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Characterization and Purification of H1TF2, a Novel CCAAT-Binding Protein That Interacts with a Histone H1 Subtype-Specific Consensus Element

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Definition of mechanisms regulating human histone H1 gene transcription during the cell cycle requires the isolation and biochemical characterization of protein factors which interact with specific promoter elements. Two distinct binding activities have been identified in nuclear extracts from HeLa cells and mapped within a 180-base-pair (bp) region of a cell cycle-regulated H1 gene promoter. H1TF1 bound to an H1-specific A+C-rich sequence (AC box), 100 bp upstream of the cap site; H1TF2 interacted with the H1 subtype-specific consensus element and was dependent on the presence of an intact CCAAT box for binding. H1TF2 was purified through a combination of ion-exchange and oligonucleotide affinity chromatographies. Analysis of purified fractions by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and UV crosslinking showed that H1TF2 was a single polypeptide of 47 kilodaltons. This factor was distinct from previously characterized CCAAT-binding proteins in both molecular size and binding properties. Fractions containing H1TF2 activity activated transcription in vitro only if programmed with an H1 DNA template carrying an intact H1TF2-binding site.

The role of histones as the fundamental structural unit for packaging eucaryotic DNA requires that they be synthesized at elevated rates during the S phase of the cell cycle. A variety of in vivo (1, 4, 26) and in vitro (13, 18, 21, 32) studies have established that transcriptional control is an important component of the S-phase regulation of histone gene expression and have revealed the complexity of mammalian histone gene promoters and their cognate transcription factors. In particular, the DNA-binding transcription factors important for expression of the human histone H4 (9) and H2b (13) genes are distinct. In the case of the H2b gene, it has been directly demonstrated that the subtype-specific consensus element mediates S-phase transcriptional regulation in vivo (26). This regulation has been reproduced in vitro, and a 90-kilodalton (kDa) factor (OTF1) which stimulates H2b transcription through utilization of the H2b regulatory sequence has been purified and characterized (13). These observations, and the existence of subtype-specific consensus elements in the H4, H2b, and H1 promoters (for a review, see reference 36), have led to the proposal that these genes may be organized in a functionally analogous manner in which proteins interacting with the subtype-specific elements are crucial components of the S-phase transcriptional regulatory apparatus (for discussion, see references 20 and 26). In this model, a common mechanism modulates the activity of each of the distinct subtype-specific transcription factors, resulting in the induction of histone gene transcription during S phase.

It is apparent that identification of the molecular mechanisms regulating expression of each of the mammalian histone genes will be required to define this model further. In the case of the histone H1 gene, information is available about promoter elements involved in transcription, although very little is known about the proteins interacting with these elements. Coles and Wells (8) first noted that an H1-specific "AC box," approximately 100 base pairs (bp) upstream from the chicken histone H1 cap site, can be found in all histone H1 genes but is not present in the related chicken H5 gene. An initial analysis of a possible role for this element in histone H1 transcription indicated that deletion of the AC box from the H1 promoter had no effect on transcription, assayed by injection into *Xenopus* oocytes or in transient expression assays in HeLa cells (39). More recently, Dalton and Wells (10) have reported that this sequence has a major effect on H1 transcription when the gene is stably introduced into the HeLa cell genome and that it may be directly involved in cell cycle regulation. Finally, both Dalton and Wells (10) and van Wijnen et al. (34) have reported specific interactions of nuclear proteins with the H1 promoter in HeLa cell nuclear extracts, although no information about the identity of these proteins is available.

In this paper we report the initial characterization of a human histone H1 promoter and the proteins interacting in vitro with the two H1 subtype-specific domains. Mutation of the H1 AC box reduces transcription both in vivo and in vitro, confirming the observations of Wells and colleagues that this element acts positively to increase H1 transcription. A second H1-specific element which is very highly conserved among H1 genes for 18 bp and includes a CCAAT motif also acts positively to increase transcription in vivo and in vitro. Proteins which specifically interact with each of these sites are present in the nuclear extract, and we have extensively purified each of them by using a combination of ion-exchange and affinity chromatographies. We have identified the factor H1TF2 interacting with the proximal histone H1-specific promoter element as a 47-kDa polypeptide. H1TF2 is distinct from previously characterized CCAAT-binding proteins in both molecular weight and DNA-binding properties, suggesting that it is not a general CCAAT factor and may be utilized preferentially for histone H1 transcription in vivo.

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MATERIALS AND METHODS

Construction of fusion genes. The chloramphenicol acetyltransferase (CAT) coding region *PstI-BamHI* fragment from the ppCAT vector (16, 26) was inserted at the *PstI* site 60 nucleotides downstream of the mRNA start site of a human histone H1 (Hh9) gene. Deletion mutants of the Hh9 promoter were generated by a series of Bal 31 digestions as described previously (18). Oligonucleotide-directed mutagenesis (40) was used to generate the CCAAT box point mutant in an M13 clone containing a −180 H1-*cat* fusion gene.

Cell culture, transfection, and S1 analysis. Human 293 cells were maintained and calcium phosphate transfected essentially as described previously (26). Sixteen hours after transfection, cell medium was replaced, and total RNA was collected 48 h after transfection. S1 analysis of *cat* mRNA was performed according to established procedures (2) as described previously (26). The probe used in S1 experiments to detect H1-*cat* mRNAs was made by digesting and 5′-end-labeling the −180 H1-*cat* plasmid at the *NcoI* site, 550 nucleotides downstream of the *cat* AUG site. Quantitation of transcription was accomplished by densitometric scanning of at least two exposures of films from each experiment, with a Beckman Du 8 spectrophotometer equipped with a gel-scanning accessory.

In vitro transcription of H1 templates. HeLa cell nuclear extracts were prepared essentially as described before (21). Each 10-μl transcription reaction mix contained 25 μg of HeLa nuclear extract protein, 200 ng of circular H1-*cat* DNA template, 20 mM HEPES (*N*-2-hydroxethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7.9), 0.6 mM each ATP, CTP, and UTP, 20 μM GTP, 2 mM creatine phosphate, and 10 mM $MgCl_2$. In vitro transcription assays with purified H1TF2 were performed in 20-μl reaction mixtures containing 40 μg of HeLa nuclear extract protein, 200 ng of H1-*cat* DNA, 100 ng of H4-*cat* internal control (Δ2606) (18), and 0, 2, or 4 μl of the input from the H1TF2 oligo column. The KCl concentration was adjusted to 55 mM in all samples. After incubation at 30°C for 30 min, transcription reactions were terminated by the addition of 50 μg of carrier RNA and 0.5% Sarcosyl. S1 analysis of protected *cat* mRNAs, after phenol chloroform extraction, was performed with a −37 H1-*cat* DNA probe labeled at the *NcoI* site and the conditions described above. The samples were electrophoresed on a 5% polyacrylamide denaturing gel, and the dried gel was exposed to X-ray film. Quantitation of transcription was achieved by densitometric scanning of the films, as described above.

Gel shift assay. Gel shift assays were performed essentially as originally described (14, 15) with minor modifications. DNA oligonucleotides (see Fig. 2A) were synthesized by the Rockefeller University Protein Sequencing Laboratory with an Applied Biosystems DNA synthesizer and 3′-end labeled with [α-^{32}P]dNTPs by using the Klenow fragment of DNA polymerase I. Each 20-μl binding reaction mixture contained 1 ng (34 fmol) of oligonucleotide probe (20×10^4 to 50×10^4 cpm) and 5 to 10 μg of crude extract protein or 0.5 ng (10 fmol) of affinity-purified H1TF2 protein. From 2 to 4 μg of poly(dI-dC):(dI-dC) (Pharmacia) was included to minimize nonspecific binding to the DNA probe. The reaction mixtures were incubated in a binding buffer containing 20 mM HEPES (pH 7.9)–4% (wt/vol) Ficoll–40 mM KCl–2.5 mM $MgCl_2$ for 20 to 30 min at room temperature; 10 μl from each sample was then electrophoresed on a 4% polyacrylamide gel in 22.25 mM Trizma base–22.25 mM boric acid–0.2 mM EDTA at 150 V for 1.5 h at room temperature. Then 0.01% Nonidet P-40 (NP-40) was added to samples, gels, and electrophoresis buffer for analysis of highly purified H1TF2 to prevent aggregation. Gels were then dried and exposed to X-ray film.

Chromatography. All chromatography buffers (BC buffers) contained 20 mM Tris hydrochloride (pH 7.9 or 7 as indicated), 20% glycerol, 0.1 mM EDTA, 0.01% NP-40, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and the indicated concentrations of KCl (e.g., BC100 contained 100 mM KCl).

The fractionation procedure used for H1TF2 purification was derived from that devised for OTF1 purification (13), since these two activities cochromatograph until the last, OTF1-specific oligonucleotide affinity column. Typically, 200 ml of HeLa nuclear extract (10 mg of protein per ml from approximately 4×10^{10} nuclei) in BC100 (pH 7.9) were applied to a 100-ml phosphocellulose (P11, Whatman) and a 50-ml DEAE-Sepharose Fast Flow (Pharmacia) column linked in tandem at flow rates of 1 and 2 column volumes per h, respectively. The flowthrough was collected, dialyzed against BC100 (ph 7), and loaded at 3 column volumes per h on a 50-ml Biorex 70 column equilibrated in the same buffer. Bound material was eluted with 350 mM KCl, dialyzed against BC60 (pH 7.9), and loaded on a 1.5-ml double-stranded DNA column at 4 column volumes per h. Again, the bound protein was eluted with 350 mM KCl, dialyzed to 60 mM KCl, and loaded on a 500-μl OTF1-specific oligo-affinity column (13), and the flowthrough was collected and loaded on the H1TF2-specific oligo-affinity column at 5 column volumes per h. After the column was washed with several column volumes of BC60, proteins bound with low affinity were eluted with 6 column volumes of 200 mM KCl, and H1TF2 was eluted with a single 800 mM KCl step. The DNA columns were prepared essentially as described by the manufacturer (Pharmacia) with 250 μg of DNA coupled per ml of CnBr-activated Sepharose CL-4B resin. The two nonspecific DNA columns consisted of sonicated salmon sperm DNA (Pharmacia) and the H2b oligonucleotide previously described (13), respectively. The H1TF2-specific DNA affinity column consisted of the synthetic H1TF2 oligonucleotide shown in Fig. 2A.

Oligonucleotide affinity column fractions containing the peak of H1TF2-binding activity were pooled, and a portion was analyzed by silver staining (ICN) of sodium dodecyl sulfate (SDS)-polyacrylamide gels (27).

UV crosslinking in situ. A modified protocol combining UV crosslinking of protein-DNA complexes (5, 37) with the gel shift assay was developed based on those described previously. The probe was prepared by hybridizing an oligonucleotide of the coding strand of the H1 promoter, positions −64 to −33, to a complementary oligonucleotide which contained sequence −44 to −33. This oligonucleotide was made completely double stranded by incubation with the Klenow fragment of DNA polymerase I in the presence of [α-^{32}P]dGTP and the three other cold deoxynucleotides. Each 100-μl binding reaction mixture containing 5 ng of oligonucleotide probe (6.5×10^4 cpm), 10 μg of poly(dI-dC):(dI-dC), and 10 μl (5 ng) of the H1TF2 affinity-purified preparation was incubated for 30 min at room temperature in the presence of a 100-fold molar excess of specific (H1TF2) or nonspecific (H1TF1) cold competitor oligonucleotides. The samples were electrophoresed on a 4% polyacrylamide nondenaturing gel at 150 V for 1.5 h. The gel was then UV-irradiated (1,600 W, 260 nm) for 20 to 30 min at 4°C and autoradiographed at 4°C overnight. The region correspond-

ing to the specific H1TF2-DNA complex in the sample with nonspecific competitor was identified and excised from the gel. Then, 20 μl of a mixture containing DNase I (1 μg/μl; Sigma) and micrococcal nuclease (1 U/μl; Bethesda Research Laboratories) was spread over the gel slice and incubated for 10 min at room temperature. A 20-μl solution of 50 mM $CaCl_2$ and 50 mM $MgCl_2$ was added, nuclease digestion proceeded at room temperature for 30 min, and the reaction was terminated by adding 20 μl of 0.25 M EDTA–0.25 M EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid]. The crosslinked complex was electroeluted from the gel in an Elutrap (Scheicher and Schuell) at 150 V for 16 h in Tris-glycine SDS-polyacrylamide gel electrophoresis (PAGE) running buffer (27) containing 0.05% SDS. The eluate (about 600 μl) was then precipitated with 7 volumes of acetone for 1 h in an ethanol-dry ice bath and centrifuged at 12,000 rpm for 30 min. The dried pellet was suspended in 20 μl of SDS-PAGE sample buffer, heated, and loaded on a 10% polyacrylamide-SDS gel along with a portion of the H1TF2 purified preparation and molecular mass protein markers (Bio-Rad and Sigma). After electrophoresis, the gel was silver stained, dried, and exposed to X-ray film.

DNase I footprinting and methylation interference. The noncoding strand of a 220-bp *Eco*RI-*Pst*I fragment of the H1 promoter, extending between positions −155 and +65, was labeled at the 3′ end by [α-^{32}P]dATP with the Klenow fragment of DNA polymerase I and used as a probe in both DNase I footprinting and methylation interference analysis.

For DNase I footprinting, 2 ng of DNA probe was mixed with affinity-purified H1TF2 and 100 ng of poly(dI-dC):(dI-dC) in a 20-μl volume. The samples were incubated for 30 min at 30°C before addition of 1 μl of a 5-mg/ml solution of DNase I (Sigma). After 30 s of digestion at 30°C, the samples were put in dry ice, phenol-chlorophorm and chlorophorm extracted, and ethanol precipitated. After centrifugation, dried pellets were suspended in 10 μl of formamide loading buffer (29) and electrophoresed on an 8% polyacrylamide sequencing gel. The gel was then exposed to X-ray film.

For methylation interference assays, the DNA probe was partially methylated on guanine residues by dimethylsulfate (DMS) (29) and used for gel shift experiments with affinity-purified H1TF2 as described above. The positions of the resultant free and protein-bound DNAs, separated by electrophoresis, were identified by autoradiography of the wet gel and purified with an Elutrap (Schleicher and Schuell). The isolated DNAs were cleaved at modified G residues with 10% piperidine and ethanol precipitated. The dried pellets were suspended in loading buffer so that each sample contained a comparable amount of radioactivity (3×10^3 cpm in 5 μl). Samples were analyzed on an 8% polyacrylamide sequencing gel.

RESULTS

DNA constructions and S1 mapping probes. Workers in our laboratory have cloned and characterized two human histone H1 genes. We have previously reported that one of these genes (Hh8 H1) is expressed and cell cycle regulated only in human KB cells, but is not expressed in several other human cell lines (HeLa and 293) (25). For this analysis, we have chosen to examine a second human H1 gene (Hh9 H1) which is also cell cycle regulated and which is widely expressed in all human cell lines analyzed, including the HeLa cells from which we prepared our in vitro transcription extracts and the 293 cells we used in transfection experiments (unpublished observations). This gene was isolated from the Hh9 lambda genomic clone, which does not contain other histone genes (R. Zhong and N. Heintz, unpublished data).

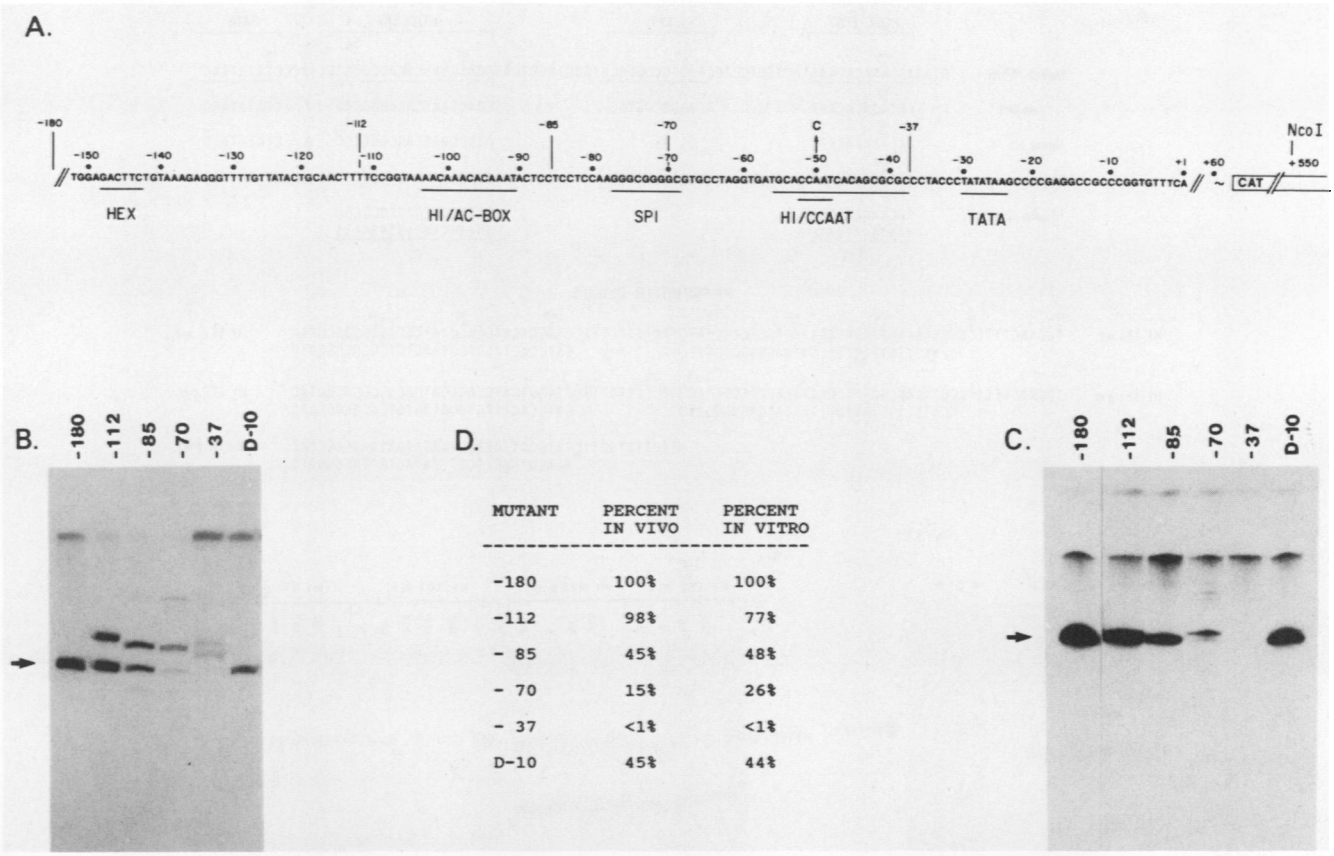
To identify the promoter elements responsible for transcription of the human histone H1 gene in vitro and in vivo, a series of 5′ deletion mutants were generated by Bal 31 nuclease digestion of the −180 parental clone, as diagrammed in Fig. 1A and detailed in Materials and Methods. In addition, a single nucleotide change was introduced into the CCAAT sequence by oligonucleotide-mediated mutagenesis (Fig. 1A). Each promoter was coupled to the bacterial *cat* gene (16) and tested for transcription both in vitro and in vivo. In both types of assay, the RNA transcripts produced from the H1 promoter-*cat* fusion genes were analyzed by quantitative S1 nuclease mapping (26) with a probe 5′-end labeled 610 bp from the initiation site of H1 gene transcription.

Identification of H1 promoter elements necessary for transcription in vivo and in vitro. To assay the contribution of each of the H1 promoter elements to transcription in vivo, the H1-*cat* fusion genes were transfected into 293 cells and the level of fusion mRNA produced 48 h posttransfection was measured by S1 nuclease analysis (26), with the −180 H1-*cat* construct used as the probe.

Figure 1B shows the effect of the various promoter deletions and of the point mutation on the level of H1 fusion transcript production. It is clear both from inspection of the autoradiograph shown in Fig. 1B and from the quantitation of a number of independent transfection assays (Fig. 1D) that several discrete elements within the H1 promoter contributed to maximal transcription in vivo. Deletion of H1 promoter DNA sequences between −180 and −112 did not affect the efficiency of transcription. In contrast, deletion to −85 resulted in a decrease in the level of transcript produced which was approximately 50% of that produced from the wild-type template (this most distal region in the H1 promoter contains the H1 AC box). Deletion to −70, which removed the putative SP1-binding site, further reduced the amount of steady-state mRNA detected to approximately 15% of the wild-type level. Finally, removal of the proximal H1 consensus element, by deletion to −37, reduced the amount of transcript produced to an undetectable level.

To investigate further the discrete effect of the proximal H1 consensus sequence in the context of the wild-type promoter, a single nucleotide change was introduced in the CCAAT box. This point mutation resulted in a twofold reduction in expression of the fusion RNA compared with the wild-type template. Together, these results show that the H1-proximal promoter element (containing the CCAAT motif), the G+C-rich SP1 consensus binding site, and distal promoter DNA sequences including the H1 AC box all contribute significantly to expression of this gene in vivo.

In order to determine whether the same H1 promoter elements are functional in vitro, the wild-type H1 and mutant H1-*cat* fusion genes were tested in an in vitro transcription assay with HeLa cell nuclear extract. Since our analysis of the histone H4 (18) and H2b (32) promoters indicated that closed circular templates were transcribed more efficiently than linear templates in vitro, supercoiled plasmid DNA was used for these assays. The RNAs produced in vitro were analyzed by S1 nuclease mapping with the −37 H1-*cat* construct as the probe. The autoradiogram presented in Fig. 1C shows that the efficiency of in vitro transcription decreased incrementally as the H1 AC box, the putative SP1



D.

MUTANT	PERCENT IN VIVO	PERCENT IN VITRO
-180	100%	100%
-112	98%	77%
- 85	45%	48%
- 70	15%	26%
- 37	<1%	<1%
D-10	45%	44%

FIG. 1. In vivo and in vitro transcriptional analysis of Hh9 H1 gene promoter. (A) Diagram of Hh9 H1-*cat* fusion gene. Sequences and positions of the upstream elements of the H1 promoter are shown with the relevant deletion mutants used in this study. The A to C transversion which inactivates the CCAAT box is indicated by an arrow. The transcription initiation site is indicated by +1. The *cat* coding region is depicted by an open box. (B) Expression of H1 promoter mutants in 293 cells. A 10-μg amount of total RNA was extracted from transfected 293 cells and S1 mapped as described in the text. Names of the transfected mutants are indicated above each lane. D10 refers to the CCAAT point mutant. The arrow on the side of the autoradiogram points to the H1-*cat*-protected band (610 bp). The presence of bands larger than those initiated at the H1 cap site which decrease in length with increasing 5′ deletion is due to transcripts initiated within vector sequences that are discontinuous with the −180 H1-*cat* probe at the position of the deletion. (C) S1 analysis of H1-*cat* in vitro transcription products. Equal concentrations of each of the mutants tested in vivo were transcribed as described in the text, and RNAs were subjected to S1 mapping with the −37 H1-*cat* construct as the probe. Arrow, Specific 610-bp H1-*cat* transcripts. (D) Quantitation of in vivo and in vitro transcriptional analyses. The quantitative results obtained from several independent repetitions of these assays are summarized. Quantitation was performed densitometrically on at least two different autoradiographic exposures of each assay to ensure that scans were in the linear range of the film and the densitometer.

site, and the H1-proximal subtype-specific consensus element were progressively removed from the H1 promoter. In addition, the single nucleotide change introduced in the CCAAT sequence clearly affected the level of H1-*cat* RNA transcripts. The quantitation of several independent in vitro transcription experiments is shown in Fig. 1D. Comparison with in vivo data indicates that the utilization of the H1 promoter elements in vitro was essentially indistinguishable from their use in the transient assay system in vivo. The simple conclusion, therefore, is that the H1 promoter is complex and requires the activity of several sequences for efficient expression either in vivo or in vitro.

Proteins binding to the H1 subtype-specific consensus sequences in vitro. As mentioned in the Introduction, it has previously been noted that all histone H1 genes so far analyzed contain an A+C-rich consensus sequence (5′-AAACACA-3′) approximately 100 bp upstream from the transcription initiation site (36). Inspection of the mammalian histone H1 promoter DNA sequences which are presently available reveals a second very highly conserved consensus element which extends for 18 bp immediately upstream from the TATA box and includes a CCAAT motif (Fig. 2A). The very high conservation of this sequence, its precise positioning in the H1 promoter at a site analogous to the H4 and H2b subtype-specific elements, and its absence in the other histone promoters suggested that it may also have a subtype-specific role. It was of interest, therefore, to identify factors that interact with each of these elements and characterize their roles in H1 gene expression.

Figure 2A shows the oligonucleotides which we used in gel shift assays to detect proteins that interact with these H1-specific elements in vitro and the mutant competitor oligonucleotides which were used to demonstrate the specificity of these interactions.

As shown in Fig. 2B, incubation of the HeLa nuclear extract with oligonucleotide probes containing the distal (−120 to −77) or proximal (−65 to −33) H1 subtype-specific elements and separation of the resulting protein-DNA complexes on a nondenaturing gel (14, 15) produced two distinct protein-DNA complexes. Addition of a 500-fold molar ex-

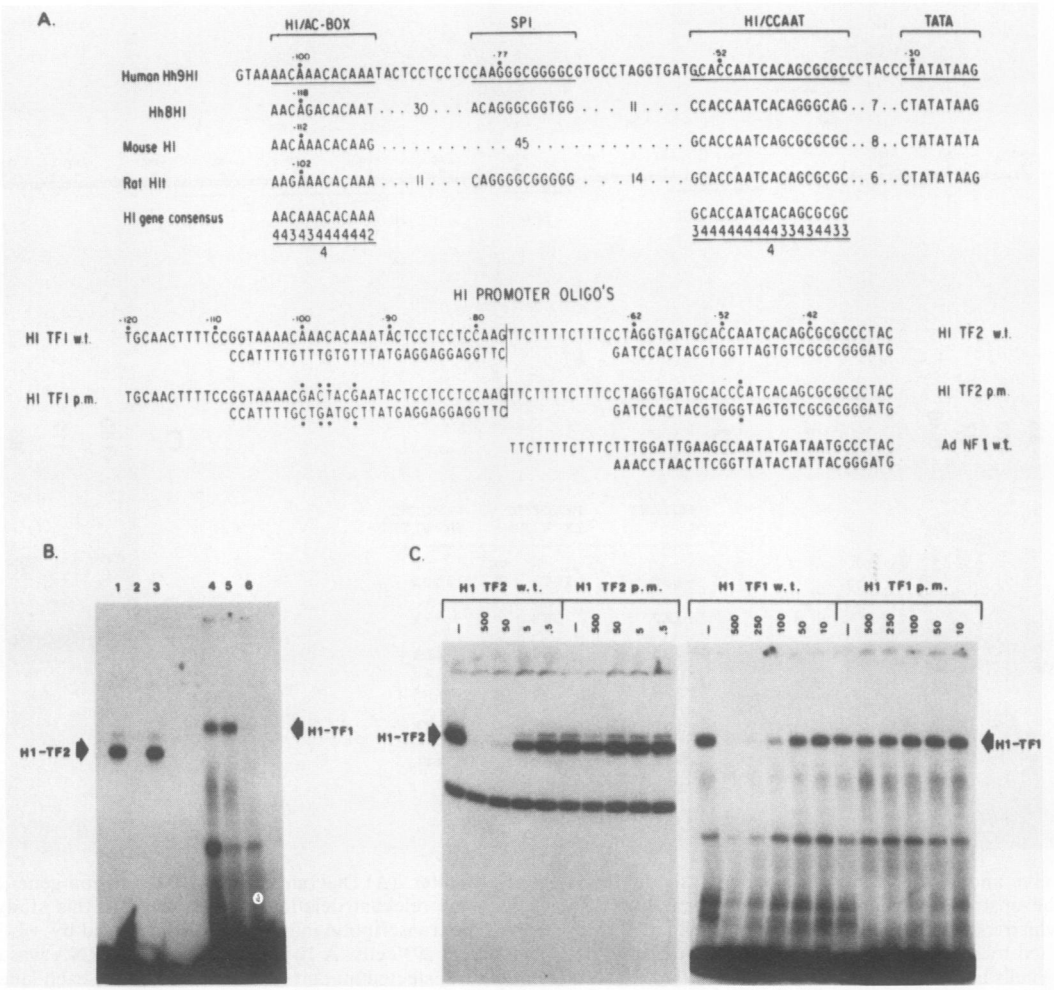


FIG. 2. *cis*- and *trans*-acting elements on the H1 gene promoter. (A) H1 gene conserved 5′ elements. H1 promoter sequences from human, mouse (38), and rat (7) are shown. Distances between conserved regions are in base pairs, and numbering of the promoter elements is relative to the cap sites. H1-specific consensus sequences derived from the comparison of human and cross-species H1 promoters are indicated. The 44-mer wild-type (w.t.) oligonucleotides (H1TF1 and H1TF2), containing the AC box and the CCAAT box, respectively, and the corresponding point mutants (p.m.) are also shown. AdNFI is an oligonucleotide which contains the NFI-binding site from adenovirus type 2 (11, 28). (B) Identification of H1TF1 and H1TF2 binding activities in a crude HeLa nuclear extract by gel shift assay. A 1-ng (34 fmol) amount of labeled H1TF1 or H1TF2 oligonucleotide was incubated with HeLa nuclear extract as described in the text and electrophoresed on a 4% polyacrylamide gel. Bands in lanes 1 and 4 representing specific complexes were identified by competition with a 500-fold molar excess of unlabeled H1TF2 (lanes 2 and 5) or H1TF1 (lanes 3 and 6) oligonucleotide. (C) Identification of H1TF1 and H1TF2 target sequences by competition with point mutant oligonucleotides. Binding assays were performed as described in the text, adding to the reaction serial dilutions of the wild-type or the corresponding point mutant unlabeled oligonucleotide. The molar excess of competitor DNA is indicated at the top of each lane.

cess of the appropriate specific oligonucleotide competitor DNA abolished detectable binding of each factor to the radioactive probe, while incubation in the presence of a similar excess of nonspecific competitor had no effect. This result indicates that in HeLa nuclear extracts, two distinct binding activities (H1TF1 and H1TF2) interact with the H1 AC box and the H1 subtype-specific consensus element, respectively.

To confirm the assignment of the binding sites for these two different proteins, mutant oligonucleotides were used in a quantitative competition assay. As shown in Fig. 2C, the single point mutation in the H1-CCAAT oligonucleotide reduced its competitive ability approximately 100-fold. The protein interacting with this domain of the H1 promoter (H1TF2) is therefore dependent on the CCAAT box for DNA binding. The four sequence alterations that we introduced into the H1 AC box (Fig. 2A) destroyed the ability of the mutant oligonucleotide to compete effectively with the distal probe for binding. H1TF1 must therefore require an intact AC box for binding to the H1 promoter.

H1TF2 is distinct from NFI/CTF. Since H1TF2 interacts with a DNA consensus element containing a CCAAT box, it was important to determine whether this protein is related to the previously described CCAAT-binding factor NFI/CTF (23). A synthetic oligonucleotide was constructed (AdNFI, Fig. 2A) in which 25 bp of the original H1 promoter sequence were replaced with the binding site for NFI/CTF present in the terminal repetition of adenovirus type 2 (11, 28). The H1TF2 and AdNFI oligonucleotides were then either end labeled and used as substrates in gel retardation assays or used as unlabeled competitors. A third oligonucleotide containing the proximal consensus element from a different

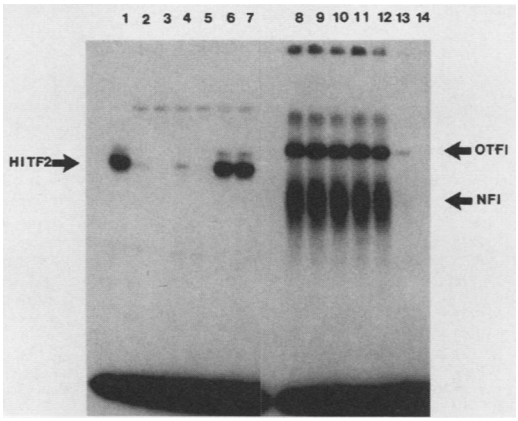


FIG. 3. Analysis of cross-competition between H1TF2- and NFI/CTF-binding proteins by gel shift assay. A 1-ng amount of H1TF2 (lanes 1–7) or AdNFI (lanes 8–14) end-labeled oligonucleotide (see Fig. 2A) was tested by gel shift assay in the presence of three different unlabeled oligonucleotides. Lanes 1 and 8, no competitors added; lanes 2, 3, 9, and 10, 10 ng (lanes 2 and 9) or 40 ng (lanes 3 and 10) of cold H1TF2 oligonucleotide; lanes 4, 5, 11, and 12, 10 ng (lanes 4 and 11) or 40 ng (lanes 5 and 12) of cold Hh8 oligonucleotide containing the subtype-specific domain from the Hh8 H1 gene; lanes 6, 7, 13, and 14, 10 ng (lanes 6 and 13) or 40 ng (lanes 7 and 14) of cold AdNFI oligonucleotide.

human H1 gene (Hh8 H1) (25) was also included in this study.

As shown in Fig. 3, protein-DNA complexes formed by H1TF2 and NFI/CTF in HeLa nuclear extracts with probes containing the different CCAAT DNA sequences migrated differently on nondenaturing polyacrylamide gels. The retarded band observed with the H1TF2 oligonucleotide migrated more slowly than that obtained with the AdNFI probe. The slightly faster-migrating smear of complexes observed with the AdNFI probe is probably due to interaction with NFI/CTF protein, since it resembles the pattern of bands obtained with affinity-purified preparations of NFI/CTF (23). The upper band observed with the AdNFI probe is due to binding of the previously characterized octamer-binding protein (OTF1) (13) to an imperfect octamer DNA sequence present in the AdNFI probe, as indicated by competition experiments with a cold OTF1-specific DNA oligonucleotide (data not shown).

The cross-competition assays shown in Fig. 3 unambiguously indicated that the H1TF2 and the AdNFI probes specifically bound different proteins present in the extract. Thus, excess H1 competitor oligonucleotide derived from either of two human histone H1 genes competed efficiently for binding of H1TF2 to the H1 consensus element (Fig. 3, lanes 2 to 5), whereas excess AdNFI DNA did not compete (Fig. 3, lanes 6 and 7). Conversely, excess NFI/CTF-specific oligonucleotide efficiently competed for binding of both the NFI/CTF and the OTF1 DNA complexes (Fig. 3, lanes 13 and 14), whereas DNA sequences able to bind H1TF2 did not compete (Fig. 3, lanes 9 to 12). These data indicate that H1TF2 is distinct from NFI/CTF in DNA-binding properties and support the possibility that it is an H1-specific CCAAT-binding factor.

Purification and identification of H1TF2 as a 47-kDa CCAAT-binding protein. To further characterize H1TF2, it was extensively purified from HeLa nuclear extracts by sequence-specific DNA affinity chromatography (31, 37). Purification was achieved by using the fractionation scheme previously described (13) for purification of the octamer-binding factor OTF1, since H1TF2 cochromatographs with OTF1 until the last step of this procedure. As shown in Fig. 4A, the scheme used includes several conventional ion-exchange chromatographic fractionations, two steps through nonspecific DNA columns (sonicated salmon sperm DNA and H2b octamer oligonucleotide affinity columns), and a final purification through a DNA resin containing the H1TF2 recognition site. The specific DNA affinity matrix was prepared by coupling a synthetic oligonucleotide containing H1 promoter DNA sequences between −65 and −33 (Fig. 2A) to CnBr-activated Sepharose CL-4B (Pharmacia).

Figure 4B shows the elution profile of H1TF2 binding activity from the oligonucleotide affinity column, as analyzed by gel shift assay, which indicates the presence of H1TF2 in fractions 45 to 55. These fractions were pooled, and a portion was analyzed by SDS-PAGE and silver staining. As shown in Fig. 4C, the pooled fractions from the oligonucleotide affinity column contained a prominent polypeptide with an apparent molecular mass of 47 kDa. Further purification of this fraction by reverse-phase high-pressure liquid chromatography (HPLC) established that the 47-kDa band evident in the silver-stained gel represented a single polypeptide species (data not shown). Repeated attempts to restore binding activity by renaturation from the HPLC gradient were unsuccessful.

To prove that the 47-kDa protein accounts for H1TF2 DNA-binding activity, we used UV crosslinking to transfer isotope from the radiolabeled nucleic acid probe to the specifically bound protein. For these experiments, a portion of the affinity-purified preparation was incubated with an appropriate H1TF2-specific oligonucleotide probe which was uniformly labeled throughout the H1TF2 DNA-binding site. Reactions were performed in the presence of specific or nonspecific competitor oligonucleotides. The H1TF2-DNA complex was separated from unbound probe on a nondenaturing gel and crosslinked in situ by UV irradiation for 20 to 30 min. The specific complex, recognized by competition, was excised from the autoradiographed gel, nuclease digested, eluted from the gel slice, acetone precipitated, and analyzed on an SDS-polyacrylamide gel adjacent to the purified H1TF2 fraction and standard molecular mass markers. Figure 4D shows the autoradiogram of such an SDS gel, demonstrating that only a 47-kDa polypeptide was specifically labeled by this procedure. Thus, the major 47-kDa protein species in the affinity-purified preparation is present in the protein-DNA specific complex resolved by the gel shift assay and can account for the specific DNA-binding properties of H1TF2.

Delineation of nucleotides which interact with purified H1TF2. The extensive conservation of the proximal H1 subtype-specific consensus element which we have noted in Fig. 2A extends for 10 bp 3′ to the CCAAT motif. If the protein we have purified as H1TF2 is the factor which interacts with this sequence in vivo, one might expect that it would protect this extended region upon interaction with H1 DNA. To determine whether this is the case, a 220-bp *Eco*RI-*Pst*I fragment of H1 gene promoter extending between positions −155 and +65 was end labeled at the *Eco*RI terminus by the Klenow fragment and used as the substrate in DNase I protection and methylation interference experiments. Figure 5A shows the results of DNase I footprinting analyses performed on the H1 DNA probe incubated with increasing amounts of affinity-purified H1TF2. This fraction protected a region between positions −55 and −33 that was asymmetrically positioned relative to the CCAAT box, extending further 3′ into the conserved downstream flanking

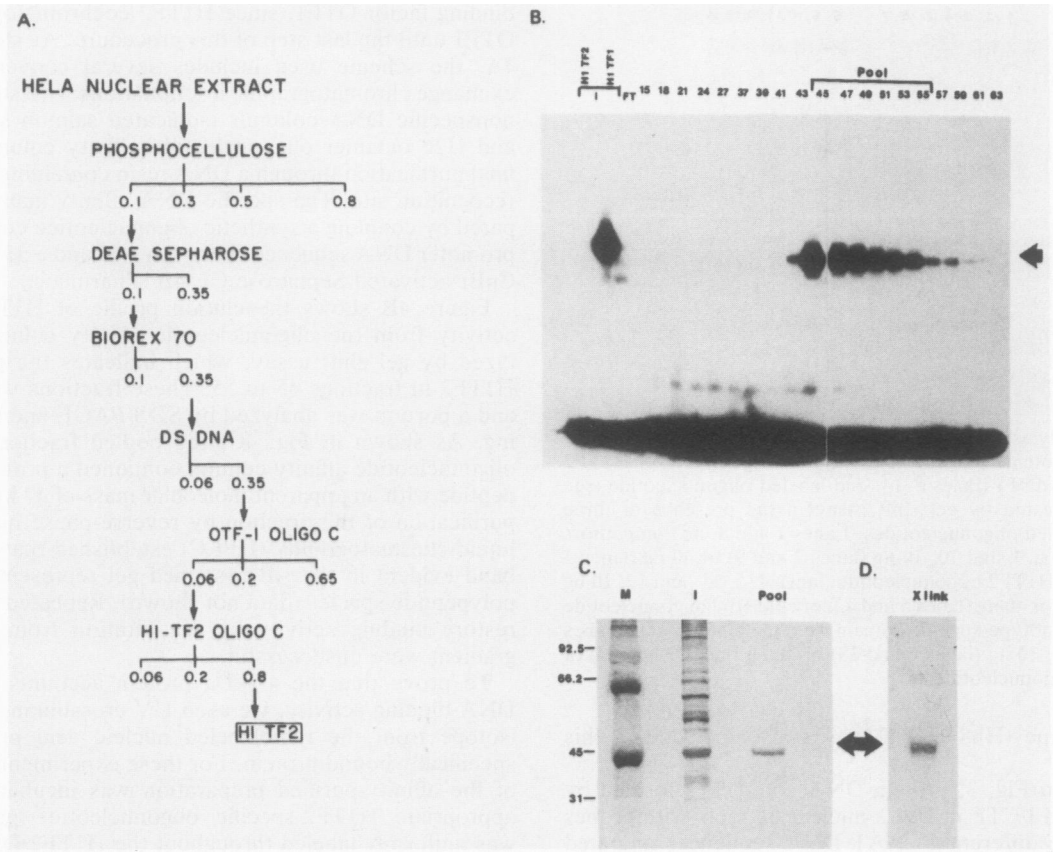


FIG. 4. Purification and identification of H1TF2. (A) Scheme for the purification of H1TF2. Nuclear extract preparation from HeLa cells was performed as described before (21), and the fractionation procedure was derived from that devised for OTF1 purification (13) as detailed in the text. (B) Gel shift analysis of fractions from the H1TF2-specific oligonucleotide affinity column. The OTF1 oligonucleotide affinity column flowthrough was loaded at 60 mM KCl onto an H1TF2-specific oligonucleotide affinity column, and H1TF2 was eluted as described in the text. Samples (1 μl) from the input (I), flowthrough (FT), every third fraction of the 0.2 M KCl step (fractions 15 to 27), and every second fraction of the 0.8 M KCl step (fractions 37 to 63) were analyzed by gel shift assay. Fraction numbers are indicated at the top of each lane. Specific and nonspecific competitors added to the input binding reaction mixes are also indicated. The H1TF2-specific shift is indicated by an arrow. Fractions 45 to 55, containing the peak of H1TF2 binding activity, were pooled and analyzed further. (C) SDS-PAGE. Five-microliter volumes of the column input (I) and of the pooled affinity-purified material (Pool) were loaded on a 10% polyacrylamide–SDS gel, and polypeptides were visualized by silver staining. The arrow indicates the prominent band coeluting with H1TF2 binding activity. Lane M, Protein molecular mass markers (in kilodaltons). (D) In situ crosslinking and SDS-PAGE analysis of the H1TF2-DNA specific complex. A 10-μl sample of the H1TF2 affinity-purified preparation was incubated with the appropriate oligonucleotide probe and processed as described in the text. The specific H1TF2-DNA crosslinked complex (identified by competition) was isolated from the native gel as detailed in the text and analyzed on a 10% polyacrylamide–SDS gel, silver stained to reveal positions of protein molecular mass markers, and autoradiographed. The arrow indicates the band on the autoradiogram corresponding to protein covalently labeled by crosslinking.

region. Thus, the protected region corresponds exactly to the H1 subtype-specific consensus element. Furthermore, the footprint observed on H1 promoter with affinity-purified H1TF2 differed markedly from the DNase I protection patterns described for the previously characterized CCAAT-binding proteins NFI/CTF (23) and C/EBP (17, 22). Both these factors generate footprints that, due to the dyad symmetry of their binding site, extend asymmetrically 5′ to the CCAAT motif.

More detailed information about H1TF2 contact sites on the DNA was derived from methylation interference mapping. The DNA fragment, end labeled on the noncoding strand containing the majority of G residues within the footprint, was partially methylated with DMS and incubated with affinity-purified H1TF2, and the resulting free and bound DNAs were separated on a nondenaturing gel. The DNA bands were then purified from the gel and incubated with piperidine to cleave modified G residues, and the products were displayed on a sequencing gel. Comparison of the free and bound lanes in Fig. 5B reveals that G-52 and G-51, complementary to the two C's of the CCAAT motif, were severely underrepresented in the bound sample, indicating that they are strong contact points for H1TF2. Furthermore, several of the G residues further 3′ in the consensus element within the region protected from DNase digestion by binding of H1TF2 also appeared to be important for H1TF2 binding, although methylation at these positions interfered with H1TF2 binding less strongly than did the two residues within the CCAAT motif.

Fractions containing H1TF2 activity stimulate H1 gene expression in vitro. In order to determine whether H1TF2 can function as a sequence-specific transcription factor, functional assays for in vitro transcription were performed by adding fractions containing H1TF2 activity to HeLa nuclear extracts.

To ascertain that transcriptional stimulation requires the DNA sequence recognized by H1TF2, we compared the effect of H1TF2 addition on transcription from both wild-

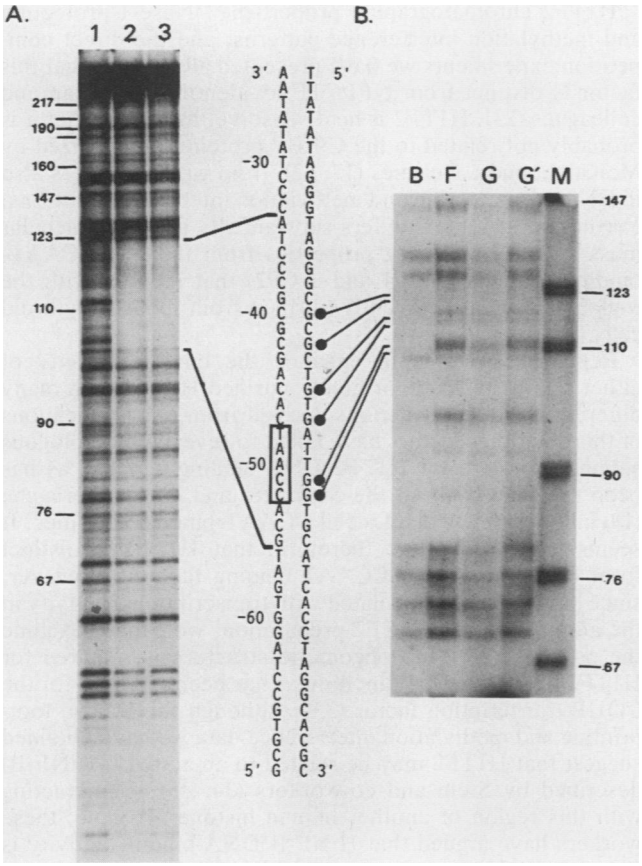


FIG. 5. Analysis of the H1TF2 binding site by DNase I footprinting and methylation interference. (A) DNase I footprinting. The end-labeled *Eco*RI-*Pst*I DNA fragment of the H1 promoter (2 ng) was incubated either without (lane 1) or with 0.5 ng (lane 2) and 1 ng (lane 3) of affinity-purified H1TF2, as detailed in the text. Following DNase I digestion, products were analyzed on an 8% polyacrylamide sequencing gel. Boundaries of the protected region on the complementary coding strand are indicated by bars. The size of pBR322 *Hpa*II fragments (in nucleotides) used as markers is also indicated. (B) Methylation interference. Labeled DNA fragment was partially methylated (29) as described in the text and incubated with affinity-purified H1TF2, and free (F) and bound (B) DNAs were isolated after electrophoresis. Modified G residues were cleaved with piperidine and resolved on a sequencing gel. Lanes: B, DNA bound by factor; F, DNA derived from free probe; G and G', standard G reactions (29) of the same DNA probe; M, DNA size markers (in nucleotides). Dots indicate G residues whose methylation interfered with H1TF2 binding, resulting in the decrease of the corresponding bands in the B sample.

type and CCAAT point mutant templates. A histone H4-*cat* fusion gene in which all promoter elements upstream of the TATA box have been deleted (18) was included as an internal control. As shown in Fig. 6, addition of increasing amounts of the input of the H1TF2-specific oligonucleotide affinity column to a crude HeLa cell nuclear extract resulted in a three-to fourfold induction of transcription from the template containing wild-type H1 promoter. In contrast, addition of this protein fraction to the extract did not result in enhanced transcription from the mutant template containing the single nucleotide change, which was shown to decrease the affinity of H1TF2 binding approximately 100-fold. Thus, this chromatographic fraction contains a sequence-specific, positively acting transcription factor. However, in several separate experiments we have been unable to achieve the same result with the affinity-purified H1TF2 preparation.

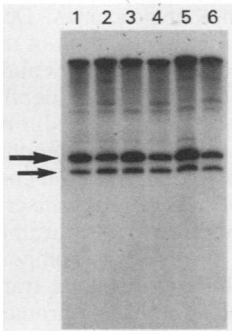


FIG. 6. In vitro transcription activity of purified H1TF2. The wild-type (lanes 1, 3, and 5) or the CCAAT point mutant (lanes 2, 4, and 6) H1 promoter-*cat* construct was transcribed as circular templates in HeLa cell nuclear extracts in the absence or presence of the input of the H1TF2 affinity oligonucleotide column. Reactions were performed as described in the text, and RNA products were S1 mapped and electrophoresed on a 5% polyacrylamide denaturing gel. Lanes 1 and 2, 4 μl of BC60; lanes 3 and 4, 2 μl of protein and 2 μl of BC60; lanes 5 and 6, 4 μl of protein. Large and small arrows indicate the positions of H1-*cat* and H4-*cat* protected bands, respectively.

Although the fractions eluted from the affinity matrix were significantly more active in DNA-binding assays than the input fraction (data not shown), no positive or negative effect on transcription in the reconstitution assay used in Fig. 6 was observed. To determine whether H1TF2 could be a complex transcription factor composed of heterologous subunits (6, 19), several types of reconstitution assays were attempted. However, supplementation of the affinity-purified H1TF2 DNA-binding activity with either the flowthrough or the low-salt fractions from the oligonucleotide column and with fractions derived from earlier steps in the purification procedure (i.e., P11, DEAE, and Biorex 70) did not restore transcription activity. Presently, we do not know the reason for the discrepancy between DNA-binding and transcription activity in the final affinity-purified H1TF2 preparation.

DISCUSSION

The definition of sequence elements and transcription factors regulating histone gene expression in mammalian cells has indicated that subtype-specific factors are crucial for regulation of histone gene transcription (4, 9, 13, 18, 26, 32). This raises several interesting questions about mammalian histone gene expression which can only be addressed by identification of the mechanisms controlling transcription of each histone gene subtype. Thus, it is important to elucidate the detailed molecular interactions which control cell cycle regulation of transcription of individual histone genes, to determine the nature of the biochemical cascade which ultimately results in activation of these genes as cells progress from G1 to S phase, and to discover the point in the cascade where the transcriptional regulation of the five histone gene subtypes becomes coordinated. It will also be interesting to determine whether the transcription factors which are not required for cell cycle regulation serve any role other than to constitutively increase transcription. Finally, it is important to understand whether the mechanisms for histone gene regulation are utilized for S-phase induction

of other cellular genes or processes. Definitive answers to these questions require the isolation of transcription factors involved in expression of these genes and the preparation of specific antibodies to them for in-depth analysis of their chemical structure and interactions during the cell cycle.

This study of the histone H1 promoter is an initial step towards achieving that goal and both confirms and extends previous analyses of histone H1 transcription (10, 34, 39). Thus, both in vivo and in vitro transcription assays demonstrated that the H1 promoter is composed of several elements which contribute to maximal transcription and that the functional organization of this promoter is analogous to that of the histone H4 and H2b genes. However, it is not possible from these data to assign the relative importance of these elements for transcription of the endogenous H1 genes in vivo because both of these assays are done in conditions of DNA excess. It is conceivable, for example, that sequences which contribute in a minor way to total transcription under these experimental conditions are major determinants for transcription under physiological conditions.

The presence of two subtype-specific consensus elements, the H1 AC box and the proximal H1TF2-binding site, raises the question of which of these controls cell cycle regulation of this gene. Dalton and Wells (10) have demonstrated that the H1 AC box is essential for cell cycle regulation of a chicken histone H1 gene. However, no definitive evidence was presented that this single element can confer cell cycle regulation or that other more proximal elements in the promoter are not essential for cell cycle regulation. Although it seems likely that the AC box is indeed a histone H1 transcriptional regulatory element, it is certainly possible that the proximal H1 subtype-specific consensus element is also crucial for temporal control during the cell cycle. More precise mutagenesis experiments will be required to unequivocally resolve this issue.

In this paper we report the identification in HeLa crude nuclear extracts of two distinct proteins, H1TF1 and H1TF2, which bind to the H1 AC box and the H1 proximal subtype-specific consensus element, respectively. It is difficult to determine whether these two proteins are related to those reported in previous studies of the histone H1 promoter. Although in this study we do not present data on the purification and characterization of H1TF1, its chromatographic properties indicate that it is probably not the HiNF-A protein reported by Stein and colleagues (34). On the other hand, it seems likely that the complex we have observed is related to the H1-SF complex reported by Dalton and Wells (10) in their analysis of proteins binding to the chicken histone H1 promoter in vitro. A preliminary analysis of highly purified fractions containing H1TF1 activity indicates that a 90-kDa protein coelutes from the H1TF1 DNA affinity matrix with the specific DNA-binding activity, although we do not have definitive renaturation or crosslinking data to prove this point.

The present study focuses on the purification and the characterization of H1TF2, which interacts with the H1-proximal subtype-specific consensus domain containing a CCAAT motif. By in situ UV crosslinking, we have identified this factor as a single polypeptide species with an apparent molecular mass of 47 kDa. It depends on an intact CCAAT sequence for binding but protects from DNase I digestion essentially the entire conserved sequence between −55 and −33 in this H1 promoter. Furthermore, partially purified H1TF2 preparations contain a positively acting transcription factor which interacts specifically with the CCAAT box on the H1 promoter.

H1TF2 chromatographic properties, DNase I protection and methylation interference patterns, and the direct competition experiments we have presented all indicate that this factor is distinct from NFI/CTF as identified by Tjian and colleagues (23). H1TF2 is heat sensitive, indicating that it is probably not related to the C/EBP protein characterized by McKnight and colleagues (17, 22), from which it differs also in DNase I protection and methylation interference patterns. Furthermore, H1TF2 differs substantially in both molecular mass and DNA-binding properties from the two CCAAT-binding proteins (α-CP1 and α-CP2) that interact with the α-globin promoter, recently purified from mouse erythroid cells (2, 24).

Repeated attempts to enhance the binding activity of either relatively crude or highly purified H1TF2 with many different HeLa cell fractions derived from our purifications of this and other factors have failed to reveal a heterologous subunit structure for H1TF2 DNA-binding activity, as has been reported by both the Sharp (6) and de Crombrugghe (19) laboratories for HeLa cell CCAAT-binding activities. It seems highly probable, therefore, that H1TF2 is distinct from these oligomeric CCAAT-binding factors. However, since binding is not correlated with transcriptional activity in the affinity-purified H1TF2 preparation, we cannot exclude the possibility that an oligomeric structure is required for H1TF2 transcriptional function, as has been reported for the COUP transcription factor (33). Although the DNase footprinting and methylation interference data we have obtained suggest that H1TF2 may be related to an activity (HiNF-B) described by Stein and co-workers (34, 35) as interacting with this region of another human histone H1 gene, these workers have argued that HiNF-B DNA-binding activity is composed of two components which can be reconstituted from P11 fractions, which is not the case for H1TF2. Finally, the information available for the other CCAAT-binding activities which have been reported (12, 30, 33) is not sufficient for us to determine whether they are related to H1TF2.

Characterization of the chemical and biochemical properties of H1TF1 and H1TF2 as cells progress through the cell cycle will require preparation of appropriate molecular and immunological reagents to each of these factors. Our present efforts are directed toward this goal and toward determining whether the mechanisms modulating this H1 subtype-specific factor(s) are identical to those regulating the histone H2b and H4 cell cycle-regulatory factors.

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