Identification of a 68 kDa protein species as a specific DNA-binding component of the H3abp complex interacting with the histone H3.2 G1/S regulatory domain

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ABSTRACT

The hamster histone H3.2 promoter contains a protein binding site (referred to as site X) required for G1/S transcriptional activation. We report here that nuclear extracts prepared from serum synchronized cells at various stages of the cell cycle show a biphasic increase in the H3.2 specific complex, H3abp, binding to site X. An increase in binding activity occurs as cells first enter the cell cycle and later at the G1/S border. The H3.2 specific binding activity is enhanced by Mg^{2+} and Ca^{2+} *in vitro*, but is inhibited by Zn^{2+} . Site X resembles a Jun/AP-1 site, but previously it has been shown that the H3abp complex is immunologically distinct from the characterized AP-1 proteins. Here, we identify the size of the hamster nuclear protein(s) that bind specifically to the H3abp site by ultra-violet crosslinking and renaturation of specific protein bands following gel electrophoresis. In addition, we purify H3abp by affinity chromatography and show that the purified H3abp has a different methylation interference profile from AP-1. Our results indicate that a protein species around 68 kDa is the major DNA binding component of the H3abp complex and it binds specifically to the histone promoter site required for G1/S regulation.

INTRODUCTION

The molecular events preceding DNA synthesis occur in a highly coordinated fashion. Early in G1, cell surface signals which induce proliferation are transduced to the nucleus. A cascade of genes becomes activated which stimulates the cell to proceed through G1 in preparation for DNA synthesis. The amplified signals that reach the nucleus can affect the regulation of numerous genes and may be the result of signals converging from multiple pathways. The control of these events is an absolute requirement for establishing regulated proliferation. To better understand the mechanisms through which cell surface signals regulate these genes, analysis of their transcriptional regulation at

the onset of DNA synthesis is being studied. Work on the promoters of S-phase regulated genes has identified protein-binding elements involved in directing the G1/S phase increase in transcription and currently the factors interacting at these sites are being identified and characterized (1-5).

Within the promoters of the coordinately regulated replication dependent histone genes the individual histone subtypes have conserved regulatory elements between species, however there is no obvious consensus sequence in the genes attributed to their coordinate transcriptional induction at S phase (6-9). The histone H2B subtype specific promoter element ATTTGCAT and the H1 subtype specific AC and CCAAT sequences are a few examples of unique cell cycle regulatory elements associated with the individual histones (7). The study of histone gene transcription through the cell cycle provides a system in which the mechanism of action for multiple proliferation signaling events on their respective targets can be described.

We have previously characterized a site within a footprinted region of the promoter of the replication-dependent hamster H3.2 histone gene. Through mutational analysis, it has been established that a 10 base pair (bp) region within this footprinted region is required for the S-phase increase in transcription of this gene (10-12). The 10 bp protein binding site (site X) contains an element (5'-CGAGTCA-3') from -238 to -231 that resembles, with the exception of the underlined bases, the consensus AP-1 site, TGA^G/CTCA, as well as the cyclic AMP response element, TGACGTCA. As a 19 bp oligonucleotide (Table 1, X monomer) this site mimicked a weak AP-1 site. However, the binding characteristics are notably different when site X is flanked by 10 bp on either side with H3.2 promoter sequence (XY oligomer). The H3.2 binding activity, H3abp, yields a distinct complex from that which binds to an AP-1 binding site. The H3abp complex requires an intact site X but cannot be competed by site X or a consensus AP-1 site in the presence of the required promoter flanking sequence (12). Furthermore, monospecific antibodies against the characterized AP-1 proteins are not effective at inhibiting the formation of the H3abp complex.

To characterize the composition of H3abp and to understand further how H3abp functions to control the G1/S transcriptional increase of the H3.2 gene we conducted the following studies to:

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Table 1.

Oligomer	Sequence*	Size (nt)	Used as:
X monomer	tcaTGG CGAGT CAGCC ACCG CTCAGT CGGagt	19	Competitor in EMSAs
XY	ggcaagcttGCAGAACCTTGG CGAGT CAGCCAGCCGCGGGGCTGGACAA aCGTCTTGGAAACCG CTCAGT CGGTTCGGCGCCCCGACCTGTT	49	Probe and competitor in EMSAs
X ^m Y	CCCTCGCAGAACCTTttaaagcttctCCAGCCGCGGGGCTG GGGAGCGTCTTGGAAaattcgaagaGGTTCGGCGCCCCGAC	40	Competitor in EMSAs
H3abp core oligomer	ctagaGCAGAACCTTGG CGAGT CAGCCAGCCGCGGGGCTGGACAA tCGTCTTGGAAACCG CTCAGT CGGTTCGGCGCCCCGACCTGTTgatc	45	Plasmid 3X[XY]CAT construction Affinity column construction Methylation interference analysis
Collagenase AP-1 site	agcttAAAGCAT TAGT CAGACAGg aTTTCGT ACTCAGT CTGTGcatcg	29	Probe and competitor in EMSAs
grp78 core	ctcgaGCCGCTTCGAATCGGCAGCGGCCAGCTTGGTGGCAT CGGCGAAGCTTAGCCGTCGCCGGTTCGAACCACCGTAcagctg	41	Probe in EMSAs

* Lower case letters indicate polylinker DNA and bold indicates sequence of AP-1 or AP-1-like sites. In the mutated X^mY oligomer the italic letters indicate the mutated bases substituted in the original location of the AP-1-like site.

(i) characterize the binding properties of H3abp following serum stimulation, (ii) identify the sizes of the hamster nuclear proteins that bind specifically to the H3abp binding site by ultra-violet crosslinking and renaturation of specific protein bands following gel electrophoresis, (iii) purify H3abp by affinity chromatography, and (iv) compare the contact sites of H3abp and AP-1. These combined results establish that the major DNA binding species of H3abp is a protein species of about 68 kDa distinct from characterized AP-1 family members and that it binds specifically to the histone promoter site required for G1/S regulation.

MATERIALS AND METHODS

Cell culture

Conditions for culturing and synchronization of the Chinese hamster K12 cell line have been described (12,13). Cells were maintained in DMEM with 10% cadet calf serum and 1% antibiotics (PSN: 50 µg/ml of penicillin and streptomycin, 100 µg/ml of neomycin). To synchronize cells, subconfluent cultures were washed with phosphate buffered saline (PBS) and grown for 24–48 h in DMEM with 0.1% serum and 1% antibiotics to allow cells to growth arrest at the G₀/G₁ phase of the cell cycle. Cells were released from arrest by addition of fresh medium containing 10% serum and harvested at various points in the cell cycle. Progression of synchronized populations was monitored as a function of DNA synthesis as determined by [³H]thymidine incorporation (10).

Nuclear extracts

For cell cycle binding analysis simplified nuclear extracts (SNEs) were prepared as described in (12) with the addition of 1 mM PMSF. Extracts were prepared from two confluent (15 cm

diameter) cell culture dishes (~3 × 10⁷ cells/dish) for each time point following addition of fresh serum to synchronized cells. Nuclear extracts (NE) for ultra-violet crosslinking, methylation interference and affinity purification were prepared as described by Shapiro *et al.* (14) from ~3 × 10⁹ cells with a final recovery of ~70 mg. The final extracts contain the following protease inhibitors: 1 µg/ml each of pepstatin, leupeptin and aprotinin, with 0.5 mM benzamidine and 1 mM PMSF. All protein concentrations were determined with the Bio-Rad micro protein assay.

Electrophoretic mobility shift assays (EMSAs)

Analytic electrophoretic mobility shift assays were performed essentially as described in (12). Briefly, 0.5–1 µg of SNE or NE were used with 1 ng of probe and 100 ng of poly dI-dC as a non-specific competitor. The sequences and usage of the oligonucleotides are described in Table 1. Where indicated, varying molar excesses of unlabeled homologous or heterologous competitor DNAs, or divalent metal cations were included in the binding reaction. All probes were labeled by filling in the 5' overhang ends with the Klenow DNA polymerase fragment using [α-³²P]dATP, [α-³²P]dCTP (50 µCi each at 3000 Ci/mmol), dTTP and dGTP. Labeled oligomers were gel purified on 12% nondenaturing polyacrylamide gels in 1 × TBE (89 mM Tris-HCl, pH 7.5, 89 mM boric acid and 0.2 mM EDTA). All binding reactions were conducted at room temperature for 15 min in binding buffer containing 10 mM Tris-Cl, pH 7.5, 50 mM KCl, 1 mM DTT, 0.05% NP-40, 2 µg of bovine serum albumin (BSA). Complexes were resolved on 6% nondenaturing polyacrylamide gels (39:1 acrylamide:bisacrylamide) electrophoresed in 1 × TBE at 150 V for 90–120 min at room temperature. The gels were dried and subjected to autoradiography.

Methylation interference

Methylation interference analyses were performed as described (15). The coding or non-coding strand of the H3abp core oligomers were separately end-labeled with [γ - 32 P]ATP and T4 polynucleotide kinase. The labeled strand was reannealed with the corresponding unlabeled complementary oligonucleotide and purified on a 12% nondenaturing polyacrylamide gel in 1 $\times$ TBE. Radiolabeled oligomers were excised from the gel and electroeluted in 1 $\times$ TBE. Approximately 10^7 c.p.m. of the labeled oligomers were methylated in 200 μ l of 50 mM Na Cacodylate, pH 8.0, 1 mM EDTA with 1 μ l of dimethyl sulfate for 5 min. The reaction was stopped with 50 μ l of stop buffer (1 M β -mercaptoethanol, 1.5 M sodium acetate) and ethanol precipitated. A scaled up EMSA was performed with 10^6 c.p.m. of the methylated probe, and either 1 μ l AP-1 (Promega, Madison, WI) with 500 ng of poly dI-dC or 15 μ l of affinity purified H3abp in a 60 μ l binding reaction as described above. The wet gel was exposed to X-ray film for 2 h at room temperature. The resulting complexes and remaining free probe were excised from the gel, electroeluted in 0.5 $\times$ TBE, passed through a 0.2 μ m Z-Spin filter (Gelman Sciences, Ann Arbor, MI) and ethanol precipitated. The isolated complexes and free probe were treated with 100 μ l of 10% piperidine for 30 min at 90°C. Samples were diluted with 100 μ l distilled H₂O and lyophilized. The H₂O wash and lyophilization was repeated two additional times. An equivalent number of c.p.m. of piperidine digested DNA samples were analyzed on 12% acrylamide, 8 M urea sequencing gels. The gels were dried and subjected to autoradiography.

Ultra-violet crosslinking

A bromo-deoxyuridine (BrdU) substituted probe for a preparative ultra-violet crosslinking EMSA was generated using a 16mer oligonucleotide (TTGTCCAGCCCCGCGG) reannealed to the coding strand of the XY oligonucleotide pair and extended with the Klenow DNA polymerase fragment using [α - 32 P]dCTP, BrdU, dATP and dGTP at 37°C for 1 h. The labeled DNA was purified on a 12% nondenaturing polyacrylamide gel in 1 $\times$ TBE followed by electroelution in 1 $\times$ TBE. An 8-fold scale-up EMSA was performed with $\sim 4 \times 10^5$ c.p.m. (8 ng) of BrdU substituted probe, 8 μ g nuclear extract, 800 ng poly dI-dC in an 80 μ l reaction as described above. Where indicated, 100-fold molar excess of cold competitors were included in the binding reaction. The 0.4 M affinity fraction (8 μ l) was crosslinked in the absence of poly dI-dC. Complexes were resolved on 6% nondenaturing polyacrylamide gels in 1 $\times$ TBE run at room temperature for 4 h to remove uncomplexed probe. The wet gel was crosslinked with 500 mJ in a Stratagelinker (Stratagene, La Jolla, CA), exposed to X-ray film at room temperature for 1.5 h and the resulting complexes were excised from the gel and electroeluted in 1 $\times$ TBE. The isolated complexes were precipitated with 20 μ g of insulin as a carrier and 5 vol of ice-cold acetone. After 30 min at -70°C the precipitates were centrifuged for 20 min at 4°C in an Eppendorf microfuge. The pellets were subsequently washed in 95% EtOH, centrifuged for 10 min and dried. Pellets were resuspended in 40 μ l of 2 $\times$ SDS-sample buffer (625 mM Tris, pH 6.8, 10% glycerol, 2% SDS, 710 mM β -mercaptoethanol), boiled for 5 min and assayed on a denaturing 10% Laemmli (16) SDS-polyacrylamide gels. The gels were dried and subjected to autoradiography. The ultra-violet crosslinking analysis was

performed with and without BSA in the binding reaction using unfractionated NE without any apparent effect on the protein species crosslinked.

Binding reconstitution

All steps were conducted at 4°C. Sixty μ g of K12 nuclear extract were size separated on a 10% polyacrylamide gel with (20 μ g/lane) in 1 $\times$ Tris-glycine-SDS buffer (25 mM Tris-HCl, pH 8.3, 190 mM glycine, 0.1% SDS), with the samples applied onto the gel in 2 $\times$ SDS-sample buffer without boiling. The gel was cut into 6 mm slices, placed in a microfuge tube with 200 μ l of renaturation buffer (50 mM Tris, pH 7.9, 100 μ g/ml bovine serum albumin, 100 mM KCl, 5 mM DTT, 0.1 mM EDTA, 0.1% NP-40, 20% glycerol), and crushed with a conical teflon pestle. The crushed acrylamide slurry was passed through a 0.2 μ m Z-spin microfuge filter. The slurry was rinsed with an additional 200 μ l of renaturation buffer. Approximately 200 μ l of filtrate was recovered and precipitated for 30 min at -70°C with 5 vol ice cold ethanol and 20 μ g bovine serum albumin as a carrier. The precipitate was centrifuged for 20 min at 4°C and the resulting pellet was washed with 95% EtOH and centrifuged for an additional 10 min. The supernatant was decanted and the pellet was briefly dried under vacuum. The protein pellets were resuspended in 30 μ l of 1 $\times$ EMSA binding buffer with 15% glycerol and 1-3 μ l were used in an EMSA binding reaction as described above without poly dI-dC.

Preparation of DNA-Sephrose columns

Calf-thymus DNA-Sephrose. Ten mg of calf thymus DNA (Sigma, St Louis, MO) were sonicated and coupled to 10 ml of CNBr-activated Sepharose 4B (Pharmacia, Piscataway, NJ) as described by the manufacturer and Kadonaga (17). Coupling efficiency was monitored by following the OD₂₆₀ of the calf-thymus DNA solution before and after the coupling reaction. Based on DNA recovered after washing the coupled resin, there was an 80% coupling efficiency yielding ~ 1 mg of sonicated calf-thymus DNA/ml hydrated Sepharose 4B.

H3abp core affinity sepharose. Two mg of reannealed H3abp core oligonucleotide were kinased and ligated into multimeric molecules. Ligated oligomers were coupled to ~ 4.5 ml of CNBr-activated Sepharose 4B as described above. Coupling efficiency was monitored by following the recovery of a tracer of H3abp oligonucleotide labeled with kinase and [γ - 32 P]ATP. Coupling efficiency of the multimeric H3abp core oligo was $\sim 73\%$, yielding ~ 500 μ g DNA/ml of resin.

Affinity purification. All fractionations were performed at 4°C using nuclear resuspension buffer (NRB, 20mM HEPES, pH 7.9, 0.2 mM EDTA, 0.2 mM EGTA, 20% glycerol and 2 mM DTT) with varying concentrations of KCl.

Bio-Rex70. Seventy mg of K12 nuclear extract in NRB with 0.1 M KCl (0.1 M NRB) was applied to a 10 ml bed volume Bio-Rex70 column pre-equilibrated with 0.1 M NRB. Following the elution of void volume (7.5 ml), the flow through protein (FT) was re-applied and washed with another void volume. The column was then washed with 15 ml of 0.2 M NRB. Proteins were eluted with a step gradient using 10 ml for each salt concentration: 0.4, 0.5, 0.6, 0.8 and 1.0 M NRB. One ml fractions were collected

for each step of the gradient following elution of the FT. Fractions containing XY binding activity were identified by EMSAs using 1 ng XY probe and 100 ng of poly dI-dC.

DNA-sepharose column. Positive fractions (14 mg) from Bio-Rex70 were pooled and dialyzed against 40 vol of 0.1 M NRB. Dialyzed protein (20 ml) was applied to a 8 ml bed volume (7.5 ml void volume) calf-thymus DNA-Sepharose column pre-equilibrated with 0.1 M NRB. Following complete flow through of the protein, the 20 ml was re-applied to the column. FT protein was collected as a 20 ml fraction and the column was subsequently washed with 16 ml of 0.1 M NRB. Two 8 ml fractions were collected as the wash. The protein was eluted with a step gradient as described for Bio-Rex70 using 8 ml of each salt concentration and collecting 1 ml fractions. Positive fractions were again assayed by EMSA, pooled, and dialyzed against 0.1 M NRB.

H3abp core affinity column. The dialyzed DNA-Sepharose fraction (3.3 mg in 10 ml) was mixed with 40 ng of poly dI-dC/ μ g of protein and applied to a 3 ml H3abp core affinity column (void volume $\sim$ 2.7 ml). The 10 ml loaded was collected as FT and the column was washed with 3 ml 0.1 M NRB followed by 9 ml of 0.2 M NRB. The protein was eluted with a step gradient as described above with 3 ml for each salt concentration and collecting 1 ml fractions. Positive fractions were assayed as above. Positive fractions (7 ml) were pooled and dialyzed as before and re-applied to the same H3abp core affinity column without poly dI-dC and eluted as above. Positive fractions from the second affinity elution were pooled and diluted to 0.25 M KCl (3.6 ml) and applied to a 1 ml H3abp core affinity column. The FT was collected and the column was washed with 2 ml of 0.25 M NRB followed by 3 ml of 0.3 M NRB to remove proteins which co-eluted with the H3abp activity that eluted at 0.4 M KCl. The H3abp activity was eluted with 1 ml of 0.4, 0.5 and 0.6 M NRB. 500 μ l fractions were collected and assayed by EMSA without poly dI-dC. The protein profile of the DNA affinity purified fractions was analyzed by silver staining (Bio-Rad) SDS-polyacrylamide gels containing eluted fractions.

RESULTS

Temporal regulation of H3abp binding following serum stimulation

Using the H3abp binding site as probe, the binding profile of H3abp was examined with synchronized K12 nuclear extract following release of the starved cells with fresh serum. Nuclear extracts were prepared at 2–4 h intervals after serum stimulation and the progression of the synchronized cells was concomitantly monitored by incorporation of 3 H-thymidine. The results, as shown in Figure 1A, demonstrate that the H3abp complex was composed of a cluster of multiple bands [I', I and II]. Two distinct peaks of H3abp binding activity were detected. The first peak occurred at the 2 h time point, immediately after the serum release. The second peak occurred at 12 h which coincided with the onset of the DNA synthetic phase (Fig. 1B). Quantitation of the two peak areas in multiple experiments showed that the magnitude of the two peaks was similar, although in some preparations the first peak was slightly more predominant. The temporal fluctuations of the H3abp binding activity were specific

Figure 1. Protein binding profile of the H3abp complex in serum synchronized cells. (A) Gel mobility shift assays performed with nuclear extracts prepared from serum synchronized cells and the H3abp wild type probe (XY) (top panel) or the grp78 core as control probe (bottom panel). (B) Synchrony of the cell cultures used for nuclear protein extraction as monitored by 3 H-thymidine incorporation. H3abp relative binding activity as measured by densitometry of the autoradiogram is represented by the open triangles.

to the H3 binding site and were not due to intrinsic differences among the individual extracts since the binding profile to another probe derived from the promoter of a 78 kDa glucose regulated gene (grp78 core) was relatively constant throughout the cell cycle (Fig. 1A).

The specificity of the complexes in the 2 and 12 h extracts was assayed by subjecting the extracts to competition in an EMSA. The results are shown in Figure 2 and demonstrate that the H3abp complex [I', I and II] is specifically competed by the histone promoter sequence but not by a similar oligomer with a mutated site X. The collagenase AP-1 oligomer was able to compete complex I' but not I and II. We note that complexes I and II in early G1 have the same specificity at the G1/S border demonstrating that the basic composition of the bound H3abp is similar for each time point. Interestingly, the I' complex is prominent in the G1-phase (2 h) extract but is less evident in the S phase (12 h)

Figure 2. Binding properties of H3abp. (A) Competition analysis of H3abp in 2 (G1) and 12 (S) h extracts from serum synchronized cells. EMSA using 250 ng of SNE from the indicated time points complexed with the radiolabeled XY probe in the presence of 100 ng poly dI-dC. The complex was competed with 50- and 100-fold molar excess of the indicated competitors, the sequences of which are shown in Table 1. Complexes I', I and II are indicated. (B) Effect of Ca^{2+} and Zn^{2+} on H3abp binding. The EMSA was performed by incubating 250 ng of nuclear extract (NE) with 1 ng of probe, 100 ng poly dI-dC and increasing amounts (μM) of Mg^{2+} and Zn^{2+} in the binding buffer at room temperature for 15 min. For the lane labeled 10⁺, the NE was preincubated with poly dI-dC and 10 mM Mg^{2+} in the binding buffer for 20 min at 37°C prior to the addition of the probe. The reaction mixture was further incubated for 15 min at room temperature. The positions of complexes I and II are indicated. (C) EMSA using 750 ng of SNE from the indicated time points with 100 ng poly dI-dC and 1 ng of XY probe in binding buffer alone (0) or buffer containing either 10 mM Mg^{2+} , 5 mM Ca^{2+} or 2 mM Zn^{2+} as indicated. The reaction mixtures were incubated for 15 min at room temperature. The positions of complexes I', I and II are indicated.

70extract. The I' binding activity tapers off in mid-G1 and does not increase at the onset of S-phase as observed with complexes I and II (Fig. 1A).

In determining the parameters which would affect the H3abp binding activity, we discovered that H3abp binding to the H3 site is sensitive to divalent cations. Previously, it has been reported that Mg^{2+} and Ca^{2+} stimulated mammalian cells to progress through G1/S transition whereas Zn^{2+} did not (18–20). Using nuclear extracts from exponentially growing cells which formed predominantly complexes I and II, the binding activity was enhanced with increasing amounts of Mg^{2+} but inhibited by Zn^{2+} (Fig. 2B). At 10 mM Mg^{2+} , there was a 10-fold increase in H3abp binding activity. We next determined whether the synchronized nuclear extracts behaved the same and whether Ca^{2+} could exert similar effects. The results, as shown in Figure 2C, revealed that 10 mM Mg^{2+} and 5 mM Ca^{2+} stimulated H3abp binding by 2- and 3-fold respectively, while 2 mM Zn^{2+} inhibited the binding by 5-

to 10-fold. Preincubation of the cell extracts with Mg^{2+} or ATP at 37°C showed no additive effect (Fig. 2B and data not shown). Interestingly, when the G1 phase extracts (2 and 6 h) were treated with Mg^{2+} or Ca^{2+} , complex I' with affinity for AP-1 was diminished whereas the H3 specific complexes I and II were much enhanced. For the S phase extract (12 h), the major complexes I and II increased in binding activity. Thus, by enhancing complexes I and II binding, selective cations can convert the G1 binding pattern to one characteristic of S phase.

Identification of the sizes of H3abp components by ultra-violet crosslinking

Initially we performed ultra-violet (UV) crosslinking experiments with unfractionated nuclear extracts to get a preliminary indication of the molecular weights for the DNA binding species which compose H3abp. Figure 3A shows the results of a typical UV crosslinking experiment. When conducting UV crosslink experiments, extended run times (≥ 4 h) of the EMSA gels revealed that the H3abp complexes I' and I could be resolved from complex II (Fig. 3B). When complexes I' and I were crosslinked to the BrdU substituted XY probe and analyzed on an SDS-PAGE (Fig. 3A, panel [I + I']) two major species, around 45 and 68 kDa in size, were observed. Interestingly, when the oligonucleotide mutated at the AP-1-like site (X^{mY}) was used as competitor there was a reproducible increase in intensity of the 45 kDa protein. When the collagenase AP-1 oligonucleotide was used as the competitor, the 45 kDa protein was eliminated and the 68 kDa protein was not efficiently competed. The wild type competitor eliminated crosslinking of both proteins. These results indicate that the 45 kDa protein has a high affinity for the consensus AP-1 site and correlates with the general size of the known AP-1 family members. Furthermore, when the 68 kDa protein was competed by the X^{mY} probe containing the intact flanking sequence it allowed for an increased access to the relatively weak H3abp site for the 45 kDa protein, resulting in an increase in the amount of protein crosslinked. For negative control, gel slices from preparative gel mobility shift assays devoid of complexes were excised after UV cross-linking and analyzed for labeled protein. No labeled protein bands were detected (data not shown).

In a standard analytical EMSA run for 4 h (Fig. 3B) it appears that the 45 kDa protein may attributed to the complex designated I'. Relative to the wild type competitor, the X^{mY} probe did not compete the I' complex to the same extent, whereas the collagenase AP-1 probe was very effective at eliminating its formation, even at concentrations as low as 25-fold molar excess. The predominant protein crosslinked from complex II is the 68 kDa protein (Fig. 3A, panel II) and the competition analysis correlates with what was observed in the analytic EMSA. The increase in binding activity observed, particularly in complex II, following competition with the collagenase AP-1 site in the 'extended' EMSA (Fig. 3B) was consistently observed in multiple independent experiments. One explanation is that when the AP-1 like complexes were effectively removed by the presence of excess consensus AP-1 binding sites more H3abp could bind resulting in an apparent increased binding activity. This interpretation suggests that the I' complex is generated by the 45 kDa protein(s) and is related to the AP-1 family with respect to binding specificity.

Figure 3. UV crosslink analysis of H3abp. (A) SDS-PAGE analysis of ultra-violet crosslinked complexes from crude nuclear extract and affinity purified H3abp bound to BrdU substituted wild type oligomer. Left panel, UV crosslinked complex I and I'. Right panel, UV crosslinked complex II. Lanes 1-4 in each panel are from crude nuclear extract and 100-fold molar excess of indicated competitors (lanes 2-4). Lane 5 is from affinity purified H3abp binding activity. (B) Competition analysis of complexes I', I and II with increasing molar excess of the indicated competitors (10-, 25-, 50- and 100-fold molar excess).

Detection of H3 specific binding activity from renatured proteins of ~68 kDa

The significance of a protein species around 68 kDa as a major binding component of the H3abp complex was confirmed by reconstitution of specific binding of nuclear proteins separated on 10% SDS-PAGE gels. Gel slices containing protein ranging in size from 200 to 14 kDa (slice 1 through 8) were assayed for binding to the H3abp site (Fig. 4A). Only proteins excised from slices 3 and 5 showed binding activity. Slice 3 co-migrated with the 68 kDa size marker and contained proteins ranging from 90 to 60 kDa. Slice 5 co-migrated with the 45 kDa size marker and contained proteins ranging from 45 to 35 kDa.

Binding activity associated with proteins from slice 5 showed one complex migrated slightly slower than the predominant complex I and was similar to the position of the complex I' previously observed. Another complex with a much faster electrophoretic mobility was also observed. When the specificities of these complexes were assayed by competition, the slower migrating complex was much more efficiently competed by the collagenase AP-1 site than the wild type XY or X^mY oligomers whereas the fast migrating complex was competed efficiently by

Figure 4. Reconstitution of H3 binding activity from nuclear proteins. (A) K12 nuclear extract was size separated on a 10% polyacrylamide gel and protein slices corresponding to the following sizes were excised. 1:200 to 160 kDa; 2:160 to 90 kDa; 3:90 to 60 kDa; 4:60 to 45 kDa; 5:45 to 35 kDa; 6:35 to 28 kDa; 7:28 to 20 kDa and 8:21 to 14 kDa. The proteins were extracted and subjected to acetone precipitation. The protein pellet was resuspended in 50 µl of binding buffer with 15% glycerol and 2 µl was assayed for binding activity by EMSA in parallel with a reaction using the unfractionated nuclear extract (NE). The positions of complexes I', I and II and the free probe (F) are indicated for the unfractionated NE. The protein slices 3 and 5 which showed reconstituted binding activity were indicated by (*). (B) Reconstituted complex from slice 3 was assayed for specificity by competition with 5-, 10- and 20-fold molar excess of the indicated competitors. In lane 0, no competitor was added. (C) Reconstituted complex from slice 5 was assayed for specificity by competition with 10-fold molar excess of the indicated competitors.

all three oligomers (Fig. 4C). These results suggest that the slower migrating complex reconstituted from slice 5 consisted primarily of members of AP-1 family with minimal specificity for the XY site and the slower migrating complex consisted of non-specific DNA binding protein(s).

Proteins from slice 3 in the 90-60 kDa range bound to the XY probe and generated a shifted band that resembles complex II from crude extract (Fig. 4A). Although the reconstituted band migrated with a slightly faster electrophoretic mobility, its binding specificity was analogous to that seen with crude nuclear extract such that it was competed by the homologous XY oligomer and not by the X^mY or AP-1 oligomers (Fig. 4B). Presumably, upon extraction from polyacrylamide gels, co-factor(s) to the proteins necessary to generate complexes I and II were missing. We further noted that the electrophoretic mobility of this reconstituted band was dependent on the amount of protein recovered from the gel. The more dilute preparations formed a

Figure 5. (A) Schematic diagram for affinity purification of H3abp binding activity. The fractions enriched for the binding activity are boxed. (B–D) Comparative gel mobility shift assay with affinity purified fraction containing H3abp activity. The collagenase AP-1 or the XY oligomers described in Figure 1 were used as probes with 1 μ l of the H3abp affinity fraction. The relative binding activity with decreasing amounts of poly dI-dC (100–0 ng) was compared with 250 ng of K12 nuclear extract containing 100 ng dI-dC. (B) Binding activity of the H3abp fraction to the Collagenase AP-1 probe. (C) Binding activity to the XY probe using the same fraction. A new H3.2 specific complex, enriched for by affinity chromatography, is indicated by ($\blacktriangle$). (D) Competition analysis of affinity purified H3abp activity. Using 1 μ l of the affinity fraction and the radiolabeled XY oligomer, the complex was competed with 5-, 10- and 50-fold molar excess of the indicated unlabeled competitors.

complex which migrated even faster and could presumably be a monomeric form of the 68 kDa protein binding to the XY probe (data not shown).

Chromatographic purification of H3abp

To further characterize the role of the 68 kDa protein in the H3abp complex we purified the protein from K12 nuclear extract using the scheme outlined in Figure 5A. Nuclear lysate was subjected to precipitation with 33% ammonium sulfate and the pellet was resuspended in nuclear resuspension buffer (0.1 M KCl–NRB) and used as the starting material in the purification. Seventy mg of ammonium sulfate fractionated nuclear extract was applied to a weak-cation exchange column, Bio-Rex 70, and eluted with a step gradient of increasing KCl molarity. Positive fractions were pooled, dialyzed to 0.1 M KCl–NRB, and applied to a calf-thymus DNA–Sepharose column to remove proteins which bind DNA non-specifically and with low affinity. Proteins were eluted with a step gradient and positive fractions were pooled. After dialysis, these proteins were mixed with poly dI-dC (40 ng/ μ g of protein) and applied to an affinity column containing concatamers of the H3abp binding site coupled to Sepharose. Proteins were eluted with a similar step gradient. Fractions were analyzed by EMSA and silver staining of SDS–polyacrylamide gels, pooled, dialyzed and re-applied to the same affinity column. Positive fractions from the second affinity purification step were loaded on to a third affinity column. This column had a bed volume of 1 ml and the proteins were loaded with 0.25 M KCl–NRB. The last step allowed the H3abp to elute in a more

concentrated form and be separated from other proteins which began eluting at 0.25 M KCl.

As a confirmation that we were enriching for a protein specific for the H3abp site we did a comparative EMSA with the affinity purified fractions using the radiolabeled collagenase AP-1 probe and the wild type XY probe (Fig. 5B and C). The affinity purified fraction demonstrated a significant enrichment in H3abp binding activity compared to the enrichment of the AP-1 binding activity in the wide range of poly dI-dC concentrations used as a non-specific competitor. A quantitative comparison of the number of c.p.m. complexed with 250 ng of unfractionated nuclear extract to that of $\sim$ 1 ng of the affinity purified fraction shows that there is an $\sim$ 4000-fold purification of H3abp binding activity. Competition analysis of the affinity purified fraction (Fig. 5D) shows that the enriched protein retained its specificity for the XY probe. We note that after DNA affinity purification the I' complex attributed to AP-1 was not evident in the H3abp complex. A new H3 specific band with faster mobility than complex I and II was also observed in the DNA affinity fraction. To ascertain which protein(s) were enriched for during affinity purification we performed a UV crosslink analysis with the affinity purified fraction. Figure 3A shows for both complexes I and II a prominent 68 kDa band that was crosslinked to the BrdU XY probe. The UV crosslink data indicates that the 68 kDa protein is the major DNA binding species occupying the H3abp site on the XY probe and supports the idea that the 45 kDa crosslinked species is generated from AP-1 proteins binding to the XY probe which were purified away from the H3abp following affinity purification.

Comparison of the H3abp and AP-1 contact points in the H3abp binding site

To characterize further the relationship between H3abp and AP-1 binding to the H3abp site, we conducted methylation interference analysis (Fig. 6). Using purified AP-1 and both the upper and lower strands of the H3abp core oligomers as probes, we observed that the AP-1 contact sites on the upper and lower strands are symmetrical and cluster around the AP-1 like site (Fig. 6A). Two hypersensitive bases were consistently observed in the lower strand. Furthermore, two protected G residues within the boxed AP-1-like site are similar to those described in previous methylation protection and interference assays for the AP-1 site (21,22). The results we obtained with the affinity purified H3abp complex are distinct from the AP-1 complex and show predominant protection of only the lower strand (Fig. 6B). The protected residues are inside the AP-1 like site but extend beyond the AP-1 consensus region (boxed) into the two sets of guanine doublets in the flanking palindromic residues. Further, H3abp binding does not generate the hypersensitive sites associated with AP-1 binding. The methylation interference data are summarized in Figure 6C. We note that the H3abp site has a six base palindromic sequence (TGGCGAGTCAGCCA) as compared to the three base sequence in an AP-1 consensus site (TGAGTCA). Interestingly, the protected bases in the H3abp site appear to be symmetrical for both AP-1 and H3abp, except that the configuration of protection is different. These results demonstrate that H3abp and AP-1 exhibit different binding properties to the H3.2 site and further support the novel nature of the H3abp complex.

DISCUSSION

At the onset of DNA synthesis the transcriptional induction of the hamster H3.2 histone gene is regulated in part through an activator site found 170 bp upstream of the TATA element. In this report we describe the characterization and purification of the complex binding to this site, H3abp. This complex exhibits a biphasic binding increase in extracts prepared from serum stimulated cells. The second peak of binding activity, parallels the onset of DNA synthesis, and correlates with the initiation of transcription of the endogenous H3.2 gene (13). This suggests that the increase in binding activity may be involved in the mechanism of gene activation. The initial peak of binding activity was surprising since it was not observed when a larger histone promoter fragment was used as probe (10). It is possible that when the H3abp site is removed from the context of the promoter, repressing activities able to suppress the early G1 binding may also be lost. We discovered here that the binding of H3 specific binding activities to the H3 site is enhanced by Mg^{2+} and Ca^{2+} . It has been shown in mammalian cells that millimolar range of Mg^{2+} stimulates the rate-limiting steps in the cell cycle, including that of the transit through G1 and S and onset of DNA synthesis (18,19,23). Ca^{2+} /calmodulin has also been implicated in the control of cell cycle progression from the response to growth factors in G1 to DNA replication in S phase (20). These earlier observations coupled with our *in vitro* observation that Mg^{2+} and Ca^{2+} enhanced binding of the H3abp complex specific to the H3 site led us to speculate that fluctuations in nuclear Mg^{2+} or Ca^{2+} during the cell cycle could influence the binding of some G1/S regulatory transcription factors to their target sites and induce the

onset of transcription of the responsive S phase genes. This hypothesis can be tested further for H3abp when it is purified.

The H3abp complex is immunologically distinct from the characterized Jun and Fos family members (12), suggesting novel protein components may be involved. This hypothesis is consistent with results from co-transfection studies in F9 cells where c-Jun and Fos expression vectors are able to activate the collagenase promoter containing a consensus AP-1 site but fail to activate the H3.2 promoter (G.S.Naeve and A.S.Lee, unpublished results). To determine the composition of the H3abp complex, we utilized several techniques. UV crosslinking studies demonstrate that the major DNA binding component of H3abp is a protein species of about 68 kDa. Since DNase I was not used in our assays and the cross-linked protein band was broad, this protein size is approximate. Nonetheless, previous studies have shown that proteins with or without crosslinked oligonucleotides migrated with similar electrophoretic mobility in SDS-PAGE (24,25). In spite of these considerations, the molecular size estimated for H3abp is distinct from the characterized members of the Jun and CREB families of DNA binding proteins. The gel shifted doublet, which can be resolved into three complexes with extended gel run times, shows in UV crosslink analysis that the 68 kDa component is present in both complexes I and II, whereas complex I' is primarily due to the presence of a 45 kDa component. The I' DNA binding protein has a molecular weight characteristic of the known Jun family members and is readily competed by the collagenase AP-1 probe. The UV crosslinking data is supported by the reconstitution experiments where only proteins extracted from SDS-PAGE gels in the 90–60 kDa and 45–35 kDa molecular weight ranges bind to the H3abp site. The proteins in the 90–60 kDa range have binding specificity that is analogous to the bona fide H3abp however the migration pattern in an EMSA is not identical to that from crude nuclear extract. This altered migration could be due to the binding of the protein in the absence of the accessory factors which help to generate complexes I and II. The 45–35 kDa proteins have binding characteristics which more closely resemble AP-1 activity and could bind as a minor component to the H3abp site.

Evidence that the 68 kDa protein is the predominant DNA binding species of H3abp is further confirmed by analysis of the affinity purified proteins. The enriched complex migrates and competes identically with H3abp from crude extract and UV crosslink analysis demonstrates that the 45 kDa AP-1 binding activity normally associated with complex I and I' is missing. From these data it is clear that the major DNA binding protein component of H3abp is a protein of about 68 kDa that has distinct binding activity from AP-1 factors and is specifically enriched for by affinity chromatography using the H3abp binding site. The size of p68 is similar to the recently identified 66 kDa ATF-2, a member of the CREB-site binding protein (26). We therefore tested whether antisera raised against CREB capable of recognizing ATF protein members (27, kindly provided by Dr M. Montminy) and specifically against ATF-2 (kindly provided for by Dr J. Hoeffler) were able to interfere with the formation of H3abp. We observed that none of the antisera tested exhibited any effect (data not shown). Additionally, bacterially expressed ATF-2 (gift of Dr J. Hoeffler) has little or no binding affinity for the H3abp site.

The novel nature of the H3abp complex relative to an AP-1 complex is further seen in the unique binding characteristics of H3abp in footprint analyses. We have previously shown that site

Figure 6. Methylation interference assay of the H3abp site by purified AP-1 and H3abp. (A) Purified AP-1 was bound to methylated H3abp core oligomers labeled on either the upper or lower strand. Remaining free DNA was separated from bound DNA by EMSA. Free and bound DNA was electroeluted, piperidine digested, and separated on a 12% polyacrylamide gel with 8 M urea. F: Free, uncomplexed DNA. B: Bound, protein complexed DNA. Protection of guanine residues is indicated by arrows and the hypersensitive sites by stars. The AP-1 like sequence is boxed. (B) Affinity purified H3abp bound to methylated oligomers labeled either in the upper or lower strand. The assay was performed as described in (A). The protected bases are indicated by arrows. (C) Diagrammatic representation of protected bases observed with the H3abp site. Large horizontal arrows depict palindromic sequence from the central G residue (●). Open portion of arrow in H3abp site reflects a non-palindromic base pair. The G:C base pair unique to the H3abp site (■) is indicated.

X is capable of binding members of the AP-1 family and it is the addition of promoter sequence flanking site X that generates an H3.2 specific complex (11,12). Using nuclear extracts from HeLa or K12 cells in a DNase I footprint assay the H3abp complex protects a region that includes the AP-1-like site but the pattern is notably different from that of purified AP-1, recombinant

v-Jun, or chicken c-Jun when bound to the same promoter fragment (11). In this report we show by methylation interference that the affinity purified H3abp generates a distinct pattern of protected guanine residues on the H3.2 promoter compared to those protected by purified AP-1. The symmetrical protection pattern observed with purified AP-1 and the histone AP-1-like

site is consistent with methylation interference data from a consensus AP-1 element (22). The predominant protection of the lower strand that extends into the flanking region differs from the established AP-1 pattern and indicates that the binding contacts of H3abp are distinct from an AP-1 complex and supports the observation that the DNA binding factor is unique to the H3.2 promoter. In addition, the methylation interference analysis allows us to more accurately evaluate the boundary of the H3abp binding site. We note that the protection of the G residue specific for H3abp binding site is palindromic to the protected G found on the coding strand just 3' to the site X homology with the AP-1 site. This palindromic pair of residues reveals an extended palindrome which is unique to the H3.2 promoter and flanks the centered G residue common to most AP-1 palindromes. The AP-1-like site X may therefore be considered the core binding element of H3abp where the flanking sequence, which constitutes a larger palindrome than the AP-1 site, generates the unique specificity for the H3.2 gene.

The binding properties of the 68 kDa H3abp protein are similar to recently identified proteins that bind AP-1-like sites as independent protein complexes or in conjunction with different Jun family members (22,28). These proteins, NF-E2 and LRF-1, do not share significant structural homology with any characterized immediate early gene family with the exception of the bZIP motif but require an AP-1 binding site to specifically bind DNA. The 45 kDa NF-E2 protein is a hematopoietic specific protein that is postulated to regulate globin synthesis (22). It co-purifies with Jun and CREB proteins by affinity chromatography but is able to bind DNA independently in the presence of an 18 kDa bZIP containing accessory protein. Its role in the transcriptional activation of the globin genes and the temporal expression of the factor has not been established. The LRF-1 factor has been extensively characterized with regard to its expression and transactivation properties in regenerating liver (28). LRF-1 is a 21 kDa bZIP protein that is capable of binding DNA alone but preferentially forms heteromeric complexes with c-Jun and JunB. The heterocomplexes bind with highest affinity to CRE elements but also have affinity for related AP-1 and ATF sites. The activation kinetics of LRF-1 in regenerating liver show that the factor accumulates late in G1 and is implicated in the control of delayed G1 gene expression through various complex interactions among LRF-1, JunB, c-Jun and c-Fos. The degree of transactivation or repression is dependent upon the heterocomplex composition and the site being bound. The composition of the complex is regulated by the relative levels of the various components at a particular stage in the cell cycle and may represent the mode of action through which the H3abp activates the H3.2 gene at the G1/S border. The studies in regenerating liver do not address the possible biphasic expression profiles of LRF or the various Jun and Fos proteins. Only a few members of Jun and Fos family members have been analyzed with regard to temporal expression at these delayed time points (29,30). The role these proteins play and the combinations required for transcriptional activation during late G1 are not understood. Those studies would provide additional insight regarding the role played by the immediate early gene products and their associated proteins in coordinating the transcriptional events preceding DNA synthesis. While the extreme low abundance of H3abp precludes its direct isolation at the present time, the identification of the 68 kDa H3abp binding protein provides an additional tool

for addressing this mechanism and extend our understanding of the transcriptional regulation of replication dependent histones.

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