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Bipartite Structure of the Proximal Promoter of a Human H4 Histone Gene

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Abstract The proximal promoter of the human H4 histone gene FO108 contains two regions of in vivo protein-DNA interaction, Sites I and II. Electrophoretic mobility shift assays using a radiolabeled DNA probe revealed that several proteins present in HeLa cell nuclear extracts bound specifically to Site I (nt-125 to nt-86). The most prominent complex, designated HiNF-C, and a complex of greater mobility, HiNF-C′, were specifically compatable by an Sp1 consensus oligonucleotide. Fractionation of HiNF-C using wheat germ agglutinin affinity chromatography suggested that, like Sp1, HiNF-C contains N-acetylglucosamine moieties. Two minor complexes of even greater mobility, designated HiNF-E and F, were compatable by ATF consensus oligonucleotides. A DNA probe carrying a site-specific mutation in the distal portion of Site I failed to bind HiNF-E, indicating that this protein associated specifically to this region. UV cross-linking analysis showed that several proteins of different molecular weights interact specifically with Site I. These data indicate that Site I possesses a bipartite structure and that multiple proteins present in HeLa cell nuclear extracts interact specifically with Site I sequences. © 1995 Wiley-Liss, Inc.

Key words: ATF, Sp1, transcription factors, cell cycle

Complex arrays of protein/DNA interaction sites are involved in the transcriptional regulation of genes involved in cell growth (reviewed in reference 1). To elucidate the pathways of regulation that support growth control, we have analyzed transcriptional mechanisms regulating a cell cycle controlled human H4 histone gene. Human histone genes constitute a moderately repeated, multi-gene family which encode five histone subtypes critical for the packaging of newly replicated DNA into chromatin (2–4). The human genes are organized in clusters of core (H2A, H2B, H3, H4) or core with H1 histone genes (5–10) and have been identified on at least two different chromosomes (11,12). The majority of histone genes is expressed in a cell cycle regulated manner, tightly coupled both temporally and functionally to DNA replication (13,14).

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Cell cycle dependent histone genes are transcribed at a basal level and exhibit a 2–5-fold induction of transcription during the initial period of DNA synthesis (15–17). This induction of transcription is accompanied by a disruption of the chromatin structure and nucleosome organization in the promoter region (18,19). Histone mRNA accumulates rapidly during S-phase to a level 20–100-fold higher than that detected in non S-phase cells (15–17,20–22). These results demonstrate that both transcriptional and post-transcriptional control mechanisms are critical in regulating the cell cycle dependent expression of histone genes.

Transcription of the cell cycle regulated FO108 human H4 histone gene has been shown to be modulated by both proximal and distal promoter elements (17,23), and the promoter of this gene supports cell growth regulated transcription in transgenic mice (24). Our laboratory has previously shown that the proximal promoter consists of two regions of in vivo protein-DNA interaction, Sites I and II (25). Site II is located between −64 and −24 bp upstream from the transcription initiation site and contains the TATA box and an H4 histone specific element (5′-RGTYYTCAAYYNGGTCCG-3′). Oc-

cupancy of Site II in vivo and in vitro has been correlated with rendering the gene transcribable in proliferating cells (24,26–30). Furthermore, Site II sequences are involved in the regulation of histone gene transcription during the cell cycle (31).

The FO108 H4 histone promoter Site I is positioned at −124 to −86 bp upstream of the initiation site. In this study, we begin to characterize the protein/DNA interactions which occur at Site I. We report that Site I possesses a bipartite structure, binding specifically to several distinct nuclear proteins.

MATERIALS AND METHODS

Preparation of Nuclear Extracts

Nuclear extracts were prepared from 1×10^9 exponentially growing HeLa S3 cells essentially as described by Dignam et al. (32), except 2 ml of 600 mM KCl was used to extract the nuclei. All steps were performed at 4°C and all buffers except the phosphate buffered saline contained fresh 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 0.5 mM dithiothreitol (DTT), and 5 μg/ml each of pepstatin A, antipain, leupeptin, and chymostatin (Sigma Chemical Co., St. Louis, MO).

Gel Mobility Shift Analysis

The gel mobility shift analysis was performed essentially as described by Staudt et al. (33) using a TGE buffer system (50 mM Tris-HCl, 380 mM glycine, 2 mM EDTA, pH 8.5). Binding reactions were as described previously (31), except that the Site I probe extended from −156 to −72 bp (plus 14 bp of pUC19 polylinker) and poly(dl-dC)poly(dl-dC) (3 μg) was used as the non-specific competitor. The specific oligonucleotide competitor for distal Site I has been described previously (29) and the ATF oligonucleotides were the generous gift of Dr. Michael Green (34–35). The H3 histone distal site II oligonucleotide (H3-DSII) has been described previously (36).

Site-Directed Mutagenesis

Selected base substitutions were introduced into the promoter of the H4 histone gene by oligonucleotide-directed mutagenesis without phenotypic selection, essentially as described by Kunkel (37). Single-stranded M13 DNA carrying the 1.85 kB EcoRI/HindIII fragment of FO108 and containing uracil was prepared by amplification in the *Escherichia coli* strain CJ236 (*dut*⁻ *ung*⁻ F) (a gift of Dr. Barbara Bachmann, *E. coli* Genetic Stock Center, Yale University, New Haven, CT). Mutations were introduced in complementary synthetic oligonucleotides containing the base substitutions in either distal Site I, 5′-GAGGAAAACAGAAAAGAcATcACtAAATGTCGAG-3′, or proximal Site I, 5′CGAGAGttCGGGGAC-3′. The mutations were recovered by transfection into wild type *E. coli* strain XL1-Blue (Stratagene, La Jolla, CA), confirmed by enzymatic sequencing (Sequenase™, U.S. Biochemical Corp., Cleveland, OH), and the 1.85 kB EcoRI/HindIII histone gene containing fragment was cloned back into pUC19.

DNase I Protection

DNase I footprinting was performed as described by Augereau and Chambon (38) and used the same Site I and mutant probes as used in the gel mobility shift assay. The relative affinity of the factors for the binding sites was assessed by determining the degree of change in the intensity of the footprint protection as measured by densitometry.

Wheat Germ Agglutinin Affinity Chromatography

Wheat germ agglutinin affinity chromoatography was carried out essentially as described (39) using HeLa cell nuclear extracts.

UV-Cross Linking of Site I Proteins

Molecular mass determination by UV cross-linking was performed as described (40).

RESULTS

The FO108 human H4 histone gene proximal promoter consists of two regions of protein-DNA interaction in vivo, Sites I (−124 to −86 bp; Fig. 1) and II (−64 to −24 bp). To begin to understand the molecular mechanisms by which Site I influences transcription, we initiated the characterization of the protein/DNA interactions which occur at this sequence in vitro.

We first examined the interactions at Site I by DNase I footprint analysis (Fig. 2). Nuclear proteins incubated with the Site I probe protected a region of 36 bp (−122 to −87 bp). These results match the boundaries of this protein/DNA interaction region determined in vivo (25) (Fig. 2). As observed previously (25), this protected region contains both ATF (distal) and Sp1 (proximal)

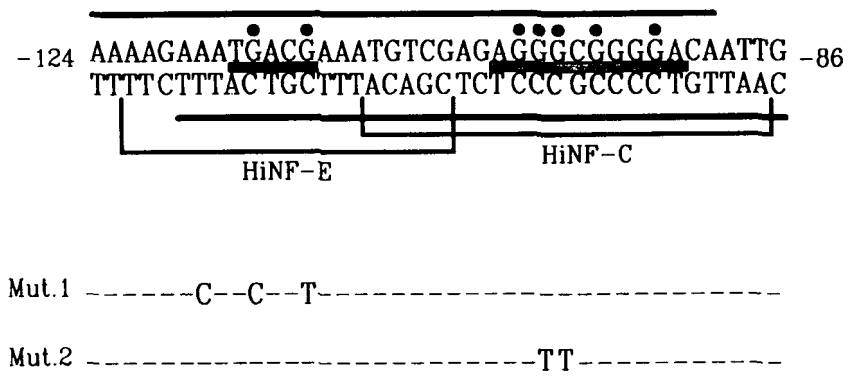

Fig. 1. Schematic of the Site I domain. Dark lines indicate the extent of the in vivo DNase I footprint and filled circles denote the methylation protected guanine residues. Brackets define the in vitro DNase I footprints of HiNF-E and HiNF-C determined in the absence of binding by the other factor. Filled boxes designate the ATF and Sp1 consensus recognition sequence. The base substitutions in Mutation 1 and Mutation 2 are indicated below, at the bottom of the figure.

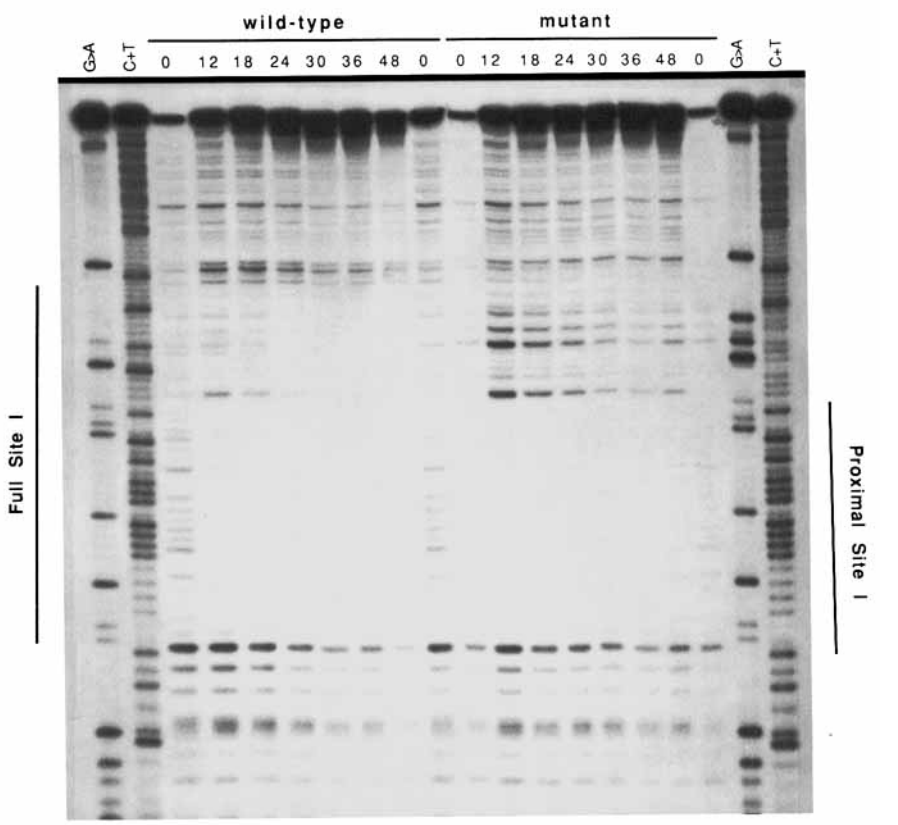

Fig. 2. DNase I footprint of Site I proteins. Amounts of nuclear protein extract (μg) present in the reaction are shown above each lane. The probe used, either wild-type or mutant (mutant for the distal ATF site), is shown at the top of the figure. Boundaries of the footprints are indicated along the sides.

consensus sequences. A mutation designed to disrupt the ATF-like binding site was created (from 5'AATGACG3' to 5'CATCACT3; see below). This mutation resulted in a reduced footprint (−108 to −87 bp), indicating that a protein(s) binds to the distal portion of the wild-type sequence. A search of the published gene sequences revealed that a number of histone gene promoters contain potential ATF binding sites (Fig. 3).

Distinct Binding Events Occur at Site I

To address the mechanisms by which Site I contributes to transcription, we studied the number and types of protein/DNA interactions that

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                    -121          -107
Human H4 FO108:      5'GAAATGACGAAATGTC3'

                    -170          -155
Human H4 H4/h:       5'TTGGTGACGTCATCCA3'

                    -198          -183
Human H3 ST519:      5'AAAATGACGTCAGAGT3'

                    -157          -142
Human H3 FF435:      5'CGGGTGACGTCACAGC3'

                    -106           -91
Human H2B:           5'CCTCTGACGTTACCCT3'
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Fig. 3. Comparison of ATF binding sequences found in the promoter region of several human histone genes. The underlines delineate the ATF consensus sequences. The histone H4 FO108 sequence is from reference 25; the H4/h sequence is from reference 47 (D. Doenecke, unpublished data); the H3 ST519 sequence is from references 43 and 44; the H3 FF435 sequence from Charles Stewart (unpublished data); and the H2B sequence from reference 47.

occur at this site in vitro by employing the gel mobility shift assay. A radioactive DNA probe (−156 to −72 bp) spanning Site I was incubated with the same nuclear protein preparations from HeLa cells used in the DNase I footprinting studies. This probe formed protein-DNA complexes that resulted in several prominent shifted bands in TGE gels (Fig. 4A). The specific interactions in each complex were further defined by DNA binding site competition with synthetic oligonucleotides (Fig. 4B). An oligonucleotide representing the distal half of Site I (−125 to −101 bp), which was used at a 500-fold molar excess over the probe, eliminated the two lower complexes, designated HiNF-E and F, and partially reduced the intensity of the upper prominent complex, HiNF-C + E (Fig. 4B, lane 2). In addition, we examined whether either a consensus ATF recognition sequence or a natural ATF binding site from the adenovirus E4a promoter would compete for the Site I interactions. Both ATF recognition sites displayed competition patterns similar to that of the distal Site I oligonucleotide when a 500-fold molar excess was used (Fig. 4B, lanes 3 and 4). An unrelated oligonucleotide was unable to compete specifically for any of the bands at these concentrations (lane 5). These results suggest that HiNF-E and F interact specifically with ATF consensus sequences. Competition of both HiNF-E and F by the ATF consensus oligonucleotide suggests that these complexes contain ATF-like factors. The factors designated as HiNF-C and C′ were not competed by any of these ATF consensus oligonucleotides, but were compatable by an Sp-1 consensus dimer oligonucleotide (lane 6).

Distal ATF-Like Site Is Required for Binding

We defined the binding site requirements of protein/DNA interactions at Site I by site-directed base substitution analysis. Previously, we had identified by in vivo genomic sequencing (25) and in vitro dimethylsulfate (DMS) fingerprinting (29) a series of highly specific protein/guanine residue contact points at Site I. These contacts are arranged into two groups, one in the proximal half over the region of similarity to the Sp1 consensus recognition site and the other in the distal half within the consensus ATF recognition sequence, and were used to guide the construction of the site-directed mutation of the distal region.

The mutation (described above) alters the two guanine contacts in the distal half of Site I as well as one adjacent nucleotide and disrupts the ATF binding site consensus core, 5′-TGACG-3′. Examination of the protein-DNA interactions formed with this mutant probe revealed that the complex designated HiNF-E was diminished by the mutation (Fig. 4C). The F complex was not affected by this mutation, suggesting that this complex may represent a distinct, and perhaps non-specific interaction with the site. The binding of factors HiNF-C and C′ was also not affected by the mutation (Fig. 4C).

Partial Purification of HiNF-C

Since the binding of HiNF-C was specifically decreased by both Sp1 consensus oligonucleotide competition (Fig. 4B) and by mutation of the G-rich sequence located in the proximal portion of Site I (4C), we postulated that HiNF-C might be related to Sp1 itself. Sp1 is known to characteristically bind to a wheat germ agglutinin affinity matrix because of covalently attached N-acetylglucosamine (GlcNAc) moieties (39). When a HeLa cell nuclear extract was passed over this matrix, electrophoretic mobility shift analysis of fractions using the Site I probe revealed that, like Sp1, HiNF-C displayed binding behavior indicative of the presence of GlcNAc (data not shown).

UV Cross-Linking of Site I Proteins

In order to further analyze the protein/DNA interactions at Site I, we estimated the molecu-

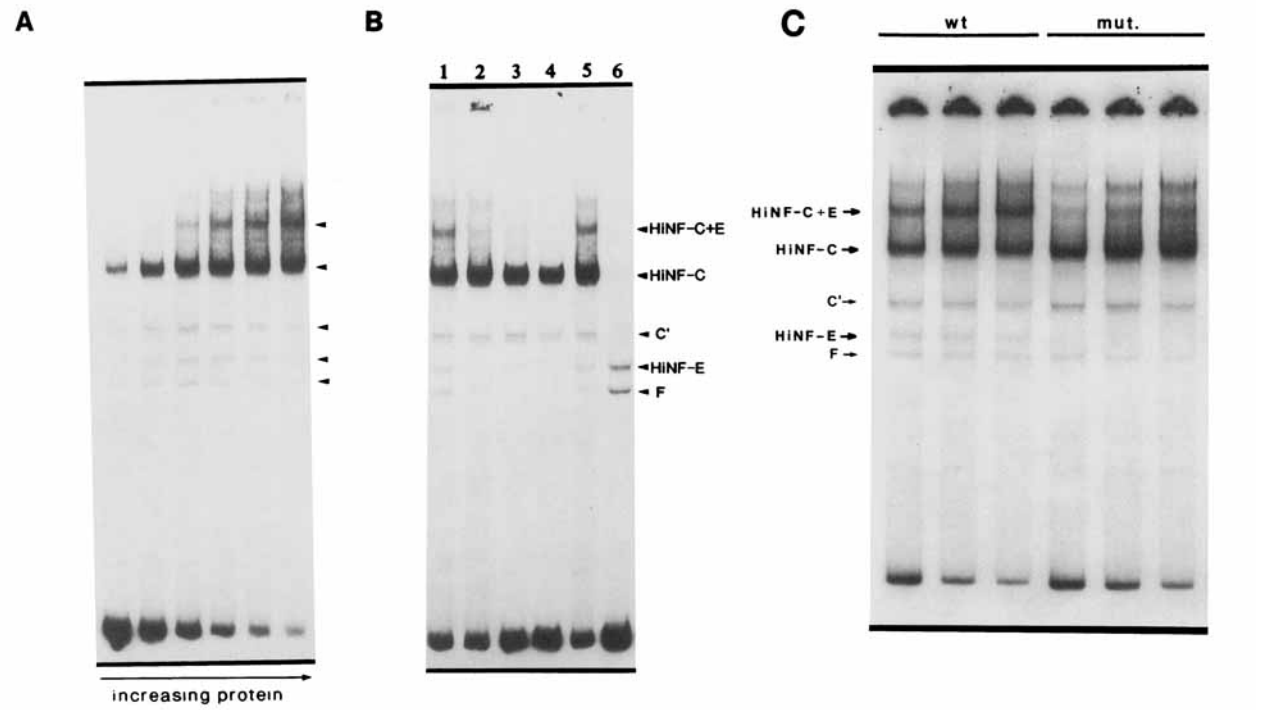

Fig. 4. Gel mobility shift analysis of the Site I protein-DNA interactions. **A:** A radiolabeled Site I probe (−156 to −72 bp plus 14 bp of pUC19 polylinker) was incubated with increasing concentrations (0.5 to 8 μg) of HeLa nuclear extract and resolved on a polyacrylamide gel as described (see Materials and Methods). The arrowheads to the right of the figure represent the protein/DNA complexes observed reproducibly. **B:** Oligonucleotide competition of the Site I protein-DNA complexes. Gel mobility shift analysis of the Site I probe incubated with 4 μg of HeLa nuclear extract and 500-fold molar excess of specific oligonucleotides. **Lanes: 1,** no competitor; **2,** distal Site I (DS-I); **3,** consensus ATF (CATX3) (42); **4,** ATF site from the E4 promoter (pE427X1) (41); **5,** non-specific oligonucleotide from the histone H3 promoter (H3-SII); **6,** Sp1 consensus dimer. **C:** Effect of specific base substitutions on the Site I protein-DNA interactions. Gel mobility shift analysis of Site I probes (−156 to −72 bp plus 14 bp of pUC19 polylinker) containing either the wild type sequence (wt) or mutated (distal ATF site mutant shown) as described in Materials and Methods. Each probe was incubated with three concentrations of HeLa nuclear extract (4, 6, 8 μg). Complexes have been designated HiNF-C + E, HiNF-C, HiNF-C', HiNF-E, and HiNF-F.

lar mass of the contributing proteins by UV cross-linking with a bromodeoxyuridine-containing Site I probe. In addition, because the UV cross-linking procedure results in stabilization of DNA binding proteins to their cognate sequence, this method potentially facilitates detection of short-lived protein/DNA interactions that would not be detectable in gel mobility shift assays or DNase I footprint analysis. The results shown in Figure 5 indicate that three proteins of approximately 61, 91, and 95 kDa are specifically cross-linked to Site I. Other bands of approximately 43–45 kDa are also detected after 60 min of UV treatment. All of the bands detected were dependent on the addition of nuclear factors and UV irradiation and could be eliminated by digestion with Proteinase K (Fig. 5). Competition with the distal Site I oligonucleotide specifically eliminated the 61, 92, and 95 kDa bands described above, but only minimally decreased the signal of non-specific bands in the 43–45 kDa range. A large excess of an unrelated oligonucleotide generally lowered the signal but did not specifically abolish any of the bands. These competition results suggest that multiple proteins are capable of specifically interacting with Site I. Our UV cross-linking experiments using bromodeoxyuridine failed to demonstrate the binding of specific proteins to Site I that were compatable by the Sp1 consensus oligonucleotide (data not shown). Identical results were obtained with a Site I cross-linking probe containing a non-functional HiNF-C binding site (data not shown). This result, that no Sp1-like proteins were cross-linked to the Site I probe, is not surprising since it is likely due to the lack of nucleotides within the HiNF-C binding site that can be substituted with bromodeoxyuridine.

DISCUSSION

The FO108 human H4 histone gene contains two regions of in vivo protein-DNA contact,

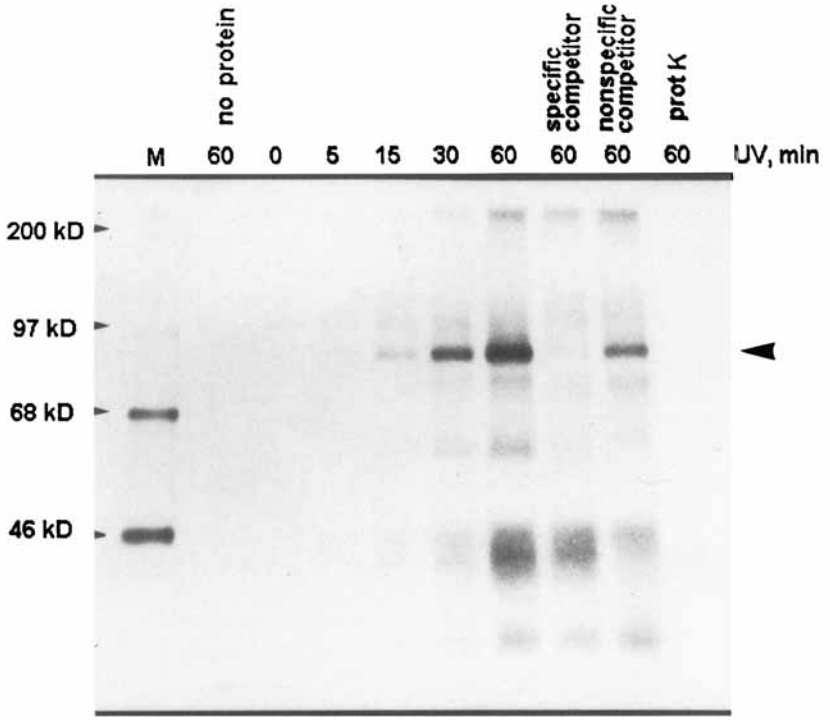

Fig. 5. Molecular weight determination of Site I proteins using UV cross-linking analysis. UV cross-linking to Site I was performed with BrdU-substituted, uniformly labeled Site I DNA probes (−156 to −72 bp plus 14 bp of pUC19 polylinker). After DNase I digestion, the samples were resolved on a 10% SDS-polyacrylamide gel and autoradiographed. Length of UV treatment is indicated above each lane. Specific competitor, DS-1; nonspecific competitor, H3-DSII; prot K indicates sample was digested with proteinase K prior to electrophoresis. The major cross-linked protein is indicated by an arrowhead in the right margin.

Sites I and II, located within the first 200 bp upstream of the transcription initiation site (25). Several Site II binding factors have been described previously (see reference 17). The results presented in this report contribute to the understanding of this histone gene proximal promoter by describing the bipartite structure of Site I.

Electrophoretic mobility shift analysis of unfractionated (Fig. 4) or wheat germ agglutinin affinity matrix-purified nuclear proteins (data not shown) suggest that the proximal factor, HiNF-C, might be related to the transcription factor Sp1. Indeed, antibody supershift studies confirm that HiNF-C is Sp1 (41). Further analysis of Site I both in vitro and in vivo demonstrates conclusively that the proximal portion of Site I functions as an Sp1 site which is essential for the maximal expression of this histone gene (41).

The binding site found in the distal portion of Site I contains a sequence with strong similarity to the ATF consensus recognition element, 5′-GTGACGT$^{AA}_{CG}$-3′ (42). While no other vertebrate histone gene promoter has been described to bind ATF, similar sequences are found in several other histone gene promoters (see Fig. 3). Notably, the ST519 human H3 histone gene promoter contains an ATF consensus sequence element that is involved in protein-DNA interactions both in vivo and in vitro (43,44). Additionally, Tabata et al. have identified ATF-related factors that bind the promoter of a wheat H3 histone gene (45,46). Therefore, ATFs may play an important role in orchestrating the coordinated expression of several different histone genes. Mobility shift studies using the entire FO108 Site I sequence show that the binding of one protein complex is effectively reduced by mutation of the ATF consensus core sequence present in Site I and is specifically competed by ATF consensus oligonucleotides (Fig. 4C). When the assay was optimized for the detection of ATF family members using a probe encompassing only the distal portion of the site, we observed that several ATF-related proteins either in the HeLa nuclear extract, or in recombinant form, bound specifically to this region (41). Tran-

sient expression studies in HeLa cells indicate that the mutation of this asymmetric ATF binding site causes a significant decrease in reporter activity only in the absence of the proximal Sp1 site (41). Therefore, we postulate that while Sp1 plays a dominant role in bringing about the maximal expression of the histone H4 FO108 gene, ATFs probably perform some auxiliary role in the process. How these proteins might interact with the other components of the proximal promoter of this gene is currently under examination.

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