Human H4 Histone Gene Transcription Requires the Proliferation-specific Nuclear Factor HiNF-D

AUXILIARY ROLES FOR HiNF-C (Sp1-LIKE) AND HiNF-A (HIGH MOBILITY GROUP-LIKE)*

(Received for publication, November 23, 1988)

Andre J. van Wijnen, Kenneth L. Wright, Jane B. Lian, Janet L. Stein, and Gary S. Stein

From the Department of Cell Biology, University of Massachusetts Medical School, Worcester, Massachusetts 01655

The proximal promoter of the human H4 histone gene F0108 contains two in vivo protein binding domains, sites I and II. In this report we show that these sequences interact with three nuclear factors: HiNF-D, HiNF-C, and HiNF-A. HiNF-C and HiNF-A bind independently to the distally located site I, possibly in conjunction with other proteins, and deletion of site I reduces transcription rates 4- to 6-fold in vitro. Factor HiNF-D binds to an H4 histone-specific element (5'-dGGTPyPyTCAATCNGGTCCG, where Py indicates pyrimidine) present in site II that has previously been shown to be essential for in vitro expression of this H4 histone gene. All three binding activities are present in human HeLa S3 cells throughout the cell cycle and in exponentially growing mouse C127 and human HL60 cells. This result is consistent with the transcription of H4 histone genes throughout the cell cycle. However, unlike HiNF-A and HiNF-C, HiNF-D is not present in terminally differentiated HL60 cells, in which histone transcription is down-regulated. These findings suggest a crucial role for HiNF-D, with an auxiliary role for HiNF-C and possibly HiNF-A, in the regulation of H4 histone gene transcription. Furthermore, the conservation of potential HiNF-D binding sites in mammalian H4 histone gene promoters suggests that HiNF-D has an essential role in the coordinate transcriptional down-regulation of the H4 histone multigene subfamily during the shutdown of proliferation.

Histone genes comprise a heterogeneous multi-copy family with gene products that are an essential component of eukaryotic chromatin. The expression of the most abundant class of histone genes (designated cell cycle-regulated) is tightly coupled to DNA replication and, hence, highly specific for proliferating cells (reviewed in Refs. 1-3). Thus, understanding the control mechanisms that influence the expression of the proliferation-specific histone genes should provide insight into gene regulatory events occurring in both normally dividing and transformed cells as a function of the cell cycle (1-3), cell aging (4, 5) and the onset of differentiation (6, 7).

Cell cycle-dependent histone gene expression has a prominent post-transcriptional component (reviewed in Refs. 8, 9) as evidenced by a 15-20-fold increase in histone mRNA levels when DNA replication initiates and a rapid destabilization of histone mRNA at the natural (or chemically induced) termination of DNA synthesis (10-15). Histone mRNA levels parallel the extent of histone protein synthesis, consistent with histone mRNA abundance as a rate-limiting step in histone gene expression. Histone mRNA turnover requires continued protein synthesis (16, 17) and may involve 3' end processing events (18, 19), particular histone mRNA sequences (20-23), a histone mRNA degrading exonuclease (24, 25), and possibly specific subcellular structures (26, 27).

The transcriptional component of histone gene expression during the cell cycle involves a 3- to 5-fold transient increase of histone mRNA synthesis rates at the onset of S-phase (10-15) and contributes to the accumulation of histone mRNA in the early half of S-phase. However, in dividing cells replication-dependent histone mRNAs are synthesized throughout the cell cycle (albeit rapidly degraded) and only in terminally differentiated cells that have lost the ability to proliferate is there a complete shutdown of histone mRNA transcription (6).

Vertebrate histone gene promoters have a modular organization, and the transcriptional regulation of each individual histone gene involves multiple, distinct promoter elements (28-34). Several DNA binding proteins interacting in vitro with such elements in the promoters of H1 (33, 35-37), H2B (38, 39), H3 (34, 40), and H4 (40-43) histone genes have been characterized. These studies suggest that transcriptional control of histone gene expression involves a plethora of promoter factors that either are histone-specific or recognize a broad spectrum of gene promoters. A common principle underlying the coordinate transcriptional regulation of the histone genes remains to be established. Recently, our laboratory has established the in vivo sites of DNA/protein interactions of human H3 and H4 histone gene promoters (44-46). From these in vivo DNA/protein interaction data it has become apparent that the histone gene promoter factors studied thus far (33-43) comprise only a subset of the full complement of factors that potentially can associate with each individual histone gene.

The active expression of the H4 histone gene F0108 in proliferating cells involves a dynamic cell cycle-dependent chromatin structure (47-50) and both distal and proximal promoter elements (28-30). We have previously shown that the H4 histone proximal promoter interacts in vitro with a factor designated HiNF-A (40) that also binds to H3 (40) and H1 histone gene promoters (35). The H4 histone proximal promoter contains two in vivo DNA/protein interaction sites (sites I and II) (44). In this study we have examined the binding in vitro of three distinct nuclear factors (HiNF-A, HiNF-C, and HiNF-D) to these DNA sequences. The binding

* This work was supported by National Institutes of Health Grant GM32010, National Science Foundation Grant DCB88-96116, and March of Dimes Birth Defects Foundation Grant 1-1091. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
sites of the factors were defined by deletion analysis, DNasel footprinting, and/or DMS\(^1\) fingerprinting. We have explored the functional significance of these DNA/protein interaction sites by in vitro transcription assays and by monitoring the presence of the factors involved in various cell lines and biological situations. We propose that the interaction of HiNF-D with the proximal promoter is crucial in the regulation of H4 histone mRNA synthesis and is implicated in the down-regulation of histone gene transcription at the onset of differentiation.

**MATERIALS AND METHODS**

Fractionation of Nuclear Extracts—Undialyzed nuclear extracts (UNE) from exponentially growing HeLa S3 cells (35) and nuclear extracts of mouse C127 monolayer cells (68) were prepared by the procedure of Chailly and Kelberg (51). Dialyzed nuclear extracts (DNE) from HeLa S3 cells synchronized by double thymidine block (40) were prepared according to Dignam et al. (52) at multiple hourly intervals after release from the blockade, extending into the second S-phase (up to 25 h). The preparation of nuclear extracts from proliferating and differentiated human HL60 cells was done according to the Dignam procedure (52). Exponentially growing HL60 cells were induced to differentiate by treatment with 16 nM of the phorbol ester tetradecanoylphorbol-13-acetate.

The functional significance of these DNA/protein interaction sites was determined essentially as described previously (40). Binding reactions containing 0.5 ng of a \(32P\)-labeled DNA fragment and 2 pg of poly(dI-dC) DNA (Pharmacia LKB Biotechnology Inc.) and the extract were incubated for an additional 15 min on ice in dilution buffer (20% glycerol, 20 mM NaCl, 0.1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 25 mM Hepes/NaOH, pH 7.5) containing various concentrations of chelators; the preincubated extracts were diluted 2-fold during the binding reaction. For reconstitution experiments, undialyzed nuclear extracts were preincubated for 15 min on ice in nuclear extraction buffer (10% sucrose, 0.4 M KCl, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 50 mM Hepes/NaOH, pH 7.5) containing 0.5 mM 1,10-phenanthroline (stock solution: 0.5 M in isopropanol alcohol). The mixture was diluted 10-fold in dilution buffer containing the indicated amounts of divalent cations, incubated for an additional 15 min on ice, and finally diluted 2-fold in the binding reaction.

In Vitro Transcription Analysis—In vitro transcription experiments were performed with supercoiled plasmids pUC8 and JC50 essentially as described by Dignam et al. (32). RNA transcripts were analyzed by S1 nuclease protection using a single end-labeled probe overlapping the transcriptional initiation site. The reaction products were analyzed on a 6% sequencing gel and the intensity of a 390-nt fragment, corresponding to accurately initiated F0108 H4 histone mRNA, was quantitated by densitometry.

**RESULTS**

The proximal promoter of the human H4 histone gene F0108 contains multiple consensus regulatory sequences that can potentially function as recognition sites for distinct DNA binding proteins (28, 44; refer to Fig. 5 for details). Several of these elements appear to interact in vivo with factors present in HeLa S3 cells and are located in two domains designated site I (nt -151 to -114) and site II (nt -97 to -58) (44). Site I contains AT-rich sequences (distal), an AAATGACG motif (distal), and an Sp1 consensus sequence (proximal); site II consists of an H4 histone-specific element (distal) and the TATA box (proximal). An additional Sp1 box located immediately upstream of site II does not appear to be bound by a factor in vivo in HeLa S3 cells.

**Factor HiNF-A Binds to the Distal Part of Site I**—The binding of HiNF-A to the promoter of the F0108 H4 histone gene (Fig. 1) was studied using several deletion mutants and synthetic DNA fragments in order to define the binding site requirements for this factor. The binding of HiNF-A to a radiolabeled DdeI/TaqI fragment (nt -183/-133) was established in a gel-retardation assay (Fig. 1A). Incubation of this probe with increasing amounts of nuclear extracts (lanes 1–5) or partially purified HiNF-A (P550–1000 fraction) (lanes 6 and 7) shows a fast migrating complex characteristic of HiNF-A. The binding of HiNF-A was also examined by the gel-retardation assay using BAL31 deletion mutants of the H4 histone promoter (Fig. 1B). HiNF-A binds to the -182/13 probe (lanes 1–3), but not the -147/13 or -141/13 fragments (lanes 4–6 and 7–9). The binding of HiNF-A to synthetic oligonucleotides spanning the distal part of site I (DSI: nt -152/-128) and the distal part of site II (DSS: nt -91/-64) was studied (Fig. 1C). HiNF-A binds to the site I fragment in the presence of E. coli DNA (lane 1), although not efficiently in the presence of poly(dI)-d(C) DNA (lane 2), and the HiNF-A complex is not detected with the site II fragment in uitro.
Two Additional Factors, HiNF-C and HiNF-D, Interact with the H4 Histone Promoter—The DNA sequences in the proximal promoter of the F0108 H4 histone gene that correspond to the two in vivo sites of DNA/protein interaction have the potential to bind at least four distinct factors, based on the presence of consensus promoter elements and the distribution of protein/nucleotide contacts (Ref. 44; see Fig. 5). At least six in vitro DNA-protein complexes that occur in the H4 histone proximal promoter between nt -152 and nt -13, which coincides with the distal part of site I. However, several lines of evidence suggest that HiNF-A may not be the only factor binding to this region (see below).

Two Additional Factors, HiNF-C and HiNF-D, Interact with the H4 Histone Promoter—The DNA sequences in the proximal promoter of the F0108 H4 histone gene that correspond to the two in vivo sites of DNA/protein interaction have the potential to bind at least four distinct factors, based on the presence of consensus promoter elements and the distribution of protein/nucleotide contacts (Ref. 44; see Fig. 5). At least six in vitro DNA-protein complexes that occur in the H4 histone proximal promoter between nt -152 and nt -13, which coincides with the distal part of site I. However, several lines of evidence suggest that HiNF-A may not be the only factor binding to this region (see below).

Two Additional Factors, HiNF-C and HiNF-D, Interact with the H4 Histone Promoter—The DNA sequences in the proximal promoter of the F0108 H4 histone gene that correspond to the two in vivo sites of DNA/protein interaction have the potential to bind at least four distinct factors, based on the presence of consensus promoter elements and the distribution of protein/nucleotide contacts (Ref. 44; see Fig. 5). At least six in vitro DNA-protein complexes that occur in the H4 histone proximal promoter between nt -152 and nt -13, which coincides with the distal part of site I. However, several lines of evidence suggest that HiNF-A may not be the only factor binding to this region (see below).

Two Additional Factors, HiNF-C and HiNF-D, Interact with the H4 Histone Promoter—The DNA sequences in the proximal promoter of the F0108 H4 histone gene that correspond to the two in vivo sites of DNA/protein interaction have the potential to bind at least four distinct factors, based on the presence of consensus promoter elements and the distribution of protein/nucleotide contacts (Ref. 44; see Fig. 5). At least six in vitro DNA-protein complexes that occur in the H4 histone proximal promoter between nt -152 and nt -13, which coincides with the distal part of site I. However, several lines of evidence suggest that HiNF-A may not be the only factor binding to this region (see below).

Two Additional Factors, HiNF-C and HiNF-D, Interact with the H4 Histone Promoter—The DNA sequences in the proximal promoter of the F0108 H4 histone gene that correspond to the two in vivo sites of DNA/protein interaction have the potential to bind at least four distinct factors, based on the presence of consensus promoter elements and the distribution of protein/nucleotide contacts (Ref. 44; see Fig. 5). At least six in vitro DNA-protein complexes that occur in the H4 histone proximal promoter between nt -152 and nt -13, which coincides with the distal part of site I. However, several lines of evidence suggest that HiNF-A may not be the only factor binding to this region (see below).

Two Additional Factors, HiNF-C and HiNF-D, Interact with the H4 Histone Promoter—The DNA sequences in the proximal promoter of the F0108 H4 histone gene that correspond to the two in vivo sites of DNA/protein interaction have the potential to bind at least four distinct factors, based on the presence of consensus promoter elements and the distribution of protein/nucleotide contacts (Ref. 44; see Fig. 5). At least six in vitro DNA-protein complexes that occur in the H4 histone proximal promoter between nt -152 and nt -13, which coincides with the distal part of site I. However, several lines of evidence suggest that HiNF-A may not be the only factor binding to this region (see below).

Two Additional Factors, HiNF-C and HiNF-D, Interact with the H4 Histone Promoter—The DNA sequences in the proximal promoter of the F0108 H4 histone gene that correspond to the two in vivo sites of DNA/protein interaction have the potential to bind at least four distinct factors, based on the presence of consensus promoter elements and the distribution of protein/nucleotide contacts (Ref. 44; see Fig. 5). At least six in vitro DNA-protein complexes that occur in the H4 histone proximal promoter between nt -152 and nt -13, which coincides with the distal part of site I. However, several lines of evidence suggest that HiNF-A may not be the only factor binding to this region (see below).
Fig. 3. Localization of the binding sites of HiNF-C and HiNF-D. A, DNaseI footprint of isolated HiNF-C complex; lane 1: DNaseI digestion pattern of unbound probe (U); lane 2: pattern of the isolated HiNF-C complex using UNE protein (P); lane 3: guanine-sequence ladder of probe (nt -240/-13; EcoRI/BanII fragment of pFP-1); lanes 1-3: DNA sequence ladder of probe (nt -240/-13; EcoRI/BanII fragment of pFP-1). B, DNA sequence ladder of probe (nt -240/-13; EcoRI/BanII fragment of pFP-1). C, guanine residues near the footprint; arrows point at the positions of bottom strand (nt -130/-40) and 2 pg of DNE protein using a site I1 fragment as competitor (nt -98/-40; NaeI/HindIII fragment of pFP-1); lanes 1-5: respectively, 0-, 5-, 10-, 20-, and 50-fold molar excess of the site II fragment.

Fig. 4. Summary of binding site deletion experiments. Shown are the H4 histone proximal promoter with the in vivo DNA/protein interaction sites I and II (top) and the various fragments used for binding studies (bottom). Oligonucleotide Dd/Tq refers to a natural DNA fragment spanning nt -183 to -130. Formation of the A, C, and D complexes with the various fragments is indicated on the right (plus, binding of factor; minus, no binding; ND, not determined).

Fig. 5. Sequence of the proximal promoter of the F0108 H histone gene (see text for details and references). Lanes under the sequence represent the in vivo DNaseI footprints of sites I and II, and circles represent the combination of in vivo DMS fingerprints of guanines on the top and the bottom strand. Dashes between the strands represent various consensus sequences as detailed in the text, the distal part of site I contains both an AT-rich repeat (5'dAAAT[N4]AAAT) and an AAATGACG element that partially overlap. Solid brackets show the DNaseI footprint boundaries of HiNF-C and HiNF-D, while bracket with long dashes represents the binding domain of HiNF-A as established by deletion analysis; brackets with short dashes indicate the possible binding domains of uncharacterized factors. Arrowheads indicate positions of restriction sites used in binding site deletion analysis.

* A. J. van Wijnen, J. L. Stein, and Gary S. Stein, unpublished data.
site I may interact with HiNF-E or a related factor.

**Factor HiNF-D Binds to the Distal Part of Site II**—The interaction of HiNF-D with sequences at or near the histone-specific consensus elements in site II was confirmed and analyzed in more detail by DNase I footprinting experiments. HiNF-D was bound to a probe (nt -130 to -40) labeled on the bottom (anti-sense) strand and the binding mixture treated with DNase I. After electrophoretically separating the HiNF-D complex from unbound DNA on native gels, the DNase I cleavage products were analyzed on a denaturing gel. Fig. 3C demonstrates that HiNF-D clearly protects a number of phosphodiester bonds between nt -66 to -90 on the bottom strand from DNase I cleavage. Some nucleotides were less well protected than others, and we also observed apparent partial protections beyond nt -66 near the TATA box (not indicated); however, only between nt -66 and -90 did we observe contiguous protections. This region is entirely contained within the distal part of the in vivo DNase I footprint designated site II and encompasses the histone-specific element (see Fig. 5 for summary).

The binding of HiNF-D to site II was further established by competition analysis using a DNA fragment spanning the entire site II (nt -98 to -40). The inclusion of 20- to 50-fold molar excess of this DNA fragment resulted in a corresponding decrease in the intensity of the band representing the HiNF-D complex, but not in the band representing the HiNF-C complex (Fig. 3D). Competition experiments with the distal site II oligonucleotide (nt -91 to -64) showed a similar decrease in HiNF-D complex formation, although competition was less efficient, possibly due to the smaller size of the DNA fragment used (data not shown).

A number of vertebrate H4 histone gene promoters (31, 44, 55–58) contain sequences remarkably similar to the HiNF-D binding site. These sequences are located at analogous positions within these promoters and conform to the mammalian consensus sequence: 5′-dGgPtyPtyTcaAtcGngTcG (Py is pyrimidine).

**Effect of Site I and Site II on H4 Histone Gene Transcription**—The functional properties of H4 histone promoter elements were studied utilizing BAL31 deletion mutants in an in vitro transcription system. We used supercoiled templates (containing the intact H4 histone gene with promoter segments truncated at nt -182 (K8) and nt -100 (J56)), incubated these in the presence of nuclear extracts, and subjected the transcription products to S1 nuclease analysis (Fig. 6). The amount of accurately initiated transcription obtained with mutant K8, comprising both sites I and II, was 4.2 arbitrary densitometry units (n = 4, S.D. = 0.5), whereas mutant J56, which has a deletion spanning site I, yielded 0.83 units (n = 4, S.D. = 0.33). No significant effect was observed in vitro when deletions were introduced into site II (data not shown). Thus, the site I DNA/protein interactions are capable of augmenting H4 histone gene transcription rates 3-5-fold.

The in vivo expression of the human H4 histone gene F0108 has been studied in a heterologous murine cell system (mouse C127 cells) (29). The cross-species comparability of mouse and human promoter-binding factors is evidenced by gel retardation assays performed with mouse C127 nuclear extracts. Complexes are detected that are similar to those formed by HiNF-D, HiNF-C (Fig. 7, lanes 1–4), or HiNF-A (lanes 5–8), and the binding sites of the murine factors coincide with those of the human counterparts as established by footprinting analysis using human and mouse nuclear proteins in parallel (data not shown). Although no effects with site II deletions up to nt -74 were observed in the homologous in vitro system, Kroeger et al. (29) have reported a profound effect of the same deletions extending into site II in the murine in vivo system. Accurate initiation of H4 mRNA transcription in vivo was observed when sequences up to nt -100 are present, but when sequences upstream of nt -74 were deleted no human H4 histone mRNAs were detected. The latter deletion perturbs the HiNF-D binding site, strongly indicating that the interaction of HiNF-D with site II, although dispensable under our in vitro conditions, is essential for the in vivo regulation of human H4 histone gene transcription.

**HiNF-D Is Specific for Proliferating Cells Synthesizing Replication-dependent H4 Histone mRNAs**—Human H4 histone mRNAs are transcribed throughout the HeLa S3 cell cycle with a transient 3-5-fold increase in transcription rates occurring at the G1/S-phase boundary (10, 11). One of the mechanisms by which the cell modulates histone mRNA transcription rates during the cell cycle could involve, for instance, alterations in the DNA binding activity of HiNF-A, HiNF-C, or HiNF-D. However, the overall DNA binding activity of these factors as a function of the HeLa S3 cell cycle does not vary (Fig. 8). The factors can be detected both in early S-phase and G1-phase HeLa S3 cells (A, lanes 1–8;
**Human H4 Histone Gene Promoter Factors**

5-8, 81, procedure) operating HL60 cells (DNE-protein added) and terminally differentiated HL60 cells. Factor HiNF-A is present in nuclear extracts from HeLa cells. Similar DNA binding activities in S-phase (nuclear extract prepared in nuclear extracts of terminally differentiated HL60 cells) were shown with no significant changes in DNA binding activity. The level of detection following treatment of HL60 cells with factors HiNF-C (Fig. 8B) and HiNF-D (Fig. 8D) is related to the transcription of this gene.

Factors HiNF-C (Fig. 8F) and HiNF-A (Fig. 8D) are present in both proliferating and terminally differentiated HL60 cells. Most interestingly, although HiNF-D activity is clearly present in exponentially growing cells (Fig. B), this activity is selectively lost during differentiation. Hence, the site II binding protein HiNF-D is specific for proliferating cells. These results suggest a principal role for HiNF-D in the regulation of transcription of the cell cycle-dependent H4 histone genes. In support of this are *in vivo* DMS protection experiments in the living cell (6) in which protection of sites I and II was observed in proliferating HL60 cells, but only protection of site I, and not site II, was seen in terminally differentiated HL60 cells.

**Temperature and Detergent Stability of Factor HiNF-D**—The binding site specificity of HiNF-D is very similar to that of factor H4TF-2, isolated by Dailey et al. (41, 42), which binds to a different H4 histone promoter (designated Hu4a). However, the migration rate of the HiNF-D complex in gel retardation assays is very different from the H4TF-2 complex. We have studied the temperature and detergent stability of factor HiNF-D to investigate its physical properties and to facilitate a comparison with H4TF-2. Nuclear extracts were subjected to various mild denaturing conditions and subjected to the gel retardation assay (Fig. 9). The addition of low concentrations of SDS (above 0.002%) prevented the formation of complex HiNF-D (Fig. 9B), but the addition of various concentrations (up to 1%) of non-ionic detergents such as Nonidet P-40 and Triton X-100 had no effect on the binding.

When nuclear extracts were gently heated during the binding reaction (Fig. 9A), we observed a decrease in the formation of the HiNF-D complex with increasing incubation temperatures from 37 to 55°C (lanes 1–9). In a second experiment, nuclear extracts were preincubated at the same temperatures prior to the binding reaction (10–18). In this case we observed that HiNF-D remains relatively stable up to 50°C and becomes rapidly destabilized at 52°C and higher temperatures. These data establish that HiNF-D is heat-labile in a small temperature range, and this property may aid in a comparison between HiNF-D and H4TF-2. Interestingly, we observed an increase in the formation of a complex with higher mobility (designated D') with increasing temperatures when proteins were heated during the binding reaction (compared lanes 1–9 with lanes 10–18). The nature of the factor involved is currently not known.

**Salt Dependence of HiNF-C and HiNF-D Complex Formation**—The salt stability of *in vivo* DNA/protein interactions

![Fig. 8. Gel retardation analysis showing selective loss of HiNF-D DNA binding activity in terminally differentiated cells and constitutive presence of the H4 histone promoter factors during the cell cycle.](image)

![Fig. 9. Temperature, detergent, and salt stability of HiNF-D complex formation in the gel retardation assay.](image)
at sites I and II has been tested in isolated human HeLa S3 nuclei by a salt extraction procedure (44). The H4 proximal promoter factors gradually dissociate in vivo at salt concentrations between 120 and 200 mM KCl. We studied the salt dependence of HiNF-C and HiNF-D complex formation in gel-retardation assays to allow a comparison between in vivo and in vitro salt stability, as an initial measure of the relative binding affinity of these factors in vivo and in vitro. The results (Fig. 9D) show that HiNF-D binding is unstable at KCl concentrations between 80 and 110 mM but that HiNF-C binding is relatively stable up to 150 mM KCl (Fig. 9D). From these experiments it appears that HiNF-D binding is much more sensitive to increased ionic strength conditions than HiNF-C binding.

Factor HiNF-C Requires Divalent Cations for Site-specific Binding—We observed a significant loss of HiNF-C DNA binding activity upon dialysis of nuclear extracts against EDTA during chromatographic fractionation, and mixing of various fractions did not lead to restoration of HiNF-C activity (data not shown). A number of DNA binding proteins have been described that contain amino acid motifs capable of divalent cation binding (e.g. zinc binding proteins; 59). Therefore, we considered the possibility that HiNF-C would have a similar divalent cation requirement for its DNA binding activity and possibly its stability.

Titration experiments were performed in which the binding of HiNF-C was studied in the presence of various concentrations of metal ion chelating agents. HiNF-C activity was virtually undetectable when nuclear extracts were preincubated in the presence of 1 mM 1,10-phenanthroline (Fig. 10A). The binding of HiNF-C partially decreased after preincubation with 10 mM EDTA but was not affected by EGTA (10 mM). These data strongly suggest that HiNF-C has a requirement for divalent cations. Pretreatment with 1,10-phenanthroline had a gradual, inhibitory effect at a range of concentrations between 0.1 and 1.0 mM (Fig. 10B, lanes 1–8) and its effect was also observed if the preformed HiNF-C complex was challenged with the chelator after formation in the binding mixture (lanes 9 and 10).

The metal ion requirement of HiNF-C was further explored by reconstitution experiments in which HiNF-C was first partially inactivated by 1,10-phenanthroline and subsequently incubated in the presence of various amounts of divalent cations (Fig. 10C). Binding activity could not be restored by adding Mg2+ or Ca2+ (data not shown), but the addition of an optimal amount of Zn2+ (approximately 100 μM) restored HiNF-C binding. This result implies that HiNF-C has a selective metal ion requirement and that Zn2+ ions are sufficient for the restoration of binding activity.

Factor HiNF-D Is 1,10-phenanthroline Sensitive at Elevated Temperatures—Chelation experiments aimed at showing a divalent cation requirement for factor HiNF-D did not result in decreased binding activity under the assay conditions described above. Dailey et al. (60) have reported that H4TF-2 under similar conditions is very sensitive to 1,10-phenanthroline and has a divalent cation requirement (Zn2+ or Fe2+). We reasoned that the metal ion in HiNF-D might be very tightly bound and therefore protected from chelation. The possibility was investigated whether HiNF-D would display sensitivity toward chelators at elevated temperatures.

HiNF-D was preincubated with 1,10-phenanthroline at temperatures close to the point where its DNA binding ability is thermally inactivated. The binding of HiNF-D gradually decreased when the factor was preincubated at temperatures between 48 and 55°C (Fig. 11). No effect was observed when nuclear extracts were preincubated with EDTA or EGTA (10 mM) (data not shown). Interestingly, when 1,10-phenanthroline was added at these elevated temperatures HiNF-D displayed sensitivity toward this chelator (range 1–10 mM) that became more pronounced as temperature increased. These results demonstrate that HiNF-D and H4TF-2 are differentially sensitive toward divalent cation chelators and suggest that the protein structures of these factors are different.

DISCUSSION

The proximal promoter of the human H4 histone gene F0108 has been shown to contain two in vivo sites of DNA/protein interaction (sites I and II) (44). In this report, we have demonstrated that the DNA sequences of this promoter (nt −30 to −240) form at least three different, specific DNA/protein complexes in vitro, the complexes of HiNF-A, HiNF-C, and HiNF-D. Factor HiNF-A binds to AT-rich sequences in the distal part of site I. HiNF-A has a moderate sequence specificity as its binding in gel-retardation assays is competed...
out by a very small amount of the nonspecific competitor poly(dI-dC) DNA (<100 ng) and a moderate amount of E. coli DNA (>1 μg). The detection of HiNF-A in gel-retardation assays, despite its limited sequence specificity, may be a reflection of its abundance in nuclear extracts, thus excluding a role for HiNF-A as a limiting transcription factor. HiNF-A may belong to a class of DNA binding proteins that bind to A/T-rich DNA sequences such as human high mobility group I (61) or monkey α-protein (62) and may have a chromatin structural role as proposed for these and other high mobility group proteins. In this regard, it must be noted that HiNF-A binding sites are present in the proximal promoters of human H4, H3, and H1 histone genes (35, 40) and that the chromatin structure of these promoters is subject to dynamic changes during the cell cycle (47-50).

Factor HiNF-C binds to a DNA fragment (nt -99 to -130) containing an Sp1 consensus binding site (54; match; 8 of 10 basepairs) present in the proximal part of site I. The DMS protection pattern of total nuclear protein in this region shows only protection of nucleotides coinciding with the Sp1 box. This in vitro DMS protection pattern is identical to the DMS footprint observed in vivo in the F0108 H4 histone promoter (44) and very similar to DMS protections observed in vitro for Sp1 binding to Sp1 boxes (63). The estimated DNaseI footprint of HiNF-C spans approximately 20 nt, which is in close agreement to the DNaseI footprint size of Sp1. Factor HiNF-C has a selective, divalent cation requirement for DNA binding, and Zn2+ ions are sufficient in regenerating HiNF-C activity after treatment with the metal ion chelator 1,10-phenanthroline (<1 mM). Factor Sp1 has been shown to contain cysteine-rich motifs assumed to be involved in binding of Zn2+ ions (zinc-fingers; reviewed in Ref. 59), although Sp1 binding was influenced by the addition of EDTA (50 mM), but not 1,10-phenanthroline (25 mM) (64). We propose that HiNF-C is a general promoter factor similar or identical to Sp1.

Factor HiNF-D binds to the distal part of in vivo site II (nt -50 to -90) containing a mammalian histone-sense consensus element (5′-dGGTPyPyTCAATC(N)GGTCCG) of H4TF-2 and HiNF-D appear to be different. We conclude that although the binding site specificities of HiNF-D and H4TF-2 are very similar, the factors are distinct in many other respects.

The proximal part of site II contains a TATA element that is protected in vivo in conjunction with the HiNF-D site. The distance between potential HiNF-D sites in several mammalian H4 histone genes and the adjacent TATA box sequence is strictly conserved (9 basepairs). This strict spacing constraint suggests that HiNF-D binds in vivo in conjunction with a putative TATA box factor (65) to site II. Consistent with this hypothesis is that HiNF-D binding by itself in vitro is stable only at moderate ionic strength conditions. This hints at the possibility that HiNF-D binding in vivo requires a stabilizing factor(s). In this regard, the fact that HiNF-D is required for accurate transcription initiation in vivo but not in vitro could indicate that the factor is involved in recruiting a TATA box factor that may be limited in vivo, but not in vitro.

In conclusion, the H4-Site I binding proteins HiNF-A and HiNF-C are not proliferation specific. The functional significance of binding events at site I is evidenced by a 4-6-fold reduction in the efficiency of in vitro histone mRNA synthesis upon deletion of this site. We propose that the interaction of HiNF-C with site I, possibly in conjunction with HiNF-A and/or other uncharacterized factors, has an auxiliary (housekeeping) function in augmenting H4 histone mRNA synthesis rates and that the roles of these factors are not confined to H4 histone gene transcription. The H4-site II binding factor HiNF-D is proliferation specific and the binding of HiNF-D is essential for in vivo expression of the F0108 human H4 histone gene. We postulate that the interaction of HiNF-D with site II performs an essential function (on/off switch) without which no transcription can occur. Because the HiNF-D binding site is conserved in a number of mammalian H4 histone genes and the DNA binding activity of the factor is down-regulated during differentiation, it is conceivable that the factor is involved in the coordinate regulation of the proliferation-specific H4 histone multigene subfamily during the cell cycle and becomes a rate-limiting factor during the coordinate shut down of histone gene transcription at the onset of terminal differentiation.

Acknowledgments—We thank Dr. Steven Dworetzky and Gerard Zambetti for critically reviewing the manuscript.

REFERENCES
1. Stein, G. S., Stein, J. L., and Marzluff, W. F. (eds) (1984) Histone Genes, John Wiley & Sons, New York
2. Schumperli, D. (1986) Cell 45, 471-472
3. Stein, G., and Stein, J. (1984) Bioessays 1, 202-205
4. Zanetti, G., Dell’Orco, R., Stein, G., and Stein, J. (1987) Exp. Cell Res. 172, 397-405
5. Rittling, S. R., Brooks, K. M., Cristofalo, V. J., and Baserga, R. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 3313-3320
6. Stein, G., Lian, J., Stein, J., Briggs, R., Shalhoub, V., Wright, K., Pauli, V., and van Wijnen, A. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 1865-1869
7. Collart, D., Wright, K. L., van Wijnen, A. J., Ramsey, A. L., Lian, J., Stein, J. L., and Stein, G. S. (1988) J. Biol. Chem. 263, 15880-15883
8. Marzluff, W. F., and Pandey, N. B. (1988) Trends Biochem. Sci. 13, 49-52
9. Stein, G. S., and Stein, J. L. (1984) Mol. Cell. Biochem. 64, 105-110
10. Plumb, M., Stein, J., and Stein, G. (1983) Nucleic Acids Res. 11, 2391-2410
11. Heintz, N., Sive, H. L., and Roeder, R. G. (1983) Mol. Cell. Biol. 3, 539-550
Human H4 Histone Gene Promoter Factors

12. Artishevsky, A., Delegane, A. M., and Lee, A. S. (1984) Mol. Cell. Biol. 4, 2964–2969
13. DeLisle, A. J., Graves, R. A., Marzluff, W. F., and Johnson, L. F. (1983) Mol. Cell. Biol. 3, 1920–1929
14. Dalton, S., Coleman, J. R., and Wells, J. R. E. (1986) Mol. Cell. Biol. 6, 601–606
15. Baumbach, L., Stein, G., and Stein, J. (1987) Biochemistry 26, 6178–6187
16. Baumbach, L., Marashi, F., Plumb, M., Stein, G., and Stein, J. (1984) Biochemistry 23, 1618–1625
17. Graves, R. A., and Marzluff, W. F. (1984) Mol. Cell. Biol. 4, 351–357
18. Gick, O., Kramer, A., Keller, M., and Birnstein, M. L. (1986) EMBO J. 5, 1319–1326
19. Luscher, B., and Schumperli, D. (1987) EMBO J. 6, 1721–1726
20. Luscher, B., Stauber, C., Schindler, R., and Schumperli, D. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 4389–4393
21. Pandey, N. B., and Marzluff, W. F. (1987) Mol. Cell. Biol. 7, 4557–4559
22. Morris, T., Marashi, F., Weber, L., Hickey, E., Greenspan, D., Bonner, J., Stein, G., and Stein, J. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 981–985
23. Capasso, O., Bleecker, G. C., and Heintz, N. (1987) EMBO J. 6, 1825–1831
24. Ross, J., Peltz, S. W., Kobs, G., and Brewer, G. (1986) Mol. Cell. Biol. 6, 4362–4371
25. Peltz, S. W., and Ross, J. (1987) Mol. Cell. Biol. 7, 4345–4356
26. Zambruti, G., Schmidt, W., Stein, G., and Stein, J. (1985) J. Cell. Physiol. 125, 345–353
27. Zambruti, G., Stein, J., and Stein, G. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 2683–2687
28. Sierra, F., Stein, G., and Stein, J. (1983) Nucleic Acids Res. 11, 7069–7086
29. Kroeger, P., Stewart, C., Schaap, T., van Wijnen, A., Hirshman, J., Helms, S., Stein, G., and Stein, J. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 3982–3986
30. Helms, S., van Wijnen, A., Kroeger, P., Shiba, A., Stewart, C., Hirshman, J., Stein, J., and Stein, G. (1987) J. Cell. Physiol. 13, 552–558
31. Hanly, S. M., Bleecker, G. C., and Heintz, N. (1985) Mol. Cell. Biol. 5, 380–389
32. Sive, H. L., Heintz, N., and Roeder, R. (1986) Mol. Cell. Biol. 6, 3329–3340
33. Dalton, S., and Wells, J. R. E. (1988) EMBO J. 7, 49–56
34. Artishevsky, A., Woodin, S., Sharma, A., Resendez, E., Jr., and Lee, A. S. (1987) Nature 328, 823–827
35. van Wijnen, A. J., Wright, K. L., Massung, R. F., Gerretsen, M., Stein, J., and Stein, G. (1988) Nucleic Acids Res. 16, 571–590
36. van Wijnen, A. J., Massung, R. F., Stein, J. L., and Stein, G. S. (1988) Biochemistry 27, 6534–6541
37. Gallinari, P., LaBella, F., and Heintz, N. (1989) Mol. Cell. Biol. 9, 1966–1977
38. Sive, H. L., and Roeder, R. G. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 6382–6386
39. Fletcher, C., Heintz, N., and Roeder, R. G. (1987) Cell 51, 773–781
40. van Wijnen, A. J., Stein, J. L., and Stein, G. S. (1987) Nucleic Acids Res. 15, 1679–1688
41. Dailey, L., Hanly, S. M., Roeder, R. G., and Heintz, N. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 7241–7245
42. Dailey, L., Boseman Roberts, S., and Heintz, N. (1988) Genes & Dev. 2, 1700–1712
43. Pauli, U., Chiu, J.-P., D’Ullio, P., Kroeger, P., Shalhoub, V., Rowe, T., Stein, G., and Stein, J. (1989) J. Cell. Physiol. 139, 320–328.
44. Pauli, U., Chrysogelos, S., Stein, J., Stein, G., and Nick, H. (1987) Science 236, 1308–1311
45. Pauli, U., Chrysogelos, S., Nick, H., Stein, G., and Stein, J. (1989) Nucleic Acids Res. 17, 2333–2350
46. Pauli, U., Chrysogelos, S., and Stein, G. (1989) Proc. Natl. Acad. Sci. U. S. A. 85, 21–25
47. Chrysogelos, S., Riley, D. E., Stein, G., and Stein, J. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 7535–7539
48. Moreno, M. L., Chrysogelos, S. A., Stein, G. S. and Stein, J. L., (1986) Biochemistry 25, 5364–5370
49. Moreno, M. L., Pauli, U., Chrysogelos, S., Stein, G. S. and Stein, J. L. (1988) Biochem. Cell Biol. 66, 132–137
50. Chrysogelos, S., Pauli, U., Stein, G. S. and Stein, J. (1989) J. Biol. Chem. 264, 1232–1237
51. Challberg, M. D., and Kelly, T. J., Jr. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 655–659
52. Dignam, J., Lebovitz, R., and Roeder, R. (1983) Nucleic Acids Res. 11, 1475–1489
53. Fried, M., and Crothers, D. M. (1981) Nucleic Acids Res. 9, 6505–6525
54. Kadonaga, J. T., Jones, K. A., and Tjian, R. (1986) Trends Biochem. Sci. 11, 20–23
55. Grimes, S., Wein-Carrington, P., Daum, H., III, Smith, J., Green, L., Wright, K., Stein, G., and Stein, J. (1987) Exp. Cell Res. 173, 534–545.
56. Wolfe, S. A., Anderson, J. V., Grimes, S. R., Stein, G. S., and Stein, J. L. (1989) Pharm. Biophys. Acta 1007, 140–150
57. Seiler-Tuyns, A., and Birnstein, M. L. (1981) J. Mol. Biol. 151, 607–625
58. Wells, D. E. (1986) Nucleic Acids Res. 14, (suppl.) r119–r149
59. Evans, R. M., and Hollenberg, S. M. (1988) Cell 52, 1–3
60. Dailey, L., Boseman Roberts, S., and Heintz, N. (1987) Mol. Cell. Biol. 7, 4582–4584
61. Russnak, R. H., Candido, E. P. M., and Astell, C. R. (1988) J. Biol. Chem. 263, 6392–6399
62. Solomon, M. J., Strauss, F., and Varshavsky, A. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 1276–1280
63. Gidoni, D., Dynan, W. S., and Tjian, R. (1984) Nature 312, 409–413
64. Kadonaga, J. T., Carner, K., Masiarz, F. R., and Tjian, R. (1987) Cell 51, 1079–1090
65. Parker, C. S., and Topol, J. (1984) Cell 36, 357–369