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# Protein/DNA Interactions Involving ATF/AP1-, CCAAT-, and HiNF-D-Related Factors in the Human H3-ST519 Histone Promoter: Cross-Competition With Transcription Regulatory Sites in Cell Cycle Controlled H4 and H1 Histone Genes

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**Abstract** Protein/DNA interactions of the H3-ST519 histone gene promoter were analyzed in vitro. Using several assays for sequence specificity, we established binding sites for ATF/AP1-, CCAAT-, and HiNF-D related DNA binding proteins. These binding sites correlate with two genomic protein/DNA interaction domains previously established for this gene. We show that each of these protein/DNA interactions has a counterpart in other histone genes: H3-ST519 and H4-F0108 histone genes interact with ATF- and HiNF-D related binding activities, whereas H3-ST519 and H1-FNC16 histone genes interact with the same CCAAT-box binding activity. These factors may function in regulatory coupling of the expression of different histone gene classes. We discuss these results within the context of established and putative protein/DNA interaction sites in mammalian histone genes. This model suggests that heterogeneous permutations of protein/DNA interaction elements, which involve both general and cell cycle regulated DNA binding proteins, may govern the cellular competency to express and coordinately control multiple distinct histone genes.

**Key words:** gene expression, transcription, histone gene, cell cycle, development, DNA/protein interaction

The orchestrated expression of groups of genes specifying the synthesis of structural and enzymatic proteins is required for the execution of global cellular functions. Duplication of the genome during the cell cycle occurs in every eukaryotic organism and involves expression of genes that are directly or indirectly associated with DNA replication [1,2]. The regulated expression of this S phase related gene program is initially controlled at the transcriptional level.

Five classes of cell cycle dependent histone genes (H1, H2A, H2B, H3, and H4) are coordinately expressed during S phase and are required for the assembly of DNA into chromatin. Each class represents a functional multi-gene family encoding the same or closely related proteins [3]. Transcriptional regulation of individual histone genes has been studied in diverse organisms ranging from yeast to man [4–15]. Nonetheless, our understanding of how these genes are jointly rendered competent for transcription in proliferating cells from diverse cell lineages in higher eukaryotes is limited. The principles that govern the transcriptional competency of this group of structurally and functionally related genes are fundamental to the intricate mechanisms regulating batteries of cell type specific genes during development [16–19].

Parallel studies on in vitro transcription and protein/DNA interactions of several distinct human H1 [20–24], H2B [25–26], and H4 [27–30] histone genes in different laboratories have resulted in the definition of cis-acting elements that are functionally involved in transcription of these genes and represent sites for transcription factor binding in vitro. Elements have been described for Sp1 [27], OTF-1 [25], ATF- [29], and CCAAT-box related DNA binding proteins [20,24]. Recently, the available repertoire of histone gene 5′ flanking sequences has been expanded [31–36], which allows analysis of the evolutionary conservation of these elements in the 5′ regions of closely related mammalian species. Moreover, we have performed an in-

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depth analysis of regulatory sequences and protein/DNA interactions required for in vivo and in vitro transcription of the F0108 human H4 histone gene during the cell cycle and differentiation [27–29,37–44]. We have also established genomic protein/DNA interaction domains in vivo for the human H4-F0108 and H3-ST519 histone genes [45,46].

These detailed analyses of molecular and cellular parameters that regulate several human histone genes raise a key issue in histone gene expression: What are the determinants that mediate coordinate transcriptional regulation of multiple histone genes in cells of diverse ontogeny. Direct identification of protein/DNA interactions in additional histone gene promoters may contribute to our understanding of this problem. In the present study, we show that the H3-ST519 histone promoter mediates multiple protein/DNA interactions in vitro that correlate with two genomic protein/DNA interaction domains previously established for this gene [46]. One of these protein/DNA interactions involves the cell cycle regulated DNA binding activity HiNF-D3 [47]. Competition analysis suggests that this protein/DNA interaction involves a DNA binding activity that also binds to three different H4 histone genes. We have also established binding sites for ATF- and CCAAT-box binding proteins. Competition experiments and sequence alignments using extended consensus sequences indicate that each of these protein/DNA interactions has a counterpart in other, but not all, histone gene classes. Our results are discussed within the context of established and putative protein/DNA interaction sites in mammalian histone genes. Current data suggest that heterogeneous permutations of protein/DNA interaction elements in histone promoters, which involve both general and cell cycle regulated DNA binding proteins, may govern the cellular competency to express, and perhaps coordinately control, multiple distinct histone genes.

## MATERIALS AND METHODS

### Gel Retardation Assays, Stairway Assays, and Competition Analysis

Nuclear extraction and chromatographic fractionation procedures, as well as gel retardation assays, have been documented previously [20,28,47,48]. Radio-labelled DNA fragments (5′ end-label) were derived from pST519ΔH or pTP-1 [47]. These fragments span the proximal promoter of the human H3 histone gene ST519 and are uniquely labelled at one DNA terminus. The probes were prepared by site-specific endonuclease cleavage, dephosphorylation using calf intestinal phosphatase, and subsequent $^{32}$P-labelling using T4 polynucleotide kinase [49,50]. DNA fragments were then cleaved using a secondary restriction enzyme and purified by gel electrophoresis.

The following probes were derived from pST519ΔH using the pair of restriction endonucleases indicated in brackets. The terminus created by the first enzyme is $^{32}$P-labelled. Nucleotides are measured relative to the protein coding region and represent dsDNA sequences associated with the labelled fragment: respectively, HX- and XH-probes (nt −256/−20; HindIII/XmnI(=Asp700) fragment), and HHp- and HpH-probes (nt −200/−20; HindIII/HpaI fragment). The other probes were derived from pTP-1: respectively, EH- and HE-probes (nt −200/−20; EcoRI/HindIII fragment), EB-probe (nt −200/−143; EcoRI/BstNI fragment), and HB-probe (nt −139/−20; HindIII/BstNI fragment). Deletion analysis (stairway assay) [48,51] was performed by shortening the above probes, using a panel of restriction enzymes (indicated in the figure legends). Protein/DNA interactions mediated by each set of deletion mutants were analyzed by electrophoresis of binding reactions in parallel in native polyacrylamide gels.

Competition analysis was carried out with a panel of oligonucleotides (summarized in Results) and several unlabelled plasmid DNA fragments isolated from pST523B (a 0.74 kB EcoRI/NcoI fragment containing the 5′ portion of this H4 histone gene and a 1.1 kB NcoI/EcoRI fragment containing the 3′ portion) and pST512 (1.0 kB NcoI/NcoI fragment).

### DNaseI Footprinting, DMS Fingerprinting, and Methylation Interference Analysis

DNaseI and dimethylsulfate (DMS) protection analysis, as well as methylation interference assays, were executed as described previously [20,52]. The probes used for these experiments are indicated in the figure legends; the non-specific competitor in all cases was poly (dI-dC)*(dI-dC). Results with DNaseI and DMS protection experiments were obtained by direct analysis on denaturing gels of the processed reaction products derived from binding mixtures containing fractionated nuclear proteins. Methylation interference analysis was performed by electrophoretic separation of free and com-

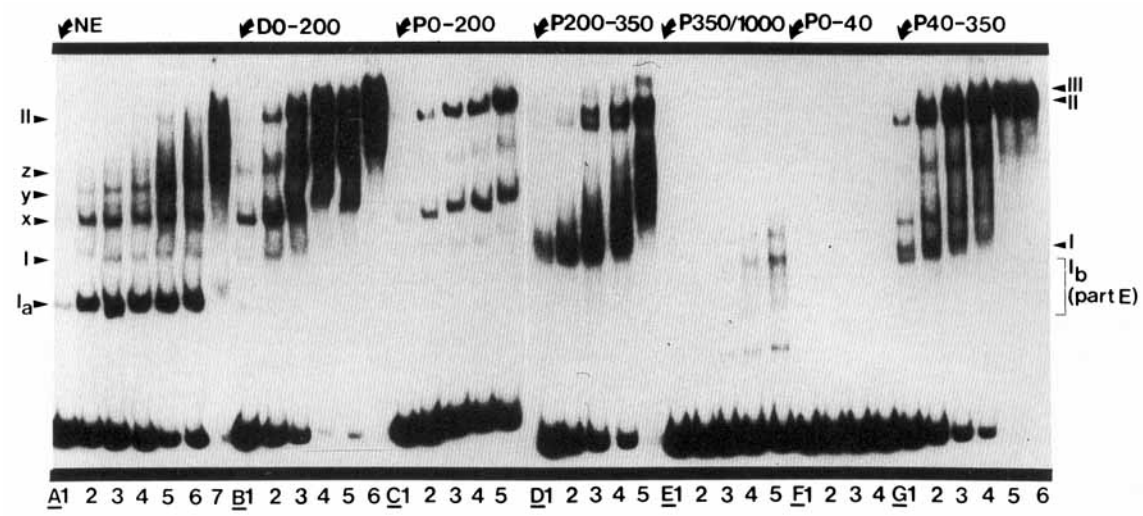



**Fig. 1.** The human H3-ST519 promoter mediates formation of multiple DNA/protein complexes in vitro. Gel retardation assays were performed with the XH-probe (Asp700/HindIII; nt −256/−20) and unfractionated (lanes A1–A6) or fractionated nuclear proteins from HeLa S3 cells (lanes B1–G6) using poly (dI–dC)*(dI–dC) (2 μg) as non-specific competitor. Chromatography fractions were obtained as described previously [20,48] using DEAE Sephacel ("D")(partB) and phosphocellulose ("P") (parts C to G). Proteins were eluted using the indicated amounts of KCl (in mM) (e.g., P0-200 represents the flow through fraction of a phosphocellulose column at 200 mM KCl in chromatography buffer, whereas P200-350 represents the subsequent fraction obtained by elution with 350 mM KCl). Fractionated proteins throughout this study were used solely to facilitate detection of DNA binding activities. Electrophoresis was performed using a 4% (80:1) polyacrylamide gel. Amounts of protein added per reaction range between 1–10 μg. Arrows indicate the positions of the various complexes. The bracket indicates the position of a series of complexes jointly designated Ib in In E1–E5; these interactions clearly resolve into five distinct complexes upon electrophoresis in 5% (30:1) polyacrylamide gels and compete equally efficiently with unlabelled probe (data not shown).

plexed DNA on native polyacrylamide gels, and examination of processed reaction product on denaturing gels.

## RESULTS

### Deletion Analysis of Binding Sites for Multiple Factors Interacting With the H3 Histone Gene Promoter

The proximal promoter (nt −260 to nt −40) of the human H3 histone gene ST519 comprises two in vivo domains of DNA/protein interaction [46]. One of these, designated H3-Site I (nt −223 to nt −174), coincides with a genomic DNaseI footprint. We have defined the other domain, H3-Site II, in this and a previous study [40] as the region between nt −138 and −50, based on sequence similarities with short consensus elements (e.g., 5′dGGTCC, 5′dCCAAT and 5′dTATA) that are present in the analogous region, designated H4-Site II, of the human H4-F0108 histone gene. The distal portion of H3-Site II coincides with a well-defined genomic DNaseI footprint (nt −138/−112), whereas the proximal part of H3-Site II displays an altered reactivity pattern for dimethylsulfate (DMS) and DNaseI in vivo [40]. To examine nuclear factors of HeLa S3 cells interacting with the H3-ST519 promoter in vitro, we analyzed protein binding to the HX-probe (nt −256/−20) in gel retardation assays (Fig. 1). The results show a variety of protein/DNA complexes that are observed using either unfractionated or chromatographically fractionated nuclear proteins. We have designated these complexes I, Ia, Ib, II, and III, x, y, and z, with complex Ib representing a set of five, and focus here on the complexes with Roman numerals.

Localization of the sequences required for binding of protein/DNA complexes I to III was accomplished by deletion analysis (stairway assay) (Fig. 2; summarized in Fig. 3). Sets of deletion mutants were derived from either distally labelled DNA fragments (XM-probe, nt −256/−20; HpH-probe, nt −200/−20) (parts A-C), or proximally labelled DNA fragments (Hp- and HE- probes, both comprising nt −200/−20) (parts D-F). These proximal and distal series of deletion mutants were each incubated with fixed amounts of protein from various chromatographic fractions and then were electrophoresed

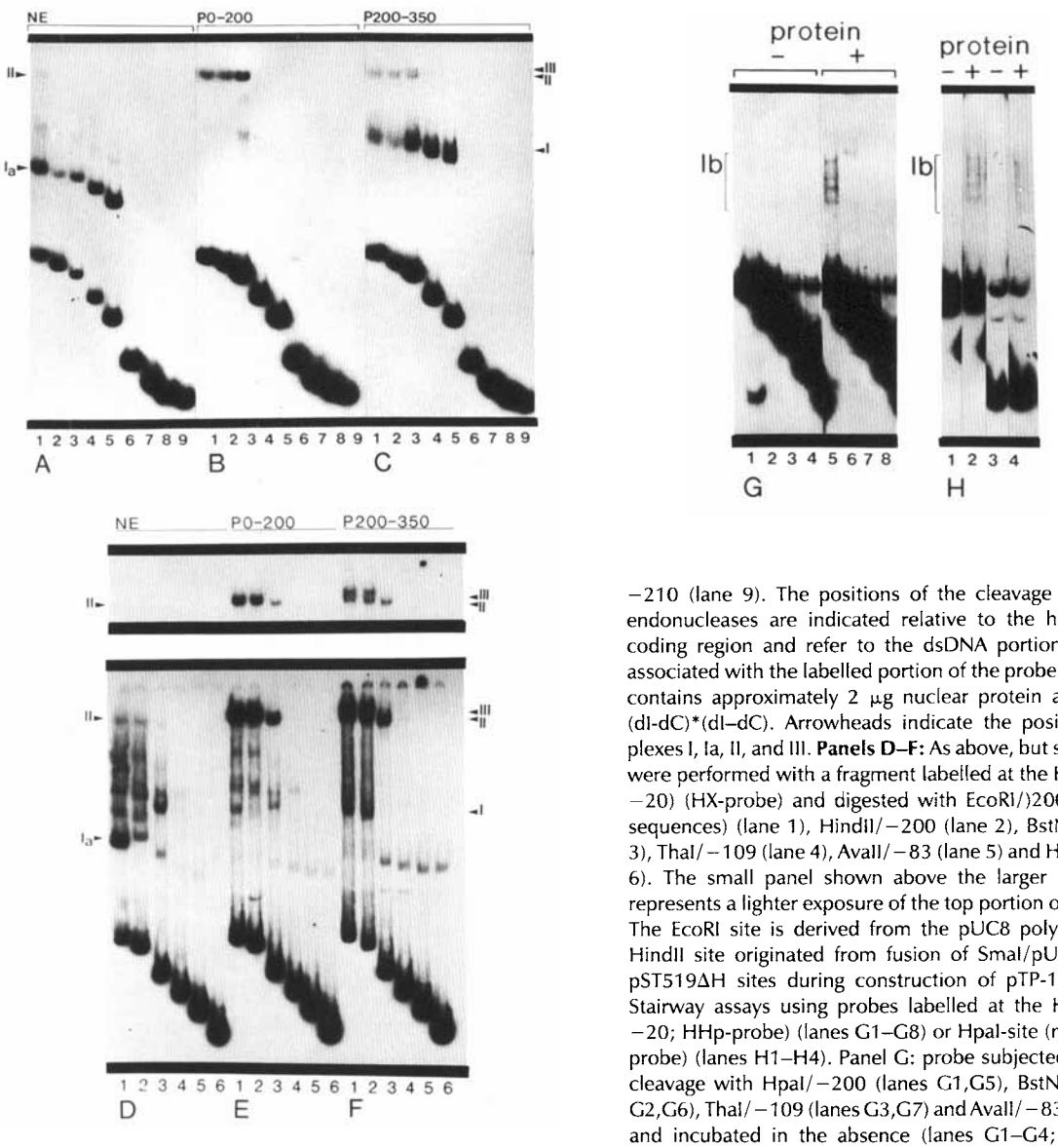



**Fig. 2.** Bidirectional deletion analysis of protein/DNA complexes in the H3-ST519 promoter. **Panels A–C:** Stairway assays with fragments derived from the H3-ST519 promoter and labelled at the Asp700 site (nt −256) (XH-probe) and digested with HindIII/−20 (lane 1), MboII/−43 (lane 2), HinfI/−67 (lane 3), Tha I/−110 (lane 4), AvaII/−129 (lane 5), BstNI/−143(lane 6),AatII/−192 (lane 7), HpaI/−201 (lane 8), AluI/−210 (lane 9). The positions of the cleavage sites of these endonucleases are indicated relative to the histone protein coding region and refer to the dsDNA portion that remains associated with the labelled portion of the probe. Each reaction contains approximately 2 μg nuclear protein and 2 μg poly (dI-dC)*(dI–dC). Arrowheads indicate the positions of complexes I, Ia, II, and III. **Panels D–F:** As above, but stairway assays were performed with a fragment labelled at the Hind III site (nt −20) (HX-probe) and digested with EcoRI/)200(+ polylinker sequences) (lane 1), HindII/−200 (lane 2), BstNI/−139 (lane 3), ThaI/−109 (lane 4), AvaII/−83 (lane 5) and HinfI/−63 (lane 6). The small panel shown above the larger autoradiogram represents a lighter exposure of the top portion of the same gel. The EcoRI site is derived from the pUC8 polylinker and the HindII site originated from fusion of SmaI/pUC8 and HpaI/pST519ΔH sites during construction of pTP-1. **Panels G,H:** Stairway assays using probes labelled at the HindIII site (nt −20; HHp-probe) (lanes G1–G8) or HpaI-site (nt −200; HpH-probe) (lanes H1–H4). Panel G: probe subjected to secondary cleavage with HpaI/−200 (lanes G1,G5), BstNI/−139 (lanes G2,G6), ThaI/−109 (lanes G3,G7) and AvaII/−83 (lanes G4,G8) and incubated in the absence (lanes G1–G4; "−" sign) or presence (lanes G5–G8; "+" sign) of 2 μg P350–1000 protein; panel H: same, but digestion occurred with HindIII/−20 (lanes H1–H2) and BstNI/−139 (lanes H3–H4) in the absence (lanes H1,H3) or presence (lanes H2,H4) of P350–1000 protein. Poly (dI–dC)*(dI–dC) (2 μg) was used as non-specific competitor. The series of complexes designated Ib are indicated by a bracket.

in parallel in native polyacrylamide gels. The pattern of complexes upon deletion of specific DNA segments was assessed to define sequences that are either required or sufficient for formation of protein/DNA complexes.

The results of these experiments (Fig. 2) show that complexes I (In C5–C6 and F2–F3), Ia (In A5–A6 and D1–D2) and Ib (In G1–G2 and H3–H4) require sequences between −200 and −143. Also, although sequences between nt −256 and −143 mediate formation of complexes I and Ia, a DNA fragment spanning nt −256 to − 190 does not. Deletions perturbing binding of complexes I, Ia, and Ib coincide with the proximal part of H3-Site I (nt −223/−174). These results suggest that complexes I, Ia, and Ib represent distinct H3-Site I protein/DNA interactions.

Formation of complex II requires sequences between nt −139 and nt −67 (In B3–B4 and E3–E4). Binding was below the level of detec-

tion when sequences between nt −139 and −110 (In E4) or −109 and −67 (In B4) were removed. This suggests that formation of complex II requires an extended DNA sequence that overlaps with the entire in vivo DNA/protein interaction region H3-Site II (nt −138/−50). Formation of complex III was abolished upon deletion of H3-Site II (In C3–C4) or H3-Site I sequences (In F1–F2; top exposure). These findings are consistent with complex III representing binding events involving factors that interact with both H3-Site I and H3-Site II.

In summary (Fig. 3), these results show that several sequence-specific protein/DNA interactions occur in the H3-ST519 proximal promoter. The proximal part of H3-Site I that is required for formation of complexes I, Ia, and Ib, contains several short palindromic sequences (e.g., 5′TGACGTCA). This suggests that this region interacts in vivo, and in vitro (see below), with symmetrical factors (e.g., dimeric proteins). Almost the entire H3-Site II region is required for complex II suggesting that the factor interacts with an elongated sequence.

### Competition Between H3 and H4 Histone Genes for a Cell Cycle Regulated Factor

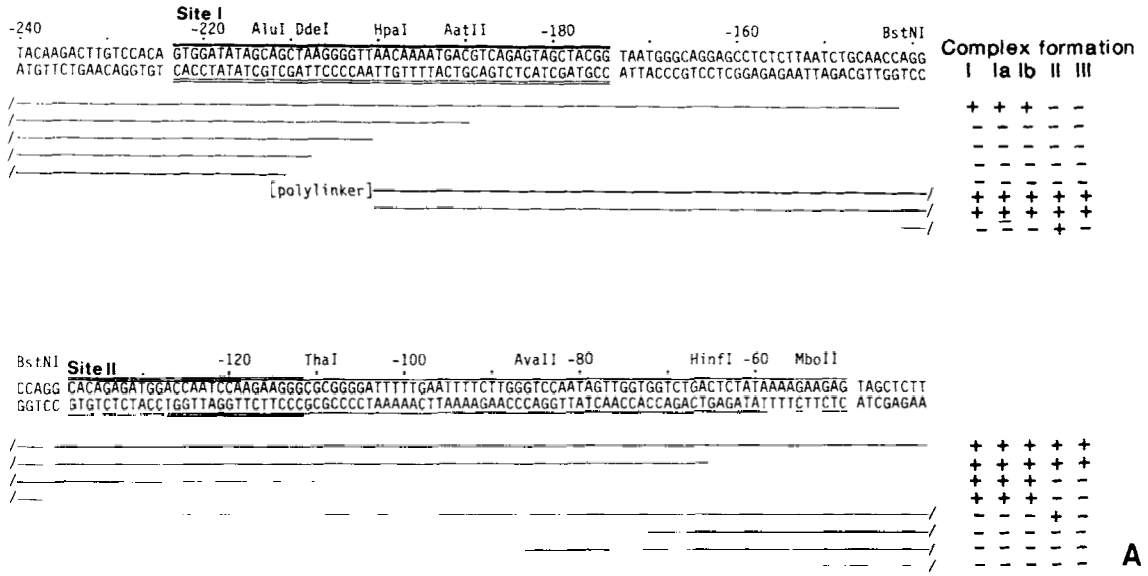
We have previously assigned gel retardation complex II to a DNA binding activity designated HiNF-D3 [47]. This factor competes with an oligonucleotide that spans sequences of the analogous in vivo protein/DNA interaction domain H4-Site II of the H4-F0108 histone gene, and the same oligonucleotide also competes for the H4-Site II:HiNF-D interaction (Fig. 4a). Factors HiNF-D3 and HiNF-D can be detected in a wide variety of mammalian cell types that actively transcribe histone genes [42,47]. Moreover, these analogous H3 and H4 histone gene protein/DNA interactions are regulated in parallel during differentiation [40], the cell cycle, development, and tumorigenesis [47]. To determine whether or not the H3-Site II:HiNF-D3 and H4-Site II:HiNF-D3 protein/DNA interactions have counterparts in other H4 histone genes, we performed competition experiments using cloned genomic DNA fragments spanning the 5′ regions of H4-ST523 and H4-ST512 histone genes (Fig. 4b). Indeed, specific competition is observed for both HiNF-D3 and HiNF-D with these analogous H4 histone genes. This suggests that these H3 and H4 histone genes have corresponding proliferation-specific protein/DNA interactions, which may involve a common DNA binding activity.

### Protein/DNA Interactions With the ATF-Consensus Element of H3-Site I

The H3-Site I sequence 5′dTGACGTCA represents an ATF-consensus sequence [53]. ATF binding activity is mediated by dimeric proteins comprised of distinct subunits encoded by members of the ATF-multigene family [54,55]. Thus, some of the multiple complexes detected here may represent distinct ATF factors. We systematically analyzed chromatography fractions for ATF-like DNA binding activities using DNaseI protection analysis (Fig. 5). The use of chromatographic fractions facilitates direct detection of DNA binding activities mediating DNaseI footprints. Stairway assays were performed with these same fractions (data not shown) to allow initial correlation between the gel retardation complexes described in Fig. 2 and DNaseI footprint activities.

On the antisense-strand we observed a DNaseI footprint between nt −197 and −178 (Fig. 5A) and on the sense-strand between nt −200 to −174 (Fig. 5B). This DNaseI footprint activity coelutes with the factor mediating complex I, but not with gel retardation activities mediating the other H3-Site I complexes (Ia and Ib) (data not shown). Using DMS protection analysis (Fig. 5C), we observed protection of two guanines (nt −190 and −187) located in the ATF element. The in vitro DNaseI footprints coincide with the proximal part of the in vivo genomic DNaseI footprint H3-Site I (nt −223/−174), and the guanines protected in vitro are identical to the in vivo DMS fingerprint. Comparison of these in vitro and in vivo results indicates that the proximal part of H3-Site I interacts with an ATF-related DNA binding activity.

In vivo protein/DNA interaction domain H4-site I of the H4-F0108 histone gene [45] also contains an ATF element that interacts with a factor (HiNF-E/ATF-84) related to the ATF family of DNA binding proteins [29]. The in vitro and in vivo DMS fingerprints of the H4-Site I element are very similar to those of the ATF sequence in H3-Site I (Fig. 3). To address whether or not each site interacts with the same binding activity, we performed competition analysis of the H3-Site I protein/DNA complexes (Fig. 5D). Using total nuclear protein we observed that protein/DNA complexes I and Ia competed specifically with an oligonucleotide


Site I
-240
-220
AluI DdeI
HpaI
AatII
-180
-160
BstNI
TACAAGACTTGTCCACA GTGGATATAGCAGCTAAGGGGTTAACAAAATGACGTCAGAGTAGCTACGG TAATGGGCAGGAGCCTCTCTTAATCTGCAACCAGG
ATGTTCTGAACAGGTGT CACCTATATCGTCGATTCCCCAATTGTTTTACTGCAGTCTCATCGATGCC ATTACCCGTCCTCGGAGAGAATTAGACGTTGGTCC
Complex formation
I Ia Ib II III
[polylinker]
BstNI Site II
-120
ThaI
-100
AvaII -80
HinfI -60
MboII
CCAGG CACAGAGATGGACCAATCCAAGAAGGGGCGCGGGGATTTTTGAATTTTCTTGGGTCCAATAGTTGGTGGTCTGACTCTATAAAAGAAGAG TAGCTCTT
GGTCC GTGTCTCTACCTGGTTAGGTTCTTCCCGCGCCCCTAAAAACTTAAAAGAACCCAGGTTATCAACCACCAGACTGAGATATTTTCTTCTC ATCGAGAA
A


Competition analysis:
I Ia B3 D3
DS-I : gatcCGGAAAAGAAATGACGAAATGTCGAGA
GCCTTTTCTTTACTGCTTTACAGCTCTctag
NMP-I : gatcTGGGATTCGCTGACGTCCATGAGAAAG
ACCCTAAGCGACTGCAGGTACTCTTTCctag
DS-II : ctagCTTTCGGTTTTCAATCTGGTCCGATACT
GAAAGCCAAAAGTTAGACCAGGCTATGAgatc
DD-1 : gatcCGCTTTCGGTTTTCGCGCTTTCGGTTTTCT
GCGAAAGCCAAAAGCGCGAAAGCCAAAAGActag
PD-2 : gatcTCAATCTGGTCCGATTCAATCTGGTCCGAT
AGTTAGACCAGGCTAAGTTAGACCAGGCTActag
H3-II : gatcTCACAGAGATGGACCAATCCAAGAGGG
AGTGTCTCTACCTGGTTAGGTTCTCCCctag
MYB (291b) : gatcAGTAATCCAAACTGCCACAGTTCATAAG
TCATTAGGTTTGACGGTGTCAAGTATTCcatg
B

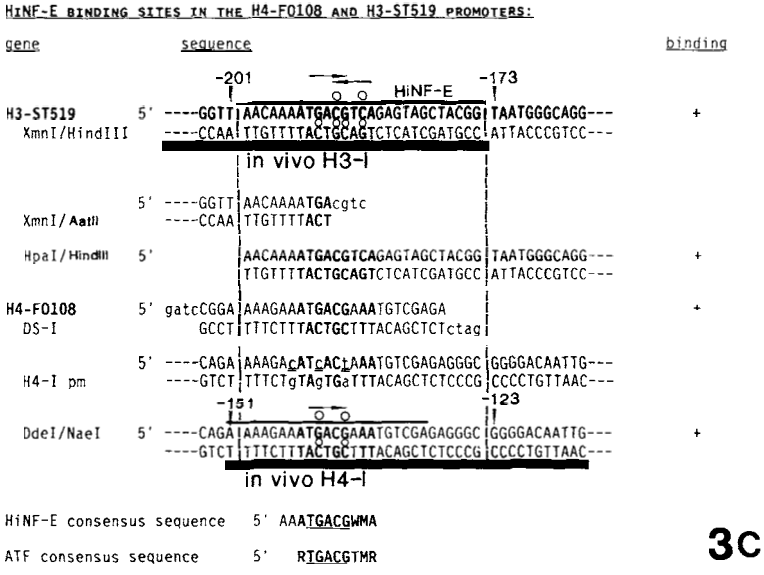

HiNF-E BINDING SITES IN THE H4-FO108 AND H3-ST519 PROMOTERS:
gene
sequence
binding
-201
-173
HiNF-E
H3-ST519 5' ----GGTT AACAAAATGACGTCAGAGTAGCTACGG TAATGGGCAGG--- +
XmnI/HindIII ----CCAA TTGTTTTACTGCAGTCTCATCGATGCC ATTACCCGTCC---
in vivo H3-I
5' ----GGTT AACAAAATGAcgtc
XmnI/AatII ----CCAA TTGTTTTACT
HpaI/HindIII 5' AACAAAATGACGTCAGAGTAGCTACGG TAATGGGCAGG--- +
TTGTTTTACTGCAGTCTCATCGATGCC ATTACCCGTCC---
H4-FO108 5' gatcCGGA AAAGAAATGACGAAATGTCGAGA +
DS-I GCCT TTTCTTTACTGCTTTACAGCTCTctag
5' ----CAGA AAAGAcATcACtAAATGTCGAGAGGGC GGGGACAATTG---
H4-I pm ----GTCT TTTCTgTAgTGaTTTACAGCTCTCCCG CCCCTGTTAAC---
-151
-123
DdeI/NaeI 5' ----CAGA AAAGAAATGACGAAATGTCGAGAGGGC GGGGACAATTG--- +
----GTCT TTTCTTTACTGCTTTACAGCTCTCCCG CCCCTGTTAAC---
in vivo H4-I
HiNF-E consensus sequence 5' AAATGACGWMA
ATF consensus sequence 5' RTGACGTMR
3C

Figure 3.

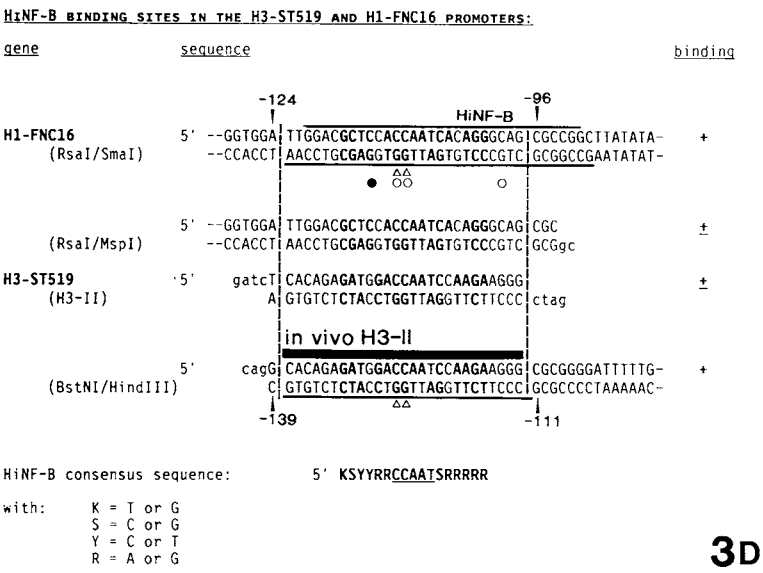



**Fig. 3.** **(A)** Summary of stairway assay results. Horizontal lines depict restriction fragments (restriction enzymes above sequence) used in binding reactions; brackets denote a small segment of polylinker sequences derived from pUC8. The table on the right indicates the ability of the various DNA fragments to form the specific complexes I to III ("+": binding, "−": no binding, "±": decreased binding). Slanted dashes on left and right indicate that the sequence extends to nt-256 or nt-20, respectively. Double lines beneath and above the sequence reflect in vivo genomic DNaseI footprints of H3-Site I and H3-Site II [46], whereas the single lines beneath and above the sequence represent the general area that we refer to as the H3-Site II region that is analogous to H4-Site II. The DNA sequence of the H3-ST519 histone gene [64] has been revised at nt −140, −118, −92 and −79 using chemical sequencing data obtained with pTP-1. The revised sequence differs at nt −121 from the genomic DNA [46]. **(B)** Summary of the results obtained using specific competitor oligonucleotides ("+": competition, "−": no competition) for complexes I and Ia (both complexes compete with the DS-I oligonucleotide that spans the binding site for HiNF-E/ATF-84) [29] (Fig. 5D), as well as for the complexes of HiNF-B3 (Fig. 6C) and HiNF-D3 (Fig. 4A). DS-I [27] spans the distal portion of H4-Site I [45], whereas DS-II, DD-1 and PD-2 [28] span portions of H4-Site II [45]. NMP-1 spans the ATF-like binding site of a nuclear matrix protein that binds to the H4-F0108 gene (S. Dworetzky, JS & GS, unpublished data), whereas MYB represents a binding site (designated 291b) [65] for the MYB protein.H3-II spans the sequences of the in vivo genomic footprint within the H3-Site II region. Sequences in bold, underlined lettering represent core consensus sequences for, respectively, ATF (5'dTGACG) and CP1/NF-Y (5'dCCAAT) (see text). **(C and D)** Sequence similarity between in vivo and in vitro protein/DNA interaction sites in the human H4 [45], H3 [46], and H1 [20] promoters. Indicated in C are sequence similarities between the distal part of H4-F0108 Site I and the proximal part of H3-ST519 Site I, whereas Figure 3D shows similarities between H3-ST519 Site II and the H1-FNC16 promoter binding site of CCAAT-box binding protein HiNF-B [20]. The following symbols are used: sequences protected from DNaseI digestion in vitro (thin horizontal lines) or in vivo (thick line) [45,46], guanines protected during DMS fingerprinting experiments in vitro (open circles above or below sequences) and in vivo (open circles between strands) [45,46], and methylation interference contacts (open triangles) [20]. Also shown are restriction fragments that have been used in deletion analysis, with "+" and "−" indicating the ability to mediate electrophoretically stable binding or competition. The generic designation "HiNF-E" refers to complexes I and Ia (for the H3-ST519 gene) and binding of HiNF-E/ATF-84 (for the H4-F0108 gene). Similarities with extended ATF (part C) [53] or CCAAT-box consensus sequences (part D) (located at the bottom of each panel) are indicated by bold lettering (W = A or T, M = A or C, K = T or G, S = C or G, Y = C or T, R = A or G). H4-I pm is a point mutant that has lost the ability to interact with HiNF-E/ATF-84 [29].

(DS-I) containing the H4-Site I ATF element. Competition also occurred, albeit to a lesser degree, with a short DNA fragment designated NMP-1 (S. Dworetzky, J.S. and G.S.; unpublished data) (Fig. 5D). The NMP-1 oligonucleotide contains another ATF-like sequence located within a sequence context different from the DS-I oligonucleotide. Taken together, the results of in vivo [45,46] and in vitro (Fig. 5) approaches are in agreement with the ATF elements of H3-Site I and H4-Site I interacting with a DNA binding protein that is a member of the ATF family of transcription factors.

### Protein/DNA Interactions Involving the CCAAT-Box of H3-Site II

Deletion analyses of the multiple complexes mediated by the H3-ST519 histone promoter in vitro (Fig. 2) suggest a partition between distal and proximal binding events, thereby reflecting the bimodular structure of this promoter as established by in vivo analysis of protein/DNA

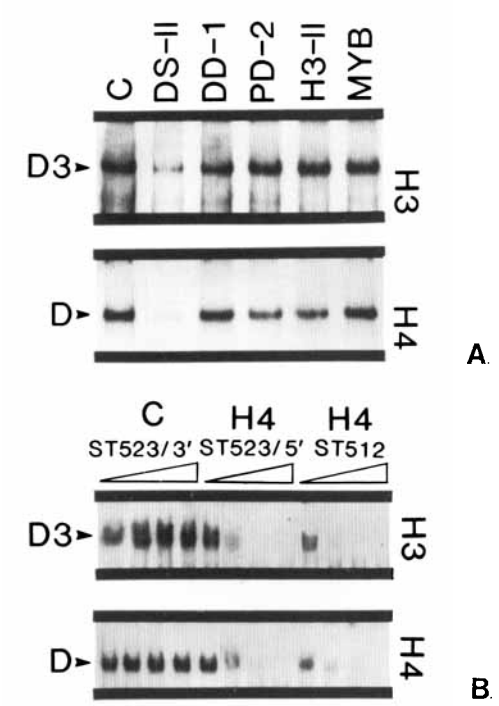



**Fig. 4.** **(A)** Competition analysis of the HiNF-D3:H3-Site II **(upper panel)** and HiNF-D:H4-SiteII **(lower panel)** protein/DNA interactions using a 400-fold molar excess of oligonucleotides spanning binding sites of HiNF-D (DS-II), HiNF-M (DD-1), short consensus elements within H4-Site II (PD-2), the H3-Site II CCAAT-box, and a MYB binding site [28,64]; the first lane does not contain specific competitor DNA (C). The probes used for detection span promoter sequences of, respectively, H3-ST519 (nt −139/−20) and H4-F0108 (nt −97/−38). Assay conditions were as described in [28]. **(B)** Competition analysis of HiNF-D and HiNF-D3 with DNA fragments spanning human H4 histone genes ST523 and ST512. Competition was performed by the inclusion of restriction fragments spanning the 3' region of ST523 **(C; left four lanes)**, or the 5' regions of the human H4 histone genes ST523 **(middle section)** and ST512 **(right four lanes)**. Lanes in each case contain, respectively, 0, 5-, 10-, and 20-fold molar excess of each fragment. Binding reactions each contain 2 μg nuclear protein and 2 μg poly (dI–dC)*(dI–dC). DNA fragments spanning the promoters of H3-ST519 (nt −200/−20; top) and H4-F0108 (nt −130/−38) were used as probes.

interactions. In vitro DNaseI protection analysis of the proximal promoter region using nuclear protein shows that the same area that is protected in vivo is also protected in vitro (Fig. 6A). To relate this DNaseI footprint activity to gel retardation complexes, we investigated the interactions of H3-Site II in closer detail using stairway assays with a DNA fragment spanning nt −139 to −20 (Fig. 6B). With these short DNA fragments, we can clearly observe the HiNF-D3 (="II") complex, and two other complexes designated B3 and F3 (mediated by HiNF-B3 and HiNF-F3). Deletion of nt −139 to −109 abolishes detection of complexes B3 and D3, but not F3, whereas detection of F3 requires nt −109 to −83 (Fig. 6B). Hence, this analysis indicates that these complexes require different sequences (F3 versus B3 and D3), or require DNA binding activities eluting in separate chromatographic fractions (D3 versus B3 and F3). These results suggest that these complexes are mediated by distinct entities.

To examine in vitro protein/DNA interactions mediated by the H3-Site II sequences that correspond to the in vivo genomic DNaseI footprint (nt −138/−112), we assessed binding of total nuclear protein to an oligonucleotide (nt −139 to −112) spanning H3-Site II and the region (nt −139 to −109; see Fig. 6B) required for complexes B3 and D3 (Fig. 6C). Competition analysis shows that this short DNA fragment mediates formation of complex B3, but not the HiNF-D3 complex. These results are consistent with HiNF-D3 interacting with a more extended DNA sequence (see Fig. 2), and are similar to results for the analogous H4-Site II:HiNF-D interaction that also requires an elongated sequence [28]. These results also demonstrate that nt −139 to −112 are sufficient for sequence-specific binding of HiNF-B3.

The H3-Site II footprint (nt −138/−112) contains a CCAAT-motif that resembles the consensus binding site of the CP1/NF-Y class of transcription factors [56–58] suggesting that HiNF-B3 is a CCAAT-box binding protein. The protein/DNA contacts of complex B3 were established by methylation interference (Fig. 6D), and we observed that methylation of guanines nt −126 and −125 is inhibitory for binding. These nucleotides coincide with the H3-CCAAT element (nt −126 to −122) and are equivalent to protein/DNA contacts of the heteromeric H1 histone CCAAT-box factor HiNF-B [20,21]. Therefore, it is possible that the DNA binding activities interacting with the H3- and H1-CCAAT boxes are the same.

To address this question more directly, we electrophoresed probes spanning the H3-ST519 (nt −139/−20) and H1-FNC16 (−213/−78) histone promoters in parallel in gel retardation assays (Fig. 7). The complexes of HiNF-B3 and HiNF-B have similar relative mobilities and indistinguishable competition behavior. Specifically, the HiNF-B:H1-CCAAT box interaction competes with the H3-Site II CCAAT-box oligonucleotide that also competes for HiNF-B3 binding to the H3 histone gene. Thus, we have

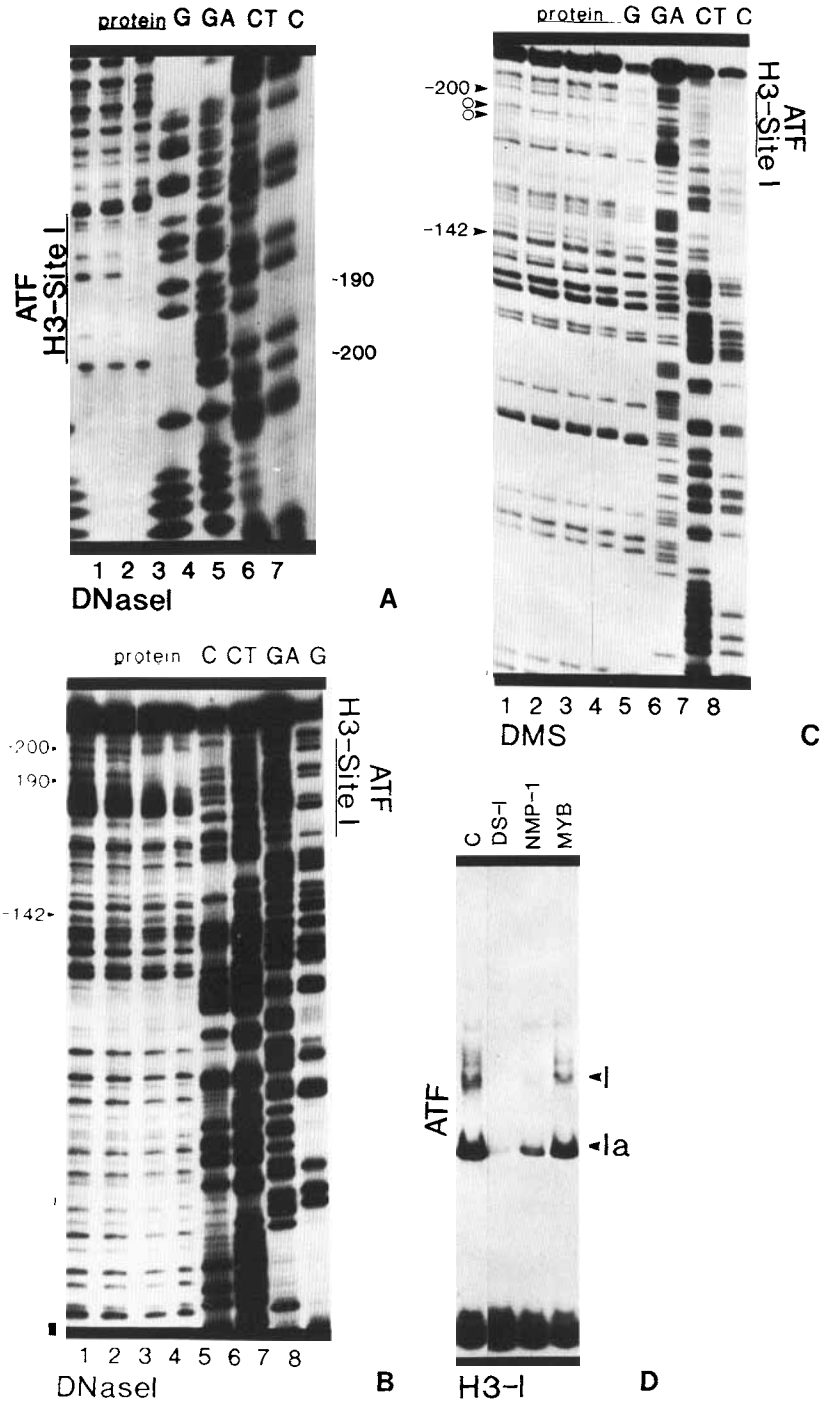



**Fig. 5.** *In vitro* DNaseI footprint and DMS fingerprint analysis of the ATF element in the proximal portion of H3-Site I. Binding mixtures containing fractionated nuclear proteins (D100–250 fraction) [28 and unpublished data] were treated with DNaseI and DMS (and piperidine) and the reaction products were directly analyzed on denaturing gels. **(A)** DNaseI footprint (sense strand) obtained with EcoRI/HindIII fragment of pTP-1 as the probe (labelled at the EcoRI site) in a 10-fold scaled up binding reaction (final volume 200 μl). **Lanes 1–3:** respectively, no protein (control), 25 and 100 μl protein added; **lanes 4–7:** chemical sequencing reactions as indicated. The area of DNaseI protection is indicated by a thin line adjacent to the gel; the locations of restriction sites are indicated for reference. **(B)** DNaseI footprint (anti-sense strand) obtained using the same fragment as above, but labelled at the HindIII site. **Lane 1:** DNaseI control; **lanes 2–4:** respectively, 10, 50 and 100 μl protein; **lanes 5–8:** chemical sequencing reactions as indicated. **(C)** DMS fingerprint (sense strand); **lanes 1–4:** identical binding reactions as in middle panel, but samples treated with DMS (and piperidine) instead of DNaseI; **lanes 5–8:** chemical sequencing reactions as indicated. This fraction mediates formation of complex I, but not complexes Ia and Ib, in gel retardation assays (data not shown). **(D)** Competition analysis of H3-Site I protein/DNA complexes (I and Ia) by gel retardation analysis using a 200-fold molar excess of oligonucleotides spanning the ATF binding site of H4-F0108 Site I (DS-I), a palindromic ATF binding site (NMP-1), and a MYB binding site [28,64]. The probe spans nt −200 to −143 of the H3-ST519 promoter.

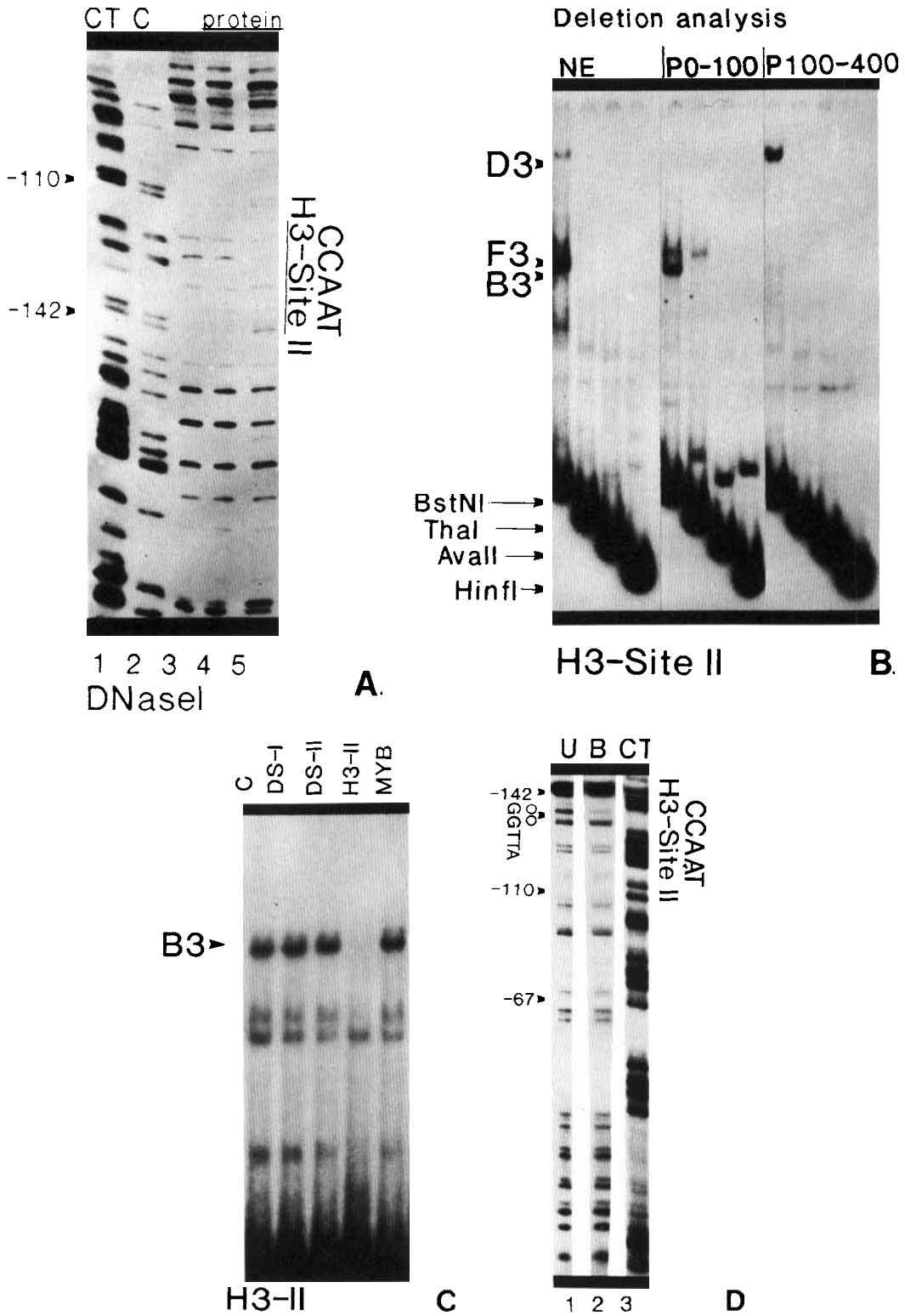

CT C protein
-110
-142
CCAAT
H3-Site II
1 2 3 4 5
DNaseI
A.
Deletion analysis
NE
P0-100
P100-400
D3
F3
B3
BstNI
TthI
AvaII
HinfI
H3-Site II
B.
C
DS-I
DS-II
H3-II
BMW
B3
H3-II
C
U B CT
-142
GGTTA
-110
-67
CCAAT
H3-Site II
1 2 3
D

Figure 6

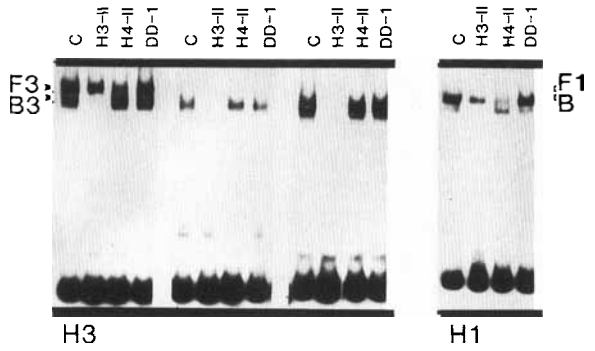



Fig. 7. Competition analysis of protein/DNA complexes interacting with the H3-ST519 (nt −139/−20) and H1-FNC16 (nt −213/−78) promoters using the oligonucleotides indicated above the lanes (see Fig. 3B). The lane designated "H4-II" refers to binding reactions containing the DS-II oligonucleotide. Binding reactions were performed using nuclear protein from human HeLa S3 cells **(left panel,** H3: lanes 1–4; and **right panel,** H1: lanes 1–4), mouse fetal liver (H3: lanes 5–8) or mouse fetal brain (H3: lanes 9–12). The complexes with similar migration rates and competition behaviors are indicated by arrowheads and equivalent letters. Complex B represents the complex of CCAAT-box binding protein HiNF-B [20].

observed similarities in electrophoretic mobility, size, and sequences of the DNA binding domain, methylation interference contacts, and competition behavior. In addition, both the H3 and H1 CCAAT-box binding activities are regulated in parallel and are constitutively present during tissue development from fetus to adult [47]. We postulate that the CCAAT-boxes of the H3-ST519 and H1-FNC16 histone genes interact with the same binding activity.

Interestingly, the H3 promoter (nt −139/−20) also mediates formation of complex F3, which has an apparent counterpart of similar migration in the H1 promoter (complex F1) (Fig. 7). The intensities of complexes F3 and F1 relative to complexes B3 and B1, respectively, are dependent on the non-specific competitor DNA present in the binding reaction (compare Figs. 6 and 7). The results of specific competition analysis using a panel of oligonucleotides show that these complexes compete specifically with a DNA fragment spanning sequences of the H4 histone promoter. However, HiNF-F3 and HiNF-F1 can not be detected in nuclear protein preparations from fetal mouse tissues (Fig. 7) that actively transcribe histone genes [44]. Thus, the significance of these protein/DNA complexes is unclear at present.

Fig. 6. **(A)** In vitro DNaseI footprint analysis of the CCAAT-element located in the genomic DNaseI footprint of H3-Site II. The experiment was performed as described in Figure 5A, but samples were electrophoresed for a longer time. **Lanes 1–2:** chemical sequencing reactions as indicated; **lanes 3–5:** respectively, no protein (control), 25 and 100 μl protein added. The area of DNaseI protection spanning the CCAAT-box (= 5'AT-TGG on the anti-sense strand) is indicated by a thin line adjacent to the gel. **(B)** Stairway assays of the H3-Site II region using unfractionated (NE) and fractionated nuclear proteins (PO-100 and P100–400) [28] were performed with fragments labelled at nt −20 (HindIII site) which span sequences up to the following sites: BstNI/−139 **(lanes 1,5,9),** ThaI/−109 **(lanes 2,6,10),** AvaII/−83 **(lanes 3,7,11)** and HinfI/−63 **(lanes 4,8,12).** Indicated by arrowheads are complexes mediated by HiNF-B3, −D3 and −F3. **(C)** Competition analysis of HiNF-B3 binding to a radio-labelled oligonucleotide (H3-II, 0.5 ng; see Fig. 3) spanning the in vivo and in vitro footprint of H3-Site II. Binding reactions were performed with 10 μg nuclear protein and a 200-fold exces of the indicated oligonucleotides (see Figs. 5d and 3). **(D)** Methylation interference analysis of HiNF-B3. Results were obtained by isolating the HiNF-B3 complex from the gel after DMS treatment of binding reactions containing PO-100 protein and a probe labelled at the anti-sense strand (HindIII/BstNI fragment, nt −142 to −13). **Lanes 1–2:** DNA cleaved at purines (G > A); lane 1: unbound DNA (U), lane 2: bound DNA (HiNF-B3 complex) (B), **lane 3:** DNA cleaved at pyrimidines (C + T).

## DISCUSSION

### Involvement of ATF/AP1 Related Proteins in Transcriptional Regulation of Several Histone Gene Classes

In this work, we have established the binding site of an TGACG)box protein-that binds to a portion of genomic protein/DNA interaction domain H3-Site I of the H3-ST519 histone gene. This activity competes with the binding site of factor HiNF-E/ATF-84 located in the analogous domain (H4-Site I) of the H4-F0108 histone gene. Deletion of the HiNF-E/ATF-84 binding site in H4-Site I results in a decrease, but not abolishment of H4 histone gene transcription both in vitro [29] and in vivo [39]. This indicates that HiNF-E/ATF-84 is a positively acting, auxiliary transcription factor, consistent with the postulated roles of other members of the ATF family [53–55]. Based on comparison of DNaseI and DMS protection patterns in vivo [45,46] and in vitro, as well as cross-competition, we suggest that H4-Site I and H3-Site I interact with similar DNA binding activities. Hence, HiNF-E/ATF-84 may be involved in a mechanism that coordinately stimulates transcription of both H4 and H3 histone genes.

This conclusion does not exclude a role for ATF sites in other histone genes. For instance, Sive et al. [59] have shown that mutation of

upstream sequences (nt −115 to −100) of a human H2B histone gene reduces in vitro and in vivo transcription. This region appears to coincide with an ATF element, suggesting that ATF factors may also influence transcription of H2B histone genes. Interestingly, Tabata et al. [5] have shown that a similar ATF element is functionally involved in the transcription of a wheat H3 histone gene. These results suggest that ATF sites may represent an ancient component of a highly conserved, histone gene regulatory transcription mechanism that is operative in all eukaryotic species.

Sharma et al. [13] have shown that a hamster H3 histone gene interacts with a factor that is immunologically related to the JUN oncoprotein (with JUN representing a component of the AP1 transcription factor). Recently, Hai and Curran [60] have shown that certain members of the ATF family (ATF-1, ATF-2, etc.) [55] may form selective cross-family heterodimers with members of the AP1 family (Jun, Fos, FRA-1, etc.) [19], suggesting that the various ATF and AP1 members represent a superfamily of related transcription factors [60]. Consistent with the close sequence similarity between AP1 (5′dTGACTCA) and ATF (5′dTGACGTCA) elements, which differ only by the spacing between two half-sites, cross-family heterodimers may recognize both elements, albeit with different affinities [60]. Thus, it is clear that at least one member of the ATF/AP1 superfamily may be involved in regulation of several distinct histone gene classes. However, the subunit composition of the specific ATF/AP1 species that is physiologically most relevant to histone gene expression remains to be established.

### Interactions of Heteromeric CCAAT-Box Binding Proteins With Several Different Histone Gene Classes

We have also defined the binding site of a CCAAT-box binding protein that corresponds to the in vivo DNaseI footprint of H3-Site II and have presented evidence here that this site interacts with the heteromeric H1 histone CCAAT-box binding protein HiNF-B [20,21]. The H3 and H1 histone CCAAT-boxes display extensive sequence similarities (see Fig. 3) with elements in these and other histone subtypes. These elements resemble the binding sites of heteromeric CCAAT-box proteins of the CP1/NF-Y class that have been evolutionarily conserved from yeast to man [56–58]. Gallinari et al. [24] and Sive et al. [59] have shown that mutation of identical CCAAT elements (i.e., conforming to an extended consensus sequence) in H1 and H2B histone genes decreases transcription, and similar "CP1/NF-Y type" CCAAT boxes have been shown to influence transcription of many other genes [19,56–58]. We deduce that the CCAAT boxes of human H3 and H1 histone promoters are conserved transcriptional elements interacting with equivalent DNA binding activities that may collectively enhance transcription of H3, H2B, and H1, histone genes.

### Histone Promoters Contain Unique Permutations of Shared Promoter Elements

Parallel studies on transcriptional regulation or protein/DNA interactions in distinct human H1 [20,21,23,24], H2B [26,59], H3 (this work), and H4 histone genes [27–30] by our laboratory and others have resulted in the definition of several protein/DNA interaction elements. The list of nuclear factors from human HeLa S3 cells characterized to date include OTF-1 [25], CP1/NF-Y related CCAAT-box factors (HiNF-B/H1-TF2) [20,24], H1-SF/H1-TF1 [14,23,61], Sp1/H4-TF1 [27,30], and ATF-84/HiNF-E [29], and we have shown for the H4-F0108 [45] and H3-ST519 [46] histone gene that several of these elements represent protein/DNA interaction sites in vivo.

We aligned these binding sites with the immediate 5′ regions of recently characterized mammalian histone genes for which only sequence information is available [31–36,62,63] (data not shown). We observed in each individual gene a limited number of extended sequence similarities that overlap with putative core consensus sequences for the aforementioned factors. The most striking observations are the following: (I) the H2B subtype specific element (OTF-1 binding site) [25,26] is associated with both H2B *and* H2A histone genes; (II) an H1 histone consensus sequence (H1-SF binding site) [14] in one case is also found associated with an H4 histone gene; (III) extensive CCAAT-box similarities (of the "CP1/NF-Y" type) [20,56–58], and referred to as a secondary "H1 subtype specific element" [23,24], are present in H1, H2A, H2B, and H3 histone genes, but are usually not associated with H4 histone genes; (IV) the Sp1 consensus sequence [19] is frequently associated with H1, H3, and H4 histone genes, but Sp1 consensus sequences with the same high degree of similarity are not found in H2A and H2B histone genes;

(V) ATF/AP1 elements are usually present in H3 and H4 histone promoters, and less frequently in other histone genes. Based on the apparent occurrence of putative H1-subtype [23,24] and H2B-subtype specific consensus elements in more than one subtype, the function of these elements in mediating binding of histone gene transcription factors is most likely not confined to specific classes of histone genes.

We conclude from this analysis that individual histone genes are associated with a limited number of protein/DNA interaction elements. These elements represent a permutated subset of the full complement of histone promoter binding sites. Although the subset of putative factor binding sites is unique for each individual gene, these 5′ regions frequently have one or more elements in common. Consequently, the modular organization of the prototypical histone promoter is reflected by a mosaic of cis-acting elements that is to various degrees similar to that of another histone gene. These heterogeneous permutations may govern the cellular competency to transcribe histone genes by increasing the likelihood that stimulatory factors interact with these genes. Shared protein/DNA interaction elements may reflect regulatory coupling at the transcriptional level of multiple distinct histone genes by a relatively small number of transcription factors.

### Constitutive and Proliferation-Specific Protein/DNA Interactions

In vivo H4-Site I protein/DNA interactions involving ATF and Sp1 factors are not downregulated during differentiation of HL60 cells at the cessation of histone gene transcription [3,15]. Moreover, we have shown that histone CCAAT-box binding activity (HiNF-B) remains constitutive during hepatic development (reflecting the onset of in vivo quiescence and differentiation in the intact animal) [47], although downregulation of histone gene transcription occurs during this process as shown, using transgenic mice [44]. Thus, permutated sets from a fixed complement of shared promoter elements including ATF, Sp1, and CCAAT-box factors may be constitutive components of a mechanism by which common regulatory factors selectively stimulate groups of histone genes. Another component is represented by a set of analogous proliferation-specific protein/DNA interactions in the promoters of the H4-F0108, H3-ST519, and H1-FNC16 histone genes involving HiNF-D [27,28] and related factors. This set is coordinately controlled during the cell cycle, differentiation and development, and is collectively deregulated during tumorigenesis [47]. Together, these findings suggest that the interplay of constitutive and cell cycle regulated components may mediate coordinate control of five histone multi-gene families.

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