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Human H1 Histone Gene Promoter CCAAT Box Binding Protein HiNF-B Is a Mosaic Factor†

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ABSTRACT: Vertebrate histone gene promoters in many cases contain an upstream element, 5′dCCAAT, that has been implicated in modulating the efficiency of transcription of a broad spectrum of genes. We have previously isolated a nuclear factor (HiNF-B) that binds specifically to the CCAAT element of a cell cycle regulated human H1 histone gene. This factor shows similarities with other CCAAT box binding proteins in that it recognizes the same sequence but shows a distinct chromatographic behavior. In the present study, we have employed the gel retardation assay to demonstrate that HiNF-B is a cell cycle independent DNA binding protein that is conserved in both human and mouse cells. Using a series of reconstitution experiments with partially purified HiNF-B fractions, we show that this factor requires association of at least two components for site-specific binding. The composite structure of HiNF-B suggests that binding of at least some CCAAT elements in vertebrates may require cooperative interaction of CCAAT box binding proteins with other factors.

Sequence-specific DNA binding proteins play key roles in the transcriptional regulation of eukaryotic gene expression (Dynan & Tjian, 1985). Vertebrate promoter DNA sequences acting as binding sites for such proteins include the TATA element (Parker & Topol, 1984), the CCAAT element (Graves et al., 1986), the GGGCGG element (Dynan & Tjian, 1983), and the ATTTGCAT element (Singh et al., 1986). The CCAAT element can be found in a variety of vertebrate promoters and has been implicated in the regulation of a number of genes, such as the Herpes Simplex Virus thymidine kinase gene (Jones et al., 1985) and mouse α-globin (Mellon et al., 1981) and β-globin genes (Dierks et al., 1983). This transcriptional element has been shown to act as a recognition site for CCAAT box binding proteins (McKnight & Tjian, 1986; Cohen et al., 1986; Myers et al., 1986; van Wijnen et al., 1988). In addition, the CCAAT element has been implicated in the initiation of adenovirus DNA replication (Jones et al., 1987).

The eukaryote-specific H1 and core (H2A, H2B, H3, and H4) histone genes encode a set of proteins that are essential for maintaining the integrity of the eukaryotic genome and are encoded in multiple clusters in the vertebrate genome. Coordinate regulation of this multigene family occurs in tight conjunction with DNA replication, and control is exerted at both transcriptional and posttranscriptional levels [reviewed in Stein et al. (1984) and Schumperli (1986)]. Vertebrate histone genes are constitutively transcribed at a basal rate in actively dividing cells, and at the onset of S phase in the cell division cycle, a 3-5-fold transient enhancement of the transcription rate occurs (Plumb et al., 1983; Heintz et al., 1983; Sittman et al., 1983; Artishevsky et al., 1987; Baumbach et al., 1987).

The transcriptional regulation of human H1 and core histone genes is mediated by a battery of cis-acting elements (Sierra et al., 1983; Kroeger et al., 1987; Helms et al., 1987; Sive et al., 1986; Dailey et al., 1986), some of which are histone specific and others that are shared by a broad spectrum of human promoters. Several of these histone promoter elements coincide with DNA/protein interaction sites in vitro (van Wijnen et al., 1987, 1988; Dailey et al., 1986; Sive & Roeder, 1986) and with regions of chromatin hypersensitive to DNaseI, MNase, and S1 nuclease (Pauli et al., 1988; Moreno et al., 1986; Chrysogelos et al., 1985). Moreover, our laboratory has established in vivo sites of DNA/protein interaction in the proximal promoter region of two cell cycle regulated core (H3 and H4) histone genes. Both the H4 and H3 histone genes have two major regions of in vivo DNA/protein interactions (site I and site II) that overlap in vitro protein binding sites (Pauli et al., 1987; unpublished data). Sequences encompassing the entire H4 promoter site II, which spans both a (distal) histone-specific element and a (proximal) TATA box, are required and sufficient for accurate H4 histone mRNA transcription initiation in vivo (Kroeger et al., 1987). Although various transcriptional elements and associated nuclear factors have been identified, the role of these in the coordinate increase in histone mRNA synthesis in early S phase is not clear.

The proximal promoter region of a cell cycle regulated human H1 histone gene contains two target sites for the binding of nuclear factors in vitro (van Wijnen et al., 1988). A distal domain is bound by HiNF-A, a factor that binds to at least two other (H3 and H4) histone gene promoters (van Wijnen et al., 1987). A proximal domain (analogous in position to the H4 promoter site II) functions as a binding site for HiNF-B, a novel CCAAT box binding protein. In this work, we present evidence that both factors have a cell cycle

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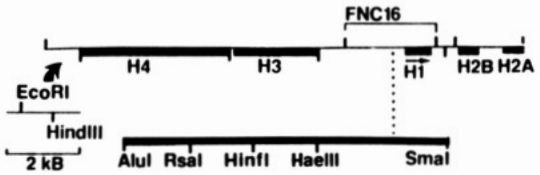

FIGURE 1: Restriction map of the human genomic histone gene cluster 415 containing the FNC16 H1 histone gene (top), showing the orientation of transcription (arrow) and the promoter region (bottom) used for the generation of probes.

independent DNA binding activity and are conserved in different human and mouse cell lines. Moreover, using reconstitution experiments in combination with the gel retardation assay, we show that HiNF-B requires the association of at least two components in order to interact specifically with its binding site.

Materials and Methods

Vectors and DNA Probes. Probes were derived from pOX001 (van Wijnen et al., 1988), a plasmid based on Bluescript KS (+) (Stratagene) that contains a *SmaI/PvuII* fragment spanning the first 0.5 kb upstream of a human H1 histone gene designated FNC16 (Carozzi et al., 1984) present in the genomic histone gene cluster λ 415 (also designated 49) (Figure 1). Single-end-labeled DNA probes for DNA/protein binding assays were made by digesting pOX001 with the restriction enzymes *RsaI* [nucleotide (nt) −213] or *SmaI* (nt −76) and end-labeling the dephosphorylated DNA fragments with [γ-^{32}P]ATP. The probes were subsequently cleaved with, respectively, *SmaI* (nt −76) and *AluI* (nt −233) and purified on native 4% polyacrylamide gels.

Nuclear Extracts and Fractionation. Nuclear extracts for in vitro DNA binding studies, fractionation of these extracts by liquid chromatography into DEAE-Sephacel salt fractions (D0–200, D200–1000), and separation of the D0–200 fraction on phosphocellulose (yielding P0–40, P40–350, and P350–1000 fractions) have been described previously (scheme I; van Wijnen et al., 1987) (numbers refer to millimolar KCl in the elution buffer). Using an alternative elution procedure, we have obtained three different phosphocellulose fractions (P0–200, P200–350, and P350–1000 fractions) (scheme II; van Wijnen et al., 1988). Mouse nuclear extracts from C127 monolayers were prepared by using a modification of the procedure of Challberg and Kelly (1979) by extracting 5×10^8 nuclei with 10 mL of sucrose buffer (50 mM Hepes, pH 7.5, and 10% sucrose) containing 0.6 M KCl. The procedure for the preparation of nuclear extracts from HeLa S3 cells synchronized by double thymidine block was identical with a previous procedure (van Wijnen et al., 1987).

Gel Retardation Assays and Stairway Assays. Electrophoretic mobility shift assays (gel retardation assays) were performed as before (van Wijnen et al., 1987). Binding reactions were composed of 0.5 ng of probe, 2 μg of sonicated *Escherichia coli* DNA [or poly(I/C) DNA] as competitor DNA, and protein amounts as indicated in the figure legends in a final volume of 20 μL of binding buffer (12% glycerol, 12 mM Hepes/NaOH, pH 7.9, 4 mM Tris-HCl, 60 mM KCl, 10 mM NaCl, 5 mM $MgCl_2$, and 0.5 mM EDTA). Binding mixtures were subjected to electrophoresis in native, low ionic strength polyacrylamide gels and analyzed by autoradiography.

Reciprocal restriction site deletion analysis (stairway assay) (van Wijnen et al., 1987, 1988) was performed by digesting a single-end-labeled probe with the enzymes indicated in the figure legends. This yields a series of probe deletion mutants that were separately incubated with protein and electrophoresed in parallel in low ionic strength gels. Stairway assays

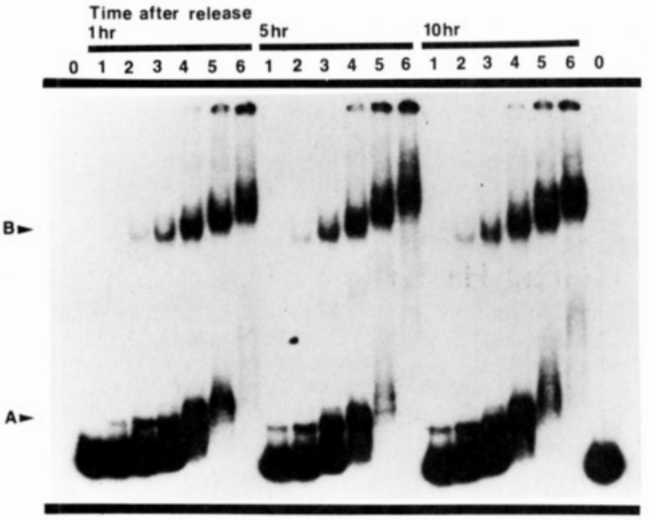

FIGURE 2: Gel retardation assay using nuclear extracts from human HeLa S3 cells prepared at the indicated time points after release from a double thymidine block (2 μg of *E. coli* DNA as competitor). Amounts of protein added: for each block (lanes 1–6), respectively, 0.3, 0.6, 1.5, 2.4, and 3 μg. Lane 0, probe only.

are a convenient procedure to localize the binding of sequence-specific factors to short DNA segments. The resolution of this approach is roughly 2-fold lower than DNaseI footprinting but allows detection of secondary (lower affinity) binding sites and correlates a DNA/protein complex with a specific DNA fragment on a single gel. Reconstitution experiments were performed by preincubating the indicated protein fractions for 15 min on ice prior to the addition of probe DNA and competitor DNA.

Quantitation of Reconstitution Experiments. Reconstitution experiments were quantified by excising gel segments corresponding to free probe, specific DNA/protein complexes, and areas between free and complexed DNA. Radioactivity present in these gel segments was determined by Cerenkov counting. Radioactivity present in the areas between free and complexed DNA was subtracted as background. Subsequently, the values were expressed as a percentage of radioactivity present in a gel segment containing free probe from a lane that contained probe DNA only. One hundred percent radioactivity (arbitrarily set as 100 units) corresponds approximately to 5 fmol of probe DNA fragments.

Results and Discussion

Factor HiNF-B Is a Constitutive DNA Binding Activity. Factor HiNF-B is a sequence-specific DNA binding protein that interacts with a CCAAT box in the promoter of a cell cycle regulated human H1 histone gene (van Wijnen et al., 1988) and potentially may bind to many other histone gene promoters containing a similar DNA sequence. The presence or absence of this activity in cells at different stages in the cell division cycle may reveal clues to the type of transcriptional regulation in which it is involved (constitutive transcription versus S-phase specific enhancement of transcription).

Cell cycle stage specific nuclear extracts from human HeLa S3 cells synchronized by a double thymidine block procedure have been employed in the gel retardation assay in order to study the DNA binding activity of HiNF-B as a function of the cell cycle (Figure 2; time points as indicated). The probe used for these experiments was a *SmaI/RsaI* restriction fragment spanning nucleotides (nt) −76 to −213 measured from the human H1 histone gene FNC16 translational start codon. When the H1 probe was used in the presence of nuclear extract protein and *E. coli* DNA as nonspecific competitor, two

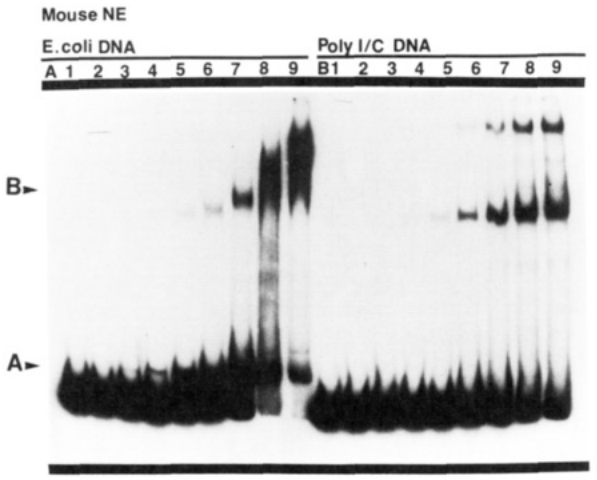

FIGURE 3: Gel retardation assay using nuclear extracts from C127 monolayers in the presence of 2 μg of *E. coli* DNA (part A) or 2 μg of poly(I/C) DNA (part B). Amounts of protein added for lanes 1–9, respectively, were 0, 0.1, 0.2, 0.5, 0.75, 1.0, 2.0, 3.0, and 4.0 μg.

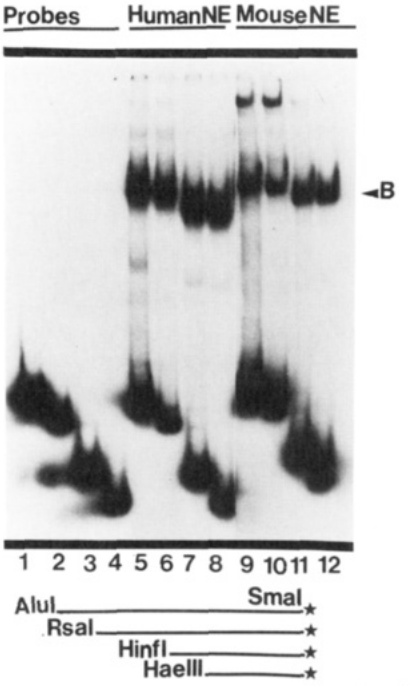

FIGURE 4: Stairway assay using probes end-labeled at the *Sma*I site (nt -76) (indicated by star) and digested with, respectively, *Alu*I (nt −233), *Rsa*I (nt −213), *Hin*fI (nt −160), and *Hae*III (nt −138) (lanes 1–4). Binding reactions contain 3 μg of human (lanes 5–8) or mouse (lanes 9–12) nuclear extract protein. Poly(I/C) DNA is used as competitor (2 μg).

complexes were formed (designated complex A and complex B; Figure 2). These complexes are detected in nuclear extracts representing early S phase (1-h extracts; lanes 1–6), mid S phase (5-h extracts; lanes 1–6), and non S phase (10-h extracts; lanes 1–6). Complex A is mediated by a factor designated HiNF-A that binds to analogous regions in the H4 and H3 histone promoters containing ATTT repeat elements (van Wijnen et al., 1987); complex B has been shown to be mediated by HiNF-B (van Wijnen et al., 1988). Detection of both HiNF-A and HiNF-B in the promoter region of the H1 histone gene FNC16 at the three cell cycle times examined suggests that these factors may be involved in the constitutive transcription of this gene that occurs throughout the cell cycle.

Mouse Nuclear Extracts Contain HiNF-A and HiNF-B Homologues. Human histone gene expression has been studied in heterologous, murine cell lines (Kroeger et al., 1987; Helms et al., 1987; Green et al., 1986; Capasso & Heintz, 1985) as a means of identifying cis-acting elements involved in the regulation of histone mRNA synthesis. These experiments were performed with the assumption that trans-acting factors in mouse cells are compatible with cis-acting elements in human histone gene promoters. In order to confirm the validity of this assumption, we have used the gel retardation assay to probe for the existence of such factors. Nuclear extracts prepared from mouse C127 monolayer cells were complexed to the human H1 promoter probe to detect factors similar to HiNF-A and HiNF-B (Figure 3). When *E. coli* DNA was used in the assay, two very similar DNA/protein complexes were detected (designated A and B; Figure 3A, lanes 1–9); when poly(I/C) DNA was included in the binding reaction, only formation of complex B, and not complex A, was observed. Because HiNF-A cannot be detected in the presence of poly(I/C) DNA (van Wijnen et al., 1987), this indicates that mouse complex A is formed by a factor similar to HiNF-A.

Human and mouse nuclear extracts were also used in parallel in stairway assays in order to show that formation of human and mouse complex B requires the same DNA segment. Probes end-labeled at the *Sma*I site were prepared with various deletions extending toward the indicated restriction sites (Figure 4; lanes 1–4) and incubated with human (lanes 5–8) or mouse (lanes 9–12) nuclear extracts in the presence of poly(I/C) DNA. These results show that sequences upstream of a *Hae*III site (nt −138) are dispensable and that the *Hae*III/*Sma*I fragment (a 53 bp fragment containing the CCAAT element bound by HiNF-B) is sufficient for formation of mouse complex B. This result indicates that mouse complex B is formed by a factor similar to HiNF-B. The detection of similar factors in murine and primate nuclear extracts shows that HiNF-A and HiNF-B DNA/protein complexes are conserved in evolution, and is compatible with the notion that murine and primate transcription factors are interchangeable in regulating human histone gene expression.

Purification of Factor HiNF-B by Phosphocellulose Chromatography. We have fractionated nuclear extracts by passage over DEAE-Sephacel and phosphocellulose in order to separate the various histone promoter associated DNA binding activities (van Wijnen et al., 1987, 1988). Preliminary experiments aimed at partially purifying HiNF-B by phosphocellulose chromatography showed that HiNF-B binds to phosphocellulose at a salt concentration of 40 mM KCl and is released when the elution buffer contains 350 mM KCl (scheme I; data not shown). However, the recovery of HiNF-B binding activity in these experiments was low. We suspected that dialysis steps prior to and during purification could be related to the low recovery of HiNF-B and used an alternative fractionation scheme (scheme II) in which no dialysis was performed between chromatography steps.

In this procedure, nuclei were extracted with 0.4 M KCl, and the nuclear extract was diluted to 0.2 M KCl prior to passage over a DEAE-Sephacel column equilibrated with buffer containing 0.2 M KCl and the flow-through collected. Subsequently, this flow-through (designated D0–200) was applied to a phosphocellulose column that is equilibrated at the same salt concentration and again the flow-through collected (designated P0–200). Material bound to the phosphocellulose column was recovered by increasing the salt concentration of the elution buffer to 350 mM KCl (P200–350 fraction) and then to 1000 mM KCl (P350–1000).

The DEAE-Sephacel and phosphocellulose fractions were analyzed for the presence of HiNF-B by using the gel retar-

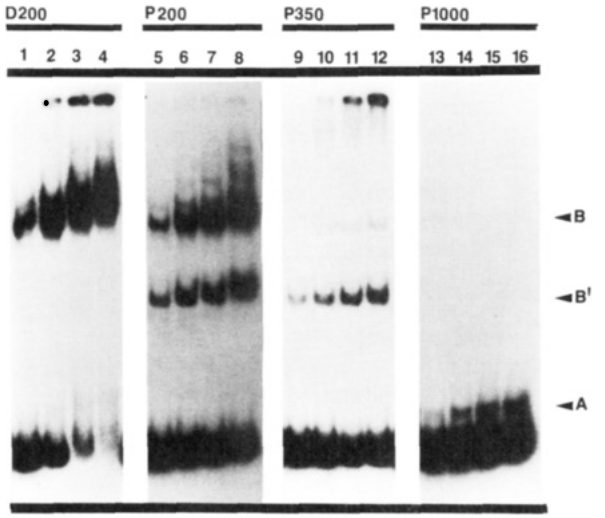

FIGURE 5: Monitoring of column fractions for the presence of HiNF-B with *E. coli* DNA as competitor (2 μg). Lanes 1–4: D0–200 fraction; 2.1, 2.8, 4.2, and 5.6 μg of protein added, respectively. Lanes 5–8: P0–200 fraction; 0.9, 1.2, 2.2, and 2.5 μg of protein. Lanes 9–12: P200–350 fraction; 1.2, 1.5, 2.2, and 2.9 μg of protein. Lanes 13–16: P350–1000 fraction; 0.5, 0.7, 1.1, and 1.4 μg of protein.

dation assay (Figure 5). These results showed that HiNF-B activity elutes almost quantitatively in the D0–200 fraction and predominantly in the P0–200 fraction (Figure 5; lanes 5–8); HiNF-B appears well separated from HiNF-A, which elutes in the P350–1000 fraction (Figure 5; lanes 13–16). Again we observed a substantial loss of HiNF-B DNA binding activity during chromatography (Figure 5; compare lanes 5–8 with lanes 1–4). Interestingly, we noted a new complex (designated B′), and the putative factor involved appeared to elute in both the P0–200 and P200–350 fractions but was not detectable in the D0–200 fraction. The loss of HiNF-B DNA binding activity (complex B) during chromatography and the appearance of a new DNA/protein complex with a higher relative mobility (complex B′) hint at a direct relationship between complex B′ and complex B.

Formation of Complex B′ Requires Sequences That Overlap the HiNF-B Binding Domain. In order to identify the DNA segment involved in the formation of complex B′, we performed stairway assays with the D0–200, P0–200, and P200–350 fractions by using a set of probes that were single-end-labeled at the *Rsa*I site (nt −213) and progressively shortened by restriction enzymes (Figure 6). Substantial formation of complex B′ was observed when the full-length probe (nt −213 to −78) was used in the gel retardation assay (Figure 6, lanes 5 and 9), but deletion of an oligonucleotide DNA fragment by *Msp*I digestion (nt −93) (Figure 6, lanes 6 and 10) resulted in a strong reduction in the formation of complex B′ in all experiments. [Residual formation of complex B′ was observed with the shortest probe (*Rsa*I/*Hin*fI; nt −213 to −176) which implies that the factor present in complex B′ might have a second (lower affinity) binding site within this DNA segment.] Interestingly, similar results were obtained for complex B (Figure 6, lanes 1 and 2 and lanes 5 and 6), although no residual formation of complex B was observed with the shorter probes.

The result obtained with complex B is in agreement with DNaseI footprinting data for this complex that show that DNaseI protection by HiNF-B extends to nt −89, hence encompassing the *Msp*I site (Figure 7). Because complex B′ requires the same DNA sequences for optimal formation as complex B, this demonstrates that complex B′ contains a DNA binding activity whose binding domain overlaps the binding

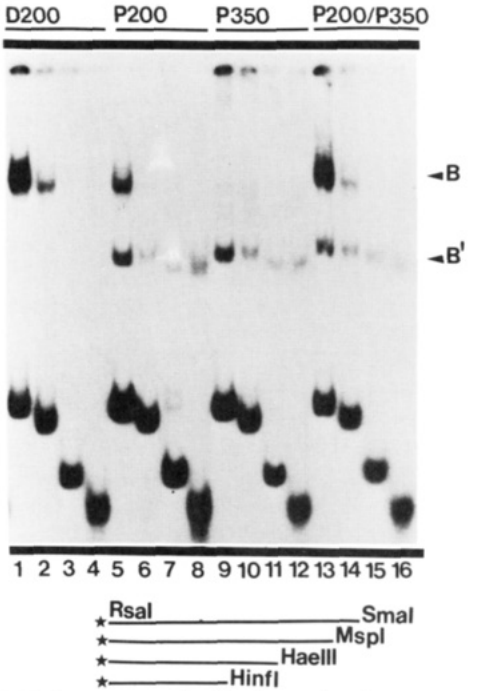

FIGURE 6: Stairway assay using fractionated nuclear extracts. Probes are end-labeled at the *Rsa*I site and digested with the indicated restriction enzymes; *E. coli* DNA (2 μg) is used as competitor. Lanes 1–4, 2.8 μg of D0–200 protein added; lanes 5–8, 2.4 μg of P0–200 protein; lanes 9–12, 2.8 μg of P200–350 protein; lanes 13–16, mixture of 1.2 μg of P0–200 and 1.4 μg of P0–200 and P200–350.

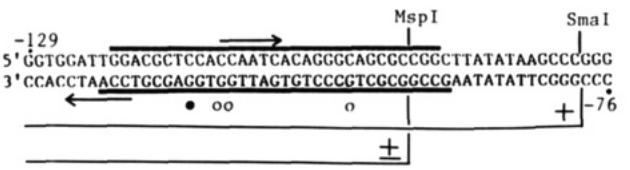

FIGURE 7: Partial sequence of the FNC16 H1 histone promoter (nt −129 to −76) spanning the DNaseI footprint (overlined and underlined) and DMS fingerprint (open circles, DMS protection; closed circles, DMS enhancement) of HiNF-B. Indicated by arrows are two CCAAT boxes, only one of which is contained within the DNaseI footprint. Digestion of the probe (labeled at the *Rsa*I site at nt −213) with *Msp*I reduces binding (indicated by ±) as compared to binding to the probe-containing sequences up to the *Sma*I site (indicated by +).

domain of HiNF-B. This suggests that complex B′ is an intermediate in the formation of the HiNF-B complex.

The possibility was considered that formation of complex B′ and formation of complex B are mutually exclusive binding events and that the factor present in complex B′ is unrelated to complex B (and with the assumption that this factor has become activated during chromatography). To address this question, we mixed the P200–350 fraction (containing primarily the factor involved in complex B′) with the P0–200 fraction (containing both the factors involved in formation of complex B′ and complex B) in order to see if adding the former fraction would decrease formation of complex B in the stairway assay.

Most interestingly, combining the P0–200 fraction with the P200–350 fraction resulted in a dramatic increase, rather than a decrease, in the formation of complex B (Figure 6, lanes 13–16) as compared to similar amounts of P0–200 and P200–350 fractions alone (Figure 6, lanes 5 and 9). This shows that formation of complex B and formation of complex B′ are not mutually exclusive. In addition, the fact that optimal formation of complex B requires factors present in two

Table I: Percentage Conversion of ^{32}P-Labeled DNA into Specific DNA/Protein Complexes and Specific Activity

fraction added:		D0–200	D0–200	P0–200	P0–200	P200–350	P200–350	P0–200/P200–350	P0–200/P200–350
μg of protein:		2.8	5.6	1.2	2.4	1.4	2.8	1.2/1.4	2.4/2.8
					Absolute Activity[a]				
complex B		32.2	34.6	2.0	8.1	0.5	1.6	17.1	23.2
complex B′		0.0	0.0	3.0	8.4	3.0	7.0	6.0	6.7
free DNA	100.0	34.9	19.3	69.4	68.3	67.6	47.1	40.1	14.5
nonspecific		32.9	46.1	25.6	15.2	28.9	44.3	36.8	45.6
concn[b]		0.14	0.28	0.06	0.12	0.07	0.14	0.13	0.26
					Specific Activity[c]				
complex B		11.5	6.2	1.7	3.4	0.4	0.6	6.6	4.5
complex B′		0.0	0.0	2.5	3.5	2.1	2.5	2.3	1.3

[a] Absolute activity in binding reactions containing the indicated amounts of protein. Values represent the percentage of radioactivity measured in a gel segment corresponding with a specific DNA/protein complex as compared to radioactivity in a lane containing DNA only. [b] Protein concentration in milligrams per milliliter. [c] Specific activity expressed as the percentage of radioactivity in a complex per microgram of protein.

different fractions reveals that HiNF-B consists of more than one component.

Reconstitution of HiNF-B DNA Binding Activity. The possibility that HiNF-B has a subunit composition and that these components elute in two different phosphocellulose fractions was further explored by mixing various amounts of protein of the P0–200 and the P200–350 fractions in order to find optimal combinations of factors for formation of complex B. To this end, a series of titration experiments were performed in which constant amounts of one phosphocellulose fraction were preincubated with increasing amounts of the other phosphocellulose fraction and the mixtures subjected to the gel retardation assay (Figure 8).

One set of experiments was performed with fixed amounts of protein from the P0–200 fraction (Figure 8; lanes A1, B1, and C1) and increasing amounts of protein from the P200–350 fraction (lanes A2–A4, B2–B4, and C2–C4) that were identical with the protein amounts used in the control lanes (D1–3). Samples in lanes B1, D2, and A2 (and C1, D3, and B2) have equivalent amounts of protein. A global impression of the results can be obtained, for instance, by superimposing lanes D1–3 on lane A1 to yield lanes A2–A4 (or on lane B1 to yield lanes B2–B4, or on lane C1 to yield lanes C2–C4). The results show that all combinations of the two fractions yield a substantial increase in complex B formation over similar amounts of protein of either fraction alone. Hence, this cooperative effect supports the notion that HiNF-B is composed of at least two physically separable factors.

Quantitation of Reconstitution Experiments. Quantitation of these reconstitution experiments was carried out by excising out gel segments corresponding to free probe and complexes B′ and B and determining the amounts of radioactivity in these segments by Cerenkov counting. Values were expressed as the percentage (units) of radioactivity found in these gel segments as compared to a control lane containing probe only (Table I, top section) and were corrected for the amount of protein present in the binding reaction to obtain a measure for specific activity (Table I; bottom section).

The results obtained with the P0–200 fraction showed a severalfold decrease in both the absolute and the specific activity of factors mediating complex B as compared to the D0–200 fraction. (We noted that the specific activity of factors involved in complex B present in the P0–200 fraction increased with increasing protein concentration in the binding reaction; discussed in the next section.) The specific activity of factors mediating complex B in the P200–350 fraction is hardly significant (Table II). The specific activity of the factor present in complex B′ is almost equal in both the P0–200 fraction and the P200–350 fraction and is comparable to the specific activity measured in the combination of both fractions (Table II).

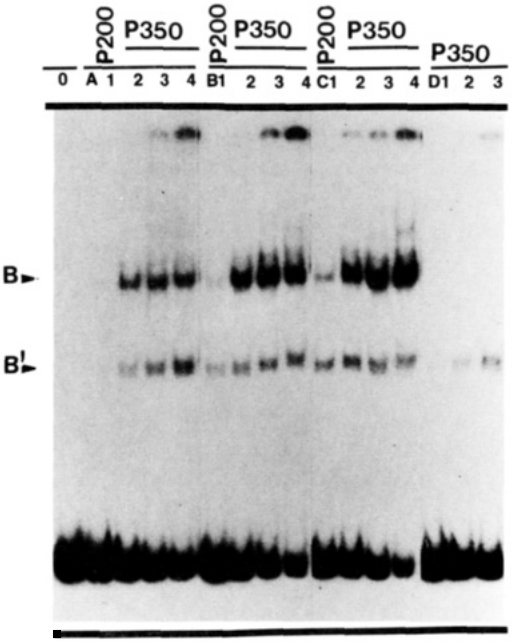

FIGURE 8: Reconstitution of HiNF-B activity by combination of the P0–200 fraction with the P200–350 fraction. Lanes A1, B1, and C1 contain, respectively, 0.6, 1.5, and 2.4 μg of P0–200 protein alone. Lanes D1–D3 contain, respectively, 0.7, 1.4, and 2.8 μg of P200–350 protein alone. Lanes A2–A4 contain a mixture of 0.7, 1.4, and 2.8 μg of P200–350 protein, respectively, with 0.6 μg of P0–200 protein; lanes B2–B4, same increasing amounts but reconstituted with 1.5 μg of P0–200 protein; lanes C2–C4, same increasing amounts but reconstituted with 2.4 μg of P0–200 protein. *E. coli* (2 μg) is used as competitor DNA.

However, the absolute activity and the specific activity of factors present in complex B increase up to 4-fold in the mixture of the P0–200 and P200–350 fractions. These data are in agreement with the visual observations described above (see Figure 8).

We hypothesize that complex B′ is an intermediate in the formation of complex B and that detection of complex B′ and the loss of specific activity of HiNF-B are related to separation of HiNF-B components during phosphocellulose chromatography. We propose that complex B′ is formed by a DNA binding factor (designated B1) that binds near the *Msp*I site located downstream of the CCAAT box (see Figure 7) and that formation of complex B requires association of a second component (designated B2).

Presence of Excess Unbound HiNF-B Components in the Binding Reaction. The proposal that complex B′ (mediated by B1) is an intermediate in the formation of complex B (in part mediated by B2) implies that B1 is also present in complex B. The unit definition used in this study is related to the

Table II: Distribution of Components B1 and B2 over Complexes B′ and B and Units Unbound

	fraction added						
	P0–200		P200–350		P0–200/P200–350		unbound units[c]
μg of protein	1.2		1.4		1.2/1.4		
complex	B	B′	B	B′	B	B′	
units of B1[a]	2.0	3.0	0.5	3.0	17.1	6.0	14.6
units of B2[a]	2.0		0.5		17.1		14.6
μg of protein	2.4		2.8		2.4/2.8		
complex	B	B′	B	B′	B	B′	
units of B1[a]	8.1	8.4	1.6	7.0	23.6	6.7	4.8
units of B2[a]	8.1		1.6		23.6		13.5
unbound units of B1[b]	6.5		1.6				
unbound units of B2[b]	4.1		0.6				

[a] Units of B1 and B2 present in complex B (left) and complex B′ (right); values were obtained by converting percentage formation of complex B′ into units of B1 and percentage formation of complex B into units of B1 and B2 (1% is 1 unit of B1 and 1 unit of B2). [b] Units of B1 and B2 unbound per 2.4 μg of P0–200 protein (left) or 2.8 μg of P200–350 protein (right). Values were obtained according to the following formula (in units): P0–200(2.4 μg) − 2[P0–200(1.2 μg)] (left) and P200–350(2.8 μg) −2[P200–350(1.4 μg)] (right). [c] Units of B1 and B2 unbound per 2.6 μg of P0–200/P200–350 protein (top) or 5.2 μg of protein (bottom). Values were obtained according to the following formula (in units): P0–200/P200–350(1.2/1.4 μg) − P0–200(1.2 μg) − P200–350(1.4 μg) (top) and P0–200/P200–350(2.4/2.8 μg) − P0–200(2.4 μg) − P200–350(2.8 μg) (bottom).

(femto)moles of probe DNA bound. If we assume a constant stoichiometry of factors bound per mole of DNA fragments, then our unit definition is a direct measure of (femto)moles of protein molecules bound. Hence, if we detect 1 unit of complex B′ (=B1) and 1 unit of complex B (=B1 + B2), then we detect in total 2 units of component B1.

The mixture of 1.2 μg of P0–200 fraction, containing 3.0 units of complex B′ and 2.0 units of complex B (see Table I; in total, 5.0 units of component B1), and 1.4 μg of P200–350 fraction, containing 3.0 units of complex B′ and 0.5 unit of complex B (in total, 3.5 units of B1), yields in combination 17.1 units of complex B and 6.0 units of complex B′ (in total, 23.1 units of B1). From these data, it is clear that a severalfold molar excess of component B1 must be present in the binding reactions containing either fraction alone. Table II gives an estimation of free units of components B1 and B2 for the protein fractions tested. These data show that not only a severalfold molar excess of B1 must be present in the binding reactions containing either the P0–200 or the P200–350 fraction by itself but also a severalfold molar excess of component B2.

Cooperativity of HiNF-B Components. The specific activity of factors involved in formation of complex B present in the P0–200 fraction increases as a function of protein concentration. For instance, if the amount of P0–200 protein is doubled (from 1.2 to 2.4 μg), then the absolute activity increases 4-fold and, hence, the specific activity 2-fold (see Table I). We have used this observation to obtain an estimation of unbound components B1 and B2 per microgram of P0–200 protein and P200–350 protein (Table II). First, these data suggest that the majority of unbound components B1 and B2 is present in the P0–200 fraction. In addition, these data suggest that the increase in specific activity observed with increasing P0–200 protein concentration is possibly related to cooperative interaction between unbound B1 and B2 molecules that are present in excess in the binding reaction.

However, the amounts of unbound B1 and B2 molecules as calculated from each fraction by itself (Table II, bottom section) are approximately 2-fold lower than the amounts of unbound B1 and B2 molecules as calculated from the reconstitution experiments (Table II, right column). Hence, apart from the cooperativity between factors present in the P0–200 fraction, additional cooperativity is caused by the combination of factors present in both the P0–200 and the P200–350 fraction.

At Least One Component of HiNF-B Loses Specificity during Isolation. The P0–200 fraction used in this study has also been analyzed in the presence of poly(I/C) DNA instead of *E. coli* DNA as competitor (van Wijnen et al., 1988). The results obtained with poly(I/C) DNA showed a severalfold increase in the formation of complex B and a severalfold decrease in the formation of complex B′ when compared to the results obtained with *E. coli* DNA (data not shown). However, when crude extracts (or the D0–200 fraction) containing unfractionated HiNF-B were used, there was no difference in the use of either nonspecific competitor. This means that the conversion factor necessary for the formation of complex B (component B2) has a DNA binding activity that loses specificity during chromatography. Apparently, the loss of specificity renders this component susceptible to nonspecific competition by *E. coli* DNA.

Component B1 appears to have a second, lower affinity binding site in a distal probe segment that becomes apparent only when fractionated nuclear extracts are used (see Figure 6). This indicates that component B1 also loses specificity during chromatography. We propose that the loss of HiNF-B activity is related to the loss of specificity of both components B1 and B2 upon phosphocellulose chromatography and that the severalfold molar excess of "unbound" components B1 and B2 in binding reactions containing the P0–200 fraction actually is caused by nonspecific binding to *E. coli* DNA.

The initial purification scheme used for the isolation of HiNF-B (scheme I; data not shown) included a dialysis step prior to phosphocellulose chromatography and gave a lower yield of this mosaic factor than the subsequent scheme in which no dialysis was performed beforehand. This could suggest that stability of HiNF-B during chromatography (or site specificity during DNA binding reactions) requires a dialyzable ligand. We initiated experiments to address whether metal ions were involved by preincubating nuclear extracts with metal-chelating agents such as EDTA or 1,10-phenanthroline prior to the gel retardation assay. Although the results of a similar experiment provided evidence for metal dependency of an H4 histone promoter specific factor (HiNF-C), the binding of HiNF-B was not altered by the addition of these chelators (unpublished observations). Nevertheless, it is possible that HiNF-B is a metalloprotein that contains a nonchelatable metal ion.

Presence of a Conversion Factor in the P350–1000 Fraction. Reconstitution experiments with the P0–200 and P200–350 fractions showed a dramatic increase in the formation of

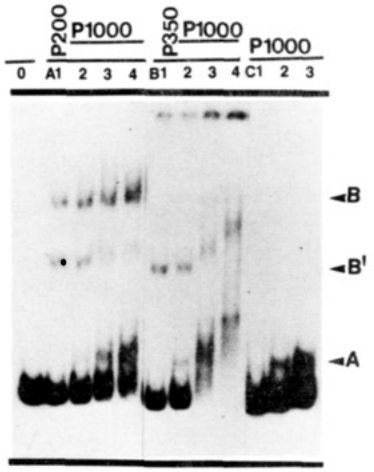

FIGURE 9: Reconstitution of HiNF-B activity by combination of the P350–1000 fraction with the P0–200 fraction (lanes, A2–A4) but not with the P200–350 fraction (lanes B2–B4). Lane A1, 1.5 μg of P0–200 protein alone; lane B1, 1.8 μg of P200–350 protein alone; lanes C1–C3, 0.6, 1.5, and 2.4 μg of P350–1000 protein alone, respectively. Samples of lanes A2–A4 (containing 1.5 μg of P0–200 protein) and lanes B2–B4 (containing 1.8 μg of P200–350 protein) are mixed in each case with the same increasing amounts of P350–1000 protein as present in lanes C1–C3.

complex B in the mixture of these fractions, but we did not observe full conversion of complex B′ to complex B in any of the combinations tested. Therefore, reconstitution experiments were carried out with combinations of the P350–1000 fraction with either the P200–350 or the P0–200 fraction (Figure 9) in order to test the possibility that an additional conversion factor would be present in the P350–1000 fraction. These data show that the addition of increasing amounts of P350–1000 protein (lanes C1–C3) to a fixed amount of P0–200 protein (lane A1) results in an increase in the formation of complex B, while a decrease in the formation of complex B′ occurs (lanes A2–A4). This suggests that P350–1000 contains a factor involved in the conversion of complex B′ to complex B.

However, when the same amounts of P350–1000 protein were added to the P200–350 fraction, no conversion of complex B′ to complex B occurred (lanes B1–B4). Apparently, the mixture of component B1 present in complex B′ (eluting in both the P0–200 and P200–350 fractions) and the putative conversion factor present in the P350–1000 fraction are not sufficient for the formation of complex B. This observation indicates that the conversion factor present in the P350–1000 fraction (designated B3) is different from the conversion factor designated B2 (eluting predominantly in the P0–200 fraction), both in elution behavior and in the ability to convert complex B′ to complex B. Considering that both B2 and B3 are required for conversion of complex B′ to complex B, this implies that the formation of complex B using the P0–200 fraction involves component B3. The heterogeneous elution behavior of component B3 (eluting both in the P0–200 and in the P350–1000 fraction) could suggest that this factor is associated with either component B1 or component B2 and dissociates during chromatography. Because component B3 affects the conversion of complex B′ to complex B and does not increase formation of complex B′, we suggest that this factor could be involved in promoting the association of component B2 with B1.

CONCLUSIONS

In this work, we have presented evidence that a factor designated HiNF-B, which binds specifically to a CCAAT box element in the promoter region of a cell cycle dependent human H1 histone gene, is a mosaic protein consisting of at least two nonidentical components (B1 and B2), at least one of which has a demonstrated DNA binding activity. In addition, the presence of the second and a third component (B3) with different chromatographic behaviors is inferred from various reconstitution experiments. We have shown that formation of HiNF-B depends on cooperative interaction of its components.

Mouse C127 cells appear to contain a HiNF-B homologue that binds the same DNA segment as human HiNF-B, as evidenced by stairway assays. This indicates that both the interaction between HiNF-B subunits and the interaction of the HiNF-B complex with DNA are conserved in both species. This is in agreement with the notion that trans-acting factors in either mouse Ltk$^-$ or C127 cells are capable of mediating transcriptional control of human histone gene expression in vivo (Kroeger et al., 1987). HiNF-B DNA binding activity can be detected both during and outside of S phase, suggesting that the factor at least may be involved in the constitutive synthesis of histone mRNA that occurs throughout the cell cycle in proliferating cells.

We have discussed the difference between HiNF-B and other CCAAT box binding proteins before (van Wijnen et al., 1988). While this paper was in preparation, yet two other CCAAT box binding proteins have been reported. The similarity between HiNF-B and one of these, CRF (Goding et al., 1987), is presently unclear. Interestingly, a large, ubiquitous metalloprotein (NF-Y) that binds to a CCAAT box in the promoter of a major histocompatibility complex class II gene (E-α) has been isolated (Hooft van Huijsduijnen et al., 1987). These workers have obtained indications that NF-Y (M_r 250K–300K) may be composed of multiple peptides (M_r 50K–70K). Moreover, Cohen et al. (1986) observed an increased mobility of the complex mediated by a mouse α-globin-specific CCAAT box binding protein (64–68 kDa) upon chromatography, hinting at a dissociation event during purification. Currently, we are attempting to purify HiNF-B using DNA affinity chromatography in order to evaluate its polypeptide composition and to assess the similarities of its subunits with these and other CCAAT box binding proteins.

In summary, the CCAAT box is a promoter element that is present in the 5′ flanking region of both tissue-specific and cell growth related genes and usually flanked by several other promoter elements that act as DNA/protein interaction sites. It is becoming increasingly clear that this element is not recognized by a single protein, but rather by a class of proteins whose interrelationship is unclear. The finding that the human histone promoter CCAAT box binding protein HiNF-B is a mosaic factor adds further complexity to the CCAAT element and the heterogeneous, modular structure of eukaryotic RNA polymerase II promoters. The intriguing possibility that the selective function of a CCAAT box may be determined by a subunit of CCAAT box binding proteins warrants further investigation.

ADDED IN PROOF

The existence of multisubunit CCAAT box binding proteins is supported by recent findings by Chodosh et al. (1988) and Hatamochi et al. (1988).

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