Purification and Characterization of a Protein from HeLa Cells That Binds with High Affinity to the Estrogen Response Element, GGTCAGCGTGACC

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ABSTRACT: A non-histone protein, NHP1, that binds with high affinity to the estrogen response element (ERE), GGTCAGCGTGACC, has been purified ~45 000-fold from HeLa cells by a combination of chromatography on Sephacryl S-300, heparin–Sepharose, Mono Q (FPLC), and sequence-specific oligonucleotide–Sepharose. The native protein has a molecular weight of 170 000 and is composed of two polypeptides of 85 and 75 kDa. The two polypeptides are different as judged by peptide mapping, and only the 85-kDa polypeptide can be cross-linked to the bromodeoxyuridine-substituted synthetic ERE by UV irradiation. The native protein binds to the ERE with an apparent K_D of 1×10^{-11} M and has a p*I* of 5. The contact points of the protein with individual bases of the ERE have been determined by using partially depurinated and depyrimidinated synthetic oligonucleotides. The strongest contact points of NHP1 with the ERE are 5′AGCG3′ in the center of the palindrome and differ from those of the estrogen receptor. NHP1 appears to produce specific nicks around the central CpGs of the ERE, thereby suggesting that it may play a role in active demethylation of mCpGs.

The regulation of gene expression by steroid hormones is mediated by the interaction of hormone receptor complexes with specific sequences upstream of the target gene (Yamamoto, 1985). Sequence analysis of the upstream regions of the estrogen-inducible *Xenopus* and chicken vitellogenin genes revealed a region of homology consisting of the dyad symmetry sequence GGTCANNNTGACC (Walker et al., 1984). Transfection studies carried out with deletion mutants have demonstrated that a 35 base pair sequence containing this palindrome is sufficient to confer estrogen inducibility (Seiler-Tuyns et al., 1986; Klein-Hitpass et al., 1986; Martinez et al., 1987; Burch et al., 1988).

It has been shown that the partially purified estrogen receptor complex binds preferentially to a region 600 base pairs upstream of the avian vitellogenin II gene that contains this consensus sequence (Jost et al., 1984). Further evidence for the estrogen receptor binding to the dyad symmetry sequence (ERE)[1] has been described (Kumar et al., 1987; Maurer et al., 1987; Klein-Hitpass et al., 1988a,b, 1989; Peale et al., 1988; Metzger et al., 1988; Kumar & Chambon, 1988).

The expression of the vitellogenin gene is under tissue-specific and hormonal control. During activation of the chicken vitellogenin II gene by estradiol, the whole region including the ERE becomes reversibly associated with the nuclear matrix (Jost & Seldran, 1984). Moreover, two CpGs within the ERE become demethylated in a strand-specific manner and in the absence of DNA replication (Saluz et al., 1986). The demethylation of the mCpG in the upper strand correlates well with the induction of vitellogenin mRNA synthesis whereas the demethylation of the mCpG in the complementary DNA strand lags by 24 h. More recently, protein–DNA interactions at the ERE have been visualized by electron microscopy (ten Heggeler-Bordier et al., 1987, 1988), and the large complexes formed suggest that more than one protein binds to this region. This paper describes the purification and characterization of a protein different from the estrogen receptor that binds with high affinity to the ERE.

Materials and Methods

DNA–Protein Binding Assays. Gel shift assays were carried out as previously described (Jost et al., 1987) with the addition of 0.25 mg/mL bovine serum albumin. The end-labeled double-stranded synthetic oligonucleotide 5′TCCTGGTCAGCGTGACCGGA3′ was used as the substrate. For the quantitative determination of NHP1 present in the various chromatographic fractions, it was assumed that the labeled DNA and NHP1 bind with a 1:1 stoichiometry.

Preparation of Affinity Column. The oligonucleotides 5′GATCCCTGGTCAGCGTGACCGGA3′ (upper strand) and 5′GATCTCCGGTCACGCTGACCAGG3′ (lower strand) were annealed, phosphorylated, and ligated essentially as described by Kadonaga and Tjian (1986). The ligation products were coupled to CNBr-activated Sepharose 4B (Pharmacia) according to the manufacturer's instructions.

Purification of NHP1. Cell extracts were prepared from 30 g of HeLa cells as described by Jiricny et al. (1988). Ammonium sulfate was added to 70% saturation to the supernatant, and the precipitate was collected by centrifugation at 15000*g* for 30 min at 4 °C. The pellet was resuspended in buffer A [25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid–KOH, pH 8.0, 1 mM EDTA, 1 mM benzamidine, 10 mM 2-mercaptoethanol, and 10% glycerol] containing 0.1 M KCl and applied at a flow rate of 1 mL/min onto a Sephacryl S-300 column (Pharmacia, 3 × 100 cm) equilibrated with the same buffer. The S-300 fractions having ERE binding activity were pooled and loaded onto a column containing 10 mL of heparin–Sepharose (Pharmacia, HR10/10) equilibrated with buffer A, containing 0.1 M KCl. After the column was washed with buffer A, containing 0.1 M KCl, proteins were eluted at 1 mL/min over 30 min using a linear gradient of 0.1–0.6 M KCl, and finally the column was washed with 10 mL of buffer A, containing 1 M KCl. Fractions containing NHP1 were pooled and diluted to 0.1 M KCl before being loaded onto a 1-mL Mono Q FPLC column (Pharmacia) equilibrated with buffer A, containing

[1] Abbreviations: ER, estrogen receptor; ERE, estrogen response element; mCpG, 5-methylcytosine, guanosine dinucleotide; NHP1, non-histone protein 1; PMSF, phenylmethanesulfonyl fluoride; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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0.1 M KCl. Following a 10-min wash with the same buffer, elution was carried out at 1 mL/min using a linear gradient of 0.1–1.0 M KCl over 50 min. The Mono Q fractions with NHP1 activity were pooled and diluted with buffer A to 0.1–0.15 M KCl. *Escherichia coli* DNA was added as nonspecific competitor at 0.5 mg/5 mg of protein before being loaded onto a 4-mL oligonucleotide affinity column. The column was washed with buffer A containing 0.1, 0.2, 0.3, 0.6, and 1 M KCl.

Protein concentrations were measured according to Bradford (1976) using bovine serum albumin as the standard.

Estimation of Native Size of NHP1 by Gel Filtration. Gel filtration was carried out on a Superose 12 column (Pharmacia, 24 mL) equilibrated with buffer A, containing 0.3 M KCl. A heparin–Sepharose fraction (2.5 mg of protein in 200 μL) containing NHP1 was loaded onto the column, and protein was eluted at 0.5 mL/min.

Determination of the pI of NHP1. A Mono Q fraction containing NHP1 was diluted to 10 mM KCl and loaded onto a Mono P column (HR5/20, Pharmacia) equilibrated with 25 mM Bis-Tris-HCl, 10 mM KCl, 2% glycerol, 5 mM 2-mercaptoethanol, and 1 mM phenylmethanesulfonyl fluoride (PMSF), pH 7.0. The protein was eluted with 10% Polybuffer 74 (Pharmacia) in 10 mM KCl, 2% glycerol, 1 mM EDTA, 5 mM 2-mercaptoethanol, and 1 mM PMSF, pH 4, at a flow rate of 0.5 mL/min. The pH of the fractions was measured, and samples of each fraction were taken for gel shift assay and analysis by SDS–polyacrylamide gel electrophoresis (SDS–PAGE).

Peptide Mapping with V8 Protease. Affinity-purified NHP1 was ^{125}I labeled as described by Bolton and Hunter (1973). After SDS–PAGE and autoradiography, the 85- and 75-kDa polypeptides were removed and equilibrated in 0.126 M Tris-HCl/0.1% SDS, pH 6.8. The polypeptides were digested with *Staphylococcus aureus* V8 protease (Miles Laboratories) during electrophoresis in the second dimension (Hames & Rickwood, 1985). The products were separated on a 17% SDS–polyacrylamide resolving gel after which the gel was subjected to autoradiography.

Cross-Linking Experiments. The oligonucleotides 5′TAAGGACCAGTCGCACTGGCCTCGACTTTCTTGTGTAACTAGGGG3′ and 5′TAGTTACACAAGAAAGTCG3′ were annealed, and the NHP1 binding site was synthesized by using Klenow fragment in the presence of dGTP, dCTP, [α-^{32}P]dATP, and 5-bromo-dUTP. It has previously been shown that NHP1 has a reduced affinity for a mutant oligonucleotide where the three bases at the center of the palindrome are missing (Feavers et al., 1987). The mutant oligonucleotide was synthesized after annealing the oligonucleotide 5′TAAGGACCAGTACTGGCCTCGACTTTCTTGTGTAACTAGGGG3′ to the 20-mer described above. The protein–DNA complexes were formed as described (Jost et al., 1987) and were irradiated and nuclease treated essentially as described by Chodosh et al. (1986). The reaction mixture was then separated on a 10% SDS–polyacrylamide gel and subjected to autoradiography.

Missing Contact Probing of DNA–Protein Interactions. Double-stranded synthetic oligonucleotides containing the NHP1 binding site were end-labeled in a strand-specific manner with T4 polynucleotide kinase (Biofinex) using adenosine 5′-[γ-^{32}P]triphosphate (3000 Ci/mmol; Amersham) (Maniatis et al., 1982). Base modifications were performed essentially as described (Brunelle & Schleif, 1987), except that the reactions were carried out with 10 ng of labeled DNA in the presence of 30 μg of carrier *E. coli* DNA. Dimethyl sulfate (0.02% v/v) was utilized for the G reaction, and for the G + A reaction, the DNA was dissolved in 10 μL of water and incubated with 25 μL of formic acid at 20 °C for 2.5 min. Upon ethanol precipitation, the sediment was dissolved in 10 μL of water, and 8 μL was used to form a protein–DNA complex with HeLa cell lysate containing 20 μg of protein (Jost et al., 1987). The complex was separated from free DNA on a 1% low gelling temperature agarose gel in 89 mM Tris–borate, 89 mM boric acid, and 2 mM EDTA buffer. After autoradiography of the gel, the protein–DNA complex was cut out and the DNA extracted (Maniatis et al., 1982). The purified bound and free DNAs were subjected to piperidine cleavage (Maxam & Gilbert, 1977), separated on a sequencing gel, and autoradiographed.

Demonstration of Specific Nicking Activity of NHP1. Affinity-purified NHP1 (1 ng) and 25 μg of bovine serum albumin (enzyme grade, BRL) were incubated with 5′ end-labeled oligonucleotides (200 000 cpm) in 30 μL of 50 mM Tris, pH 8.0, 100 mM sucrose, 100 mM KCl, 3 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM dithiothreitol, 0.5 mM spermidine, and 0.2 mM spermine. Both the ERE and the mutant oligonucleotide lacking the central GCG were tested. Upon incubation at 37 °C for 2 h, 3-μL aliquots were mixed with 5 μL of 95% formamide, 10 mM EDTA, 0.05% xylene cyanol, and 0.05% bromophenol blue. The samples were heated at 95 °C for 3 minutes, quick-chilled, and loaded onto a 20% polyacrylamide sequencing gel.

Table I: Purification of NHP1

fraction	protein (mg)	sp act. (fmol/μg)	total binding act. (fmol)	yield (%)	x-fold purification
crude extract	800	0.11	88 000	100	1
Sephacryl S-300	205	0.22	45 000	51	2
heparin–Sepharose	37	0.67	24 800	28	6
Mono Q	11	1.75	19 200	22	16
oligo-Sepharose	0.003	5111	15 500	17	46 000

Results

Purification of NHP1. The non-histone protein NHP1, which binds with high affinity to the ERE, was purified from HeLa cells since it is at least 10 times more abundant in HeLa cell extracts than in chicken liver nuclear extracts. The proteins from both sources are probably very similar since both gave identical results when tested by the proteolytic clipping bandshift assay as described by Schreiber et al. (1988). Moreover, the NHP1 from both sources had the same chromatographic behavior (data not shown). At each step of the purification, NHP1 was identified by its specific binding to the ERE by the gel shift assay. Table I shows that with a combination of four steps (Figures 1 and 2), the protein could be purified ~45 000-fold with a 17% recovery of the total ERE binding activity. When analyzed by SDS–PAGE, the highly purified NHP1 fraction gave upon silver staining (Burk et al., 1983) two bands corresponding to 85 and 75 kDa (Figure 3). NHP1 was initially thought to be a single polypeptide of 70 kDa (Feavers et al., 1987); however, this polypeptide probably resulted from proteolytic degradation during processing which was carried out in the absence of protease inhibitors.

Determination of the Native Molecular Weight of NHP1. Following chromatography on Superose 12, each fraction was assayed for NHP1 DNA binding activity by the gel shift assay. As shown in Figure 4, NHP1 eluted with a K_{av} corresponding to a molecular weight of ~170 000. This indicates that NHP1 is a dimer in its native state.

Peptide Mapping of the 85- and 75-kDa Polypeptides. To test whether the 75-kDa polypeptide is a degradation product

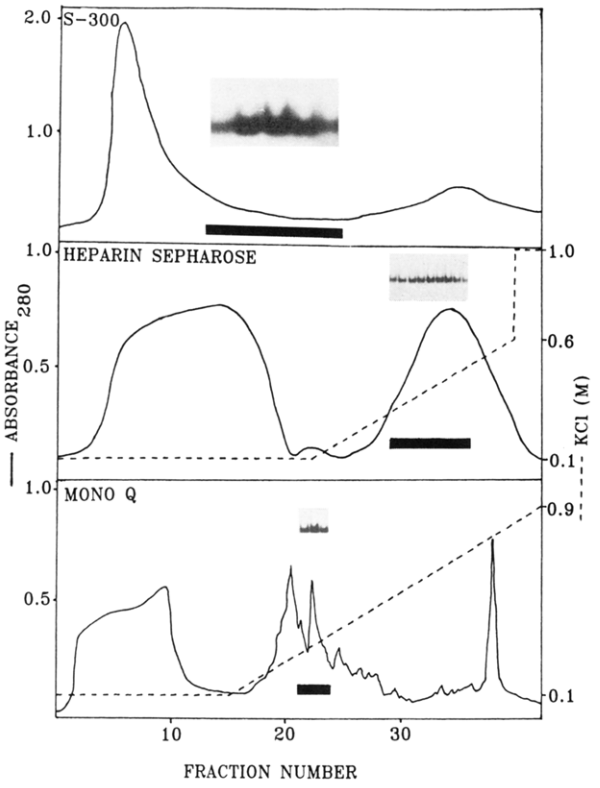

FIGURE 1: Elution profiles of proteins on Sephacryl S-300 (upper panel), heparin–Sepharose (middle panel), and Mono Q (lower panel). The inserts show gel shift assays of the fractions with activity (protein–DNA complex), and the solid bars correspond to the pooled fractions containing NHP1. Gel shift assays were carried out by incubating 0.5 ng of end-labeled oligonucleotide with 5 μL of each protein fraction in the presence of 2.5 μg of *E. coli* DNA. Protein concentration was followed by monitoring A_{280}.

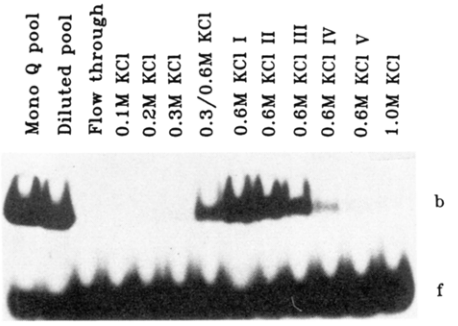

FIGURE 2: Gel shift assay of the protein fractions eluted from the DNA affinity column. Chromatography was carried out as described under Materials and Methods. Gel shift assays were carried out by incubating 0.5 ng of end-labeled oligonucleotide with 5 μL of protein fraction in the presence of 750 ng of *E. coli* DNA. The labels above the gel indicate the fraction from which the protein was taken for the gel shift assay. The 0.6 M KCl fractions were collected in 1-mL aliquots as indicated by the Roman numerals. Bands b and f are bound and free DNA, respectively.

of the 85-kDa polypeptide, peptide maps were carried out with *S. aureus* V8 protease. Figure 5 shows that the polypeptides are different, suggesting that the native NHP1 is a heterodimer and that the 75-kDa polypeptide is not a degradation product of the 85-kDa polypeptide.

Isoelectric Point of NHP1. Proteins eluted from a Mono P column were analyzed by SDS–PAGE and gel shift assay. Two polypeptides of molecular weight 85000 and 75000 eluted at pH 5.1 together with the ERE binding activity (data not shown).

Identification of the Polypeptide Binding to the ERE. As described above, the molecular weight of the native NHP1 is

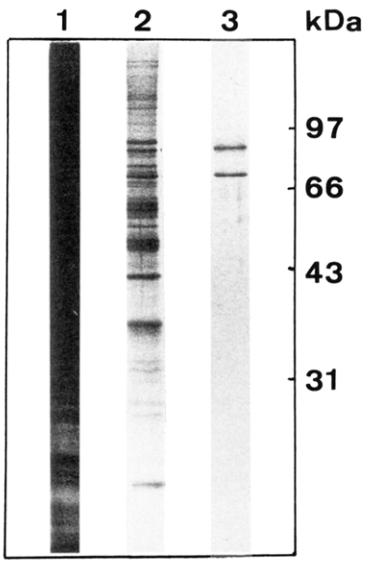

FIGURE 3: SDS–PAGE at different stages of the purification. Fractions were subjected to 10% SDS–PAGE and stained with silver. Lane 1 represents 2 μg of heparin–Sepharose fraction, lane 2, 0.4 μg of Mono Q fraction, and lane 3, 100 μL (~100 ng) out of 1 mL of purified protein fraction from the affinity column. The latter fraction was precipitated with a final concentration of 15% trichloroacetic acid and then washed with acetone before loading on the gel. Size markers are indicated in kilodaltons.

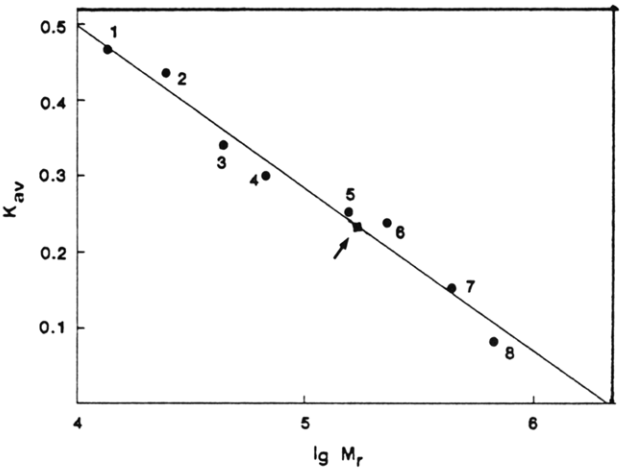

FIGURE 4: Determination of the native molecular weight of NHP1. The native size of NHP1 was determined by gel filtration on Superose-12. Each fraction was tested for ERE binding activity by gel shift assay. The standard curve was determined by following the elution of a set of proteins: ribonuclease (1), chymotrypsinogen A (2), ovalbumin (3), albumin (4), aldolase (5), catalase (6), ferritin (7), and thyroglobulin (8). The molecular weight of NHP1 is indicated by the arrow on the standard curve.

170000, and when analyzed by SDS–PAGE, two polypeptides of 85 and 75 kDa were seen. It was therefore of interest to identify which polypeptide(s) bind(s) to the ERE. This was carried out by UV cross-linking of the native protein to the bromodeoxyuridine-substituted ERE with subsequent separation of the reaction products by SDS–PAGE. As shown in Figure 6, lane 2, after binding to the ERE, UV cross-linking, and nuclease digestion, only the 85-kDa polypeptide was retained by the ERE. The apparent double band seen in lane 2 is probably due to incomplete digestion of the complex with DNase I. The mutant ERE lacking the GCG at the center of the dyad symmetry sequence was much less effective in binding the protein (lane 1).

DNA Substrate Specificity of NHP1. The binding specificity of partially purified NHP1 with the ERE was tested by titrating the protein with a constant amount of end-labeled

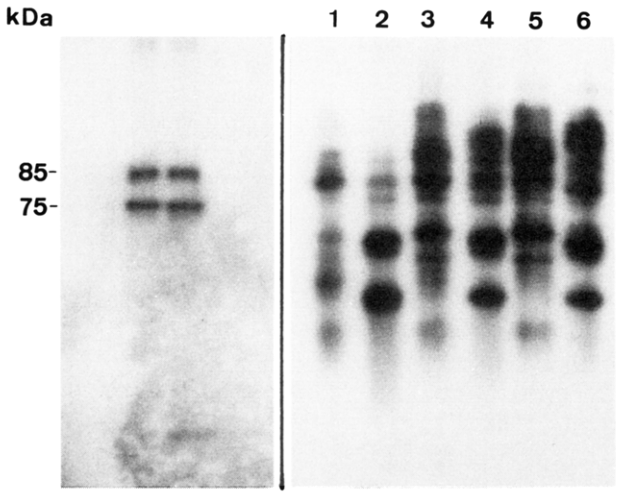

FIGURE 5: Peptide mapping of the 85- and 75-kDa polypeptides. Purified NHP1 was labeled with ^{125}I, and the polypeptides were subjected to peptide mapping as described under Materials and Methods. The 85- and 75-kDa ^{125}I-labeled polypeptides are shown in the left panel. The peptide maps are shown in the right panel. Lanes 1, 3, and 5 contained digests carried out with the 85-kDa polypeptide and lanes 2, 4, and 6 with the 75-kDa polypeptide. Lanes 1 and 2, 3 and 4, and 5 and 6 contained digests carried out with 100, 50, and 10 ng of V8 protease, respectively.

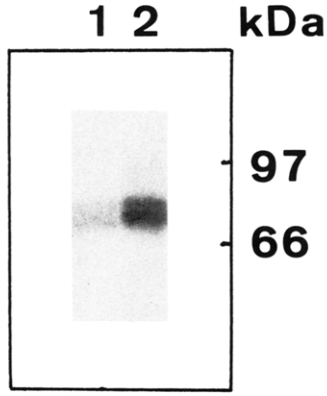

FIGURE 6: UV cross-linking of NHP1 to the ERE. UV cross-linking was carried out as described under Materials and Methods. Lane 1 shows faint bands corresponding to polypeptide cross-linked to the mutant oligonucleotide lacking the GCG at the center of the wild-type palindrome whereas lane 2 shows distinct bands corresponding to the 85-kDa polypeptide cross-linked to the wild-type ERE. Size markers are shown in kilodaltons.

oligonucleotides. The following oligonucleotides were used: single-stranded ERE 5′TCCTGGTCAGCGTGACCGGA3′; double-stranded ERE (wild type); and two mutants, one having the central GCG deleted and the other having the central GCG replaced by AGCGA. Figure 7 demonstrates that single-stranded DNA is not a substrate for NHP1 and that deletion of the 3 base pair spacer substantially decreases NHP1 binding whereas binding to the oligonucleotide with the 5 base pair spacer is increased.

Binding Constant of NHP1 with the ERE. The equilibrium binding constant was determined by measuring the amount of complex formed between NHP1 and an oligonucleotide containing the ERE as a function of increasing DNA concentration. Scatchard analysis of the data yielded a straight line (Figure 8), and assuming that the ERE and NHP1 bind in a 1:1 stoichiometry, an apparent dissociation constant of 1×10^{-11} M was obtained.

Contact Points of NHP1 with the ERE. Missing contact probing, as described by Brunelle and Schleif (1987), was used to determine the contact points of the native NHP1 with the

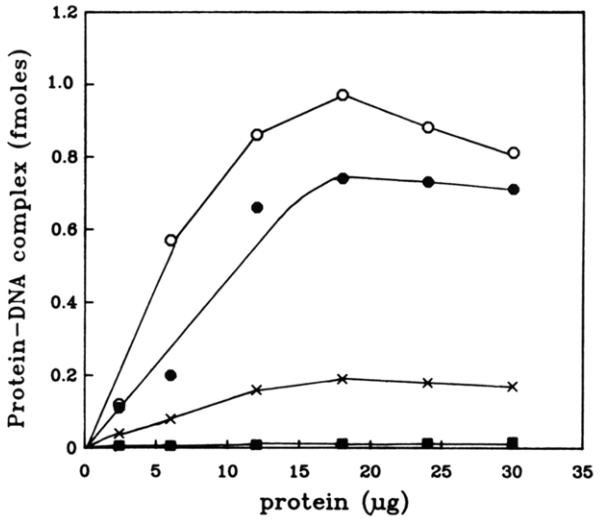

FIGURE 7: Specificity of DNA binding. Protein eluted from a Mono Q column and containing ERE binding activity was titrated with oligonucleotides, each labeled to the same specific activity, as described under Materials and Methods. Wild-type ERE (●), ERE containing AGCGA spacer (○), ERE with no spacer (+), and the upper strand of the ERE (■) were used as substrates. After electrophoresis on a 4% native polyacrylamide gel, the bands corresponding to the protein–DNA complex were cut out and assayed for radioactivity.

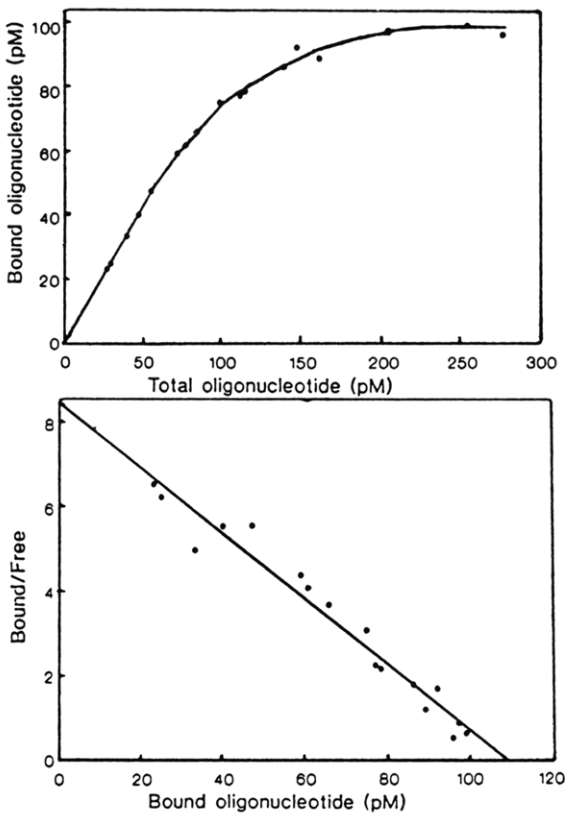

FIGURE 8: Dissociation constant of NHP1 with the ERE. Purified protein eluted from the oligonucleotide affinity column (0.3 ng) was titrated with increasing concentrations of ^{32}P-labeled ERE in the presence of 1 ng of *E. coli* DNA in a total volume of 7 μL. After a 15-min incubation at room temperature, the complex was separated from the free oligonucleotide on a 6% native gel. After autoradiography, the free and bound oligonucleotides were cut out of the gel and assayed for radioactivity. The titration curve is shown in the upper panel. The K_D was determined from a Schatchard plot of the data (lower panel).

ERE. Figure 9 shows the effect of partial depyrimidination (C + T), acid depurination (G + A), and methylation depurination (G only) on the binding. When the intensities of bands

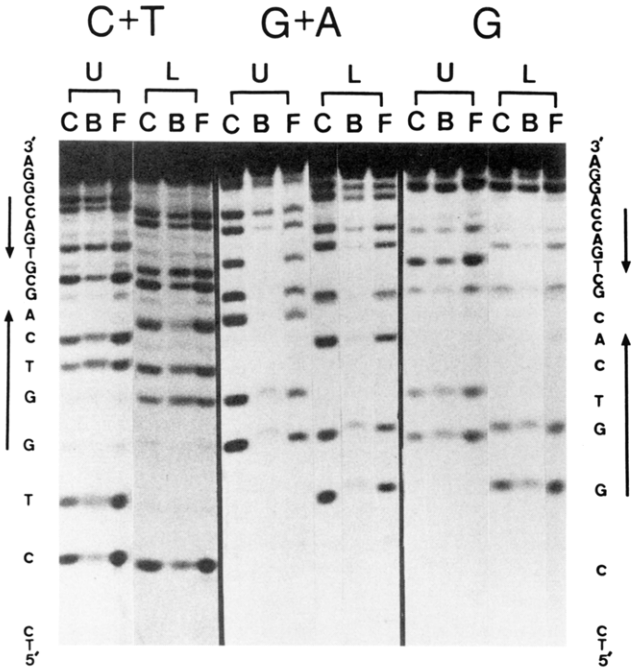

FIGURE 9: Missing contact probing of NHP1 with the ERE. The partial depyrimidination and depurination reactions and subsequent processing are described under Materials and Methods. The results of the C + T, G + A, and G reactions are shown for the upper (U) and lower (L) strands of the ERE. The bound DNA (B), eluted from the NHP1–ERE complex, can be compared with control DNA (C) and the pretreated free DNA (F). The upper and lower strand oligonucleotide sequences are indicated at the side of the figure, and the dyad symmetry region is indicated by arrows.

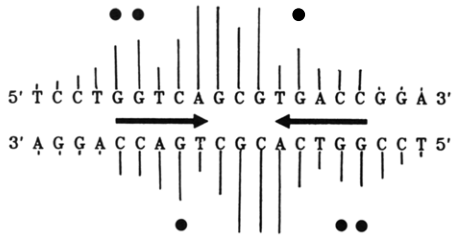

FIGURE 10: Summary of contact points of NHP1 with ERE. The diagram summarizes the results shown in Figure 9. The lengths of the vertical lines indicate the importance of the corresponding base for the binding of NHP1. The horizontal arrows emphasize the dyad symmetry of the ERE. The filled circles indicate which bases are important for the binding of the ER homodimer (Kumar & Chambon, 1988; Klein-Hitpass et al., 1989).

in lanes corresponding to bound and free DNA are compared, a significant effect is seen when the three bases in the spacer and the neighboring A residues are missing. Weaker effects are seen when bases are missing in the GGTC and CCAGT sequences of both strands. All other positions show either no or very weak effects. The results are summarized in Figure 10. These results are consistent with the titration experiments presented in Figure 7, where the importance of the three-base spacer in the palindrome is shown.

Demonstration of Specific Nicking Activity of NHP1. NHP1 appears to produce specific nicks in oligonucleotides containing the ERE where the CpGs are methylated (Figure 11) when compared to the same oligonucleotide incubated with BSA alone. No specific nicks were seen to be produced by NHP1 when it was incubated with a mutant oligonucleotide lacking the central GCG of the wild-type ERE.

Discussion

The simultaneous binding of two different proteins, the

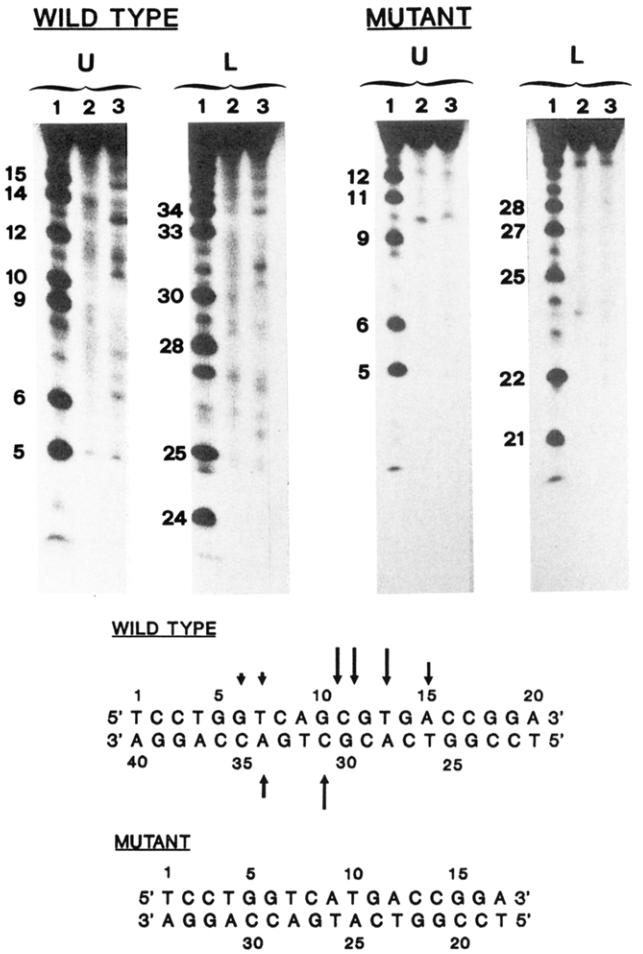

FIGURE 11: Demonstration of specific nicking activity of NHP1. Incubations and reaction conditions were as described under Materials and Methods. In each case, lane 1 represents the G + A reactions of the oligonucleotide, lane 2 incubation of the oligonucleotide with BSA alone, and lane 3 incubation of the oligonucleotide with BSA and affinity-purified NHP1. The effect of NHP1 on the upper (U) and lower (L) strands of the wild-type ERE is compared to that on both strands of the mutant ERE. The numbers at the side of the tracks correspond to the number of the base on the summary diagram shown in the lower panel. The residues at positions 11, 17, 23, and 29 were methylcytosine in the wild-type ERE. The arrows indicate the extent and position of nicking of the ERE in the presence of NHP1.

estrogen receptor (ER) and NHP1, to the ERE can be accounted for by the differences in their DNA footprints (Figure 10; Kumar & Chambon, 1988; Kelin-Hitpass et al., 1989). The homodimer of the ER could bind to the arms of the palindrome in the major groove on one side of the double helix, whereas NHP1 could be tightly associated to the spacer GCG of the palindrome in the major groove on the opposite side of the double helix. NHP1 is most likely not related to the ER since it does not bind to 17β-[^{3}H]estradiol and does not cross-react with a monoclonal antibody directed against the calf uterus ER (Feavers et al., 1987; Hughes and Jost, unpublished results). Moreover, peptide sequences derived from the 85-kDa polypeptide of NHP1 show no homology to the sequence of the ER (Hughes, unpublished results), and HeLa is a human ER-negative cell line (Walter et al., 1985).

The question arises as to what kind of role NHP1 plays in the estrogen induction of vitellogenin gene expression. The binding of NHP1 to the ERE could tag the DNA for subsequent binding of the ER (Feavers et al., 1987), or it may contribute to the biology of estrogen action in general by contacting and stabilizing specific binding of the receptor with its enhancer element (Klein-Hitpass et al., 1989). Finally,

NHP1 could have a direct enzymatic function essential for the active demethylation of the mCpG present in the spacer of the palindrome. We have previously shown that upon stimulation of the chicken vitellogenin II gene by estradiol the mCpGs present in the ERE and neighboring sequences are demethylated in a strand-specific manner (Saluz et al., 1986) and that the demethylation is parallel to the onset of gene transcription. It has also been suggested that the demethylation caused by estrogen is an active process where no DNA replication is required (Wilks et al., 1984; Saluz et al., 1986). A similar active demethylation in differentiating Friend leukemia cells has been suggested by Razin et al. (1986). In our present case, it is interesting to note that the sequence AGCG containing the methylation site plays an important role in the binding of NHP1 to the ERE (Figure 9). Moreover, in experiments where this sequence was deleted or replaced by CAG, very poor binding of NHP1 was observed, thus emphasizing the importance of the CpG for the binding of NHP1 to the ERE (Figure 7; Feavers et al., 1987). The possible involvement of NHP1 in a general mechanism of active demethylation would account for its apparent ubiquity. Preliminary experiments carried out with affinity-purified NHP1 indicate that this protein has specific nicking activity when tested with the ERE compared to the mutant oligonucleotide lacking the GCG in the center of the palindrome. This could represent one of the first steps in the process of the active demethylation of mCpGs. However, the functional relationship between ER and NHP1, if any, still remains to be firmly established.

Acknowledgments

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