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H1TF2A, the Large Subunit of a Heterodimeric, Glutamine-Rich CCAAT-Binding Transcription Factor Involved in Histone H1 Cell Cycle Regulation

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H1TF2 is a CCAAT transcription factor that binds to the histone H1 subtype-specific consensus sequence, which has previously been shown to be necessary for temporal regulation of histone H1 transcription during the cell cycle (F. La Bella, P. Gallinari, J. McKinney, and N. Heintz, Genes Dev. 3:1982–1990, 1989). In this study, we report that H1TF2 is a heteromeric CCAAT-binding protein composed of two polypeptide doublets of 33 and 34 kDa and 43 and 44 kDa that are not antigenically related. The 33- and 34-kDa species were not detected in our previous studies (P. Gallinari, F. La Bella, and N. Heintz, Mol. Cell. Biol. 9:1566–1575, 1989) because of technical problems in detection of these heavily glycosylated subunits. The cloning of H1TF2A, the large subunit of this factor, reveals it to be a glutamine-rich protein with extremely limited similarity to previously cloned CCAAT-binding proteins. A monospecific antiserum produced against bacterially synthesized H1TF2A was used to establish that HeLa cell H1TF2A is phosphorylated in vivo and that, in contrast to the H2b transcription factor Oct1 (S. B. Roberts, N. Segil, and N. Heintz, Science 253:1022–1026, 1991; N. Segil, S. B. Roberts, and N. Heintz, Cold Spring Harbor Symp. Quant. Biol. 56:285–292, 1991), no gross change in H1TF2A phosphorylation is evident during the cell cycle. Further immunoprecipitation studies demonstrated that H1TF2 is heterodimeric in the absence of DNA in vivo and identified several H1TF2-interacting proteins that may play a role in H1TF2 function in vivo.

The temporal control of gene expression is a fundamental feature of the eukaryotic cell cycle. In most actively cycling eukaryotic cells, a relatively small but important population of genes is transcriptionally controlled. The first example of a cell cycle-regulated gene family is the S-phase-specific expression of histone genes (24). A great deal of effort has been expended to delineate the mechanisms responsible for the accumulation of histone mRNAs during S phase, most notably with *Saccharomyces cerevisiae* and with cultured animal cells. From these studies it has become clear that many of the general features of transcriptional and posttranscriptional regulation of histone genes have been conserved throughout evolution.

One aspect of histone gene expression that is particularly intriguing is the increased complexity of vertebrate histone gene transcriptional regulation during the cell cycle. For example, yeast histone gene transcriptional induction during S phase is dependent on a single set of conserved sequence elements that are present in the promoters for each of the eight histone genes (24). Presumably, coordinate transcriptional regulation of the *S. cerevisiae* histone genes is achieved through the agency of a single set of DNA-binding proteins operating through those shared consensus elements. In contrast, mammalian histone transcription is controlled by subtype-specific consensus elements that are not shared between genes encoding different histones. For example, the histone H2b octamer element is crucial for S-phase-specific transcription of replication variant H2b genes (19), whereas two highly conserved and unrelated subtype-specific consensus elements are important for S-phase transcriptional induction of histone H1 genes (4, 17). In this case, therefore, the temporal regulation of histone gene transcription during the cell cycle involves distinct, functionally equivalent, subtype-specific consensus sequences that bind to entirely distinct transcription factors (12).

To understand the biochemical mechanisms that participate in cell cycle regulation of mammalian histone gene transcription and to relate them to those controlling histone gene expression in simpler eukaryotes, it will be necessary to analyze several distinct transcription factors and their interactions as cells progress through the division cycle. Thus far, detailed biochemical studies have been possible only for the histone H2b transcription factor Oct1 (OTF1). These studies have demonstrated that Oct1 is differentially phosphorylated during the cell cycle (25) and that this program of phosphorylation ultimately results in complete loss of Oct1 DNA-binding activity during mitosis (26). Upon entry into the G_1 phase of the cell cycle, Oct1 DNA-binding activity is restored, possibly by removal of the inhibitory phosphates that were put onto Oct1 late during the previous cell cycle (27). We have not yet identified a posttranslational modification of Oct1 or an interacting protein that can explain the transcriptional induction of histone H2b genes by Oct1 as cells transit the G_1/S-phase border.

To determine whether induction of mammalian histone genes during the cell cycle is coordinated by mechanisms that

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directly modulate the activities of the distinct transcription factors that interact with the subtype-specific consensus sequences, we have characterized H1TF2 as a heteromeric CCAAT-binding transcription factor composed of 43- and 44-kDa and 33- and 34-kDa polypeptides. We have identified a cDNA clone (H1TF2A) encoding a large subunit of H1TF2 that predicts an extremely glutamine-rich protein with little similarity to previously cloned CCAAT-binding transcription factors. H1TF2 is both glycosylated and phosphorylated in vivo. Immunoprecipitation experiments demonstrate that H1TF2 is heterodimeric in the absence of DNA and that several polypeptides interact with H1TF2. These are candidates for functionally important cofactors of H1TF2 activity in vivo. In contrast to the case of the H2b transcription factor Oct1, in vivo studies demonstrate that H1TF2A is not hyperphosphorylated late during the HeLa cell cycle.

MATERIALS AND METHODS

Chromatography. HeLa nuclear extracts were prepared essentially as described by Dignam et al. (6). All chromatography buffers (BC buffers) contained 20 mM Tris-HCl (pH 7.9), 20% glycerol, 0.1 mM EDTA, 0.01% Nonidet P-40 (NP-40), 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and the indicated concentration of KCl. Protein concentrations were determined by the Bradford assay, and the chromatography fractions were assayed by electrophoretic mobility shift assay (EMSA) for H1TF2 binding activity (9). Typically, 100 ml of HeLa nuclear extract (5 mg of total protein per ml) was applied to a 4-ml wheat germ agglutinin (WGA) column (Vector) at a flow rate of 5 column volumes/h. After an extensive wash with BC100, H1TF2 was eluted with BC100 containing 0.3 M *N*-acetylglucosamine. Peak fractions were pooled and loaded at a rate of 5 column volumes/h onto a 0.5-ml H1TF2-specific oligonucleotide column. After being washed with 10 volumes of BC100, proteins bound to the column were eluted with a 6-column-volume gradient from 0.1 to 0.8 M KCl, and H1TF2 was eluted with a 2.5-column-volume step of 0.8 M KCl. Peak fractions were pooled, dialyzed to 0.1 M KCl in BC0, and then loaded at a rate of 2 column volumes/h onto a 0.5-ml nonspecific oligonucleotide column. The flowthrough was then loaded onto a second H1TF2 oligocolumn, and the H1TF2 activity was eluted as described before. Aliquots of the second oligonucleotide gradient fractions were analyzed by EMSA and by double staining of sodium dodecyl sulfate (SDS)-polyacrylamide gels (Coomassie brilliant blue followed by silver stain [ICN]).

Isolation of recombinant clones encoding H1TF2A. The HeLa expression library in the vector lambda ZapII was purchased from Stratagene and was screened by using the polyclonal antibody GRM336 raised against the purified HeLa cell protein H1TF2. *Escherichia coli* cells, strain XL-1 blue, were infected with the bacteriophage, plated on NZY plates, and incubated at 42°C until plaques became visible. Induction of bacterial protein was obtained by overlaying the plates with 10 mM isopropyl-β-D-thiogalactopyranoside (IPTG)-impregnated nitrocellulose filters (Millipore; Triton-free HATF). Transfer was allowed to proceed overnight at room temperature. Filters were then washed with phosphate-buffered saline (PBS) and blocked by incubation for 1 h at room temperature in a solution of PBS, 5% nonfat dry milk, 1% normal goat serum, and 0.2% Triton X-100. The polyclonal antiserum was added to the hybridization buffer (diluted 1:400) and incubated at room temperature for 2 h. After a wash with PBS, filters were incubated at room temperature for 1 h with goat anti-rabbit horseradish peroxidase-conjugated immunoglobulin G (TAGO) diluted 1:400 in the hybridization solution. After an extensive wash with PBS, the secondary antibody was detected by incubation of the filters in the developing solution containing 500 μg of diaminobenzidine per ml, 0.03% H_2O_2, and 50 mM Tris-HCl (pH 7.5). The reaction was stopped by washing the filter with water.

Gel retardation assays. Gel retardation assays were performed as described previously (9). For antibody competition, nuclear extracts were incubated with antibody for 30 min on ice before the gel shift reaction. For antibody supershifts, 1 μl of antibody was added to the gel shift reaction mixture and incubated for 10 min at room temperature before separation on a 4% polyacrylamide gel.

For preparative gel shifts, each 40-μl binding reaction mixture contained 200 ng of H1TF2-specific oligonucleotide and 0.5 mg of a highly enriched fraction of H1TF2. After fractionation on a 4% native polyacrylamide gel, the specific H1TF2 complex was identified by autoradiography. This and several control slices were excised from the gel, boiled in 2× SDS sample buffer, and loaded onto an SDS–10% polyacrylamide gel. After separation, the gel was double stained with Coomassie brilliant blue and silver staining.

Cell cycle synchronization and metabolic labeling. Synchronized HeLa cells were isolated by centrifugal elutriation as described previously (17). The cell cycle stage of each fraction was monitored by flow cytometry (5). Early G_1-phase cells were reinoculated into culture. Aliquots were removed at 3-h intervals and incubated for 1 h in methionine- or phosphate-free Dulbecco's modified Eagle's medium and 5% dialyzed fetal bovine serum.

For each extract 2×10^7 cells were labeled for 1 h at 37°C with 800 μCi of [^{35}S]methionine per ml or with 500 μCi of $^{32}P_i$. Mitotic cells were obtained by reinoculating a G_1 population of elutriated cells into a culture containing 40 ng of nocodazole per ml for 14 h. The mitotic index, usually greater than 90%, was determined by staining of the cell pellet with Hoechst 33258 (0.01 μg/ml).

Immunochemical techniques. Immunoblotting of protein was carried out as described previously (25). For immunoprecipitations, 2×10^7 HeLa cells were labeled with [^{35}S]methionine or $^{32}P_i$, washed in ice-cold PBS, and immediately resuspended in lysis buffer. The lysis buffers are indicated in the figure legends, and their compositions are as follows. Buffer A is 0.25 M NaCl, 0.1% NP-40, and 0.05 M *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7); buffer B is 0.12 M NaCl, 0.5% NP-40, and 0.05 M Tris-Cl (pH 8); buffer C is 0.15 M NaCl, 1% NP-40, 0.5% sodium deoxycholate (DOC), 0.1% SDS, and 0.05 M Tris-Cl (pH 7.5); and buffer D is 1% NP-40, 0.5% DOC, 0.4% SDS, 2 mM EDTA, and 0.1 M Tris-Cl (pH 8.3). All buffers contained the following cocktail of protease and phosphatase inhibitors: 50 mM NaF, 0.1 mM sodium orthovanadate, 5 mM sodium PP_i, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 10 mg of aprotinin per ml, and 10 mg of leupeptin per ml. After 30 min on ice, extracts were sonicated and centrifuged for 10 min in a microcentrifuge. Supernatants were incubated with 20 μl of PRM337 or preimmune serum at 4°C for 3 h. A total of 100 μl of 50% (vol/vol with the appropriate buffer) protein A-Sepharose and 2% bovine serum albumin was added, and the incubation continued for 2 h. The immune complexes were collected by centrifugation and washed three times with the appropriate buffer and once with water. Dried pellets were resuspended in 60 μl of 2× SDS sample buffer, boiled for 5 min, and centrifuged for 20 s, and the supernatants were

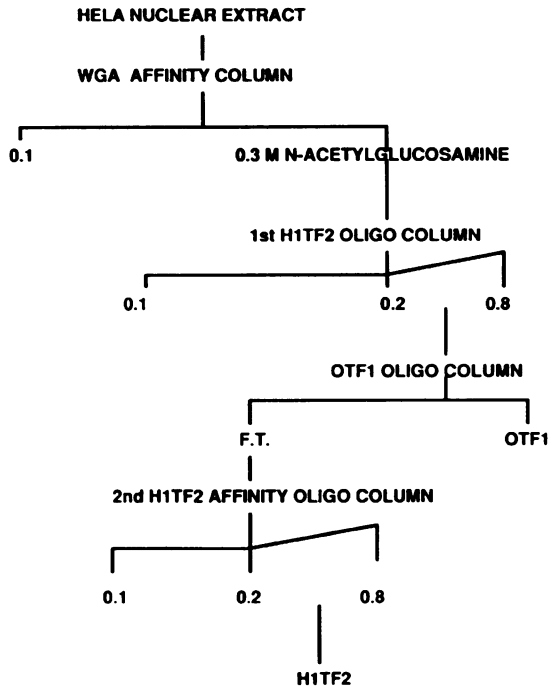


FIG. 1. Purification scheme for H1TF2.

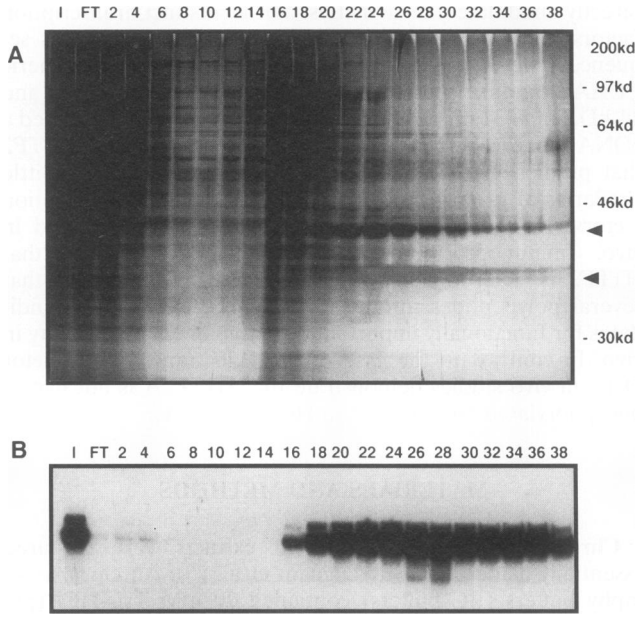


FIG. 2. Analysis of H1TF2 oligonucleotide gradient fractions. (A) SDS-PAGE analysis. Aliquots (15 μl) of input (I), flowthrough (FT), and fractions from the second H1TF2-specific oligonucleotide gradient column were electrophoresed on an SDS–10% polyacrylamide gel and double stained with Coomassie brilliant blue and silver stain, as described in Materials and Methods. The numbers of the fractions are indicated at the top of each lane. The positions of molecular mass markers are indicated. (B) Gel shift analysis. Aliquots (1 μl) from each fraction were assayed by using the H1TF2-specific gel shift assay as previously described (9).

analyzed by SDS–8 to 10% polyacrylamide gel electrophoresis (PAGE).

Antibody production. The GRM336 polyclonal antiserum was raised against the HeLa nuclear extract affinity-purified H1TF2. Affinity-purified H1TF2 (10 to 20 mg) was mixed with Alu-gel S as an adjuvant and injected intradermally into a New Zealand White rabbit (Giacomino) at intervals of 3 weeks. PRM337 antiserum was raised against the H1TF2A fusion protein obtained by cloning the *Eco*RI fragment of pBS1 into the pGEX-3X bacterial expression vector (29). The fusion protein was purified on a glutathione column as described by Smith and Johnson and then loaded on a SDS-polyacrylamide gel. After electrophoresis, proteins were detected by Coomassie staining, and the polyacrylamide gel was used to immunize the rabbit (Peppinella) as described by Harlow and Lane (10). Bleeding was done 10 days after each boost, and the sera were tested for functional activity by competition with H1TF2 binding activity and by Western blot (immunoblot).

RESULTS

Identification of H1TF2 as a heterodimeric DNA-binding protein. Our initial biochemical characterization of H1TF2 demonstrated that its DNA-binding characteristics are distinct from those of previously identified CCAAT-binding proteins (9). Purified HeLa H1TF2 appeared as a single polypeptide of approximately 43 kDa that could be specifically cross-linked to the H1TF2 DNA-binding site. As initially demonstrated in the case of the transcription factor SP1 (16), our subsequent analysis showed that H1TF2 DNA-binding activity is efficiently retained on a WGA column, presumably because of the presence of O-linked *N*-acetylglucosamine residues, allowing purification of large quantities of HeLa H1TF2 by using the simple strategy shown in Fig. 1.

To assess which proteins coelute with H1TF2 DNA-binding activity from the final DNA oligoaffinity column, aliquots from each gradient fraction were assayed by EMSA and proteins were analyzed by SDS-PAGE. Since heavily glycosylated proteins often stain poorly by standard silver staining techniques, we combined a brief prestain with Coomassie blue and silver stain to improve detection. As shown in Fig. 2, a polypeptide doublet with an apparent molecular mass of 43 and 44 kDa and another of 33 and 34 kDa that coelute quantitatively with H1TF2 binding activity are evident by using this detection method. These results strongly suggested that the 33- and 34-kDa polypeptides went undetected in our original study because of the poor staining of glycosylated proteins by conventional methodology.

To test the possibility that H1TF2 is composed of heterologous subunits, we analyzed the sensitivity of H1TF2 binding activity to the dissociating agent DOC. As shown in Fig. 3, incubation of WGA step fractions or total nuclear extract (data not shown) with increasing amounts of DOC results in a drastic reduction or complete inactivation of H1TF2 binding activity even at low concentrations (Fig. 3, compare lanes 15, 16, and 19). Subsequent addition of an excess of the nonionic detergent NP-40, which is known to sequester DOC and effectively remove it from the reaction, results in restoration of H1TF2 activity. The rapid reversibility of H1TF2 DNA binding in response to this type of detergent treatment strongly suggests that the loss of activity in the presence of DOC results from dissociation of a heteromeric complex. This result is also consistent with our inability to detect H1TF2 activity by using Southwestern (DNA-protein) blotting procedures (data not shown).

The definitive demonstration that H1TF2 is a multimeric

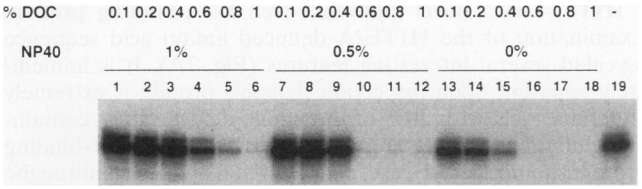


FIG. 3. Effects of the dissociating agent DOC on H1TF2 binding activity. HeLa nuclear extract, after partial purification by WGA affinity chromatography, was treated with the indicated concentrations of DOC. After 1 h of incubation on ice, NP-40 was added at the final concentrations of 1% (lanes 1 to 6) or 0.5% (lanes 7 to 12) or was not added (lanes 13 to 18).

binding protein is shown in Fig. 4A. In this case, highly purified H1TF2 was used in a preparative gel shift analysis to identify a polypeptide(s) present in the native DNA-binding complex (see Materials and Methods for the experimental details). Elution of the DNA-protein complex from the native gel followed by SDS-PAGE analysis of proteins present in that complex resulted in detection of both the 33- and 34-kDa and 43- and 44-kDa polypeptides (Fig. 4A, lane 3+). These proteins were seen only in the sample derived from the region of the gel containing the H1TF2 DNA-binding complex (lane 3+), and not in the adjacent lane which did not contain a specific H1TF2 oligonucleotide binding site (lane 3−). Elutions from two other regions of the gel, either in the presence or in the absence of specific H1TF2 binding sites, did not reveal specific polypeptides (lanes 1 and 2, both + and −). Thus, the native H1TF2 DNA-binding complex contains all four proteins that coelute with H1TF2 activity from the final affinity column. These experiments cannot distinguish whether H1TF2 binds as a mixed population of heterodimeric proteins containing different combinations of these four polypeptides or if it is a single tetrameric complex containing all four species.

To establish the native molecular mass of H1TF2, crude HeLa cell nuclear extracts were resolved by gel filtration. As shown in Fig. 4B, H1TF2 eluted from the gel filtration chromatography at a native molecular mass of approximately 70 kDa. These combined results establish that H1TF2 is a heterodimeric DNA-binding protein, as has previously been established for other CCAAT transcription factors.

Cloning of H1TF2A. Since our previous studies suggest that H1TF2 is a novel CCAAT factor but that its biochemical properties are quite similar to those of other CCAAT-binding proteins, we sought to clone H1TF2 to provide its definitive identification. The initial step in this process was the preparation of specific rabbit polyclonal antiserum to purified HeLa cell H1TF2. This was accomplished after five injections of approximately 5 to 10 μg of purified protein. Figure 5A shows the specificity of the GRM336 antibody raised against purified HeLa H1TF2. Preincubation of HeLa nuclear extract with the GRM336 antibody results in a complete inhibition of H1TF2 binding and formation of a supershift due to interaction of the antiserum with the native H1TF2 DNA-binding complex (Fig. 5A, compare lanes 1 and 5). Furthermore, specific depletion of H1TF2 binding activity from the HeLa nuclear extract can be obtained by preincubation of the GRM336 serum linked to protein A beads (Fig. 5A, lane 7). No effect is observed after nuclear extract depletion with preimmune serum protein A-linked antibodies (Fig. 5A, lane 6) or by protein A alone (Fig. 5A, lane 8). In order to test the specificity of the GRM336 antibody against H1TF2 and to exclude the possibility of a nonspecific inhibition of transcription factor binding activity, we tested its effect on Oct1, a biochemically distinct transcription factor. Preincubation of HeLa nuclear extract with GRM336 antibody does not affect the binding activity or the mobility shift of Oct1. Furthermore, Oct1 activity is not depleted with GRM336 immune serum protein A-linked antibodies. These results clearly establish the specificity of the GRM336 antiserum.

To determine which polypeptides are detected by the GRM336 antiserum in crude HeLa nuclear extract, Western blots (immunoblots) were performed (Fig. 5B). An increasingly high dilution of the antiserum results in specific detection of those proteins present in the purified H1TF2 preparation. However, in addition to the 43- and 44-kDa and 33- and 34-kDa H1TF2 doublets, the antiserum also specifically recognizes a 58-kDa protein that is not present in the H1TF2 DNA-binding complex.

Since the GRM336 antibody interacts both with the native DNA-binding complex and the denatured H1TF2 subunits, we used it to clone H1TF2. A HeLa cell cDNA expression library was screened for recombinant clones that encode proteins specifically recognized by the GRM336 antiserum. Several overlapping cDNA clones were obtained in this screen as candidates for H1TF2. To establish which of these clones encodes the correct protein, antibodies were raised against fusion protein produced from clone PBS1. Purified glutathione *S*-transferase (GST)-pBS1 fusion protein was used for immunization (see Materials and Methods). After each boost, we tested the functional activity of the rabbit serum PRM337 compared with that of preimmune serum for competition for H1TF2 binding activity in EMSA and for specificity by Western blot analysis. As shown in Fig. 6, the addition of fusion protein-derived antiserum (PRM337) to native HeLa cell H1TF2 DNA-binding reaction mixtures resulted in retarded migration of the specific H1TF2 complex. The slower migration of the supershifted complexes observed with the fusion protein antiserum (PRM337) versus antiserum raised against native HeLa cell H1TF2 (GRM336) (Fig. 6, compare lanes 3 and 7) is consistent with it recognizing more epitopes than are recognized by the GRM336 antiserum.

To determine which H1TF2 polypeptide(s) is encoded by the H1TF2A cDNA, the PRM337 antiserum was used to detect HeLa cell proteins by Western blotting (Fig. 6B). The fusion protein-derived antibody specifically interacts with the 43- and 44-kDa H1TF2 doublet, and with a 58-kDa protein that does not copurify with H1TF2 DNA-binding activity but is also recognized by the GRM336 antibodies. That the antiserum raised against bacterially expressed -pBS1 fusion protein can interact with native HeLa cell H1TF2 in a specific DNA-binding complex and that it recognizes the 43- and 44-kDa H1TF2 polypeptides on Western blots establishes the identity of H1TF2A as one of these two polypeptides. Furthermore, these results indicate that the 43- and 44-kDa proteins are closely related and that they are antigenically distinct from the 33- and 34-kDa H1TF2 polypeptides.

We have not yet been able to establish the difference between the 43- and 44-kDa H1TF2A polypeptides. Since we have evidence for alternative splicing of H1TF2A (data not shown), it is possible that these polypeptides derive from different H1TF2A transcripts. The 43- and 44-kDa doublet could also arise by posttranslational modifications to the H1TF2A protein, since H1TF2 is known to be both glycosylated and phosphorylated in vivo. Resolution of this issue will require a detailed biochemical analysis of the possible alternative forms of H1TF2A and their posttranslational modifications.

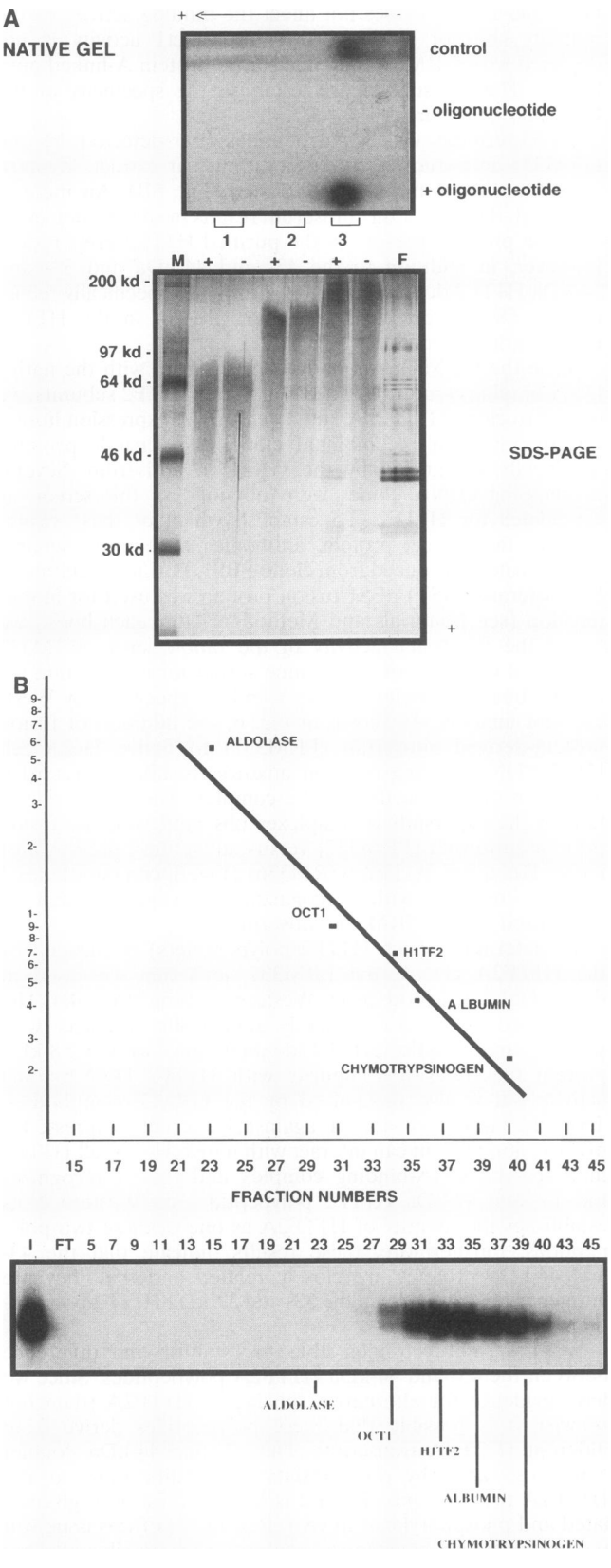


FIG. 4. Preparative gel shift analysis of H1TF2. (A) Partially purified fraction of H1TF2 (second oligonucleotide affinity column, fraction 24) was incubated with an excess of H1TF2-specific oligonucleotide, and the binding reactions were fractionated on a 4% polyacrylamide native gel. To identify the binding complex, 5 ml of a control reaction mixture was added to the preparative binding mixture just before loading. Gel slices corresponding to the H1TF2 binding complex (lane 3+), the same region from the neighboring lane in which no oligonucleotide was used in the DNA-binding reaction (lane 3−), and four additional control gel slices taken from regions not containing the H1TF2 complex, in the absence and presence of specific oligonucleotide, (lanes 1 + or − and lanes 2 + or −) were excised and loaded directly onto an SDS–10% polyacrylamide gel. After the electrophoretic separation, the gel was double stained as described in Materials and Methods. Lane F, input fraction. (B) Elution profile of H1TF2A DNA-binding activity from a Superdex 200 fast protein liquid chromatography column. Vertical lines indicate the positions of standards of known molecular weight.

H1TF2A is a novel glutamine-rich DNA-binding protein. Examination of the H1TF2A deduced amino acid sequence revealed several interesting features (Fig. 7A). It is immediately apparent upon inspection that this protein is extremely glutamine rich, with 20% of the amino acid residues containing glutamine. This is reminiscent of the CCAAT-binding protein subunit A (NF-YA) characterized by de Crombrugghe and colleagues, which is also extremely glutamine rich (11, 22). However, direct comparisons of primary nucleic acid or protein sequences for these two genes revealed very little identity. In fact, discounting similarities that occur simply because of the glutamine-rich nature of these proteins, there was only a single match that appeared significant (Fig. 7B). We found this quite surprising given the fact that these proteins each compose the largest subunit of a heterodimeric human CCAAT-binding protein. The region of similarity that is of interest is a 48-residue region extending between positions 157 and 209 in H1TF2A that is 35% identical and 54% similar to residues 119 to 171 of the rat and mouse NF-YA protein (Fig. 6). Although one possible reason for similarity in this domain is involvement in DNA binding, it has been argued on the basis of evolutionary conservation of NF-YA domains across metazoan kingdoms that the DNA-binding domain of NF-YA proteins resides more C terminal than residue 171 (20).

H1TF2 polypeptides interact in the absence of DNA. Major questions posed by the results of our biochemical analysis of HeLa cell H1TF2 is whether an H1TF2 heterodimer forms in the absence of DNA and whether it might be found associated with other proteins in vivo. To answer these questions, we have immunoprecipitated H1TF2A and its associated proteins from extracts of metabolically labeled HeLa cells with the H1TF2A-specific polyclonal antibody, PRM337. As shown in Fig. 8, immunoprecipitation of ^{35}S-labeled HeLa cell extract demonstrates that the PRM337 antiserum effectively precipitates the cellular 43-kDa polypeptide that we have designated H1TF2A. At least five additional proteins coprecipitate with H1TF2A under mild conditions. As expected, the 33-kDa H1TF2B subunit is readily detected in association with H1TF2A in immunoprecipitations done at a low stringency (lanes 2 and 4), but is not detected if the extracts are prepared with high concentrations of detergent (lane 6 and 8). These results agree with the gel filtration experiment shown in Fig. 4B, and together they demonstrate that H1TF2A and H1TF2B associate in vivo in the absence of DNA to constitute the native heterodimeric H1TF2 DNA-binding activity.

Four additional proteins immunoprecipitate with the

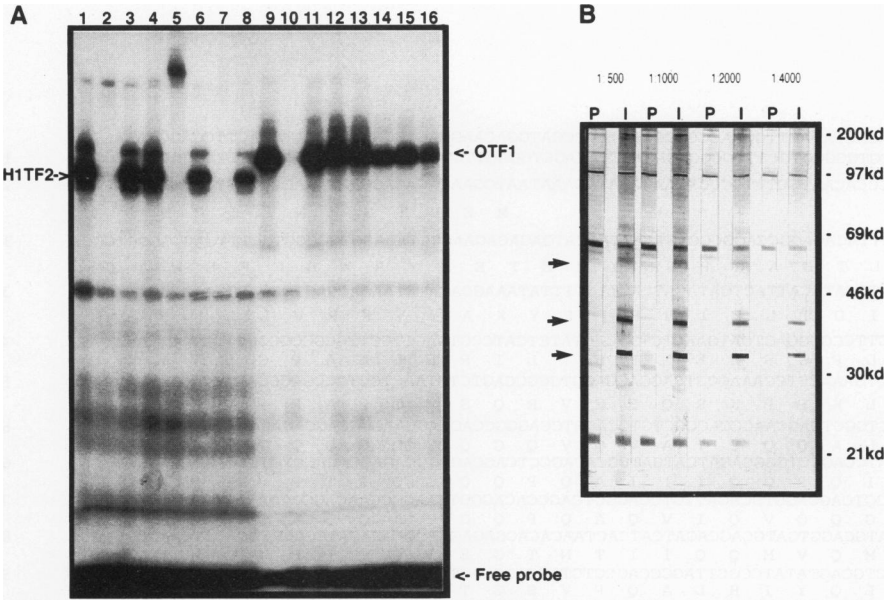


FIG. 5. Specificity of GRM336 antibody raised against HeLa purified H1TF2. (A) Gel shift assay. H1TF2 binding activity present in HeLa nuclear extracts: lane 1, no additions; lane 2, plus specific competitor; lane 3, plus nonspecific competitor; lane 4, as in lane 1 plus preimmune serum; lane 5, as in lane 1 plus GRM336 antiserum; lane 6, HeLa nuclear extract depleted with protein A beads; lanes 7 and 8, protein A beads loaded with GRM336 immune serum (lane 7) or preimmune serum (lane 8); lanes 9 to 16, the same extracts and additions used in lanes 1 to 8, respectively, but employing the H2b Oct1 binding site as a probe. (B) Western blot analysis of HeLa nuclear extract fractionated by SDS–10% PAGE and blotted onto nitrocellulose, with dilutions of preimmune (P) or GRM336 serum (I) ranging from 1:500 to 1:4,000. Polypeptides specifically recognized were visualized by biotinylated secondary antibody and avidin-conjugated alkaline phosphatase.

H1TF2A fusion protein-derived antiserum. Two of these (140 and 130 kDa) behave similarly to H1TF2B, as they are coimmunoprecipitated under gentle conditions but not in the presence of more stringent buffers (Fig. 8, compare lanes 2 and 4 with lanes 6 and 8). These results suggest that the 140- and 130-kDa proteins interact with H1TF2 in vivo and identify these proteins as obvious potential regulators of H1TF2 transcriptional activity. In-depth analysis of these proteins and their potential relationship to H1TF2 transcriptional activity is currently in progress. In contrast to H1TF2B, the 140-, and

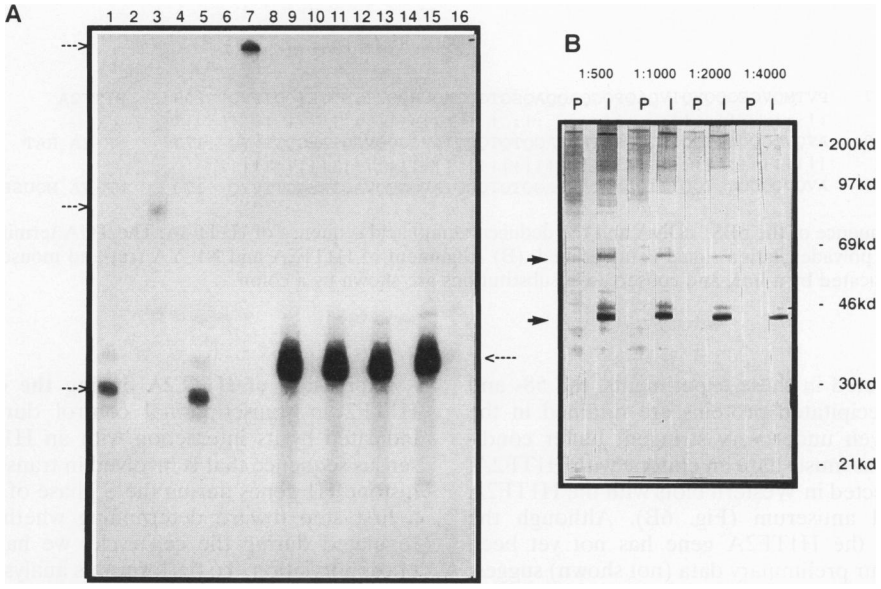
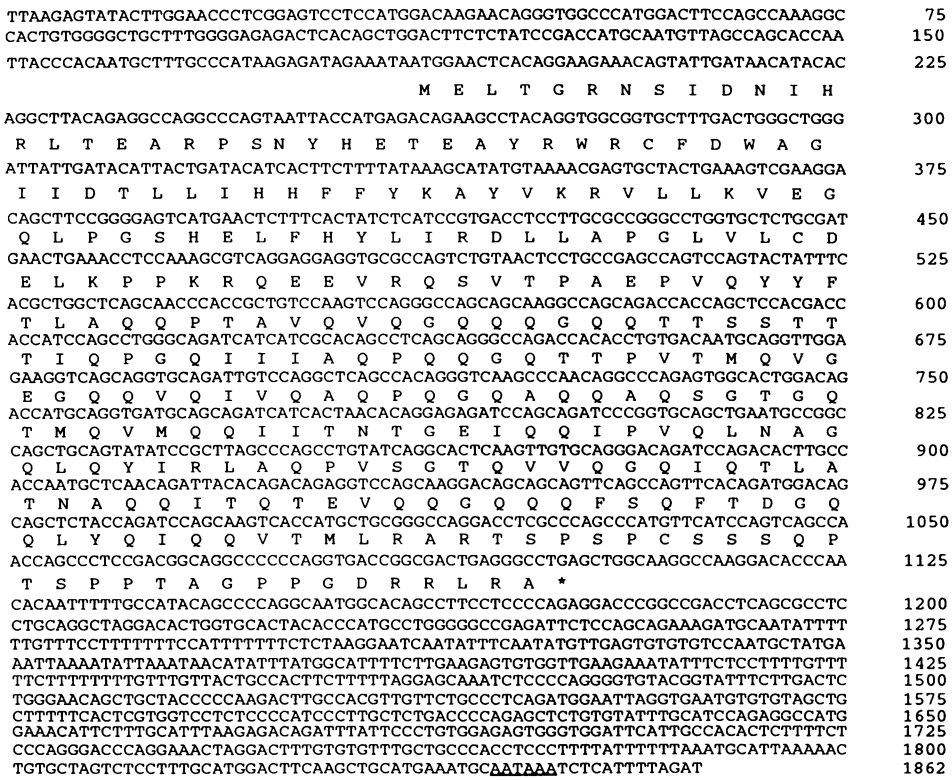


FIG. 6. Specificity of PRM337 antibody raised against the fusion protein GST-H1TF2A. (A) Lanes 1 to 8, H1TF2 specific DNA-binding reactions; lanes 9 to 16, Oct1-specific DNA-binding reactions. Even-numbered lanes contain specific competitor DNA; odd-numbered lanes nonspecific competitor. Additions to each lane are as follows: lanes 1 and 9, preimmune serum; lanes 3 and 11, GRM336 antiserum against HeLa H1TF2; lanes 5 and 13, preimmune serum from the second rabbit; lanes 7 and 15, GRM337 antiserum against H1TF2A fusion protein. (B) Western blot analysis with preimmune (P) or PRM337 immune (I) serum to H1TF2A produced in bacteria.

A



B

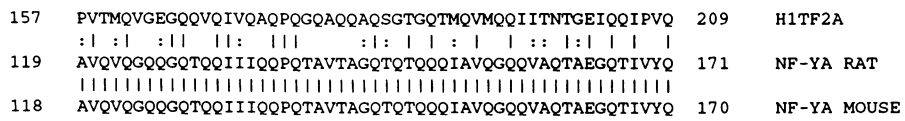


FIG. 7. Nucleotide sequence of the pBS1 cDNA and the deduced amino acid sequence of H1TF2A. The TGA termination codon is indicated with an asterisk, and the polyadenylation signal is underlined. (B) Alignment of H1TF2A and NF-YA (rat and mouse) amino acid sequences. Identical residues are indicated by a line, and conservative substitutions are shown by a colon.

130-kDa proteins detected in these experiments, the 58- and 200-kDa coimmunoprecipitated proteins are retained in the immunoprecipitates even under very stringent buffer conditions. These two proteins must share an epitope with H1TF2A, since they are also detected in Western blots with the H1TF2A fusion protein-derived antiserum (Fig. 6B). Although the alternative splicing of the H1TF2A gene has not yet been thoroughly analyzed, our preliminary data (not shown) suggest that the 58-kDa cross-reacting polypeptide derives from an alternatively spliced H1TF2A transcript. We have not yet established the identities of these polypeptides, although they are also candidates for proteins that could play a role in H1TF2 function or regulation.

Expression of H1TF2A during the cell cycle. A role for H1TF2 in transcriptional control during the cell cycle is indicated by its interaction with an H1-subtype-specific consensus sequence that is involved in transcriptional induction of histone H1 genes during the S phase of the cell cycle (18). As a first step toward determining whether H1TF2 is directly regulated during the cell cycle, we have analyzed H1TF2A phosphorylation. To perform this analysis, growing HeLa cells were centrifugally elutriated, and the G_1 population (time = 0) was reinoculated into culture. At 3-h intervals, cells were removed for fluorescence-activated cell sorter analysis and pulse-labeled with $^{32}P_i$ (Fig. 9A). In these experiments, it was necessary to prepare cell extracts at high SDS concentrations

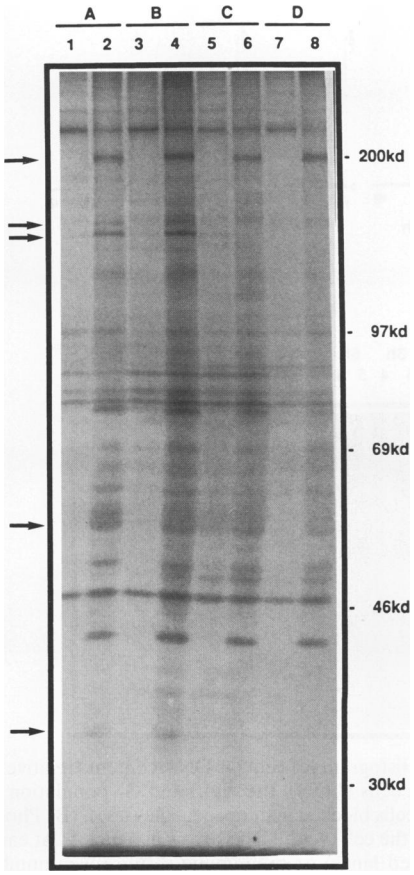


FIG. 8. Immunoprecipitation with antibody to H1TF2A. HeLa cells were metabolically labeled with [^{35}S]methionine, and lysates were prepared by using four different buffers, A to D, of increasing stringency (see Materials and Methods for details). Immunoprecipitations were performed with PRM337 serum (even-numbered lanes) and with preimmune serum as a control (odd-numbered lanes). The arrows indicate the polypeptides that are specifically coimmunoprecipitated with the PRM337 antiserum.

because immunoprecipitation under milder conditions resulted in very high background. H1TF2A was immunoprecipitated with the antiserum raised against bacterially produced H1TF2A, limiting our analysis to those polypeptides which share epitopes with H1TF2A. As shown in Fig. 9B and C, four phosphorylated proteins were precipitated with the H1TF2A fusion protein antiserum under stringent conditions. That the 43-kDa H1TF2A polypeptide was evident in this experiment establishes that it is a phosphoprotein in vivo. In contrast to our previous analysis of the histone H2b transcription factor Oct1 (25), H1TF2A does not appear to be differentially phosphorylated during the HeLa cell cycle. It remains possible that hyperphosphorylated H1TF2A is present in mitotic HeLa cells but has escaped our detection because of technical limitations in the experiment.

In addition to H1TF2A, 200-, 90-, and 58-kDa phosphoproteins are evident in this experiment. The 58- and 200-kDa proteins have been noted above as antigenically related to H1TF2A because of their cross-reaction with the H1TF2A antiserum on Western blots. Given the stringent conditions for preparation of these extracts, it is probable that the 90-kDa phosphoprotein observed in this type of experiment is also antigenically related to H1TF2A. It is interesting that each of these proteins migrated more slowly in the mitotic extract, suggesting that they are hyperphosphorylated at this time in the cell cycle. Peptide mapping experiments of the interphase and mitotic forms of the 200-kDa protein confirmed this conclusion (data not shown).

DISCUSSION

Coordinate production of core and H1 histones during S phase is a fundamental feature of most eukaryotic cell cycles. Mutagenesis experiments have located subtype-specific consensus elements in histone H1, H2b, and H4 genes that are critical for proper transcription of these genes (12). In the histone H2b and H1 genes, subtype-specific elements precisely positioned immediately upstream from the TATA box have been demonstrated to be required for transcriptional induction of these genes upon entry into S phase (17, 19). Thus, the transcription factors interacting with these elements, Oct1 and H1TF2, are directly involved in an S-phase-specific transcriptional regulatory event. Although other transcription factors have been identified in both yeast and animal cells that participate in the transcriptional induction of genes involved in production of precursors for DNA synthesis in late G_1 phase, Oct1 and H1TF2 are the only transcription factors thus far identified that play a direct role in a transcriptional event that is restricted to the S phase of the cell cycle. We have undertaken an in-depth analysis of these proteins and their interacting partners in the hope of uncovering molecular mechanisms that are fundamentally important for the control of macromolecular synthesis during the cell cycle.

In this study, we report the characterization of H1TF2 as a heterodimeric DNA-binding protein composed of 43- and 44-kDa (H1TF2A) and 33- and 34-kDa (H1TF2B) subunits. The dimeric nature of H1TF2 is supported by the copurification of H1TF2 binding activity with these subunits, by their presence in the native H1TF2 binding complex, by their coelution from gel filtration columns, and by their coimmunoprecipitation from HeLa cell extracts. The failure of H1TF2A polyclonal antiserum to cross-react with H1TF2B demonstrates that these proteins are not antigenically related. Cloning of the H1TF2A subunit cDNA revealed a predicted protein that is extremely glutamine rich. Although these properties of H1TF2 are reminiscent of previously characterized CCAAT-binding proteins, direct comparisons of the H1TF2A amino acid sequence with these proteins clearly establish that H1TF2A is only distantly related to these other factors. Since NF-YA is extremely highly conserved within the animal kingdom and is even very highly conserved within specific domains between yeasts and humans (1, 15), H1TF2A evidently does not belong to the NF-YA transcription factor family despite their similar roles as the large subunits of heteromeric human CCAAT-binding transcription factors. It will be interesting to explore the structure-function relationships of these different CCAAT factors and compare their subunit interactions, their modes of interaction with DNA, and their mechanisms of transactivation.

It is clear from both the purification and the immunoprecipitation experiments presented here that the 43- and 44-kDa and 33- and 34-kDa H1TF2 subunits assemble in the absence of DNA. Since our previous cross-linking studies of H1TF2 (9) demonstrate that it is the 43- and 44-kDa H1TF2A subunits that are in close contact with DNA and since our efforts to demonstrate specific DNA binding with bacterially expressed H1TF2A have failed, we believe that this interaction is critical for DNA binding. It seems unlikely that proteins other than

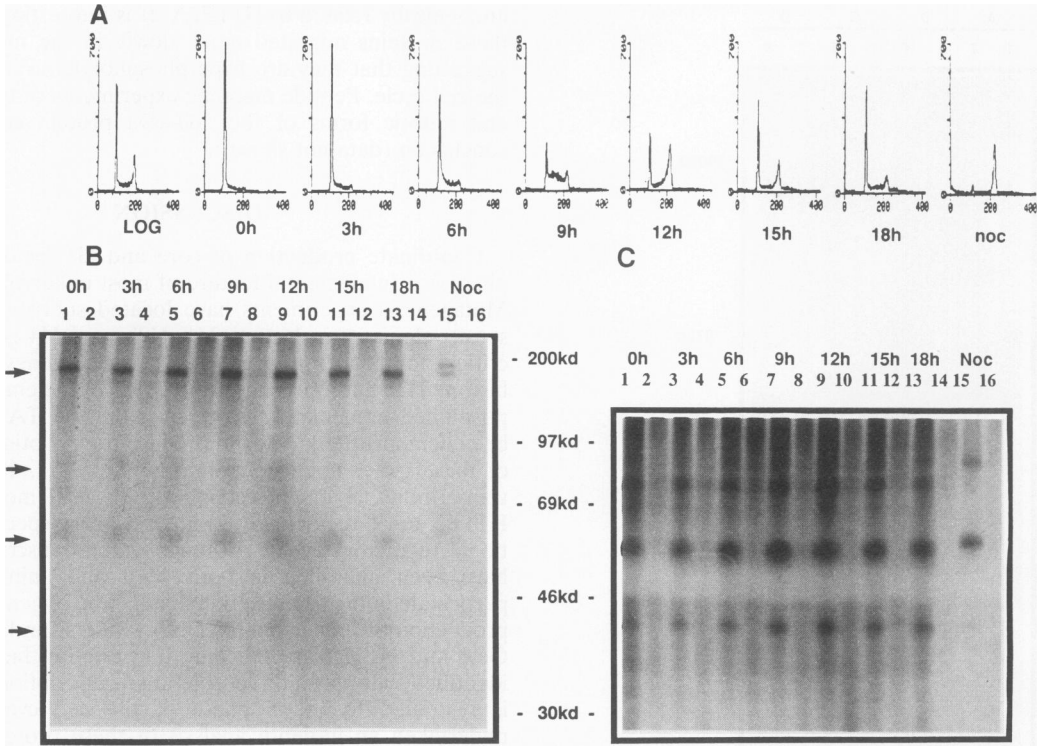


FIG. 9. Cell cycle analysis of H1TF2A. (A) Flow cytometry plot of cell cycle analysis. Histograms of cellular DNA content (relative fluorescence on the abscissa, and the cell number on the ordinate) of logarithmically growing HeLa cells (LOG), the elutriated G_1 population (0h), the G_1 population progressing through the cell cycle (3h, 6h, 9h, 12h, 15h, and 18h) and mitotic cells blocked with nocodazole (noc). (B) Phosphoproteins detected with H1TF2A antiserum (PRM337) in $^{32}P_i$ pulse-labeled HeLa cells throughout the cell cycle. Times are as in panel A. At each time point cells were labeled for 1 h and immunoprecipitated with PRM337 serum (odd-numbered lanes) or preimmune serum (even-numbered lanes). Products were separated by electrophoresis on an SDS–10% polyacrylamide gel and detected by autoradiography. (C) Longer exposure of the low-molecular-weight portion of the gel shown in panel B.

H1TF2A and H1TF2B are required for DNA binding, since no additional polypeptides are present in the native H1TF2 complex (Fig. 4A). Furthermore, gel filtration experiments have established that the native molecular mass of H1TF2 is approximately 70 kDa, precluding additional proteins as stable components of H1TF2 in the absence of DNA (Fig. 4B).

The failure of H1TF2A to bind DNA in the absence of H1TF2B is not surprising, given the precedent that CCAAT-binding factors require several subunits for DNA binding. Thus, although initial studies suggested that the human CCAAT-binding factor CBF consisted of only two subunits (CBF-A and CBF-B) (3, 11, 22), more recent evidence suggests that a third subunit (CBF-C) is also required for DNA binding (22). In the case of the yeast CCAAT-binding factor, which is both functionally and evolutionarily related to the CBF/NF-Y transcription factors, it is thought that the HAP-2 and HAP-3 subunits are sufficient for DNA binding but that an additional component (HAP-4) is necessary for transcriptional activation (23). In both of these instances a third component is required for the function of these heteromeric CCAAT transcription factors. In light of these precedents, it would not be surprising if H1TF2 function also were to require an additional component(s). On the basis of the immunoprecipitation experiments, the 140- and 130-kDa H1TF2-associated proteins are possible candidates for a HAP4-like activity in this system.

Although preparation of antibodies to highly purified H1TF2 was critical in the cloning of the H1TF2A cDNAs, antiserum to the C-terminal bacterial fusion protein has proven to be a much more useful tool. This is principally because the PRM337 antiserum is of a very high affinity and, since it is prepared against only the cloned H1TF2A subunit, it recognizes directly only those proteins that share epitopes with H1TF2A. In our attempts to discover mechanisms regulating H1TF2 activity during the cell cycle, we have failed to detect a modification that could explain its role in modulating histone H1 transcription. We have previously considered a model in which the involvement of a non-DNA-binding, *trans*-acting protein is required for different histone gene-specific DNA-binding proteins to activate transcription of their respective histone gene subtypes (17). The existence of such a shared activity would provide a mechanism for coordinate regulation of mammalian histone gene expression during the cell cycle. In an initial effort to detect such an activity, we have utilized the fusion protein antiserum to search for proteins that interact with H1TF2. In addition to the 33- and 34-kDa H1TF2B subunits, five proteins (200, 140, 130, 90, and 58 kDa) were detected by immunoprecipitation of [^{35}S]methionine-labeled (Fig. 8) and $^{32}P_i$-labeled (Fig. 9) HeLa cell extracts. Although these proteins have not yet been identified, the fact that they either interact with H1TF2A or are antigenically related to it has focused our interest on them as possible regulatory factors.

Two issues seem particularly worthy of further experimentation. First, we would like to know whether any of these proteins can be found in association with Oct1, providing a direct link between histone H1 and H2b transcription and a possible biochemical mechanism for coordinate control of

histone gene expression. Although recent evidence clearly demonstrates the presence of Oct1 in stable multimeric complexes in vitro (18), our present tools have not yet allowed direct comparisons of proteins interacting with H1TF2 and Oct1. The second issue we would like to address is whether mechanisms for direct posttranslational modification of these two histone gene transcription factors are shared and whether these shared mechanisms are critical for cell cycle regulation.

In the case of Oct1, we have previously demonstrated a complex program of phosphorylation during the cell cycle (25) that ultimately results in complete inactivation of Oct1 DNA-binding activity during mitosis (26). Both cdc2 and PKA-like protein kinases have been implicated in these phosphorylation events (25). In the case of PKA, it has been directly demonstrated that the kinase can phosphorylate Oct1 Ser-385 in vitro and that this phosphorylation mimics the loss of Oct1 DNA binding observed in vivo (26). In this study, we have not detected changes in H1TF2A phosphorylation during the cell cycle. Although this establishes that the mechanisms regulating Oct1 phosphorylation during the cell cycle do not pertain in the case of H1TF2A, two possibilities remain for further investigation. First, we have not yet been able to analyze the modifications to the 33- and 34-kDa H1TF2 subunit (H1TF2B) during the cell cycle and cannot, therefore, rule out phosphorylation as a direct participant in the regulation of H1TF2 transcription activity during the cell cycle. Second, we have observed hyperphosphorylation of H1TF2A-related proteins during mitosis. This is especially intriguing given the precedent that alternative forms of known transcriptional activator proteins can act as transcriptional repressors. One particularly interesting illustration of this point is the alternative splicing of glutamine-rich domains in the cyclic AMP response element binding protein (CREB) and its closely related antagonist, the cyclic AMP response element modulatory protein (CREM). In this case, alternative splicing is thought to be responsible for the different regulatory roles of these two forms of the protein (7). Thus, removal of two glutamine-rich domains from the CREB activator results in production of the transcriptionally inactive CREM protein that may act as a repressor (8). Similar regulatory schemes have been described in the cases of Oct1 and AP2 (2, 29).

It seems evident that the detailed biochemical approach we have taken toward understanding histone gene transcription during the cell cycle can uncover mechanisms that might be of general importance in the regulation of transcriptional activity during the cell cycle. For example, several other transcription factors have recently been shown to be hyperphosphorylated during the cell cycle (15, 21), suggesting that the inactivation of transcription during mitosis might in part be regulated by direct modification of upstream transactivating proteins. Given the possibility that transcription and DNA replication might be mechanistically similar (discussed in reference 13), we believe that these studies will also be critical in understanding the close coupling of histone gene transcription and DNA replication.

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