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The ubiquitous nuclear protein, NHP1, binds with high affinity to different sequences of the chicken vitellogenin II gene

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ABSTRACT

In gel shift assays, affinity chromatography-purified NHP1 forms a stable complex with different sequences of the chicken vitellogenin II gene. The apparent K_D of NHP1 with the estrogen response element (ERE) containing 5-methylcytosine is 1×10^{-11}M. NHP1 does not form a complex with the *Xenopus* vitellogenin ERE where the GCG bases are replaced by CAG. NHP1 is closely related if not identical to the other ubiquitous proteins NHP2, NHP3 and NHP4 that bind specifically to different sequences. All four proteins behave identically on chromatography and give identical patterns in proteolytic bandshift assays. NHP1, NHP2 and NHP3 have a native molecular weight of 170 000 and are composed of two polypeptides of 85 and 75 kDa. The possible function of NHP1 is discussed.

INTRODUCTION

The chicken vitellogenin II gene provides an ideal model system to investigate the molecular mechanisms of gene regulation since both tissue- and hormone-specific regulation of transcription can be studied. During *in vivo* primary activation of the gene with estradiol, the gene and its flanking regions (1.5−2 kb) have been shown to associate selectively and reversibly to the nuclear matrix (1). In addition, three DNase I-hypersensitive sites are induced near the 5′ end of the gene (2,3), in parallel to a strand-specific demethylation of several CpG dinucleotides located upstream of the gene (4, 5).

We have previously identified proteins that interact specifically with sequences upstream and within the chicken vitellogenin II gene (Fig. 1). The specific interaction of two nonhistone proteins, NHP1 and NHP2, with the estrogen response element (ERE) and a neighbouring sequence of the chicken vitellogenin II gene was demonstrated by use of gel shift assays, dimethylsulphate protection and competition experiments. NHP1 and NHP2 were shown to be neither tissue nor species specific and enhanced the binding efficiency of the estrogen receptor complex to the ERE (6).

The non-histone protein, NHP3, was shown to bind preferentially to a sequence within the third intron of the chicken vitellogenin II gene. NHP3 bound its substrate specifically with an apparent dissociation constant of 3.5×10^{-10}M (7).

The sequence of the DNase I-hypersensitive site at the transcription start site is highly conserved among the vitellogenin genes of chicken, *Xenopus laevis* and *Caenorhabditis elegans* (8,9,10). The protein-DNA interactions at this site have been studied *in vivo* by genomic footprinting and *in vitro* by DNase I protection experiments and gel shift assays. The results showed that several proteins bind to the transcription start site. One of these proteins, NHP4, bound to this sequence irrespective of the methylation state of the CpG dinucleotide within this sequence and also appeared to be ubiquitous, being found in many different organs and species (5).

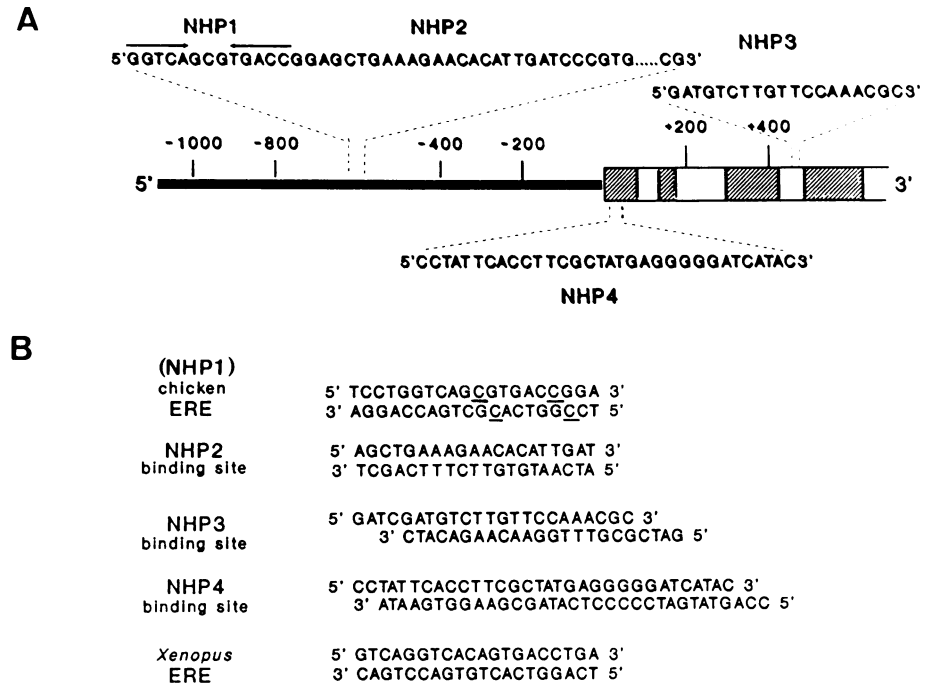

Fig. 1. *The nonhistone proteins NHP1, NHP2, NHP3 and NHP4 bind to DNA sequences upstream and within the chicken vitellogenin II gene.* The location of these sequences are summarized in panel A and the oligonucleotides used as substrates for gel shift assays are summarized in panel B. Chicken ERE and *Xenopus* ERE represent the vitellogenin EREs in each case. The underlined cytosine residues were replaced by 5-methylcytosine where applicable.

More recently, NHP1, which binds with high affinity to the ERE, has been purified from HeLa cells and extensively characterized (11). Here we show that NHP1 is capable of binding with high affinity to different sequences and that it is related if not identical to NHP2, NHP3 and NHP4.

MATERIALS AND METHODS

DNA-Protein Binding Assays

Gel shift assays were carried out as previously described (7) with the addition of 0.25 mg/ml bovine serum albumin. End-labelled double-stranded synthetic oligonucleotides were used as substrates. The sequences of these oligonucleotides are summarized in Fig. 1b.

Preparation of Cell Extracts and Purification of the Nonhistone Proteins

Cell extracts were prepared from 30 g of HeLa cells and chromatography was carried out essentially as described (12). However, heparin-Sepharose (50 ml) was incubated batchwise with the diluted HeLa cell lysate and chromatography of the protein on the Mono Q column was carried out in the presence of benzamidine as the only protease inhibitor. NHP1 was purified as described (11).

Purification of NHP2 and NHP3 by Affinity Chromatography

The affinity matrix for NHP2 was synthesized using the concatomerized oligonucleotides

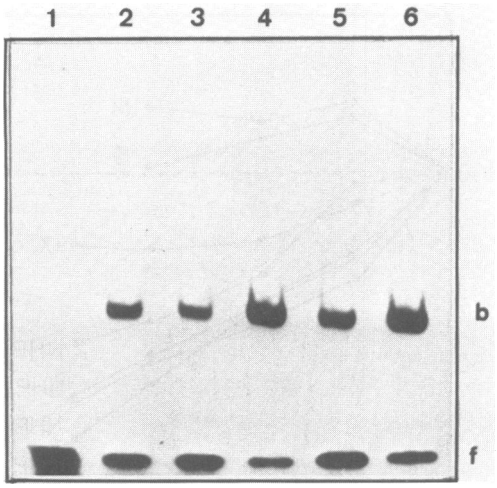

Fig. 2. *Gel shift assay of purified NHP1 with different oligonucleotides.* Gel shift assays were carried out with purified NHP1 as described (11). The oligonucleotides were *Xenopus laevis* vitellogenin ERE (lane 1), chicken vitellogenin II ERE (lane 2), chicken vitellogenin II ERE containing 5-methylcytosine (lane 3), NHP2 binding site (lane 4), NHP3 binding site (lane 5) and NHP4 binding site (lane 6). Bands b and f are bound and free DNA respectively.

5′ GATCAGCTGAAAGAACACATTGAT 3′ and 5′ GATCATCAATGTGTTCTTT-CAGCT 3′ and the matrix for NHP3 was synthesized using 5′ GATCGATGTCTTGTT-CCAAACGC 3′ and 5′ GATCGCGTTTGGAACAAGACATC 3′ essentially as described (13). Affinity chromatography was carried out as described for NHP1 (11), however in the case of NHP3, the non-specific *Escherichia coli* DNA was reduced to a third.

Estimation of Native Size of the Nonhistone Proteins by Gel Filtration

Gel filtration was carried out on a Superose 12 column as described (11).

RESULTS

Binding Specificity of Purified NHP1

It has previously been shown that NHP1 binds very weakly to an oligonucleotide homologous to the ERE except that the central GCG is deleted (11). The gel shift assay in Fig. 2 shows that affinity purified NHP1 forms a stable complex with the ERE, the ERE containing 5-methylcytosine as well as the binding sites of NHP2, NHP3 and NHP4. However, NHP1 does not form a complex with the palindromic *Xenopus laevis* vitellogenin ERE, that lacks the CpG (Fig.2, lane 1). Cross-competition bandshift experiments shown in Fig. 3 confirm that NHP1 binding to the chicken vitellogenin ERE binds also to the other oligonucleotides at the exclusion of single-stranded DNA and the mutated ERE lacking the CpG in the spacer of the palindrome.

The Binding Constant of NHP1 with the ERE containing 5-methylcytosine

The equilibrium binding constant was determined as described (11). Scatchard analysis of the data yielded a straight line (Fig. 4). Assuming that NHP1 and the ERE containing 5-methyl cytosine bind in a 1:1 stoichiometry, an apparent dissociation constant of 1×10^{-11}M was obtained. This is identical to that obtained with NHP1 and the unmethylated ERE (11).

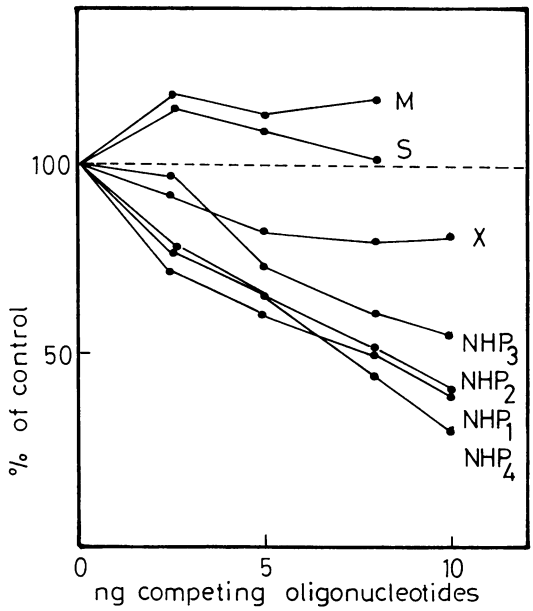

Fig. 3. *Cross-competition bandshift assays of the protein-DNA complex, NHP1-ERE, with oligonucleotides containing the binding sites of NHP2, NHP3 and NHP4.* The upper strand of the ERE is indicated by SS, whereas M and X are the mutated ERE lacking the GCG in the middle of the palindrome and the *Xenopus* vitellogenin ERE respectively. Five nanograms of the ^{32}P-labelled ERE-oligonucleotide were incubated with 4 μg of partially purified NHP1 from HeLa cells (post heparin-Sepharose fraction) and increasing concentrations of unlabelled oligonucleotide. The reaction product was separated on gels and the protein-DNA complexes were cut out and counted for radioactivity (100% represents the radioactivity in the protein-DNA complex in the absence of competing oligonucleotides, usually 50 000 CPM).

Chromatographic Behaviour of NHPs 1, 2, 3 and 4

The HeLa cell extract was incubated batchwise with heparin-Sepharose and the proteins were step-eluted. NHP1, NHP2, NHP3 and NHP4 were eluted with 0.5 M KCl. Initially, NHP3 was thought to bind both its single-stranded and double-stranded substrate (7) but only the NHP3 binding to double-stranded oligonucleotides was eluted with 0.5 M KCl.

After ammonium sulphate precipitation (70% saturation, final concentration), the proteins eluted with 0.5 M KCl were subjected to gel filtration on a Sephacryl S-300 column. NHP1, NHP2, NHP3 and NHP4 co-eluted after the major protein peak.

The S-300 fractions containing NHP1, NHP2, NHP3 and NHP4 were pooled and further fractionated by anion exchange chromatography on a Mono Q FPLC column. The gel shift assays in Fig. 5 show that NHP1, NHP2, NHP3 and NHP4 elute in the same fractions between 0.25 and 0.3 M KCl. We have previously indicated that NHP1 from HeLa cells and chicken liver nuclear extracts have the same chromatographic behaviour and give identical patterns when tested by the proteolytic clipping bandshift assay (11).

Purification of NHP2 and NHP3 by Affinity Chromatography

Both NHP2 and NHP3 are eluted from their respective affinity columns with 0.6 M KCl and fractions containing NHP2 and NHP3, when analyzed on silver-stained SDS-

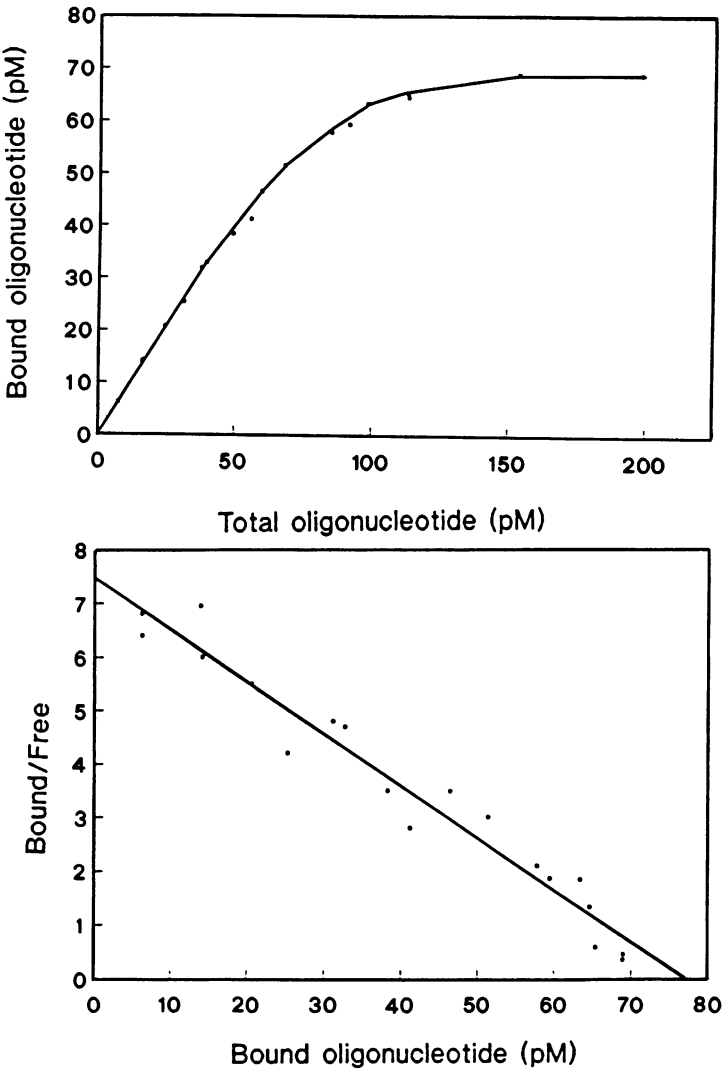

Fig. 4. *Dissociation constant of NHP1 with the ERE containing 5-methylcytosine.* The titration curve of NHP1 with the ERE containing 5-methylcytosine was obtained as described (11) and is shown in the upper panel. The K_D was determined from a Scatchard plot of the data (lower panel).

polyacrylamide gels, contain the 85 and 75 kDa polypeptides (Figs. 6 and 7). Lane 9 in Figs. 6 and 7 shows the proteins eluted from ethanolamine-inactivated CNBr-Sepharose that had been treated in an identical manner as the NHP2 and NHP3 affinity columns. This indicates that most of the bands between 55 and 66 kDa seen on the SDS-polyacrylamide gels are non-specific.

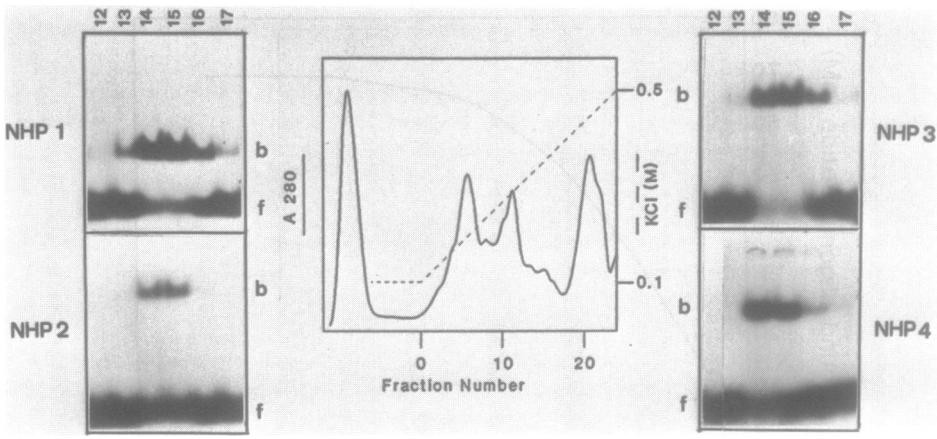

Fig. 5. *NHP1, NHP2, NHP3 and NHP4 coelute on ionexchange chromatography.* After chromatography on heparin-Sepharose and Sephacryl S-300, the nonhistone proteins were subjected to anion exchange on Mono Q. Gel shift assays carried out with oligonucleotides containing the four different binding sites and the elution profile are shown. The numbers above each lane correspond to the fraction number. Bands b and f are bound and free DNA respectively.

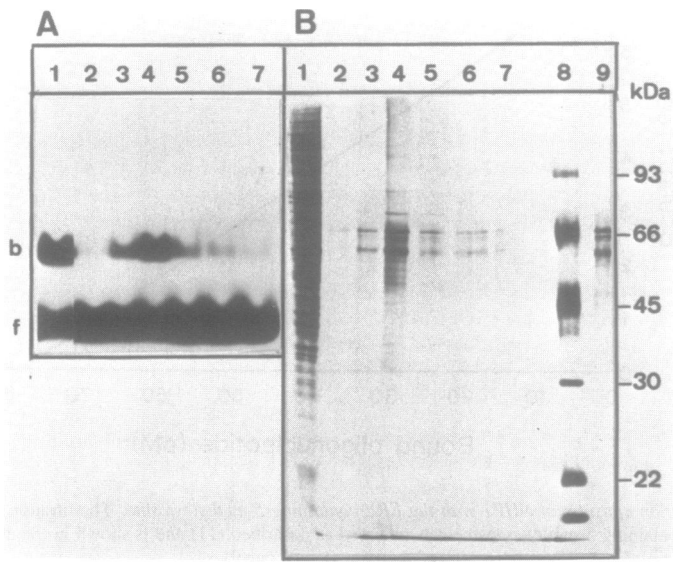

Fig. 6. *Purification of NHP2 by affinity chromatography.* A Mono Q fraction was enriched for NHP2 by chromatography on oligonucleotide-Sepharose as described in Materials and Methods. Gel shift assays and SDS-polyacrylamide gel electrophoresis was carried out as described (11). Panel A shows a gel shift assay with an aliquot of the diluted Mono Q fraction (lane 1), the 0.2 M KCl eluate (lane 2), the 0.3 M KCl eluate (lane 3) and fractions (1 ml) of the 0.6 M KCl eluate (lanes 4–7) from the affinity column. The oligonucleotide containing the NHP2 binding site was used as substrate in each case. Bands b and f are bound and free DNA respectively. Panel B shows a silver-stained SDS-polyacrylamide gel. Lanes 1–7 are as above, size markers are in lane 8 (and indicated in kDa on the right side of the gel) and lane 9 contains protein eluted from inactivated CNBr-Sepharose.

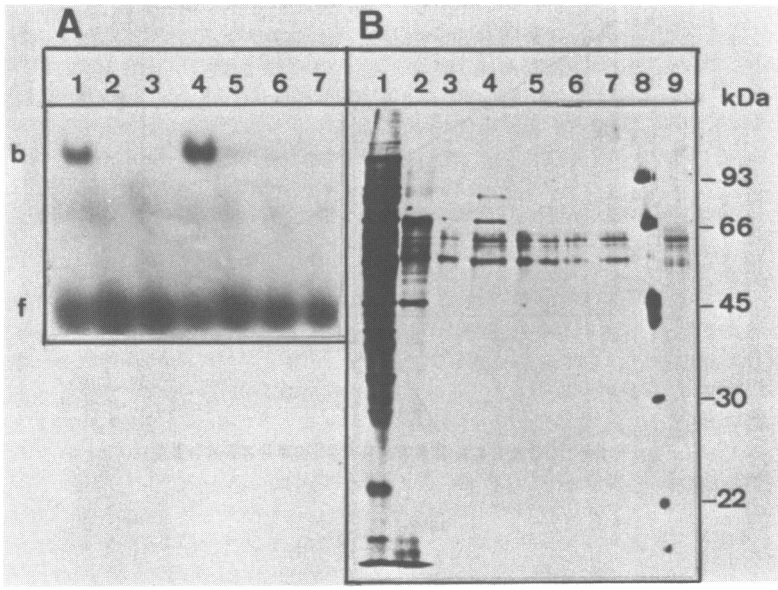

Fig. 7. *Affinity chromatography of NHP3.* A Mono Q fraction was enriched for NHP3 by chromatography on oligonucleotide-Sepharose as described in Materials and Methods. The gel shift assay (Panel A) and SDS-polyacrylamide gel (Panel B) are as described for NHP2 (Fig. 5) except that the oligonucleotide containing the NHP3 binding site was used as substrate.

Determination of the Native Molecular Weights of NHP1, NHP2 and NHP3

Following chromatography on Superose 12, each fraction was assayed for the DNA-binding activities of NHP1, NHP2 and NHP3 by gel shift assay. All three proteins elute in the same fractions and have a K_{av} corresponding to a molecular weight of ~170 000 (Fig. 8).

Proteolytic Clipping Bandshift Assays

Gel shift assays were carried out with purified NHP1 and five different oligonucleotides in the presence of increasing amounts of *Staphylococcus aureus* V8 protease. Identical patterns are seen with all substrates indicating that the protein binding to these substrates is most likely the same (Fig. 9). The small differences seen in the migration of the respective protein-DNA complexes among panels A, B, C, D and E are probably not significant and are most likely the result of small differences in acrylamide concentration.

DISCUSSION

The results presented in this study show that NHP1, NHP2, NHP3 and NHP4 are closely related if not identical although the DNA sequences that they bind do not show any homologies. Recent reports have indicated other DNA-binding proteins that bind to motifs of unrelated sequences. Purified TEF-1 binds specifically to the two motifs, GT-IIC and Sph, of the simian virus 40 enhancer (14) whilst each of the octamer binding factors, Oct-1, Oct-2A and Oct-2B bind to both the heptamer and octamer motifs of the immunoglobulin heavy chain genes (15). We have previously shown that NHP1, NHP2, NHP3 and NHP4 are neither tissue- nor species-specific (5, 6, 11, M. J. H. unpublished results). The

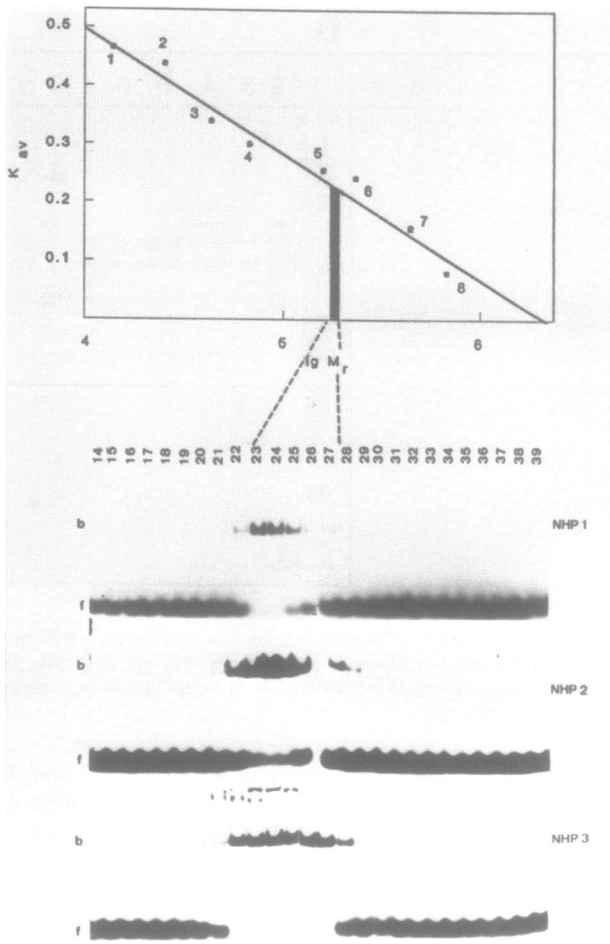

Fig. 8. *The native molecular weights of NHP1, NHP2 and NHP3 are the same.* The native size of NHP1, NHP2 and NHP3 was determined by gel filtration on Superose-12 as described (11). Gel shift assays of the eluted proteins were carried out as described in Materials and Methods. Bands b and f are bound and free DNA respectively. The standard curve was determined by following the elution of a set of proteins: ribonuclease (1), chymotrypsinogen (2), ovalbumin (3), albumin (4), aldolase (5), catalase (6), ferritin (7) and thyroglobulin (8). The filled bar indicates the lg M_r of the nonhistone proteins.

precision and complexity of gene regulation could be achieved by different combinations of ubiquitous factors with a small number of tissue-specific factors. A protein, like NHP1, could provide even more flexibility by binding to different sequences, perhaps with different affinities.

The nonhistone protein(s) could also be involved in tagging DNA for the subsequent binding of important transcription factors (6). The pseudorabies immediate early protein,

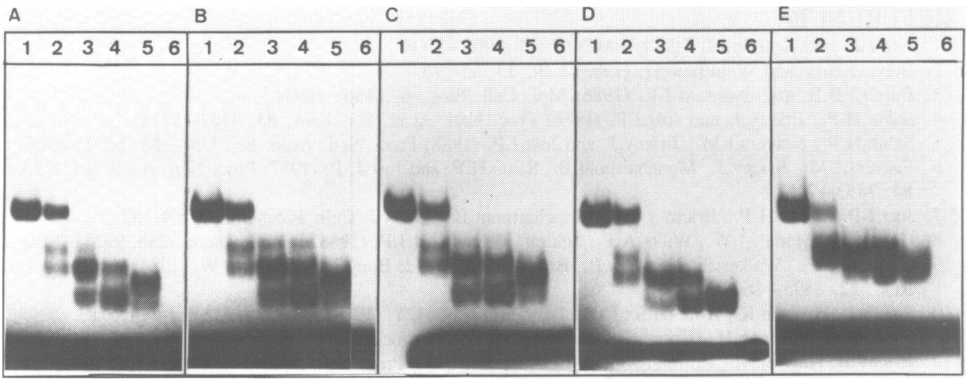

Fig. 9. *Protein binding to oligonucleotides containing the binding sites of NHP1, NHP2, NHP3 and NHP4 give identical patterns in proteolytic clipping bandshift assays.* Purified NHP1 was incubated with different oligonucleotide substrates in the presence of increasing amounts of *Staphylococcus aureus* V8 protease and 5 μg bovine serum albumin. Panels A, B, C, D and E correspond to gel shift assays with the ERE, ERE containing 5-methylcytosine, NHP2 binding site, NHP3 binding site and NHP4 binding site, respectively. In each panel, lane 1 is a gel shift assay carried out in the absence of V8 protease, lanes 2,3,4 and 5 are with 0.1 μg, 0.5 μg, 1 μg and 10 μg V8 protease, respectively, and lane 6 is a gel shift assay with 5 μg V8 protease in the absence of NHP1. The samples were incubated for 10 minutes at room temperature before electrophoresis on a 5% native gel. The lower band is free DNA, the upper bands are bound DNA.

together with TFIID, have been shown to facilitate the assembly of viral promoters into stable active complexes by preventing nucleosome assembly (16).

From genomic sequencing studies of the upstream region of the chicken vitellogenin II gene, we know that upon estrogen stimulation there is an active demethylation of specific 5-methylcytosine-guanosine dinucleotides (^{5m}CpG) (4,5). As shown in Fig. 1, the NHP1, NHP2, NHP3 and NHP4 binding sites all contain CpG dinucleotides in or flanking their sequence, thereby suggesting that the nonhistone protein(s) may be linked to the active demethylation of 5-methylcytosine. The binding constant of NHP1 with both the methylated and unmethylated ERE has been shown to be very low (1×10^{-11}M) i. e. high affinity (11, Fig. 4). Moreover, NHP1 binds only very weakly to the *Xenopus* vitellogenin ERE which lacks the CpG dinucleotide (Fig. 2) suggesting that NHP1 may be involved in the active demethylation of ^{5m}CpGs, presumably by base excision-repair. Such an enzyme would be expected to be ubiquitous and sequence non-specific except for the CpG dinucleotide. *In vitro* studies have indicated that affinity purified NHP1 may introduce nicks around the ^{5m}CpG of a synthetic oligonucleotide (11). NHP1 may be only part of a complex of enzymes involved in active demethylation. The precise function of NHP1 in the active demethylation of ^{5m}CpGs, if any, now remains to be established unambiguously.

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