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Overlapping and CpG Methylation-Sensitive Protein-DNA Interactions at the Histone H4 Transcriptional Cell Cycle Domain: Distinctions between Two Human H4 Gene Promoters

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Transcriptional regulation of vertebrate histone genes during the cell cycle is mediated by several factors interacting with a series of *cis*-acting elements located in the 5′ regions of these genes. The arrangement of these promoter elements is different for each gene. However, most histone H4 gene promoters contain a highly conserved sequence immediately upstream of the TATA box (H4 subtype consensus sequence), and this region in the human H4 gene FO108 is involved in cell cycle control. The sequence-specific interaction of nuclear factor HiNF-D with this key proximal promoter element of the H4-FO108 gene is cell cycle regulated in normal diploid cells (J. Holthuis, T. A. Owen, A. J. van Wijnen, K. L. Wright, A. Ramsey-Ewing, M. B. Kennedy, R. Carter, S. C. Cosenza, K. J. Soprano, J. B. Lian, J. L. Stein, and G. S. Stein, Science, 247:1454–1457, 1990). Here, we show that this region of the H4-FO108 gene represents a composite protein-DNA interaction domain for several distinct sequence-specific DNA-binding activities, including HiNF-D, HiNF-M, and HiNF-P. Factor HiNF-P is similar to H4TF-2, a DNA-binding activity that is not cell cycle regulated and that interacts with the analogous region of the H4 gene H4.A (F. LaBella and N. Heintz, Mol. Cell. Biol. 11:5825–5831, 1991). The H4.A gene fails to interact with factors HiNF-M and HiNF-D owing to two independent sets of specific nucleotide variants, indicating differences in protein-DNA interactions between these H4 genes. Cytosine methylation of a highly conserved CpG dinucleotide interferes with binding of HiNF-P/H4TF-2 to both the H4-FO108 and H4.A promoters, but no effect is observed for either HiNF-M or HiNF-D binding to the H4-FO108 gene. Thus, strong evolutionary conservation of the H4 consensus sequence may be related to combinatorial interactions involving overlapping and interdigitated recognition nucleotides for several proteins, whose activities are regulated independently. Our results also suggest molecular complexity in the transcriptional regulation of distinct human H4 genes.

Stringent cell cycle regulation (41) of S-phase-related genes, including thymidine kinase, thymidylate synthase, dihydrofolate reductase, and histone genes, occurs at many transcriptional and posttranscriptional levels (reviewed in reference 22). Functional redundancy of these multilevel gene regulatory processes ensures a tight coupling between availability of enzymes required for DNA synthesis, histone proteins, and DNA replication in proliferating cells. The stringency with which control of gene expression is mediated is abrogated during neoplastic transformation, which may ultimately affect diverse transcriptional components regulating S-phase-related genes. For example, the interactions of nuclear factor Yi (14) with the thymidine kinase promoter and of nuclear factor HiNF-D (64) with the histone H4 promoter are regulated differently in several normal diploid and tumor cells (4, 23). HiNF-D is a proliferation-specific DNA-binding activity (64), and its nuclear abundance is cell cycle regulated in normal diploid cells (23). Interestingly, HiNF-D binding activity is constitutive throughout the cell cycle in several tumor cells (23, 64), consistent with this factor representing a molecular target during neoplastic transformation.

Mammalian histone H4 mRNAs are transcribed from multiple distinct genes (32) that encode very similar proteins (66). We have focused on the human gene H4-FO108 (49), which is located in a histone gene cluster at chromosomal region 1q21 (1, 54). This gene is expressed in a cell cycle-dependent manner both as an endogenous gene in human cells (44) and when introduced as an episomal gene into murine cells (16). The 5′ region of this gene mediates cell cycle-regulated transcription in cell culture (44), contributes to proliferation-specific expression in transgenic mice (58), and contains two in vivo domains of protein-DNA interactions (designated H4 site I and H4 site II) that were established in the intact cell at single-nucleotide resolution (42). In vivo occupancy of H4 site II is lost during the onset of differentiation, concomitant with selective downregulation of H4 gene transcription (51). Although sequences up to at least −1.0 kb are capable of modulating the extent of transcription (21, 26, 44, 70), H4 site II (nucleotides [nt] −97 to −47) mediates both cell cycle control and the basal level of H4-FO108 gene transcription (26, 44, 50). H4 site II contains a long (27 bp), highly conserved H4 subtype consensus sequence (64) and interacts with several factors, including HiNF-D (62).

Other investigators have focused on the regulation of the human histone gene H4.A (18–20), and two factors, H4TF-1 and H4TF-2, interacting with this gene have been characterized (8–10). H4TF-2 interacts with the H4.A gene promoter at a position analogous to a segment of H4 site II in the H4-FO108 gene. The DNA-binding activity of H4TF-2 is constitutive during the cell cycle of normal diploid human WI38 cells (28); however, the protein preparations used (28)

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did not appear to contain HiNF-D binding activity. In this report, we compare protein-DNA interactions at the H4 subtype consensus sequence of the H4-FO108 and H4.A genes. Our results suggest that HiNF-D is distinct from HiNF-P/H4TF-2 and that mechanistic differences may exist in the transcriptional regulation of the H4.A and H4-FO108 genes. The absence of a HiNF-D binding site in the analogous region of the H4.A gene, the distinction between HiNF-D and HiNF-P/H4TF-2, and procedural variations may reconcile differences in published results (23, 28).

MATERIALS AND METHODS

Plasmid DNA fragments and synthetic oligonucleotides. Sequences of several synthetic oligonucleotides (DS-II, DD-1, PD-2, H3-II, ALRW-4, ALRM-5, and TM-3) have been documented previously (62). The TM-3 fragment spans nt −93 to −53 of the H4-FO108 gene and has 5′ dGATC single-stranded overhangs on both termini. The design of oligonucleotides MC-7, ST-8, GT-9, INS-10, SUB-11, and TCN-12 is based on that of TM-3, but these fragments contain specific substitution and insertion mutations (summarized in Results; see Fig. 6). Fragment NH-6 spans the analogous region (nt −99 to −58) of the H4.A gene. Nucleotides are measured relative to the protein-coding region. Single-stranded oligonucleotides were purified by gel electrophoresis and quantitated by UV spectroscopy, using the individual extinction coefficient of each DNA fragment according to standard procedures (2, 45). Stock solutions were adjusted with TE buffer (10 mM Tris, 1 mM EDTA, pH 7.6) to the same concentration (10 pmol/μl) and stored at −20°C. DNA fragments for competition assays were made by mixing equimolar ratios of sense and antisense strands and were annealed by boiling for 2 min and slow cooling to 24°C. Labelling of single-stranded oligonucleotides was performed by phosphorylating 10 pmol of each DNA fragment with [γ-^{32}P]ATP (150 μCi) and T4 polynucleotide kinase (10 U) in a 20-μl volume as described previously (45). After incubation (30 min at 37°C), 80 μl of TE buffer was added and the enzyme was heat inactivated (68°C for 10 min). The unphosphorylated opposite strand was annealed, 10 μg of glycogen was added, and free [γ-^{32}P]ATP was removed by two consecutive ethanol precipitations. The final pellets were quantitated by liquid scintillation counting, and the volume of each sample was adjusted to a DNA concentration of 10 fmol/μl.

Nuclear protein preparations. Nuclear proteins and chromatographic fractions were obtained from HeLa S3 cells exactly as described previously (62), with buffers including 1 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 0.2 mM EDTA, 0.75 mM spermidine, 0.15 mM spermine, and a broad-spectrum protease inhibitor cocktail (phenylmethylsulfonyl fluoride, leupeptin, pepstatin, trypsin inhibitor, tolylsulfonyl phenylalanyl chloromethyl ketone [TPCK], EDTA, and EGTA; Boehringer Mannheim) (Fig. 1). Desalting of nuclear extracts was accomplished by ultrafiltration using Centricon-10 units (Amicon) and dilution with KN0 buffer (KN100 storage buffer without KCl; see below). Protein concentrations were quantitated by Bradford analysis (Pierce).

In vitro protein-DNA interaction analysis. Gel retardation and methylation interference experiments were performed as described previously (2, 43, 65). Methylation interference was performed with the plasmid-derived EH probe, which was prepared from construct pFP202 (62). Protein-DNA binding reactions for detecting HiNF-D were carried out by combining 10 μl of a protein mixture (in KN100 storage buffer: 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 0.01% Nonidet P-40, 1 mM dithiothreitol, 25 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid] NaOH, pH 7.5) with 10 μl of a DNA mixture containing 1 μl of probe DNA (10 fmol in TE buffer), 2 μl poly(dG-dC) · (dG-dC) (2 μg in TE buffer), and 1 μl of poly(dI-dC) · (dI-dC) (0.2 μg in TE buffer). Alternatively, binding reactions for detecting HiNF-P were prepared by mixing 10 μl of a protein solution (in KN100/MZ buffer, which is like KN100 but includes 0.2 mM $MgCl_2$ and 0.2 mM $ZnCl_2$) with 10 μl of a DNA mixture containing 1 μl of probe DNA (10 fmol in TE buffer), 1 μl of salmon sperm DNA (Sigma) (2 μg in TE buffer), and 1 μl of a divalent cation solution (containing 2 mM $MgCl_2$ and 2 mM $ZnCl_2$).

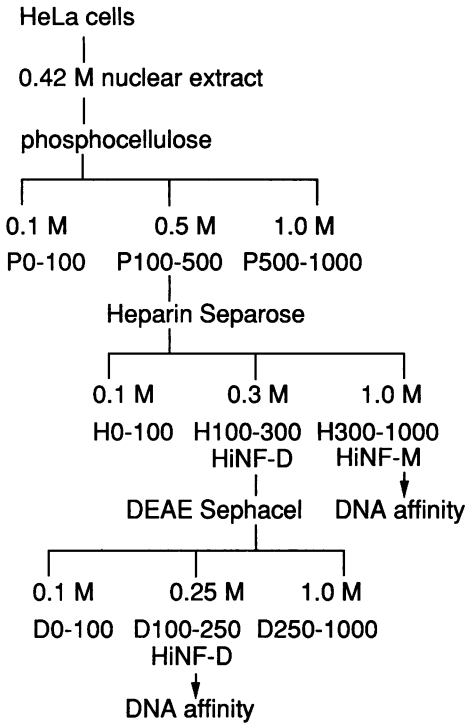


FIG. 1. Chromatographic fractionation of H4 site II-binding activities. Nuclear extracts were prepared from HeLa cells by extraction with 0.42 M KCl and fractionated by using the indicated chromatographic matrices as described previously (62). The D100-250 fraction was used as a partially purified HiNF-D preparation in this study.

Competition analyses were performed by mixing 10 μl of DNA mixture containing 10 fmol of probe and 125 to 1,000 fmol of unlabelled specific competitor double-stranded DNA with 10 μl of protein mixture containing approximately 10 to 20 μg of HeLa nuclear protein, followed by incubation for 15 to 30 min at 24°C and electrophoresis. The effects of preincubation temperatures, chelation, and monovalent cations on binding conditions were assayed as described previously (64). Electrophoresis was performed with low-ionic-strength conditions (1 mM EDTA, 3.3 mM sodium acetate, 6.6 mM Tris HCl, pH 7.9) in a buffer-cooled electrophoresis unit (Hoefer, model SE600) linked to a waterbath maintained at 4°C. Samples were loaded without tracking dye onto a 4% (80:1, acrylamide-bisacrylamide) polyacrylamide gel, and electrophoresis was performed for 2 to 2.5 h at 200 V. Gels were dried and subjected to autoradiography.

Site-specific methylation of CpG dinucleotides in H4-gene-derived probe DNA fragments was performed by incubating (16 h, 37°C) approximately 5 ng of DNA with either 10 or 0 U (control) of CpG methylase (M.*SssI*; New England Biolabs) as described by the supplier. After the reaction, samples were incubated for 10 min at 68°C and purified by organic extraction and ethanol precipitation (in the presence of 10 μg of glycogen). The extent of methylation was monitored by resistance to restriction endonuclease cleavage (*Msp*I, *Hha*I, and/or *Ava*II) of treated and untreated samples.

RESULTS

H4 site II is a multipartite protein-DNA interaction domain mediating sequence-specific binding of HiNF-D, HiNF-M, and HiNF-P. H4 site II interacts in vitro with three nuclear factors designated HiNF-D, HiNF-M, and HiNF-P (62). Methylation interference analyses of the HiNF-M and HiNF-D protein complexes showed that these factors contact H4 site II at a series of distinct, partially overlapping nucleotides that are distributed over three distinct sequence motifs (M, C, and P boxes) comprising a highly conserved H4 consensus sequence (Fig. 2). To compare the protein-DNA contacts of HiNF-M and HiNF-D with those of HiNF-P, we performed methylation interference analysis of the HiNF-P–H4 site II complex (Fig. 3). Factor HiNF-P interacts with H4 site II at dG nt −86, −85, −74, −73, and −69 on the sense strand and at nt −71 and −70 on the antisense strand. The methylation interference contacts of HiNF-P are distinct from those of both HiNF-M and HiNF-D (Fig. 2), demonstrating that at least three sequence-specific DNA-binding activities are capable of interacting independently with H4 site II in vitro. The methylation interference contacts of HiNF-P with the H4-FO108 gene are highly similar to those observed by Dailey et al. (10) for H4TF-2 binding to the H4.A gene. This suggests that HiNF-P and H4TF-2 are closely related DNA-binding activities.

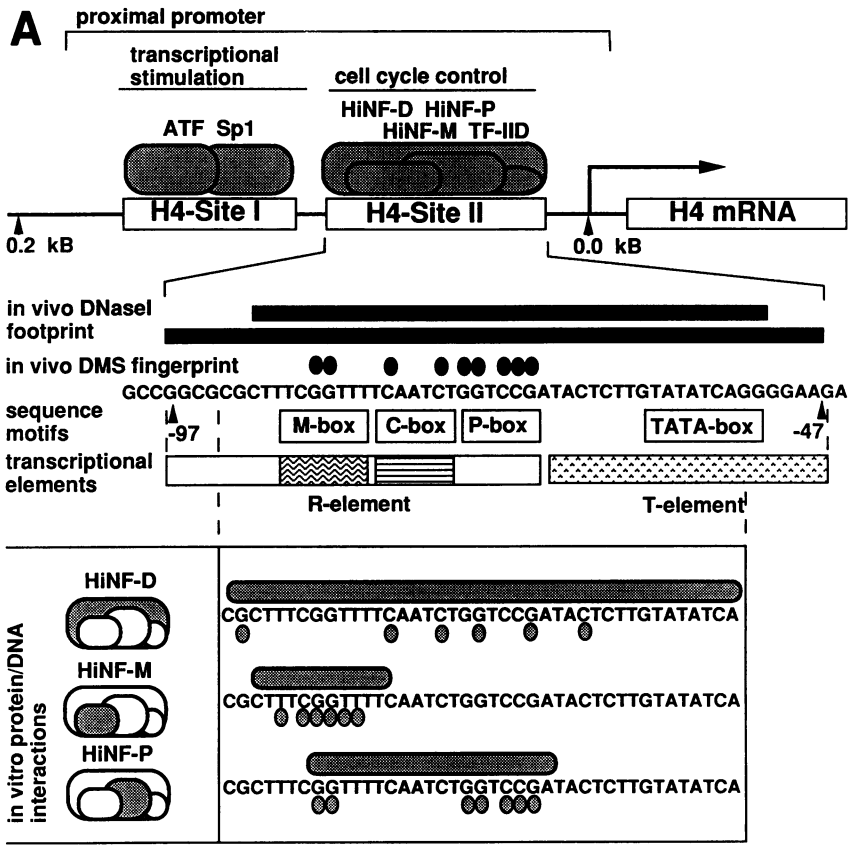
Biochemical distinctions between H4 site II binding activities suggest that HiNF-D, HiNF-M, and HiNF-P are independent entities. We have assessed the biochemical characteristics of the different H4 site II DNA-binding proteins (Fig. 4). First, the temperature ranges at which HiNF-D (48 to 52°C) (64), HiNF-M (20 to 50°C), and HiNF-P (37 to 45°C) are thermally inactivated are different for each factor (Fig. 4A). While HiNF-P is relatively insensitive to KCl concentrations of <200 mM, the optimal monovalent ion concentration for HiNF-D is below 100 mM KCl (64), and HiNF-M binding activity decreases gradually over a wide KCl range (80 to 200 mM KCl) (Fig. 4B). Both HiNF-M and HiNF-P (Fig. 4C), unlike HiNF-D (64), are differentially sensitive to several distinct chelators (at 10 to 25 mM) at low temperature. For example, the order of chelator sensitivity for HiNF-M is (1, 10)-*ortho*-phenanthroline > EGTA = EDTA, whereas for HiNF-P this order is (1, 10)-*ortho*-phenanthroline = EDTA > EGTA. This suggests that HiNF-M and HiNF-P have distinct cryptic divalent cation requirements. The chelation sensitivity of HiNF-P is comparable to that observed for H4TF-2 by Dailey et al. (9), further suggesting similarities between these factors.

A number of other characteristics also distinguish factors HiNF-D, HiNF-M, and HiNF-P. For example, each of these factors has a distinct intrinsic nonspecific DNA-binding activity; therefore, the type of nonspecific competitor DNA used in binding reactions is critical in the detection of sequence-specific H4 site II DNA-binding proteins (e.g., see Fig. 9). Also, the relative mobility of the HiNF-D complex, but not the HiNF-M complex, is dramatically reduced with decreasing pore size (Fig. 4D) in 4% polyacrylamide gels using acrylamide-bisacrylamide cross-linking ratios ranging from 80:1 to 20:1. This may prevent the detection of HiNF-D in gel retardation assays and the discrimination of HiNF-D from other protein-DNA complexes (data not shown). The pore size dependence of relative mobility suggests that the HiNF-D–H4 site II complex is a large, high-molecular-weight nucleoprotein complex. Hence, these analyses (Fig. 4) establish that factors HiNF-D, HiNF-M, and HiNF-P are biochemically distinct.

Mutations affecting the interactions of HiNF-M, HiNF-P, and HiNF-D with H4 site II. To define the boundaries of the recognition elements of H4 site II-binding proteins, we performed a competition analysis with a series of oligonucleotides containing specific mutations of the H4 site II sequences. Interactions of HiNF-D, HiNF-M, and HiNF-P with a synthetic H4-FO108 DNA probe spanning nt −93 to −53 (TM-3) were measured in the presence of increasing concentrations of specific competitor oligonucleotides by using conditions optimized for the detection of each factor (62). Mutation of both the M box and C box (MC-7; nt −89, −86, −85, −83, −80, −79, and −76) (Fig. 5A; summarized in Fig. 6) abolished competition for HiNF-M and reduced competition for HiNF-P severalfold. Mutation of the P box (GT-9; nt −75 to −67) abolished competition for HiNF-P but did not affect competition for HiNF-M. No effect on either factor was observed by complete substitution-mutation of the entire T element (ST-8; nt −66 to −56) located in the proximal portion of H4 site II. This mutational analysis indicates that HiNF-M and HiNF-P are distinct sequence-specific DNA-binding activities with partially overlapping binding sites, consistent with methylation interference analyses of these factors (Fig. 2 and 3). These results also demonstrate that mutation of nucleotides in the P box, encompassing proximal methylation interference contacts for HiNF-P, are more critical for HiNF-P binding than nucleotides encompassing the distally located M and C boxes.

Competition analysis of HiNF-D binding activity using the same set of specific competitors (Fig. 5A) indicates that competition for HiNF-D is influenced to a limited extent by mutation of both the M and C boxes (MC-7; nt −89, −86, −85, −83, −80, −79, and −76) or the T element (ST-8; nt −66 to −56). However, no effect was observed on HiNF-D binding by complete substitution-mutation of the P box (GT-9; nt −75 to −67). These competition results corroborate previous findings (62) that the HiNF-D binding site spans an extended polynucleotide sequence (a length of 27 to 41 bp on the basis of deletion analyses, with methylation interference contacts distributed over 28 bp). Specific sequences in both the distal (nt −93 to −87) and proximal (nt −58 to −53) region (62) contribute to the binding of HiNF-D to H4 site II (Fig. 6). Because extensive substitutions in several sequence motifs only partially inhibit HiNF-D binding, this suggests that HiNF-D does not recognize a simple consensus sequence. Similarities and differences in competition behaviors of HiNF-M, HiNF-P, and HiNF-D for each of these oligonucleotides suggest that the recognition sites of these factors partially overlap.

The binding domains of HiNF-M and HiNF-P were further analyzed by using a set of shorter oligonucleotides spanning subsets of sequences present in the TM-3 oligonucleotides (Fig. 5B). The recognition sequence of HiNF-M is clearly



B

Histone H4 gene promoter sequence-similarities:

human H4/a[1,2]	C G CtT T T C A G T C T C - - - T g T - G T C C G C T
human H4.A[3](distal)[2]	C T a T T T C G G T T T g - - - - - - - - - - - - - -
human H4.A[3](proximal)	C T C T T T C a G G T T C T C A g T T c G G T C C G C C
human H4/g[1]	C T C a T G T t G T T T T C A A T C A G G T C C G C C
human H4/h[1]	C G C T T T C A G T C T T C A A T T c G G T C C G A A
human H4-FO108[4]	C G C T T T C G G T T T T C A A T C T G G T C C G A T
rat H4-t[5]	C G C C T G T G G T C T T C A A T C A G G T C C G C A
rat H4-s[6]	T c C C T G C t G T T T T C A A a C A G G T C C G C T
mouse H4-AST[7]	C G C T T T C A G T T T T C A A T C T G G T C C G A T
mouse H4-12[8]	C G C T C - T A G T T T T C A A C C A G G T C C G C A
mouse H4-53[8]	C a C T T G a A G T T C T C A A C C A G G T C C G A T
Xenopus H4 (#212)[7]	a a t g T c C A G T T C C C t A T C A G G T C - G A C
Xenopus H4 (#213)[7]	T T g T T c C A G T T C C C t A T C A G G T C - G A C
Xenopus H4 (#214)[7]	T G C T T G C t G T T C C C t A T C A - G T C a G C C
Xenopus H4 (#215)[7]	T G g a g T g A G T T C T C t g T C A G G T C C t C T
chicken H4 (#206)[7]	C G C C C c C T G G T T T - C A A T C - G G T C C G A A
chicken H4 (#207)[7]	C G C C C c C T G G T T T - C A A T C A G G T C C G A C
trout H4 (#218)[7]	T G C C a - C T G G g C T - C A A T C A G G T C C a C A
maize (#232)[7]	a G C T C T C - G T T C T C A c C C - - G T C C G t C
vertebrate H4 consensus :	**Y K C Y Y K Y R G T Y Y T C A A Y Y W G G T C C G M H**
yeast UAS consensus[9] :	**G T T C T C A N W W T - - T T C G C**

CpG-3 CpG-2 CpG-1

FIG. 2. (A) Regulatory organization of the H4-FO108 gene promoter. Depicted is a schematic diagram emphasizing the regulatory organization of the first 0.2 kb of the H4-FO108 gene promoter, although additional transcriptional and conformational information (reflected by regions hypersensitive to DNase I [5] and micrococcal nuclease [36, 37]) may reside in sequences further upstream (52). Indicated are two domains of in vivo protein-DNA interaction (black bars, genomic DNase I footprints on sense and antisense strands; black ovals, genomic DMS fingerprints) that have been established in the intact cell (H4 site I, nt −150 to −113; H4 site II, nt −97 to −47) (42, 43). Promoter domain H4 site I is a bipartite *cis*-activating element that interacts distally with ATF-84 (72), a member of the ATF family of transcription factors,

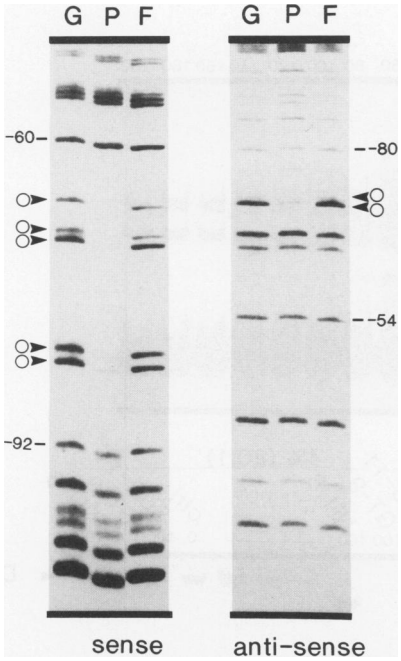


FIG. 3. Methylation interference analysis of the HiNF-P complex. The HiNF-P complex was electrophoretically fractionated by gel retardation assays using conditions described in the legend to Fig. 4. Indicated are the guanine-specific reaction (G) of the methylated input DNA (EH probe) of the sense (left panel) or antisense (right panel) strand, piperidine cleavage products of the HiNF-P–H4 site II complex (P), and free DNA (F). Methylation interference contacts are indicated by open circles, and dashes indicate the locations of adjacent nucleotides for reference.

defined by competition for this factor with a DNA fragment encompassing the DNase I footprint (nt −97 to −78) and methylation interference contacts (nt −89, −87, −86, −85, −84, and −83) of this factor (62) and containing a duplication of the M box (DD-1; nt −93 to −80) (Fig. 6). No competition for HiNF-M is observed for DNA fragments lacking the M box (PD-2; nt −82 to −66) or containing a truncation of this sequence motif (ALRW-4 and ALRM-5; nt −86 to −59). The binding site of HiNF-P includes all three sequence motifs in the distal part of H4 site II (M, C, and P boxes), as determined by strong competition for HiNF-P by oligonucleotide DS-II (nt −91 to −64) (Fig. 5b), reduced competition of oligonucleotides with truncations of the HiNF-P binding site (ALRW-4 [nt −86 to −59] and PD-2 [nt −82 to −66]), and the absence of competition by a DNA fragment containing both a truncation (nt −86 to −59) and point mutations (nt −74, −73, and −71) of the HiNF-P binding site (ALRM-5). The point mutations in the ALRM-5 fragment that affect HiNF-P binding are contained within the P box and are analogous to mutations influencing binding of H4TF-2 to the H4.A gene (10). These results further support both the importance of the P box for binding of HiNF-P and similarities in the binding activities of HiNF-P and H4TF-2.

Two independent sequence variants prevent binding of HiNF-M and HiNF-D with the H4.A gene. The sequences of the human H4-FO108 gene (42) are highly similar to those of a mouse H4 gene which we call H4-AST and which has been characterized by Seiler-Tuyns and Paterson (47). This suggests that these genes represent homologous mammalian counterparts. Interestingly, the proximal promoters of these genes have been clearly shown to mediate cell cycle-regulated transcription in vivo (44, 47). The 5′ region of the human H4.A (20) and H4-FO108 (42) genes are less similar to each other than are the human H4-FO108 and mouse H4-AST genes. Thus, the two distinct human H4-FO108 and H4.A genes may have evolved independently from a common ancestor, which is reflected by several nucleotide differences between H4 site II and the analogous region in the H4.A gene (Fig. 2B).

To address the extent to which natural sequence variation influences the interactions of H4 site II binding activities, we designed two synthetic DNA fragments spanning the analogous regions of the H4-FO108 (TM-3) and H4.A genes (NH-6) (Fig. 6). These DNA fragments either were assayed as radiolabelled probes to determine the ability to mediate

and proximally with a GC-box-binding protein, HiNF-C (64, 72), most likely identical to Sp1, with both factors capable of synergistically mediating a five fold stimulation of transcription. Cell cycle control domain H4 site II represents a mosaic of functional domains (long boxes underneath sequence motifs) that have been defined by in vivo (26, 44) and in vitro (50, 72) transcriptional assays, as well as protein-DNA interaction analyses (62, 64). The proximal region of H4 site II (nt −68 to −47) spans a TATA motif and is sufficient to mediate accurate transcription initiation in vitro (50) (T element), presumably facilitated by interaction with the TATA box-binding protein TF-IID. The distal region of H4 site II (nt −97 to −69) is required for accurate transcription initiation in vivo (26) and influences both the timing and extent of H4 gene transcription (44) (R element). The R element contains several distinct sequence motifs (small boxes) that either stimulate the basal level of H4 gene transcription (C box) or influence periodic levels of transcription (M box) (44). Apart from this, H4 site II contains a pentameric sequence (P box) that may contribute to H4 gene transcription (10). The M, C, and P boxes together are components of the H4 subtype-specific consensus sequence (64). The H4 consensus element displays significant sequence similarity with the UAS elements of cell cycle-controlled yeast histone genes (38, 39) (see panel B). The minimal binding sites of HiNF-D, HiNF-M, and HiNF-P as established by mutational analyses are indicated by grey rounded boxes above the sequences in the lower portion of the diagram, whereas grey ovals represent methylation interference contacts for these factors (62). (B) The H4 consensus element. Sequences spanning analogous regions immediately upstream of the TATA box in vertebrate H4 genes (13, 17, 20, 34, 42, 66, 68) were aligned to display the significant sequence similarity of the H4 consensus element. A vertebrate consensus sequence (27 bp), shown in boldface at the bottom of the alignment, spans nt −93 to −67 of the human H4-FO108 gene. Each of the H4 sequences shown in the diagram (with the exception of the first two lines) matches this H4 consensus sequence on average in 24 of 27 nucleotides (counting each substitution or insertion as one mismatch). Also indicated is a consensus sequence of yeast histone UAS elements (38, 39), as well as the location of CpG dinucleotides in the human H4-FO108 and H4.A genes (see Fig. 9). Dashes were inserted into the diagram to maximize similarity between sequences, lowercase letters represent mismatches with the vertebrate H4 consensus sequence, and capital letters other than A, C, G, and T represent redundant nucleotides (Y = C or T; R = A or G; K = G or T; W = A or T; M = A or C; H = A, C, or T). Footnotes are as follows: 1, the sequences of the H4/a, H4/g, and H4/h genes are from reference 13, and H4/a (13) and H4.A (20) are distinct genes; 2, location of this sequence is further upstream (20 to 30 bp) than the other sequences; 3, the published sequences of the H4.A gene (20) suggest that this gene is identical to the H4/3 gene and highly similar to the H4/d gene (13); 4, reference 42; 5, reference 17; 6, reference 69; 7, reference 66, with numbers (#) referring to the gene numbering used in reference 66; 8, reference 34; 9, references 38 and 39.

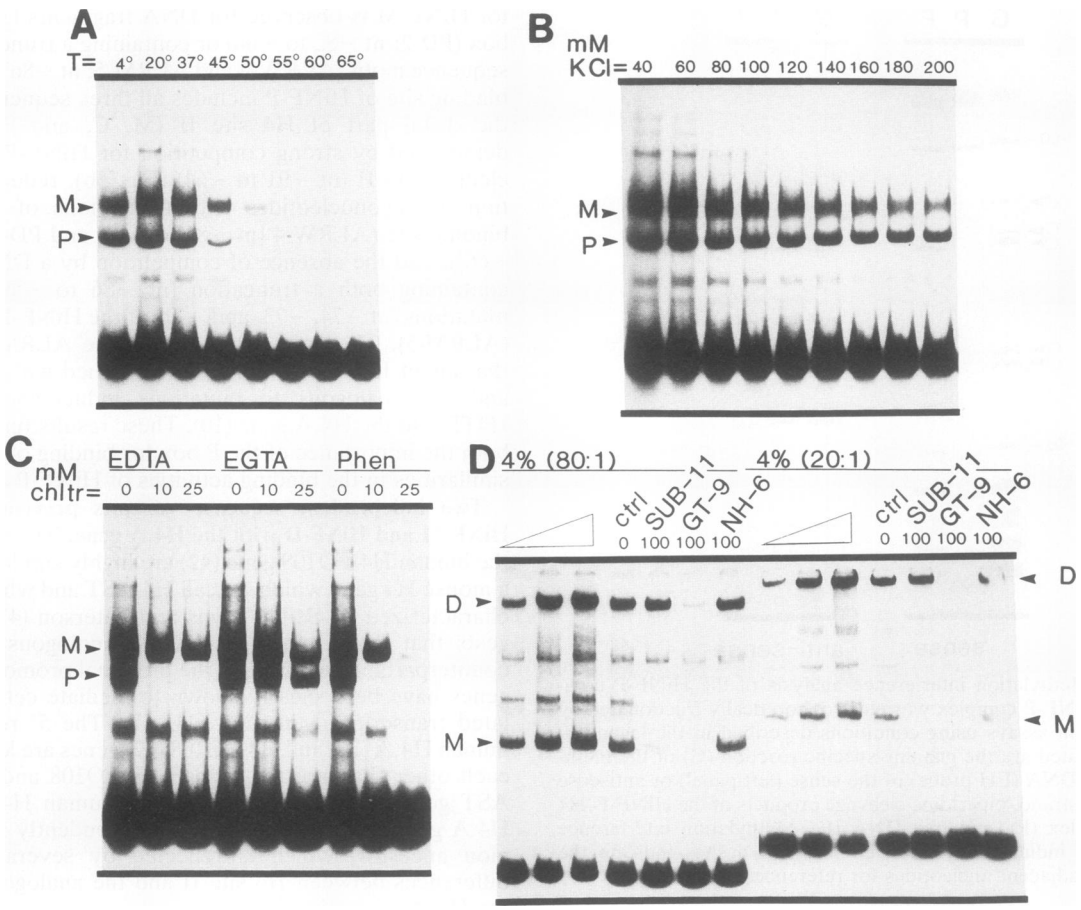


FIG. 4. Biochemical distinctions between H4 site II-binding proteins. (A) Thermal inactivation profiles of HiNF-M and HiNF-P protein-DNA complexes (indicated by arrowheads); protein samples were preincubated for 10 min at the indicated temperature (T) prior to being added to a standard binding reaction containing random DNA (from salmon sperm), 20 μg of nuclear protein, and 10 fmol of the TM-3 probe. (B) KCl concentration dependence of the HiNF-M and -P protein-DNA interactions. Binding reactions were performed at increasing KCl concentrations (indicated above the lanes). (C) Chelation sensitivity. Undialysed nuclear proteins (20 μg) were incubated for 10 min on ice in the presence of the indicated chelators at 0, 10, and 25 mM [Phen, (1, 10)-*ortho*-phenanthroline). (D) Pore size dependence of the HiNF-D and HiNF-M protein-DNA complexes (indicated by arrowheads). Binding reactions were performed by using the EH probe in the presence of poly(dG-dC) · poly(dG-dC) DNA and loaded onto 4% polyacrylamide gels with different acrylamide-bisacrylamide cross-linking ratios (lanes 1 to 7, 80:1; lanes 8 to 14, 20:1). Lanes 1 to 3 and 8 to 10 contain 4, 6, and 8 μg of protein, respectively, whereas lanes 4 to 7 and 11 to 14 each contain 5 μg of protein. Different mutant oligonucleotides (indicated above the lanes) were added in 100-fold molar excess to assess the sequence specificity of protein-DNA complexes (lanes 4 to 7 and 11 to 14) (refer to Fig. 6 and accompanying legend for details of mutations).

electrophoretically stable binding (Fig. 7) or were used as unlabelled specific competitors to assess interference with formation of stable protein-DNA complexes (Fig. 8). We observed that the radiolabelled DNA fragment spanning the H4.A gene (NH-6) was not able to mediate the formation of protein-DNA complexes of either HiNF-M (Fig. 7A and D) or HiNF-D (Fig. 7B and C). The failure of the NH-6 fragment to mediate the HiNF-D protein-DNA complex in gel retardation assays is absolute. For example, whereas reduced electrophoretically stable binding of HiNF-D is observed with the radiolabelled mutant fragments MC-7 and ST-8 (Fig. 7C), strong binding is observed with the H4-FO108 fragment (TM-3) (Fig. 7B and C) and no binding is observed for the NH-6 fragment (Fig. 7B and C). Moreover, the NH-6 oligonucleotide does not compete for HiNF-D and HiNF-M, even at high concentrations (Fig. 8), but only reduced competition for both HiNF-D and HiNF-M is observed with mutant fragments MC-7 and ST-8 (Fig. 5). These results indicate that the nucleotide differences between the analogous regions of the H4-FO108 and H4.A genes abolish the affinity of HiNF-D and HiNF-M for the H4.A gene.

However, both H4 DNA probes (NH-6 and TM-3) mediate a protein-DNA complex with the electrophoretic mobility and competition behavior of HiNF-P (Fig. 7D and E). The cross-competition we observe for the binding of HiNF-P to the H4-FO108 and H4.A genes indicates that this factor is capable of interacting with both of these H4 genes. Similar observations have been made for the binding of H4TF-2 to both the H4.A and H4-FO108 genes (28). The fact that HiNF-P and H4TF-2 have similar methylation interference contacts, interact with both genes, are not present in HiNF-M or HiNF-D containing chromatography fractions, and have similar chelator sensitivities strongly suggests that HiNF-P and H4TF-2 are identical, or at least closely related, DNA-binding activities. Although at present we cannot dismiss the possibility that these factors may represent

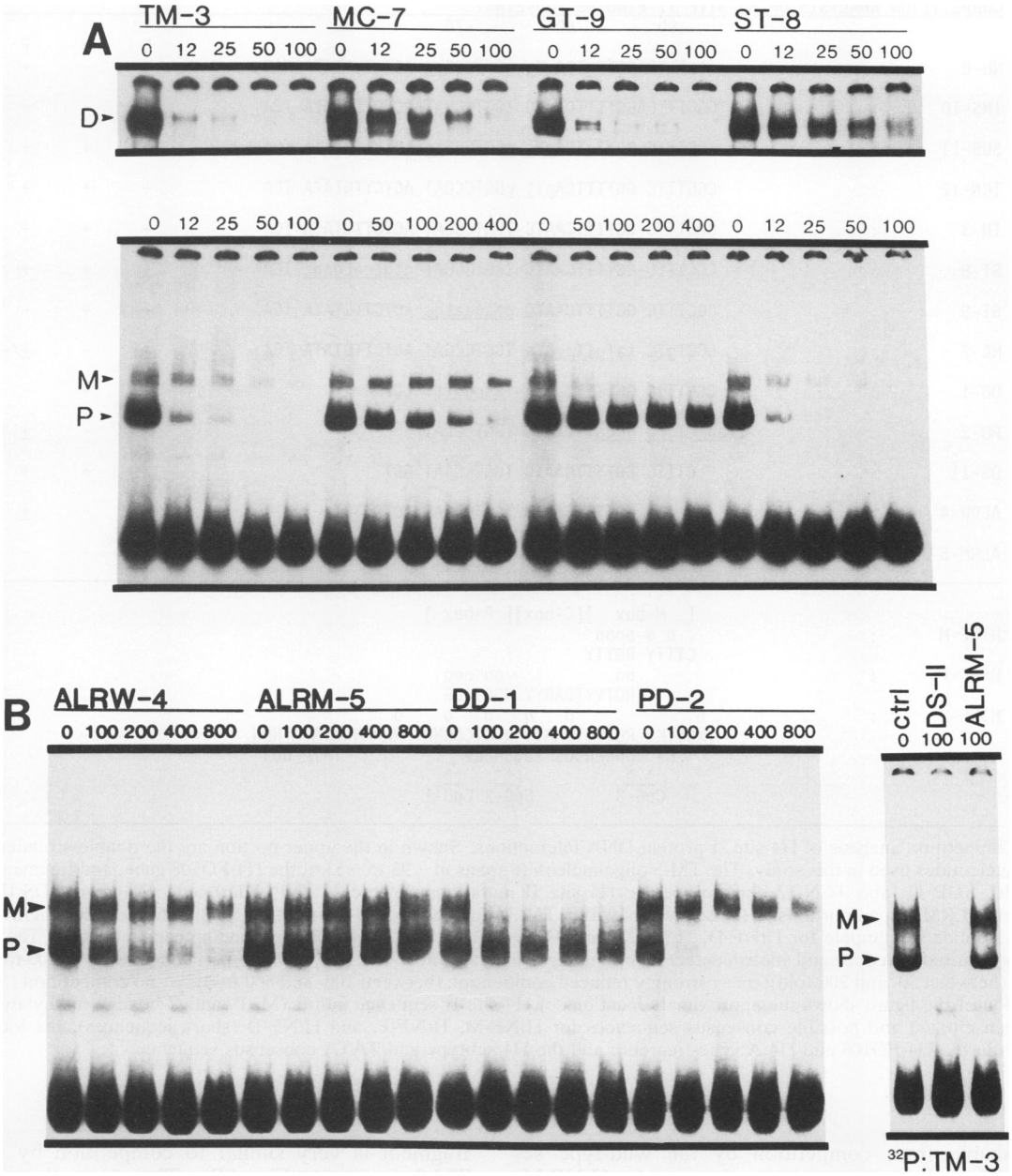


FIG. 5. Mutational analysis of H4 site II protein-DNA interactions. (A) Competition assays using increasing concentrations (shown as fold molar excess) of different oligonucleotides containing specific substitution mutations, as indicated above the lanes (see also Fig. 6). Standard reactions for HiNF-D (upper panel) were performed in the presence of poly(dG-dC) · poly(dG-dC) DNA (2 μg) and D100-250 protein (HiNF-D fraction; 10 μl), whereas those for HiNF-M and HiNF-P (lower panel) were performed with random DNA (2 μg) and 20 μg of unfractionated nuclear protein. The TM-3 probe was used to monitor complex formation, and 100-fold molar excess represents 1 pmol of synthetic DNA fragments (approximately 30 ng per 20-μl reaction; concentration, 50 nM). Complexes of HiNF-D, HiNF-P, and HiNF-M are indicated at left (D, P, and M, respectively). (B) Same as in panel A, using different sets of specific competitor DNAs.

distinct members of the same transcription factor family, we will refer to these factors as HiNF-P/H4TF-2 to reflect their relatedness.

We designed three specific mutants (INS-10, SUB-11, and TCN-12) (Fig. 6) to determine the contribution of precise nucleotide differences between the analogous elements of the H4-FO108 and H4.A genes to the binding of HiNF-D and HiNF-M. One of these fragments (INS-10) contains two separate single-nucleotide insertions (near nt −86 and −67) that influence spacing relative to the internal region of the probes spanning the H4 consensus sequence. The INS-10 probe displays additional uninterrupted sequence similarities between the H4-FO108 and H4.A genes at both 5′ and 3′ terminal sequences. The SUB-11 fragment contains six specific point mutations (nt −82, −78, −76, −75, −68, and −67) in the internal region of the TM-3 probe. These point mutations represent all nucleotide differences between the H4-FO108 and H4.A genes in the internal region (nt −86 to −67) of the H4 consensus sequence. The results (Fig. 8A) show that competition for HiNF-D and HiNF-P by INS-10 is

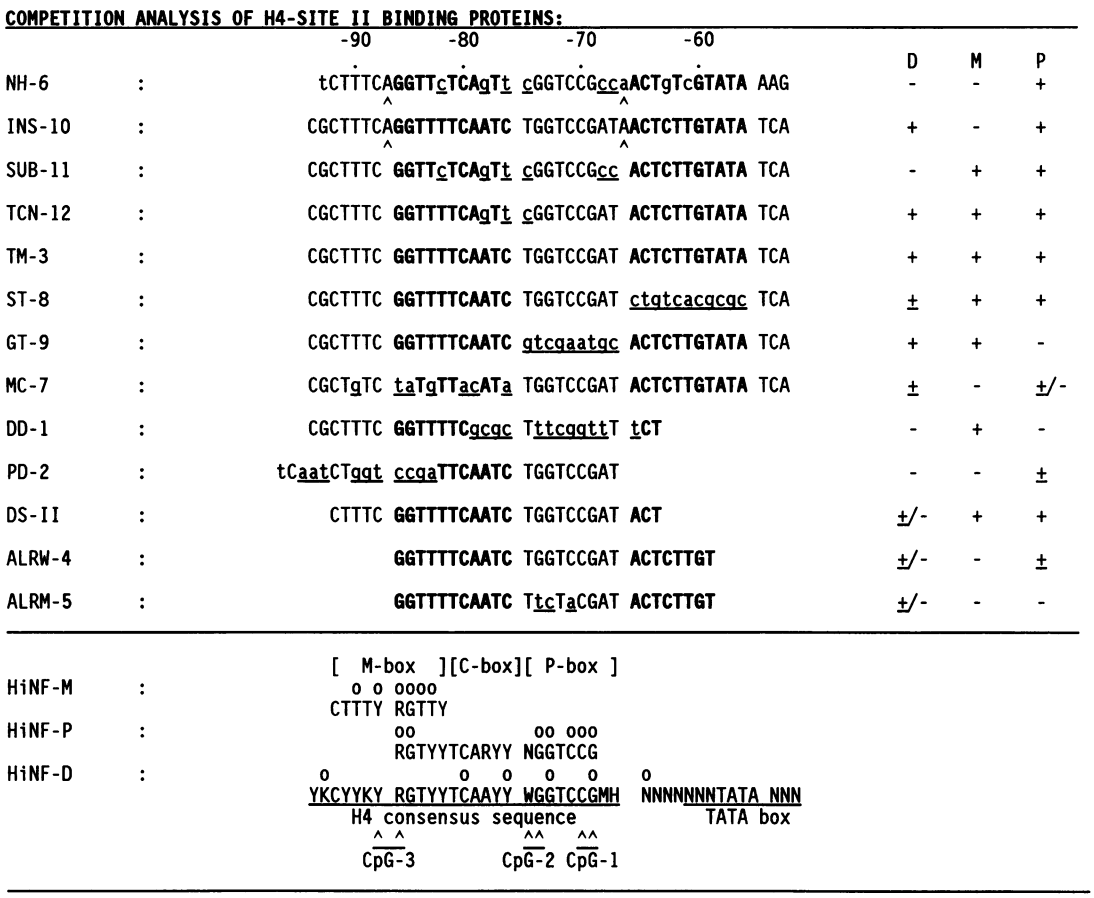


FIG. 6. Competition analysis of H4 site II protein-DNA interactions. Shown in the upper portion are the double-stranded DNA regions of the oligonucleotides used in this study. The TM-3 oligonucleotide spans nt −93 to −53 of the H4-FO108 gene, and fragments MC-7, ST-8, GT-9, INS-10, SUB-11, and TCN-12 contain specific H4 site II mutations (lowercase and underlined). Fragments DS-II, DD-1, PD-2, ALRW-4, and ALRM-5 span portions of the sequences within TM-3. The columns to the right give a qualitative assessment of the ability of each oligonucleotide to compete for HiNF-D, HiNF-M, and HiNF-P (abbreviated with corresponding capital letters). The range (fold) of molar excess required to reduce and abolish detection of binding is indicated as follows: +, "wild-type" competition (<100-fold); ±, reduced competition (between 50- and 200-fold); ±/−, strongly reduced competition (between 100- and 800-fold); −, no competition (>800-fold). The bottom portion of the figure shows the approximate locations of H4 site II sequence motifs (M, P, and C boxes); methylation interference contacts (open circles) and possible consensus sequences for HiNF-M, HiNF-P, and HiNF-D (short sequences); the locations of CpG dinucleotides in the H4-FO108 and H4.A genes (carets); and the H4 subtype and TATA consensus sequence.

indistinguishable from competition by the wild-type sequence (TM-3), but no competition is observed for HiNF-M. Thus, the spacing between 5′ and 3′ terminal sequences (distal of nt −86 and proximal of nt −67, respectively) relative to the H4 consensus sequence, which is different for the two H4 genes, does not affect HiNF-D binding. However, the insertion of a single nucleotide in the M box (present in INS-10) is sufficient to abolish HiNF-M binding. Thus, the presence of an additional nucleotide in the M box equivalent of the H4.A gene represents a specific nucleotide variant that precludes binding of HiNF-M.

Interestingly, whereas competition for HiNF-D by mutant fragment TCN-12 (point mutations at nt −78, −76, and −75; Fig. 6) is very similar to that by TM-3, the mutant fragment SUB-11 does not compete even at high concentrations (Fig. 8B). This result identifies a set of six nucleotide mutations (nt −82, −78, −76, −75, −68, and −67, contained within SUB-11; Fig. 6) that is sufficient to eliminate affinity of HiNF-D for the H4.A gene. In contrast, competition for HiNF-M and HiNF-P by either the SUB-11 or TCN-12 fragment is very similar to competition by the wild-type TM-3 fragment (Fig. 8B). Therefore, these results with HiNF-M and HiNF-D have established two independent sets of natural nucleotide variants each of which represents a unique set of critical recognition nucleotides for these factors. Moreover, these findings provide a clear molecular explanation for differences in protein-DNA interactions between the two human histone H4 genes, H4-FO108 and H4.A.

Selective inhibition of HiNF-P binding by CpG methylation. Both the H4-FO108 and H4.A genes contain several CpG dinucleotides within the H4 consensus sequence (Fig. 2B) each of which coincides with methylation interference contacts for H4 site II-binding proteins: CpG doublets are found at nt −87/−86 (CpG-3; present in H4-FO108), nt −75/−74 (CpG-2; present in H4.A), and nt −70/−69 (CpG-1; present in both genes). To explore whether CpG methylation influences the interactions of HiNF-D, HiNF-M, and HiNF-P/H4TF-2 with H4 site II, we used a DNA fragment (EH probe; H4-FO108, nt −97 to −38) that was incubated either

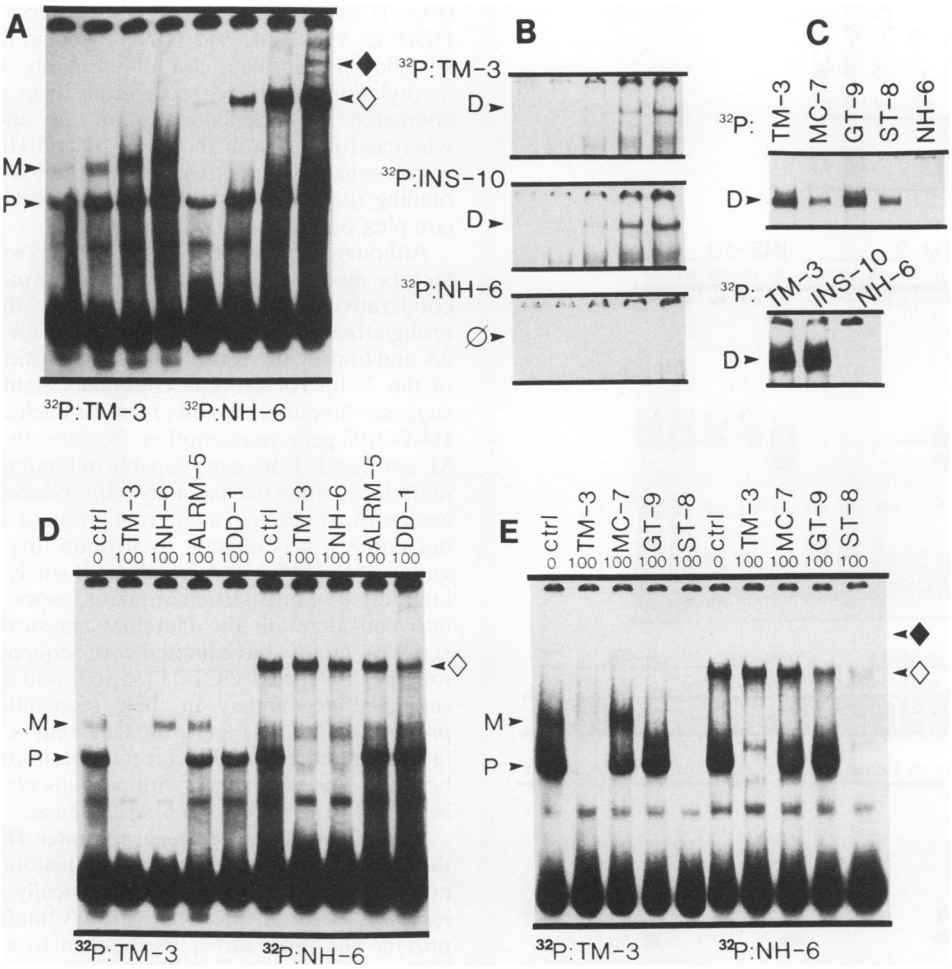


FIG. 7. Distinctions in binding events of the paralogous H4-FO108 and H4.A genes. Shown are gel retardation assays revealing differences and similarities in electrophoretically stable protein-DNA interactions in the analogous regions of the H4-FO108 and H4.A genes. Assays depicted in panels A, B, and C measure binding to the H4-FO108 (TM-3 probe) and the H4.A (NH-6 probe) genes, as well as to probes containing specific mutations (see Fig. 6) as indicated, using conditions optimal for detection of each factor (see Materials and Methods). Panels D and E show cross-competition of HiNF-P binding to both the H4-FO108 and H4.A genes. (A) Lanes 1 to 4 and 5 to 8 contain 4, 8, 12, and 16 μg of unfractionated nuclear protein, respectively. A faint nonspecific band with a migration rate slightly greater than that of the HiNF-M complex can be observed in lanes 5 to 8 (not indicated). This complex does not compete for HiNF-M (see panel D). The open diamond refers to a strong band for which no competition was observed (see panels D and E). (B) Reactions in the upper, middle, and lower panels contain 4, 8, 12, 16, and 20 μg of nuclear protein in lanes 1 to 5, respectively. D, the HiNF-D complex; slashed circle, absence of detection. (C) Reactions in the upper and lower panels all contain a fixed volume (5 μl) of the D100-250 (HiNF-D) fraction, with different probes as indicated. (D and E) Cross-competition analysis of HiNF-P with identical sets of oligonucleotides (see Fig. 6), using the indicated probes (lanes 1 to 5, TM-3; lanes 6 to 10, NH-6). Numbers above the lanes indicate fold molar excess of competitor DNA fragments.

in the absence (control) or presence of a large excess of CpG methylase, and both DNA samples were processed in parallel under identical conditions. The extent of methylation was monitored by resistance of both methylated and nonmethylated DNA aliquots to restriction enzymes sensitive to cytosine methylation (Fig. 9). The results (Fig. 9A) show that binding of either HiNF-D or HiNF-M to H4 site II is not significantly influenced by CpG methylation but that binding of HiNF-P/H4TF-2 was severely reduced. Hence, CpG methylation selectively interferes with the binding of HiNF-P to the H4-FO108 gene.

Using a panel of oligonucleotides (TM-3, INS-10, TCN-12, and NH-6) containing only the three CpG doublets discussed above, we monitored the binding of HiNF-P/H4TF-2 to each of these methylated or unmethylated DNA fragments (Fig. 9B), with the methylated DNA fragments being completely resistant to *Ava*II cleavage (Fig. 9C). The results show that methylation of CpG-1, CpG-2, and CpG-3 (TCN-12) completely abolishes HiNF-P/H4TF-2 binding and that methylation of either CpG-1 and CpG-3 (TM-3) or CpG-1 and CpG-2 (NH-6) strongly reduces HiNF-P/H4TF-2 binding (Fig. 9B). Thus, although the effect of methylation is more severe as the number of CpG methylation sites increases, two CpG doublets are sufficient to impair HiNF-P/H4TF-2 binding. Moreover, methylation of INS-10 also decreased HiNF-P binding (Fig. 9B). This DNA fragment contains only the one CpG doublet (CpG1) that is conserved in both the H4-FO108 and H4.A genes, as well as a number of other H4 histone genes (Fig. 2B). Thus, CpG methylation interferes with HiNF-P/H4TF-2 binding to both the H4-FO108 and

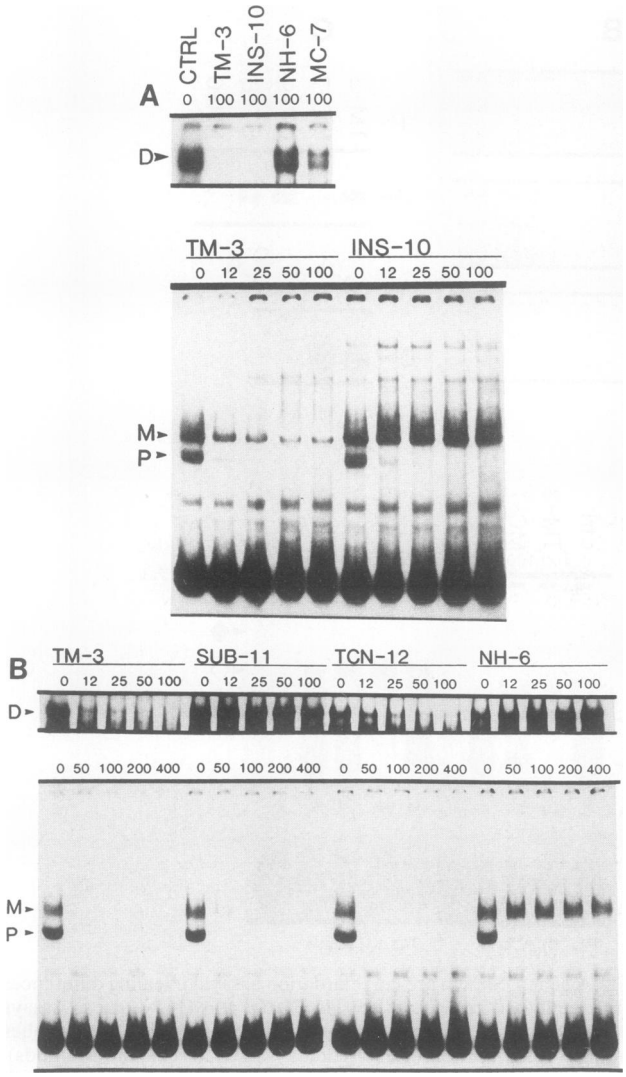


FIG. 8. Unique nucleotide variants prevent interaction of HiNF-M and HiNF-D with H4.A. Shown are competition assays (essentially performed as described in Fig. 5) using different concentrations of oligonucleotides, as summarized in the legend to Fig. 6. Reactions contained D100-250 (HiNF-D) fraction (upper panel of part A) or unfractionated nuclear protein (remaining panels). Numbers above the lanes indicate fold molar excess of competitor DNA fragments.

H4.A genes, and methylation of a single, highly conserved CpG dinucleotide (nt −70/−69) is sufficient to decrease the affinity of HiNF-P/H4TF-2 for H4 site II.

DISCUSSION

The histone H4 site II transcriptional cell cycle domain containing the H4 subtype consensus sequence represents a multipartite protein-DNA interaction site. In this study, we have shown that occupancy of H4 site II in vitro occurs by several distinct nuclear factors, including HiNF-D, HiNF-M, and HiNF-P, with each of these recognizing distinct nucleotides within the H4 consensus element. The conclusion that these H4 site II DNA-binding proteins are biochemically distinct (Fig. 4) is supported by the extensive chromatographic separation of H4 site II binding activities (Fig. 1) (62), as well as by the observations that factors HiNF-D, HiNF-M, and HiNF-P recognize unique (factor-specific) nucleotides, are differentially sensitive to CpG methylation of shared recognition base pairs, and can be competed for independently of one another. Moreover, whereas formation of the HiNF-D and HiNF-M complexes is phosphatase sensitive, as determined by inhibition of binding (62), no sensitivity was observed for the HiNF-P complex (unpublished observations).

Although we cannot dismiss the possibility that these factors mediate mutually exclusive protein-protein and/or cooperative DNA-protein interactions, the overlap and interdigitation of recognition nucleotides for these factors (Fig. 2A and 6) provides a rationale for evolutionary conservation of the 27-bp H4 subtype consensus element (Fig. 2B) and suggests functional involvement of each of these factors in H4-FO108 gene transcription. Specifically, HiNF-D, HiNF-M, and HiNF-P are each capable of binding to distinct sets of mutations, albeit occasionally with reduced affinity. Because each protein requires a different group of critical recognition nucleotides, it is difficult to attribute the extensive conservation of the H4 consensus element solely to a protein-DNA interaction of one particular factor, especially when we take into consideration the fact that classical eukaryotic transcription factors have limited core sequences (e.g., 4 to 8 bp for SP1, AP1, and OCT-1) (35, 67) and allow considerable sequence degeneracy in their recognition elements. We propose that the H4 consensus element is a one-dimensional lattice directing spatially correct protein-protein associations between several factors, with perhaps none of these factors being H4 gene specific in a strict sense.

Regulation of histone gene promoter DNA-binding activities. Transcriptional regulation of histone gene expression may occur at a series of mechanistically distinct molecular levels. Control at the level of DNA-binding activity would provide the most direct mechanism to alter occupancy of histone promoter protein-DNA interaction sites. In a series of studies (Table 1), we have assessed the correlation between modulations in histone gene expression and alterations in histone gene DNA-binding activities (see footnote to Table 1 for references containing experimental details and supporting data). Factor HiNF-D has been isolated from a broad spectrum of proliferating cell types in our laboratory (3, 23, 40, 57, 58, 64, 71), as well as other laboratories (23, 58, 71) in collaborative studies (Table 1). Competition and deletion analyses (references 57, 61, and 62 and unpublished data) have confirmed the detection of HiNF-D binding activity in nuclear protein preparations of at least three distinct mammalian species (human, mouse, and rat), supporting cross-species compatibility of histone gene DNA-binding activities. Hence, HiNF-D is an evolutionarily conserved and reproducibly detectable nuclear factor.

Studies using synchronized human HeLa S3 cervical carcinoma cells indicate that at least four histone promoter factors (HiNF-D, -C, -B, and -A) (60, 64) are present throughout the cell cycle, consistent with the persistence of histone gene transcription throughout the cell cycle in tumor cells (27, 29, 38, 44). Strikingly, HL60 promyelocytic leukemia cells that have been induced to differentiate with the phorbol ester tetradecanoyl phorbol acetate display selective downregulation of nuclear factor HiNF-D, while levels of at least two other DNA-binding activities (HiNF-C/Sp1 and HiNF-A/HMG-I) remain constant during the onset of differentiation (64). This process is accompanied by loss of in vivo H4 site II protein-DNA interactions and persistent occupancy of H4 site I inside the intact cell (51). The vacancy of

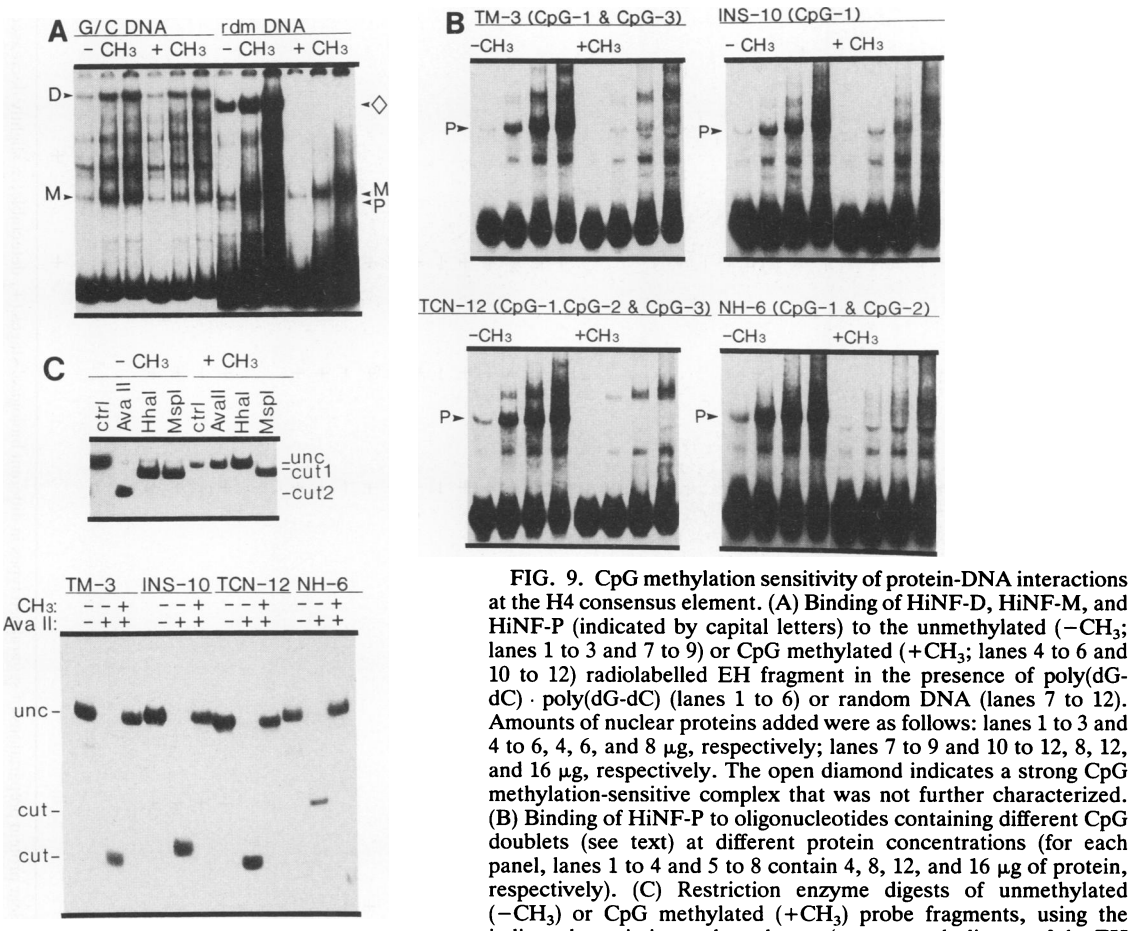


FIG. 9. CpG methylation sensitivity of protein-DNA interactions at the H4 consensus element. (A) Binding of HiNF-D, HiNF-M, and HiNF-P (indicated by capital letters) to the unmethylated ($-CH_3$; lanes 1 to 3 and 7 to 9) or CpG methylated ($+CH_3$; lanes 4 to 6 and 10 to 12) radiolabelled EH fragment in the presence of poly(dG-dC) · poly(dG-dC) (lanes 1 to 6) or random DNA (lanes 7 to 12). Amounts of nuclear proteins added were as follows: lanes 1 to 3 and 4 to 6, 4, 6, and 8 μg, respectively; lanes 7 to 9 and 10 to 12, 8, 12, and 16 μg, respectively. The open diamond indicates a strong CpG methylation-sensitive complex that was not further characterized. (B) Binding of HiNF-P to oligonucleotides containing different CpG doublets (see text) at different protein concentrations (for each panel, lanes 1 to 4 and 5 to 8 contain 4, 8, 12, and 16 μg of protein, respectively). (C) Restriction enzyme digests of unmethylated ($-CH_3$) or CpG methylated ($+CH_3$) probe fragments, using the indicated restriction endonucleases (upper panel, digests of the EH probe used in panel A; lower panel, digests of oligonucleotides used in panel B). The uncut full-length fragments (unc) and cleavage products (cut) are indicated.

H4 site II correlates with transcriptional downregulation of cell cycle-controlled H4 (51), H2B (6), and H1 (7) genes, although expression of a variant poly(A)$^+$ H2B gene is increased (6). Therefore, these results for H4 site II in vivo and HiNF-D in vitro demonstrate that selective modifications occur in protein-DNA interactions when histone gene transcription is modulated.

The correlation between cessation of cell proliferative activity, transcriptional downregulation of histone gene expression, and loss of HiNF-D binding activity is also evident in other cell culture systems, including during differentiation of primary rat calvarial osteoblasts (40) and mouse 3T3 preadipocytes (3), serum deprivation of CF-3 fibroblasts (71), and density-inhibited growth of rat osteosarcoma (ROS 17/2.8) cells (data not shown). Moreover, in transgenic mice during hepatic development, HiNF-D binding activity declines in parallel with downregulated expression of both endogenous mouse H4 genes and an introduced reporter transgene driven by the human histone H4-FO108 gene promoter (58). Because of this well-established and consistent relationship between HiNF-D and cell proliferation, the observation by Holthuis et al. (23) that HiNF-D is cell cycle regulated in normal diploid human WI-38 fibroblasts and primary rat calvarial osteoblasts, but not in four different tumor cell types, is consistent with deregulation of the cell cycle-dependent DNA-binding activity of this factor during neoplastic transformation.

In contrast, the binding activities of factors H4TF-2, which is similar to HiNF-P, and H4TF-1 are constitutive throughout the cell cycle in both normal diploid and tumor-derived cells (28). Similarly, nuclear factor HiNF-A, which recognizes A/T-rich DNA sequences in histone H4, H3, and H1 promoters, is a cell cycle-independent DNA-binding protein irrespective of cell type (61). With normal diploid cells, but not with tumor cells, we also have observed cell cycle-dependent sequence-specific protein-DNA interactions in human histone H3 and H1 gene promoters, and regulation of these interactions is remarkably similar to that of the HiNF-D–H4 site II interaction (61). We have discussed previously (61) that in all cell cycle experiments in which HiNF-D binding activity was found to change, we included several controls and precautions which indicate that trivial explanations of our findings are extremely unlikely. We conclude from the currently available data (23, 27–29, 60, 61, 64) that only a subset of human histone gene protein-DNA interactions is regulated during the cell cycle.

Deregulation of HiNF-D binding activity and regulation of histone gene expression in normal and tumor cells. Deregulation of HiNF-D binding activity during the cell cycle in tumor cells (23) could suggest that this aberration is functionally significant for histone gene transcription during neoplastic transformation. However, irrespective of the role of putative transcription factors, multilevel regulation of histone gene expression (33, 38) is operative in proliferating

TABLE 1. Cellular distribution of histone gene promoter DNA-binding activities[a]

Cell type	Biological stage	H4UA-1	HiNF-A	HiNF-B	HiNF-C	HiNF-E	HiNF-D1	HiNF-D3	HiNF-D	HiNF-M	HiNF-P
Proliferating and nonproliferating cells											
Human HeLa cervical carcinoma	Proliferating	+	+	+	+	+	+	+	+	+	+
Mouse C127 fibroblasts	Proliferating		+	+	+				+		
Human HL60 promyelocytes	Proliferating	+	+		+				+	+	
	Differentiated	±	+		+				−	+	
Mouse 3T3 preadipocytes	Proliferating								+	+	
	Differentiated								−	+	
Rat calvarial osteoblasts	Proliferating	+		+	+	+	+	+	+	+	+
	Differentiated	−		+	+	+	−	−	−		
Rat ROS 17/2.8 osteosarcoma	Proliferating	+		+	+	+	+	+	+	+	+
	Density inhibited			+	+	+	−	−	−		
Human CF-9 fibroblasts	Serum deprived				+				−		
	Serum stimulated				+				+		
Human WI-38 fibroblasts	Serum deprived	+									
	Serum stimulated	+									
Cell cycle synchronization											
Human HeLa cervical carcinoma	S phase	+	+	+	+		+	+	+		
	G_2, M, and G_1 phases	+	+	+	+		+	+	+		
Human HL60 promyelocytes	S phase						+	+	+		
	G_2, M, and G_1 phases						+	+	+		
Human WI-38 fibroblasts	S phase		+				+	+	+		
	G_2, M, and G_1 phases		+				−	−	−		
Human WI-38/SV40 transformed fibroblasts	S phase						+	+	+		
	G_2, M, and G_1 phases						+	+	+		
Rat calvarial osteoblasts	S phase		+				+	+	+		
	G_2, M, and G_1 phases		+				−	−	−		
Rat ROS 17/2.8 osteosarcoma	S phase						+	+	+		
	G_2, M, and G_1 phases						+	+	+		
In vivo tissue distribution											
Mouse liver	Fetal			+			+	+	+	+	
	Adult			+			−	−	−	+	
Mouse brain	Fetal			+			+	+	+	+	
	Adult			+			+	+	+	+	
Mouse spleen	Adult								+	+	
Mouse thymus	Adult								+	+	
Rat liver	Adult	+							−	+	
Rat brain	Adult	+							+	+	

[a] Shown is a qualitative assessment of the presence of the indicated histone promoter factors in nuclear protein preparations of several cell types in different biological stages (+, detectable; ± slightly decreased presence; −, severely reduced presence). Details of these experiments and supporting data have been documented in several references (23, 40, 56, 58, 60, 61, 64, 71). The sequence-specific histone promoter DNA-binding activities shown here each have been characterized by several of a number of techniques, including competition, mutation, and deletion analyses; DNAse I and DMS protection; methylation and depurination interference, and chromatographic separation. Factors H4UA-1 (56, 70), HiNF-C/Sp1 (64, 72), HiNF-D (62, 64), HiNF-P, and HiNF-M (62) have been shown to interact with the H4-FO108 gene. Factors HiNF-D3 and HiNF-D1 (61) are HiNF-D-related proteins binding to the H3-ST519 and H1-FNC16 genes, respectively. Other DNA-binding activities appear to interact with at least two histone genes, including HiNF-A (H4-FO108, H3-ST519, and H1-FNC16) (63, 65), HiNF-B (H3-ST519 and H1-FNC16) (59, 65), and HiNF-E/ATF-84 (H4-FO108 and H3-ST519) (59, 72). Related data on other histone gene promoter-binding activities and *cis*-acting elements characterized in other laboratories have been reviewed recently (38) or were presented in several references (e.g., see references 11, 12, 15, 24, 25, 30, 31, 48, 53, 55, and 69).

cells regardless of the extent to which cells display cell growth properties of the transformed phenotype. Thus, the absence of histone mRNA accumulation, despite the persistence of histone gene transcription outside of S phase and in the absence of DNA synthesis, appears to reflect posttranscriptional rate-limiting steps (33, 38), ensuring maintenance of the coupling between histone gene expression and DNA replication.

The biological regulation of HiNF-D suggests that it is a critical histone gene promoter factor. On the basis of differences in cell cycle regulation of HiNF-D binding activity in cell types with distinct cell growth characteristics, this raises the possibility that differences may exist in the relative importance of transcriptional and posttranscriptional regulatory levels between normal and tumor cells. However, the DNA-binding activities and *trans*-activating properties of transcription factors can be controlled separately (35); thus, deregulation of HiNF-D binding activity does not exclude cell cycle regulation of a putative transcriptional activity (e.g., involving interactions with other proteins). Regardless, because the loss of cell cycle-dependent modulation of HiNF-D binding activity in tumor cells does not affect the periodic accumulation of histone mRNAs (23), it appears that deregulation of HiNF-D binding activity may reflect a phenotypic molecular event, rather than a consequential (or causative) mechanism during neoplastic transformation.

Detection of HiNF-D in cellular protein preparations. In a recent study (28), HiNF-D could not be detected in the protein preparations used by the investigators, a result which prevents comparison with results from our laboratory. This inability to detect HiNF-D is surprising, because our laboratory routinely measures HiNF-D activity (e.g., see references 23, 62, and 64; see also Table 1). However, the data shown by these authors were obtained by using experimental conditions for protein isolation, binding reactions, and electrophoresis that were different from those optimized for detection of HiNF-D (23, 62). For example, our laboratory assays HiNF-D from nuclear extracts, whereas LaBella and Heintz used whole cell extracts from WI-38 cells in their study. The presence of cytosolic proteins influences the specific activity of nuclear proteins which may adversely affect detection of nuclear factor HiNF-D. Also, the possibility must be considered that fluctuations of HiNF-D activity in cell cycle stage-specific nuclear extracts could reflect cell cycle-dependent alterations in compartmentalization of (inhibitory) factors. With respect to the possibility that factor HiNF-D is "extremely labile" (28) and therefore difficult to isolate or maintain, we have observed that this activity is stable for several weeks at 4°C and for longer than 1 year at −70°C in protein storage buffer but is very sensitive to oxidation and phosphatase activity (reference 62 and unpublished observations).

Concluding remarks. In this study, we have shown that HiNF-D, HiNF-P/H4TF-2, and HiNF-M are distinct entities and that the H4 subtype consensus sequence in the H4.A gene contains unique nucleotide variations that preclude binding of HiNF-D and HiNF-M. We propose that a compounding series of procedural differences and biochemical distinctions between HiNF-D and HiNF-P/H4TF-2, as well as the absence of HiNF-D and HiNF-M binding sites in the analogous region of the H4.A gene, may have resulted in the inability (28) to detect HiNF-D and HiNF-M. However, because these DNA-binding activities have been reproducibly and independently assayed by several investigators (Table 1), failure to detect HiNF-D activity may not represent a compelling argument to construe the findings by Holthuis et al. (23) as either "provisional" or "in error" (28).

The HiNF-D–H4 site II interaction reflects a nucleoprotein complex with an apparent high molecular weight. Therefore, rather than referring to DNA-binding activity of a single polypeptide in a restricted sense, we consider the possibility that HiNF-D binding activity is composed of distinct subunits. Cell cycle modulations of the HiNF-D complex in normal diploid cells may reflect periodic and reversible phosphorylation-dependent (62) protein association events between putative constitutive DNA-binding activities and/or regulatory subunits. Similar mechanisms have been proposed for other human histone gene promoter factors (46, 60, 62). However, because we have shown in this study that HiNF-D contains a unique sequence specificity distinct from that of other H4 site II-binding activities, the recruitment of this activity into an H4 site II protein-DNA complex is a cell cycle-controlled event irrespective of the mechanism by which this recruitment occurs.

We have probed the regulatory mechanism of H4-FO108 gene transcription by a systematic series of in vitro approaches, including extensive biological characterization, chromatographic separation, recognition site analyses of H4 site II-binding proteins, and cell free transcription assays (e.g., see references 23, 50, 62, 64, 71, and 72), as well as by in vivo transcriptional analyses (26, 44) and genomic protection assays (42, 51). These results have increased our understanding of the transcriptional regulation of the H4-FO108 gene (52). However, we agree with LaBella and Heintz that further studies on histone H4 gene-binding activities HiNF-D, HiNF-M, and HiNF-P/H4TF-2 are required to reveal the specific contributions of each of these in the cell cycle regulation of gene transcription. Indeed, these studies are currently in progress. Also, the selective inhibitory effect of CpG methylation on histone gene-binding activities deserves further consideration. We conclude that variations in the distribution of transcription factor sites in the 5′ regions of distinct human histone H4 genes and the multipartite nature of the H4 consensus sequence located in H4 site II suggest that transcriptional regulation of the H4 multigene family occurs by heterogeneous and intricate mechanisms.

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