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Protein-DNA Interactions at the H4-Site III Upstream Transcriptional Element of a Cell Cycle Regulated Histone H4 Gene: Differences in Normal Versus Tumor Cells

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Abstract Upstream sequences of the H4 histone gene FO108 located between nt −418 to −213 are stimulatory for in vivo transcription. This domain contains one protein/DNA interaction site (H4-Site III) that binds factor H4UA-1. Based on methylation interference, copper-phenanthroline protection, and competition assays, we show that H4UA-1 interacts with sequences between nt −345 to −332 containing an element displaying sequence-similarity with the thyroid hormone response element (TRE). Using gel retardation assays, we also demonstrate that H4UA-1 binding activity is abolished at low concentrations of Zn^{2+} (0.75 mM), a characteristic shared with the thyroid hormone (TH) receptor DNA binding protein. Interestingly, phosphatase-treatment of nuclear proteins inhibits formation of the H4UA-1 protein/DNA complex, although a complex with higher mobility (H4UA-1b) can be detected; both complexes share identical protein-DNA contacts and competition behaviors. These findings suggest that phosphorylation may be involved in the regulation of H4-Site III protein/DNA interactions by directly altering protein/protein associations. H4-Site III interactions were examined in several cell culture systems during cell growth and differentiation. We find that H4UA-1 binding activity is present during the cell cycle of both normal diploid and transformed cells. However, during differentiation of normal diploid rat calvarial osteoblasts, we observe a selective loss of the H4UA-1/H4-Site III interaction, concomitant with an increase of the H4UA-1b/H4-Site III complex, indicating modifications in the heteromeric nature of protein/DNA interactions during downregulation of transcription at the cessation of proliferation. Transformed cells have elevated levels of H4UA-1, whereas H4UA-1b is predominantly present in normal diploid cells; this alteration in the ratio of H4UA-1 and H4UA-1b binding activities may reflect deregulation of H4-Site III interactions in transformed cells. We propose that H4-Site III interactions may contribute, together with protein/DNA interactions at proximal regulatory sequences, in determining the level of H4-FO108 histone gene transcription.

Key words: DNA-binding proteins, differentiation, distal promoter elements, proliferation, cell growth

Histones constitute a family of highly conserved basic proteins responsible for packaging chromosomal DNA into nucleosomes. The expression of the five classes of cell cycle regulated histone genes (H1, H2A, H2B, H3, and H4) is functionally and temporally coupled to DNA replication, and hence highly specific for proliferating cells (Stein et al., 1984). The rate-limiting step in histone protein synthesis is the abundance of histone mRNA, which is modulated at multiple gene regulatory levels (Osley, 1991; Heintz, 1991; Stein et al., 1989a). Transcriptional control of histone gene expression contributes significantly to the regulation of histone mRNA levels during the cell cycle. In proliferating cells, histone gene transcription occurs constitutively throughout the cell cycle and exhibits a 3–5-fold increase at the onset of S-phase (Osley, 1991; Heintz, 1991; Stein, et al., 1989a), whereas in differentiated cells which have ceased proliferative activity, histone mRNA synthesis is completely downregulated (Stein et al., 1989a).

Multiple distinct regulatory elements are involved in transcriptional control of histone gene expression. The modular organization of his-

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tone gene promoters is reflected by distal and proximal promoter regions containing general promoter elements common to genes transcribed by RNA polymerase II, as well as histone gene selective elements (Pauli et al., 1987, 1989; Osley et al., 1986; Lai et al., 1988; Hinkley and Perry, 1991; Lee et al., 1991; Tung et al., 1990; Kroeger et al., 1987; van Wijnen et al., 1987, 1988, 1989, 1991a,b; Dalton and Wells, 1988; Artishevsky et al., 1987; Sharma et al., 1989; Ito et al., 1989; Gallinari et al., 1989; Fletcher et al., 1987; Dailey et al., 1988; Levine et al., 1988; Wright et al., 1992a). Studies in our laboratory have established that several of these elements in the human *H3* and H4 promoters function as in vivo sites of protein/DNA interaction in the intact cell (Pauli et al., 1987, 1989). In vitro protein/DNA interaction assays using the proximal promoters (up to 0.2 kB from the histone mRNA coding region) of the cell cycle regulated histone genes H1 (Dalton and Wells, 1988; Gallinari et al., 1989; van Wijnen et al., 1988), H2B (Ito et al., 1989; Fletcher et al., 1987), H3 (Artishevsky et al., 1987; Sharma et al., 1989; van Wijnen et al., 1991b) and H4 (Lee et al., 1991; van Wijnen et al., 1987, 1989, 1991a,b; Dailey et al., 1988; Wright et al., 1992a) have identified several transacting DNA binding proteins. Several of these activities are either related to or identical to general transcription factors (reviewed in Mitchell and Tijan, 1989; Wingender, 1990), including AP-1 (Sharma et al., 1989), Sp1 (van Wijnen et al., 1989; Wright et al., 1992a), CP1/CP2 (Gallinari et al., 1989; van Wijnen et al., 1988, 1991b), OTF (Fletcher et al., 1987), and ATF (Wright et al., 1992a; van Wijnen et al., 1991b). Others, such as H1-SF (Dalton and Wells, 1988; Gallinari et al., 1989), HiNF-P/H4TF-2 (van Wijnen et al., 1991a; Dailey et al., 1988) and HiNF-D (van Wijnen et al., 1989, 1991a), interact selectively with histone promoter sequences. These protein/DNA interactions are in part gene-specific, while others are operative in many members of the histone multigene family. The interplay of general and gene-specific protein/DNA interactions represent a component of the mechanism by which the cell coordinately regulates the timing, extent, and selectivity of histone gene transcription. These findings suggest that several factors acting in close proximity of the mRNA initiation site are involved in transcriptional control of histone gene expression.

Recently, our laboratory has demonstrated for the human histone H4 gene FO108 that sequences upstream of the proximal promoter (nt −418 to −213) contribute to the regulation of histone gene expression by significantly enhancing the efficiency of transcription in vivo (Ramsey-Ewing et al., 1991; Wright, 1990; Wright et al., 1992b). This distal domain contains one protein/DNA interaction site (H4-Site III) and binds factor H4UA-1 (Ramsey-Ewing et al., 1991), indicating that the transcriptional regulatory mechanisms operative at the proximal promoter of the H4-FO108 histone gene (Ramsey-Ewing et al., 1991; van Wijnen et al., 1991c) occur within the context of distally located trans- and cis-acting components. In this study, we have systematically characterized the H4UA-1/H4-Site III interaction in detail and defined the biological regulation of the protein/DNA interactions at this distal promoter element. Our results are consistent with possible contributions of H4UA-1 and H4UA-1b in the physiological control of H4 histone gene transcription, with modifications in the ratio of these binding activities reflecting differences between the normal diploid and transformed phenotype.

MATERIAL AND METHODS

DNA Fragments and Synthetic Oligonucleotides

The FO108 human H4 histone promoter is numbered from the transcription initiation site. The radiolabeled DNA restriction fragment encompassing the H4-Site III region of the H4 (FO108) histone gene (*Hind*III/*Msp*I fragment spanning nt −418 to −278) was prepared from plasmid pGW-2. Plasmid pGW-2 was constructed by ligation of the *Hind*III/*Eco*RI fragment (nt −418 to −213) of plasmid pFO005 (Kroeger et al., 1987) into pGEM7ZF+(Promega).

Oligonucleotides (for sequences see Results) used for competition assays span portions of H4-Site III: H4 IIId (= distal) spans nt −367 to −348 and H4 IIIp (= proximal) spans nt −354 to −326. H4 IIIp-mut is a substitution mutant of H4 IIIp and contains 5 point mutations (at nt −345, −344, −339, −338, and −337). We also used oligonucleotides spanning the rat osteocalcin VDRE- and OC-box sequences (Owen et al., 1990a), as well as synthetic DNA fragments encompassing the MHC-TRE sequences (Flink and Morkin, 1990; nt −158 to −138) and hMT-IIa AP-1 sequences (Owen et al., 1990a).

Nuclear Protein Preparation and Protein Phosphatase Treatment

Nuclear protein extracts used in gel retardation assays were derived from human HeLa S3, HL-60, and WI-38 cells, rat calvarial osteoblasts, rat osteosarcoma cells (ROS 17/2.8), and Swiss mouse 3T3 fibroblasts, as well as from nuclei isolated from homogenized rat tissues, and were prepared as described previously (van Wijnen et al., 1987, 1991d; Holthuis et al., 1990). Exponentially growing promyelocytic HL-60 cells were induced to differentiate by treatment with 16 nM of the phorbol ester TPA (12-O-tetradecanoylphorbol-13-acetate) (Rovera et al., 1979; Huberman and Callahan, 1979). Protein concentrations were quantitated by Bradford analysis (Biorad). Phosphatase treatment of nuclear proteins was performed as described previously (van Wijnen et al., 1991a).

Gel Retardation Assays

Gel retardation assays were performed as described previously (van Wijnen et al., 1991d, 1992), using radioactively labeled DNA fragments spanning the distal H4 histone promoter region between nt −418 and −278. These probes were prepared by site-specific endonuclease cleavage, dephosphorylation using calf intestinal phosphatase, and subsequent ^{32}P-labeling using T4 polynucleotide kinase and ^{32}P-gamma ATP (Ausubel et al., 1987; Sambrook et al., 1989). DNA fragments were then cleaved using a secondary restriction enzyme and purified by gel electrophoresis. Binding reactions (20 μl) were performed with 2 ng of ^{32}P labeled DNA in diluted storage buffer (10% glycerol, 0.1 mM EDTA, 50 mM KCl, 1 mM DTT, and 12.5 mM Hepes/NaOH pH 7.5). Included in the binding reaction as non-specific competitor DNA were 2 μg of poly(dA-dT) • poly(dA-dT) and 1 μg of poly(dI-dC) • poly(dI-dC), unless indicated otherwise. Binding reactions were incubated for 20 min at room temperature and electrophoresed at 4°C on 4% (20:1 acrylamide:bisacrylamide) polyacrylamide gels for 3 hr at 200 V. Gels were dried and subjected to autoradiography.

Protein Analysis of Eluted Protein/DNA Complexes

For analysis of proteins involved in the H4UA-1/H4-Site III complex standard gel retardation binding reactions were scaled up to 50 μl. Two binding reactions were performed in parallel, one of which included a radioactively labeled DNA fragment, whereas the second reaction was performed without probe. The H4UA-1 protein/DNA complex was electrophoretically separated from the unbound probe DNA and identified after wet exposure to X-ray film at 4°C. Both the radioactive protein/DNA complex and the proteins present at the same level in the samples containing no radioactive-labeled probe DNA were eluted in 0.5 × TGE buffer (50 mM Tris/HCl pH 8.4, 40 mM glycine, and 1 mM EDTA) at 4°C and concentrated with Centricon-10 units (Amicon). The eluted proteins of both samples were resolved by 10% denaturing sodium dodecyl sulfate (SDS) discontinuous gel electrophoresis and subjected to silver staining.

Methylation Interference

The probe used for methylation interference (Pauli et al., 1990) was ^{32}P 5′ end-labeled with T4 polynucleotide kinase as described above or ^{32}P 3′ end-labeled using the fill-in procedure with the Klenow fragment of *E. coli* DNA polymerase I. Incubation with 1 μl undiluted dimethylsulphate (DMS) was carried out for 6 min at 18°C. After removal of the DMS, the partially methylated probe was used in large scale binding reactions (50 μl), similar to those in gel retardation assays. Free and complexed probe DNA was separated by preparative native gel electrophoresis. The samples were eluted as described previously (Pauli et al., 1990), treated with piperidine and guanine-specific cleavage products were analyzed on denaturing polyacrylamide gels followed by autoradiography.

Copper-Phenanthroline Protection Analysis

In situ footprinting using the 1,10-phenanthroline-copper ion (Kuwabara and Sigman, 1987; Sigman, 1990) was performed within the polyacrylamide matrix after electrophoretic separation of protein/DNA complexes and free DNA, using standard gel retardation assay conditions. This method involves immersion of the *entire* gel in a 1,10-phenanthroline-copper solution. Chemical cleavage was allowed to proceed for 8 minutes at room temperature, and the gel segment corresponding to the protein/DNA complex of interest was localized by autoradiography (4–16 hr of exposure at 4°C). Subsequently, the nicked DNA products were eluted from this

gel segment and directly analyzed on denaturing polyacrylamide gels, followed by autoradiography.

RESULTS

The H4UA-1/H4-Site III Protein/DNA Interaction Occurs at Sequences Between NT −358 and −332

The upstream promoter of the cell cycle controlled human histone H4 FO108 gene has been shown to be involved in the regulation of transcription (Ramsey-Ewing et al., 1991; Wright, 1990; Wright et al., 1992b). Specifically, using deletion analysis, our laboratory has identified a strong activating element (designated H4-Site III, located between nt −418 and −213) that is capable of stimulating in vivo transcription up to 10-fold (Ramsey-Ewing et al., 1991), and in this study we examine the protein-DNA interactions at the H4-Site III element (Fig. 1). Figure 2 shows the electrophoretically stable protein/DNA complex of H4UA-1 interacting with H4 Site III. Using methylation interference analysis, we established the contacts of H4UA-1 with its recognition site at single nucleotide resolution (Fig. 2). The guanine residues in close proximity with H4UA-1 are nt −342, −341, −339, −338, −332 on the lower strand and nt −344 at the upper strand consistent with previous findings (Wright, 1990; Wright et al., 1992b).

To further define the binding site of H4UA-1, we performed copper-phenanthroline (CuPhen) protection analysis. The 1,10-phenanthroline-cuprous complex is a small chelate capable of interacting with both the minor and major groove, with only three base pairs involved in the nicking of the sugar-phosphate backbone (Sigman, 1990). The CuPhen-footprint of H4UA-1 spans nt −358 to −332 (Fig. 3). Two distinct regions (nt −358 to −346 and −341 to −332) are protected from attack by CuPhen, suggesting that these nucleotides are in close contact with factor H4UA-1. In addition, we observed enhanced susceptibility to cleavage of residues at either border of the CuPhen-footprint. This enhanced cleavage may be due to conformational changes in the H4UA-1 interaction sequences following binding. The CuPhen-footprint is contained within the DNase I-foot-

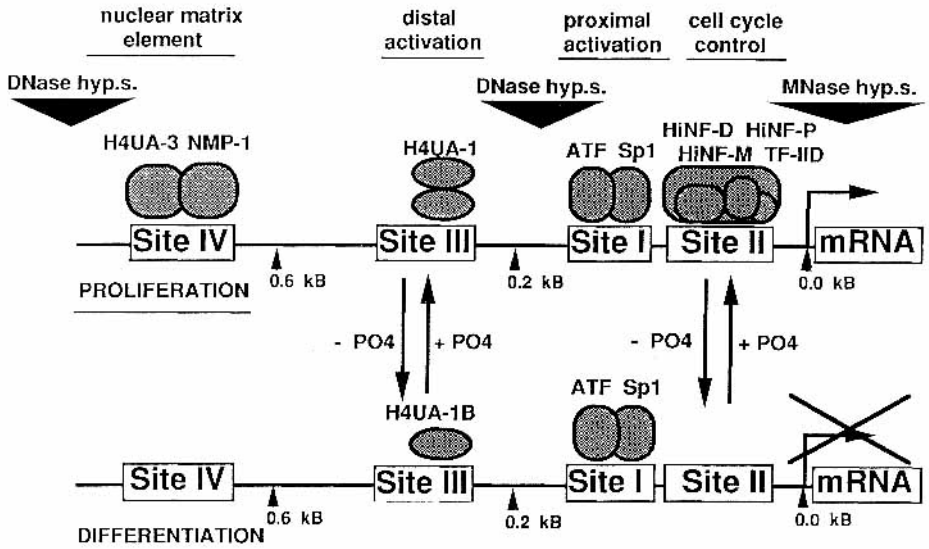

Fig. 1. A model of H4 histone promoter elements. Schematic drawing showing the promoter region of the H4 histone gene FO108 with the positions of H4-Site I (nt −151 to −114), H4-Site II (nt −97 to −47), and H4-Site III (nt −367 to −326), and the factors interacting at these sequences. The numbers refer to the distance in nucleotides from the translational start codon. Also shown are possible modifications in phosphorylation-dependent protein/protein and protein/DNA interactions that may influence H4-FO108 gene transcription during the cell cycle and cessation of proliferation at the onset of differentiation. This postulated model involves differential occupancy of H4-Site II by HiNF-D, HiNF-M, HiNF-P, and TF-IID during the S and G2/M/G1 phases of the cell cycle resulting in basal levels (thin arrow), or maximal levels (thick arrow) of transcription. Shutdown of H4-FO108 gene transcription at the cessation of proliferation (thin arrow covered by X) coincides with modifications of protein/protein interactions at H4-Site III and downregulation of H4-Site II occupancy by its cognate factors. The potential contribution of phosphorylation to protein/DNA interactions at H4-Site II and protein/protein interactions at H4-Site III are indicated (see text for references).

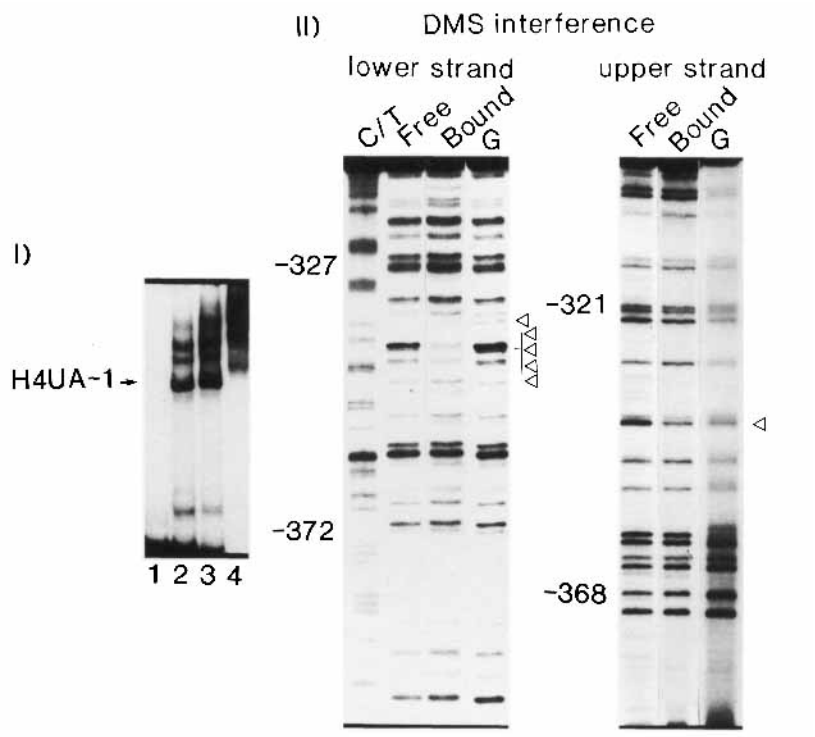

Fig. 2. Protein/DNA interactions at H4-Site III. **Panel I:** Gel retardation assay showing the formation of the H4UA-1/H4-Site III complex, using the *HindIII/MspI* probe (nt −418 to −278) and increasing amounts of nuclear proteins from exponentially growing HeLa S3 cells (Lanes 1 to 4: respectively, 0, 2.5, 5, and 10 μg of nuclear proteins added). **Panel II:** Methylation interference pattern at the lower and the upper strand with factors present in nuclear extract from HeLa S3 cells. C/T: cytosine + thymidine-sequence ladder of probe (nt −418 to −278); Free: DMS reactivity pattern of unbound probe; Bound: DMS reactivity pattern of bound probe; G: guanine-sequence ladder of the probe. Open arrowheads represent the guanine residues interfering with H4UA-1 binding.

print of the H4UA-1/H4-Site III complex (Wright, 1990; Wright et al., 1992b), further supporting that H4UA-1 interacts with sequence-specificity to a subset of nucleotides (nt −358 to −332) in the H4-Site III element.

H4UA-1 Binding Activity Displays a Selective Divalent Cation Sensitivity

To determine the effect of pH, temperature, detergents, chelators, and concentrations of mono- and divalent cations on the formation of the H4UA-1/H4-Site III protein-DNA complex, we performed gel retardation assays with binding conditions in which each of these parameters was altered separately (Fig. 4). Our results show that the H4UA-1 interaction with H4-Site III sequences is destabilized above 37°C and has a binding optimum at a lower temperature. The pH optimum for binding is between pH 7.5 and pH 9.0, although a substantial amount of binding occurs over a wide pH range (between pH 4.5 and pH 10). Inclusion of low concentrations (between 0.005% and 0.05%) of the anionic denaturing detergent SDS abolishes binding, whereas the nonionic detergent Nonidet P-40 (NP-40) has no effect on binding up to 0.5%. Interestingly, the addition of the zwitterionic detergent Empigen shows an optimum for complex formation around 0.0005%, but binding is decreased above 0.05%. This detergent apparently facilitates the solubilization of nuclear proteins at low concentrations, thereby optimizing the formation of the protein/DNA complex at H4-Site III, but may affect protein structure at higher concentrations resulting in decreased H4UA-1/H4-Site III complex formation.

These results (Fig. 4) also show that H4UA-1 has a monovalent ion optimum (100 mM KCl), although the protein-DNA complex is partially stable up to 200 mM. The addition of the divalent cation Ca^{2+} does not influence binding, but a moderate increase in binding activity was observed in the presence of micromolar concentrations of Mg^{2+}. This divalent cation possibly acts

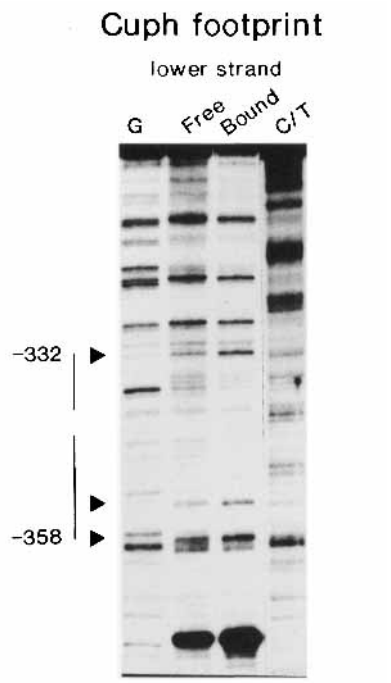

Fig. 3. Copper-phenanthroline (CuPh) protection analysis. Protected sequences with factors present in nuclear extracts from HeLa S3 cells. C/T: cytosine + thymidine-sequence ladder of probe (nt −418 to −278); Free: CuPhen reactivity pattern of unbound probe; Bound: CuPhen reactivity pattern of bound probe; G: guanine-sequence ladder of the probe. Arrowheads represent the residues showing enhanced susceptible to cleavage by CuPhen.

as a co-factor that stabilizes the H4UA-1/H4-Site III protein/DNA complex; alternatively, Mg^{2+} ions may enhance specific complex formation indirectly by quenching interactions of non-specific DNA binding proteins with H4-Site III. Inhibitory effects on H4UA-1 binding activity are observed when either EDTA or EGTA are included in the binding reaction, suggesting that H4UA-1 is sensitive to divalent cation chelators. The sensitivity towards both chelators indicates a cryptic requirement for a divalent cation different from the Mg^{2+} ion and is consistent with the involvement of at least one type of divalent cation in the stabilization of the H4UA-1/H4-Site III interaction. However, the addition of micromolar concentrations of Zn^{2+} (750 μM final concentration in the binding reaction) abolishes binding. Although the basis for this inhibitory effect of Zn^{2+} on protein-DNA interactions is unclear, this observation has also been made for the TH receptor DNA binding protein interacting with its cognate TRE (Lavin et al., 1988) which indicates that H4UA-1 shares this property.

Competition Analysis of the H4UA-1/H4 Site III Complex

The H4UA-1/H4-Site III interaction was further analyzed by oligonucleotide competition analysis. We used oligonucleotides spanning specific segments of H4 Site III (nt −367 to −326) as defined by stairway assays (Wright et al., 1992b; van Wijnen et al., 1992) and the DNaseI protection pattern (Wright et al., 1992b) (Fig. 5). Figure 5 shows that sequences between nt −367 and −348 do not compete, even at extremely high molar excess, whereas the sequences between nt −350 and −326 (H4-IIIp) effectively compete for binding. Using this fragment as a probe in gel retardation assays, only one electrophoretically stable complex is detectable which is specifically competed by the unlabeled DNA fragment (Fig. 6), implying that the sequences of H4 IIIp alone are sufficient for the formation of the H4UA-1/H4-Site III protein/DNA complex.

Thus, these results confirm that the binding site for H4UA-1 is located between nt −350 and −326. This region comprises a putative half element of the TRE which consists of an imperfect direct repeat containing 5 of the 6 guanine contacts. These sequences display significant similarity to the high affinity TH receptor binding site 5′-CTGGAGGT-3′ as described for the human alpha myosin heavy chain promoter (Flink and Morkin, 1990) (Fig. 5). To directly explore a possible relation between H4UA-1 and a hormone receptor-like DNA binding activity, we performed competition assays with the human alpha myosin heavy chain thyroid hormone response element (MHC-TRE) and the vitamin D receptor element (VDRE) (Owen et al., 1990a), considered to be the same class of hormone receptor promoter binding sequences as the TRE (Evans, 1988; Beato, 1989). Whereas the MHC-TRE oligonucleotide does not compete, the DNA fragment containing the VDRE competes for binding, albeit at a moderately specific molar ratio. These results demonstrate that the TRE consensus sequence is not sufficient for competition of H4UA-1, suggesting that H4UA-1 binding requires more than the putative TRE half-element. Close examination of the sequences of the VDRE oligonucleotide reveals that competition with this DNA fragment is reflected by limited primary sequence-similarity. The region of direct similarity extending outside the hormone response element, indicating that this se-

quence may serve as a low affinity binding site determinant.

The sequences also show an interesting motif of two symmetric TGG elements possibly involved in binding of a separate class of transcription factors (Gil et al., 1988). To assess the importance of these two motifs for the binding of H4UA-1, we constructed a mutant oligonucleotide replacing the TGG element in the upper strand by ACG and the TGG element in the lower strand by GCT (H4IIIp-mut). This mutant oligonucleotide does not display obvious similarity to any known consensus transcription factor binding sequence and does not compete for binding even at high molar ratios (Fig. 5). We conclude from these results that the mutated nucleotides at positions −345, −344, −339, −338, and −337 are directly involved in and are essential for the binding of H4UA-1.

A Factor of 36 KD Is Part of the H4UA-1/H4-Site III Complex

Initial indications of the proteins involved in the formation of the H4UA-1/H4-Site III complex were obtained by simple elution of the protein/DNA complex as detected in gel retardation assays. Although detection is complicated by comigrating proteins not associated with the target DNA, and the low quantities of protein present in protein/DNA complexes, this method provides a direct way to identify proteins in protein/DNA complexes. The standard binding reaction used for gel retardation assays (containing radioactively labeled probe DNA) was scaled up, the bound and unbound probe were electrophoretically separated, and the H4UA-1/H4-Site III complex was electro-eluted. As control, the same binding reaction was carried out in the absence of probe DNA and electrophoresis of this sample was performed in parallel, followed by elution of proteins exhibiting the same electrophoretic mobility as the H4UA-1/H4-Site III

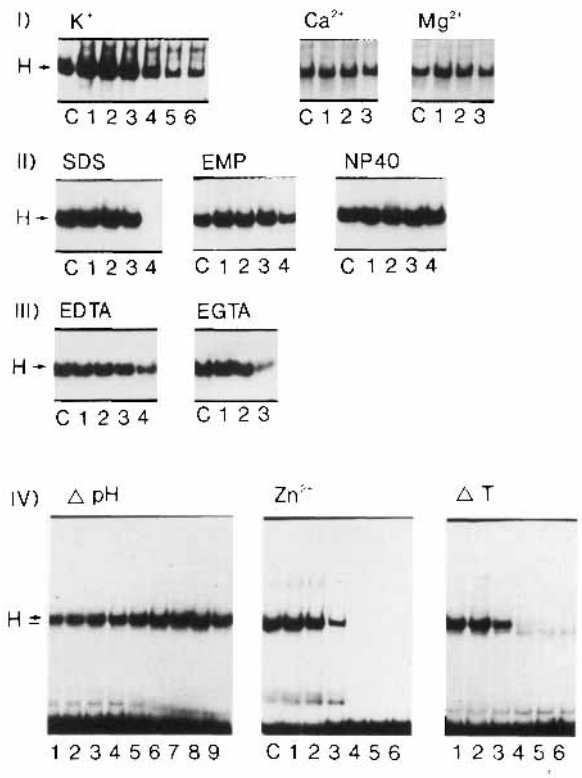

Figure 4.

Fig. 4. Influence of salt, detergent, chelators, pH, and temperature stability of H4UA-1/H4-Site III complex formation in gel retardation assays using nuclear extracts from HeLa S3 cells. The arrows indicate the H4UA-1/H4-Site III complex; the dash indicates an inconsistently observed complex with slightly higher mobility than H4UA-1/H4-Site III. **Panel I:** Salt dependence of H4UA-1/H4-Site III complex formation: Lane C: (in all panels) 5 μg of nuclear proteins with no salt added. Left panel: Effect of monovalent cation concentration in the binding reaction. Lanes 1–6: same as C, but KCl added to total concentrations of, respectively, 75, 100, 125, 150, 175, and 200 mM. Middle and right panel: Effect of divalent cation concentrations in the binding reaction. Middle panel: Lanes 1–3: same as C, but $CaCl_2$ added to total concentrations of, respectively, 0.2, 1.0, and 5.0 mM. Right panel: Lanes 1–3: same as C, but $MgCl_2$ added to total concentrations of, respectively, 0.2, 1.0, and 5.0 mM. **Panel II:** Detergent stability of the H4UA-1/H4-Site III complex: Lane C: (in all panels) 5 μg of nuclear proteins with no detergent added. Left panel: Anionic detergent stability of complex H4UA-1/H4-Site III. Lanes 1–4: same as C, but 0.00005%, 0.0005%, 0.005%, and 0.05% sodium dodecyl sulphate (SDS) added. Middle panel: Zwitterionic detergent stability of complex H4UA-1/H4-Site III. Lanes 1–4: same as C, but 0.0005%, 0.005%, 0.05%, and 0.5% Empigen added. Right panel: Nonionic detergent stability of complex H4UA-1/H4-Site III. Lanes 1–4: same as C, but 0.0005%, 0.005%, 0.05%, and 0.5% Nonidet P-40 (NP-40) added. **Panel III:** Divalent cation requirement for the formation of the H4UA-1/H4-Site III complex: Lane C: (in both panels) 5 μg of nuclear proteins with no chelators added. Left panel: Effect of chelator EDTA included in the binding reaction. Lanes 1–4: same as C, but EDTA added to total concentrations of, respectively, 0.5, 1.0, 2.0, and 5.0 mM. Right panel: Effect of chelator EGTA included in the binding reaction. Lanes 1–3: same as C, but EGTA added to total concentrations of, respectively, 0.5, 1.0, 5.0 mM. **Panel IV:** pH, Zn^{2+} and temperature sensitivity of the H4UA-1/H4-Site III complex. Left panel: Lanes 1–9: gradual pH increase in the binding reaction from, respectively, 4.5, 5.0, 5.5, 6.0, 6.5, 7.5, 8.5, 9.0 to 10.0. Middle panel: Lane C: binding reaction with no Zn^{2+} added. Lanes 1–6: same as C, but Zn^{2+} added to final concentrations of, respectively, 0.10, 0.25, 0.50, 0.75, 1.0, and 1.5 mM. Right panel: Lanes 1–6: binding reaction incubated at increasing temperature from, respectively rT, 30, 37, 42, 45 to 50°C.

complex. Both protein samples were concentrated and analyzed in a denaturing SDS polyacrylamide gel. Repeatedly, we observed a 36 kD factor in the fraction containing the protein/DNA complex (Fig. 7), suggesting that this factor is an intrinsic component of H4UA-1 binding activity.

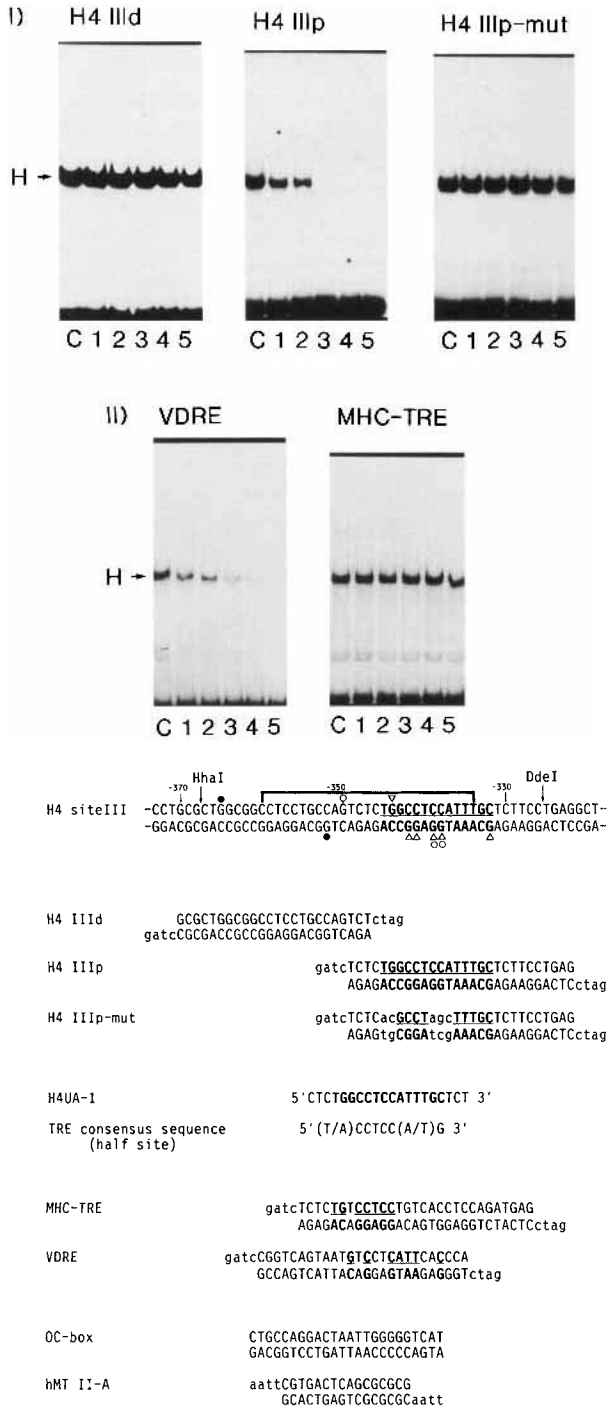

Figure 5.

Modifications of Protein/DNA Interactions at H4-Site III During Differentiation

To investigate the biological significance of H4UA-1, this DNA binding activity was measured during the entry into and progression through the cell cycle, as well as during differentiation. Factor H4UA-1 is constitutive throughout the cell cycle of synchronized HeLa S3 cells, following release from a double thymidine block, as well as of 3T3 cells, following release from serum-deprivation (Fig. 8). Similar observations have been made using cell cycle stage-specific nuclear extracts of rat osteosarcoma cells (data not shown). These results are consistent with histone gene transcription occurring constitutively in actively proliferating cells.

Fig. 5. Competition of the H4UA-1/H4-Site III protein/DNA complex with oligonucleotides spanning segments of H4-Site III and consensus sequences for the TRE and VDRE using gel retardation assays. Lane C: (in all panels) 5 μg of nuclear proteins without competitor added. Arrow indicates the H4UA-1/H4-Site III complex. **Panel I:** H4-Site III related oligonucleotides. Left panel: Lanes 1–5: same as C, but H4 IIId oligonucleotide (spanning nt −367 to −348 of H4-Site III) included in the binding reaction in, respectively, 50-, 100-, 250-, 500-, and 1,000-fold molar excess. Middle panel: Lanes 1–5: same as C, but H4 IIIp oligonucleotide (spanning nt −354 to −326 of H4-Site III) included in the binding reaction in, respectively, 50-, 100-, 250-, 500-, and 1,000-fold molar excess. Right panel: Lanes 1–5: same as C, but H4 IIIp-mut oligonucleotide (derived from H4 IIIp, mutated at nt −345,−344, −339, −338 and −337) included in the binding reaction in, respectively, 50-, 100-, 250-, 500-, and 1,000-fold molar excess. **Panel II:** Steroid hormone response element consensus oligonucleotides. Left panel: Lanes 1–5: same as C, but rat osteocalcin VDRE oligonucleotide included in the binding reaction in, respectively, 50-, 100-, 250-, 500-, and 1,000-fold molar excess. Right panel: Lanes 1–5: same as C, but oligonucleotide containing the human myosin heavy chain TRE sequence included in the binding reaction in, respectively, 50-, 100-, 250-, 500-, and 1,000-fold molar excess. **Panel III:** Summary of the protein/DNA interaction pattern at H4-Site III and the oligonucleotides used in the competition assays. Line over the H4-Site III sequence indicates the DNase I footprint mediated by the H4UA-1 protein/DNA complex; thin line under the H4-Site III sequences indicate CuPhen-footprint; open arrowheads represent the guanine residues in close proximity of H4UA-1 as detected by methylation interference; open circles represent those guanine residues shielded from DMS methylation after binding of nuclear proteins, whereas closed circles represent those showing an enhanced methylation by DMS after binding of nuclear proteins (Wright, 1990). Arrows indicate positions of restriction sites which border H4-Site III as detected by stairway assays (Wright, 1990). The minimal sequence required for H4UA-1 binding is written in bold and underlined. Homologous sequences in the oligonucleotides used are indicated in the same manner. Mutations in the oligonucleotide are given in lowercase letters.

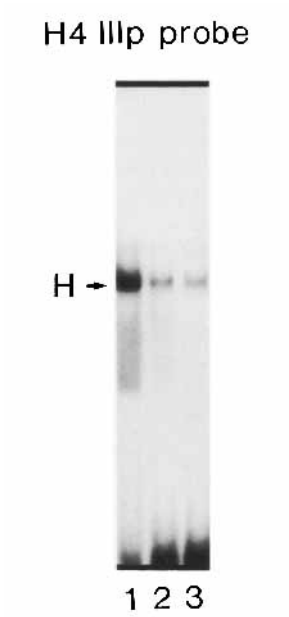

Fig. 6. H4 IIIp is sufficient to mediate binding of the H4UA-1/H4-Site III protein/DNA complex formation in gel retardation assays. Lane 1: Binding reaction with 5 μg nuclear proteins from HeLa S3 cells without competitor added. Lanes 2 and 3: same, but with respectively 100- and 200-fold excess unlabeled H4 IIIp added. H indicates the H4UA-1/H4-Site III complex.

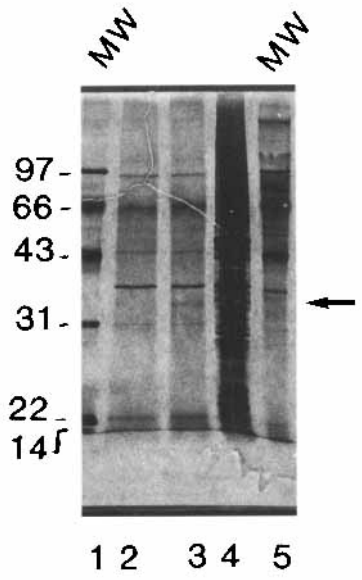

Fig. 7. A 36 kd factor is a component of the H4UA-1/H4-Site III complex. Denaturing (SDS), discontinuous poly-acrylamide gel. Arrow indicates the 36 kd protein found to be associated with the H4UA-1/H4-Site III complex. Lanes 1 and 5: molecular weight markers. Lane 2: proteins migrating at the level of the H4UA-1/H4-Site III complex without radioactive probe included in the binding reaction. Lane 3: same as 2, but with radioactive labeled probe added, thus forming the H4UA-1/H4-Site III complex. Lane 4: 0.5 μg nuclear proteins of HeLa S3 cells.

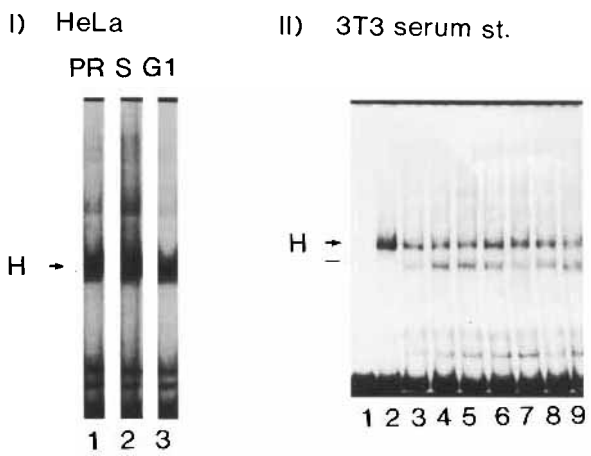

Fig. 8. The H4UA-1 binding activity is constitutive in tumor-derived cells both during the cell cycle (HeLa S3 cells), as well as after release from serum-deprivation induced growth arrest (Swiss 3T3 mouse fibroblasts). **Panel I:** HeLa S3 cells were synchronized by double thymidine block and harvested prior to release from the second thymidine block (PR), during S-phase (S, 4 hours after release) and during G1 phase (G1, 10 hours after release). Nuclear protein extracts were assayed for H4UA-1 binding activity as described previously. **Panel II:** Swiss 3T3 mouse fibroblasts were serum deprived for 2 days, after which the cells were stimulated by addition of 10% fetal bovine serum. Nuclear protein extracts were assayed for H4UA-1 binding activity. Lane 1: unbound probe, Lane 2: 2 μg nuclear proteins from exponentially growing HeLa S3 cells, Lanes 3–9: 2 μg nuclear proteins from Swiss 3T3 mouse fibroblasts harvested, respectively, 0, 1, 3, 10, 16, 18, and 24 hours after serum stimulation.

In contrast to the rather subtle fluctuation of histone gene transcription during the cell cycle, histone gene transcription dramatically declined after phorbol ester induced differentiation of the human promyelocytic leukemia HL-60 cells into monocyte-like cells (Stein et al., 1989b) and following the downregulation of proliferation in osteoblasts (Owen et al., 1990b). This transcriptional downregulation suggests a rate-limiting role for transcription factor/DNA interactions in the regulation of histone gene transcription during the cessation of proliferation. To investigate the regulation of H4UA-1 during differentiation, we monitored the level of H4UA-1 binding activity following TPA-induced differentiation of HL-60 cells, as well as during the onset and progression of osteoblast phenotype development. We find that in terminally differentiated HL-60 cells binding of H4UA-1 persists at a decreased level, while a complex of higher mobility, mediated by a factor designated H4UA-1b, is observed (Fig. 9). In extracts of undifferentiated proliferating rat calvarial osteoblast (day 5) that do not express the bone-

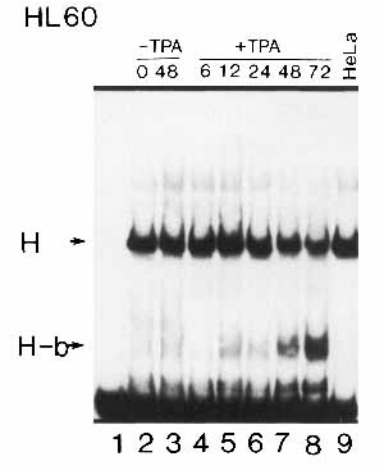

Fig. 9. Altered H4UA-1/H4-Site III interactions during TPA induced differentiation of HL-60 cells in gel retardation assays. Lane 1: unbound probe; Lanes 2 and 3: binding reaction with nuclear proteins from human promyelocytic HL-60 cells, respectively, 0 and 48 hours after plating without 12-O-tetradecanoylphorbol-13-acetate (TPA) treatment (negative controls); Lanes 4–8: nuclear proteins from HL-60 cells respectively 6, 12, 24, 48, and 72 hours after treatment with 16 nM TPA; Lane 9: nuclear proteins from exponential growing HeLa S3 cells. H indicates the H4UA-1/H4-Site III complex, H-b indicates the H4UA-1b/H4-Site III complex.

specific osteocalcin gene, factor H4UA-1 binding is present, but in differentiated osteoblasts (day 25), H4UA-1 binding activity is no longer detectable even at increased proteins concentrations (Fig. 10). At the same time, the levels of H4UA-1b are elevated in the differentiated compared with proliferating rat calvarial osteoblasts. Thus, it appears that H4UA-1 is associated with the proliferative state of these bone-derived cells and its downregulation occurs concomitant with differentiation. In contrast, H4UA-1b remains detectable in post-proliferative, differentiated osteoblasts.

Histone genes are highly conserved in all vertebrates, and this evolutionary conservation is reflected by cross-species compatibility of H4-Site III protein/DNA complexes occurring at the human H4-FO108 gene promoter, based on competition results obtained with human and rodent nuclear proteins (Fig. 10). In addition, the protein/DNA interactions observed with rat nuclear proteins have identical characteristics with respect to binding parameters of the H4UA-1/H4-Site III complex (data not shown). These results are similar to those obtained previously in several other studies on histone gene transcriptional regulation (Kroeger et al., 1987; Dalton and Wells, 1988; van Wijnen et al., 1991d;

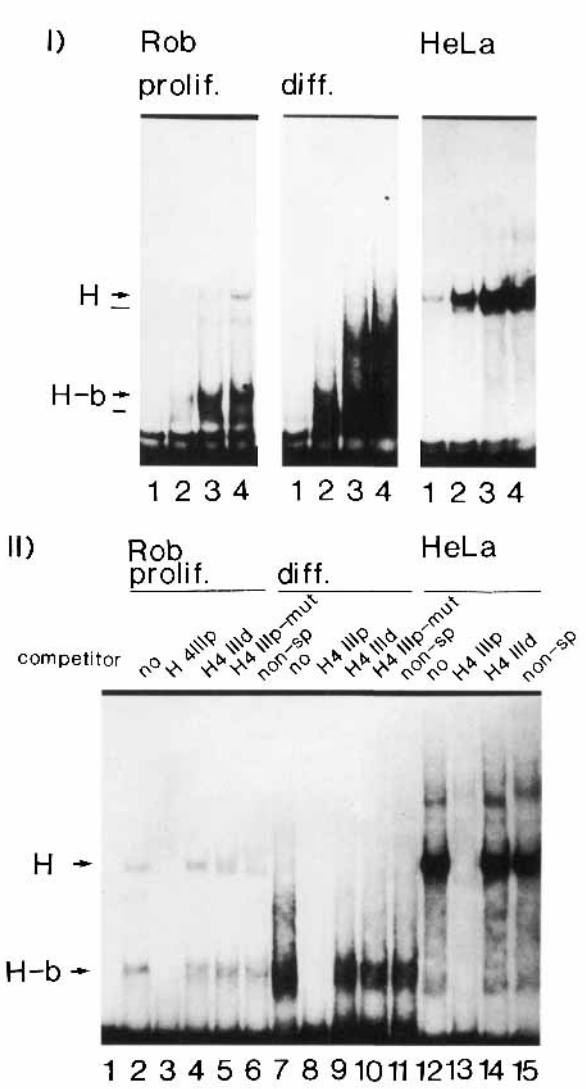

Fig. 10. Downregulation of H4UA-1 binding activity during differentiation of rat calvarial osteoblasts. H indicates the H4UA-1/H4-Site III complex, H-b indicates the H4UA-1b/H4-Site III complex, assayed for in gel retardation assays as described previously. **Panel I:** H4UA-1 is downregulated and H4UA-1b is upregulated during differentiation of normal diploid rat calvarial osteoblasts. Lanes 1–4: respectively 2.0, 5.0, 7.0, and 10.0 μg nuclear proteins of proliferating rat calvarial osteoblasts (Rob prolif., harvested at day 5), differentiated rat calvarial osteoblast (Rob diff., harvested at day 25), and proliferating HeLa S3 cells (HeLa) were assayed for H4UA-1/H4UA-1b binding activity. **Panel II:** The protein/DNA interactions detected with nuclear extracts of rat calvarial osteoblasts are identical to H4UA-1 and H4UA-1b in HeLa S3 cells as shown with competition assays. Lanes 1–5, 6–10, 11–15: respectively, no competitor, H4 IIIp, H4 IIId, H4 IIIp-mut, and a non-specific (non-sp) competitor oligonucleotide added in 500-fold molar excess to a regular binding reaction with 7 μg nuclear proteins of proliferating rat osteoblasts (Rob prolif. harvested at day 5), 3 μg nuclear proteins of differentiated rat osteoblasts (Rob diff., harvested at day 25), and 5 μg nuclear proteins of proliferating HeLa S3 cells.

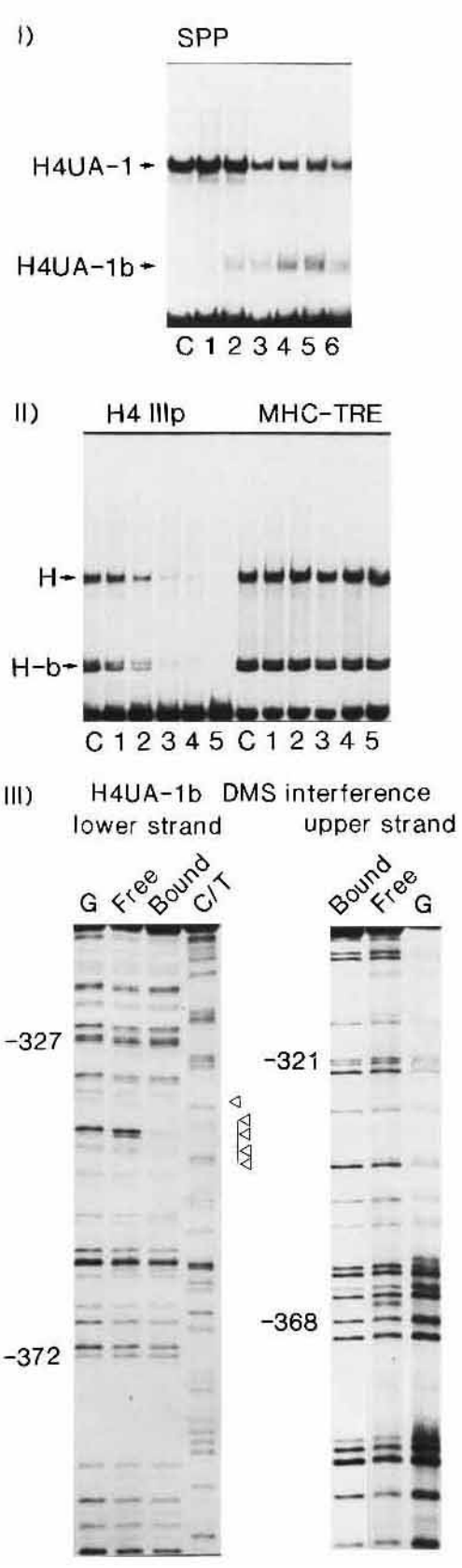

Figure 11.

Holthuis et al., 1990), and together firmly support cross-species compatibility of trans-acting factors and cis-acting elements of histone gene expression in diverse vertebrate species.

De-Phosphorylation Alters H4-Site III Protein/DNA Interactions

To address the possibility that phosphorylation participates in the regulation of the H4UA-1/H4UA-1b binding activities, we incubated nuclear proteins in the presence of sweet potato acidic phosphatase. Acidic phosphatase, a broad spectrum phosphomonoesterase, is capable of removing phosphate moieties of phosphorylated amino acids. We followed the same protocol previously used to establish phosphatase sensitivity of the multipartite protein complex interacting with H4 Site II (van Wijnen et al., 1987), with which no degradation of the nuclear proteins has been observed. Figure 11 shows that treatment of nuclear proteins with acidic phosphatase inhibits the formation of the low mobility protein/DNA complex mediated by H4UA-1, whereas H4UA-1b binding activity is clearly enhanced. This demonstrates that factor H4UA-1 is phosphatase sensitive and suggests that H4UA-1b is a post-translational modified form of H4UA-1. Because the difference in relative migration rates of the H4UA-1 and the *H4UA-1b* mediated protein-DNA complexes are substan-

Fig. 11. Phosphatase treatment of nuclear extracts inhibits H4UA-1/H4-Site III complex formation and induces the formation of a high mobility complex in gel retardation assays, mediated by H4UA-1b, which has identical binding characteristics. **Panel I:** Lane C: binding reaction with 5 μg nuclear proteins from HeLa S3 cells. Lane 1: same, but the nuclear extracts are incubated with sweet potato phosphatase incubation buffer for 15 min at 37°C before using them in the binding reaction. Lanes 2–6: same as 1, but, respectively, 0.01, 0.05, 0.10, 0.20, and 0.50 units sweet potato phosphatase is included in the incubation prior to the binding reaction. **Panel II:** Lane C: binding reaction with 5 μg nuclear proteins from HeLa S3 cells. Left panel: Lanes 1–5: same, but oligonucleotide H4-IIIp added in, respectively, 50-, 100-, 250-, 500-, and 1,000-fold molar excess. Right panel: Lanes 1–5: same, but oligonucleotide MHC-TRE added in, respectively, 50-, 100-, 250-, 500-, and 1,000-fold excess. H indicates the H4UA-1/H4-Site III complex; H-b indicates the H4UA-1b/H4-Site III complex. **Panel III:** Methylation interference pattern of the H4UA-1b/H4-Site III complex at the lower and the upper strand with factors present in nuclear extract from rat calvarial osteoblasts (day 25). C + T: cytosine + thymidine-sequence ladder of probe (nt −418 to −278); Free: DMS reactivity pattern of unbound probe; Bound: DMS reactivity pattern of bound probe; G: guanine-sequence ladder of the probe. Arrowheads represent the guanine residues interfering with H4UA-1b binding.

tial, it is unlikely that these complexes solely represent phosphatase-induced conformational changes in protein structure of the same factor. Rather, it is possible that H4UA-1 and H4UA-1b are directly related by a post-translational modification involving protein/protein associations.

To test the possibility that H4UA-1 and H4UA-1b represent post-translational modifications of the same binding activity, we performed competition assays and methylation interference analysis. The H4 IIIp oligonucleotide competes with identical specificity for binding of both protein complexes (Fig. 11), whereas the MHC-TRE fragment does not, implying that H4UA-1 and H4UA-1b exhibit indistinguishable binding affinity for H4-Site III. Methylation of guanine residues at nt −342, −341, −339, −338, −332 on the lower strand interferes with H4UA-1b binding, but at the upper strand no interference was detectable (Fig. 11). Most interestingly, the contacts of H4UA-1 and H4UA1-b at H4-Site III display striking similarities, although there are subtle differences in the H4UA-1b interaction at the upper strand. Hence, we propose that H4UA-1 and H4UA-1b are related by a post-translational modification involving the association of a secondary protein.

The indistinguishable binding activities of H4UA-1 and H4UA-1b and the phosphatase mediated transition from the low mobility to the high mobility complex led us to further investigate the interrelation between these two protein/DNA complexes. We monitored the extent to which increasing amounts of nuclear proteins from exponentially growing HeLa S3 cells reconstitute the H4UA-1 mediated low mobility complex when added to a constant amount of nuclear proteins of differentiated cells (rat calvarial osteoblasts, day 25) that mediate formation of the high mobility complex H4UA-1b. We observed a negative correlation in H4UA-1b binding activity when increasing amounts of HeLa S3 nuclear proteins were added (Fig. 12), indicating that a specific activity capable of reconstituting H4UA-1 is present in nuclear extracts of actively proliferating cells.

The possibility must be considered that the putative shift from the high to low mobility protein/DNA interaction in the gel retardation pattern is due to mutually exclusive binding events. However, the amount of free probe decreases substantially with increasing protein concentration, providing evidence that more protein becomes bound to the DNA, rather than

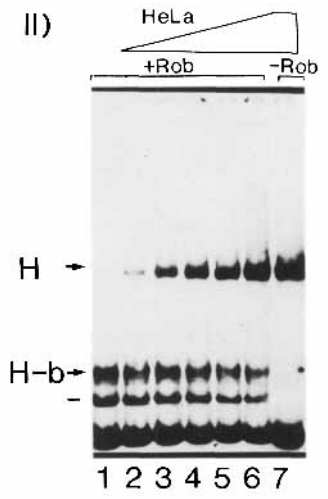

Fig. 12. Reconstitution of H4UA-1 by addition of nuclear proteins of proliferating HeLa S3 cells to nuclear proteins of differentiated rat calvarial osteoblasts. Gel retardation assays as described previously. H indicates the H4UA-1/H4-Site III complex; H-b indicates the H4UA-1b/H4-Site III complex. Lane 1: binding reaction with nuclear proteins of differentiated rat calvarial osteoblasts (Rob); lane 7: proliferating HeLa S3 cells (HeLa) only. Lanes 2–6: 1.0, 2.0, 3.0, 4.0, and 5.0 μg nuclear proteins of HeLa added to the constant amount of 5 μg nuclear proteins of Rob.

displaces protein/DNA interactions. Therefore, we prefer the interpretation that H4UA-1 and H4UA-1b are related by a post-transcriptional modification involving the association of a secondary protein.

H4UA-1/H4UA-1B Binding Is Deregulated in Transformed Cells

We have demonstrated that H4UA-1 is downregulated in differentiated rat calvarial osteoblasts, and at the same time the levels of H4UA-1 in proliferating rat calvarial osteoblasts are low compared with H4UA-1b. In proliferating tumor-derived cells (HeLa S3, Figs. 2, 8; HL-60, Fig. 9) we find predominantly H4UA-1. To address the possibility that differences in the representation of the binding activities of H4UA-1 and H4UA-1b in distinct cell types reflect phenotypic differences between normal diploid compared with tumor-derived cells, we monitored the presence of these DNA binding activities in nuclear protein extracts of exponentially growing rat calvarial osteoblasts and WI-38 human fetal lung fibroblasts. Factor H4UA-1 is present as expected in both types of proliferating cells, but we also observe a high level of H4UA-1b binding activity. In contrast, in tumor-derived rat osteosarcoma and human HeLa S3 cells the levels

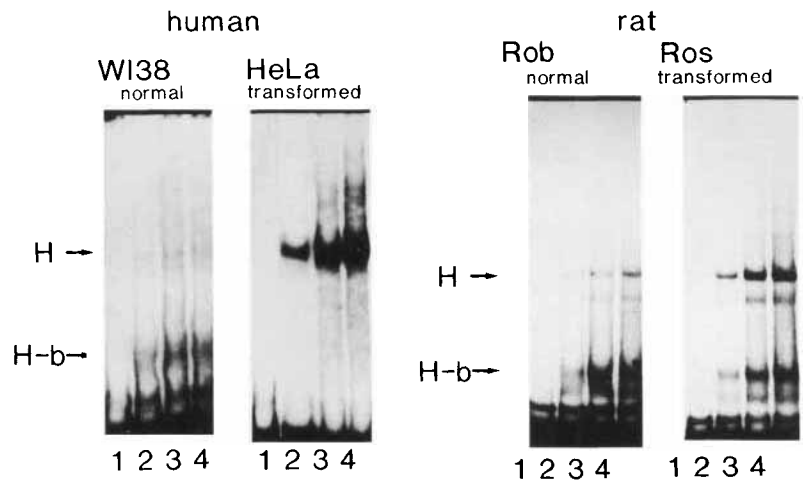

Fig. 13. Deregulation of H4UA-1 in tumor-derived human and rodent cells. Gel retardation assays were performed as described. H indicates the H4UA-1/H4-Site III complex; H-b indicates the H4UA-1b/H4-Site III complex. **Left panel:** Lanes 1–4: 1.0, 2.0, 5.0, and 7.0 μg nuclear protein of, respectively, normal diploid human WI-38 lung fibroblasts (WI38) and human HeLa S3 cervical carcinoma cells. **Right panel:** Lanes 1–4: 1.0, 2.0, 5.0, and 7.0 μg nuclear protein of, respectively, normal diploid primary rat calvarial osteoblasts (Rob) and transformed rat osteosarcoma cells (Ros).

of H4UA-1 activity are elevated compared with H4UA-1b (Fig. 13). These data indicate that the ratio of H4UA-1 and H4UA-1b is regulated in an analogous manner in human and rodent cells, and that this ratio may be deregulated in tumor-derived cells reflecting loss of stringent control of cell growth regulation.

H4UA-1/H4UA-1B Binding Activity in Tissues

Occupancy at H4-Site III was measured using nuclear proteins derived from different tissues to explore the regulation of H4-Site III binding activities in vivo in the intact animal. For example, H4UA-1 binding activity is not detectable in adult liver (Fig. 14), whereas H4UA-1b is present. The representation of H4UA-1 and H4UA-1b binding activities in this tissue parallels that of post-proliferative cells in culture (see Fig. 10). Because we have previously shown that histone mRNA levels are barely detectable in adult liver, consistent with the differentiated and quiescent state of cells in this tissue (van Wijnen et al., 1991c), these results support the hypothesis that the ratio of H4UA-1 and H4UA-1b binding activities is tightly linked to cell growth regulation.

Similarly, nuclear protein preparations from fetal brain contain primarily H4UA-1 binding activity (Fig. 14). RNA samples derived from rodent fetal brain contain measurable levels of H4 histone mRNA (van Wijnen et al., 1991c,d) consistent with the presence of both non-proliferating neurons and proliferating glial cells. Thus, the distribution of H4UA-1 binding activity in fetal brain may reflect the presence of proliferating cells in this tissue.

Competition assays (Fig. 14) were performed to show that the H4-Site III protein/DNA complexes detected with proteins from these tissues are identical to the H4UA-1/H4UA-1b complex from HeLa S3 cells. The observed protein/DNA interactions in rat liver and brain tissues exhibit identical competition patterns as those observed with extracts of cultured cells, consistent with ubiquitous roles for H4UA-1 and/or H4UA-1b in the regulation of histone gene transcription in a broad spectrum of cell types.

DISCUSSION

Studies on transcriptional control of vertebrate histone gene expression (van Wijnen et al., 1987, 1988, 1989, 1991b; Artishevsky et al., 1987; Sharma et al., 1989; Ito et al., 1989; Gallinari et al., 1989; Fletcher et al., 1987; Dailey et al., 1988; Wright et al., 1992a; and reviewed in Osley, 1991) have placed primary emphasis on the characterization of protein/DNA interactions occurring at regulatory sequences located in the initial 0.2 kB of 5′ flanking sequences (proximal promoter) of the five principal histone gene classes. However, the rate of both human H4-FO108 and H3-ST519 histone gene transcription, when solely supported by proximal cis-activating sequences, is several-fold lower than observed when transcription is under the influence of an exogenous enhancer

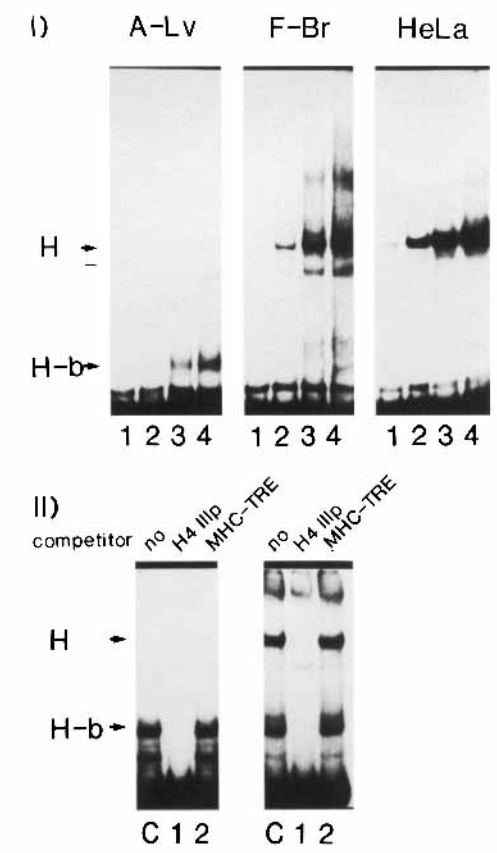

Fig. 14. H4UA-1 binding activity in tissues. Gel retardation assays as described above. H indicates the H4UA-1/H4-Site III complex; H-b indicates the H4UA-1b/H4-Site III complex. **Panel I:** Lanes 1–4: 1.0, 2.0, 5.0, and 7.0 μg nuclear protein of, respectively, adult rat liver (A-Lv), fetal rat brain (F-Br), and proliferating HeLa S3 cells (HeLa). **Panel II:** Lanes C, 1, and 2: no competitor, H4 IIIp, and MHC-TRE oligonucleotide added as competitor in 500-fold molar excess to the binding reaction with nuclear extracts of, respectively, adult rat liver and fetal rat brain.

element (see Kroeger et al., 1987). Moreover, in histone genes from lower eukaryotes, including yeast (Sturm et al., 1988 and reviewed in Osley, 1991) and sea urchin (Tung et al., 1990), distally located sequences are functionally involved in modulating the periodic expression of histone genes during the cell cycle. Mutational analyses carried out in our laboratory of the 5′ flanking region of the histone H4-FO108 gene has led to the identification of several distal transcriptional elements (Ramsey-Ewing et al., 1991; Wright, 1990; Wright et al., 1992b), including H4-Site III, which is capable of enhancing histone gene transcription by several-fold (Ramsey-Ewing et al., 1991). Thus, histone gene transcription is controlled by the integrated activities of a series of regulatory sequences that operate in close proximity of the mRNA initiation site, as well as in distal promoter sequences.

In this study, we have characterized molecular components of the distal regulatory mechanism controlling H4-FO108 gene transcription and have defined the recognition sequences of the protein/DNA interactions mediated by factor H4UA-1 at H4-Site III using methylation interference, copper-phenanthroline protection, and (mutational) competition analysis. We observed alterations in H4-Site III protein/DNA interactions that correlate with changes in H4 gene transcription during several biological processes. In addition, we have obtained initial indications that regulation of the H4UA-1/H4-Site III interaction may involve two distinct post-translational modifications of H4UA-1, including phosphorylation and protein/protein heteromerization.

The H4UA-1 recognition site is located in the proximal part of H4-Site III (nt −358 and −332), and displays extensive and repeated sequence similarity with the TRE and the interaction site for the TH receptor (Evans, 1988; Beato, 1989). The region of similarity coincides with 5 out of 6 methylation interference contacts observed for H4UA-1 and corresponds to some extent with those observed for the TH receptor/TRE interaction. Similar to the TH receptor/TRE interaction, the H4UA-1/H4 Site III interaction is inhibited by low concentration of Zn^{2+} ions. However, sequences spanning a prototypical TRE element do not compete for the H4UA-1/H4-Site III interaction, suggesting that H4UA-1 is distinct from the TH receptor, but may belong to a class of hormone receptor-like DNA binding factors sharing similar DNA binding parameters.

Hormone receptor elements are composed of short repeated sequence motifs and are often found in close proximity to, or overlap with, binding sites for other classes of transcription factors, as has been shown for hormone response elements upstream of several genes, including the chicken lysozyme (Steiner et al., 1987), human metallothionein IIa (Lee et al., 1987), rat osteocalcin (Owen et al., 1990a), and tryptophane oxygenase genes (Schuele et al., 1988a). The multipartite nature of H4-Site III sequences is evidenced by the representation of the TRE direct repeat, two TGG elements which may interact with NF-1 like factors (Gil, et al., 1988), and a region displaying a 6 out of 8 match with an octamer motif that potentially represents an OTF-1 binding site (reviewed in Mitch-

ell and Tijan, 1989; and Wingender, 1990) (see Fig. 5). The presence of several consensus transcription factor binding site sequences adjacent to, or overlapping with, the TRE repeat in H4-Site III is consistent with the observation that hormone receptors stimulate transcription synergistically in association with other factors, including NF-1, SP-1, and OTF-like factors (reviewed in Schuele et al., 1988b). Recently, specific-enhanced DNA binding of TH-receptor has been shown to be due to heteromerization of the TH-receptor with other factors (Lazar et al., 1991). Thus, it is reasonable to postulate that similar protein/protein associations occur at H4-Site III. The potential for regulatory complexity at H4-Site III is further reflected by evidence that phosphorylation dependent protein/protein heteromerization events modify the H4UA-1/H4-Site III interaction.

Dephosphorylation of factor H4UA-1 results in formation of a high mobility protein/DNA complex which is mediated by H4UA-1b. Because H4UA-1 and H4UA-1b display identical interference contacts, competition behavior and Zn^{2+} inhibition, this suggests that H4UA-1b and H4UA-1 involve the same DNA binding activity and are directly related by protein/protein association. Because factor H4UA-1b is most abundant in post-proliferative cells that have ceased to transcribe histone genes, whereas H4UA-1 is present in actively dividing cells when these genes are transcribed, this suggests that H4UA-1 may be the H4-Site III binding activity with trans-activating potential. We postulate that at the cessation of proliferation, H4UA-1b may arise from H4UA-1 by dephosphorylation, and this may occur concomitantly with the dissociation of a secondary protein (e.g., with an intrinsic transcription stimulatory activity in conjunction with H4UA-1b). Alternatively, we can not dismiss the possibility that the H4UA-1b/H4-Site III complex may be functionally related to the downregulated state of the gene.

While comparing nuclear factor distribution in exponentially growing cell populations, we observed that tumor-derived cells contain higher levels of factor H4UA-1 than normal diploid cells, suggesting that H4UA-1 binding activity and the phosphorylation state of this factor are deregulated in tumor cells. Interestingly, we also observed deregulation in tumor cells of the cell cycle controlled histone gene transcription factor HiNF-D (Holthuis et al., 1990), that interacts with the proximal promoter element H4-Site II (van Wijnen et al., 1989). Moreover, the Pardee laboratory has shown that the binding activity of factor Yi interacting with the thymidine kinase promoter is deregulated in cells where stringent growth control has been abrogated (Bradley et al., 1990; Dou et al., 1991). Thus, deregulation of transcription factor/promoter interactions in cell growth regulated genes possibly reflects a component of mechanisms associated with the sequential loss of proliferation control operative in normal diploid cells.

The deregulation of H4UA-1 may reflect the elevated levels of protein kinase activities generally operative in transformed cells that may increase the extent to which certain transcription factors are phosphorylated. As a consequence of the transformation associated upregulation of kinase activities, the levels of H4UA-1 observed in proliferating normal diploid cells may support the requirement for enhancement of histone H4 gene transcription during the S-phase of the cell cycle to provide histone proteins for packaging newly replicated DNA as chromatin. It may not solely be a transformation related increase in level of phosphorylation capacity, but a selective upregulation of a cell cycle related phosphorylation pathway. The p34/cdc2 related kinase pathway is an example of a mechanism that may support upregulation of transcription factors associating with proliferation specific genes (reviewed in Murray and Kirschner, 1990). The function of H4UA-1 may not be restricted to H4-Site III of the H4 gene promoter, but the factor may interact in a sequence-specific manner with other cell cycle regulated genes. Hence, the elevated abundance of H4UA-1 activity in nuclear extracts may directly reflect utilization of H4UA-1 at multiple gene regulatory sequences of a series of growth regulated genes.

A relationship between oncogene expression and H4UA-1 activity is suggested by shared but not identical properties of H4UA-1 with the TH receptor. Interestingly, the TH receptor is related to the c-erb oncogene (Green and Chambon, 1986), which permits us to consider the possibility that a component of H4UA-1 is an oncogene encoded phosphoprotein exhibiting modified expression, heteromerization potential, or activity with deregulation of cell growth.

Results of this and previous studies have established the composite organization of the H4 gene promoter that contains a series of distinct elements each contributing to the timing and

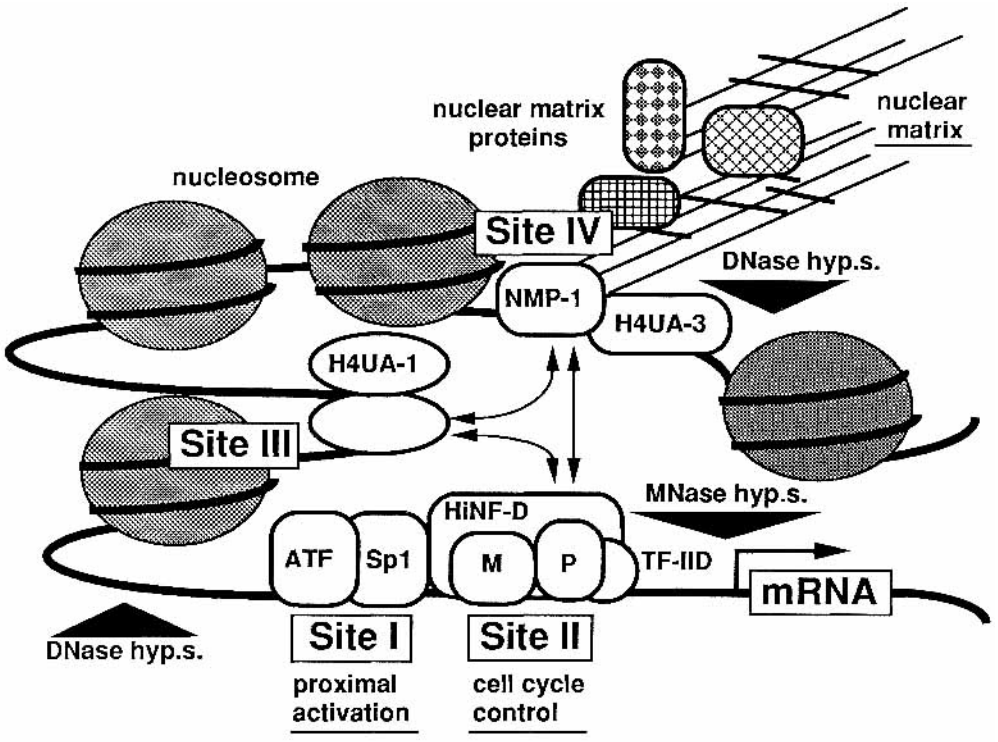

Fig. 15. Schematic representation of a model for the three-dimensional organization of the H4-FO108 promoter depicting a relationship of distal and proximal protein/DNA interaction sites when H4-FO108 promoter DNA (solid black line) is packaged into nucleosomes (open ovals). Indicated are possible cooperative and/or mutually exclusive higher-order nucleoprotein interactions (thin arrows) between various DNA bound trans-acting factors. The presence of a putative attachment site (Site IV) with the nuclear matrix (network of lines) containing matrix associated sequence-specific DNA binding proteins (rounded boxes filled with alternative symbols), provides a basis for restricted mobility of the promoter to a confined position within the nucleus as well as for the concentration and localization of transcription factors.

extent of H4-FO108 gene transcription. The integration of intracellular signals that independently act upon these multiple elements may in part reside in the three-dimensional organization of the promoter within the context of nuclear architecture (Fig. 15). A nuclear matrix attachment site has been identified in the upstream region (−1.0 kB) of the H4-FO108 gene promoter (Dworetzky et al., 1990) which may serve two functions: imposing constraints on chromatin structure, as well as concentrating and localizing transcription factors. The presence of nucleosomes in the H4 promoter (Chrysogolos et al., 1985; Moreno et al., 1986) increases proximity of independent regulatory elements and supports synergistic and/or antagonistic cooperative interactions between histone gene transcriptional DNA binding activities. In addition, nucleosomal organization varies as a function of the cell cycle (Moreno et al., 1986) which may enhance and restrict accessibility of transcription factors, and modulate the extent to which DNA bound factors are phosphorylated. The specific mechanism by which the H4UA/H4-Site III complex participates in regulating the extent to which the histone H4-FO108 gene is transcribed remains to be determined. However, regulation is unquestionably operative within the context of the complex series of proximal and distal regulatory elements and their cognate factors, which are responsive to a broad spectrum of biological signals.

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