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Transcriptional Element H4-Site II of Cell Cycle Regulated Human H4 Histone Genes Is a Multipartite Protein/DNA Interaction Site for Factors HiNF-D, HiNF-M, and HiNF-P: Involvement of Phosphorylation

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Abstract Cell cycle regulated gene expression was studied by analyzing protein/DNA interactions occurring at the H4-Site II transcriptional element of H4 histone genes using several approaches. We show that this key proximal promoter element interacts with at least three distinct sequence-specific DNA binding activities, designated HiNF-D, HiNF-M, and HiNF-P. HiNF-D binds to an extended series of nucleotides, whereas HiNF-M and HiNF-P recognize sequences internal to the HiNF-D binding domain. Gel retardation assays show that HiNF-D and HiNF-M each are represented by two distinct protein/DNA complexes involving the same DNA binding activity. These results suggest that these factors are subject to post-translational modifications. Dephosphorylation experiments in vitro suggest that both electrophoretic mobility and DNA binding activities of HiNF-D and HiNF-M are sensitive to phosphatase activity. We deduce that these factors may require a basal level of phosphorylation for sequence specific binding to H4-Site II and may represent phosphoproteins occurring in putative hyper- and hypo-phosphorylated forms. Based on dramatic fluctuations in the ratio of the two distinct HiNF-D species both during hepatic development and the cell cycle in normal diploid cells, we postulate that this modification of HiNF-D is related to the cell cycle. However, in several tumor-derived and transformed cell types the putative hyperphosphorylated form of HiNF-D is constitutively present. These data suggest that deregulation of a phosphatase-sensitive post-translational modification required for HiNF-D binding is a molecular event that reflects abrogation of a mechanism controlling cell proliferation. Thus, phosphorylation and dephosphosphorylation of histone promoter factors may provide a basis for modulation of protein/DNA interactions and H4 histone gene transcription during the cell cycle and at the onset of quiescence and differentiation.

Key words: phosphorylation, cell cycle, proliferation, transcription, histone, development

Cell cycle regulation of gene expression [1–3] is fundamental to cell growth control during embryonic development and throughout the life of an organism. During tumorigenesis aberrations in stringent regulation result in deregulation of the proliferative process. The histone multigene family is a paradigm for regulatory mechanisms operative during the eukaryotic cell cycle. Synthesis of histone proteins is a prerequisite for the assembly of newly replicated DNA into chromatin and is essential for the ordered progression through the cell division cycle. Histone mRNAs are among the most highly abundant gene transcripts expressed during S-phase. Histone mRNA levels are coordinately regulated and tightly coupled to DNA replication and histone protein synthesis. Histone gene expression is regulated at multiple levels with an important contribution of transcriptional control [4].

Transcriptional cis-acting elements involved in regulation of histone genes, and the cognate trans-acting factors, have been defined in some detail in diverse eukaryotic species [5–26]. The human H4 histone gene FO108 contains a proliferation-specific protein/DNA interaction site (H4-Site II) [11,27–29] that is essential for its transcription [10] and interacts with the cell cycle regulated factor HiNF-D [12,30]. The involvement of HiNF-D in rendering this gene competent for transcription is supported by downregulation of this factor during hepatic development in transgenic mice [29]. This downregulation is coincident with the cessation of H4

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histone gene transcription and the onset of in vivo quiescence and differentiation. Moreover, HiNF-D appears to be a component of a mechanism that coordinately modifies protein/DNA interactions in the promoters of human core (H4 and H3) and H1 histone genes during the cell cycle, tumorigenesis, and development [31]. Recently, Dailey et al. [18] have characterized a different factor (H4-TF2) that interacts with the analogous region of the human H4 histone gene Hu4A. The role of H4-TF2 in cell cycle regulation of the H4-Hu4a gene and its relationship with HiNF-D are unclear at present.

In the present study, we have assessed the complement of factors that can interact with H4-Site II of the human H4 histone gene FO108 in vitro. Our results show that apart from HiNF-D two other distinct DNA binding activities (HiNF-M and HiNF-P) interact with H4-Site II. Based on sequence specificity and conditions required for detection, it is possible that HiNF-P is directly related, or may be identical, to H4-TF2. The characterization of three distinct H4-Site II binding proteins for the H4-FO108 histone gene, and a single factor for the analogous element of the H4-Hu4a histone gene [18] could imply heterogeneity in transcriptional regulation of individual H4 histone genes. Alternatively, these data suggest that multiple distinct factors from HeLa S3 cervical carcinoma cells recognize H4-Site II sequences in vitro, and perhaps in vivo. Interestingly, HiNF-D and HiNF-M are each represented by two distinct protein/DNA complexes, and binding of these factors to H4-Site II is sensitive to phosphatase activity in vitro. These and other findings suggest a viable mechanism for modulation of these factors during the cell cycle based in part on post-translational modifications that influence DNA binding activity.

MATERIALS AND METHODS

DNA Fragments and In Vitro Protein/DNA Interactions

Plasmid FP202 was derived from the pUC19-based and H4-Site II containing construct pFP201 [31] (insert DNA fragment from nt −97 to −38; measured from the H4 histone translational start codon) by cleaving with PstI, blunt-ending with T4 polymerase, and ligating with T4 ligase in the presence of excess unphosphorylated BglII-linkers (5′dGAAGATCTTC) according to standard procedures [32,33]. The EcoRI/BglII fragment of pFP202 was inserted into EcoRI and BamHI sites of pFP202 to yield pFP203, which as a result contains a unidirectional duplication of the H4-Site II fragment. Plasmid pFP204 contains the AvaII/PstI fragment (nt −74/−38) of pFP201 and the SmaI/PstI vector fragment of pUC19.

Probes for protein/DNA interactions were derived from pFP201 by EcoRI cleavage, calf intestinal phosphatase (CIP) treatment, and T4 kinase 5′ end-labeling, followed by HindIII digestion to obtain labeled sense-strand (EH-probe; nt −97/−38). The order of the enzymes EcoRI and HindIII was reversed for labelling of the anti-sense strand (HE-probe). The EA- and AH-probes [coinciding with EcoRI/AvaII (nt −97/−74) and AvaII/HindIII (−71/−38) fragments of pFP201, respectively] represent H4-Site II deletion mutants and were prepared in an analogous manner. One DNA fragment was internally labeled at an AvaII site (AIL-probe) and prepared from pFP203 by separate actions of AvaII, CIP, and T4 kinase. The resulting mixture of DNA fragments was ligated using T4 ligase and cleaved with XhoII (or BstYI). All probe fragments were isolated by electrophoresis. The synthetic oligonucleotides (Nucleic Acid Facility of the University of Massachusetts Medical School) used in this study are summarized in the results section (see Fig. 3).

Gel retardation assays and competition analysis, DNaseI, and DMS protection analysis, as well as methylation- and depurination interference experiments were performed essentially as described [24,31,32–35]. Protein/DNA binding reactions (for detection of HiNF-D and HiNF-M) were performed by combining 10 μl of a protein mixture (in KN100 buffer; see below) with 10 μl of a DNA mixture containing probe DNA (25 pg/μl) and nonspecific competitor DNA substrates at the following concentrations: 0.1 μg/μl poly (dG-dC)*(dG-dC)(=GC-DNA), 0.01 μg/μl poly (dI-dC)*(dI-dC), (Pharmacia, Piscataway, NJ) (=IC-DNA) and 0.01 μg/μl of crude Sp1 binding site oligonucleotide [5′dGATC CGGATGGGCGGGGCCGGGGATGGGCGGG GCCGG:5′dGATCCCGGCCCCGCCCATCCCC GGCCCCGCCCATCCG]. Optimal conditions for detection of HiNF-P were similar to those of H4-TF2 [18]: binding reactions were performed as above with the exception that GC-DNA was replaced with salmon sperm DNA (0.1 μg/μl), and divalent cations were added, i.e., 0.1 mM $ZnCl_2$ and 0.5 mM $MgCl_2$ (to quench EDTA present in DNA and protein preparations).

Protein Preparations and Chromatography

Nuclear extracts prepared according to Dignam et al. [36] were obtained from HeLa S3 cells (density 7–9 × 10^5 cells/ml) as described previously [11,23,24], but magnesium salt was replaced in all buffers by 1 mM EGTA, 0.2 mM EDTA, 0.75 mM spermidine, and 0.15 mM spermine according to Shapiro et al. [37], and a broad spectrum protease inhibitor cocktail was used (PMSF, leupeptin, pepstatin, trypsin inhibitor, TPCK, EDTA, and EGTA; Boehringer, Indianapolis, IN). Also, desalting was not performed by dialysis but by dilution with storage buffer without KCl (20% glycerol, 0.2 mM EDTA, 0.01% NP40, 25 mM Hepes, pH7.5 and 1 mM DTT; KN0 buffer). Alternatively, samples were desalted by gel filtration using PD-10 columns (P-L Biochemicals), or ultrafiltration using Centricon-10 units (Amicon, Lexington, MA). Final protein concentrations were adjusted with storage buffer (KN0 buffer with 100 mM KCl = KN100 buffer).

Separation of H4-Site II DNA binding activities was performed by batch absorption of nuclear proteins to phosphocellulose resin preequilibrated with KN100. Proteins were eluted by a step-gradient using KN0 buffer with, respectively, 100 mM, 500 mM, and 1,000 mM KCl to yield P0-100, P100-500, and P500-1000 fractions. Fractions obtained in similar procedures using heparin-agarose ("H"-fractions) and DEAE Sephacel ("D"-fractions) received analogous designations. Samples were also derived from an alternative fractionation scheme in which crude nuclear protein was separated using phosphocellulose and eluted with KN-buffers containing, respectively, 100 mM, 300 mM, 500 mM, and 1,000 mM KCl. The P300-500 fraction obtained in this way was used as a partially purified HiNF-M preparation in some experiments. Nuclear protein preparations derived from synchronized cells [30] and from mouse tissues [29] were identical to those generated by previous procedures.

Protein Phosphatase Assays

Calf intestinal phosphatase (AlkP; Boehringer) and sweet potato acid phosphatase (AcP; Sigma Chemical Co., St. Louis, MO) were stored at 4°C in buffers recommended by the suppliers and diluted in KN100 buffer containing fresh protease inhibitor cocktail immediately prior to use. To use high amounts of AlkP (supplied in 3 M NaCl) it was necessary to reduce excess salt (which may interfere with HiNF-D binding) [11] by dilution of AlkP aliquots with KN0 buffer (containing protease inhibitors) and concentration with Centricon-10 units. Absence of protease activity in phosphatase preparations was examined by SDS-PAGE of treated and untreated samples (data not shown).

Phosphatase assays were performed with various protein fractions by incubating with increasing units of AcP or AlkP for 10 min at 20°C in KN100 buffer in a 10 μl volume. Binding reactions were initiated by adding 10 μl of a mixture containing 2 μg poly (dG-dC)*(dG-dC), 0.2 μg poly (dI-dC)*(dI-dC), and 0.5 ng of the AIL-probe and incubating the resulting mixture for 10 min at 20°C. Samples were then directly subjected to electrophoresis as described [28].

RESULTS

Two Distinct DNA Binding Activities, HiNF-M and HiNF-D, interact with H4-Site II Sequences

To investigate systematically the complement of factors capable of interacting with H4-Site II sequences (nt −97 to −47), we analyzed binding of nuclear proteins to DNA fragments spanning this element in the immediate proximal promoter of the H4 histone gene FO108. Protein/DNA complexes are observed that are attributed to binding of HiNF-D and HiNF-M, respectively (Fig. 1A). HiNF-D interacts with the entire H4-Site II containing fragment EH (nt −97/−38), but in agreement with previous findings (12) HiNF-D binding is not observed with fragments truncated at an AvaII-site internal to H4-Site II, i.e., fragments EA (nt −97/−74) and AH (nt −71/−38). HiNF-M binds to both the EH (nt −97/−38) and the EA (nt −97/−74) fragments, but not to the AH (nt −71/−38) fragment. These data demonstrate a novel sequence-specific protein/DNA interaction at H4-Site II involving HiNF-M.

To explore the possibility that complexes mediated by HiNF-M and HiNF-D are directly related by intermolecular association of more than one protein on the same DNA template, we analyzed binding of HiNF-D and HiNF-M using fractionated nuclear proteins (Fig. 1B). The results show that chromatography procedures using phosphocellulose, heparin-agarose, and DEAE-Sephacel successfully separate a number of H4-Site II binding proteins. More importantly, HiNF-D binding is observed in the absence of HiNF-M binding. This suggests that

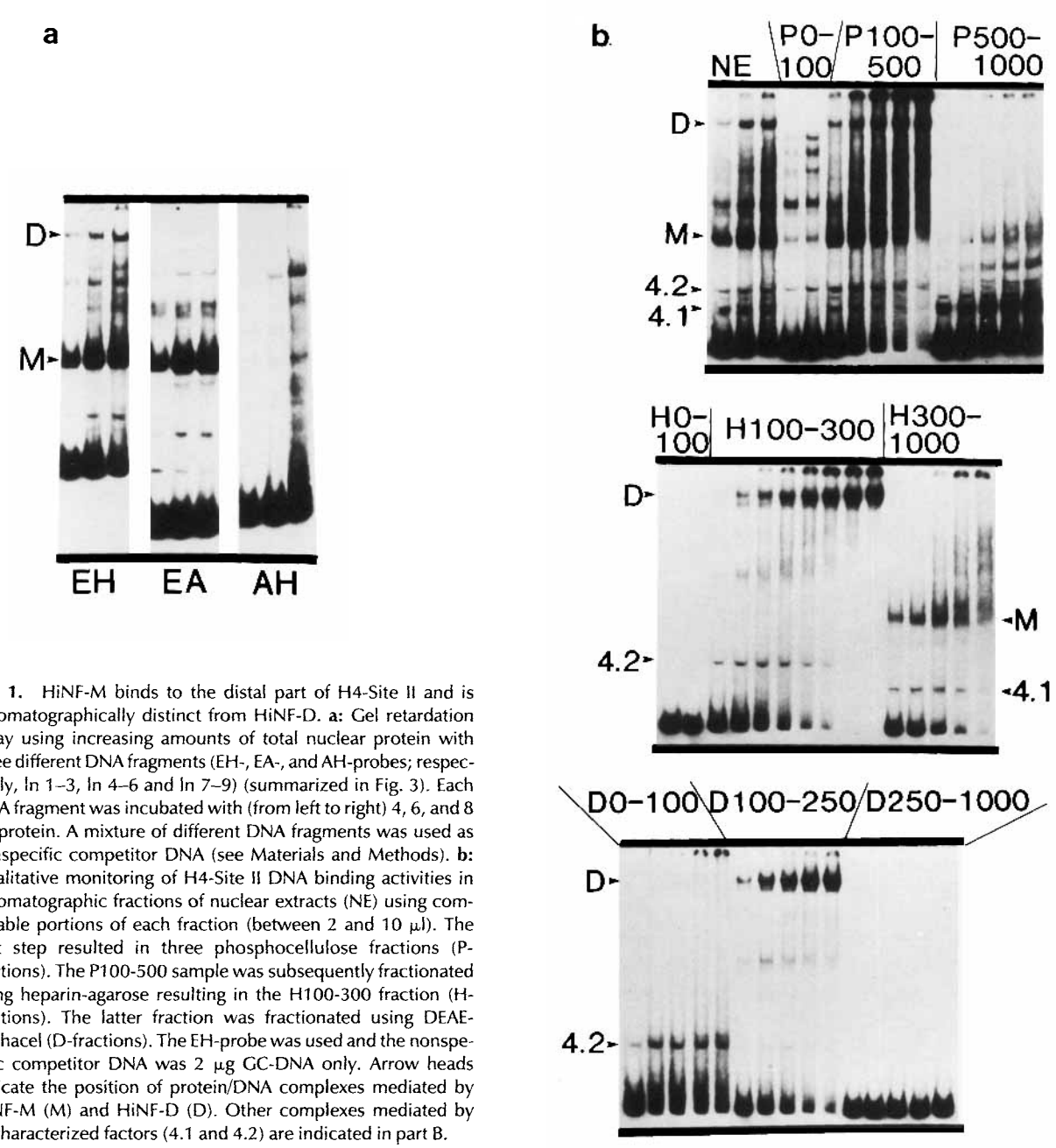

Fig. 1. HiNF-M binds to the distal part of H4-Site II and is chromatographically distinct from HiNF-D. **a:** Gel retardation assay using increasing amounts of total nuclear protein with three different DNA fragments (EH-, EA-, and AH-probes; respectively, In 1–3, In 4–6 and In 7–9) (summarized in Fig. 3). Each DNA fragment was incubated with (from left to right) 4, 6, and 8 μg protein. A mixture of different DNA fragments was used as nonspecific competitor DNA (see Materials and Methods). **b:** Qualitative monitoring of H4-Site II DNA binding activities in chromatographic fractions of nuclear extracts (NE) using comparable portions of each fraction (between 2 and 10 μl). The first step resulted in three phosphocellulose fractions (P-fractions). The P100-500 sample was subsequently fractionated using heparin-agarose resulting in the H100-300 fraction (H-fractions). The latter fraction was fractionated using DEAE-Sephacel (D-fractions). The EH-probe was used and the nonspecific competitor DNA was 2 μg GC-DNA only. Arrow heads indicate the position of protein/DNA complexes mediated by HiNF-M (M) and HiNF-D (D). Other complexes mediated by uncharacterized factors (4.1 and 4.2) are indicated in part B.

the corresponding protein/DNA complexes are not in a direct, rapidly fluctuating association in vitro. These factors elute at different salt concentrations from heparin-agarose resin and are present in two different fractions designated H100-300 (HiNF-D) and H300-1000 (HiNF-M). Similar differences were also observed when these factors were eluted from phosphocellulose using step-gradients: HiNF-M elutes at substantially higher salt concentrations than HiNF-D (data not shown). Thus, HiNF-D and HiNF-M are distinct entities with different electrophoretic and chromatographic properties.

Protein/DNA Recognition Analysis and Regulation of HiNF-M

To define the binding site of HiNF-M at single nucleotide resolution, we performed several experiments. DNaseI protection analysis (Fig. 2A) using partially purified HiNF-M preparations shows that this factor protects sequences at nt −95 to −78 on the sense-strand and nt −97 to −82 on the anti-sense strand. The specificity of nuclease protection was confirmed by competition with oligonucleotides: inclusion of an H4-Site II specific fragment (DS-II: nt −91 to −64)

decreased nuclease protection, whereas inclusion of a nonspecific DNA fragment did not (Fig. 2A). Depurination interference assays show that depurination of either guanines or adenines between nt −92 to −83 in both the sense- and anti-sense strands is inhibitory for binding (Fig. 2B), consistent with the DNaseI footprinting results. Methylation interference analysis (Fig. 2C) on sense- and anti-sense strands of the gel purified HiNF-M protein/DNA complex shows a pattern of both methylation interference (dG^{87}, dG^{85}, dA^{84}, and dA^{83}) and striking methylation enhancement (dG^{86} and dA^{89}). The pattern of methylation interference indicates that the heptameric sequence 5′dTTCGGTT (or 5′dAACCGAA) represents a minimal recognition sequence for HiNF-M.

Binding of HiNF-M to oligonucleotides containing this heptamer (DD-1: nt −93/−80; DS-II: nt −91/−64) can be directly demonstrated and confirmed by cross-competition (Figs. 2D, 3). Furthermore, HiNF-M is specifically retained on Sepharose-CL2B chromatography resins containing these oligonucleotides during DNA affinity chromatography (data not shown). However, DNA fragments containing the CCAAT-box motif, such as CTF/NF-1 or CP1/NF-Y binding sites (5′ dRRCCAAT; R = G or A) [38–40], and the oncoprotein MYB DNA binding consensus sequence (5′dYAACKG; Y = C or T, K = G or T) [41] do not compete for binding. These results further emphasize the importance of the heptamer element for HiNF-M binding.

To investigate the biological regulation of HiNF-M binding activity, we examined whether modulation of this activity occurs in relation to cell proliferation. HiNF-M was monitored in nuclear protein preparations derived from both tissue-culture cells and mammalian tissues. The HiNF-M protein/DNA complexes were identified by competition analysis [data not shown; 29]. Comparable levels of HiNF-M were observed in several cell types such as HeLa S3 cervical carcinoma cells, HL60 promyelocytic leukemia cells, as well as normal diploid and SV40 transformed WI38 lung fibroblasts, both during S and G1 phases of the cell cycle (data not shown).

Comparable levels of HiNF-M were also found *in nuclear extracts* from murine tissues, including adult liver, spleen, thymus, and brain [29]. More importantly, the levels of HiNF-M in cells from murine tissues do not correlate with those of HiNF-D. For example, the abundance of HiNF-D is in part proportional to the proliferative state of cell populations in these tissues and is downregulated during hepatic development; in contrast, HiNF-M binding activity is constitutively expressed during liver development [29]. These observations suggest that these DNA binding activities are subject to different modes of regulation.

Binding of HiNF-D and HiNF-M to Overlapping Elements of H4-Site II

To define the protein/DNA contacts of HiNF-D relative to those of HiNF-M, we performed methylation interference analysis of the gel purified HiNF-D protein/DNA complex (Fig. 4A) on the sense- and anti-sense strands. Methylation interference contacts were observed only on guanine residues (dG^{80}, dG^{76}, dG^{73}, dG^{69}, and dG^{65}) and one methylation enhancement could be detected (dG^{91}). This result shows that the HiNF-M heptamer contacts are contained within the HiNF-D binding domain and are distinct from, and complementary to, those contacts mediated by HiNF-D. This suggests that the HiNF-M and HiNF-D interactions represent binding events

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Fig. 2. Recognition site analysis of HiNF-M (see Fig. 3 for summary and description of oligonucleotides). **a:** DNaseI protection analysis of the sense (left panel) and anti-sense (right panel) strands of H4-Site II using P300-500 protein. Brackets indicate the regions of DNaseI protection (see also Fig. 3). Left panel: ln 1 and 8, G > A reaction of sense-strand; ln 2–5, respectively, 0 (=C), 12, 25, and 50 μl protein (=P) was added to a 100 μl binding reaction prior to DNaseI digestion; the binding reaction of ln 6 contained 500-fold molar excess of nonspecific (N) DNA fragment (H3-II), and that of ln 7 contained the same excess of specific (S) H4-Site II competitor fragment (DS-II). Right panel: ln 1, G > A reactions of anti-sense-strand; ln 2–3, respectively, 0 (=C) and 50 μl protein added; ln 4, same as ln 3, but DS-II (S) oligonucleotide added; ln 5, as ln 3 but H3-II (N) fragment added. **b:** Depurination interference analysis of the HiNF-M protein/DNA complex. Left panel: ln 1–3, anti-sense strand G + A reaction products of free (F), complexed (M), and input probe DNA (GA). Right panel: sense-strand, same abbreviations. **c:** Methylation interference analysis of the HiNF-M protein/DNA complex. Left panel: ln 1 and 4, G > A reaction products of input probe DNA from anti-sense-strand (G), as well as of free (F) and complexed (M) probe DNA. Right panel, same abbreviations for sense-strand. **d:** Competition analysis of HiNF-M binding to H4-Site II. Radio-labeled oligonucleotides (0.5 ng) were incubated with 10 μg nuclear protein, and each of a panel of oligonucleotides as indicated above the lanes (see also Fig. 3). Left panel: DS-II used as probe; right panel: DD-1 used as probe. Arrowhead indicates the position of the HiNF-M complex. Oligonucleotide DS-I (nt −152/−128) spans an ATF-like binding site in the distal part of H4-Site I and displays some, perhaps fortuitous, similarity with the distal part of H4-Site II.

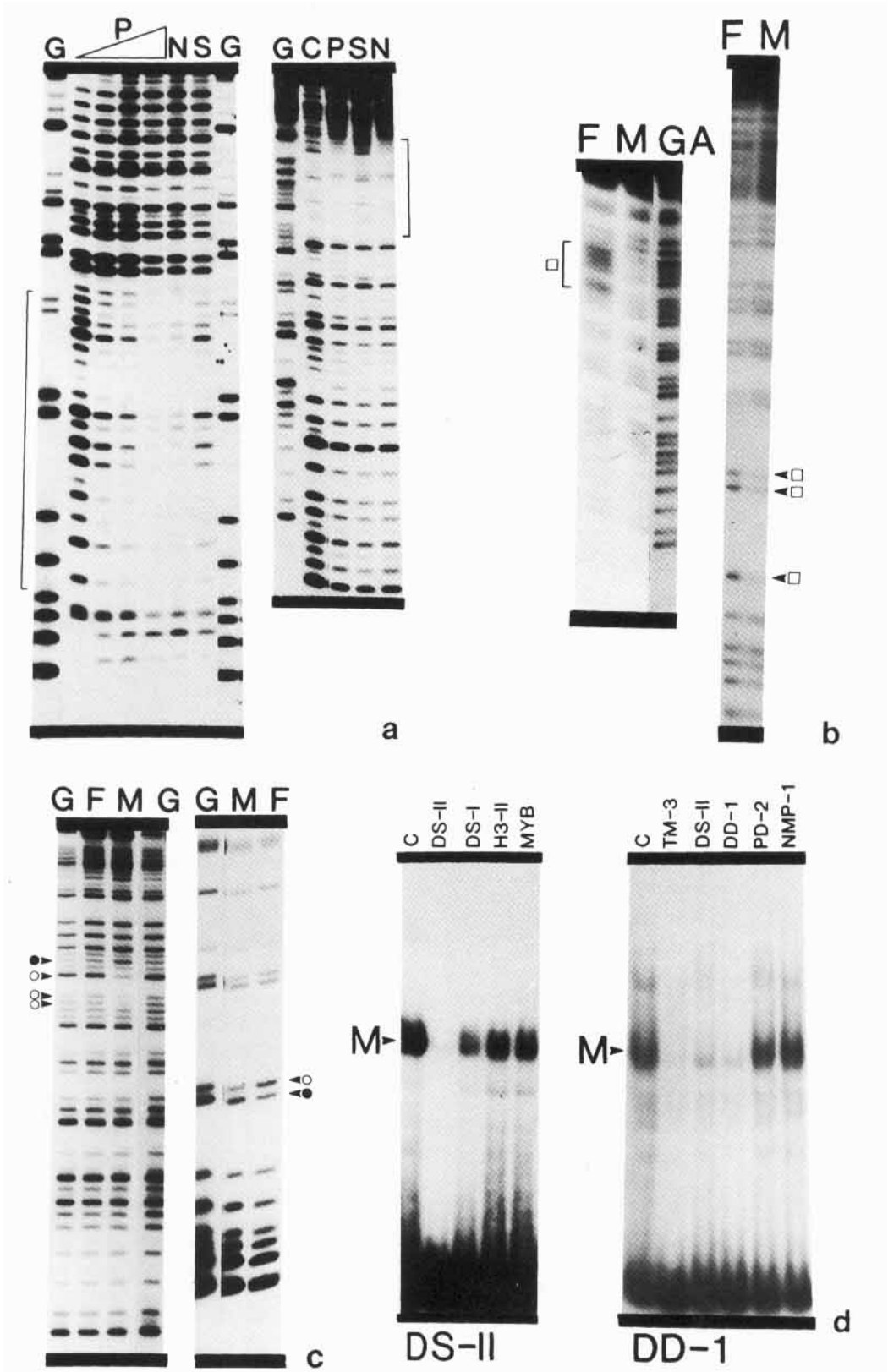

G
P
NS G
G C P S N
a
F M
F M G A
b
G F M G
G M F
c
C
DS-II
DS-I
H3-II
MYB
M
DS-II
C
TM-3
DS-II
DD-1
PD-2
NMP-1
M
DD-1
d

Figure 2.

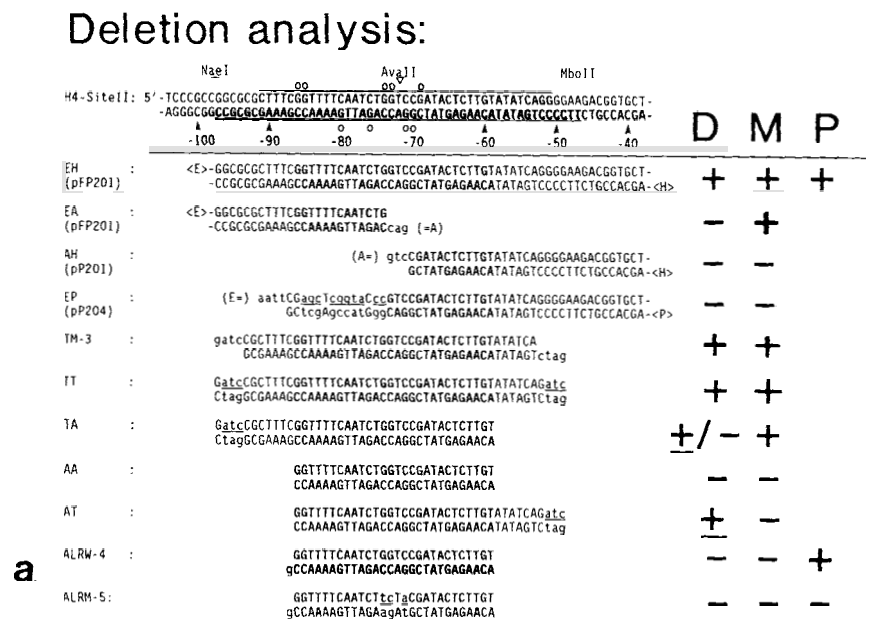
to overlapping elements in the evolutionarily conserved H4-Site II sequences.

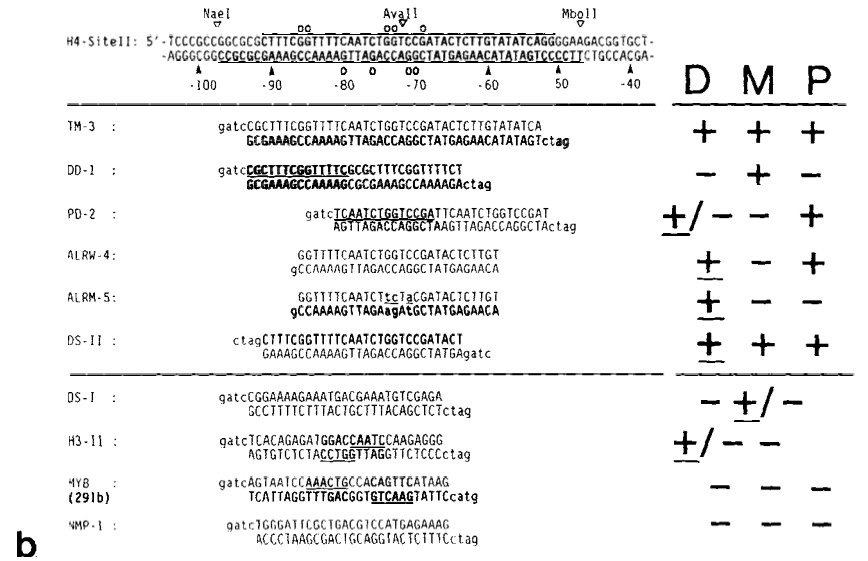
The differences in binding activities of HiNF-M and HiNF-D were further investigated by competition analysis (Fig. 4B). As noted above, HiNF-M competes specifically and very efficiently with both the DD-1 (nt −93/−80) and DS-II (nt −91/−64) oligonucleotides, but not with DNA fragment ALRW-4 (nt −86/−59), which represents a truncation of the HiNF-M heptamer. However, HiNF-D displays a more heterogeneous competition behavior and shows a reciprocal relationship between band intensity and molar ratio of competitor DNA only at high oligonucleotide concentrations. Specific competition is observed at high concentrations of the DS-II (nt −91/64) and ALRW-4 (nt −86/−59) oligonucleotides. DNA fragments PD-2 (nt −82/−66) and H3-II (sequence similarities with nt −80 to −70), which contain subsets of the H4 histone gene consensus sequence, compete in a marginally specific manner, whereas the MYB binding site 291b [41] does not compete even at extremely high molar ratios (approximately 2,000-fold) of competitor DNA (Fig. 3).

Several lines of evidence support the conclusion that the protein/DNA interactions involving HiNF-M and HiNF-D are independent binding events. Abolishment of HiNF-M activity by inclusion of excess specific competitor DNA does not influence HiNF-D binding, nor does competition of HiNF-D influence HiNF-M binding in vitro (Fig. 4B). This indicates that HiNF-M is not a direct intermediate of the HiNF-D protein/DNA complex. Moreover, these factors have distinct chromatographic behavior (Fig. 1), are subject to different modes of regulation (29), and mediate complementary protein/DNA contacts (Figs. 2, 4). Taken together, the results are consistent with these factors collectively mediating regulatory protein/DNA interactions.

To define the DNA sequences that represent a minimal HiNF-D binding domain, we constructed a series of radiolabeled oligonucleotides that embody a nested set of H4-Site II deletion mutants (Fig. 4C and data not shown). The identity of the HiNF-D protein/DNA complex was confirmed by competition analysis using both unfractionated nuclear proteins and partially purified HiNF-D preparations. Binding of HiNF-D to the AA-fragment (nt −86/−59), which spans the entire H4-Site II consensus element [5′dGGTYYTCAATCNGGTCCG; Y = T or C, N = any nucleotide; 12] was below the level of detection. Extension towards the 5′ direction of this core sequence by several nucleotides (to nt −93; TA-fragment), with inclusion of the most distal HiNF-D contact, resulted in a low, but detectable level of HiNF-D binding. Notably, extension of the 3′ terminus (to nt −53; AT-fragment) resulted in a stronger signal for

Fig. 3. Summary of H4-Site II protein/DNA interaction data (see Figs. 1 to 5 and data not shown). **a:** Deletion analysis with plasmid derived fragments (see Materials and Methods) and a nested set of oligonucleotides prepared by 5′ to 3′ polymerase or 3′ to 5′ exonuclease action of T4 DNA polymerase. The effect of deletions on binding of HiNF-D, HiNF-M, and HiNF-P (abbreviated D, M, and P, respectively) is represented as follows: +, strongest binding; ±, weaker binding; ±/−, binding barely detectable; −: no binding observed. Single letters in pointed and round brackets refer to the endonuclease sites at the termini of the plasmid derived probes (E = EcoRI, H = HindII, P = PstI, and A = AvaII). The EP-probe is the EcoRI/PstI insert of pFP204, which contains sequences derived from the pUC19 EcoRI/SmaI polylinker (nucleotides printed in lowercase depict altered nucleotides relative to H4-Site II sequences). The sequence at the top shows the region of H4-FO108 histone proximal promoter spanning in vivo protein/DNA interaction domain H4-Site II; indicated are genomic DNaseI (lines above and underneath sequence) and DMS (open circles) protection patterns, the nucleotide numbering relative to the protein coding region, and the mRNA start site. **b:** Competition results with the synthetic oligonucleotides used in this study. Shown on the right are qualitative assessments of the competition results using these oligonucleotides in gel retardation assays (+: band increase directly proportional to molar ratio of competitor and probe DNA; ±: competition only observed at higher ratio of competitor and probe DNA; ±/−: marginally specific competition; see text for details). Sequence at the top represents H4-Site II as described in A. Single-stranded overhangs are represented by lowercase letters. DD-1 and PD-2 represent duplications of elements within H4-Site II (duplicated segment in bold and underlined lettering in each case). The nucleotide substitutions in ALRM-5 relative to ALRW-4 are depicted with underlined, lowercase lettering. Sequences in H3-II and MYB(291b) that display similarity with H4-Site II sequences are indicated by bold, underlined lettering. **c:** Recognition site analysis of H4-Site II binding proteins. Summarized are the results obtained for both strands: in vitro DNaseI footprints of HiNF-M (bracketed lines), methylation interference of HiNF-M (triangles closest to the sequence: open, interference; filled, enhancement), depurination interference of HiNF-M (open squares), and methylation interference of HiNF-D (triangles connected with small arrows). The three stars refer to nucleotide substitutions in the ALRM-5 oligonucleotide that interfere with binding for HiNF-P. The thick lines with the designations HiNF-M and HiNF-P indicate the minimal elements capable of competing for these factors; the thick line with designations HiNF-D depicts the HiNF-D core sequence with the 5′ and 3′ extensions that each contribute to HiNF-D binding indicated by the dotted part of this line. Thin lines immediately above and below the H4-Site II sequence (bold) represent the boundaries of in vivo genomic DNaseI footprints; smallest dots indicate nucleotide numbering.


Deletion analysis:
NaeI
AvaII
MboII
H4-SiteII: 5'-TCCCGCCGGCGCGCTTTCGGTTTTCAATCTGGTCCGATACTCTTGTATATCAGGGGAAGACGGTGCT-
-AGGGCGGCCGCGCGAAAGCCAAAAGTTAGACCAGGCTATGAGAACATATAGTCCCCTTCTGCCACGA-
-100 -90 -80 -70 -60 -50 -40
D M P
EH (pFP201)
EA (pFP201)
AH (pP201)
EP (pP204)
TM-3
TT
TA
AA
AT
ALRW-4
ALRM-5:
a


Competition analysis:
NaeI
AvaII
MboII
H4-SiteII: 5'-TCCCGCCGGCGCGCTTTCGGTTTTCAATCTGGTCCGATACTCTTGTATATCAGGGGAAGACGGTGCT-
-AGGGCGGCCGCGCGAAAGCCAAAAGTTAGACCAGGCTATGAGAACATATAGTCCCCTTCTGCCACGA-
-100 -90 -80 -70 -60 50 -40
D M P
TM-3
DD-1
PD-2
ALRW-4:
ALRM-5:
DS-II
DS-I
H3-II
MYB (291b)
NMP-1
b

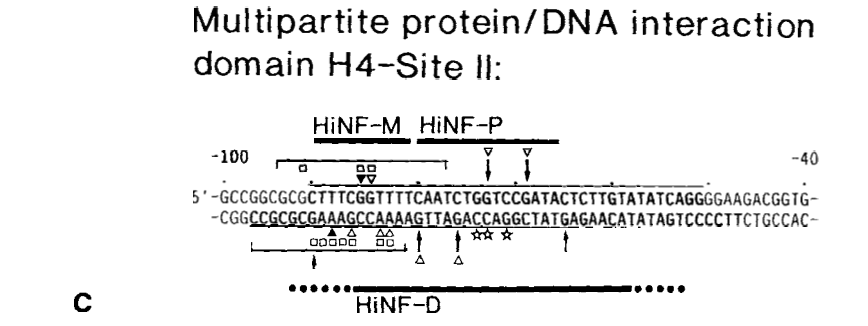

Multipartite protein/DNA interaction domain H4-Site II:
HiNF-M
HiNF-P
-100
-40
5'-GCCGGCGCGCTTTCGGTTTTCAATCTGGTCCGATACTCTTGTATATCAGGGGAAGACGGTG-
-CGGCCGCGCGAAAGCCAAAAGTTAGACCAGGCTATGAGAACATATAGTCCCCTTCTGCCAC-
HiNF-D
c

Figure 3.

the HiNF-D:H4-Site II interaction. Most efficient binding was observed using DNA fragments spanning nt −93 to −53 (TT and TM), consistent with the contribution of auxiliary sequences both at the 5′ and 3′ termini of the core sequence to HiNF-D binding.

Competition analysis with a spectrum of oligonucleotides using the TM-3 (nt −93/−53) fragment as a probe shows that HiNF-D binding is virtually abolished with a 100-fold molar excess of the unlabeled TM-3 oligonucleotide (Fig. 4D). However, only a several-fold decrease in HiNF-D binding is observed upon inclusion in the binding reaction of the same molar excess of the oligonucleotides DS-II (nt −91/64), ALRW-4 (nt −86/−59), and ALRM-5 (as ALRW-4 but containing three point mutations; see Fig. 3 and below). The difference in competition potential between TM-3 (nt −93/−53) and the shorter oligonucleotides can be directly attributed to the requirement for HiNF-D to bind to an extended DNA sequence.

Based on these data we can not discriminate whether these extensions of the core sequence provide additional protein/DNA contacts, facilitate conformational changes of the DNA, or contribute to initial nonspecific binding to the target DNA prior to stabilization at the putative HiNF-D recognition sequence. The combined results of methylation interference and competition analysis, as well as deletion analysis, strongly indicate that the HiNF-D binding site spans an extended polynucleotide sequence (between 27 and 41 nucleotides based on deletion analysis, with protein/DNA contacts distributed over 28 nucleotides) that is substantially larger than binding sites for both HiNF-M and most other eukaryotic DNA binding factors [42].

H4-Site II is a Multipartite Protein/DNA Interaction Site for Factors HiNF-D, HiNF-M, and HiNF-P

The establishment of specific binding sites for HiNF-D and HiNF-M that overlap the H4-Site *II in vivo protein/DNA interaction domain* does not exclude the possibility that additional factors may bind to these sequences. Using modified in vitro binding conditions, in particular, by replacing the nonspecific competitor GC-DNA with random DNA (from salmon sperm), we do not observe the HiNF-D complex, and HiNF-M is barely detectable (data not shown). However, we do observe a third protein/DNA complex interacting with H4-Site II, and this complex has a migration rate faster than HiNF-M (Fig. 5A). The specificity of this interaction is readily demonstrated by competition analysis: TM-3 (nt −93/−53) and ALRW-4 (nt −86/−59) compete, but DD-1 (nt −93/−80) does not. Hence, the factor involved (designated HiNF-P) is distinct from HiNF-M.

To address a potential relationship between HiNF-P and HiNF-D, we performed the following experiment. The ALRW-4 oligonucleotide spans an AvaII site that coincides with a pentameric element (5′dGGTCC; nt −74/−70) that is most strongly conserved in the mammalian H4-SiteII consensus sequence [12]. The mutant oligonucleotide ALRM-5 (Fig. 3) which contains three substitutions within this pentamer is, unlike the ALRW-4 fragment, not capable of competing for HiNF-P (Fig. 5A). However, both fragments compete with about equal (albeit moderate, relative to TM-3) efficiency for HiNF-D binding (Fig. 4D). The difference in the competition results for HiNF-D and HiNF-P suggests that these factors represent distinct DNA binding activities.

Our assay conditions for detection of HiNF-M and HiNF-D using the full length H4-Site II probe fragment (and GC-DNA as nonspecific competitor) do not allow detection of HiNF-P in gel retardation assays because, among other variables, the probe becomes saturated with HiNF-D and HiNF-M at relatively low protein concentrations. However, if we use the shorter ALRW-4 oligonucleotide (lacking the HiNF-M binding site) as a probe, and compare binding events with this fragment to those of the analogous mutated fragment ALRM-5, we can observe a very minor sequence specific protein/DNA complex at high protein concentration with a migration rate and competition properties identical to the HiNF-P complex (Fig. 5B). Moreover, HiNF-P is not only competed by the ALRW-4 fragment but also by the DS-II (nt −91/64) and PD-2 (nt −82/−66) oligonucleotides. This establishes that the minimal binding site capable of competition spans the element 5′dTTCAATC-T*GG*TCCGAT (nt −82 to −66; mutated nucleotides inhibitory for binding in italics) surrounding the 5′dGGTCC-pentamer element. The establishment of interaction sites for three distinct DNA binding activities clearly indicates that H4-Site II is a multipartite protein/DNA interaction site.

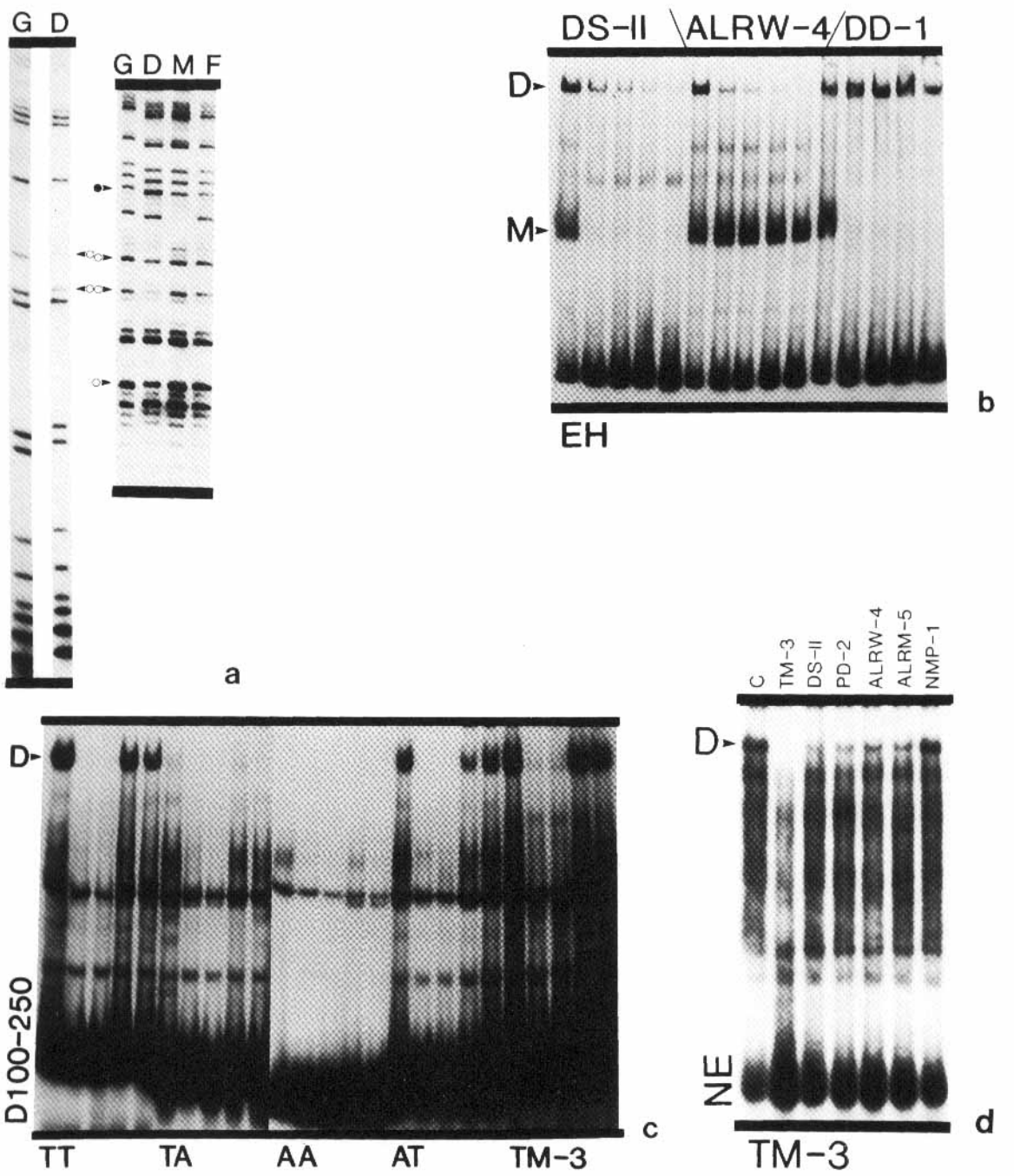


Fig. 4. Recognition site analysis of HiNF-D. **a:** Methylation interference analysis of the HiNF-D protein/DNA complex. Left panel: ln 1, G reaction of bottom-strand (G); ln 2, G reaction of HiNF-D complex (D). Right panel: G > A reaction of top-strand (G), free DNA (F), and the HiNF-D complex (D); the reaction products of the HiNF-M complex (M) are shown for reference. **b:** Competition analysis using the EH-probe (0.5 ng) and unlabeled synthetic oligonucleotides as indicated above each group of five lanes. The amounts of competitor added were in each case, respectively 0, 50, 100, 200, and 400 ng. **c:** Gel retardation assay with D100-250 protein (10 μl each) and oligonucleotides (0.5 ng each) representing a nested set of H4-Site II deletion mutants (see Fig. 3). The HiNF-D complex was identified by competition analysis (100-fold excess) with the following fragments, respectively, control (ln 1, 6, 11, 16, and 21), TM-3 (ln 2, 7, 12, 17, and 22), ALRW-4 (ln 3, 8, 13, 18, and 23), DD-1 (ln 4, 9, 14, 19, and 24), and NMP-1 (ln 5, 10, 15, 20, and 25). The NMP-1 oligonucleotide spans the unrelated binding site of the nuclear matrix protein NMP-1 (S. Dworetzky, JLS, and GSS; unpublished data) and is used as a nonspecific competitor DNA. **d:** Competition analysis with a panel of unlabeled oligonucleotides (100-fold molar excess) as indicated above the gel, using the TM-3 fragment (0.5 ng) as a probe and nuclear extract protein (10 μg). The binding reactions contain 200ng DD-1 oligeonucleotide to quench HiNF-M activity.

Dephosphorylation-Dependent Interactions of Factors HiNF-D and HiNF-M With the H4-Site II Element

The protein/DNA complexes of HiNF-D and HiNF-M each appear as a doublet band on autoradiograms of several gel retardation assays. We therefore explored the possibility that this may be attributable to post-translational modifications. Nuclear proteins were enzymatically dephosphorylated by incubation with increasing amounts of calf intestinal phosphatase (AlkP) or sweet potato acid phosphatase (AcP) (Fig. 6) to examine the effect of phosphate groups on the formation of the HiNF-D protein/DNA complex. Both AlkP and AcP are broad spectrum phosphomonoesterases that are capable of removing the phosphate moiety of phosphorylated amino acids.

Incubation of unfractionated nuclear protein or partially purified HiNF-D preparations with low concentrations of AcP results in the disappearance of the upper band of HiNF-D, whereas both HiNF-D bands are abolished at higher concentrations of AcP (Fig. 6A). The same results were observed when a fixed concentration of AlkP was used in the assay at various different temperatures (Fig. 6B). Note that in the absence of AlkP both HiNF-D species are irreversibly inactivated within an identical temperature interval (Fig. 6B, left lanes). Interestingly, low concentrations of AlkP (at fixed temperature) appear to shift the ratio of upper and lower bands of HiNF-D, while not dramatically influencing the combined binding represented by both complexes, yielding an apparent net increase in the formation of the lower HiNF-D complex (Fig. 6C). Results similar to those with HiNF-D were obtained for HiNF-M (Fig. 6A): limited phosphatase treatment resulted in the appearance of a faster migrating protein/DNA complex and more extensive treatment abolished HiNF-M binding.

Competition experiments were performed using nuclear proteins treated with phosphatases (Fig. 6D). These results demonstrate that the two sets of closely co-migrating species, corresponding to HiNF-D and HiNF-M, respectively, each have indistinguishable competition behavior, and we define each of these sets of species (in the simplest explanation) as post-translationally modified forms of the same DNA binding activity.

The possibility must be considered that alter-

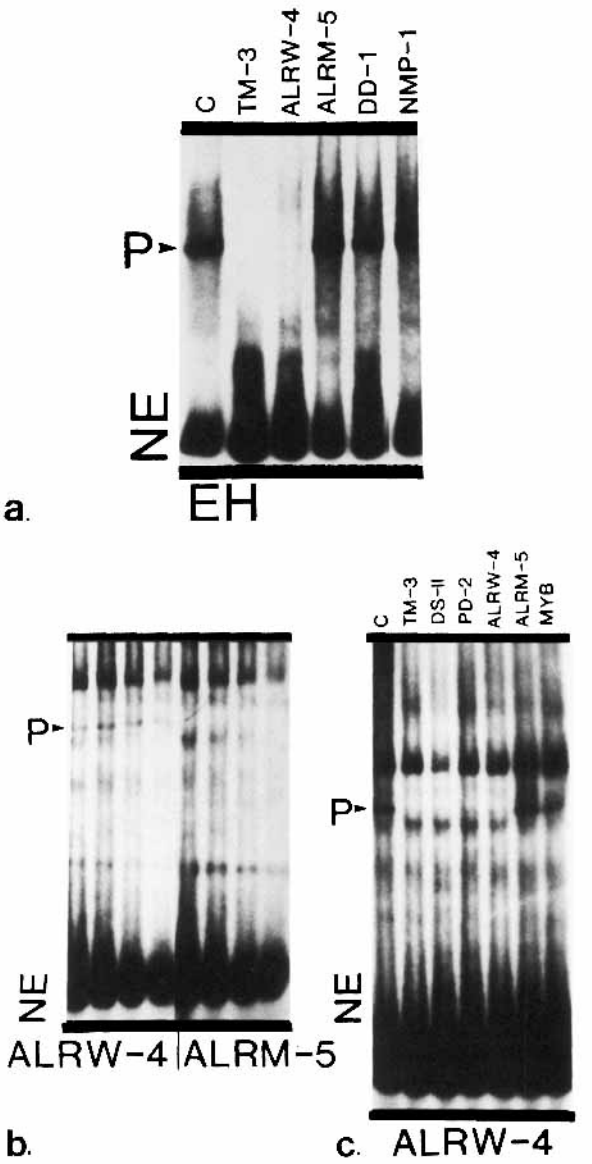


Fig. 5. Detection of a novel H4-Site II protein/DNA interaction involving HiNF-P [indicated by arrowhead (P)]. **a:** Binding and competition analysis of unfractionated and undialyzed nuclear extract proteins (25 μg in each case). The EH-probe was used and the nonspecific competitor DNA was salmon sperm DNA (2 μg); divalent cations were also added (0.1 mM $ZnCl_2$, 0.5 mM $MgCl_2$). The following oligonucleotides were present in 100-fold molar excess: ln 1–6, respectively, no specific competitor, TM-3, ALRW-4, ALRM-5, DD-1, and NMP-1. **b:** Detection of HiNF-P binding activity using oligonucleotides ALRW-4 (left panel: ln 1–4) and ALRM-5 (left panel: ln 5–8) as probes. The binding reactions contain a mixture of DNA fragments (2 μg GC-DNA, 0.2 μg IC-DNA, and 0.2 μg crude Sp1 oligonucleotide). The ALRW-4 and ALRM-5 fragments were in each case incubated with increasing amounts of nuclear protein (ln 1–4: from left to right, 20, 30, 40, and 50 μg protein; ln 5–8: same). **c:** Competition analysis of the HiNF-P complex using the ALRW-4 fragment as a probe with 25 μg nuclear protein, and 100-fold molar excess of the following oligonucleotides: ln 1–7, respectively, no specific competitor DNA, TM-3, DS-II, PD-2, ALRW-4, ALRM-5, and MYB(291b).

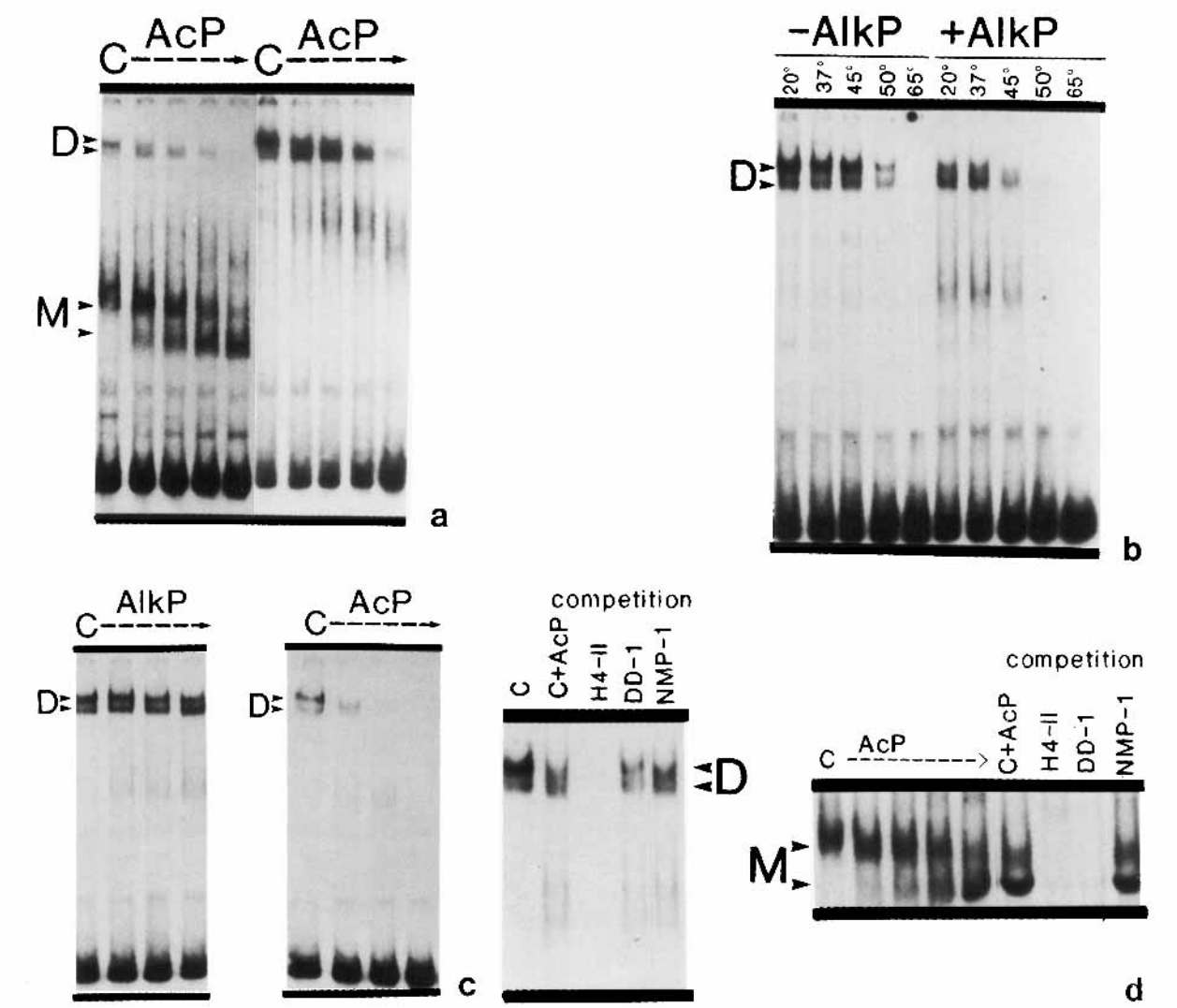


Fig. 6. Dephosphorylation of HiNF-D and HiNF-M influences binding to H4-Site II. Fractionated HeLa nuclear proteins were incubated with increasing amounts (dashed arrow) of sweet potato acid phosphatase (AcP) or calf intestinal alkaline phosphatase (AlkP). The AIL-fragment was used as probe. **a:** Incubation of P100-500 protein (4 μg) containing both HiNF-M and HiNF-D activity (In 1–5) or H100-300 protein containing primarily HiNF-D activity (In 6–10) with increasing amounts of acid phosphatase (units added in In 1–5 and In 6–10 in each case, respectively, 0 [=C], 0.05, 0.1, 0.2, and 0.5). The two sets of comigrating complexes (doublets) corresponding to HiNF-D and HiNF-M are indicated by arrowheads. **b:** Incubation of H100-300 protein in the absence (−AlkP) or presence (+AlkP) of 50 units of alkaline phosphatase at different temperatures as indicated. The two HiNF-D complexes are indicated with an arrowhead. **c:** Left panel: as in B, but incubation was performed in the absence (C) or presence of limited amounts of alkaline phosphatase (In 2 to 4, respectively, 10, 20, and 30 units AlkP added); right panel, incubation was performed in the absence (C) or presence of acid phosphatase (In 2–4, respectively, 0.1, 0.2, and 0.5 units AcP added). All reactions were performed with 5 μl D100-250 protein. **d:** Competition analysis of protein/DNA complexes using phosphatase-treated nuclear proteins. Incubations occurred in the absence of competitor DNA and phosphatase (C) or in absence of competitor DNA and presence of acid phosphatase (C + AcP), or in the presence of both 500-fold molar excess of competitor DNA (as indicated above the gels) and phosphatase (remaining lanes). Left panel: H100-300 protein incubated with 0.05 units acid phosphatase (In 2–5). Right panel: P100-500 protein incubated with 0.05, 0.1, 0.2, and 0.5 units acid phosphatase.

ations in the formation of these protein/DNA complexes are not directly related to dephosphorylation but could reflect an effect of another enzymatic activity present in our phosphatase reaction mixtures, for instance, the presence of contaminating protease activity. Protease activity would be fairly specific and cleave both HiNF-D and HiNF-M into discrete DNA binding products that exhibit faster migration of the corresponding protein/DNA complexes. We consider this unlikely because phosphatase treatment was carried out in the presence of a broad

spectrum protease inhibitor cocktail, several distinct chromatography fractions, and with two entirely different enzymatic preparations. Moreover, we did not observe protein degradation upon examination of phosphatase treated- versus untreated-protein samples using SDS/PAGE (data not shown).

We interpret these findings to indicate that both HiNF-D and HiNF-M are phosphoproteins. Based on the finding that extensive dephosphorylation abolishes binding of HiNF-D, the fast-migrating HiNF-D species must be at least partially phosphorylated, and therefore correspond to a putative hypophosphorylated species. The slower migrating form of HiNF-D appears to be a hyperphosphorylated species. A similar rationale can be applied to the results for HiNF-M. Obviously, these electrophoretic mobility assays do not discriminate between limited dephosphorylation creating a long-range structural effect on the protein conformation of a single polypeptide or (perhaps more likely) the dissociation of a loosely bound secondary molecule, both of which could contribute to a global effect on protein structure that may cause alterations in electrophoretic mobility. Extensive dephosphorylation ultimately affects protein domains that specify the DNA binding activity resulting in loss of binding. We conclude that the extent of dephosphorylation, and by deduction the state of phosphorylation, of HiNF-D and HiNF-M influences the ability to bind to DNA and alters the nature of H4-Site II protein/DNA interactions in vitro.

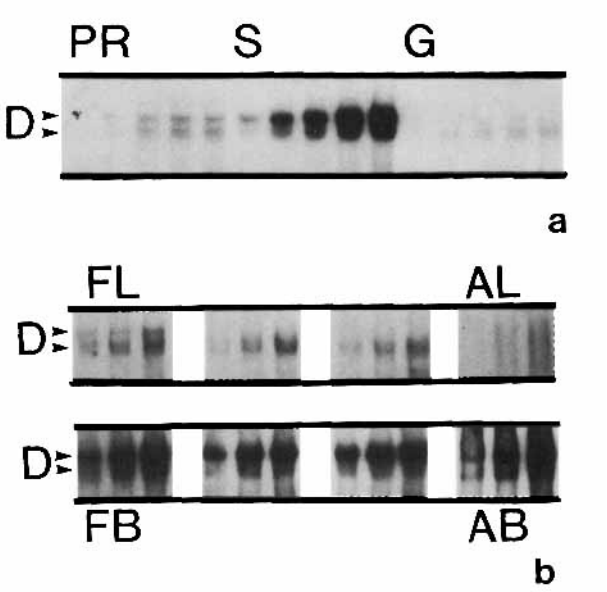


Fig. 7. Cell cycle dependent and developmental modulations in the two forms of HiNF-D. **a:** Electrophoretic migration of the two forms of HiNF-D prior to release from double thymidine block (PR), during S-phase (S), and during M/G1 (G). The alteration in HiNF-D binding DNA activity during the cell cycle of normal diploid cells (rat osteoblasts) (10), and the modulation in ratio of putative hyper- (slower migrating, upper band) and hypo-phosphorylated (lower band) forms are shown. **b:** Electrophoretic migration of the two forms of HiNF-D during liver (top part) and brain (lower part) development. Each panel shows the HiNF-D complex detected with, respectively, 2, 4, and 6 μg protein. Top part: first three panels, total nuclear protein from fetal liver (FL) at approximately day 14, day 16, and day 18 of gestation; right (and fourth) panel, protein from adult liver (AL). Bottom part, as top part but using protein from fetal brain (FB) and adult brain (AB).

Alterations in Post-Translational Modification of HiNF-D During the Cell Cycle and Development

Post-translational modification of DNA binding proteins may represent a level of regulation in the control of histone gene transcription. To address differences in the extent to which HiNF-D is post-translationally modified, we evaluated HiNF-D binding activity during the cell cycle of normal diploid cells [10]. Figure 7A shows that cells blocked at the G1/S phase boundary contain the two HiNF-D species in approximately equal quantities. Upon release into S phase, total HiNF-D binding activity increases along with the ratio of the two species. In contrast, cells in G1 phase contain lower amounts of total HiNF-D activity and only the faster migrating species can be detected. Similar results can be observed with synchronized populations of normal diploid WI38 fetal lung fibroblasts [data not shown; 10]. Hence, these results show that the phosphatase-sensitive post-translational modification of HiNF-D changes during the cell cycle in normal diploid cells.

To further explore the post-translational modification of HiNF-D during biological processes, we examined HiNF-D activity in mice during hepatic development [Fig. 7B; 29]. We observe that both forms of HiNF-D are present in fetal liver in mid to late gestation, and the downregulation of HiNF-D [29] occurs in conjunction with a decrease in the ratio of slower to faster migrating forms (Fig. 7B). However, no changes were observed with HiNF-D during brain development [discussed in 29]. Thus, these observations indicate that the phosphorylation state of HiNF-D changes during hepatic development coincident with the onset of cellular quiescence and differentiation in this tissue, further lend-

ing support to the physiological significance of multiple forms of HiNF-D.

DISCUSSION

We have performed a systematic analysis of H4-Site II protein/DNA interactions by the combined use of gel retardation deletion and competition analysis, DNaseI footprinting, methylation interference, and depurination interference. These data suggest that at least three factors (HiNF-D, HiNF-M, and HiNF-P) exhibit interactions with H4-Site II in a sequence specific and independent fashion, reflecting the multipartite nature of this protein/DNA interaction domain. The detection of three different factors binding to H4-Site II in vitro is consistent with our previous estimates of the number of H4-Site II factors that may interact with this regulatory element in vivo [11].

To date, we have not obtained indications that factor H4-TF2 characterized by Dailey et al. [18], which binds to an analogous human H4 histone gene, is identical to either HiNF-M or HiNF-D. We note that H4-TF2 has been characterized in vitro using salmon sperm DNA as nonspecific competitor, and that HiNF-D can not be detected under conditions used for detection of H4-TF2. Moreover, differences in methylation interference contacts of HiNF-D, HiNF-M, and H4-TF2 are in agreement with the assessment that these proteins are different [see also 12]. However, factor HiNF-P has interesting similarities with H4-TF2, because it requires very similar conditions for optimal detection and binds to a sequence in the H4-FO108 histone gene that is analogous to the H4-TF2 binding site in the H4-Hu4a histone gene [18]. The detection of at least three different DNA binding activities that specifically recognize the evolutionarily conserved H4-Site II sequences of H4 histone genes suggests that a multiplicity of H4-Site II binding proteins exists. The regulation of these binding activities should occur in a stringent and well-balanced manner to render the H4-Site II proximal promoter element competent for selective occupancy in vivo by biologically relevant DNA binding factors.

The functional significance of the HiNF-D:H4-Site II interaction in regulating H4 histone gene transcription is indicated by the positive correlation of biological regulation of HiNF-D binding activity, histone gene transcription, and occupancy of H4-Site II in vivo [10–12, 27–30] in a variety of biological processes. Factor HiNF-P recognizes specific sequences internal to the HiNF-D binding domain, indicating a role in conjunction with HiNF-D. The third protein, HiNF-M, binds independently of HiNF-D interactions at H4-Site II. The dissimilar distribution of these factors in a broad spectrum of cell types suggests that these DNA binding activities are differentially regulated. However, similar to HiNF-D, HiNF-M is subject to analogous post-translational modifications. Hence, the binding of post-translationally modified forms of HiNF-M and HiNF-D to overlapping sequences in H4-Site II may be functionally related to an extra regulatory dimension to accomodate cell types of diverse ontogeny in the modulation of H4 histone gene transcription during the cell cycle or the onset of quiescence and differentiation.

Phosphorylation of transcription factors involved in cell cycle regulation of histone gene expression has been proposed previously [43–48]. Here, we have used the cloned human H4 histone gene FO108, whose regulation has been well documented [10–12, 27–30]. We show that the interactions of factors HiNF-M and HiNF-D at the key proximal promoter element H4-Site II are influenced by phosphatase-sensitive post-translational modification and deduce that an apparent basal level of phosphorylation is required for these two DNA binding activities. At least one of these, HiNF-D, displays cell cycle dependent fluctuations in this post-translational modification. Hence, the present results are in support of the proposal that protein phosphorylation is involved in cell cycle regulation of histone gene transcription [43–48].

The phosphatase-sensitive post-translational modification of HiNF-D changes during the cell cycle in normal diploid cells. Interestingly, tumor-derived or transformed cells not only have constitutively elevated levels of this activity during the cell cycle [30], but the major species that can be detected is the putative hyperphosphorylated form of HiNF-D. Hence, the accumulation of this alternative form of HiNF-D in the G1-phase of four different tumor-derived and transformed cell types is consistent with modulation of HiNF-D by a fundamental oscillatory mechanism involving protein kinases and phosphatases, that is a frequent (and perhaps invariable) target of deregulation in the process of tumorigenesis.

Phosphorylation of proteins has been shown to have a role within the context of cell cycle control of gene transcription. For instance, cy-

clins and yeast cell cycle mutant derived CDC-genes specify components of a hypothetical molecular oscillator mediating a cascade of cell cycle dependent phosphorylation and dephosphorylation events [reviewed in 1–3]. In this regard, the putative hyperphosphorylated HiNF-D species is constitutively present throughout the cell cycle in four distinct tumor-derived or transformed cell lines, whereas in normal diploid cells we clearly observe cell cycle dependent fluctuations in two forms of HiNF-D. Others have reported similar cell cycle stage specific changes in phosphorylation states of proteins that are intimately associated with cell growth control, including tumor suppressors and oncoproteins such as RB, p53, MYC, and MYB [reviewed in 49–52]. Establishing the possible links between histone promoter factor HiNF-D and the genes encoding the specific oncoproteins, tumor-suppressor and/or CDC-related proteins that may be directly or indirectly associated with HiNF-D regulation will be a challenging task. This task will depend critically on the availability of genetic information and immunologic reagents for HiNF-D.

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