Functional Role for Sp1 in the Transcriptional Amplification of a Cell Cycle Regulated Histone H4 Gene†

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ABSTRACT: The promoter of the cell cycle regulated histone FO108 H4 gene is mediated by two *in vivo* protein/DNA interaction domains, sites I and II. We have shown previously that site II mediates the cell cycle controlled enhancement of H4 gene transcription at the G1/S phase boundary. Here we show that site I, an element containing both G-rich and ATF-like consensus sequences, confers maximal levels of transcription in proliferating cells. By the combined application of gel shift assays with site-directed mutagenesis, DNase I footprinting, oligonucleotide competition, *in vitro* expression of recombinant proteins, and specific antibody supershift studies, we demonstrate that the proximal G-rich sequence within site I interacts with the transcription factor Sp1, while the distal portion of site I interacts with members of the ATF family of proteins, including ATF-1. *In vitro* transcription studies as well as expression assays of transiently and stably transfected genes in HeLa cells reveal that the deletion of site I causes a dramatic decrease in expression. Mutation of the Sp1 element, which abolishes Sp1 binding, results in a 6−10-fold reduction in reporter activity. In addition, overexpression of Sp1 in Sp1-deficient cells results in the dramatic activation of the histone promoter. In contrast, mutation of the asymmetric ATF binding site, located distally within site I, has a more limited effect upon expression. Interestingly, the contribution of the Sp1 site to maximal transcription was cell type dependent. Thus, we demonstrate that the Sp1 binding site of the site I histone H4 promoter in particular is critical for maximal expression in living cells and postulate that this site may act to amplify the cell cycle response.

The histone gene family, encoding the critical structural and functional elements of eukaryotic chromatin, provides an outstanding model for the transcriptional control of gene expression during the cell cycle and cell growth [reviewed in Osley (1991) and Stein *et al.* (1992)]. While histone gene transcription occurs at all stages of the cell cycle, upon the entry into S phase, a rapid 3−5-fold increase in histone mRNA synthesis ensues (Heintz *et al.*, 1983; Graves & Marzluff, 1984; Baumbach *et al.*, 1987). Discrete functional elements involved in both cell cycle related control (Dalton & Wells, 1988; La Bella *et al.*, 1988a, 1989; Ramsey-Ewing *et al.*, 1994) and maximal expression (La Bella *et al.*, 1988b, Gallinari *et al.*, 1989; Artishevsky *et al.*, 1987; Kroeger *et al.*, 1987) have been discovered within the 5′ regulatory regions of histone H1, H2B, H3, and H4 genes. These promoters display marked heterogeneity in their modular organization.

The human H4 histone gene FO108 contains two proximal promoter regions that exhibit protein−DNA interactions *in vivo*, sites I (−124 to −86) and II (−64 to −24; Pauli *et al.*, 1987). Site II confers the enhancement of transcription at the G1/S phase boundary (Ramsey-Ewing *et al.*, 1994). This region contains the TATA box and the binding sequences for distinct nuclear factors designated HiNF-D, -P, and -M, all of which overlap with a histone H4 specific element. The formation of HiNF-D is tightly correlated with proliferation (van Wijnen *et al.*, 1989) and represents a multi-subunit complex comprised of the key cell cycle proteins cdc2, cyclinA, and an Rb-related protein (van Wijnen *et al.*, 1994). HiNF-P is a cell growth regulated protein (van den Ent *et al.*, 1993) similar or identical to H4TF-2 (van Wijnen *et al.*, 1992; Dailey *et al.*, 1988). The cell cycle control element is located within the distal portion of site II and most likely functions *in vivo* by binding its cognate factor HiNF-M to bring about S phase activation of the gene (Ramsey-Ewing *et al.*, 1994). Recently, HiNF-M has been purified and identified as the transcription factor IRF-2 (unpublished).

Located distally to site II, site I contains a proximal G-rich element, closely matching the consensus Sp1 binding sequence GGGCGGG [reviewed by Courey and Tjian (1992)], that binds HiNF-C *in vitro* (van Wijnen *et al.*, 1989). The distal half of site I encompasses an asymmetric ATF consensus sequence as well as sites for the HMG-I(Y)-like protein, HiNF-A (van Wijnen *et al.*, 1989). While G-rich and ATF-like consensus elements are found in the proximal regions of many histone H1, H3, and H4 promoters (Artishevsky *et al.*, 1987; van Wijnen *et al.*, 1989; Wells, 1986; Younghusband *et al.*, 1986; Coles *et al.*, 1987; Hanly *et al.*, 1985), that these sequences function to regulate transcription has not been examined directly. Furthermore, several recent independent lines of evidence illustrate the necessity to assess the functional contribution by G/C box elements to a given promoter. First, there are several examples where Sp1 consensus binding sites do not actually function to elevate

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transcription *in vivo* (Lang *et al.*, 1992; Abruzzo & Reitman, 1994). Second, G/C elements are now known to bind specifically to an increasing number of proteins belonging to the Sp family of transcription factors, some of which act to repress transcription (Hagen *et al.*, 1994; Imataka *et al.*, 1992). In addition, the levels of Sp1 itself are known to differ between distinct cell types by as much as 100-fold (Saffer *et al.*, 1991), and at least one study shows that G/C elements may confer a significant level of cell type specific expression to a promoter (Zhang *et al.*, 1994). Therefore, in the present study, we systematically analyze the functional contribution of the Sp1 and ATF elements in site I to the FO108 H4 histone gene in a variety of cell types using *in vitro* and *in vivo* experimental approaches. We show unequivocally that Sp1 plays a dominant role in enhancing the transcription of this cell cycle regulated histone H4 gene.

MATERIALS AND METHODS

Cell Culture. HeLa S3 cells were grown and maintained in suspension at $3-6 \times 10^5$ cells per mL at 37 °C in Joklik-modified minimum essential medium supplemented (Gibco/BRL) with 5% fetal calf serum and 5% horse serum. ROS 17/2.8 cells (rat osteosarcoma) were grown in F-12 medium with 5% fetal calf serum. *Drosophila melanogaster* Schneider's S-2 cells were grown in Schneider's insect medium (Sigma) supplemented with 10% heat inactivated fetal calf serum at room temperature.

Plasmid Constructions and Mutagenesis. The histone promoter−chloramphenicol acetyltransferase (CAT) gene fusion construct, FO108-CAT, was derived from the previously described construct FO002-CAT (Kroeger *et al.*, 1987) by *Eco*RI digestion followed by ligation of the proximal *Eco*RI site of the histone promoter (−213 bp) to the *Eco*RI site of the vector (Ramsey-Ewing *et al.*, 1994). The construct FP201-CAT was derived from FO108-CAT by deletion of the sequences between −204 (*Sma*I) and −70 (*Nae*I). The 5′ deletion mutants used for *in vitro* transcription were prepared by Bal-31 digestion as previously reported (Sierra *et al.*, 1983) and were recloned into a pUC19 vector in order to make the vector sequences uniform. Selected base substitutions were introduced into the promoter of the FO108 gene using synthetic oligonucleotide-directed mutagenesis, essentially as described (Kunkel, 1985). Uracil containing single-stranded M13 DNA, including a 1.85-kb *Eco*RI/*Hin*dIII fragment of the FO108 gene, was prepared by amplification in the *Escherichia coli* strain CJ236 (*dut*$^-$ *ung*$^-$ *F*; a gift of Dr. Barbara Bachmann, *E. coli* Genetic Stock Center, Yale University). Mutations were introduced in complementary oligonucleotides containing the base substitutions in the proximal portion of site I, $^{5'}$CGAGAGttCGGGGAC$^{3'}$, or the distal portion, $^{5'}$GAGGAAAACAGAAAAGAcATcACtAAATGTCGAG$^{3'}$. The mutations were recovered by transformation into wild-type *E. coli* strain XL1-Blue (Stratagene), confirmed by enzymatic sequencing (Sequenase, US Biochemical), and the 1.85-kb *Eco*RI/*Hin*dIII FO108 gene fragment was cloned back into pUC19. For the creation of a mutant in which both the distal and the proximal sites were substituted, the distal mutation was included in the original M13 DNA, and the mutagenesis was carried out as described above using the proximal site I mutagenic oligonucleotide. A total of 140-bp *Eco*RI/*Bbs*I fragments isolated from these wild-type and mutant pUC19 constructs were cloned into the plasmid FP2 (plasmid

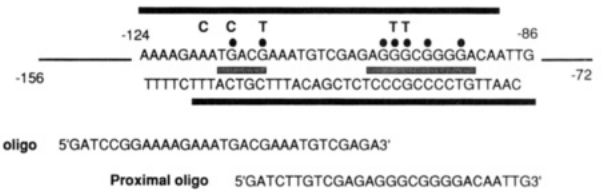

FIGURE 1: Site I of the FO108 histone H4 promoter. The top and bottom strands of site I are shown. The thick and dark lines represent the extent of the *in vivo* footprint, and the filled circles indicate the methylation protected guanine residues (Pauli *et al.*, 1987). The shaded box within the distal (left-hand) portion of site I denotes the asymmetric ATF consensus sequence, and the shaded box found in the proximal (right-hand) portion of site I designates the Sp1-like consensus element. The specific mutations used in the study are indicated in bold lettering above the wild-type sequence, and the oligonucleotides used as either probes or specific competitors are shown at the bottom of the figure (top strand only). The full site I region used as a wild-type or mutant probe contained the sequence extending from −156 to −72, as indicated.

108CAT with the 1.7-kb *Hin*dIII CAT fragment removed; Kroeger *et al.*, 1987) that had been digested with the same two restriction enzymes. A 1.7-kb *Hin*dIII fragment from the plasmid FP201-CAT (Kroeger *et al.*, 1987) containing the chloramphenicol acetyltransferase gene was cloned into the *Hin*dIII site of the resulting intermediates. Clones correct for both the proper mutations and orientation were confirmed using restriction enzyme analysis and enzymatic sequencing and have been designated pSCAT (wild type), pSICAT (distal mutant), pSIPCAT (proximal mutant), and pSIPICAT (double mutant).

Nuclear Extracts. Nuclear extracts from exponentially growing HeLa S3 cells were prepared as described previously (Dignam *et al.*, 1983), except that 600 mM KCl was used to extract the nuclear proteins.

DNase I Footprinting. DNase I footprinting was performed as described previously (Augereau & Chambon, 1986) using wild-type or mutant site I probes extending from −156 to −72 bp (plus 14 bp of pUC19 polylinker).

In Vitro Transcription. Transcription from linearized template DNA was carried out essentially as described (Dignam *et al.*, 1983). The histone templates were linearized at the unique *Nco*I restriction site to produce a runoff transcript of 279 nucleotides. Transcription from covalently closed circular template DNA was performed by preincubating the DNA and 50−100 μg of crude nuclear extract in 21 μL at 4 °C for 15 min in a buffer containing 12 mM Hepes [*N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid] (pH 7.9 with KOH), 12% glycerol, 60 mM KCl, 8 mM $MgCl_2$, 0.3 mM DTT, and 0.12 mM EDTA (ethylenediaminetetraacetic acid). Transcription was initiated by the addition of 280 μM of ATP, CTP, GTP, and UTP (Sigma) to bring the final volume to 25 μL, and the reaction was placed at 30 °C for 30 min. The reactions were terminated by adjusting the sample to 50 mM sodium acetate, 0.5% SDS (sodium dodecyl sulfate), and 0.5 mg/mL yeast t-RNA in a total volume of 100 μL. The nuclei acids were isolated by phenol/$CHCl_3$/isoamyl alcohol extraction, precipitated, and resuspended in 49 μL of DNase I digestion buffer [40 mM Tris-HCl (pH 7.9), 10 mM NaCl, and 6 mM $MgCl_2$]. After digestion with 1 unit of RNase-free DNase I (RQ1, Promega) for 15 min at 37 °C, the RNA transcripts were isolated by extraction as before and directly used for nuclease S1 protection analysis (Lichtler *et al.*, 1982). The probe used

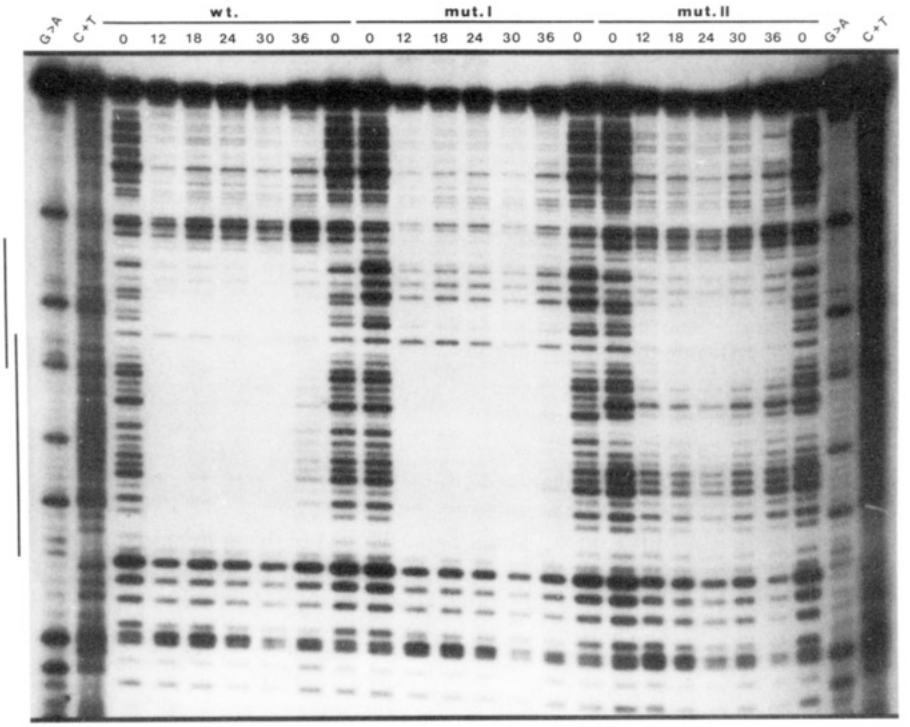

FIGURE 2: DNase I footprint of site I proteins. The amounts of nuclear protein extract (μg) present in the reaction are shown above each lane. The boundaries of the shorter distal asymmetric ATF-site footprint and the longer proximal Sp1-like footprint are indicated at the left of the panel.

was a single end-labeled DNA fragment overlapping the transcription initiation site, which yields a 279 nucleotide protected fragment corresponding to the accurately initiated FO108 H4 histone mRNA.

Electrophoretic Mobility Shift Assay. The electrophoretic mobility shift assay was performed essentially as described (Staudt *et al*., 1986) using either a TGE buffer system (50 mM Tris-Cl, 380 mM glycine, and 2 mM EDTA, pH 8.5) or a TBE buffer system (50 mM Tris-HCl, 50 mM borate, and 2 mM EDTA, pH 8.5). Binding reactions were as described previously (van Wijnen *et al*., 1989), except that 3 μg of poly(dI-dC)•poly(dI-dC) was added, and the ^{32}P-labeled site I probes utilized included the wild-type or mutant DNAs extending from −156 to −72 (see Figure 1) or a double-stranded oligonucleotide comprised of only the proximal portion of site I (top strand: $^{5'}$GATCTT-GTCGAGAGGGCGGGGACAATTG$^{3'}$; bottom strand: $^{5'}$GATCCAATTGTCCCCGCCCTCTCGACAA$^{3'}$) or the distal portion of site I (van Wijnen *et al*., 1989). The specific competitors included the above proximal and distal site I oligonucleotides, an Sp1 consensus oligonucleotide (top strand: $^{5'}$ATTCGATCGGGGCGGGGCGAGC$^{3'}$; bottom strand: $^{5'}$GCTCGCCCCGCCCCGATCGAAT$^{3'}$), an Sp1 consensus dimer oligonucleotide (Westin & Schaffner, 1986), and ATF consensus oligonucleotides (Hai *et al*., 1988a, 1989). The H3 histone distal site II oligonucleotide used as a nonspecific competitor has been previously described (van Wijnen *et al*., 1991).

When antibodies were included in the binding reactions, 1 μL of antiserum was mixed with 9 μL of the nuclear extract or cell lysate and incubated on ice for 1 h to allow complex formation. This mixture was then added to the rest of the reaction (in the absence of DTT) and incubated for an additional 20 min at room temperature. The Sp1 antibody Sp1 (PEP 2), a rabbit polyclonal IgG raised against residues 520−538 of the human Sp1; the ATF-1 antibody (C41-5.1), a mouse monoclonal IgA raised against recombinant human ATF-1; and the ATF-2 antibody ATF-2 (F2BR-1), a mouse monoclonal IgG1 raised against recombinant human ATF-2 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

For experiments involving recombinant ATF proteins, *in vitro* transcription/translation of the plasmids pGEM3-ATF-1 and pGEM3-ATF-2 (Michael Green, personal communications; Hai *et al*., 1989) was carried out using a coupled reticulocyte lysate system (Promega). A sample of 2 μL of the expressed protein products was assayed as described above using the gel mobility shift assay, with the exceptions that only 0.5 μg of poly(dI-dC)•poly(dI-dC) was added and that the final KCl concentration of the reaction was 120 mM.

Transient and Stable Transfection Assays. All cells were plated in six-well flat bottom plates (Corning) the day prior to transfection either at a density of 0.8×10^5 cells/35 mm well in 2 mL of Dulbecco's modified Eagle's medium supplemented as described above (for HeLa S3 cells) or F-12 media (for ROS 17/2.8 cells) or at a density of 2.5×10^5 cells/well in 2 mL of Schneider's insect medium supplemented as described above (for *Drosophila* Schneider S2 cells). Transfection of plasmids pSCAT, pSICAT, pSIP-CAT, pSIPICAT, pDSI (Ramsey-Ewing *et al*., 1994), p$^-$46CAT (Ramsey-Ewing *et al*., 1994), pPacSp1 (Courey & Tjian, 1988), or with ROS cells pRSVLuc (Brazier *et al*., 1989) was accomplished with both the HeLa and *Drosophila* cells using the calcium phosphate coprecipitation method as described (Chen & Okayama, 1988) or by the DEAE-dextran method (Ausabel *et al*., 1989). HeLa and ROS 17/2.8 cells were harvested 72 h following transfection by washing the cells twice in cold phosphate-buffered saline, scraping the cells from the plate, and centrifuging for 3 min at 6000*g*. Schneider cells were washed off the plate using a pipet,

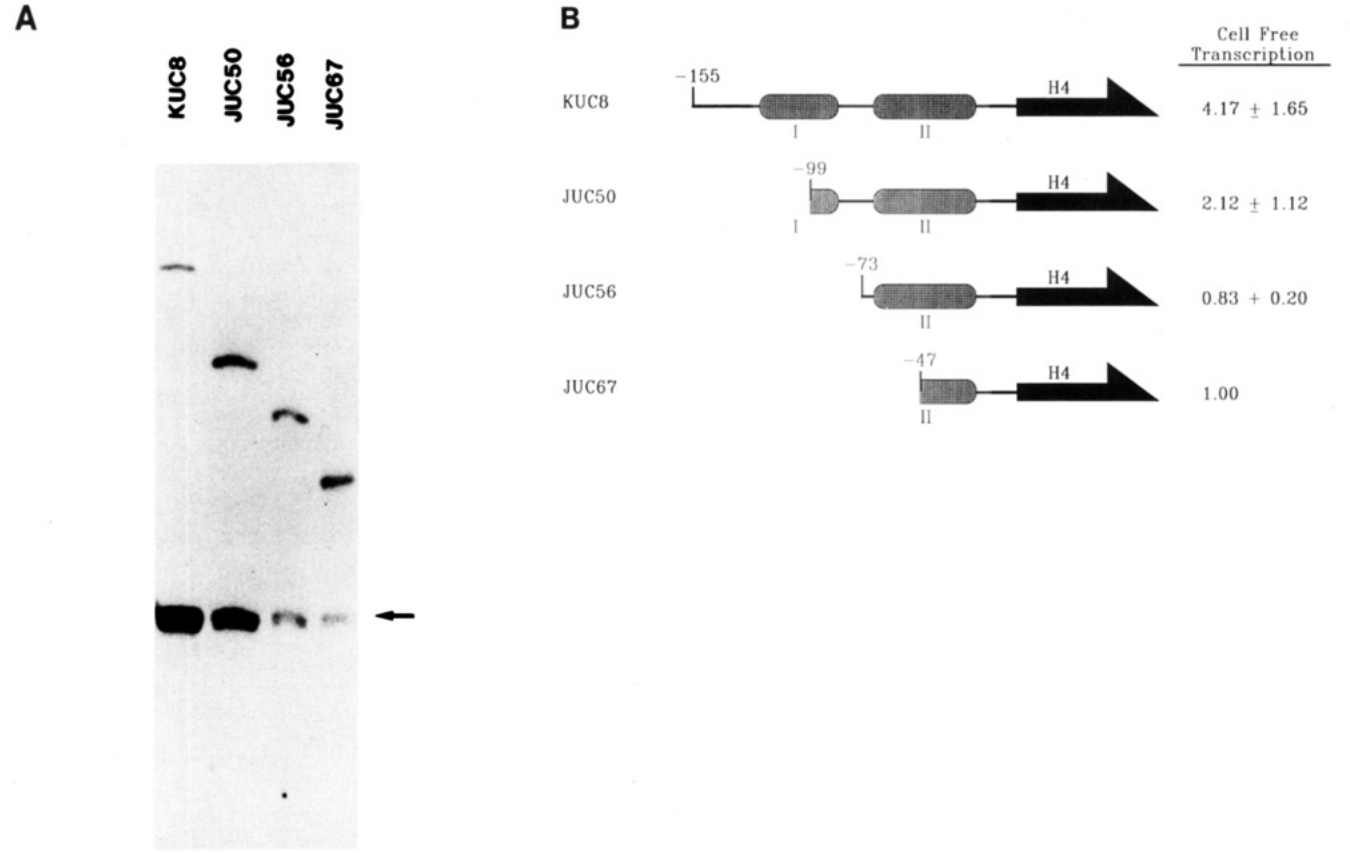

FIGURE 3: *In vitro* transcription analysis of H4 histone promoter deletions. (A) Autoradiograph of S1 nuclease protection analysis. Circular plasmids representing a series of promoter deletion mutants were transcribed in a HeLa cell nuclear extract system, and transcripts were assayed by S1 nuclease protection. The arrow indicates accurately initiated transcripts. (B) Schematic representation of the promoter deletion constructs and quantitation of the cell-free transcription analysis. Values are the average of four independent experiments; the experiment to experiment variations were controlled by normalization to expression driven by a full-length histone promoter (pFO002). The standard error of the mean is indicated.

centrifuged as above, washed in 0.5 mL of unsupplemented Schneider's insect medium, and recentrifuged. The cell pellets were stored at −80 °C until assayed for chloramphenicol acetyltransferase (CAT; Gorman *et al.*, 1982) or luciferase (Brazier *et al.*, 1989) activity. Unless otherwise noted, CAT activities were normalized for transfection efficiency using the luciferase data and are presented as percent conversion/relative light units. Statistical analysis was carried out using ANOVA, and the data were transformed to better approximate a normal distribution. Stably transfected cell lines were established by co-transfection with the neomycin resistance gene (pSV3*neo*) and were selected with500 mg/mL Geneticin (G418, Gibco) as described (Southern & Berg, 1982). Cells used for these analyses were polyclonal. No significant differences in the average gene copy number were observed between cells transfected with the different constructs. Nuclear run-on assays were utilized to measure *in vivo* the transcriptional levels of the transfected genes as described (Clayton & Darnell, 1983).

RESULTS

The in Vitro Site I Footprint Matches the in Vivo Genomic Footprint. To assess structural and functional similarities between *in vivo* and *in vitro* protein/DNA interactions at site I of the FO108 H4 histone gene, we performed DNase I footprint analysis with the same unfractionated HeLa cell nuclear extracts used for transcription *in vitro* (see below). Using a wild-type site I probe (Figure 1), the nuclear extract protected a 36 base pair region (−122 to −87 bp) identical to the boundaries of the site I DNase I footprint determined *in vivo* that spans both the proximal G-rich region and the distal ATF-like site (Figure 2). Mutation of the G-rich region (see Figure 1) produced a footprint of reduced size, unaltered on the distal side (−122) but withdrawn on the proximal side up to nucleotide −104, indicating that a distinct complex binds to the proximal sequence in the wild-type probe. Mutation of the ATF-like site (see Figure 1) also produced a footprint of reduced size (−108 to −87), indicating that a second complex binds to the distal region. The footprinting results establish that site I is a bipartite protein/DNA interaction domain.

Site I Contributes to the Level of Transcription in Vitro. Because protein/DNA interactions that occur *in vivo* could be reproduced using HeLa cell nuclear extracts *in vitro* (see above), we developed an *in vitro* transcription system using aliquots of the exact same nuclear extracts. Four 5′ deletion mutants of the FO108 H4 histone promoter were created by Bal 31 digestion and cloned into a pUC19 vector to assess the contribution of the proximal promoter *in vitro*. Figure 3, panels A and B, shows that a construct containing a deletion of site I in its entirety (JUC56) yielded a 5-fold decrease in transcription as compared to the construct in which site I was intact (KUC8). Deletion of the distal half at site I (JUC50) decreased transcriptional efficiency to a limited extent. Removal of the distal half of site II (JUC67) did not significantly alter the level of transcription *in vitro*

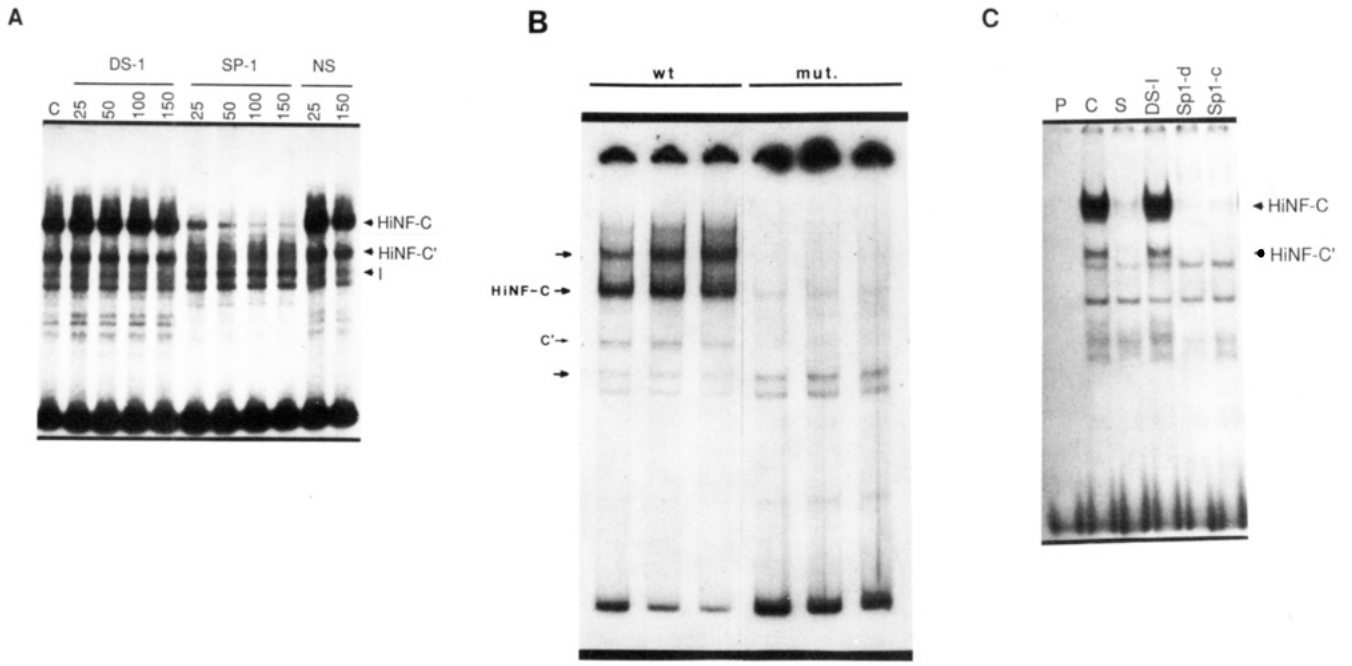

FIGURE 4: Electrophoretic mobility shift assay of site I protein−DNA interactions. (A) Specific competition of HiNF-C with an Sp1 consensus oligonucleotide. A full-length site I probe (−156 to −72 bp plus 14 bp of pUC 19 polylinker) was incubated with 5 μg of HeLa nuclear extract with increasing concentrations of either the distal site I oligonucleotide (DS-1; see Figure 1), an Sp1 consensus dimer oligonucleotide (SP-1; see Materials and Methods), or an unrelated nonspecific oligonucleotide (NS; see Materials and Methods). Lane C designates a control reaction without added competitor, and the numbers above the lanes indicate the fold molar excess of unlabeled double-stranded oligonucleotide competitor over the labeled probe. Complexes HiNF-C, HiNF-C′, and I (see text) are identified to the right of the figure. (B) Effects of a mutation in the proximal G-rich portion or site I on protein−DNA interactions. Either the wild-type full-length probe or a mutant probe in which G residues known to be critical for Sp1 binding were altered was incubated with increasing concentrations of HeLa nuclear extract (4, 6, and 8 μg). The various protein−DNA complexes (see text) are designated to the left. The presence of the uppermost prominent band varied between nuclear extracts (C) Specific binding of complex HiNF-C to the proximal portion of site I. HeLa nuclear protein (5 μg) was incubated with a radiolabeled proximal site I oligonucleotide (see Figure 1) and various competitor oligonucleotides. P, probe incubated in the absence of extract; C, extract included in the absence of competitors; S, extract incubated with a 100-fold excess of unlabeled proximal site I oligonucleotide; DS-1, extract with an excess of the DS-1 oligonucleotide; Sp1-d, extract incubated with an excess of the Sp1 dimer oligonucleotide (see Materials and Methods); Sp1-c, extract incubated with an excess of the Sp1 consensus oligonucleotide (see Materials and Methods). Complexes HiNF-C and -C′ are designated to the right of the figure.

as compared to the site I deletion (JUC56). The effectiveness of the intact promoeter *in vitro* indicates that the necessary trans-activating factors involved are contained in the nuclear extract. More importantly, these data establish that the site I region represents a functional, cis-acting element essential for maximal transcription of the H4 gene.

Transcription Factor Sp1 Binds Specifically to the FO108 Promoter. Previous work from our laboratory has demonstrated that factor HiNF-C binds to site I *in vitro* (van Wijnen *et al.*, 1989). The DNase I footprints of the HiNF-C complex in gel shift assays (van Wijnen *et al.*, 1989) coincide with the footprint observed in the proximal half of site I (Figure 2). To determine the specificity and identity of HiNF-C, we initially carried out electrophoretic mobility shift analysis using a variety of site I probes with excess amounts of unlabeled, double-stranded oligonucleotides (Figure 4). Using the entire site I as a probe, HiNF-C and -C′ present in the HeLa cell nuclear extract were specifically competed in a concentration-dependent manner by an Sp1 dimer oligonucleotide (Figure 4A). A 25-fold excess of this oligonucleotide, the lowest quantity used, was sufficient to compete most of the complexes effectively. These complexes were unaffected either by the distal site I oligonucleotide or by an oligonucleotide completely unrelated to this sequence. The distal site I oligonucleotide did compete a lower, less prominent complex, designated complex 1, that appears to be related to the ATF family of proteins (see below). The uppermost complex was not reproducible between different extracts. Binding of the HiNF-C and -C′ complexes to the full site I probe was abolished when the probe carrying a mutation in the proximal G-rich region was used (Figure 4B), indicating that HiNF-C and -C′ bind specifically to this proximal region. We corroborated these results using an oligonucleotide covering only the proximal portion of site I as a probe (Figure 4C). The two slowest migrating complexes were competable by the unlabeled proximal site I oligonucleotide or by two different Sp1 consensus oligonucleotides but not by the distal site I oligonucleotide, strongly suggesting that these complexes are related to the transcription factor Sp1. Antibodies specific for human Sp1 caused a clear supershift of the HiNF-C complex (Figure 5) but had no effect on the HiNF-C′ complex or on the probe in the absence of nuclear extract. Further, an antibody directed against ATF-2 had no effect on any of the complexes. Taken together, these data identify HiNF-C as the transcription factor Sp1. The identity of HiNF-C′ as the transcription factor Sp3 has recently been established in our laboratory (see Discussion).

ATFs Interact with the Distal Portion of Site I. To assess directly the binding of ATF-like proteins to the site I sequence, we optimized the assay conditions for the detection of ATF family members. We used an oligonucleotide probe spanning the distal portion of site I (DS-I) containing the asymmetric ATF sequence only (see Figure 1). In addition,

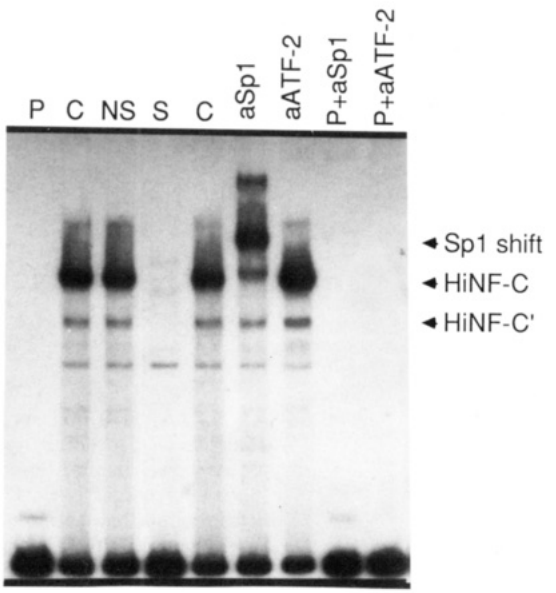

FIGURE 5: Antibody supershift identification of HiNF-C as the transcription factor Sp1. The full site I probe was incubated with 5 μg of HeLa cell nuclear extract and analyzed using the electrophoretic mobility shift assay. The Sp1 supershift and HiNF-C and -C′ are identified to the right of the figure. P, probe alone in the absence of extract; C, control reaction with the addition of nuclear extract; NS, 100-fold excess of a nonspecific oligonucleotide competitor (see Materials and Methods); S, 100-fold excess of unlabeled site I DNA competitor; aSP1, extract preincubated with 1 μL of anti-Sp1 supershift antibody; aATF-2, extract preincubated with 1 μL of anti-ATF-2 supershift antibody; P+aSp1, probe in the absence of extract but in the presence of the anti-Sp1 antibody; P+aATF-2, probe in the absence of extract but in the presence of the anti-ATF-2 antibody.

heat treatment of nuclear extracts and the TBE gel system were used to optimize extraction and DNA binding of ATF protein (Hai *et al.*, 1988a,b). This assay yielded two prominent protein/DNA complexes (Figure 6A, lane C) that were effectively competed with the wild-type distal oligonucleotide using a 100-fold molar excess (lane S). However, when the oligonucleotide carrying the mutation in the ATF core was used, little or no competition was observed (lane M), indicating that formation of these two complexes is dependent on an intact ATF core sequence. The addition of an ATF-1-specific antibody caused a supershift of the two predominant complexes (lane a1), indicating that the antibody is interacting with a component of each. An antibody generated against ATF-2 had no effect on the reaction (lane a2). These results demonstrate that ATF-1 is an endogenous HeLa cell nuclear protein that binds specifically to the distal portion of site I.

To assess which ATF family members are capable of binding to the distal portion of site I, human cDNAs encoding ATF-1 and ATF-2 were used as templates for the expression of these proteins *in vitro* and the resulting lysates tested in the gel mobility shift assay (Figure 6B). Control lysates that did not receive template DNA displayed a prominent lower band (lane L, complex 4) and a minor upper band (land L, complex 1). Lysates containing expressed ATF-1 (lane ATF1, complex 3) and ATF-2 (lane ATF2, complex 2) yielded complexes of differing mobilities, both distinct from bands formed with control lysates (lane L). The recombinant ATF-1 formed a protein/DNA complex displaying a migration very similar to the pair of ATF-1 complexes found in HeLa nuclei (Figure 6C). Both of the recombinant ATFs could be supershifted in gel retardation assays using their corresponding antibodies (data not shown). These results indicate that the distal portion of site I represents a bone fide ATF-binding sequence. Studies using a battery of individual antibodies directed against many of the ATF and AP-1 proteins failed to identify any of these proteins as additional components of the two ATF-1-containing complexes in HeLa cells (data not shown). Because recombinant ATF-1 homodimers expressed *in vitro* (lane ATF 1, see Discussion) appear to comigrate with the faster migrating complex observed using nuclear extracts (Figure 6C), we postulate that this HeLa complex represents an ATF-1 homodimer while the slower migrating band is a heterodimer consisting of ATF-1 and an unknown nuclear protein. It is interesting that, while no ATF-2 binding can be detected using HeLa cell extracts (Figure 6A, lane a2), recombinant ATF-2 can interact with this site (Figure 6B).

Transcriptional Contribution of Site I in Vivo. To examine the functional role of site I *in vivo*, we studied the effect of a total deletion of the region using transient transfection assays. Transfection of a histone promoter/CAT fusion construct (108CAT) encompassing all of site I and site II into HeLa cells yielded significant reporter activity (Figure 7, panels A and C). Deletion of site I and adjacent sequences severely reduced this activity (Figure 7, panels A and C). After normalization with respect to the transfection efficiency, the removal of site I sequences was found to decrease CAT activity 7-fold. Further deletion of the promoter, which disrupted the site II protein/DNA interaction domain, abolished all specific initiation (Kroeger *et al.*, 1987) and reduced CAT activity to background levels (Ramsey-Ewing *et al.*, 1994).

To further demonstrate a role for site I in modulating transcription and to address the effect of integration into a chromosomal structure, we established pools of stably transfected cell lines containing these constructs. Expression of each construct was assayed in several independent cell pools by nuclear runon transcription and normalized to the level of 18S ribosomal gene expression as an internal control (Figure 7, panels B and C). Consistent with the data generated using transient expression assays, the stable cell lines demonstrated a 7.0−7.5-fold decrease in transcription *in vivo* upon the deletion of site I. Therefore, the effect of site I sequences on transcription in the transient assay accurately reflects the effect observed with the genes integrated into a chromosomal structure.

The Sp1 Binding Site Is Essential for Maximal and Cell-Type Specific Promoter Activity. To directly analyze the function of the Sp1 and ATF binding sites, we generated specific mutations within these sites and performed transient gene expression assays (Figure 8). Wild-type or mutant histone promoter−CAT fusion constructs were transfected into HeLa cells, and the cells were subsequently assayed for CAT activity. As shown in Figure 8, panels A and B, mutation of the proximal Sp1 site caused an 80−90% reduction of promoter activity in HeLa cells ($P < 0.0007$). Mutation of the distal ATF site alone did not result in a statistically significant change in promoter activity. However, mutation of both sites (double mutant) was equivalent to the deletion of the entire site I region, causing an additional 50−80% decrease in promoter activity as compared to the proximal mutant ($P = 0.0224$). These data were highly reproducible in three separate experiments using three

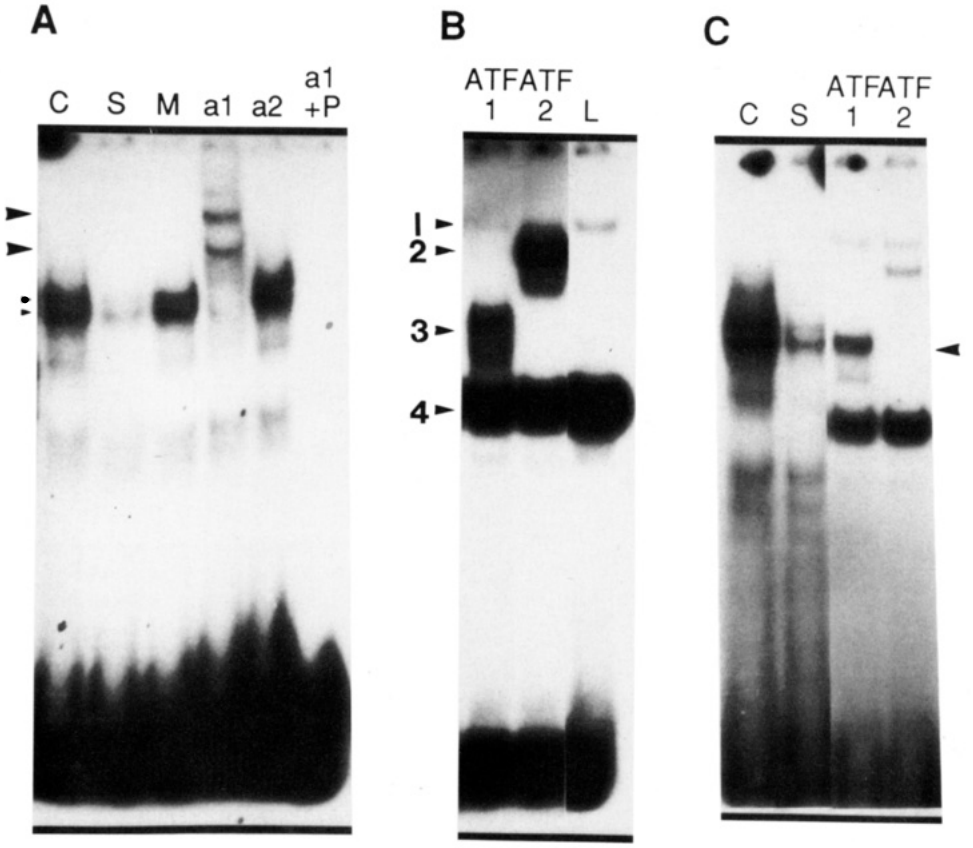

FIGURE 6: Interaction of ATF-1 and other ATF-family related proteins with the distal portion of site I. Gel mobility shift analysis was carried out using TBE gels, heat inactivated nuclear extract, and the distal site I probe (Figure 1). (A) Specific and mutated oligonucleotide competition and immunological detection of ATF-1 protein/DNA complexes. Lanes: C, control without competitor; S, 100-fold excess of distal site I specific oligonucleotide; M, 100-fold excess of mutated distal site I oligonucleotide; a1, ATF-1 specific antibody present during preincubation; a2, ATF-2 specific antibody present during preincubation; a1+P, probe alone with ATF-1 specific antibody present. The large arrowheads designate the ATF-1 antibody supershifts, and the small arrowheads designate the doublet of ATF-1 complexes. (B) Binding of recombinant ATF-1 and ATF-2 to distal site I. pGEM3-ATF-1 and pGEM3-ATF-2 were used as templates for the overexpression of ATF-1 and ATF-2 as described in Materials and Methods and analyzed in the gel mobility shift assay. Lanes: ATF1, expressed recombinant ATF-1; ATF2, expressed recombinant ATF-2; L, lysate without protein overexpressed. For an explanation of the complexes designated by arrowheads 1−4, see text. (C) Comigration of recombinant ATF-1 homodimers with ATF-1 present in HeLa cell nuclear extracts. Lanes: C, control nuclear extract without competition; S, extract with 100-fold excess of distal site specific oligonucleotide; ATF1, expressed recombinant ATF-1; ATF2, expressed recombinant ATF-2. The arrowhead at the right designates the ATF-1 homodimers.

separate DNA preparatons in which the transfection efficiency was normalized to the internal control (pRSVLuc, data not shown) and establish that the Sp1 binding site is critical for maximal H4 gene transcription. On the other hand, functionality of the ATF site was demonstrable in this assay only in the absence of the intact Sp1 binding site.

Since Sp1 activity can vary significantly between different cell types (Courey & Tjian, 1992), we also examined our battery of site I mutants in other cell types (Figure 8C). In rat osteosarcoma (ROS 17/2.8) cells, mutation of the proximal Sp1 site yielded only a reproducible 2-fold reduction in reporter activity ($P = 0.016$). Mutation of both elements resulted in a slight additional decrease in promoter function ($P = 0.0035$). These data differ ($P < 0.0007$) from those obtained with HeLa cells where the proximal mutation had a more pronounced effect (Figure 8C). Thus, it appears that the Sp1 binding site is more important to H4 gene expression in HeLa cells than in ROS 17/2.8 cells, suggesting that Sp1 may influence H4 gene transcription in a cell type specific manner.

Transactivation of the H4 Histone FO108 Promoter by Sp1 Overexpression. Because the Sp1 binding site had a significant effect on transcription in all cells, we explored directly Sp1 activation of the H4 promoter *in vivo*. We carried out co-transfection experiments using *Drosophila* Schneider S-2 cells known to lack endogenous Sp1 activity (Courey & Tjian, 1988). When the wild-type histone promoter−CAT fusion construct was used as the reporter, only a basal level of expression was observed (Figure 9). Consistent with the absence of Sp1 activity in these cells, there was no difference detected between this construct and the construct carrying the Sp1-binding site mutation. However, upon the co-transfection of the wild-type plasmid with the Sp1 overexpression plasmid pPacSp1 (Courey & Tjian, 1988), a 10-fold activation of expression was detected. This activation was not observed using the reporter plasmid carrying the mutation in the Sp1 consensus site or with a reporter plasmid in which all of site I and part of site II is deleted (p204CAT, Figure 8A). In addition, this activation was not observed when an overexpression plasmid containing the *Drosophila* actin promoter but not the Sp1 gene was used for co-transfection (data not shown). The data establish that Sp1 transactivates the H4 promoter by binding the G-rich portion of site I.

DISCUSSION

The data presented in this paper demonstrate unequivocally that the transcription factor Sp1 plays an important role in

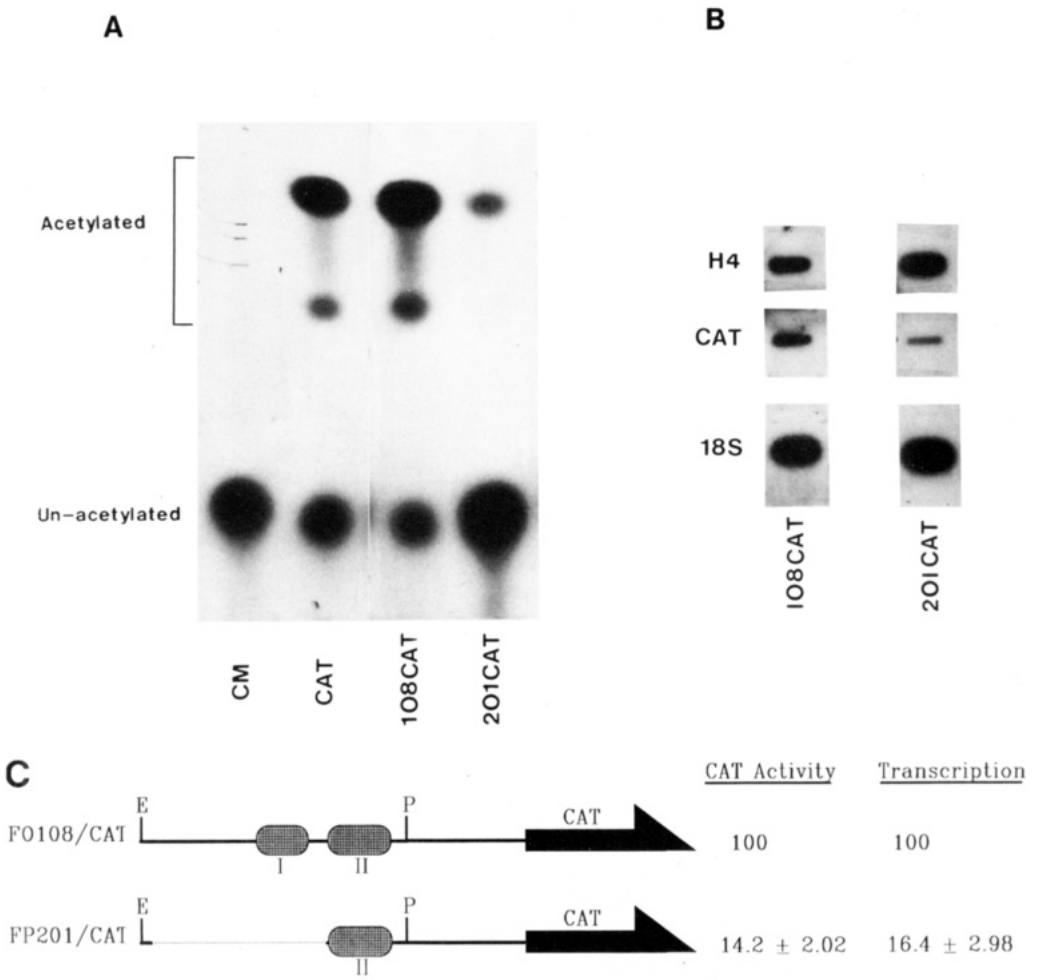

FIGURE 7: *In vivo* analysis of site I deletion mutants. (A) CAT assay of HeLa cells transiently transfected with the indicated histone promoter−CAT fusion constructs. The unacetylated and two acetylated forms of chloramphenical are designated. Lanes: CM, control without CAT enzyme; CAT, control with 0.9 unit of purified CAT enzyme; 108CAT and 201CAT, extracts from cell transfected with the indicated construct. (B) Nuclear run-on transcription analysis of stably transfected cell pools containing the indicated constructs. Radiolabeled RNA transcripts were hybridized to Southern blots containing immobilized plasmid DNA of H14 human histone gene (FO108), 18S ribosomal genes as control, and CAT coding sequence to detect the introduced test gene. (C) Schematic showing deletion constructs and quantitation of the CAT activity (average of five experiments) and nuclear runon transcription (average of three experiments). Similar relative levels of expression were obtained for both transient transfections and stable cell lines. Nuclear runon transcription was normalized to the endogenous 18S ribosomal transcripts. Standard errors are indiated.

the function of the FO108 promoter and, hence, in the expression of this cell cycle regulated histone H4 gene. While our deletion analysis illustrates that histone H4 site I as a whole is required for maximal expression, the point mutations studied here precisely define a prominent role for Sp1 in the expression of this gene in HeLa cells. Therefore, while the cell cycle element found in site II is responsible for the timing of transcriptional enhancement as cells reach the S phase (Ramsey-Ewing *et al.*, 1994), Sp1 probably acts to amplify this response.

That adjacent Sp1 and ATF binding sites may function in concert is supported by evidence from the human retinoblastoma gene promoter, where point mutation of either one of these elements results in a greater than 90% decrease in transient expression (Sakai *et al.*, 1991). However, a role for the distal asymmetric ATF binding sequence in site I is less obvious, as mutation of this region by itself results in, at best, only a 2-fold effect upon expression in any cell type examined. Only in the absence of a functional Sp1 site can the effect of the ATF mutation be reproducibly observed in HeLa cells. Because mutation of the Sp1 site is, of course, not a normal condition, any interpretation of the Sp1/ATF double mutation is complex. Nevertheless, the data do show that the ATF site can possess function in living cells. We offer the speculation that this ATF site may function in cells where Sp1 activity is low (see below). In any event, DNase I footprinting data clearly show that this site is occupied both *in vitro* (Figure 2) and *in vivo* (Pauli *et al.*, 1987). Further, our results with the gel mobility shift assay demonstrate specific binding of two ATF-1-containing complexes present in HeLa cells (Figure 6A). While ATF-1 will form homodimers, it apparently does not heterodimerize with ATF-2 or ATF-3 (Hai *et al.*, 1989) or with the AP-1 proteins Jun, Fos, or Fra-1 *in vitro* (Hai & Curren, 1991). In addition, while our data show that ATF-1 and ATF-2 homodimers are able to interact with the asymmetric binding site present in the FO108 histone H4 promoter, other work (Vallejo *et al.*, 1993) raises the possibility that some ATF family members may bind this region only as heterodimers with other, non-ATF-related proteins.

That the Sp1 motif present in site I confers cell type specificity to the H4 promoter is evidenced by the fact that a different fold-effect of the Sp1 mutation is observed between cell types. In HeLa cells, the mutation causes a

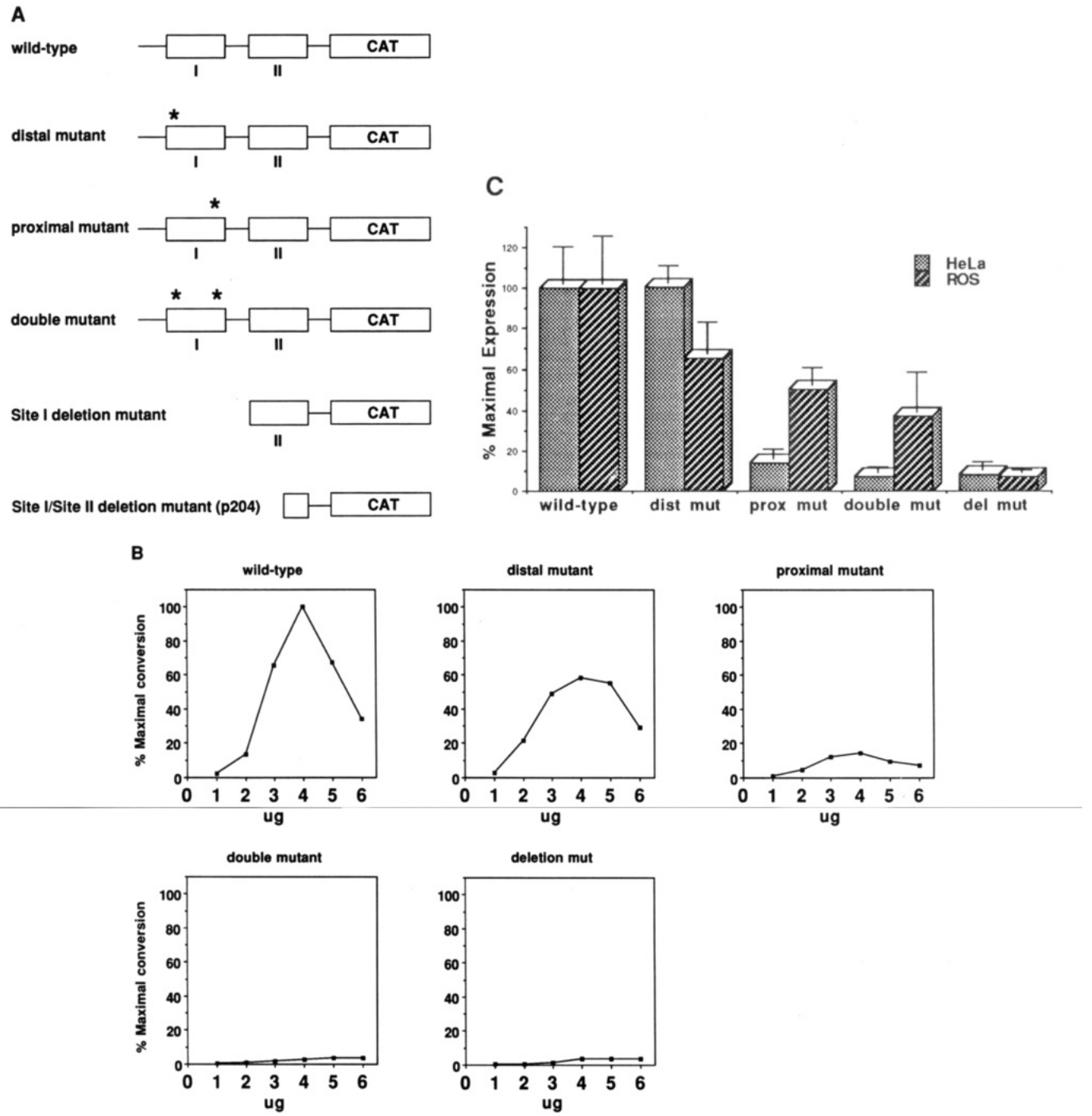

FIGURE 8: Dominant, cell type specific role for the Sp1 binding site of the FO108 histone H4 promoter in living cells. (A) Diagram of the plasmid constructions used in the transfection procedure. The distal mutation disrupts the ATF binding site while the proximal mutation disrupts the Sp1 binding site. (B) Analysis of wild-type and mutant promoter function in HeLa cells. A titration curve of each transfected plasmid is represented by an individual graph. The data show the results of a representative experiment with one set of DNA preparations. (C) Comparison of mutant promoter function in HeLa and ROS cells. The data represent the results of two transfection experiments (using 4 μg of reporter plasmid/well in triplicate) with two different sets of DNA preparations, and the data are normalized to the internal control plasmid pRSVLUC. The error bars designate the standard deviation.

significant 6−10-fold reduction in promoter activity, whereas in ROS 17/2.8 cells the same construct shows only a 2-fold decrease relative to the wild type. Further, this mutation is irrelevant in *Drosophila* cells lacking Sp1 activity. Therefore, the cellular availability of Sp1 obviously affects the function of the H4 FO108 promoter. Similarly, Sp1 has recently been shown to be important in the monocytic cell specific expression of the human CD14 gene (Zhang *et al.*, 1994). While Sp1 is believed to be a ubiquitous factor, many lines of evidence support the concept that both the expression and the activity of Sp1 are indeed regulated [reviewed by Courey and Tjian (1992)]. Levels of Sp1 expression are known to vary significantly (Saffer *et al.*, 1991) and are induced upon SV40 infection, causing the activation of several Sp1-responsive promoters (Jackson *et al.*, 1990; Saffer *et al.*, 1990). In addition, alterations in Sp1 activity are known to come about within different cell types via differential glycosylation (Jackson & Tjian, 1988; Schaufele *et al.*, 1990) and/or phosphorylation (Jackson *et al.*, 1990) of Sp1 itself or by the methylation of Sp1 binding sites (Jane

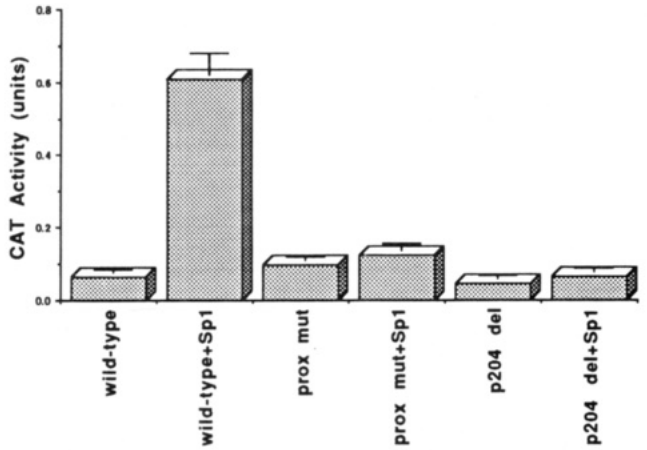

FIGURE 9: Transactivation of the FO108 histone H4 promoter by Sp1 in *Drosophila* cells. Schneider S2 cells, lacking endogenous Sp1 activity, were transfected with 2 μg of the specified reporter plasmid (see Figure 5A) in the absence or presence of 5 ng of the Sp1 overexpression plasmid pPacSp1. The data represent the results from triplicates and are normalized to the internal control plasmid pRSVLUC. The error bars designate the standard deviation.

et al., 1993). More recent work (Chen *et al.*, 1994) shows that the retinoblastoma gene product (Rb) may regulate Sp1 activity by sequestering an Sp1-binding inhibitory protein. Taken together, these findings have led to the hypothesis that Sp1 activity may indeed be present in limiting amounts in mammalian cells, and physiological events that give rise to alterations in Sp1 activity may yield changes in gene expression (Courey & Tjian, 1992). Most recently, the Sp1-like protein Sp3 was shown to inhibit the transactivating effects of Sp1 overexpression in *Drosophila* cells, probably by competing effectively for G-rich DNA binding sites (Hagen *et al.*, 1994). These data imply that a changing Sp1/Sp3 ratio between cell types during differentiation or during the cell cycle may also contribute to Sp1-mediated transcriptional control mechanisms. Recent studies in our laboratory demonstrate that Sp3 present in HeLa cell nuclear extracts binds specifically to the H4 promoter (M. J. Birnbaum, G. S. Stein, and J. L. Stein, unpublished).

While the tissue-specific expression of histone proteins and its variants has long been recognized (Zweidler, 1984), including the DNA replication-dependent variant H1 genes Hh8c (La Bella *et al.*, 1988) and FNC16 (Collart *et al.*, 1988), little is known about this phenomenon in the replication-dependent histone H4 genes. Our results with the FO108 promoter raise the interesting possibility that a single H4 histone gene may be differentially expressed in different cell types depending, at least in part, upon the activity of Sp1. It is also possible that other histone H4 genes exhibit cell type specific patterns of expression distinct from the FO108 gene. Some heterogeneity does exist between the different histone H4 promoter regions, and not all H4 genes contain a proximal Sp1-like binding motif (unpublished data). Therefore, cell type specific variations in trans-acting factors would require a battery of different H4 histone promoters that vary in their responsiveness to any particular milieu in order to ensure that all cells have a proper complement of H4 histones required for proliferation and the maintenance of the chromatin.

The function of sites I and II undoubtedly requires the interaction of specific transcriptional activating proteins with one another, with specific sites along the DNA, and with an array of more general transcription factors [for review, see Ptashne (1992)]. Recently, many of the specific proteins functioning at sites I and II have been identified, including IRFs (P. S. Vaughn, G. S. Stein, and J. L. Stein, unpublished), cdc2, cyclin A, an Rb-related protein (van Wijnen *et al.*, 1994), Sp1, ATFs, and perhaps HMG-I(Y) (van Wijnen *et al.*, (1989). Therefore, we can now begin to analyze how these multiple protein components might interact with one another to support the transcription of this cell cycle regulated histone gene.

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