second promoter. Otherwise, PR-A and PR-B have identical sequences in the DNA binding and C-terminal ligand binding domains [35]. Both PR isoforms have been shown to be capable of binding to DNA and to require ligand for induction of binding to specific PREs [34, 36–38]. Additionally, human PR is capable of forming stable dimers in solution and of binding to DNA in three dimeric forms

composed of the possible PR subunits; AA, AB and BB dimers [27, 36, 38, 39]. Previous studies from our laboratory have also shown that PR bound and activated by hormone *in vivo* (intact cells and then extracted from nuclei with 0.45 M NaCl), exhibited substantially higher binding activity (5- to 7-fold) for PREs than an equal number of cytosolic receptors bound to hormone and activated by salt treatment *in vitro* [21, 27, 34, 39]. This suggests that hormone binding modifies PR in a manner in the intact cell that is not mimicked *in vitro* or, that nuclear factors facilitate PRE binding that are either absent or in low amounts in cytosols. In support of the former possibility, PR is a phosphoprotein and hormone binding *in vivo* promotes hyperphosphorylation of PR whereas hormone-dependent phosphorylation does not occur in cell-free cytosols [39, 40]. However, these are correlative results and there are no direct data as yet to demonstrate a functional role for phosphorylation in receptor–DNA binding. As evidence that the higher DNA binding activity of *in vivo* activated nuclear PR is due in part to a nuclear factor, we found that addition of nuclear extracts (lacking PR) to cytosol receptors stimulated a 3- to 4-fold increase in specific PRE binding [21].

The influence of hormone (synthetic progestin R5020) *in vitro* and *in vivo* on DNA binding of PR from T47D human breast cancer cells is shown by gel-mobility shift assay in Fig. 1. The target ^{32}P-labeled DNA is an oligonucleotide containing a single PRE from the promoter distal partial palindromic response element of MMTV [1]. Cytosolic PR prepared from T47D cells in the absence of hormone and treated with 0.5 M NaCl to dissociate receptors from hsp90 failed to bind to the PRE and to form a retarded mobility complex [Fig. 1(A), lane 1]. Incubation of salt-treated cytosol PR with R5020 *in vitro* for 5 h was sufficient to induce PRE binding [Fig. 1(A), lane 2]. Incubation with hormone for longer periods of time did not produce a further increase in binding [Fig. 1(A), lane 3]. In contrast, PR activated *in vivo* by the addition of hormone to intact cells and then extracted from nuclei with high salt, displayed a markedly higher level of

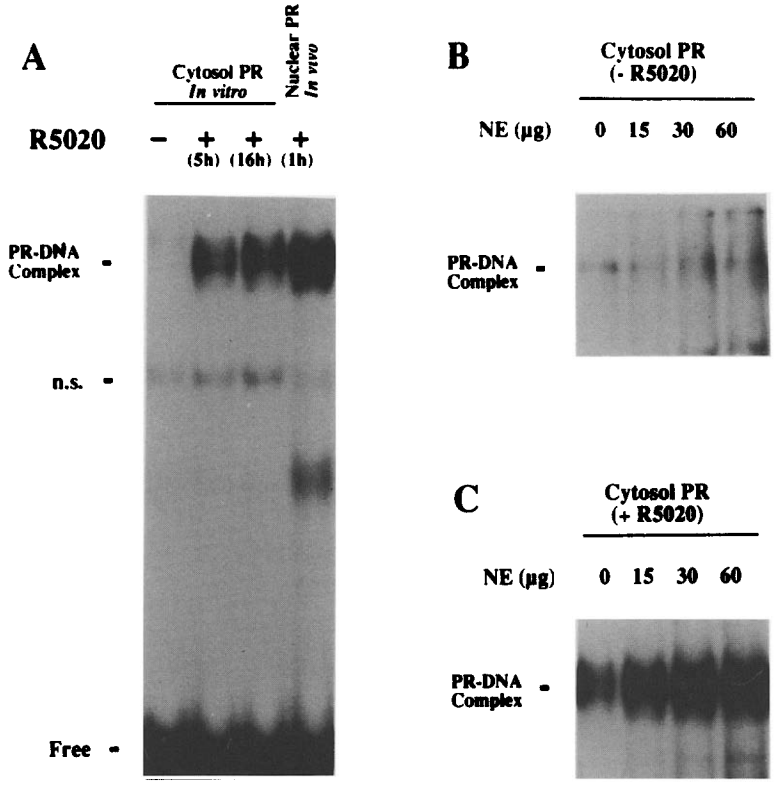

Fig. 1. PR binding to specific DNA is dependent on hormone and an accessory nuclear factor. (A) *In vitro* and *in vivo* effect of hormone on binding of T47D PR to DNA. Unliganded cytosolic PR prepared from T47D cells were treated with 0.5 M NaCl for 1 h at 4°C and incubated with R5020 (200 nM) at 4°C for the times indicated. Receptors were also bound to R5020 *in vivo* (40 nM) for 1 h at 37°C and the PR–R5020 complexes were then extracted from isolated nuclei with 0.5 M NaCl. An estimated 0.05 pmol of PR in WCEs (as assessed by hormone binding) were submitted to gel-shift assay with 0.3 ng of a [^{32}P]PRE oligonucleotide. PR–DNA complexes, non-specific protein–DNA complexes and free DNA are indicated. (B and C) Effect of receptor-depleted nuclear extract on the DNA binding of cytosolic PR. Nuclear extracts from non-hormone treated T47D cells were dialyzed and immunodepleted of PR by receptor-specific MAb affinity resins. Receptor depletion was effective as demonstrated by Western immunoblot with receptor-specific MAb (not shown). Depleted nuclear extracts (in μg of total proteins) were mixed in a DNA-binding buffer with 0.05 pmol of either unliganded (−R5020) cytosolic PR (B) or with liganded (+R5020) cytosolic PR (C) and incubated for another 30 min at 4°C. [^{32}P]PRE was then added for another 30 min at 4°C and the entire reaction mixture (20 μl) submitted to gel-electrophoresis. B and C show only the region of the gel containing the PR–DNA complex.

DNA binding (5- to 10-fold). To determine whether this difference could be due to the presence of an accessory nuclear protein mixing experiments were done. Nuclear extracts from non-hormone treated T47D cells were depleted of PR by MAb-affinity chromatography as described previously [21]. Aliquots of the PR-depleted nuclear extracts were then mixed with cytosolic PR and tested for effects on PR–DNA binding. Unliganded cytosolic PR continued to show no DNA binding activity in the absence or presence of added nuclear extracts [Fig. 1(B)]. In contrast, addition of nuclear extracts to cytosolic PR bound to R5020, stimulated binding to higher levels than that induced by the hormone alone [Fig. 1(C)]. This stimulatory effect was dependent on the amounts of protein added and appeared to reach saturation, indicating that a binding phenomenon was responsible for the enhancement activity. These results suggest that the higher DNA binding activity of PR bound to hormone *in vivo* is due, at least in part, to an auxiliary nuclear factor.

To further characterize the nuclear factor we have purified human PR to apparent homogeneity and have used the purified receptor as a substrate for addition of accessory factors. Because endogenous PR in mammalian cells is expressed at low levels, recombinant receptor overexpressed in a baculovirus insect cell system was used as a source for purification. Both PR isoforms were expressed separately as intact full-length proteins, and they have been shown previously to be structurally and functionally indistinguishable from their endogenous counterparts [28]. The recombinant PRs bind hormone with the same affinity and steroid specificity as endogenous PR and when present in whole cell extracts they also bind with high affinity to PREs in a hormone-dependent manner [28].

Each PR isoform was purified by MAb affinity chromatography from whole cell extracts of baculovirus infected Sf9 insect cells. For simplicity of presentation, most results in this paper are shown with PR-B. Essentially identical results were obtained with PR-A (see Fig. 6). PR was eluted from the MAb affinity resin by exposure to alkaline pH and was immediately neutralized with a buffer containing excess reducing agent, zinc, and glycerol to attempt to renature any unfolded PR. As shown by silver stained SDS–PAGE in Fig. 2 (left panel), WCEs contain high levels of soluble PR-B, detectable as major stainable bands at 118–120 kDa, and the affinity purified receptors are essentially homogenous. The Western blot in Fig. 2 (right panel) confirms the identity of the silver stained bands in WCEs and after purification as authentic PR-B. The band heterogeneity of PR-B on SDS-gels that is evident in Fig. 2 is due to phosphorylation [28]. It should be noted that PR was bound to hormone *in vivo* prior to cell lysis and MAb-affinity resins were washed in buffers containing 0.5 M NaCl. These are conditions that favor receptor dissociation from heat shock proteins and immunophilins (p59) that associate with the inactive cytosolic receptor complex [41]. Thus

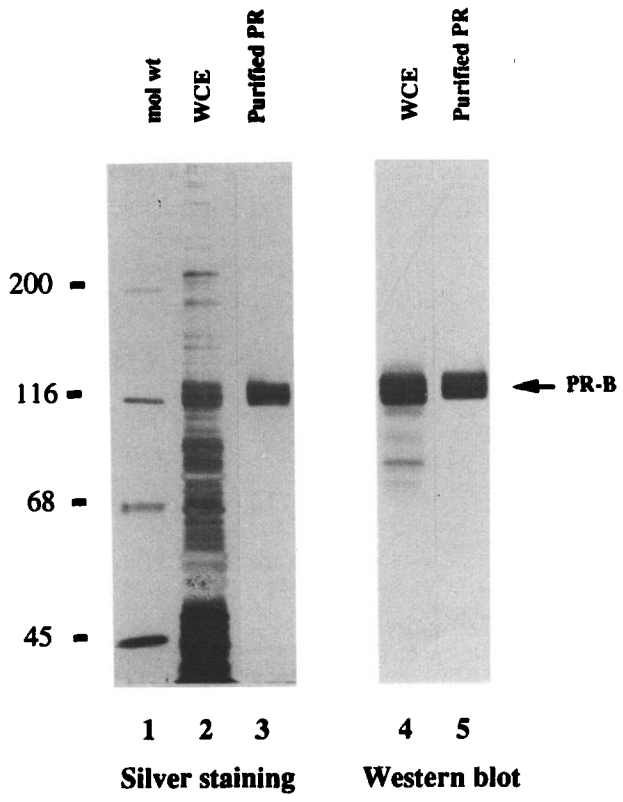

Fig. 2. Purification of recombinant PR-B by MAb affinity chromatography. WCEs from Sf9 insect cells expressing human PR-B were prepared and PR was purified by a receptor-specific MAb (B-30) linked to protein G-Sepharose as described in the Experimental section. PR in WCE (25 μg of total protein) and after purification (5 μg) were analyzed by SDS–PAGE and silver staining (left panel) and by Western blot (right panel) adapted from Ref. [22].

purified PR appears to be essentially free of contaminants and other known receptor-associated proteins. PR in WCEs and after purification was quantitated by steroid binding assay and by dot blot immunoassay. Approximately 80% of PR protein in WCEs can be accounted for as having steroid binding activity (data not shown). As found previously with endogenous T47D PR [26], exposure to alkaline pH is a relatively mild denaturation condition that maintains the majority (>60%) of the steroid originally bound to the native receptor (not shown).

PR in WCEs and after purification were also analyzed for their ability to bind to specific DNA by gel-mobility shift assay. As shown in Fig. 3 (left panel, lane 2), hormone (R5020) activated PR-B present in WCEs bound efficiently to the PRE oligonucleotide. By contrast purified PR-B exhibited no DNA binding activity under the conditions of the gel shift assay (Fig. 3, left panel, lane 3). It should be noted that equal amounts (estimated by dot blot immunoassay and steroid binding) of PR in WCEs and in purified form were added to DNA-binding reactions to ensure that differences in DNA binding were not due to different amounts of receptor protein. Remarkably, the DNA binding activity of purified PR-B was nearly fully restored to that of native PR by addition of 0.45 M NaCl nuclear extracts that lacked PR [Fig. 3, left panel,

lanes 3–8]. Moreover, the ability to stimulate DNA binding was dependent on the amount (in μg of total protein) of nuclear extract added and appeared to be saturable. The reconstituted complex contained PR-B as demonstrated by a further mobility shift induced by addition of a PR-B specific MAb ([22], not shown).

Nuclear extracts prepared from several different eukaryotic cells enhanced the DNA binding of purified PR-B including, PR-positive T47D breast cancer cells (PR was immunodepleted), PR-negative MDA-231 breast cancer cells and non-infected Sf9 insect cells (Fig. 4). Activity was also present in rabbit reticulocyte lysates (not shown), but little or no activity was detectable in *E. coli* extracts (see Fig. 6). This suggests that the PRE binding enhancement activity is associated with a ubiquitous eukaryotic factor. By subcellular fractionation the majority of activity was present in 0.45 M NaCl extracts of isolated nuclei, although some activity was found in cytosol (data not shown). Activity also appeared to be associated with a protein(s) since it was inactivated by heating or by trypsin digestion and was retained by dialysis with membranes having molecular weight cut-offs in the range of 12–14,000 (data not shown).

To determine whether enhancement of PRE binding was due to a specific protein, as opposed to a general effect of protein concentration on stabilization of highly purified receptor, we examined the ability of various purified proteins and other agents to enhance the DNA binding of purified PR-B. As shown in Fig. 3, addition of ovalbumin in amounts equal to that of the total protein of nuclear extracts, did not stimulate PRE binding. Table 1 lists other proteins and compounds tested which included charged and neutral polyamino acids, nucleotide triphosphates, excess reducing agents and 5% polyethylene glycol as a water excluding agent. Of all the agents above, only nuclear extracts were able to effectively stimulate and restore the DNA binding activity of purified PR. These results indicate that a specific protein(s) present in nuclear extracts is responsible for enhancement of PRE binding.

Nuclear accessory proteins do not affect steroid binding activity, dimerization, or DNA binding specificity

Since some of the steroid may have been stripped from receptors during affinity purification coupled with the fact that ligand is needed for DNA binding, we questioned whether exposure of purified receptor to additional ligand might have a stimulatory effect on the PRE binding. As shown in Fig. 3 (right panel), addition of excess free ligand to purified PR did not stimulate PRE binding, nor did excess ligand influence the stimulatory effect of nuclear extracts. We also questioned whether nuclear extracts might enhance DNA binding indirectly by stimulating the steroid binding activity of purified PR. However, addition of nuclear extracts did not increase the steroid binding capacity of purified PR-B (data not shown).

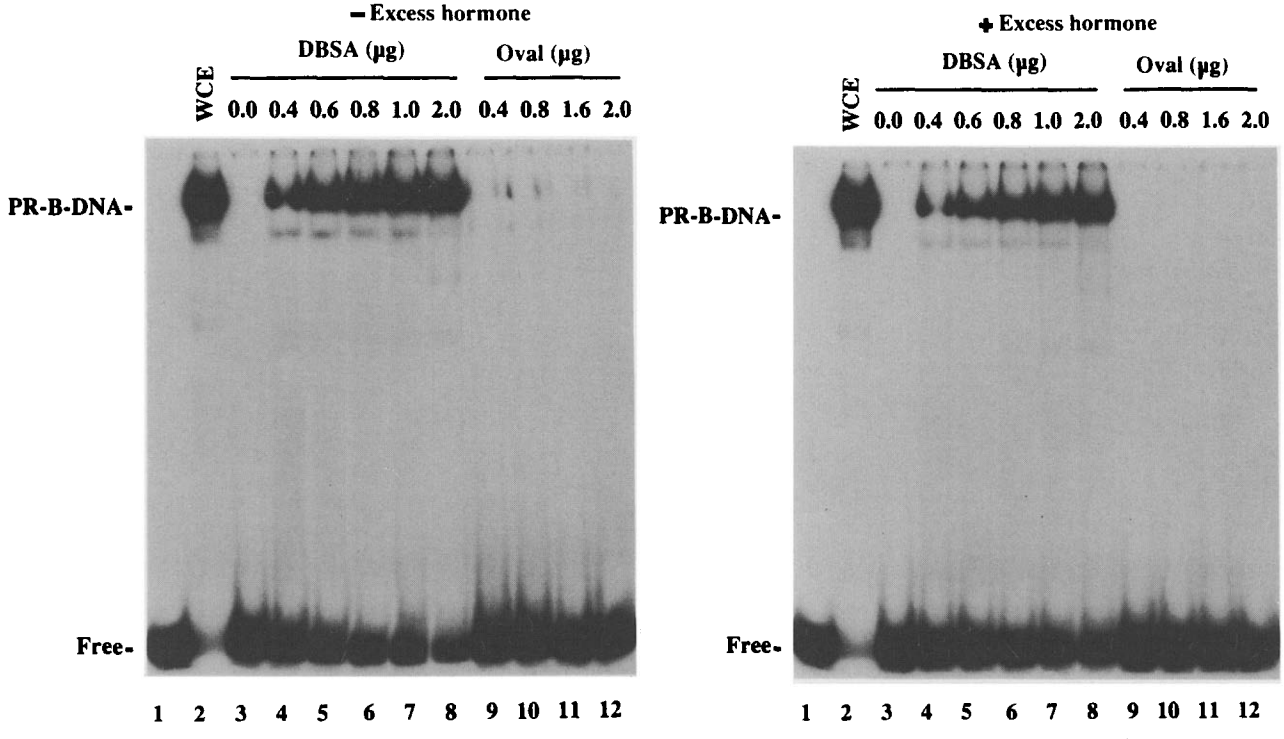

Fig. 3. DNA binding of PR-B in whole cell extracts and after affinity purification. ***Left panel*****: equal amounts (5 nm) of recombinant baculovirus expressed PR-B in WCE (lane 2) or affinity purified recombinant PR-B (lane 3) were analyzed by gel shift with an end-labeled PRE oligonucleotide as in Fig. 1. In lanes 4–8, increasing amounts (in μg of total protein) of nuclear extracts were added to purified PR-B. Nuclear extracts containing DNA-binding stimulatory activity (DBSA) were prepared from PR-negative MDA-231 breast cancer cells. A non-specific carrier protein, ovalbumin, was added in increasing amounts to purified PR-B (lanes 9–12).** ***Right panel*****: the identical samples were incubated with excess (200 nM) hormone (R5020) (adapted from Ref. [22]).**

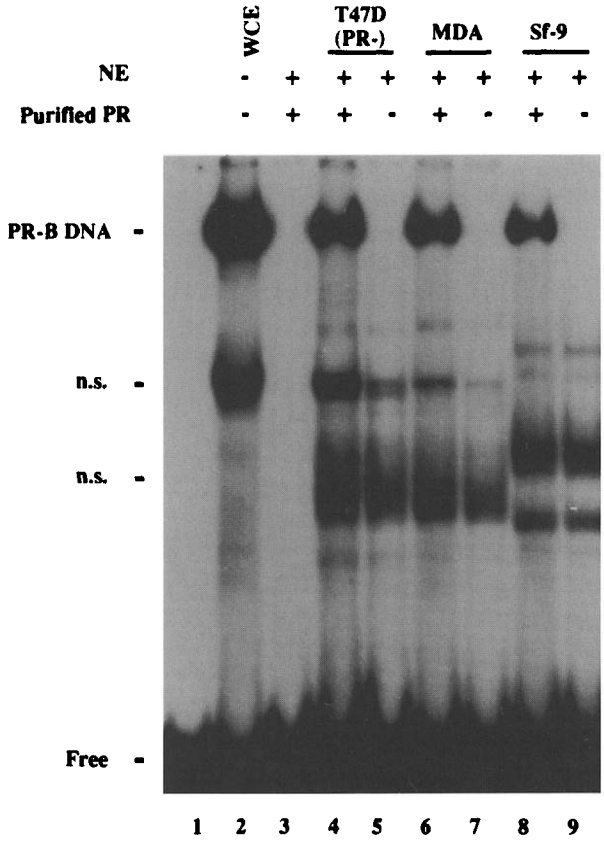

Fig. 4. PRE-binding enhancement activity is present in PR-positive and -negative cells. Nuclear extracts were prepared from two breast cancer cell lines, T47D (depleted of PR by receptor-specific MAb resins) and MDA-231 (PR-negative) and from non-infected Sf9 insect cells. Equal amounts of recombinant PR-B in WCEs (lane 2) or affinity purified PR (lane 3) were assayed for binding to a PRE oligonucleotide by gel shift. In lanes 4–9, nuclear extracts from T47D, MDA-231, and Sf9 cells were added to purified PR-B or assayed alone (adapted from Ref. [22]). Non-specific DNA complexes and free DNA are indicated.

Various steroid hormone receptors have been shown to dimerize in solution in the absence of DNA and the ability to dimerize has been correlated with DNA binding activity [36, 39, 42–44]. To investigate the possibility that the nuclear accessory protein(s) mediates its effect by modulation of PR dimerization, we have employed a coimmunoprecipitation assay described previously that detects dimerization between truncated PR-A and full-length PR-B [36, 39]. The A and B receptors were expressed separately in Sf9 insect cells and purified by MAb affinity chromatography as in Fig. 2. Varying amounts of purified PR-A and PR-B (in a constant ratio) were mixed together in the presence or absence of nuclear extracts and samples were immunoprecipitated with the B-30 MAb that recognizes an epitope present in the amino terminus unique to PR-B. Coimmunoprecipitation of PR-A with PR-B is indicative of dimerization between the A and B receptors. The amount of PR-A that specifically coimmunoprecipitated with PR-B was the same at all concentrations of purified PR whether or not nuclear extracts were added (not shown, see Ref. [22]). Thus, stimulation of PR–DNA binding activity by the nuclear factor(s) does not appear to occur by modulation of receptor dimerization.

Table 1. *Agents that do not stimulate DNA binding of purified PR*

General proteins	Nucleotides
BSA	ATP
Ovalbumin	GTP
Insulin	
RNAse	*Reducing agents*
Gelatin	βME
Histone H1	DTT
Polyamino acids	*Water excluding agent*
Poly-L-lysine	PEG
Poly-L-aspartic acid	
Poly-L-proline	

Standard gel-mobility shift assays contain excess unlabeled competitor DNA to minimize binding to the specific ^{32}P-labeled oligonucleotide by other non-specific DNA binding proteins present in crude nuclear extracts. We thought it important to determine whether nuclear extracts might stimulate the binding of purified PR to specific PREs by simply displacing PR from competitor DNA. However, when the amount of competitor DNA in gel shift assays was reduced or eliminated, purified PR still failed to bind to the PRE under the conditions of the assay and continued to require addition of nuclear proteins (data not shown). With purified PR we routinely used 80 ng of poly dA-dT : poly dA-dT as competitor DNA which is considerably reduced from the more standard amounts of 1–2 μg. The above results collectively are consistent with the presence of a specific protein in nuclear extracts that enhances the DNA binding of PR for its response element DNA.

The high mobility group protein HMG-1 functionally substitutes for nuclear extracts

Using nuclear extracts prepared from PR-negative MDA-231 breast cancer cells as a source, we attempted to purify a protein associated with PRE binding stimulatory activity. Conventional column chromatography was used and column fractions were assayed for their ability to enhance the binding of purified PR to PREs by gel shift assay. The majority of PRE enhancement activity was retained by heparin Sepharose and phosphocellulose and required high salt concentrations for elution; 0.8 M NaCl for elution from heparin Sepharose and 0.6 M for elution from phosphocellulose. Activity bound poorly to double stranded DNA–agarose and did not bind at all to a PRE oligonucleotide column. In contrast, the majority of activity bound to denatured single stranded DNA–agarose and was eluted at 0.2 M NaCl (Fig. 5). Taking advantage of these properties we used sequential column chromatography steps of heparin Sepharose, phosphocellulose and two passes over single-stranded DNA–agarose to obtain an approx. 100-fold enrichment of activity per unit of protein (not shown).

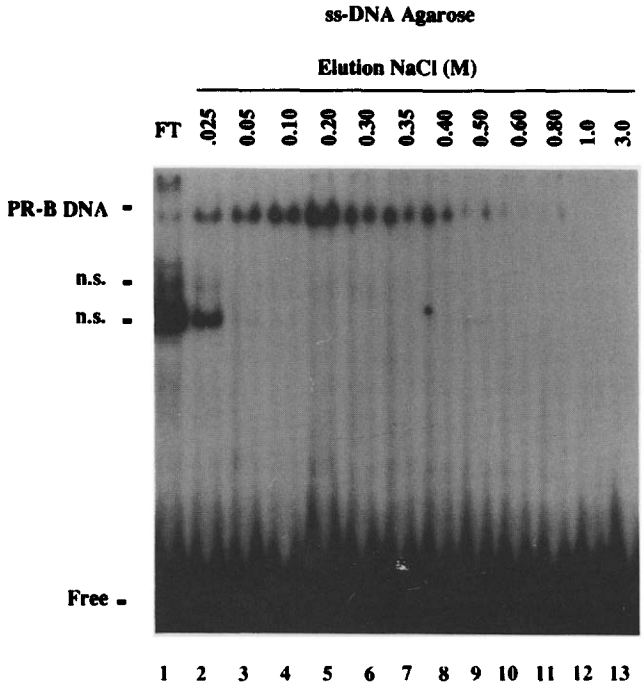

Fig. 5. Fractionation of the PRE binding enhancement activity by single-stranded DNA–agarose. Nuclear extracts prepared from PR-negative MDA-231 breast cancer cells were sequentially fractionated on heparin Sepharose, phosphocellulose and then applied to a single-stranded DNA–agarose column. Bound material was eluted stepwise with NaCl at the concentrations indicated. The column flow through (FT) and salt eluted fractions (lanes 2–13) were dialyzed and mixed with purified recombinant PR-B in a gel-mobility shift assay (adapted from Ref. [22]).

During the course of these fractionation experiments we noticed that several other known nuclear proteins have been reported to exhibit similar chromatographic behavior, in particular binding to single-stranded DNA–agarose. These proteins include the basal transcription factors TFIID [45] and TFIIB [46] and the high mobility group chromatin protein HMG-1 [47]. TFIIB was also of interest because of it was recently identified as a protein that stabilizes the DNA binding of an orphan receptor, COUP-TF, and it was shown to bind directly to PR and ER [48]. TBP (TATA binding protein of TFIID) and TFIIB were expressed as GST-fusion proteins in *E. coli* and purified by glutathione-Sepharose affinity chromatography [48]. HMG-1 was purified from calf thymus by ion exchange chromatography on polybuffer (PBE 94) chromatofocusing resins [32]. Each purified protein, compared with BSA, *E. coli* extracts and nuclear extracts prepared from MDA-231 breast cancer cells, was tested for its ability to enhance PRE-binding of purified PR. As shown in Fig. 6, only HMG-1 and nuclear extracts from MDA-231 cells had a substantial effect on enhancing PR–DNA binding. Figure 7 is a silver stained SDS–PAGE of the purified HMG-1 used in these experiments. This shows a single silver stained band at 28,000 kDa which was confirmed immunologically by Western blot to correspond to HMG-1. HMG-1 was purified from two sources, calf thymus and MDA-231 nuclear extracts. On a molar basis, HMG-1 was effective when added in stoichiometric amounts with PR. It also appeared that HMG-1 enhanced PRE binding without participating stably in the DNA–receptor complex. As evidence of this, HMG-1 alone did not directly bind to the PRE oligonucleotide, an HMG-1 antibody failed to induce a further mobility shift under conditions where a PR-specific MAb induced a supershift, and enhancement of PRE binding occurred without an obvious change in mobility of the DNA–PR complex (not shown, see Ref. [22]).

HMG-1 harbors two domains termed "HMG-A and -B boxes," that comprise structure-specific sequence-independent DNA binding motifs [49, 50]. The A and B boxes are homologous (but not identical), 80 amino acid regions located respectively in the N-terminus and central portions of the protein. HMG boxes are proposed to be a novel DNA binding motif that recognizes angles or bends in DNA [49, 50]. Regions of homology to HMG boxes have been found in several other proteins including the sequence specific transcriptional regulators, lymphoid cell enhancer factor LEF-1 [51] and testes determining factor SRY [52]. HMG-1 box domains expressed and purified from *E. coli* were tested for their ability to stimulate the DNA binding of purified PR. The A box exhibited weak enhancement of PRE binding, whereas the B box exhibited strong activity nearly equivalent to that of intact HMG-1. In contrast, HMG boxes of LEF-1 and SRY failed to enhance PRE binding of purified PR (not shown, see Ref. [22]). Thus the HMG-B box alone is sufficient for enhancement of PRE binding, but it appears that not all HMG box motifs are equivalent in this respect.

HMG-1 increases the binding affinity of PR

Because essentially no specific PRE complexes were detected with purified PR alone, it was not possible to estimate a fold enhancement of PRE binding promoted by HMG-1. Protein–DNA interactions that exhibit high rates of dissociation are generally not maintained under standard gel shift conditions, which suggested that the affinity of PR alone may be too low for detection under our conditions and that the effect of HMG-1 is to increase PR affinity to a detectable level. In order to estimate the binding affinity of purified PR in the presence and absence of HMG-1, we performed binding analysis with varying concentrations of PR against a constant ^{32}P-labeled PRE DNA present in a limiting amount. Much higher concentrations of receptor were used here than the single concentration (5 nM) of purified receptor used in previous experiments. Specific PRE–receptor complexes were quantitated by direct analysis (Phosphorimager) of the radioactivity of dried mobility shift gels. As shown in Fig. 8, binding of native PR in WCEs to a synthetic palindromic PRE was dose-responsive and saturable in the nM range. Purified PR alone, over the concentration range that gave saturable binding in WCEs, failed to exhibit any detectable PRE binding. However, some PRE complexes were obtained at much

higher concentrations of purified receptor, but the binding did not saturate and the number of complexes obtained at the highest concentration of purified receptor was less than that exhibited at saturation with much lower concentrations of native PR in WCEs. Essentially identical results were obtained with PR-A [Fig. 8(A)] and PR-B [Fig. 8(B)]. Based on the concentration of PR that gave a 50% upshift of the [^{32}P]PRE, we have estimated a kDa of 2×10^{-9} M for both PR-A and PR-B in WCE and a 10-fold higher kDa of 2×10^{-8} M for purified PR (the A and B isoforms were not significantly different). Addition of nuclear extracts or HMG-1 to purified PR resulted in a leftward shift of the binding curve that was nearly superimposable on the curve exhibited by native PR in WCEs. This indicates that HMG-1 (and nuclear extracts) increased the binding affinity of PR by approx. 10-fold. It is not shown here, but it is important to note that HMG-1 appeared to stimulate the binding affinity of PR for the partial palindromic element of MMTV by >10-fold. However, the difference in kDa in the presence and absence of HMG-1 was not precisely measurable because PR binds less well to the imperfect than to the perfect palindromic PRE. We had difficulty in obtaining any binding of purified PR alone to the PRE of MMTV, even at the highest concentrations of receptor.

DISCUSSION

The RXR appears to be a common coregulator for a subgroup of the steroid receptor family that includes TR, VDR, RAR, PPAR and COUP-TF [12–15, 23, 24], acting to enhance DNA binding by a heterodimerization mechanism. Nuclear accessory factors, distinct from RXR, have been reported to enhance the DNA binding of receptors for the classical steroid hormones including receptors for progesterone [21, 22], estrogen [18, 19], glucocorticoids [16, 17, 20] and androgen [20]. In general much less is known about the identity and mechanism of action of these accessory factors. We have demonstrated that HMG-1 can function in this capacity to enhance the binding of purified human PR to its specific PRE and this has been shown when both PR and HMG-1 were purified to apparent homogeneity. It is unclear at present whether the classical steroid hormone receptors (GR, MR, AR, ER, PR), as a group, utilize a common accessory protein such as HMG-1 or whether different accessory factors are involved for each receptor. Based on the reported differences in the literature in molecular mass, it would appear that there are multiple accessory factors [16–22]. However, from most of these studies, because they have used either crude factor and/or receptor preparations, it is difficult to interpret whether activity is associated with the same, related or different proteins. These accessory factors presumably do not function by a heterodimerization mechanism, since the classical steroid hormone receptors bind efficiently to DNA as homodimers [39, 42–44]. In order to determine whether HMG-1 has the ability to enhance the DNA binding of other steroid hormone receptors will

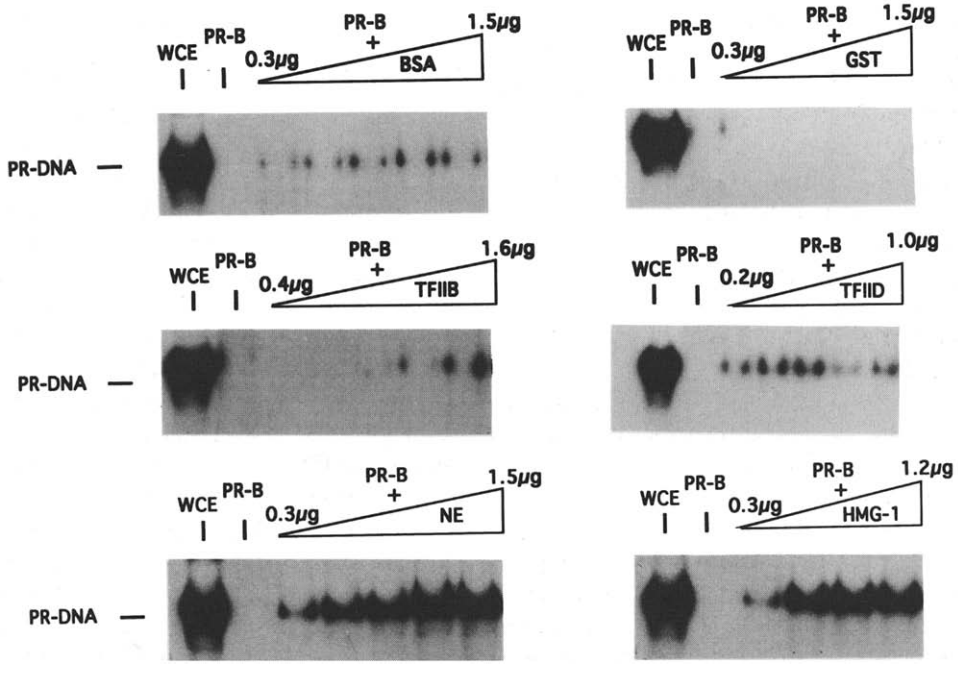

Fig. 6. HMG-1 enhanced the binding of purified PR-B to PREs. PR-B expressed in Sf9 cells and bound to hormone (R5020) *in vivo* was prepared either as a WCE or was affinity purified. Equal amounts (25 ng) of PR-B in WCE or in purified form were assayed by gel shift assay against 0.3 ng of an imperfect palindromic PRE oligonucleotide derived from MMTV. Purified PR-B was assayed alone (PR-B) or after addition of increasing amounts of *E. coli* extract, BSA, purified TFIID, purified TFIIB, nuclear extracts from MDA-231 cells or purified HMG-1 from calf thymus. The amounts in μg of protein added to purified recombinant PR-B are indicated in the figure. Each panel shows only the region of the mobility shift gel that contains the DNA–receptor complex (adapted from Ref. [22]).

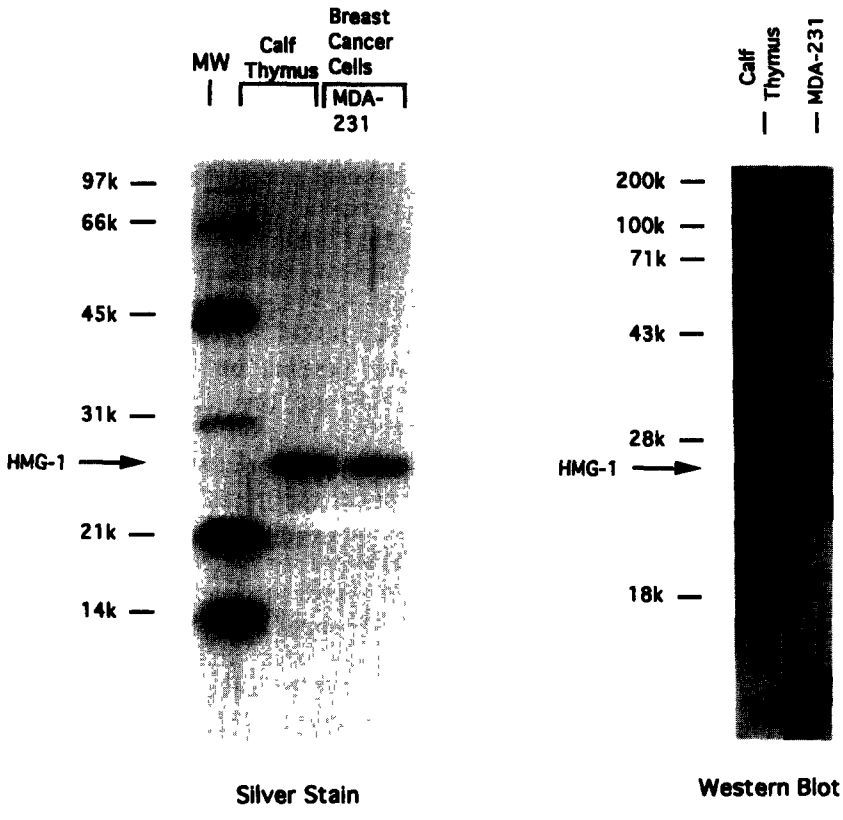

Fig. 7. Purification of high mobility group protein HMG-1. HMG-1 was purified from calf thymus and from MDA-231 breast cancer cells by fractionation on a polybuffer-resin (PBE94). The PBE94 column was used under anion exchange chromatography conditions. Bound proteins were eluted from the column by a linear 0–1.5 M NaCl gradient. HMG-1 that elutes at about 0.6 M NaCl was dialyzed and rechromatographed in the same manner on a second PBE94 column. The most purified fraction was analyzed by 12.5% SDS–PAGE and silver staining and by Western blot with a rabbit polyclonal antibody raised to calf thymus HMG-1. ^{125}I-donkey anti-rabbit IgG secondary antibody and autoradiography was used as the Western blot detection method (adapted from Ref. [22]).

require studies similar to those described here, with both purified HMG-1 and various other purified receptors.

Although our studies with HMG-1 have been conducted *in vitro*, physiological relevance is suggested by several observations. For instance, enhancement of PRE binding is not confined to recombinant PR and gel-shift as an assay method. As shown here enhancement was observed by gel-shift assay both with endogenous PR from T47D cells and recombinant PR. In earlier studies, enhancement of PRE binding by nuclear extracts was also detected by an immunoprecipitation assay [21] (immunoprecipitation of PR–DNA complexes with PR-specific MAbs). Purified PR alone is in fact able to bind to a PRE to some extent when receptor is added in high enough concentrations (Fig. 8) [22]. The effect of HMG-1 is to increase PR-binding affinity so that receptor–DNA interactions occur efficiently at low PR concentrations. Moreover, enhancement of PRE binding did not require excess HMG-1, but occurred when HMG-1 and PR were present in equal molar amounts. Also, HMG-1 stimulated a greater fold increase of PR binding affinity for an imperfect palindromic PRE, than it did for a synthetic perfect palindromic PRE [22]. In this regard, it is interesting that naturally occurring progesterone responsive genes contain imperfect palindromic response elements [1]. Although the biological effect of HMG-1 on PR function *in vivo* is not known, our data suggest that HMG-1 could modulate the affinity of receptors for specific DNA sites *in vivo* and influence transcriptional activity of PR.

HMG-1 is a sequence-independent DNA binding protein that prefers to bind to distorted DNA structures including single-stranded DNA (presumably a stable secondary structure), bends in DNA, cruciform DNA and DNA adducts [33, 47, 49, 50–53]. Recognition of DNA structure is mediated by a conserved HMG box motif and the HMG box alone is necessary and sufficient for DNA binding. The solution structure of the HMG-B box has been solved by 2-D NMR [50]. It is composed of three α helices arranged in an L shape with the two arms held at an 80° angle to each. This unusual shape has been suggested to be important for recognition of bends or kinks in DNA [50]. Regions of homology to the A and B boxes of HMG-1 have been discovered in several other proteins including, the sequence specific transcription factors SRY and LEF-1 [51, 52]. Interestingly, the HMG boxes of SRY and LEF-1 not only bind to distorted DNA structure but also promote sharp bends in DNA. As shown by cyclization assays,

HMG-1 also promotes DNA bending but does so in a sequence-independent manner [54]. In fact DNA bending may be a general effect of all proteins with HMG box motifs. The functional role of HMG-1 is not well defined. It has been suggested to be involved in replication, DNA repair, recombination and transcription [33, 47, 49–53]. Based on our results with PR, HMG-1 may be a general facilitator of the binding of sequence-specific proteins to DNA.

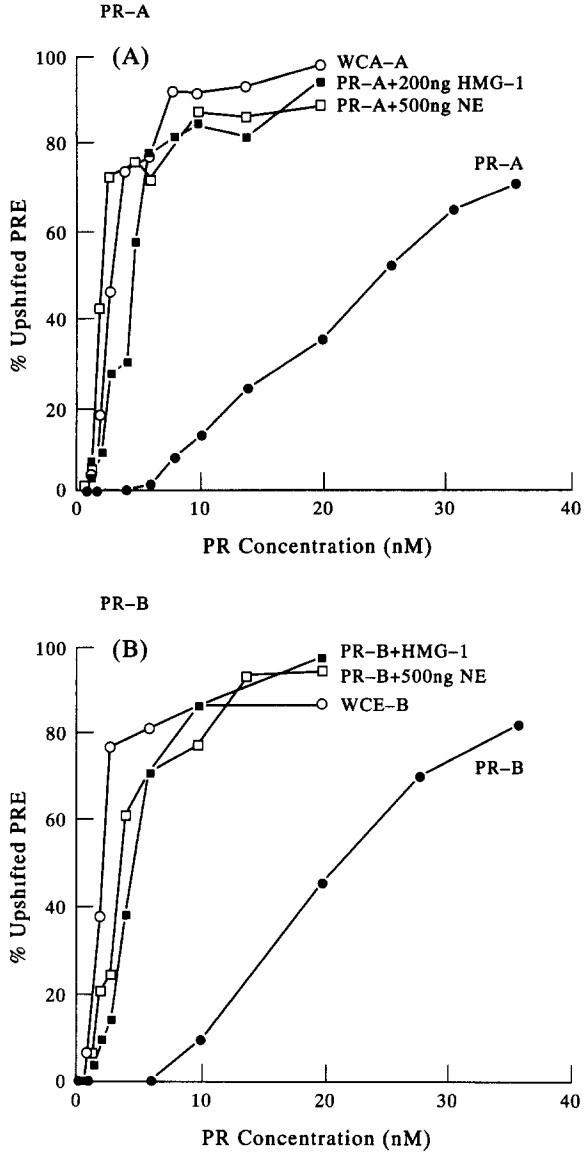

Fig. 8. HMG-1 increased the binding affinity of purified PR-A and PR-B for specific DNA. The PR concentration in gel-shift assays was varied against a constant amount of a ^{32}P-synthetic palindromic PRE oligonucleotide. Recombinant PR-A and PR-B (PR-A upper panel, PR-B lower panel) were assayed in this manner when prepared as a crude WCE or affinity purified. Affinity purified PR was assayed alone (PR-A or PR-B) or after addition of 500 ng of nuclear extract (PR-A + NE, PR-B + NE) or 200 ng of purified HMG-1 (PR-A + HMG-1, PR-B + HMG-1). The mobility shift gels were directly scanned for radioactivity (Phosphorimager, Molecular Dynamics) and the % upshifted PRE complexes were quantitated for each concentration of PR. The midpoint concentration of PR required to bind and upshift 50% of the DNA was taken as an approximation of the binding dissociation constant (reproduced from Ref. [22]).

We propose that high affinity PR binding is dependent upon both nucleotide sequence and protein induced conformational changes in DNA structure. As a working hypothesis receptor alone is predicted to bind with low affinity to PREs to produce a transient distortion in DNA structure. This marks the DNA as a binding site for the sequence-independent structure-specific HMG-1. HMG-1 increases the binding affinity of PR by either stabilizing a dynamic structural conformation in the target PRE that is favored by PR or, by promoting further distortions in DNA structure. HMG-1 interaction appears to be transient or unstable because it is not a component of the final DNA–receptor complex. This model provides an explanation for how a non-specific DNA binding protein like HMG-1 can facilitate binding of a sequence specific protein. It will be important in future studies to determine the nature of the structural changes in DNA that are promoted by the combined actions of PR and HMG-1.

Recent studies on the ability of steroid receptors to bend DNA provide support for this model. The ER when bound to an ERE was reported to bend DNA, however, this was observed with an ER–DNA binding domain fragment and partially purified full-length ER, but not with highly purified full-length ER that failed to bind to EREs at all. This suggests that an accessory factor may be required both for high affinity DNA interaction and for DNA bending [55, 56]. The thyroid hormone receptor has also been shown to bend DNA when bound to its response element and bending was found to be enhanced by nuclear factors of unknown identity [57]. This raises the possibility that HMG-1 or HMG related proteins may act to facilitate DNA bending promoted by steroid receptors and that bending may be requisite for high affinity binding.

An important unanswered question is how members of the GR subfamily (GR, MR, AR, PR) can elicit specific responses to their cognate ligands *in vivo* when each receptor can bind to and enhance transcription from the same consensus glucocorticoid response element (GRE). It has been suggested that cell-specific accessory proteins are involved in specifying responses *in vivo* on the same GRE [1]. Several proteins with a wide range in molecular mass have been discovered to have HMG box motifs and to bind to DNA structures [33, 47, 49–53]. It is possible that some of the proteins of varying size that have been reported to enhance the DNA binding of the classical steroid receptors may be related to HMG-1 by virtue of containing an HMG box motif. In theory other proteins that harbor HMG boxes may be able to functionally substitute for HMG-1. In support of this notion is the fact that enhancement of PRE binding was found to be intrinsic to the HMG-B box (see Ref. [22]). Not all HMG boxes, however, appear to be equivalent in this regard since the HMG boxes of LEF-1 and SRY failed to enhance PR–DNA binding (see Ref. [22]). Perhaps, differential utilization of HMG related proteins as accessory factors plays a role in dictating specificity within the GR

subfamily by promoting alternative structural conformations in the same GRE that will be favored by one receptor.

Acknowledgements—The authors are grateful for the generous contributions of Drs Raymond Reeves and Mark Nissen, Washington State University, Pullman, WA, for providing purified HMG-1 from calf thymus; Dr Jean O. Thomas, Cambridge University, England, for providing HMG box domain peptides; Dr Michael Bustin, National Institutes of Health, Bethesda, MD for rabbit anti-serum to HMG-1; and Drs Ming-Jer Tsai and Bert W O'Malley, Baylor College of Medicine, Houston, TX for recombinant TBP and TFIIB. We also thank Nancy Hart and Clairene Mraz for expert assistance with preparation of the manuscript and Drs Steven K. Nordeen, David Pettijohn and James Wagner, University of Colorado Health Sciences Center, for insightful discussions during the course of these studies. This work was supported in part by U.S. Public Health Service Grants CA46938, Lucille Markey Charitable Trust, and the University of Colorado Cancer Center Core Grant P30CA46934.

REFERENCES

1 Truss M. and Beato M.: Steroid hormone receptors and interaction with deoxyribonucleic acid and transcription factors. *Endocrine Rev.* 14 (1993) 459–479.
2. Gramelli-Piperno A and McHugh P.. Characterization of a protein that regulates the DNA binding activity of NF-AT, the nuclear factor of activated T cells. *Proc. Natn. Acad. Sci. U.S.A.* 88 (1991) 11431–11434.
3 Grueneberg D. A., Natesan S., Alexandre C. and Gilman M Z.: Human and *Drosophila* homeodomain proteins that enhance the DNA-binding activity of serum response factor. *Science* 257 (1992) 1089–1095
4 Abate C., Patel L., Rauscher F. J and Curran T.: Redox regulation of *fos* and *jun* DNA-binding activity *in vitro* *Science* 249 (1990) 1157–1161.
5. Toledano M. B. and Leonard W. J Modulation of transcription factor NF-kB binding activity by oxidation-reduction *in vitro* *Proc. Natn Acad. Sci. U.S.A* 88 (1991) 4328–4332.
6. Thanos D. and Maniatis T : The high mobility group protein HMG 1(Y) is required for NF-kB-dependent virus induction of the human IFN-β gene. *Cell* 71 (1992) 777–789.
7 Xanthoudakis S , Miao G , Wang F., Pan Y.-C E and Curran T.: Redox activation of *fos-jun* DNA binding activity is mediated by a DNA repair enzyme. *EMBO Jl* 11 (1992) 3323–3335.
8. Burnside J , Darling D A and Chin W. W.· A nuclear factor that enhances binding of thyroid hormone receptors to thyroid hormone response elements. *J. Biol. Chem.* 265 (1990) 2500–2504.
9. Darling D. S., Beebe J. S., Burnside J., Winslow E. R. and Chin W W.: 3,5,3′-triiodothyronine (T3) receptor-auxiliary protein (TRAP) binds DNA and forms heterodimers with the T3 receptor. *Molec. Endocr.* 5 (1991) 73–84.
10. Sone T., Ozono K. and Pike J. W. A 55-kilodalton accessory factor facilitates vitamin D receptor DNA binding. *Molec. Endocr.* 5 (1991) 1578–1586.
11 Ross T K , Moss V E., Prahl J. M. and DeLuca H F.: A nuclear protein essential for binding of rat 1,25-dihydroxyvitamin D3 receptor to its response elements. *Proc Natn Acad. Sci U.S.A.* 89 (1992) 256–260
12. Kliewer S. A , Umesono K., Mangelsdorf D. J. and Evans R. M.: Retinoid X receptor interacts with nuclear receptors in retinoic acid, thyroid hormone and vitamin D3 signalling *Nature* 355 (1992) 446–449.
13 Leid M., Kastner P. H., Lyons R., Nakshatri H., Saunders M , Zacharewski T., Chen J.-Y., Staub A., Garnier J.-M., Mader S. and Chambon P. Purification, cloning, and RXR identity of the HeLa cell factor with which RAR or TR heterodimerizes to bind target sequences efficiently. *Cell* 68 (1992) 377–395
14 Yu V C., Delsert C., Andersen B., Holloway J. M., Devary O. V., Naar A. M., Kim S. Y , Boutin J.-M., Glass C. K. and Rosenfeld M. G : RXRB. a coregulator that enhances binding of retinoic acid, thyroid hormone, and vitamin D receptors to their cognate response elements *Cell* 67 (1991) 1251–1266
15. Zhang X-K., Hoffmann B., Tran P. B -V., Graupner G and Pfahl M Retinoid X receptor is an auxiliary protein for thyroid hormone and retinoic acid receptors. *Nature* 355 (1992) 441–445.
16. Cavanaugh A. H. and Simons S. S. Jr: Glucocorticoid receptor binding to calf thymus DNA. 1. Identification and characterization of a macromolecular factor involved in receptor-steroid complex binding to DNA. *Biochemistry* 29 (1990) 989–996
17 Okamoto K., Isohashi F and Ueda K.: Purification and characterization of an adenosine triphosphate-stimulatory factor that enhances the nuclear binding of activated glucocorticoid-receptor complex from rat liver. *Endocrinology* 123 (1988) 2752–2761.
18 Feavers I. M., Jiricny J., Moncharmont B., Saluz H. P. and Jost J P. Interaction of two nonhistone proteins with the estradiol response element of the avian vitellogenin gene modulates the binding of estradiol-receptor complex. *Proc. Natn. Acad Sci. U.S.A.* 84 (1987) 7453–7457
19. Mukherjee R. and Chambon P.: A single-stranded DNA-binding protein promotes the binding of the purified oestrogen receptor to its responsive element. *Nucleic Acids Res.* 18 (1990) 5713–5716
20 Kupfer S. R., Marschke K B , Wilson E M. and French F. S.: Receptor accessory factor enhances specific DNA binding of androgen and glucocorticoid receptors *J. Biol. Chem.* 268 (1993) 17519–17527.
21. Edwards D. P., Kuhnel B , Estes P A. and Nordeen S. K.: Human progesterone receptor binding to mouse mammary tumor virus deoxyribonucleic acid: dependence on hormone and nonreceptor nuclear factor(s). *Molec. Endocr.* 3 (1989) 381–391.
22 Oñate S. A., Prendergast P , Wagner J P , Nisson M., Reeves R., Pettijohn D. and Edwards D P : Identification of the high mobility group protein HMG-1 as an accessory factor that enhances the specific DNA binding activity of progesterone receptor. *Molec. Cell Biol.* In press.
23 Kliewer S. A., Umesono K., Heyman R. A., Mangelsdorf D. J., Dyck J A and Evans R M.: Retinoid X receptor-COUP-TF interactions modulate retinoic acid signaling. *Proc. Natn Acad Sci U S.A.* 89 (1992) 1448–1452
24 Kliewer S. A., Umesono K , Noonan D. J., Heyman R. A and Evans R. M.: Convergence of 9-*cis* retinoic acid and peroxisome proliferator signalling pathways though heterodimer formation of their receptors. *Nature* 358 (1992) 771–774.
25 Heyman R. A , Mangelsdorf D. J., Dyck J. A., Stein R. B., Eichele G., Evans R. M. and Thaller C.: 9-Cis retinoic acid is a high affinity ligand for the retinoid X receptor *Cell* 68 (1992) 397–406.
26. Estes P. A., Suba E. J., Lawler-Heavner J., Elashry-Stowers D., Wei L. L., Toft D. O., Sullivan W. P., Horwitz K. B. and Edwards D. P.: Immunologic analysis of human breast cancer progesterone receptors. 1 Immunoaffinity purification of transformed receptors and production of monoclonal antibodies *Biochemistry* 26 (1987) 6250–6262
27 El-Ashry D., Oñate S A , Nordeen S K and Edwards D P.. Human progesterone receptor complexed with the antagonist RU486 binds to hormone response elements in a structurally altered form *Molec. Endocr* 3 (1989) 1545–1558.
28. Christensen K , Estes P A , Oñate S A , Beck C. A , DeMarzo A , Altmann M., Lieberman B A , St. John J., Nordeen S. K and Edwards D. P.. Characterization and functional properties of the A and B forms of human progesterone receptors synthesized in a baculovirus system. *Molec Endocr.* 5 (1991) 1755–1770
29 Elashry-Stowers D., Zava D T., Speers W. C and Edwards D. P.. Immunocytochemical localization of progesterone receptors in breast cancer with anti-human receptor monoclonal antibodies *Cancer Res* 48 (1988) 6462–6474
30. Bradford M 1976. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principles of protein-dye binding. *Analyt. Biochem.* 72 (1976) 248–254
31 Heukeshoven J. and Dernick R · A simplified method for silver staining of proteins in polyacrylamide gels and the mechanism of silver staining. *Electrophoresis* 6 (1985) 103–112
32. Adachi Y , Mizuno S. and Yoshida M.. Efficient large-scale purification of non-histone chromosomal proteins HMG1 and HMG2 by using polybuffer exchanger PBE94. *J Chromat* 530 (1990) 39–46.
33. Einck L. and Bustin M.· 1985. The intracellular distribution and function of the high mobility group chromosomal proteins. *Exp Cell Res* 156 (1985) 295–310.
34. Oñate S A , Estes P A., Welch W J., Nordeen S. K. and Edwards D P.. Evidence that hsp-70 associated with progesterone receptors is not involved in receptor-DNA binding *Molec Endocr* 5 (1991) 1993–2004
35. Kastner P , Krust A., Turcotte B., Stropp U., Tora L , Gronemeyer H and Chambon P Two distinct estrogen-regulated

promoters generate transcrips encoding the two functionally different human progesterone receptor forms A and B. *EMBO Jl* **9** (1990) 1603–1614.
36. DeMarzo A. M., Oñate S. A., Nordeen S. K. and Edwards D. P.: Effects of the steroid antagonist RU486 on dimerization of the human progesterone receptor. *Biochemstry* **31** (1992) 10491–10501.
37. Bagchi M. K., Tsai S. Y., Tsai M.-J. and O'Malley B. W.: Identification of a functional intermediate in receptor activation in progesterone-dependent cell-free transcription. *Nature* **345** (1990) 547–550.
38. Meyer M. E., Parnon A., Bocquel Ji J., Bocquel M., Chambon P. and Gronemeyer H.: Agonistic and antagonistic activities of RU486 on the functions of human progesterone receptor. *EMBO Jl* **12** (1990) 3923–3932.
39. DeMarzo A. M., Beck C. A., Oñate S. A. and Edwards D. P.: Dimerization of mammalian progesterone receptors occurs in the absence of DNA and is related to the release of 90-kDa heat shock protein *Proc. Natn. Acad. Sci. U.S.A.* **88** (1991) 72–76.
40. Beck C. A., Weigel N. L. and Edwards D P · Effects of hormone and cellular modulators of protein phosphorylation on transcriptional activity, DNA binding, and phosphorylation of human progesterone receptors. *Molec Endocr.* **6** (1992) 607–620.
41. Smith D. F. and Toft D. O.· Steroid receptors and their associated proteins. *Molec. Endocr.* **7** (1993) 4–11.
42. Fawell S. E., Lees J. A , White R. and Parker M. G.. Characterization and colocalization of steroid binding and dimerezation activities in the mouse estrogen receptor. *Cell* **60** (1990) 953–962.
43. Rodriguez R., Weigel N. L., O'Malley B. W. and Schrader W. T.: Dimerization of the chicken progesterone receptor *in vitro* can occur in the absence of hormone and DNA *Molec. Endocr.* **4** (1990) 1782–1790.
44. Kumar V. and Chambon P.: The estrogen receptor binds tightly to its responsive element as a ligand-induced homodimer. *Cell* **55** (1988) 145–156.
45. Nakajima N., Horikoshi M. and Roeder R. G.: Factors involved in specific transcription by mammalian RNA polymerase II: Purification, genetic specificity, and TATA box-promoter interactions of TFIID. *Molec. Cell. Biol.* **8** (1988) 4028–4040.
46. Ha I., Lane W. S. and Reinberg D.: Cloning of a human gene encoding the general transcription initiation factor IIB. *Nature* **352** (1991) 689–695.
47. Bustin M., Lehn D. A. and Landsman D.: Structural features of the HMG chromosomal proteins and their genes. *Biochim. Biophys. Acta* **1049** (1991) 231–243.
48 Ing N. H., Beekman J. M., Tsai S. Y., Tsai M.-J. and O'Malley B. W.: Members of the steroid hormone receptor superfamily interact with TFIIB (S300-II). *J. Biol. Chem.* **267** (1992) 17617–17623.
49. Bianchi M. E., Falciola L., Ferrari J. and Lilley D. M. J.: The DNA binding site of HMG1 protein is composed of two similar segments (HMG boxes), both of which have counterparts in other eukaryotic regulatory proteins. *EMBO Jl* **11** (1992) 1055–1063.
50. Weir H. M., Kraulis P. J , Hill C. S., Raine A. R. C., Lane E. D. and Thomas J. O.: The structure of the HMG box motif in the B-domain of HMG1. *EMBO Jl* **12** (1993) 1311–1319.
51. Giese K., Cox J. and Grosschedl R.: The HMG domain of lymphoid enhancer factor 1 bends DNA and facilitates assembly of functional nucleoprotein structures. *Cell* **69** (1992) 185–195.
52. Ferrari S., Harley V R., Pontiggia A., Goodfellow P. N., Lowell-Badge R. and Bianchi M. E.: SRY, like HMG-1, recognizes sharp angles in DNA. *EMBO Jl* **11** (1992) 4497–4506.
53 Bruhn S. L., Pil P. M., Essignmann J. M., Housman D. E. and Lippard S. J.: Isolation and characterization of human cDNA clones encoding a high mobility group box protein that recognizes structural distortions to DNA caused by binding of the anticancer agent cisplatin. *Proc. Natn. Acad Sci. U.S.A.* **89** (1992) 2307–2311.
54. Paull T. T , HayKinson M. J. and Johnson R. C.: The nonspecific DNA-binding and -bending proteins HMG-1 and HMG-2 promote the assembly of complex nucleoprotein structures *Genes Dev.* **7** (1993) 1521–1534.
55. Nardulll A. M., Greene G. L. and Shapiro D. J.· Human estrogen receptor bound to an estrogen response element bends DNA. *Molec. Endocr* **7** (1993) 331–340.
56. Nardulli A. M. and Shapiro D. J.. Binding of the estrogen receptor DNA-binding domain to the estrogen response element induces DNA bending. *Molec. Cell. Biol.* **12** (1992) 2037–2042.
57 King I. N., deSoyza T., Catanzaro D. F. and Lavin T. N · Thyroid hormone receptor-induced bending of specific DNA sequences is modified by an accessory factor. *J. Biol. Chem.* **268** (1992) 495–501.