MOLECULAR AND CELLULAR BIOLOGY, May 1994, p. 3376–3391
0270-7306/94/$04.00+0

Vol. 14, No. 5

The DNA-Bending Protein HMG-1 Enhances Progesterone Receptor Binding to Its Target DNA Sequences

SERGIO A. OÑATE,[1] PAUL PRENDERGAST,[1] JAMES P. WAGNER,[2] MARK NISSEN,[3] RAYMOND REEVES,[3] DAVID E. PETTIJOHN,[2] AND DEAN P. EDWARDS[1]*

Departments of Pathology[1] and Biochemistry, Biophysics and Genetics,[2] University of Colorado Cancer Center, Denver, Colorado 80262, and Department of Biochemistry/Biophysics, Washington State University, Pullman, Washington 99164[3]

Received 19 October 1993/Returned for modification 15 December 1993/Accepted 8 February 1994

Steroid hormone receptors are ligand-dependent transcriptional activators that exert their effects by binding as dimers to *cis*-acting DNA sequences termed hormone response elements. When human progesterone receptor (PR), expressed as a full-length protein in a baculovirus system, was purified to homogeneity, it retained its ability to bind hormonal ligand and to dimerize but exhibited a dramatic loss in DNA binding activity for specific progesterone response elements (PREs). Addition of nuclear extracts from several cellular sources restored DNA binding activity, suggesting that PR requires a ubiquitous accessory protein for efficient interaction with specific DNA sequences. Here we have demonstrated that the high-mobility-group chromatin protein HMG-1, as a highly purified protein, dramatically enhanced binding of purified PR to PREs in gel mobility shift assays. This effect appeared to be highly selective for HMG-1, since a number of other nonspecific proteins failed to enhance PRE binding. Moreover, HMG-1 was effective when added in stoichiometric amounts with receptor, and it was capable of enhancing the DNA binding of both the A and B amino-terminal variants of PR. The presence of HMG-1 measurably increased the binding affinity of purified PR by 10-fold when a synthetic palindromic PRE was the target DNA. The increase in binding affinity for a partial palindromic PRE present in natural target genes was greater than 10-fold. Coimmunoprecipitation assays using anti-PR or anti-HMG-1 antibodies demonstrated that both PR and HMG-1 are present in the enhanced complex with PRE. HMG-1 protein has two conserved DNA binding domains (A and B), which recognize DNA structure rather than specific sequences. The A- or B-box domain expressed and purified from *Escherichia coli* independently stimulated the binding of PR to PRE, and the B box was able to functionally substitute for HMG-1 in enhancing PR binding. DNA ligase-mediated ring closure assays demonstrated that both the A and B binding domains mediate DNA flexure. It was also demonstrated in competition binding studies that the intact HMG-1 protein binds to tightly curved covalently closed or relaxed DNA sequences in preference to the same sequence in linear form. The finding that enhanced PRE binding was intrinsic to the HMG-1 box, combined with the demonstration that HMG-1 or its DNA binding boxes can flex DNA, suggests that HMG-1 facilitates the binding of PR by inducing a structural change in the target DNA.

The classical intracellular receptors for sex steroids and glucocorticoids/mineralocorticoids bind to *cis*-acting DNA sequences known as hormone response elements (HREs) which consist of conserved inverted repeat hexanucleotide motifs separated by a 3-bp spacer (70). Steroid hormone receptors bind with maximal efficiency to HREs as homodimers, with each subunit contacting an HRE half site (14, 16, 21, 41). As a subgroup of the steroid receptor superfamily, the receptors for thyroid hormone (TR), vitamin D_3 (VDR), and retinoic acid (RAR) are less constrained both by the architecture of their DNA recognition sequences and by the form of receptor that can bind to DNA. In addition to palindromic arrays, recognition motifs for TR, VDR, and RAR can be arranged as direct tandem repeats, or clusters of half sites with variable spacing between canonical binding sites (71). Additionally, this subgroup of receptors can bind as either monomers (TR), homodimers, or heterodimers (27, 71, 73). A general finding from a number of studies is that homodimers of TR, VDR, and RAR bind poorly in vitro to specific DNA elements and that other nuclear proteins are required for efficient binding. This was first shown for TR and a protein termed TRAP (TR accessory protein). TRAP enhances TR binding to thyroid response elements by heterodimerization with TR such that both TR and TRAP are components of the DNA complex (5, 9, 13, 51). As in studies with TR, efficient binding of VDR to vitamin D response elements requires addition of a 55-kDa nuclear protein that directly participates in the DNA complex (47, 62, 64), and binding of RAR to retinoic acid response elements is enhanced by other eukaryotic nuclear proteins (76). Subsequent studies have identified the former orphan nuclear receptor, retinoic acid X receptor (RXR), as an accessory protein that can heterodimerize with TR, VDR, and RAR to enhance the binding of each of these receptors to their cognate direct repeat elements (39, 44, 78, 79). A stereoisomer of all-*trans* retinoic acid, 9-*cis* retinoic acid, has now been identified as the endogenous ligand for RXR (32). More recently, RXR has been shown to also heterodimerize with two orphan members of the steroid receptor superfamily, the peroxisome proliferator activator receptor (PPAR) and COUP-TF (38, 40). RXR does not appear to heterodimerize with the classical steroid hormone receptors (39, 79). As a result of these studies, it has been proposed that RXR is a coregulator for a subgroup of the nuclear receptors (TR, VDR, RAR, PPAR, COUP-TF), functioning to direct each receptor to its appropriate DNA targets. Whether proteins

* Corresponding author. Mailing address: Department of Pathology (B216), University of Colorado Health Sciences Center, 4200 East Ninth Ave., Denver, CO 80262. Phone: (303) 270-5416. Fax: (303) 270-6721.

other than RXRs can also enhance DNA binding of TR, VDR, and RAR remains to be determined.

Nuclear proteins that enhance the DNA binding of the steroid hormone class of receptors have also been described. This observation was first reported for estrogen receptor (ER) binding to the estrogen response element (ERE) of the vitellogenin gene. Two nonhistone chromosomal proteins that can bind to the spacer of palindromic EREs were shown to enhance ER binding (22). It has also been shown that the in vitro binding of highly purified human ER to an ERE requires the addition of a single-stranded-DNA-binding protein (50). The finding of a low-molecular-weight polypeptide that increases binding to DNA-cellulose (11) plus the identification of a 93-kDa protein that increases binding of activated glucocorticoid receptor (GR) to nuclei and chromatin in an ATP-dependent manner (55) provides further support for the presence of accessory proteins for enhancement of DNA binding. Whether there is a common accessory factor or different factors involved with each steroid hormone receptor is not known. The identification of the protein(s) involved and elucidation of their mechanism of action also remain to be resolved. These accessory proteins presumably do not function by a heterodimerization mechanism, since the steroid hormone receptors bind efficiently as homodimers.

It was shown earlier by one of our laboratories that the human progesterone receptor (PR) is dependent upon hormone and a nonreceptor nuclear factor for maximal binding to progesterone response elements (PREs) in vitro (17). In the present study, we have identified the high-mobility-group chromatin protein HMG-1 as the major activity responsible for enhancing PR binding to PREs in vitro. Evidence supporting the concept that HMG-1 enhancement occurs by modifying the structure of the target DNA is presented.

MATERIALS AND METHODS

Cell culture, baculovirus expression, and receptor preparation. PR-rich T47D human breast cancer cells and PR-negative MDA231 cells were cultured as previously described (19, 20). *Spodoptera frugiperda* (Sf9) insect cells were grown at 27°C in Grace's insect medium (GIBCO) supplemented with 3.3 g of yeastolate (GIBCO) per liter, 3.3 g of lactalbumin hydrolysate (GIBCO) per liter, 25 μg of gentamicin (Irvine) per ml, and 10% fetal bovine serum (HyClone). For infection with recombinant baculovirus vectors, Sf9 cells were plated at a density of 10^6 cells per 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 isoform of human PR and their functional properties have been previously detailed (12).

Recombinant receptors expressed from baculovirus were prepared as whole-cell extracts (WCEs) 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) containing a cocktail of protease inhibitors (20) 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 a 100 nM concentration 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 previously described (12, 19, 20).

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 cross-linking MAb B-30 or AB-52 to protein G-Sepharose (Pharmacia-LKB), using 10 mM dimethylpimelimidate by methods described previously (20). MAbs were purified from ascites fluids and coupled to protein G-Sepharose at a concentration of 4 to 6 mg/ml. MAb B-30, which recognizes the B isoform of PR, was used to purify PR-B. MAb AB-52, which recognizes both the A and B isoforms, was used to purify PR-A (20). Approximately 300 × 10^6 Sf9 insect cells were used for each receptor purification, and 10 ml of WCEs was prepared as described above. To monitor 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 were reduced by dialysis against TEDG. Dialyzed WCEs were 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 1,500 rpm for 5 min and washed by resuspension and centrifugation in 15 ml of TEG (TEDG minus DTT). This was followed by three 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 microcentrifuge 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 composed 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 four to six times, or until the majority of PR-[^{3}H]R5020 was released from the affinity resins, and the separate eluates were pooled. The majority of hormone (60%) 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 approximately 1 to 2 months if not repeatedly freeze-thawed.

Gel electrophoresis and Western blotting (immunoblotting). Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and Western blot analyses of PR were carried out as previously described (19, 20) except that peroxidase conjugated anti-mouse immunoglobulin G and an enzyme-linked immunosorbent assay were used as the detection method instead of radioactive protein A and autoradiography. SDS-polyacrylamide gels were silver stained 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. (2). 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 to 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 antiserum to HMG-1. Western blotting of HMG-1 was performed as for

PR except that detection was by autoradiography using an ^{125}I-labeled donkey anti-rabbit immunoglobulin G (11 μCi/μg; Amersham). A rabbit antiserum to HMG-1 (18) was diluted 1:150 in Western blot dilution buffer, and the ^{125}I-labeled secondary antibody was diluted 1:2,000 (≈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 previously described, with some minor modifications (19). 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 (twofold) in TEDG to generate a series of PR concentrations ranging from 2,500 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 Sf9 WCEs were diluted 1:100. Whatman filter paper (0.36-mm pore size) and nitrocellulose were equilibrated for 30 min at 25°C (or room temperature) in TEDG and then placed in a Bio-Rad E6-well dot blot manifold. Samples in 300-μl volumes were applied under vacuum to each well of the manifold, and each well was washed once under vacuum with 300 μl of TEDG. The nitrocellulose was then removed, blocked with 1% bovine serum albumin (BSA)–0.1% Tween 20, incubated with 10 μg of MAb AB-52 per ml, and processed in the same manner as for Western blot assays. Nitrocellulose filters were incubated with [^{35}S]protein A (at 50,000 cpm/ml; Amersham), 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, Sunnyvale, Calif.).

Receptor dimerization assay. To detect solution dimerization between truncated PR-A and full-length PR-B, a coimmunoprecipitation assay was used as previously described (14). Purified PR-A and PR-B prepared separately from R5020-treated Sf9 cells were mixed in vitro and then immunoprecipitated with the PR-B-specific MAb B-30 (the MAb was covalently coupled to Affi-Gel 10 at a ratio of 5 to 6 mg of antibody per ml of gel suspension). Receptors were incubated with MAb-coupled resins on an end-over-end rotator for 4 h at 4°C in the presence of gelatin as carrier protein. To determine the level of nonspecific binding, parallel immunoprecipitations were performed by incubation with blank Affi-Gel 10 resins. After binding, the resins were washed with TEG followed by three washes with TEG containing 30 mM NaCl as previously described (14). Immobilized PR complexes were then extracted with SDS buffer and submitted to Western blot assay with AB-52, which is specific to both PR-A and PR-B (20). The presence of PR-A indicates the formation of a PR-A/PR-B heterocomplex.

Gel mobility shift assay for PR. DNA binding by electrophoretic mobility shift assay was performed as described previously, with minor modifications (14, 19, 56). PR (in femtomoles or nanograms, as indicated in figure legends) in WCEs or in purified form was incubated for 1 h at 4°C with 0.3 ng of ^{32}P-end-labeled PRE oligonucleotide (specific activity, 20,000 to 30,000 cpm/0.1 ng of DNA). The DNA binding reaction mixture also contained poly(dA-dT) · poly(dA-dT) as nonspecific 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. It should be noted that the competitor DNA was used in the range of 40 to 80 ng per assay, which is considerably reduced from the standard 1 μg per assay (14, 19, 56). In theory, purified PR should not require competitor DNA to reduce binding of nonspecific proteins to the labeled probe. However, we found that samples tended to aggregate at the top of the gel without inclusion of small amounts of nonspecific DNA. The DNA binding reactions (25 μl) were subjected to electrophoresis and autoradiography as previously described (14, 19, 56). 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-labeled PRE and PRE complexes was carried out by direct scanning of dried gels for radioactivity, using a series 400 Molecular Dynamics PhosphorImager.

Immunoprecipitation of PR-DNA complexes. Coimmunoprecipitation of a ^{32}P-labeled PRE probe in the presence of PR was performed as previously described, with minor modifications (17). Purified PR-B (6.5 nM, final concentration) was incubated for 1 h at 4°C with a ^{32}P-labeled 145-bp DNA fragment (1 ng) from mouse mammary tumor virus (MMTV) containing the promoter-distal PRE (17). The fragment was end labeled by Klenow enzyme fill-in to a specific activity of 196,000 cpm/ng of DNA. The DNA binding reaction mixture was identical to that used for the gel mobility shift assay except that the volume of the reaction was increased to 50 μl. The PR-specific MAb AB-52, an HMG-1 serum antibody, and an unrelated control antibody (rabbit anti-mouse immunoglobulin G) were each immobilized to protein G-Sepharose (Pharmacia) at a ratio of 10 μg/100 μl of resin. After 2 h of incubation of PR with the labeled DNA probe, a 25-μl suspension of antibody-bound protein G-Sepharose was added, and samples were incubated for another 2 h at 4°C with agitation to maintain the resins in suspension. The protein G-Sepharose was then washed six times in 1 ml of TEG_{N60} buffer (10 mM Tris [pH 7.4], 1 mM EDTA, 10% glycerol, 60 mM NaCl) by centrifugation and discarding of the supernatant. Labeled DNA probe that remained bound to the beads was then eluted and electrophoresed on 12% polyacrylamide gels. The gels were dried under a vacuum and exposed to X-ray film.

PRE and PR affinity columns. A concatemerized PRE oligonucleotide was covalently coupled to cyanogen bromide-activated Sepharose (Pharmacia). A monomer 32-bp double-stranded PRE oligonucleotide corresponding to the distal HRE of MMTV was randomly ligated, generating a product consisting predominantly of decamers. The efficiency of coupling of the decamers to Sepharose, determined by inclusion of a tracer end-labeled PRE, was estimated to be 15 μg of DNA per ml of resin.

To construct a PR affinity column, baculovirus PR-B was immunoaffinity purified and chemically cross-linked to receptor-specific MAb AB-52, which in turn was immobilized to protein G-Sepharose (Pharmacia). Cross-linking was performed with 20 mM dimethylpimelimidate (Pierce) as described previously (20). The efficiency of coupling to protein G-Sepharose was estimated to be 5 to 6 mg of AB-52 and 1 mg of PR-B per ml of resin.

Ring closure assays. An assay for T4 DNA ligase-dependent cyclization of DNA fragments was performed as previously developed, with some modifications (33, 66). DNA fragments of various sizes ranging from 40 to 366 bp and terminated with cohesive ends were prepared as previously described (33). The 144-bp *Xho*I fragment was excised from plasmid pBendHRE-1 (a gift of Ben Lieberman, University of Colorado Health Sciences Center). All fragments were electrophoretically purified after digestion of vector DNA with the appropriate restriction enzyme, and DNA concentrations were determined spectrophotometrically. Fragments were end labeled with ^{32}P essentially as previously described (7) except that they were phenol-ether extracted and ethanol precipitated after treatment with alkaline phosphatase (not heated). It is not certain

that all 5′ phosphate groups were restored during the end-labeling procedure, which may explain the observation that after ring closure, many fragments retain a single-strand interruption.

The standard ring closure reaction mixture contained 10 ng of linear restriction fragment DNA (10% ^{32}P labeled) with cohesive ends and was preincubated at 30°C for 30 min with and without protein in 10 μl of ligase buffer (50 mM *N*-1-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.8], 50 mM potassium glutamate, 20 mM DTT, 10 mM $MgCl_2$, 1 mM ATP, 20 ng of BSA per μl, 5% glycerol). Forty cohesive end units (1 μl of T4 DNA ligase [40 U/μl; New England Biolabs]) was added, and ligation was allowed to proceed for 30 min at 30°C. Dilution of ligase and proteins from high-concentration stocks was made immediately before use in ligase dilution buffer (10 mM HEPES [pH 7.4], 50 mM potassium glutamate, 0.1 mM EDTA, 15 mM DTT, 200 ng of BSA per μl, 50% glycerol). Reactions were stopped by the addition of 5 μl of stop/loading solution (5 mM EDTA, 1% SDS, 25% glycerol, 1 mg of bromophenol blue per ml), and samples were electrophoresed on 3-mm-thick composite agarose gels containing 4 to 6% NuSieve agarose (FMC BioProducts)–1% electrophoresis-grade agarose (Sigma) in TAE buffer (40 mM Tris-HCl [pH 7.2], 200 mM sodium acetate, 1 mM EDTA). Electrophoresis for 3 to 4 h at 100 mA was required to separate monomer circles from linear DNA. Gels were dried for 1 h at 80°C and autoradiographed or analyzed on a PhosphorImager (Molecular Dynamics series 400). In the latter case, quantitative analysis of DNA bands was done with the ImageQuant program. Some monomer circle products of the cyclization assays were gel purified from ethidium-stained acrylamide gels for use in binding competition experiments (see Fig. 9).

Gel mobility shift assay for HMG-1. The procedure used conditions optimized to detect protein-DNA interactions that have rapid dissociation rates (25). The DNA binding buffer contained 10 mM Tris (pH 7.5), 50 mM potassium glutamate, 35 mM DTT, 2 mM $MgCl_2$, 5 ng of poly(dA-dT) · poly(dA-dT) per μl, 50 ng of gelatin per μl, and 5% glycerol. Binding and electrophoresis (in TAE buffer) were done at 4°C.

RESULTS

Purified PR in vitro requires a ubiquitous protein for efficient binding to specific DNA. We have shown previously that human PR requires ligand for binding to specific response element DNA in vitro (14, 17, 56). In the course of these studies, we also observed that receptors bound to hormone in vivo (intact cells) and translocated to the tight nuclear-bound form prior to extraction exhibited higher DNA binding activity than salt-activated cytosol receptors bound to hormone in vitro (17, 56). The higher activity of PR bound to ligand in vivo appeared to be due in part to an accessory nuclear factor, since addition of nuclear extracts (lacking PR) to cytosol receptors stimulated specific PRE binding by severalfold (17).

To further characterize the nuclear factor responsible for enhancing specific DNA binding of PR, we have purified human PR to 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. In human cells, PR is expressed as two amino-terminal variants of the same gene, termed PR-A (94 kDa) and PR-B (118 to 120 kDa). PR-A is a truncated form missing 164 amino-terminal residues and arises from alternative use of a second promoter (36). Both isoforms were expressed separately from baculovirus vectors as intact full-length proteins, and they have been shown previously to be structurally and functionally indistinguishable from their endogenous counterparts (12). The recombinant PRs bind hormone with the same affinity and steroid specificity as endogenous PR, and when present in WCEs, they also bind with high affinity to PREs in a hormone-dependent manner (12).

Each PR isoform was purified by MAb affinity chromatography from WCEs of baculovirus-infected Sf9 insect cells. Cells were incubated for 4 h with the synthetic progestin R5020 in order to bind receptors to hormone in vivo. PR was eluted from the MAb affinity resins 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 the silver-stained SDS-polyacrylamide gel in Fig. 1A, WCEs contain high levels of soluble PR-A or PR-B, detectable as major stainable bands of 94 and 118 to 120 kDa, respectively. The affinity-purified receptors are essentially homogeneous (>95% apparent purity). The Western blot in Fig. 1B confirms the identity of the silver-stained bands in WCEs and after purification as authentic PR-A and PR-B proteins. The band heterogeneity of PR-B on SDS-gels that is evident in Fig. 1 is likely due to phosphorylation (12). 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 (63). Thus, 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. In the WCEs shown in Fig. 1, values of 202 and 348 pmol/mg of protein were estimated by dot blot immunoassay for PR-A and PR-B, respectively. At this level, PR represents approximately 2 to 3% of total protein in WCEs, which is consistent with the relative intensity of the PR bands in WCEs on silver-stained SDS-gels (Fig. 1). By saturation steroid binding analysis, approximately 80% of PR protein (as estimated by dot blot immunoassay) in WCEs was accounted for as having steroid binding activity (data not shown). As found previously when we purified endogenous T47D PR (20), alkaline elution of PR from antibody affinity resins is a relatively mild condition that retains the majority (60%) of the steroid originally bound to the native receptor (not shown). It should be noted that expression levels of PR in the present study are much higher (>10-fold) than the originally reported levels, which ranged between 0.1 and 0.2% of protein for expression of PR from these same baculovirus vectors (12). The higher levels of expression are due in part to inclusion of hormone which upregulates PR in Sf9 cells and to performing viral infection of insect cells as suspension rather than as attachment cultures (12).

PRs in WCEs and after purification were also analyzed for the ability to bind to specific DNA by gel mobility shift assay. For the sake of presentation, most of the results shown in this report are for assays done with PR-B. Essentially identical results were obtained with PR-A (Fig. 4 and 6). As shown in Fig. 2A (lane 2), hormone (R5020)-activated PR-B present in WCEs bound efficiently to an end-labeled oligonucleotide containing an imperfect palindromic PRE from the MMTV promoter and produced a substantial amount of reduced-mobility DNA complexes. By contrast, purified PR-B exhibited no DNA binding under the conditions of this gel shift assay

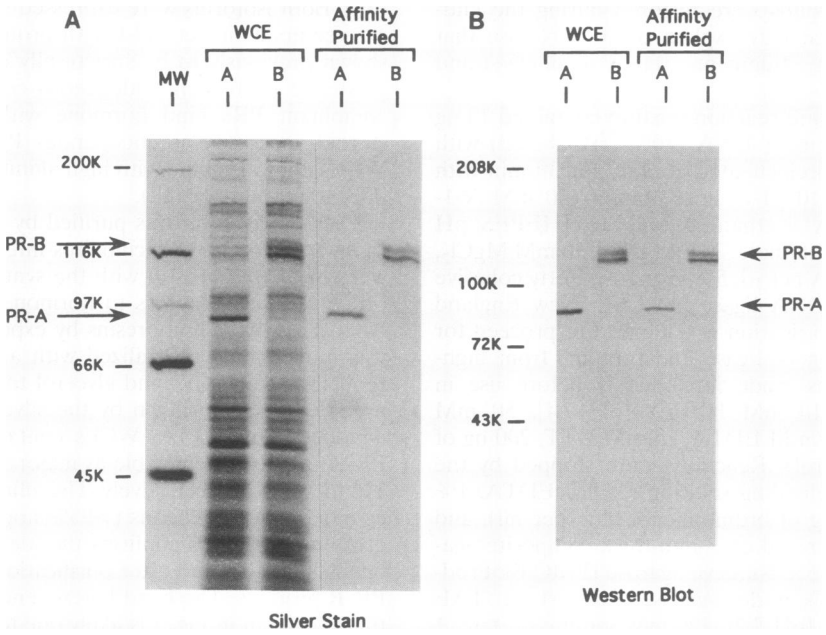

FIG. 1. Purification of recombinant PR-A and PR-B by MAb affinity chromatography. WCEs were prepared from Sf9 insect cells after infection with recombinant baculovirus vectors expressing either the A or B isoform of human PR. To bind receptor to hormone in vivo, cells were incubated with R5020 for 4 h, just prior to harvest. Receptors were purified from WCEs by MAb affinity chromatography, using B-30 for purification of PR-B and AB-52 for purification of PR-A. WCEs and purified products were analyzed by silver-stained SDS-gels (A) and by Western blotting with AB-52 (B). MW, molecular weight standards.

(Fig. 2A, 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 used to ensure that a difference in DNA binding activity was not due to variation in the amount of added receptor. Rather remarkably, the DNA binding activity of purified PR-B was nearly restored to that of native PR by addition of 0.45 M NaCl nuclear extracts that lacked PR (Fig. 2A, lanes 4 to 8). Moreover, stimulation of DNA binding was dependent on the amount of nuclear extract (in micrograms of total protein) added and appeared to be saturable (Fig. 2A, lanes 3 to 8). The supershift induced by addition of the receptor-specific MAb, B-30, demonstrated that the reconstituted DNA complex contained PR (Fig. 2A, lane 9). As shown in lanes 10 to 15 of Fig. 2A, the nuclear extract alone over the same range of added protein did not induce any detectable PR-DNA complexes. Nuclear extracts, however, produce other DNA-protein complexes of faster mobility than specific DNA-PR complexes. The pattern of these complexes was the same in the presence and absence of purified PR-B (Fig. 2A; compare left and right panels), and they were not supershifted by receptor specific antibodies. These results indicate that an accessory nuclear factor is required for efficient in vitro binding of PR to specific target DNA.

Nuclear extracts prepared from several different eukaryotic cells stimulated the DNA binding activity of purified PR-B, including PR-positive T47D breast cancer cells (PR was immunodepleted), PR-negative MDA-231 breast cancer cells, and Sf9 insect cells. Activity was also present in rabbit reticulocyte lysates, but little or no activity was detectable in *Escherichia coli* extracts (data not shown). This finding suggests that the PRE stimulatory activity is associated with a ubiquitous eukaryotic factor. 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 cutoffs in the range of 12,000 to 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 PR, we examined the ability of various proteins and other agents to enhance the DNA binding activity of purified PR. Addition of ovalbumin, insulin, and RNase, in amounts equal to that of the total protein of nuclear extracts, did not stimulate PRE binding (Fig. 2B). There are reports that high concentrations of nonspecific carrier proteins such as BSA (80) and of positively charged peptides (4) can enhance DNA binding of several eukaryotic transcription factors as detected by gel shift assay. However, we found that BSA as well as another commonly used carrier, gelatin, had minimal or no effect. Positively charged (poly-L-lysine), negatively charged (poly-L-aspartic acid), and neutral (poly-L-leucine) polyamino acids and histone 1 also failed to enhance DNA binding activity of purified PR (data not shown). It should be noted that we occasionally observed a slight enhancement of PRE binding by addition of high concentrations (0.5 to 1 mg/ml) of BSA. The level of enhancement, however, was only a fraction of that obtained with nuclear extracts at much lower protein concentrations. Nucleotides have been shown to enhance the sequence-specific binding of the transcription factor NF-κB (45), and the DNA binding of both NF-κB and c-Jun has been reported to be modulated by the oxidation-reduction state of the protein (1, 68, 75), which is mediated by a protein termed Ref-1 (75). Addition of ATP, GTP, or excess reducing agents (β-mercaptoethanol or DTT) also had no effect on PRE binding by purified PR (Fig. 2B). These results indicate that a specific protein(s) present in nuclear extracts is responsible for enhancement of the DNA binding activity of purified PR.

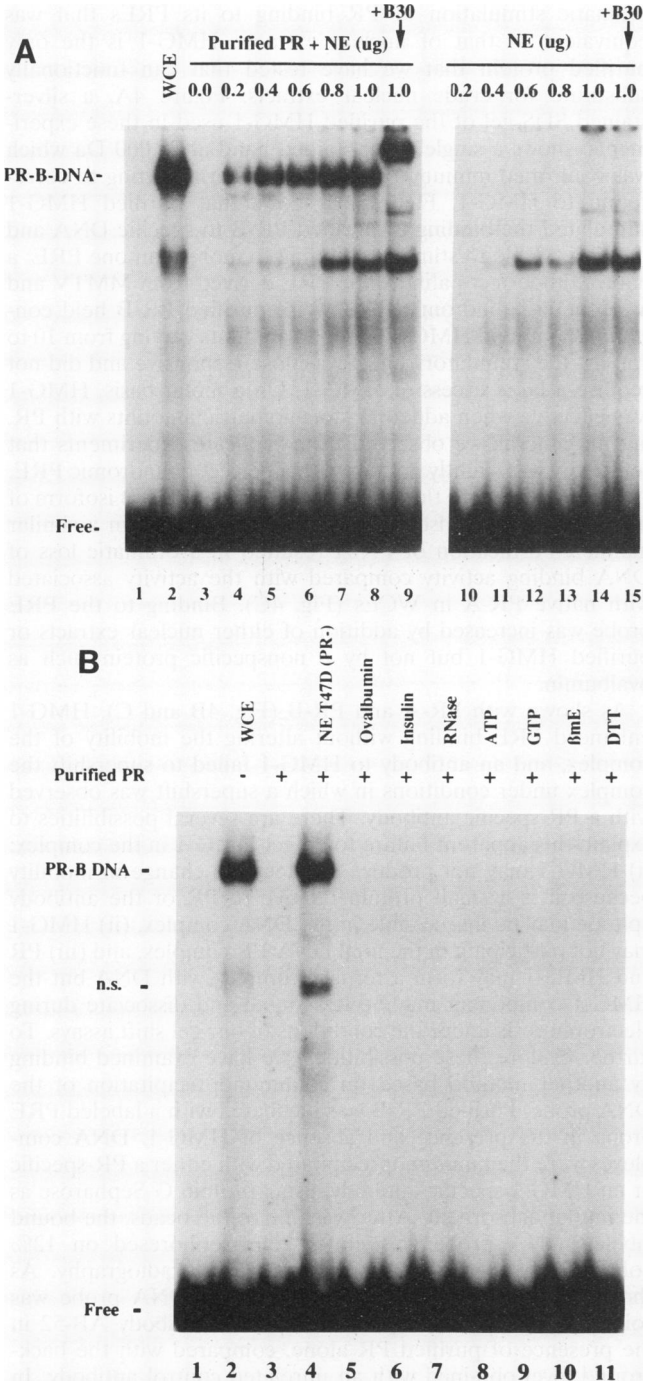

FIG. 2. Nuclear extracts contain a specific activity that enhances DNA binding by purified PR. (A) DNA binding activity of PR-B in WCEs and after affinity purification. Equal amounts (5 nM or 125 fmol per assay) of PR-B in WCE (lane 2) or affinity-purified PR-B (lane 3) were analyzed by gel shift assay using an end-labeled ^{32}P-labeled PRE oligonucleotide (14 fmol) as target DNA. The PRE corresponds to the imperfect palindromic PRE/GRE from MMTV. In lanes 4 to 8, increasing amounts of nuclear extract (in micrograms of total protein) were added to purified PR-B. The nuclear extracts were prepared from non-hormone-treated T47D cells, and any trace PR was immunodepleted as described previously (17). In lane 9, the PR-B-specific MAb B-30 was mixed with purified PR-B and nuclear extracts. Lanes 10 to 15 are immunodepleted nuclear extracts alone added in increasing amounts (micrograms of total protein). DNA-PR-B complexes and free DNA are indicated by arrows. Lane 1 is free DNA with no added protein. (B) The PRE stimulatory activity present in nuclear extracts is not mimicked by general carrier proteins, reducing agents, or nucleotides. Equal amounts (5 nM) of PR-B in WCEs (lane 2) or affinity-purified PR-B (lane 3) were analyzed by gel shift assay as described above. In lanes 4 to 11, various proteins and other factors were added to purified PR-B. These included a PR immunodepleted nuclear extract from T47D cells (lane 4), ovalbumin (lane 5), insulin (lane 6), and RNase (lane 7), each at 1 μg of total protein. Also added were 10 mM each ATP (lane 8), GTP (lane 9), β-mercaptoethanol (βmE) (lane 10), and DTT (lane 11). The mobilities of free DNA, nonspecific (n.s.) complexes, and specific DNA–PR-B complexes are indicated. Lane 1 is free DNA with no added protein.

Nuclear accessory proteins do not affect steroid binding activity or solution dimerization of PR in the absence of DNA. Human PR has been documented to require hormone for induction of specific DNA binding in vitro (3, 14, 17, 56). We questioned, therefore, whether purification may have damaged steroid binding activity such that nuclear extracts might enhance DNA binding indirectly by affecting the steroid binding activity of purified PR. However, addition of nuclear extracts did not increase the steroid binding capacity of purified PR when excess ligand was added (not shown). Nor did addition of excess ligand alone increase DNA binding of purified PR, suggesting that stripping of some hormone is not a likely explanation for the dramatic reduction of DNA binding activity that occurs during purification. We have also purified the unliganded receptor, and nuclear extracts did not stimulate PRE binding; enhancement was observed only with purified liganded PR (not shown). These results indicate that PR requires both hormone and an accessory nuclear protein for efficient binding in vitro to specific DNA.

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 (14, 15, 21, 61). To investigate the possibility that the nuclear accessory protein(s) enhances DNA binding indirectly by increasing PR dimerization, we have used a coimmunoprecipitation assay described previously that detects dimerization between truncated PR-A and full-length PR-B (14). The two PR isoforms were expressed separately in Sf9 insect cells, R5020 was added to cells at the end of the infection, and PR-A and PR-B were each purified by MAb affinity chromatography as in Fig. 1. Various 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 MAb B-30, which recognizes an epitope present in the amino terminus unique to PR-B (20). The immunoprecipitates were then analyzed by Western blotting with the A- and B-specific MAb, AB-52 (20). Coimmunoprecipitation of PR-A with PR-B is indicative of solution dimerization between the A and B receptors. As shown in Fig. 3, 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. Thus, stimulation of PR-DNA binding activity by the nuclear factor(s) does not appear to occur by increasing receptor dimerization.

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 the ability to enhance the binding of purified PR-B to specific PREs by gel shift assay. The majority of activity was retained by heparin-

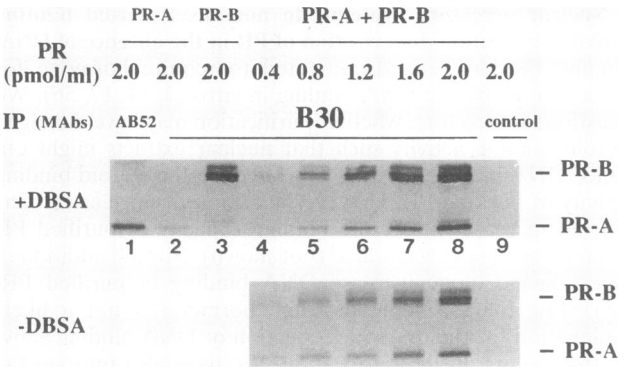

FIG. 3. The nuclear accessory factor does not affect solution dimerization of PR. Separately expressed PR-A and PR-B in Sf9 insect cells were bound to hormone (R5020) in vivo, WCEs were prepared, and each receptor isoform was purified by MAb affinity chromatography. Purified PR-A and PR-B were then mixed in vitro at the final concentrations indicated in the presence and absence of 25 μg of nuclear extracts (indicated as DNA-binding stimulatory activity [DBSA]). Mixed receptor isoforms were then immunoprecipitated (IP) with the PR-B-specific MAb B-30, and the immunoprecipitates were analyzed by Western blotting with the A- and B-specific MAb, AB-52. Coimmunoprecipitation of PR-A with the PR-B-specific MAb indicates the formation of solution dimerization between the A and B receptor isoforms. As controls for the specificity of coimmunoprecipitation, purified PR-A alone was immunoprecipitated with AB-52 (lane 1) or B-30 (lane 2), and both purified PR isoforms were immunoprecipitated with an unrelated control antibody (lane 9 [control]). The Western blot detection was done with [^{35}S]protein A and autoradiography so that the ratio of PR-A to PR-B could be quantitated directly by scanning of the nitrocellulose filter for radioactivity (PhosphorImager analyzer).

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). The 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 between 0.2 and 0.3 M NaCl. Taking advantage of these properties, we used sequential column chromatography steps of heparin-Sepharose, phosphocellulose, and single-stranded DNA-agarose to obtain an approximate 100-fold enrichment of activity per unit of protein (not shown).

During the course of these fractionation experiments, we noticed that other known nuclear proteins have been reported to exhibit similar chromatographic behavior, in particular binding to single-stranded DNA-agarose. These include the basal transcription factors TFIID (52) and TFIIB (30) and the high-mobility-group chromatin protein HMG-1 (10). TFIIB was also of interest because it was recently identified as a protein that stabilizes DNA binding of the COUP-TF orphan receptor and was shown to bind directly to PR and ER (34). TBP (TATA-binding protein of TFIID) and TFIIB were expressed as glutathione *S*-transferase fusion proteins in *E. coli* and purified by glutathione-Sepharose affinity chromatography (34). HMG-1 was purified from calf thymus by ion-exchange chromatography on polybuffer (PBE94) chromatofocusing resins (2). Each purified protein was tested in a gel shift assay for the ability to stimulate PR-DNA binding. Neither TBP nor TFIIB had a stimulatory effect whether it was purified as a glutathione *S*-transferase fusion protein or as a cleaved intact protein (data not shown). Purified HMG-1, however, caused a dramatic stimulation of PR binding to its PREs that was equivalent to that of nuclear extracts. HMG-1 is the only purified protein that we have tested that can functionally substitute for crude nuclear extracts. Figure 4A, a silver-stained SDS-gel of the purified HMG-1 used in these experiments, shows a single silver-stained band at 28,000 Da which was confirmed immunologically by Western blotting to correspond to HMG-1. Figure 4B shows that purified HMG-1 stimulated the binding of purified PR-B to specific DNA and that it was able to stimulate binding to more than one PRE: a natural imperfect palindromic PRE derived from MMTV and a synthetic palindromic PRE. With purified PR-B held constant (25 ng) and HMG-1 added in amounts varying from 10 to 150 ng, the stimulatory effect was dose responsive and did not require a large excess of HMG-1. On a molar basis, HMG-1 was effective when added in stoichiometric amounts with PR, and in general, we observed from replicate experiments that sensitivity was slightly greater with a perfect palindromic PRE. All results shown to this point have been with the B isoform of human PR. The A isoform was found to behave in a similar manner. Purification of PR-A resulted in a dramatic loss of DNA binding activity compared with the activity associated with native PR-A in WCEs (Fig. 4C). Binding to the PRE probe was increased by addition of either nuclear extracts or purified HMG-1 but not by a nonspecific protein such as ovalbumin.

As shown with PR-A and PR-B (Fig. 4B and C), HMG-1 enhanced PRE binding without altering the mobility of the complex, and an antibody to HMG-1 failed to supershift the complex under conditions in which a supershift was observed with a PR-specific antibody. There are several possibilities to explain this apparent failure to detect HMG-1 in the complex: (i) HMG-1 may not produce a detectable change in mobility because it is a small protein relative to PR or the antibody epitope may be inaccessible in the DNA complex, (ii) HMG-1 may not participate in the final DNA-PR complex, and (iii) PR and HMG-1 may form a ternary complex with DNA but the HMG-1 component may be less stable and dissociate during electrophoresis under the conditions of our gel shift assays. To further explore these possibilities, we have examined binding by another method based on coimmunoprecipitation of the DNA probe. Purified PR-B was incubated with a labeled PRE probe in the presence and absence of HMG-1. DNA complexes were then immunoprecipitated with either a PR-specific or an HMG-1-specific antibody, using protein G-Sepharose as the immunoabsorbent. After washing of the beads, the bound labeled DNA probe was eluted, electrophoresed on 12% polyacrylamide gels, and visualized by autoradiography. As shown in Fig. 5, very little of the labeled DNA probe was coimmunoprecipitated by the PR-specific antibody AB-52 in the presence of purified PR alone, compared with the background level obtained with an unrelated control antibody. In contrast, specific coimmunoprecipitation of the DNA probe was increased dramatically by the addition of HMG-1 (Fig. 5) but not by other proteins such as ovalbumin (not shown). Although to a lesser extent, the HMG-1 antibody also coimmunoprecipitated the labeled DNA probe above background but only in the presence of both PR and HMG-1 (Fig. 5). These results indicate that PR and HMG-1 are able to cobind in a ternary complex with DNA, at least under the conditions of the immunoprecipitation assay. From these results, the failure to detect HMG-1 as a component of the PR-DNA complex by gel shift assay is likely due to its dissociation during electrophoresis.

HMG-1 increases the binding affinity of PR. To attempt to quantitate the enhancement of PR-DNA binding by HMG-1,

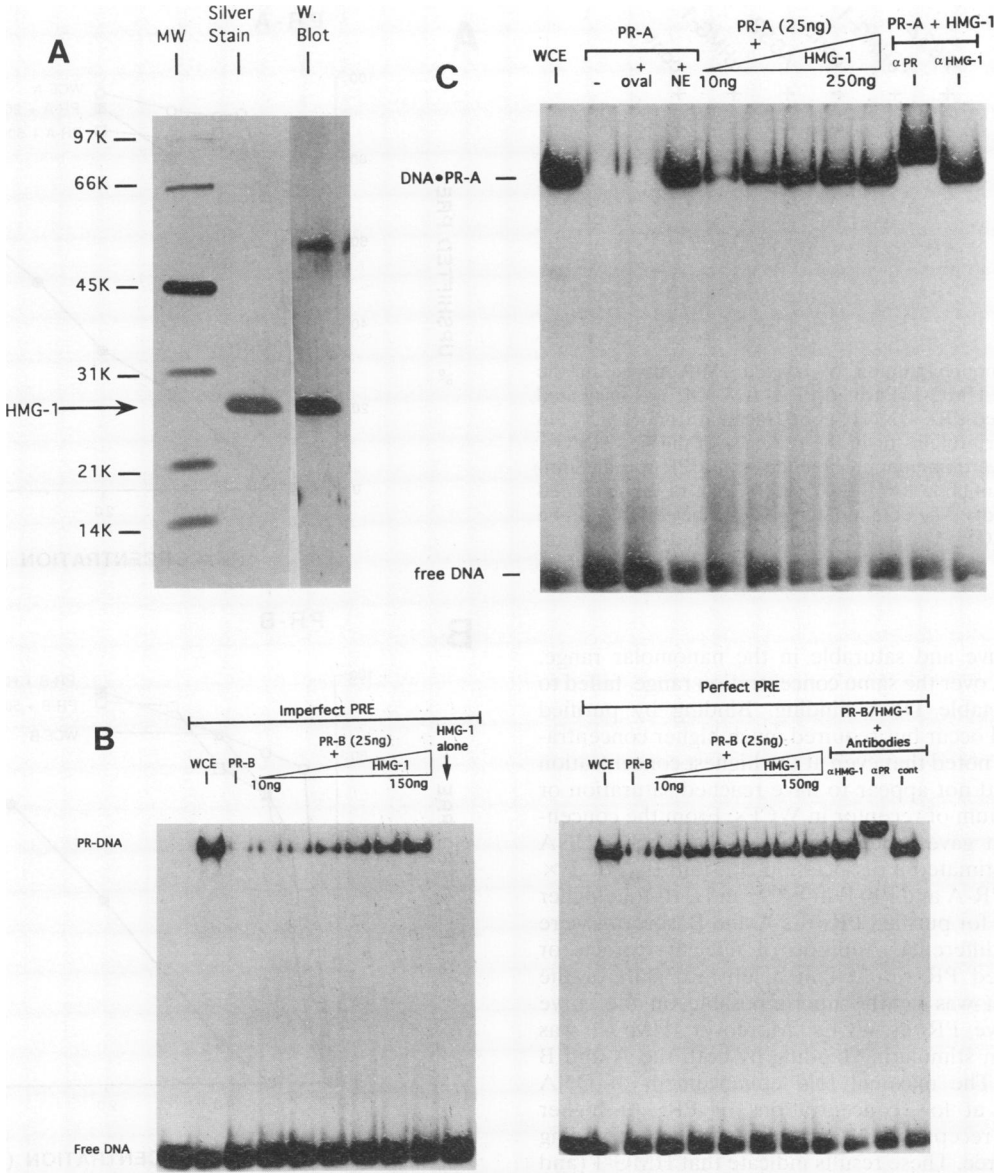

FIG. 4. The high-mobility-group protein HMG-1 enhances PR-DNA binding. (A) Purification of HMG-1. HMG-1 was purified from calf thymus by extraction of chromatin with 5% perchloric acid, dialysis of the acid-soluble protein fraction in 10 mM Tris-HCl (pH 7.8) buffer, and fractionation on a polybuffer resin (PBE94). The PBE94 was used under anion-exchange chromatography conditions. Bound proteins were eluted from the column by a linear 0 to 1.5 M NaCl gradient. Purified HMG-1 that elutes at about 0.6 M NaCl was analyzed by SDS-PAGE (12.5% gel) and silver staining and by Western (W.) blotting with a rabbit polyclonal antibody raised to calf thymus HMG-1. ^{125}I-labeled donkey anti-rabbit IgG secondary antibody and autoradiography were used for Western blot detection. MW, molecular weight standards. (B) Enhancement of binding by purified PR-B. PR-B expressed in Sf9 cells and bound to hormone (R5020) in vivo either was prepared 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 (left panel) or a synthetic palindromic PRE (right panel). Purified PR-B was assayed alone (PR-B) or after addition of increasing amounts of purified HMG-1 (PR-B + HMG-1). HMG-1 alone was analyzed for PRE binding in the right-hand lane (left panel). Purified PR-B (25 ng) was also combined with HMG-1 (150 ng) plus various antibodies (right panel), including a rabbit antiserum to HMG-1 (αHMG-1), MAb B-30 to PR-B (αPR), and an unrelated control antibody (cont). (C) Enhancement of binding by purified PR-A. PR-A expressed in Sf9 insect cells and bound to R5020 in vivo either was prepared as a WCE or was affinity purified as in Fig. 1. Equal amounts (25 ng) of PR-A in WCE or in purified form were analyzed by gel shift assay for binding to 0.3 ng of an imperfect palindromic ^{32}P-labeled PRE oligonucleotide. Purified PR-A was assayed alone (PR-A) or after addition of 1 μg of nuclear extracts (PR-A + NE), 1 μg of ovalbumin (PR-A + oval), increasing amounts of HMG-1 (PR-A + HMG-1), or HMG-1 (150 ng) plus the receptor-specific antibody MAb AB-52 (αPR) or the rabbit antibody to HMG-1 (αHMG-1).

we performed binding analysis when PR concentrations were varied and the ^{32}P-labeled PRE DNA probe was held constant at a limiting amount. PR concentrations ranged from <1 to 35 nM and thus were above and below the single concentration of 5 nM used in previous experiments. Specific PRE-receptor complexes were quantitated by direct analysis (PhosphorImager) of the radioactive bands in dried gels. As shown in Fig. 6, binding of native PR in WCEs to a synthetic palindromic PRE

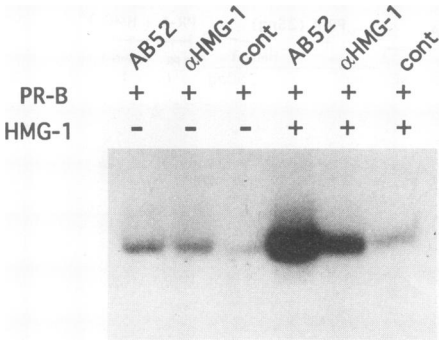

FIG. 5. Coimmunoprecipitation of labeled DNA probe in the presence of PR and HMG-1. Purified PR-B (6.5 nM) was incubated with a ^{32}P-end-labeled PRE DNA (145-bp fragment from MMTV) in the absence of other proteins or in the presence of purified HMG-1 (250 ng). Samples were immunoprecipitated with a PR-specific antibody (AB-52), an antibody to HMG-1 (αHMG-1), or an unrelated control antibody (cont). The immunoprecipitated labeled DNA probe was analyzed by PAGE (12% polyacrylamide gel) and autoradiography.

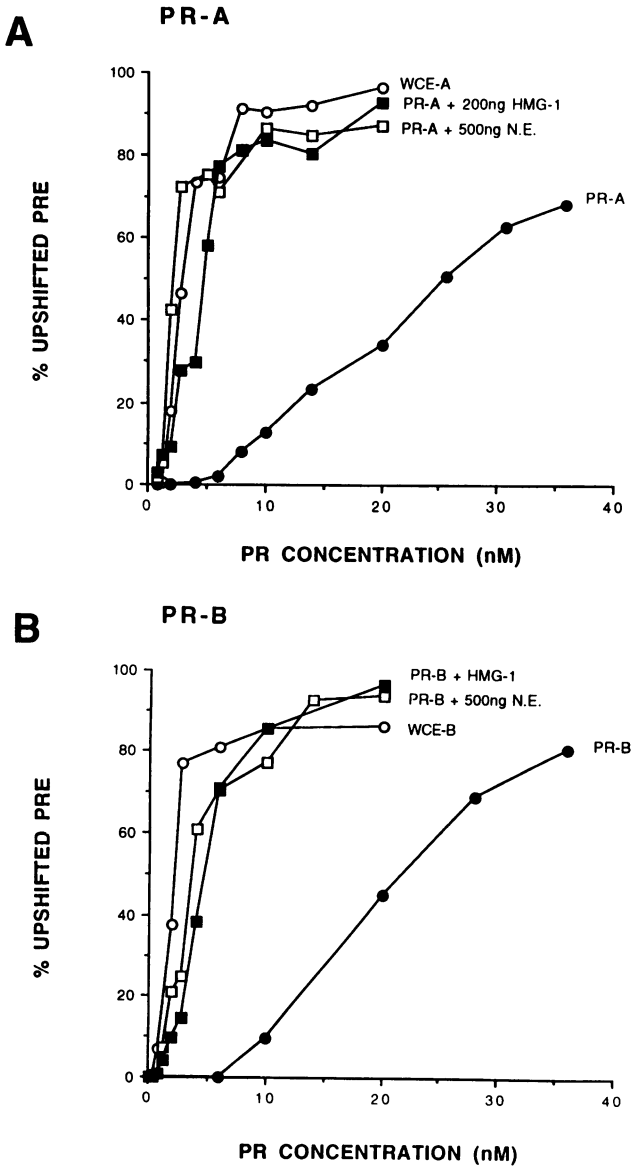

FIG. 6. HMG-1 increases the binding affinity of purified PR-A and PR-B for specific DNA. The PR concentration in gel shift assays was varied against a constant amount of ^{32}P-labeled synthetic palindromic PRE oligonucleotide. Both receptor isoforms (PR-A [A] and PR-B [B]) were assayed in this manner when prepared as a WCE or in purified form. Affinity-purified PR was assayed alone (PR-A or PR-B) or after addition of 500 ng of nuclear extract (PR-A + N.E., PR-B + N.E.) 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 percent upshifted PRE complexes was 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.

was dose responsive and saturable in the nanomolar range. Purified PR alone, over the same concentration range, failed to exhibit any detectable DNA binding. Binding by purified receptor alone did occur but required much higher concentrations. It should be noted that even at the highest concentration used, PR alone did not appear to have reached saturation or the binding maximum of receptor in WCEs. From the concentration of PR that gave a 50% upshift of the labeled DNA probe, we have estimated a dissociation constant (k_d) of 2×10^{-9} M for both PR-A and PR-B in WCE and a 10-fold higher k_d 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. Moreover, HMG-1 was equally effective in stimulating binding by both the A and B isoforms of PR. The maximal fold enhancement of DNA binding occurred at low concentrations of PR. At higher concentrations of receptor, HMG-1 stimulated DNA binding but to a lesser degree. These results indicate that HMG-1 (and nuclear extracts) increased the binding affinity of PR to synthetic palindromic PRE by approximately 10-fold.

In similar binding experiments with the imperfect palindromic PRE of MMTV, we observed that purified PR alone over the same concentration range failed to produce any detectable binding (not shown). However, PR in a WCE or in the presence of HMG-1 bound with the same affinity to MMTV PRE as to the perfect palindromic PRE. Thus, PR on its own appears less capable of binding to an imperfect PRE element of a natural gene such as MMTV than to a synthetic palindromic element. Thus, we assume that the enhancement of DNA binding promoted by HMG-1 is even greater than 10-fold with respect to the natural PRE of MMTV.

The DNA binding domain of HMG-1 is sufficient for enhancement of PR-DNA binding. HMG-1 binds to double-stranded DNA without apparent sequence specificity. It prefers to bind to distorted DNA structures, including cruciform DNA, single-stranded DNA, and bends or kinks in DNA. Thus, HMG-1 can be considered a structure-specific sequence-independent DNA-binding protein. HMG-1 contains two structural domains, termed HMG A and B boxes, that mediate this DNA binding activity. 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 novel DNA binding motifs that recognize angles or bends in DNA (6, 74). Regions of homology to HMG boxes have been found in several other proteins, including the sequence-specific transcriptional activators lym-

phoid cell enhancer factor LEF-1 (26) and testis determining factor SRY (23).

HMG-1 box domains expressed and purified from *E. coli* were tested for their effects on DNA binding by purified PR. The A box had weak PRE binding stimulatory activity compared with an unrelated control peptide (c-peptide), and the B box exhibited stronger activity nearly equivalent to that of intact HMG-1 (Fig. 7A). As with intact HMG-1, the box domains alone did not bind directly to the PRE under the conditions of our gel shift assay. Curiously, the A- and B-box domains when linked together did not enhance PR-DNA binding, but at high concentrations they bound directly to the PRE and produced a distinct reduced-mobility DNA complex. The HMG boxes of LEF-1 and SRY failed to stimulate PRE binding by purified PR-B (Fig. 7B). Both LEF-1 and SRY bound directly to the PRE and produced a ladder of reduced-mobility complexes consistent with multimers of bound peptides. Another high-mobility-group chromatin protein, HMG-I(Y), was reported to enhance DNA binding of NF-κB to the human beta interferon (67) gene. We tested HMG-I(Y) and observed that it also failed to enhance PRE binding (not shown). Thus, it appears that an HMG box alone is sufficient for enhancement of PRE binding, but it appears that not all HMG box motifs are functionally equivalent in this respect. These results suggest that the DNA binding properties of HMG-1 may be responsible for its effect on stimulation of PR-DNA interactions.

The DNA binding domains of HMG-1 induce DNA flexure, and HMG-1 has a higher affinity for tightly curved DNA than for linear DNA. It is not clear from the foregoing results how the HMG-1 protein facilitates PR-DNA binding. Recent studies have demonstrated that in addition to recognizing distortions in DNA structure, HMG-1 promotes DNA bending (57, 59). As evidence of this, HMG-1 was shown to mediate ligase-dependent cyclization of short DNA fragments by a ring closure assay. This type of assay measures the efficiency at which T4 DNA ligase can cyclize a fragment of DNA that is shorter than the DNA persistence length (*P*) (≈150 bp); the stiffness of short DNA fragments below *P* limits intramolecular alignment of the two ends so that ring closure is not detected unless an added protein bends the DNA. Purified HMG-1 was tested with a series of DNA restriction fragments ranging in size from 40 to 366 bp. HMG-1 facilitated ring closure of all tested DNA fragments from 88 bp (Fig. 8) to *P*, 150 bp, and interestingly, it also promoted the longer-range communication between widely separated sequences by enhancing cyclization of fragments with lengths greater than *P* (207- and 366-bp fragments). HMG-1 did not promote ring closure of fragments of 40 and 58 bp. For all fragments tested, maximal ring closure rates required approximately 10-fold excess of HMG-1 to DNA by mass, using the standard assay (see Materials and Methods), and there was no demonstrated preference for DNA sequence. These ring closure properties of HMG-1 (data not shown) are similar to those reported recently (57, 59) and thus confirm that HMG-1 is an effective DNA-flexing protein.

The HMG A- and B-box domain polypeptides were also tested in the DNA cyclization assay (Fig. 8). In the absence of HMG-1 or its DNA binding domains, a linear 88-bp *Eco*RI fragment was efficiently ligated into linear dimers, trimers, and higher-order structures (Fig. 8, lane 1), but we could detect no ligation products having the previously defined mobility (33) of the 88-bp circle. When purified HMG-1 protein or the purified HMG A- and B-box polypeptides were added, ring closure readily occurred; however, the specific activity of the A box was found to be severalfold higher than that of the B box (Fig. 8; compare lanes 5 and 6 with lanes 7 and 8), the significance of which is unknown. These results indicate that both of the DNA binding domains of HMG-1 are independently capable of bending DNA, suggesting that their analogous effect on stimulating DNA binding by PR may be the result of promoting flexure of the target DNA.

It is important to understand whether HMG-1, when it binds to linear DNA, increases the rate of ring closure by bending the DNA or by increasing its flexibility. This consideration is especially relevant to HMG-1–DNA interactions because it was reported that HMG-1 can locally denature the DNA double helix (48, 77), and thus the stiffness of DNA could be modified at such a site. Thermodynamically, a protein that bends a segment of DNA should bind with higher affinity to the DNA if it is prebent into the proper configuration (35). Following this line of reasoning, we tested whether HMG-1 would bind in a gel shift assay with higher affinity to DNA that was prebent in the form of tightly curved minicircles. Figure 9A shows a competition binding experiment in which approximately equal amounts of linear and circular 144-bp DNA molecules (of identical sequences) were mixed with 100 ng of purified calf thymus HMG-1. All circular DNA was taken up into reduced electrophoretic mobility complexes with HMG-1, while no linear DNA complexes were detected. This finding indicates that HMG-1 binds with a substantially higher affinity to prebent DNA than to linear DNA.

The isolated minicircular DNAs such as those in the experiment described above normally contain 15 to 25% nicked circles, probably because they are ligated only in one strand during their production in ring closure with the HMG-1 or HU protein. The 144-bp minicircles used in gel shift assays were estimated to contain 17% nicked circles by quantitative PhosphorImager scans of separated closed circular and single-stranded DNAs obtained after boiling a sample of the isolated 144-bp minicircles (Fig. 9B). The nicked circles coelectrophorese with the closed minicircles in the absence of HMG-1 and also preferentially bind the HMG-1 protein, since all detectable circular DNA is retarded at HMG-1/DNA ratios that result in little binding to the linear 144-bp DNA (Fig. 9A, lane 4). This finding argues against the possibility that the preferential binding of HMG-1 to 144-bp minicircles in Fig. 9A was attributable to the circular DNA being sealed with a linking number that corresponded to a helical over- or underwinding that favored HMG-1 binding. The nicked circles should relax this altered topology. These findings therefore suggest that the higher binding affinity of HMG-1 to the 144-bp minicircle is attributable at least partly to the tight curvature of the circular DNA and not solely to the over- or underwinding of the DNA that may be topologically constrained in covalently closed circles. Collectively, these data demonstrate that the stimulation of ring closure induced by HMG-1 is at least partly due to static bending of the DNA.

DISCUSSION

A recurring theme in the literature is the existence of nuclear accessory proteins that regulate the DNA binding activity of sequence-specific eukaryotic transcriptional activators. In several instances, this has been discovered when expressed recombinant transcriptional activators have been purified and found to suffer a loss of DNA binding activity that can be restored by addition of other proteins. Accessory proteins have been described for the activated T-cell transcription factor NF-AT (28), Fos-Jun (1), cyclic AMP response element-binding protein (CREB)/ATF (75), NF-κB (45, 68, 75), serum response factor (SRF) (29), and several steroid receptors (11, 17, 22, 39, 42, 44, 50, 64, 78, 79). It appears that

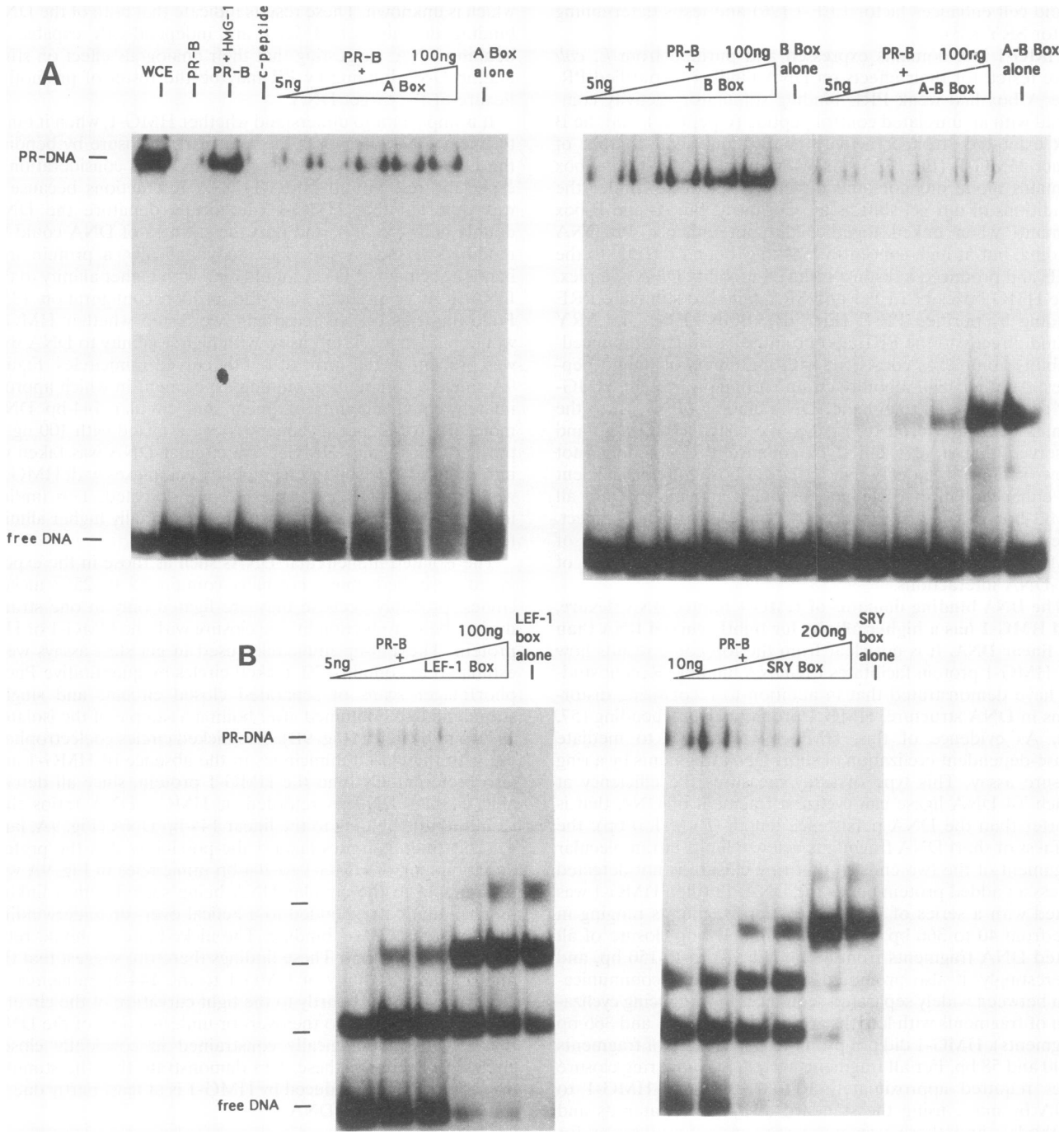

FIG. 7. The HMG B-box domain is sufficient for enhancing DNA binding by purified PR. (A) PR-B expressed in Sf9 cells and bound to R5020 in vivo was affinity purified and analyzed by gel shift assay with a synthetic palindromic PRE oligonucleotide. Purified receptor (25 ng) was assayed alone (PR-B) or after addition of 100 ng of intact HMG-1 (PR-B + HMG-1), an unrelated control peptide (+ c-peptide), or increasing amounts of HMG box peptides corresponding to the A box (PR-B + A box), B box (PR-B + B box), or A and B boxes linked together (PR-B + A-B box). Each box peptide (100 ng) was also analyzed alone. (B) HMG box motifs in other proteins, the B-lymphoid cell enhancer factor LEF-1 and testis determining factor SRY, failed to enhance the DNA binding by PR. Affinity-purified PR-B (25 ng) bound to R5020 was analyzed by gel shift assay for binding to the ^{32}P-labeled PRE (palindromic PRE) in the presence of increasing amounts of the HMG box of LEF-1 (left panel) or the HMG box of SRY (right panel). HMG boxes of LEF-1 and SRY (100 ng) were also assayed alone without the presence of purified PR-B.

there may be common accessory factors for distinct subgroups of transcriptional activators. For example, the DNA binding activities of Fos-Jun, NF-κB, Myb, CREB, and ATF-1 and ATF-2 are sensitive to oxidation-reduction of the protein, and a protein termed redox factor Ref-1 that is capable of mediating both redox regulation and enhancement of the DNA binding of these transcription factors has been isolated (75). Recently, the Tax protein of human T-cell leukemia virus type

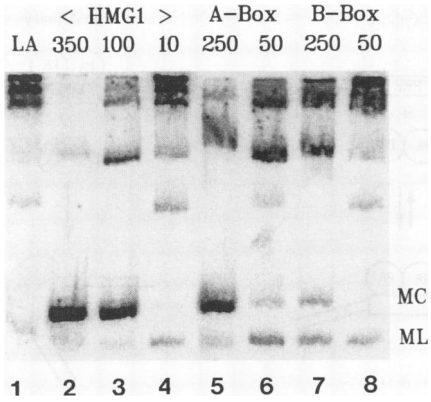

FIG. 8. The DNA binding domains A and B (HMG boxes) of HMG-1 mediate DNA ring closure. The HMG boxes of rat HMG-1 were purified as described previously (74) and assayed for stimulation of DNA ring closure of an 88-bp linear *Eco*RI fragment in the presence of T4 DNA ligase. The ligation products were analyzed by electrophoresis as described in Materials and Methods. The amount in nanograms of intact HMG-1 or the A- or B-box proteins is shown above each lane. LA, a control incubation of the 88-bp DNA with ligase alone. Positions of the monomer linear (ML) and monomer circle (MC) 88-bp DNAs are indicated on the right.

I was shown to increase the in vitro DNA binding of proteins that contain a basic region-leucine zipper motif (72) but did not affect proteins with other DNA-binding motifs. RXR, which itself is a member of the steroid receptor gene family, appears to serve as a facilitator of DNA binding for a subgroup of nuclear receptors that includes TR, VDR, RAR, PPAR, and COUP-TF (39, 44, 78, 79).

Nuclear accessory factors, distinct from RXR, have been described to enhance the DNA binding of receptors for the classical steroid hormones, including receptors for progesterone (17), estrogen (22, 50), glucocorticoids (11, 42, 55), and androgen (42). Very little is known, however, about these accessory factors, and until now, the protein(s) involved has not been identified. We demonstrate here that HMG-1, purified to apparent homogeneity, can function in this capacity to enhance the binding of purified human PR to its specific PRE. It is unclear at present whether the classical steroid hormone receptors (GR, mineralocorticoid receptor, androgen receptor, ER, and PR) as a group can utilize a common accessory protein such as HMG-1 or whether different accessory factors are involved with each receptor. Given reported differences in molecular mass, it would appear that multiple accessory factors are involved. For example, a small heat-stable polypeptide of approximately 3,000 Da was identified to enhance GR binding to DNA cellulose (11), but a much larger protein of 93 kDa was described to increase binding of GR to chromatin (55). With ER, proteins of 60 to 70 kDa (22) and 45 kDa (50) have been reported to enhance binding to EREs. While this report was being prepared, a study describing a 130-kDa receptor accessory factor that enhanced the DNA binding of recombinant androgen receptor and GR (42) and was shown to form a ternary complex with receptor and DNA was published. Because most of these studies used crude factor and/or receptor preparations, it is difficult to interpret whether activity is associated with the same, related, or different proteins. To determine whether HMG-1 has the ability to enhance DNA binding of other steroid hormone receptors, it will be necessary to conduct studies similar to those described herein, with both purified HMG-1 and various other purified receptors.

The requirement for an accessory factor for maximal in vitro DNA binding does not appear to be peculiar to recombinant receptors used in the present study or to the use of a gel mobility shift assay. In previous studies, enhancement of DNA binding by nuclear extracts was observed with endogenous cytosolic PR from T47D cells (17), and enhancement of purified recombinant PR in the present study was observed both by the gel mobility shift assay and by a coimmunoprecipitation assay. We have also shown here that purified PR alone is able to bind to a PRE to some extent when receptor is added in high enough concentrations (Fig. 6). HMG-1 appeared to increase PR binding affinity so that receptor-DNA interactions occurred efficiently at low PR concentrations. Although these assays were conducted in vitro, physiological relevance is suggested by several observations. For instance, enhancement of PRE binding did not require excess HMG-1 but occurred when HMG-1 and PR were present in equal molar amounts (Fig. 4). Additionally, HMG-1 stimulated a greater increase in PR binding for a partial palindromic PRE from MMTV than for a synthetic palindromic PRE. Naturally occurring progesterone-responsive genes contain partial palindromic response elements, not perfect palindromes. We also found that purified PR alone has a higher intrinsic affinity for a consensus palindromic PRE than for the imperfect PRE of MMTV, suggesting that HMG-1 in vivo may preferentially facilitate binding to DNA sites that have weaker affinity for PR. Lastly, enhancement of PRE binding appeared to be highly selective for HMG-1 and was not exhibited by a number of other proteins, including the DNA-binding proteins HU, HMG-I(Y), SRY, LEF-1, histone HI, TFIID, and TFIIB. Although the biological effect of HMG-1 on PR function in vivo is not known, the present results suggest that HMG-1 could modulate the affinity of receptors for specific DNA sites in vivo and influence the transcriptional activity of PR.

It is not known whether it is the DNA binding properties of HMG-1 or protein-protein interactions between HMG-1 and PR that are responsible for enhancement of PR-DNA binding. It appears that the DNA binding activity of HMG-1 is a major determinant. In support of this view, an HMG box DNA binding domain alone was sufficient both for enhancement of PR binding to PRE probes and for bending DNA (Fig. 7 and 8). Moreover, by coimmunoprecipitation assay, we were able to detect a ternary complex between HMG-1, PR, and DNA. Why the A-box domain possessed a stronger DNA bending activity and the B-box domain possessed a stronger activity for enhancement of PR-DNA binding is not known. This difference may have to do with DNA sequence, since DNA fragments of unspecific sequence were used in ring closure assays. Whether HMG-1 makes direct protein contact with PR is not certain. By use of a PR affinity column, constructed with purified PR-B coupled to an antibody-resin (see Materials and Methods), we observed little or no direct binding of purified HMG-1 to PR (not shown). These experiments were done under conditions in which we were able to observe specific binding of TFIIB to the PR affinity resin (not shown) under conditions shown by Ing et al. (34) to allow direct binding of TFIIB to either PR or ER. Thus, HMG-1 does not appear to have much affinity for PR in the absence of DNA.

We were not able to detect HMG-1 as a component of the enhanced PR-DNA complex in gel mobility shift assays (Fig. 4), yet a ternary complex of HMG-1, PR, and DNA was evident by coimmunoprecipitation assay (Fig. 5). We interpret this result to mean that HMG-1 is a less stable component of the ternary complex and likely dissociates during electrophoresis under the conditions of the PR gel mobility shift assay. There are other examples of proteins that enhance the binding

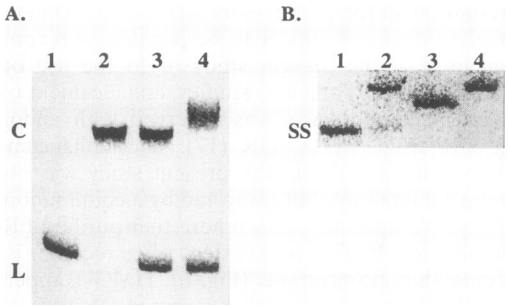

FIG. 9. HMG-1 binds with higher affinity to tightly curved circular DNA than to linear DNA. (A) HMG-1 and linear or circular 144-bp DNA were mixed (see Materials and Methods); products were electrophoresed on a 6% polyacrylamide gel and visualized by PhosphorImager analysis of the gel. Lanes: 1, linear DNA alone; 2, circular DNA alone; 3, linear and circular DNA; 4, linear and circular DNA plus 100 ng (190 nM) of HMG-1. Two nanograms of each ^{32}P-labeled DNA species was used when present. C and L, bands corresponding to uncomplexed circular and linear DNAs, respectively. (B) Samples (0.5 ng) of the same purified 144-bp linear and circular DNAs used in panel A were analyzed before and after denaturing by boiling. Lanes: 1, boiled linear DNA; 2, boiled circles; 3, linear DNA not heated; 4, circles not heated. SS, single-stranded DNA appearing after boiling.

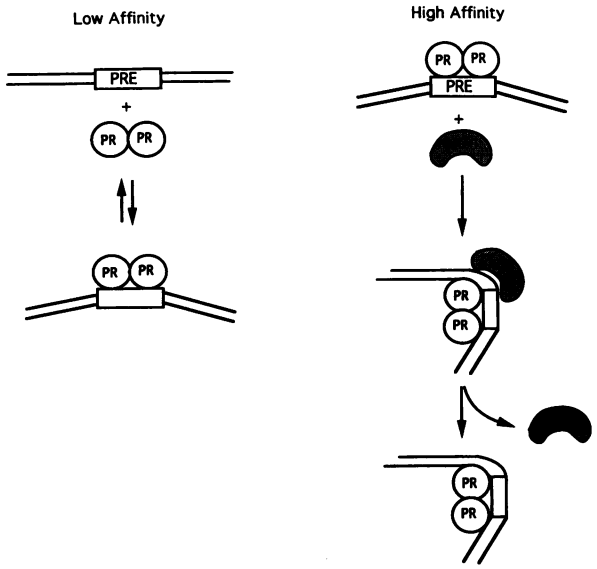

FIG. 10. Proposed mechanism of HMG-1 enhancement of PR binding to specific target DNA.

of sequence-specific transcriptional activators to their DNA sites without themselves being retained in the complex during electrophoresis on nondenaturing gels. For example, Tax enhanced DNA binding of ATF proteins without altering the mobility of ATF-DNA complexes (72). Similarly, Phox 1 enhanced binding of SRF without altering the mobility of SRF-DNA complexes (29). Tax was shown by chemical crosslinking and by coimmunoprecipitation to form a ternary complex with ATF and DNA, suggesting that the Tax component is unstable to the conditions of electrophoretic mobility shift assay (72). It is possible, however, to detect HMG-1 binding to linear nonspecific DNA by gel shift assay, under conditions optimized for detection of sequence-independent interactions that have rapid dissociation rates (25). When the gel shift conditions for PR were applied, HMG-1 binding to the 144-bp linear DNA was no longer detectable. Likewise, PR binding to a PRE could not be detected under gel shift conditions optimized for HMG-1 binding. Thus, we did not find a single gel shift condition that would maintain both HMG-1 and PR interactions with DNA. Alternatively, it is possible that HMG-1 enhances the DNA binding of PR by transient stabilization of a local structural conformation in the PRE and that binding of both HMG-1 and PR to the same DNA is mutually exclusive. There is a precedent for this type of mechanism in bacteria. HU appears to be the bacterial equivalent of HMG-1 (57), and HU has been shown to enhance DNA binding of the *lac* repressor and catabolite activator protein (24). Similar to results with HMG-1 in the present study, HU enhanced DNA binding of the *lac* repressor without participating stably in the final DNA complex. DNA structure is flexible and in equilibrium between alternative conformational states (58). HU has been proposed to work by altering the dynamic flexibility of DNA structure to periodically distort or stabilize a structural conformation favored by the sequence-specific binding protein (24, 58).

Although HMG-1 can bind to linear duplex DNA, it prefers to bind to distorted DNA structures, including single-stranded DNA (presumably secondary structures produced by unwinding), bends or kinks in DNA, cruciform DNA, four-way junction DNA, and DNA adducts (6, 8, 10, 18). Recognition of DNA structure is mediated by the conserved HMG box motif, and the solution structure of the HMG B box has been recently solved by two-dimensional nuclear magnetic resonance spectroscopy. This has revealed a novel DNA binding motif composed of three α helices arranged in an L shape with the two arms held at an 80° angle to each other (74). This unusual structure has been suggested to be important for recognition of kinks or bends in DNA (74). Regions of homology to the boxes of HMG-1 have been discovered in several other proteins, including the sequence-specific transcriptional activators SRY and LEF-1 (23, 26). The HMG boxes of SRY and LEF-1 not only bind to DNA structure but also promote sharp bends in DNA when bound to their response elements (23, 26). DNA bending in fact may be a general property of proteins that harbor HMG boxes. While this report was in preparation, two studies, based on ring closure assays, reported that HMG-1 can bend DNA in a sequence-independent manner (57, 59). The ring closure data in the present study with purified HMG-1 and its boxes confirm and extend these results. We have also shown that HMG-1 has a substantially higher affinity for prebent nicked or covalently closed DNA than linear DNA, indicating that the acceleration of ring closure in the presence of HMG-1 is the result of a static bend of the DNA as opposed to an increased flexibility due to local relaxing of the DNA double helix (Fig. 9).

The functional role of HMG-1 is not well defined. Given its ability to bind with higher affinity to DNA structures than to linear DNA sequence, and to bend DNA, it may have a regulatory role in replication, DNA repair, recombination packaging of DNA, or transcription by promoting assembly of multiprotein complex through looping of DNA (6, 8, 10, 18, 57, 59). The present study indicates that HMG-1 may also have a role as a facilitator for binding of certain sequence-specific DNA-binding proteins such as PR. Consistent with the present studies, HMG-1 likely facilitates PR binding to its DNA elements by promoting, or cooperating with receptor to promote a structural change in the target PRE DNA.

Figure 10 is a schematic of a proposed mechanism by which HMG-1 enhances PR binding to DNA. The model postulates that high-affinity PR binding is dependent on both specific

nucleotide sequence and protein-induced conformational changes in DNA structure. Receptor alone is shown to bind with low affinity to PRE and to produce a small distortion or bend in DNA structure. Since HMG-1 binds with higher affinity to bent or curved DNA, this marks the DNA as a preferred binding site for HMG-1. HMG-1 increases the binding affinity of PR either by stabilizing a dynamic structural conformation in the target PRE that is favored by PR or by promoting further distortions in DNA structure such as bending. Whether HMG-1 is retained as a stable component of the final PR-DNA complex is uncertain. This mechanism requires that HMG-1 be a component of the complex at some point. In future studies, it will be important to determine the nature of the structural changes in DNA that are promoted by the combined actions of PR and HMG-1 compared with each protein alone.

Recent studies on the ability of steroid receptors to bend DNA provide further support for this model. ER when bound to an ERE was reported to bend DNA; however, this was observed only with the DNA binding domain fragment of ER and with partially purified full-length ER. Highly purified full-length ER failed to bind to EREs at all (53, 54). This finding suggests that an accessory factor may be required both for high-affinity DNA interaction and for DNA bending. TR 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 (37). This finding raises the possibility that HMG-1 or HMG-related proteins act to facilitate DNA bending promoted by steroid receptors and that bending may be requisite for high-affinity association with DNA. In this regard, it is important to note that there are precedents, both in bacteria and in eukaryotic cells, for sequence-specific DNA-binding proteins that appear to require both nucleotide sequence and secondary structural conformations for selective recognition of DNA targets. In bacteria, this has been described for the bacterial *lac* repressor and catabolite activator protein (69) and the restriction endonuclease *Eco*RI (46). In eukaryotes, high-affinity binding of CREB has been shown to be preferred by an alternative secondary structure in the target DNA caused by a base pair mismatch (65). Secondary structural features in the ERE have also been suggested to contribute to high-affinity binding of ER (43, 49).

ACKNOWLEDGMENTS

We are grateful for the generous contributions of Jean O. Thomas, Cambridge University, Cambridge, England, for providing HMG box domain peptides; Marco Bianchi, San Raffaele Hospital, Milano, Italy, for providing HMG domain peptides and the HMG domain of SRY; R. Grosschedl, University of California at San Francisco, for providing the HMG box of LEF-1; Michael Bustin, National Institutes of Health, Bethesda, Md., for rabbit antiserum to HMG-1; Ming-Jer Tsai and Bert W. O'Malley, Baylor College of Medicine, Houston, Tex., for recombinant TBP and TFIIB; and Yvonne Hodges-Garcia, University of Colorado Health Sciences Center, and Joan Betz, Regis University, for gifts of specific DNA fragments. We also thank Nancy Hart and Clairene Mraz for assistance with preparation of the manuscript, Kurt Christensen for expert technical assistance with baculovirus expression of PR in insect cells, and Steven K. Nordeen, University of Colorado Health Sciences Center, for insightful discussions during the course of these studies.

This work was supported in part by Public Health Service grants CA46938 (D.P.E.) and GM18243 (D.E.P.), the Lucille Markey Charitable Trust, and University of Colorado Cancer Center Core grant P30CA46934.

Sergio A. Oñate and Paul Prendergast made equivalent contributions to this study.

REFERENCES

1. **Abate, C., L. Patel, F. J. Rauscher, and T. Curran.** 1990. Redox regulation of *fos* and *jun* DNA-binding activity in vitro. Science **249:**1157–1161.
2. **Adachi, Y., S. Mizuno, and M. Yoshida.** 1990. Efficient large-scale purification of non-histone chromosomal proteins HMG1 and HMG2 by using polybuffer exchanger PBE94. J. Chromatogr. **530:**39–46.
3. **Bagchi, M. K., S. Y. Tsai, M.-J. Tsai, and B. W. O'Malley.** 1990. Identification of a functional intermediate in receptor activation in progesterone-dependent cell-free transcription. Nature (London) **345:**547–550.
4. **Bannister, A. J., and T. Kouzarides.** 1992. Basic peptides enhance protein/DNA interaction in vitro. Nucleic Acids Res. **20:**3523.
5. **Beebe, J. S., D. S. Darling, and W. W. Chin.** 1991. 3,5,3′-Triiodothyronine receptor auxiliary protein (TRAP) enhances receptor binding by interactions within the thyroid hormone response element. Mol. Endocrinol. **5:**85–93.
6. **Bianchi, M. E., L. Falciola, J. Ferrari, and D. M. J. Lilley.** 1992. 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 J. **11:**1055–1063.
7. **Broyles, S. S., and D. E. Pettijohn.** 1986. Interaction of the *Escherichia coli* HU protein with DNA. Evidence for formation of nucleosome-like structures with altered DNA helical pitch. J. Mol. Biol. **187:**47–60.
8. **Bruhn, S. L., P. M. Pil, J. M. Essignmann, D. E. Housman, and S. J. Lippard.** 1992. 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. Natl. Acad. Sci. USA **89:**2307–2311.
9. **Burnside, J., D. A. Darling, and W. W. Chin.** 1990. A nuclear factor that enhances binding of thyroid hormone receptors to thyroid hormone response elements. J. Biol. Chem. **265:**2500–2504.
10. **Bustin, M., D. A. Lehn, and D. Landsman.** 1991. Structural features of the HMG chromosomal proteins and their genes. Biochim. Biophys. Acta **1049:**231–243.
11. **Cavanaugh, A. H., and S. S. Simons, Jr.** 1990. 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:**989–996.
12. **Christensen, K., P. A. Estes, S. A. Oñate, C. A. Beck, A. DeMarzo, M. Altmann, B. A. Lieberman, J. St. John, S. K. Nordeen, and D. P. Edwards.** 1991. Characterization and functional properties of the A and B forms of human progesterone receptors synthesized in a baculovirus system. Mol. Endocrinol. **5:**1755–1770.
13. **Darling, D. S., J. S. Beebe, J. Burnside, E. R. Winslow, and W. W. Chin.** 1991. 3,5,3′-Triiodothyronine (T3) receptor-auxiliary protein (TRAP) binds DNA and forms heterodimers with the T3 receptor. Mol. Endocrinol. **5:**73–84.
14. **DeMarzo, A. M., S. A. Oñate, S. K. Nordeen, and D. P. Edwards.** 1992. Effects of the steroid antagonist RU486 on dimerization of the human progesterone receptor. Biochemistry **31:**10491–10501.
15. **Drouin, J., Y. L. Sun, S. Tremblay, P. Lavender, T. J. Schmidt, A. de Lean, and M. Nemer.** 1992. Homodimer formation is rate-limiting for high affinity DNA binding by glucocorticoid receptor. Mol. Endocrinol. **6:**1299–1309.
16. **Durand, B., M. Saunders, C. Leroy, M. Leid, and P. Chambon.** 1992. All-trans and 9-*cis* retinoic acid induction of CRABP II transcription is mediated by RAR-RXR heterodimers bound to DR1 and DR2 repeated motifs. Cell **71:**73–85.
17. **Edwards, D. P., B. Kuhnel, P. A. Estes, and S. K. Nordeen.** 1989. Human progesterone receptor binding to mouse mammary tumor virus deoxyribonucleic acid: dependence on hormone and nonreceptor nuclear factor(s). Mol. Endocrinol. **3:**381–391.
18. **Einck, L., and M. Bustin.** 1985. The intracellular distribution and function of the high mobility group chromosomal proteins. Exp. Cell Res. **156:**295–310.
19. **El-Ashry, D., S. A. Oñate, S. K. Nordeen, and D. P. Edwards.** 1989. Human progesterone receptor complexed with the antagonist

RU486 binds to hormone response elements in a structurally altered form. Mol. Endocrinol. **3**:1545–1558.
20. **Estes, P. A., E. J. Suba, J. Lawler-Heavner, D. Elashry-Stowers, L. L. Wei, D. O. Toft, W. P. Sullivan, K. B. Horwitz, and D. P. Edwards.** 1987. Immunologic analysis of human breast cancer progesterone receptors. 1. Immunoaffinity purification of transformed receptors and production of monoclonal antibodies. Biochemistry **26**:6250–6262.
21. **Fawell, S. E., J. A. Lees, R. White, and M. G. Parker.** 1990. Characterization and colocalization of steroid binding and dimerization activities in the mouse estrogen receptor. Cell **60**:953–962.
22. **Feavers, I. M., J. Jiricny, B. Moncharmont, H. P. Saluz, and J. P. Jost.** 1987. Interaction of two nonhistone proteins with the estradiol response element of the avian vitellogenin gene modulates the binding of estradiol-receptor complex. Proc. Natl. Acad. Sci. USA **84**:7453–7457.
23. **Ferrari, S., V. R. Harley, A. Pontiggia, P. N. Goodfellow, R. Lovell-Badge, and M. E. Bianchi.** 1992. SRY, like HMG-1, recognizes sharp angles in DNA. EMBO J. **11**:4497–4506.
24. **Flashner, Y., and J. D. Gralla.** 1988. DNA dynamic flexibility and protein recognition: differential stimulation by bacterial histone-like protein HU. Cell **54**:713–721.
25. **Fried, M. G.** 1989. Measurement of protein-DNA interaction parameters by electrophoresis mobility shift assay. Electrophoresis **10**:366–376.
26. **Giese, K., J. Cox, and R. Grosschedl.** 1992. The HMG domain of lymphoid enhancer factor 1 bends DNA and facilitates assembly of functional nucleoprotein structures. Cell **69**:185–195.
27. **Glass, C. K., S. M. Lipkin, O. V. Devary, and M. G. Rosenfeld.** 1989. Positive and negative regulation of gene transcription by a retinoic acid-thyroid hormone receptor heterodimer. Cell **59**:697–708.
28. **Gramelli-Piperno, A., and P. McHugh.** 1991. Characterization of a protein that regulates the DNA binding activity of NF-AT, the nuclear factor of activated T cells. Proc. Natl. Acad. Sci. USA **88**:11431–11434.
29. **Grueneberg, D. A., S. Natesan, C. Alexandre, and M. Z. Gilman.** 1992. Human and *Drosophila* homeodomain proteins that enhance the DNA-binding activity of serum phox1 response factor. Science **257**:1089–1095.
30. **Ha, I., W. S. Lane, and D. Reinberg.** 1991. Cloning of a human gene encoding the general transcription initiation factor IIB. Nature (London) **352**:689–695.
31. **Heukeshoven, J., and R. Dernick.** 1985. A simplified method for silver staining of proteins in polyacrylamide gels and the mechanism of silver staining. Electrophoresis **6**:103–112.
32. **Heyman, R. A., D. J. Mangelsdorf, J. A. Dyck, R. B. Stein, G. Eichele, R. M. Evans, and C. Thaller.** 1992. 9-Cis retinoic acid is a high affinity ligand for the retinoid X receptor. Cell **68**:397–406.
33. **Hodges-Garcia, Y., P. J. Hagerman, and D. E. Pettijohn.** 1989. DNA ring closure mediated by protein HU. J. Biol. Chem. **264**:14621–14623.
34. **Ing, N. H., J. M. Beekman, S. Y. Tsai, M.-J. Tsai, and B. W. O'Malley.** 1992. Members of the steroid hormone receptor superfamily interact with TFIIB (S300-II). J. Biol. Chem. **267**:17617–17623.
35. **Kahn, J. D., and D. M. Crothers.** 1992. Protein-induced bending and DNA cyclization. Proc. Natl. Acad. Sci. USA **89**:6343–6347.
36. **Kastner, P., A. Krust, B. Turcotte, U. Stropp, L. Tora, H. Gronemeyer, and P. Chambon.** 1990. Two distinct estrogen-regulated promoters generate transcripts encoding the two functionally different human progesterone receptor forms A and B. EMBO J. **9**:1603–1614.
37. **King, I. N., T. deSoyza, D. F. Catanzaro, and T. N. Lavin.** 1992. Thyroid hormone receptor-induced bending of specific DNA sequences is modified by an accessory factor. J. Biol. Chem. **268**:495–501.
38. **Kliewer, S. A., K. Umesono, R. A. Heyman, D. J. Mangelsdorf, J. A. Dyck, and R. M. Evans.** 1992. Retinoid X receptor-COUP-TF interactions modulate retinoic acid signaling. Proc. Natl. Acad. Sci. USA **89**:1448–1452.
39. **Kliewer, S. A., K. Umesono, D. J. Mangelsdorf, and R. M. Evans.** 1992. Retinoid X receptor interacts with nuclear receptors in retinoic acid, thyroid hormone and vitamin D3 signalling. Nature (London) **355**:446–449.
40. **Kliewer, S. A., K. Umesono, D. J. Noonan, R. A. Heyman, and R. M. Evans.** 1992. Convergence of 9-*cis* retinoic acid and peroxisome proliferator signalling pathways though heterodimer formation of their receptors. Nature (London) **358**:771–774.
41. **Kumar, V., and P. Chambon.** 1988. The estrogen receptor binds tightly to its responsive element as a ligand-induced homodimer. Cell **55**:145–156.
42. **Kupfer, S. R., K. B. Marschke, E. M. Wilson, and F. S. French.** 1993. Receptor accessory factor enhances specific DNA binding of androgen and glucocorticoid receptors. J. Biol. Chem. **268**:17519–17527.
43. **Lannigan, D. A., and A. C. Notides.** 1989. Estrogen receptor selectively binds the "coding strand" of an estrogen responsive element. Proc. Natl. Acad. Sci. USA **86**:863–867.
44. **Leid, M., P. H. Kastner, R. Lyons, H. Nakshatri, M. Saunders, T. Zacharewski, J.-Y. Chen, A. Staub, J.-M. Garnier, S. Mader, and P. Chambon.** 1992. Purification, cloning, and RXR identity of the HeLa cell factor with which RAR or TR heterodimerizes to bind target sequences efficiently. Cell **68**:377–395.
45. **Lenardo, M. J., A. Kuang, A. Gifford, and D. Baltimore.** 1988. NF-Kβ protein purification from bovine spleen: nucleotide stimulation and binding specificity. Proc. Natl. Acad. Sci. USA **85**:8825–8829.
46. **Lesser, D. R., M. R. Kurpiewski, T. Waters, B. A. Connolly, and L. Jen-Jacobson.** 1993. Facilitated distortion of the DNA site enhances EcoRI endonuclease-DNA recognition. Proc. Natl. Acad. Sci. USA **90**:7548–7552.
47. **Liao, J., K. Ozono, T. Sone, D. P. McDonnell, and J. W. Pike.** 1990. Vitamin D receptor interaction with specific DNA requires a nuclear protein and 1,25-dihydroxyvitamin D3. Proc. Natl. Acad. Sci. USA **87**:9751–9755.
48. **Makiguchi, K., Y. Chida, M. Yoshida, and K. Shimura.** 1984. Mg^{2+}-dependent unwinding of DNA by nonhistone chromosomal protein HMG (1+2) from pig thymus as determined by DNA melting temperature analysis. J. Biochem. **95**:423–429.
49. **Mukherjee, R.** 1993. Selective binding of the estrogen receptor to one strand of the estrogen responsive element. Nucleic Acids Res. **21**:2655–2661.
50. **Mukherjee, R., and P. Chambon.** 1990. A single-stranded DNA-binding protein promotes the binding of the purified oestrogen receptor to its responsive element. Nucleic Acids Res. **18**:5713–5716.
51. **Murray, M. B., and H. C. Towle.** 1989. Identification of nuclear factors that enhance binding of the thyroid hormone receptor to a thyroid hormone response element. Mol. Endocrinol. **3**:1434–1442.
52. **Nakajima, N., M. Horikoshi, and R. G. Roeder.** 1988. Factors involved in specific transcription by mammalian RNA polymerase II: purification, genetic specificity, and TATA box-promoter interactions of TFIID. Mol. Cell. Biol. **8**:4028–4040.
53. **Nardulli, A. M., G. L. Greene, and D. J. Shapiro.** 1993. Human estrogen receptor bound to an estrogen response element bends DNA. Mol. Endocrinol. **7**:331–340.
54. **Nardulli, A. M., and D. J. Shapiro.** 1992. Binding of the estrogen receptor DNA-binding domain to the estrogen response element induces DNA bending. Mol. Cell. Biol. **12**:2037–2042.
55. **Okamoto, K., F. Isohashi, and K. Ueda.** 1988. Purification and characterization of an adenosine triphosphate-stimulatory factor that enhances the nuclear binding of activated glucocorticoid-receptor complex from rat liver. Endocrinology **123**:2752–2761.
56. **Oñate, S. A., P. A. Estes, W. J. Welch, S. K. Nordeen, and D. P. Edwards.** 1991. Evidence that hsp-70 associated with progesterone receptors is not involved in receptor-DNA binding. Mol. Endocrinol. **5**:1993–2004.
57. **Paull, T., M. Haykinson, and R. C. Johnson.** 1993. The nonspecific DNA-binding and -bending proteins HMG-1 and HMG-2 promote the assembly of complex nucleoprotein structures. Genes Dev. **7**:1521–1534.
58. **Pettijohn, D. E.** 1988. Histone-like proteins and bacterial chromosome structure. J. Biol. Chem. **263**:12793–12796.
59. **Pil, P. M., C. S. Chow, and S. J. Lippard.** 1993. High mobility-

group 1 protein mediates DNA bending as determined by ring closures. Proc. Natl. Acad. Sci. USA **90**:9465–9469.

60. **Pil, P. M., and S. J. Lippard.** 1992. Specific binding of chromosomal protein HMG1 to DNA damaged by the anticancer drug cisplatin. Science **256**:234–237.
61. **Rodriguez, R., N. L. Weigel, B. W. O'Malley, and W. T. Schrader.** 1990. Dimerization of the chicken progesterone receptor in vitro can occur in the absence of hormone and DNA. Mol. Endocrinol. **4**:1782–1790.
62. **Ross, T. K., V. E. Moss, J. M. Prahl, and H. F. DeLuca.** 1992. A nuclear protein essential for binding of rat 1, 25-dihydroxyvitamin D3 receptor to its response elements. Proc. Natl. Acad. Sci. USA **89**:256–260.
63. **Smith, D. F., and D. O. Toft.** 1993. Steroid receptors and their associated proteins. Mol. Endocrinol. **7**:4–11.
64. **Sone, T., K. Ozono, and J. W. Pike.** 1991. A 55-kilodalton accessory factor facilitates vitamin D receptor DNA binding. Mol. Endocrinol. **5**:1578–1586.
65. **Spiro, C., J. P. Richards, S. Chandrasekaran, R. G. Brennan, and C. T. McMurray.** 1993. Secondary structure creates mismatched base pairs required for high-affinity binding of cAMP response element-binding protein to the human enkephalin enhancer. Proc. Natl. Acad. Sci. USA **90**:4606–4610.
66. **Taylor, W. H., and P. J. Hagerman.** 1990. Application of the method of phage T4 DNA ligase-catalyzed ring-closure to the study of DNA structure. II. NaCl-dependence of DNA flexibility and helical repeat. J. Mol. Biol. **212**:363–376.
67. **Thanos, D., and T. Maniatis.** 1992. The high mobility group protein HMG 1(Y) is required for NF-κB-dependent virus induction of the human IFN-β gene. Cell **71**:777–789.
68. **Toledano, M. B., and W. J. Leonard.** 1991. Modulation of transcription factor NF-κB binding activity by oxidation-reduction in vitro. Proc. Natl. Acad. Sci. USA **88**:4328–4332.
69. **Travers, A. A.** 1989. DNA conformation and protein binding. Annu. Rev. Biochem. **58**:427–432.
70. **Truss, M., and M. Beato.** 1993. Steroid hormone receptors and interaction with deoxyribonucleic acid and transcription factors. Endocrine Rev. **14**:459–479.
71. **Umesono, K., K. K. Murakami, C. C. Thompson, and R. M. Evans.** 1991. Direct repeats as selective response elements for the thyroid hormone, retinoic acid, and vitamin D3 receptors. Cell **65**:1255–1266.
72. **Wagner, S., and M. R. Green.** 1993. HTLV-1 Tax protein stimulation of DNA binding of bZIP proteins by enhancing dimerization. Science **262**:395–399.
73. **Wahlström, G. M., M. Sjöberg, M. Andersson, K. Nordström, and B. Vennström.** 1992. Binding characteristics of the thyroid hormone receptor homo- and heterodimers to consensus AGGTCA repeat motifs. Mol. Endocrinol. **6**:1013–1022.
74. **Weir, H. M., P. J. Kraulis, C. S. Hill, A. R. C. Raine, E. D. Laue, and J. O. Thomas.** 1993. The structure of the HMG box motif in the B-domain of HMG1. EMBO J. **12**:1311–1319.
75. **Xanthoudakis, S., G. Miao, F. Wang, Y.-C. E. Pan, and T. Curran.** 1992. Redox activation of *fos-jun* DNA binding activity is mediated by a DNA repair enzyme. EMBO J. **11**:3323–3335.
76. **Yang, N., R. Schule, D. J. Mangelsdorf, and R. M. Evans.** 1991. Characterization of DNA-binding and retinoic acid binding properties of retinoic acid receptor. Proc. Natl. Acad. Sci. USA **88**:3559–3563.
77. **Yoshida, M., and K. Shimura.** 1984. Unwinding of DNA by nonhistone chromosomal protein HMG (1+2) from pig thymus as determined with endonuclease. J. Biochem. **95**:117–124.
78. **Yu, V. C., C. Delsert, B. Andersen, J. M. Holloway, O. V. Devary, A. M. Naar, S. Y. Kim, J.-M. Boutin, C. K. Glass, and M. G. Rosenfeld.** 1991. RXRB: a coregulator that enhances binding of retinoic acid, thyroid hormone, and vitamin D receptors to their cognate response elements. Cell **67**:1251–1266.
79. **Zhang, X.-K., B. Hoffmann, P. B.-V. Tran, G. Graupner, and M. Pfahl.** 1992. Retinoid X receptor is an auxiliary protein for thyroid hormone and retinoic acid receptors. Nature (London) **355**:441–445.
80. **Zhang, X.-Y., C. K. Asiedu, P. C. Supakar, and M. Ehrlich.** 1992. Increasing the activity of affinity-purified DNA-binding proteins by adding high concentrations of nonspecific proteins. Anal. Biochem. **201**:366–374.