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HiNF-P Directly Links the Cyclin E/CDK2/p220^{NPAT} Pathway to Histone H4 Gene Regulation at the G_1/S Phase Cell Cycle Transition

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Genome replication in eukaryotic cells necessitates the stringent coupling of histone biosynthesis with the onset of DNA replication at the G_1/S phase transition. A fundamental question is the mechanism that links the restriction (R) point late in G_1 with histone gene expression at the onset of S phase. Here we demonstrate that HiNF-P, a transcriptional regulator of replication-dependent histone H4 genes, interacts directly with p220^{NPAT}, a substrate of cyclin E/CDK2, to coactivate histone genes during S phase. HiNF-P and p220 are targeted to, and colocalize at, subnuclear foci (Cajal bodies) in a cell cycle-dependent manner. Genetic or biochemical disruption of the HiNF-P/p220 interaction compromises histone H4 gene activation at the G_1/S phase transition and impedes cell cycle progression. Our results show that HiNF-P and p220 form a critical regulatory module that directly links histone H4 gene expression at the G_1/S phase transition to the cyclin E/CDK2 signaling pathway at the R point.

Fidelity of genome replication in eukaryotic cells is essential for cell division and necessitates the stringent coupling of histone biosynthesis with DNA replication to ensure that nascent DNA is immediately assembled into chromatin during DNA synthesis. Cell division requires staged expression of genes in response to growth factors, which induce cell growth from quiescence or maintain competency for cell cycle progression during periods of active proliferation. Stimulation of cell proliferation initially triggers a cyclin/cyclin-dependent kinase (CDK) cascade, which activates the cyclin E/CDK2 kinase complex at the restriction (R) point (17, 19). The R point is the major cell cycle checkpoint that controls the commitment for DNA replication in late G_1 via CDK2-dependent release of E2F from Rb-related proteins. The R point is mechanistically linked through E2F to activate the gene regulatory program necessary for nucleotide metabolism and DNA replication (17, 19). Passage beyond the R point permits growth factor-independent entry into S phase and subsequent cell cycle stages. However, cell cycle progression remains constrained by multiple checkpoints, including surveillance mechanisms that monitor DNA integrity and fidelity of chromatin assembly.

We postulate that the induction of histone gene expression at the G_1/S phase transition represents a second necessary cell cycle regulatory event. The coupling of DNA synthesis with histone protein production is maintained by coordinately inducing expression of the multiple core histone gene subtypes, including the 15 distinct histone H4 genes, at the onset of S phase (1, 3, 12, 20, 23, 24). The cell cycle regulatory sequence of histone H4 genes lacks E2F binding sites (28). We have recently identified the key transcription factor of H4 genes, histone nuclear factor P (HiNF-P), which interacts with a highly conserved histone H4 subtype-specific element in the site II cell cycle regulatory domain (16). HiNF-P supports histone gene transcription at the G_1/S phase transition independently of the E2F class of regulatory factors. Antisense-mediated deficiency of HiNF-P reduces histone H4 mRNA levels and delays S phase, consistent with a rate-limiting role in cell cycle progression (16).

Through systematic analysis of cell cycle-regulated promoter elements in histone genes, our work has revealed the *cis*-acting sequences and primary transcription factors involved in replication-dependent histone gene expression (9–11, 16, 18, 21, 27–29). A longstanding question concerns how these transcription modules are linked to the basic machinery controlling the cell cycle. Insight into this linkage was initially provided by the discovery of p220^{NPAT} (*n*uclear *p*rotein mapped to the *AT*M locus) and its regulation by cyclin E/CDK2. Cyclin E/CDK2 is activated as cells traverse the G_1/S transition and has been implicated in multiple aspects of S phase entry, including DNA replication, centrosome duplication, and elimination of the CDK inhibitor p27. Cyclin E/CDK2 associates with p220, resulting in selective phosphorylation of at least five CDK2 sites in p220 (14, 33). Moreover, p220 overexpression promotes histone H2A and H4 reporter gene expression through cell cycle-regulated *cis* elements within their promoters, and this effect requires phosphorylation on CDK2 sites (14, 34). This signaling pathway is temporally and spatially regulated. During G_1 in human diploid fibroblasts, p220 is highly concentrated in two subnuclear organelles called Cajal bodies (14, 34), which

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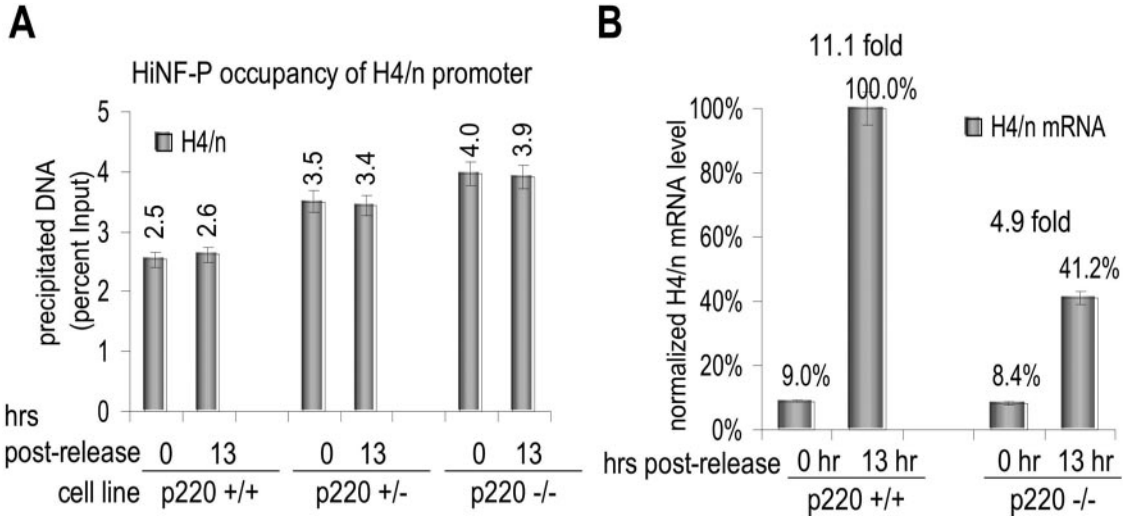

FIG. 1. p220 is a crucial coactivator of cell cycle-dependent histone H4/n expression. HCT116 cells that are wild type or conditionally null for p220 were serum deprived (0 h) and released from quiescence (13 h, G_1/S phase). The samples analyzed are the same ones used by the Harper laboratory (31) to demonstrate that p220 is a global regulator of histone gene expression. (A) HiNF-P occupancy of the H4/n promoter, as determined by ChIP-qPCR, does not depend on the presence of p220. (B) Ablation of p220 reduces the G_1/S phase-specific induction of H4/n mRNA expression, as determined by RT-qPCR. Error bars indicate standard deviations.

are physically tethered to histone gene clusters located at 6p21 (7, 22). As cells enter S phase, p220 expression is induced via the E2F pathway (8), and the protein accumulates in a new set of Cajal bodies localized at histone gene cluster 1q21 while association with 6p21 is maintained (14, 34). p220 is unphosphorylated in early G_1 and becomes phosphorylated on CDK sites in late G_1 and S phase, as cyclin E accumulates in Cajal bodies (14). This phosphorylation is maintained until metaphase, when p220-containing Cajal bodies disassemble. Studies using human somatic cells with a conditional allele of p220 revealed that p220 is required for S-phase entry and for endogenous histone gene expression independent of its role in S phase entry (31). The ability of p220 to activate histone transcription depends upon sequences near its N terminus, including a small motif referred to as a LisH motif (30). The finding that p220 and HiNF-P operate through the same H4 promoter elements suggested that these two pathways function together to coordinate replication-dependent histone gene expression. Evidence of cooperation comes from the finding that both HiNF-P and p220 are required for maximal stimulation of histone H4 gene expression (16), but whether this cooperation reflects a direct regulatory connection remains unknown.

In this study, we have tested whether p220 and HiNF-P are directly linked in a cyclin E/CDK2-dependent coactivating mechanism that regulates cell cycle control of histone H4 gene transcription at the G_1/S phase transition. HiNF-P associates with the H4 promoter independent of p220, indicating that HiNF-P is the proximal component in the pathway (16). Here we show that H4 promoter activation by ectopic p220 is abolished upon RNA interference-mediated depletion of HiNF-P, suggesting a mutual requirement for activation of H4 genes. The N terminus of p220, containing the transcriptionally important LisH-like motif (30), interacts in vivo and in vitro with the C terminus of HiNF-P, which is required for transcriptional activity, as expected if it is recruiting the p220 activator. Furthermore, HiNF-P and p220 colocalization within Cajal bodies and the HiNF-P-dependent association of both p220 and RNA polymerase with the H4/n promoter are cell cycle regulated. Our data reveal the existence of a novel signaling module linking the G_1/S regulator cyclin E/CDK2 with the histone H4 synthesis machinery through an essential HiNF-P/p220^{NPAT} protein complex.

MATERIALS AND METHODS

Cell culture, cell cycle synchronizations, and FACS analysis. HeLa, T98G, and Cos7 cells were maintained in Dulbecco's modified Eagle's medium (Gibco/Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, and 100 U/ml penicillin-streptomycin. SaOS-2 cells were cultured in McCoy's 5A medium (Gibco/Invitrogen) supplemented with 15% FBS, 2 mM L-glutamine and 100 U/ml penicillin in humidified 5% CO_2. T98G cells were synchronized by serum starvation for 72 h, followed by stimulation from quiescence with 20% FBS. HeLa cells were synchronized by the double-thymidine-block method as previously described (25). Cell cycle analysis was performed with propidium iodide-stained cells subjected to fluorescence-activated cell sorting (FACS) (University of Massachusetts Medical Core Facility). HCT116 cells carrying a conditional allele of p220 ($p220^{-/flox}$), as well as $p220^{+/flox}$ and wild-type controls (31), were treated with adenoviral Cre at a multiplicity of infection of 10:1 and analyzed at the indicated times.

Northern blots, immunoblots, and immunofluorescence microscopy. Northern and immunoblot analyses were performed as previously described (16). Immunofluorescence microscopy was performed with whole-cell preparations of HeLa cells as previously described (32). Antibodies against the following proteins were used: HiNF-P (rabbit and IgY); p220 (BD Bioscience) (14); coilin (Zymed); cyclin D1, cyclin E, cyclin A, cyclin B1, actin, and RNA polymerase II (RNAPII) with antigenic peptide (all from Santa Cruz, CA); and SC-35 (Sigma).

Yeast two-hybrid assays. Yeast two-hybrid assays were performed as described previously (2). Expression of all fusion proteins was confirmed by immunoblotting. The EGY48 yeast strain was integrated with LexA-responsive LEU2 auxotrophic marker and LacZ reporter. A two-hybrid interaction was detected by yeast growth on medium deficient for leucine or by detection of LacZ reporter activity on medium supplemented with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside).

IP assays. Cold or ^{35}S-labeled in vitro-transcribed and -translated (IVTT) proteins were produced using the TnT coupled reticulocyte lysate system (Promega, Madison WI), and protein synthesis was confirmed by immunoblotting.

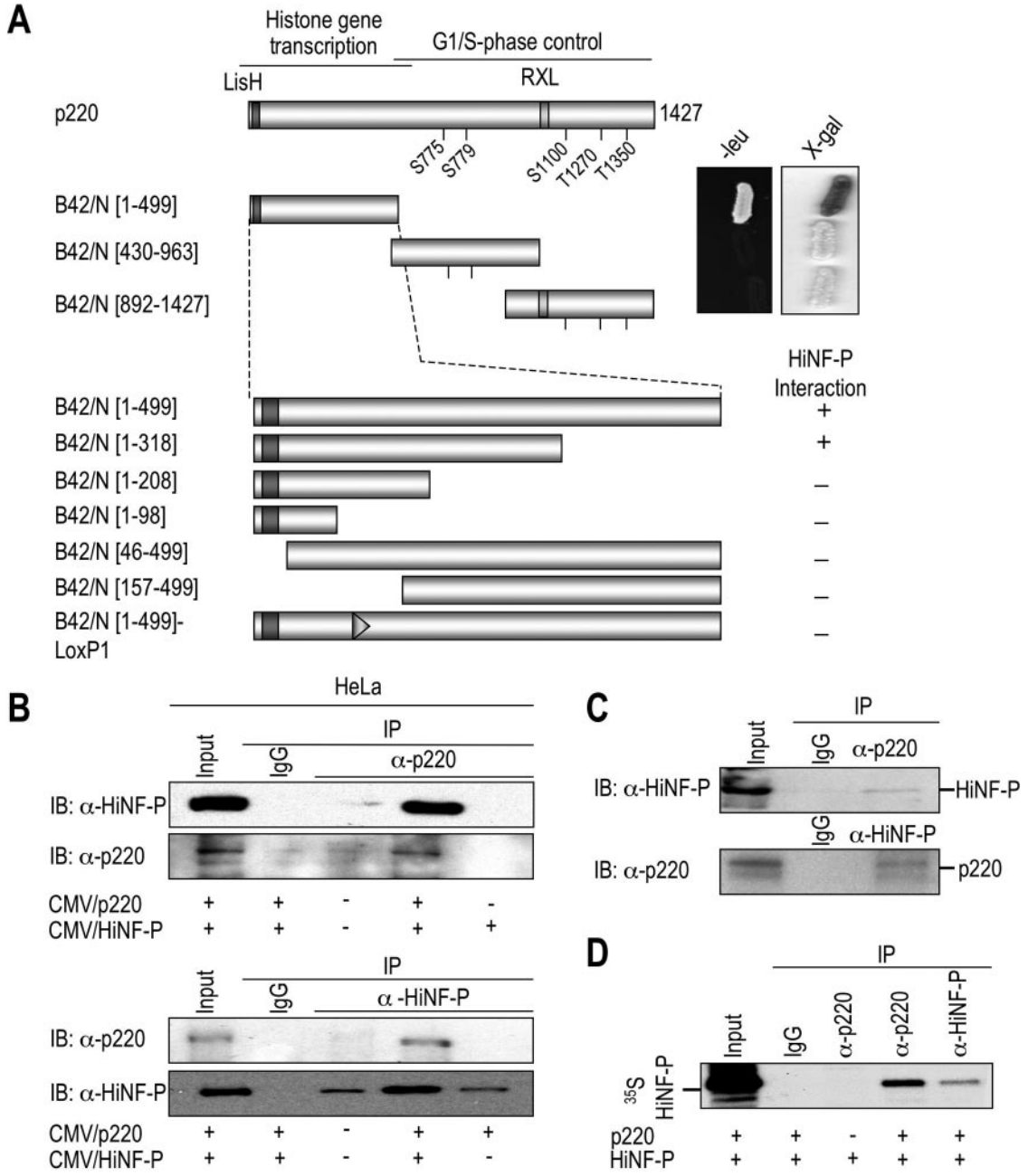

FIG. 2. Direct protein-protein interaction of HiNF-P and p220. (A) Deletion mutants of p220 fused to the B42 activation domain and LexA/HiNF-P were used in yeast two-hybrid assays (in triplicate) to determine the regions necessary for interaction. The p220 diagram shows the LisH motif critical for histone transactivation, as well as CDK2 phosphorylation sites and a cyclin E/CDK2 interaction motif (RXL) required for G_1/S phase progression. LexA/HiNF-P and B42/p220 deletion constructs were expressed and do not self-activate the reporters (data not shown). aa 1 to 46 (contains the LisH domain), 208 to 318, and 121 to 145 (LoxP1 mutant, gray triangle) are vital for p220/HiNF-P interaction. (B) Immunoprecipitations with HeLa cells transfected with p220 and/or HiNF-P vectors were analyzed by Western blotting (IB) with antibodies recognizing either p220 or HiNF-P (lanes 1 to 3, 30 μg protein [60% of input], mouse or rabbit immunoglobulin G [IgG] control, and empty vector control, respectively; lanes 3 to 5, immunoprecipitates with p220 or HiNF-P antibodies). (C) Endogenous HiNF-P and p220 proteins form reciprocal coimmunoprecipitates in HeLa cells as detected with 1,000 μg protein (lane 1, 50 μg protein [5% of input]; lane 2, nonspecific IgG control; lane 3, immunoprecipitates with p220 or HiNF-P antibodies). Blots were probed with chicken HiNF-P or rabbit p220 antibody. (D) Recombinant p220 and HiNF-P (^{35}S labeled) interact in vitro. The panel shows input HiNF-P (10%) (lane 1) and immunoprecipitates with nonreactive mouse IgG (lane 2), monoclonal anti-p220 (lanes 3 and 4), and chicken anti-HiNF-P (lane 5). Lane 3 is a control with only HiNF-P. Each set of experiments was performed at least three times.

HeLa and T98G cells were lysed with lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% NP-40, 1 mM dithiothreitol, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, and 1 μg/ml leupeptin). IVTT protein or 50 μg of cleared lysate was diluted in immunoprecipitation (IP) buffer (20 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.5% NP-40, 1× Complete protease inhibitor [Roche, Indianapolis, IN]) and incubated with the indicated antibodies overnight at 4°C. Immunoprotein complexes were pulled down with protein A/G-agarose beads (Santa Cruz), washed four times with IP wash buffer (20 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% NP-40, 1× Complete protease inhibitor), and separated by 10% sodium dodecyl sulfate-

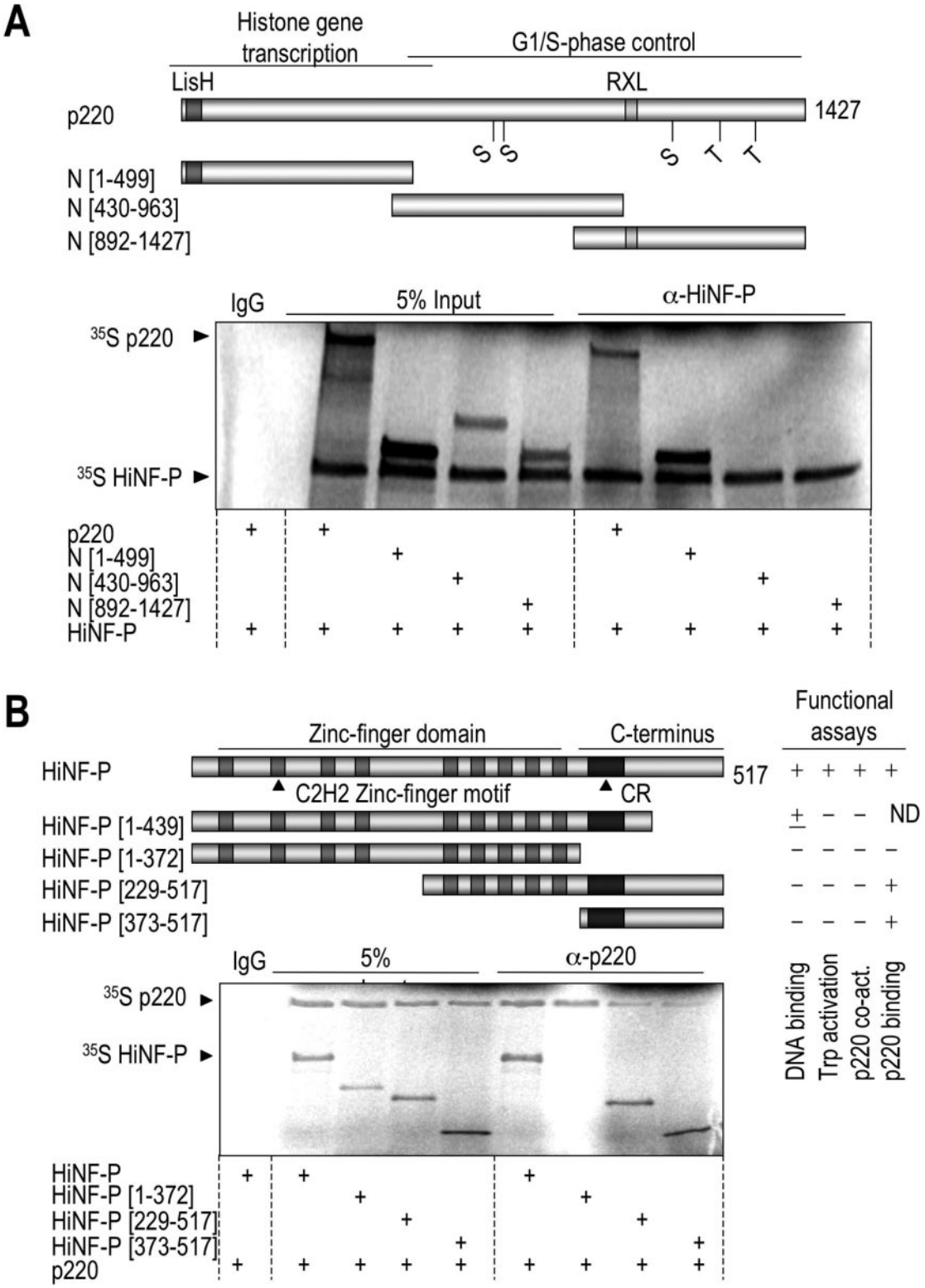

FIG. 3. Mapping the functional interaction domains of HiNF-P with p220. (A) ^{35}S-labeled full-length HiNF-P was mixed with wild-type and truncated ^{35}S-labeled p220 proteins and immunoprecipitated with anti-HiNF-P. The panel shows an SDS-PAGE gradient gel (4 to 15%) containing nonreactive rabbit IgG control with full-length proteins (lane 1), 5% input (lanes 2 to 5), and ^{35}S-labeled p220 proteins immunoprecipitated with HiNF-P antibody (lanes 6 to 9). IgG, immunoglobulin G. (B) Recombinant ^{35}S-labeled wild-type and truncated HiNF-P proteins were mixed with ^{35}S-labeled full-length p220 as indicated. The lanes represent immunoprecipitates with nonreactive mouse IgG (lane 1) or p220 antibody (lanes 6 to 9) or 5% of input protein (lanes 2 to 5), which were separated on an SDS-PAGE gradient gel (4 to 15%). Functional assays that monitor DNA binding activity (i.e., by electrophoretic mobility shift assay), transcriptional activation or p220 coactivation (i.e., by Luc reporter assay), or p220 binding (i.e., by immunoprecipitation) of the HiNF-P mutants are summarized in the table. ND, not determined.

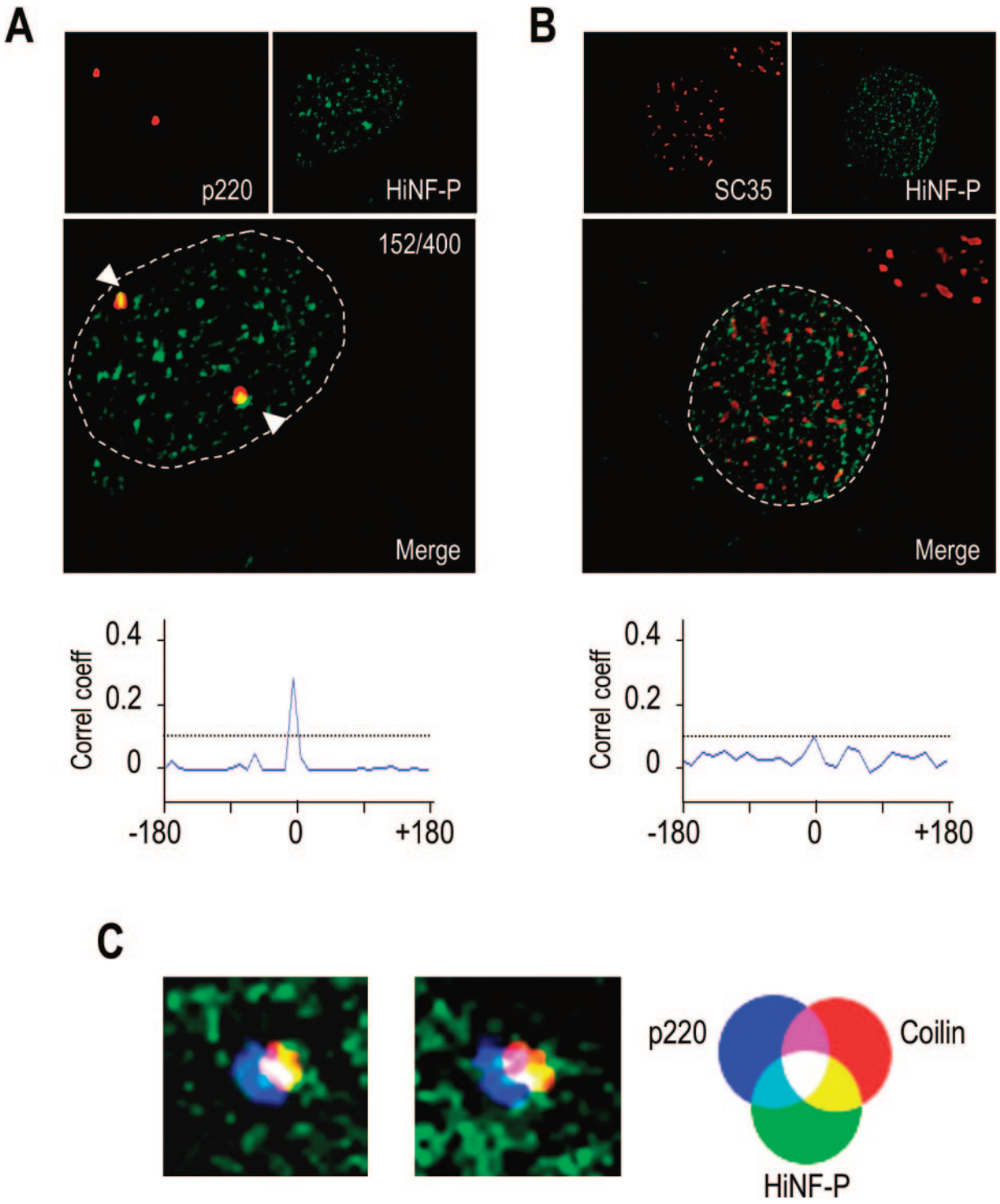

FIG. 4. Subnuclear localization of HiNF-P and p220. (A) Subnuclear localization of HiNF-P (green) and p220 (red) was assessed by in situ immunofluorescence microscopy. The outline of the nucleus (dashed line) was determined by DAPI (4′,6′-diamidino-2-phenylindole) staining. Colocalization of HiNF-P (punctate sites) with p220 foci in HeLa cells is evidenced by a yellow signal (arrowheads) in the merged image. Approximately 40% of cells (n = 400) in an asynchronous population had two or more colocalized foci. Nonrandom colocalization of the green (HiNF-P) and red (p220) of individual signals was determined by image cross-correlation analysis (9). Signal overlap is sharply reduced as the image is rotated in either direction. The single peak at 0 degrees which is depicted in the graph thus reflects specific and nonrandom colocalization. (B) Subcellular localization of HiNF-P (green) and SC-35 domains (red) shows no significant overlap. (C) Triple-label immunofluorescence microscopy indicates recruitment of the HiNF-P (green)/p220 (blue) complex to Cajal bodies (coilin/p80, red). (D) HeLa cells were synchronized at the G_1/S-phase boundary and collected at 2-hour intervals after release from double thymidine block for in situ immunofluorescence analysis of HiNF-P (green) and p220 (red) during the cell cycle. (E) In situ interactions of HiNF-P and p220 are cell cycle dependent and were quantified as the percent of cells (n = 400) displaying two or more colocalized foci at each time point. Error bars indicate standard deviations. (F) A graph of the percentage of cells in S phase (lower) based on FACS data (upper) indicates that subnuclear interaction of HiNF-P and p220 is a cell cycle-dependent event that correlates with progression into S phase.

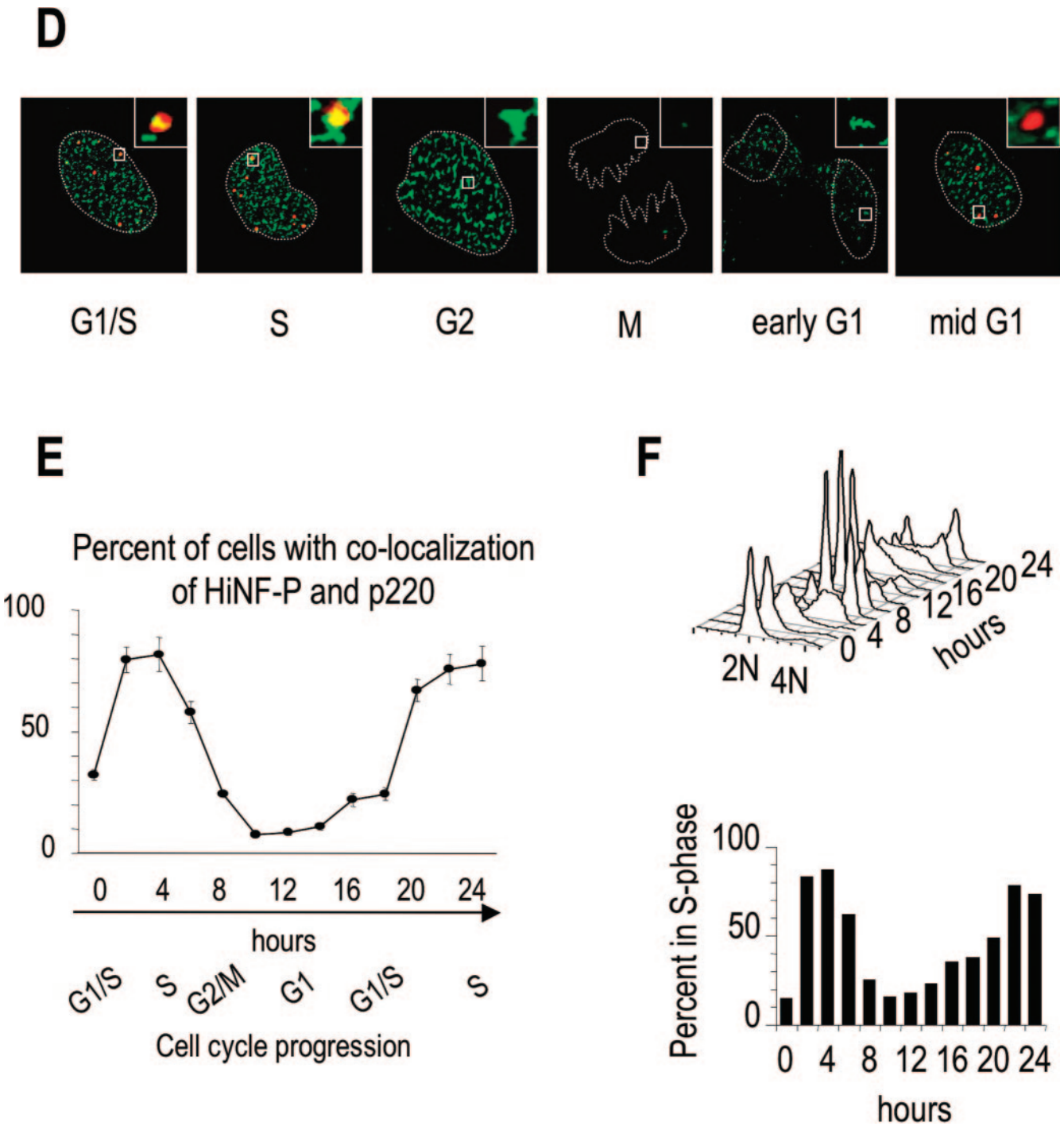

FIG. 4—*Continued.*

polyacrylamide gel electrophoresis (SDS-PAGE). Gels containing radiolabeled protein were dried and exposed to X-ray film and unlabeled proteins were subjected to immunodetection with indicated antibodies. Input protein amounts for endogenous IPs were scaled up 20-fold.

Luciferase assays in Cos7 cells. Transient transfection of Cos7 cells with Fugene 6 (Roche) was carried out in six-well plates seeded at a density of 0.2×10^6 cells per well as previously described (16). Cell lysates were measured for luciferase reporter activity (Promega) 24 h after cotransfection with the following vectors: pCMV-p220, p220 mutants (30), and/or pCMV-HiNF-P.

ChIP. Chromatin immunoprecipitations (ChIPs) were performed as previously described (11). Protein A/G-bead complexes were washed with the following buffers: low salt (20 mM Tris-Cl, pH 8.1, 150 mM NaCl, 1% Triton X-100, 2 mM EDTA, 1× Complete protease inhibitor), high salt (20 mM Tris-Cl, pH 8.1, 500 mM NaCl, 1% Triton X-100, 2 mM EDTA), LiCl (10 mM Tris-Cl, pH 8.1, 250 mM LiCl, 1% deoxycholate, 1% NP-40, 1 mM EDTA), and TE (10 mM Tris-Cl, pH 8.1, 1 mM EDTA). Protein-DNA complexes were eluted with elution buffer (1% SDS, 100 mM $NaHCO_3$). DNA was extracted, purified, precipitated, and resuspended for quantitation.

RT and qPCR. Purified total RNA was subjected to DNase I digestion and used to prepare cDNA with the iScript cDNA synthesis kit (Bio-Rad). Relative quantitation was determined using a 7000 sequence detection system (Applied Biosystems) measuring real time Sybr Green (Bio-Rad) fluorescence and calculated by the ΔΔCT method as recently described (13). Overall efficiencies of PCR were calculated from the slopes of the standard curves of serial dilutions in twofold steps and are virtually identical for each primer set. Expression profiles for H4/a mRNA were extrapolated by comparison of H4/a fluorescent minor groove binder probe-containing quantitative PCR (qPCR) reactions to reactions with both Sybr Green and minor groove binder probes specific for H4/n mRNA. The 5′ untranslated region primers used for the ChIP and reverse transcription (RT) analysis are available upon request.

Antisense and siRNA knockdown of HiNF-P mRNA. Antisense oligonucleotides were used to reduce HiNF-P levels as previously described (16). For small interfering RNA (siRNA)-mediated knockdown of HiNF-P mRNA, T98G cells were transfected in six-well plates with either Silencer Negative Control 1 or HiNF-P-specific double-stranded siRNA oligonucleotides (Ambion Inc.) according to the manufacturer's instructions with Oligofectamine (Invitrogen).

RESULTS

Functional requirement of p220 for HiNF-P-mediated control of histone H4 gene expression at the G_1/S transition. The E2F-independent activation of histone H4 genes at the G_1/S phase transition is mediated through the functional interaction of HiNF-P with the site II cell cycle control element, which has also been shown to integrate regulatory signals involving cyclin E/CDK2/p220. Consistent with the critical role of HiNF-P as a histone H4 gene regulator, we find that HiNF-P is present at endogenous H4 promoters at the G_1/S phase transition (Fig. 1A), supporting the physiologically necessary upregulation of H4/n mRNA (Fig. 1B) to accommodate nascent chromatin assembly during S phase. However, HiNF-P interaction with the H4/n locus is constitutive and occurs in both quiescent and proliferating cells (Fig. 1A). Thus, there is an obligatory requirement for a transcriptional coactivation mechanism that confers cell cycle responsiveness. Indeed, previous data, including our own, indicate that p220 is an important stimulator of histone gene transcription, including that of the H4/n gene (14, 16, 30, 31, 34). The H4/n gene is a major molecular paradigm for understanding mechanisms of cell cycle control and is located in the 1q21 cluster, which exhibits cell cycle-dependent association with Cajal bodies (14).

We directly established the importance of p220 for coactivation of histone H4/n expression by using HCT116 cells, which are conditionally null for p220 (31). In this system, $p220^{-/-}$ cells are generated upon infection of $p220^{-/flox}$ with an adenovirus expressing Cre recombinase. The histone H4/n gene is one of the most highly expressed H4 genes (see Fig. 7C), and its transcription has been extensively studied (4, 12, 16, 21, 27). Histone H4/n mRNA levels were determined in wild-type and p220 conditionally null cells (31) synchronized by serum starvation and analyzed at quiescence (0 h) or at the G_1/S phase transition (13 h after serum stimulation) (Fig. 1). Cell cycle-dependent induction of H4/n mRNA is significantly impaired in p220 null cells (Fig. 1B). Residual H4/n mRNA likely reflects incomplete removal of p220 in these transient assays, as the efficiency of recombination is only 80% (31). The extent of recombination was determined by PCR using p220-specific primers and immunofluorescence microscopy (see Fig. 1D, E, and F of reference 31). Importantly, HiNF-P occupancy on the H4/n promoter is not decreased by the lack of p220 protein (Fig. 1A). However, we observed a modest increase in the HiNF-P signal (~55%), which may result from increased detection of HiNF-P due to epitope unmasking in the absence of p220 binding. We conclude that the HiNF-P-mediated and cell cycle-dependent induction of histone H4/n mRNA at the G_1/S phase transition is regulated by p220 and perhaps involves ordered recruitment of these regulatory factors.

Phylogenetically conserved domains of p220 and HiNF-P mediate functional interactions. We examined whether the H4-regulatory factor HiNF-P and the cyclin E/CDK2-responsive protein p220 are key transcriptional components of a cell cycle-signaling cascade by initially assessing their competency for interaction in yeast two-hybrid assays. HiNF-P was linked to the LexA DNA binding domain, and three distinct segments of p220 were fused to the B42 transactivation domain (i.e., B42/N[1–499], B42/N[430–963], and B42/N[892–1427]). The three segments of p220 were designed to divide the protein into overlapping fragments of equal size (Fig. 2A) to bypass the technical difficulty of expressing this large, 220-kDa human protein in yeast (unpublished observations). We find that cotransformants expressing both LexA/HiNF-P and B42/N[1–499] exhibit growth on leucine-deficient medium and a blue color on X-Gal-containing medium (Fig. 2A). In contrast, yeast cells expressing LexA/HiNF-P and either B42/N[430–963] or B42/N[892–1427] do not grow on leucine-deficient medium and lack a blue color. Our data demonstrate a positive functional interaction between full-length HiNF-P and the N-terminal region of p220 in yeast two-hybrid assays.

The N terminus of p220 contains a LisH-like domain as well as other protein segments, including amino acids 121 to 145, that contribute to the ability of p220 to stimulate histone gene expression (30). To define the minimal region within the N terminus of p220 that interacts with HiNF-P, N-terminal and C-terminal deletion constructs of B42/p220[1–499] were cotransformed with LexA/HiNF-P and tested for activation of the LEU2 and LacZ genes in yeast. Our results show that amino acids (aa) 1 to 45, coinciding with the LisH domain of p220, and the region between aa 208 and 318 are each required for the HiNF-P/p220 interaction (Fig. 2A). Furthermore, replacement of aa 121 to 145 (i.e., B42/N [1–499]-LoxP1), which are required for H4 gene activation, causes a loss of p220 interaction with HiNF-P. Hence, N-terminal amino acids critical for histone gene transactivation by p220 are required for the interaction with HiNF-P in yeast two-hybrid assays.

We validated that p220 interacts with HiNF-P in human cells in vivo by coimmunoprecipitation experiments. Immunoprecipitates from HeLa cells coexpressing p220 and HiNF-P, obtained with highly specific affinity-purified p220 antibodies (14), contain HiNF-P, and the reciprocal immunoprecipitates obtained with affinity-purified HiNF-P antibodies contain p220 (Fig. 2B). Analogous results were obtained with endogenous proteins (Fig. 2C). Thus, p220 and HiNF-P associate in vivo. To determine whether p220 and HiNF-P can interact directly, we produced each protein separately by IVTT and then mixed them prior to immunoprecipitation. As shown in Fig. 2D, anti-p220 antibodies precipitated HiNF-P (lane 4), and this association required addition of p220 (lane 3). These data strongly suggest that p220 and HiNF-P interact directly.

The respective regions required for p220 and HiNF-P interaction were delineated by coimmunoprecipitation using ^{35}S-radiolabeled proteins (Fig. 3). The region of p220 necessary for

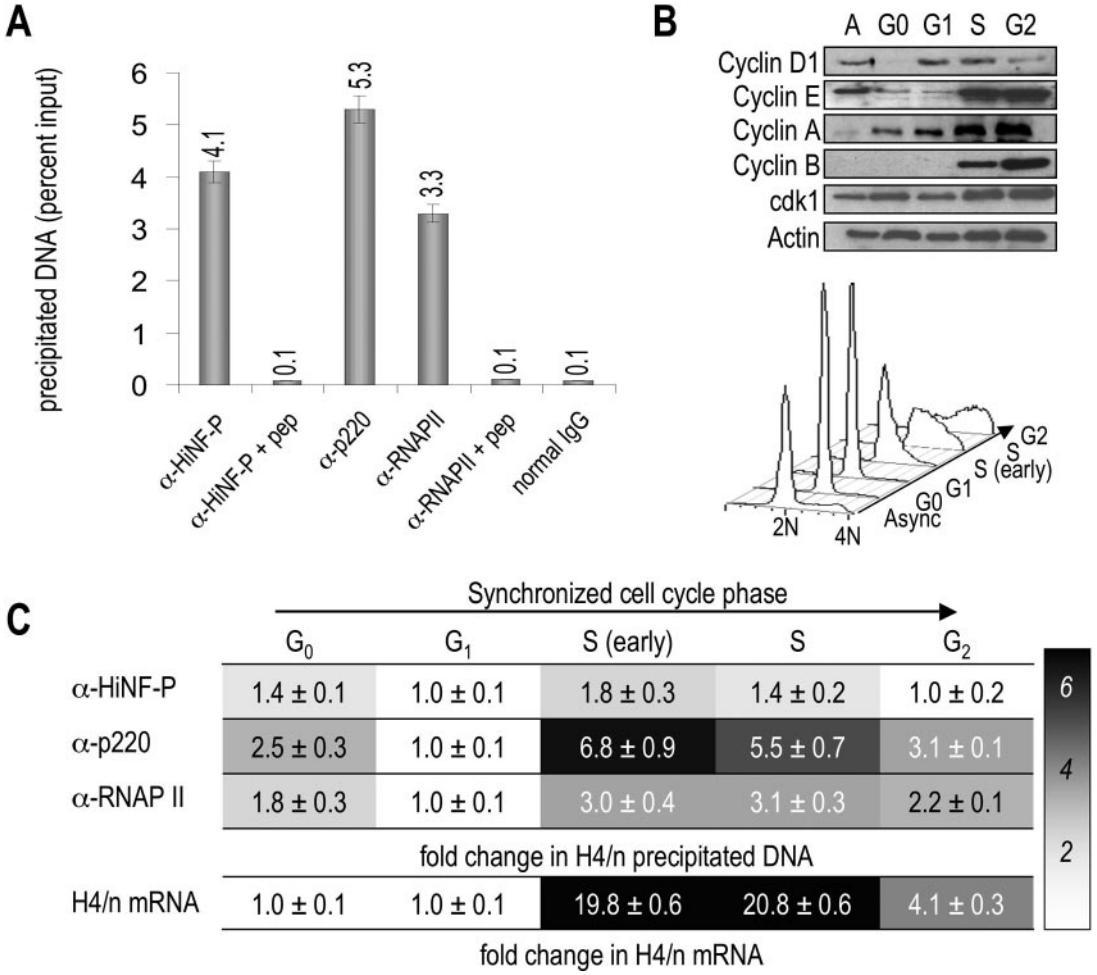

FIG. 5. Cell cycle-dependent occupancy of histone H4 promoter by HiNF-P, p220, and RNA polymerase II. ChIP assays were performed using HiNF-P, p220, and RNAPII antibodies and optimized PCR primer sets for qPCR. (A) Occupancy of the H4/n promoter by HiNF-P, p220, and RNAPII is evident in asynchronously growing human T98G cells based on fluorescent qPCR analysis of immunoprecipitated DNA. Controls for ChIP specificity include normal immunoglobulin G (IgG) and antigenic peptides. Error bars indicate standard deviations. (B) To confirm cell cycle synchrony, Western blot results for cell cycle stage specific proteins (upper) and FACS data (lower) were obtained for serum-stimulated T98G cells that were harvested at the indicated time points after release from serum deprivation. (C) Quantitative summary of ChIP-qPCR and RT-qPCRs data ($n = 5$ separate synchrony experiments) showing fold changes (numerically and by shading) in histone H4/n promoter occupancy by HiNF-P, p220, and RNAPII, as well as in H4/n mRNA levels during the cell cycle.

interaction with full-length HiNF-P was confirmed by HiNF-P antibody-mediated coimmunoprecipitations of recombinant p220 segments similar to those tested in yeast two-hybrid assays. We find that both full-length p220 and the N[1–499] mutant, but not the N[430–963] and N[892–1427] mutants, interact with HiNF-P (Fig. 3A). Thus, the region from aa 1 to 499 of p220 suffices as an HiNF-P interaction domain. Furthermore, full-length HiNF-P, as well as HiNF-P[229–517] and HiNF-P[373–517] deletion mutants, all of which contain the C-terminal region of HiNF-P, coimmunoprecipitate with p220 (Fig. 3B). However, HiNF-P[1–372], which encompasses the Zn finger region, does not interact with p220. Hence, the segment from aa 373 to 517 of HiNF-P, which contains a phylogenetically conserved region, represents a p220 interaction domain. Reporter gene analyses and electrophoretic mobility shift assays with HiNF-P deletion mutants reveal that the domain from aa 373 to 517 contains a distinct region required for transcriptional activation, while both the C and N termini of HiNF-P contribute to DNA binding to the H4 cell cycle element (Fig. 3B). Taken together, our data demonstrate that distinct domains of HiNF-P and p220 confer specificity for their functional interaction.

Cell cycle-regulated colocalization of p220 and HiNF-P in distinct subnuclear microenvironments. Based on the requirement for p220 in the HiNF-P-dependent induction of histone gene transcription at the G_1/S phase transition and our evidence for direct HiNF-P/p220 interactions, it is necessary to assess whether the assembly of HiNF-P/p220 complexes within the nucleus is cell cycle regulated. In situ immunofluorescence microscopy reveals that HiNF-P is dispersed throughout the nucleus in a punctate distribution, whereas p220 forms a limited number of subnuclear foci (Fig. 4A), consistent with previous observations (14, 16, 34). In an asynchronous population of HeLa cells, approximately 40% of cells exhibit limited, but

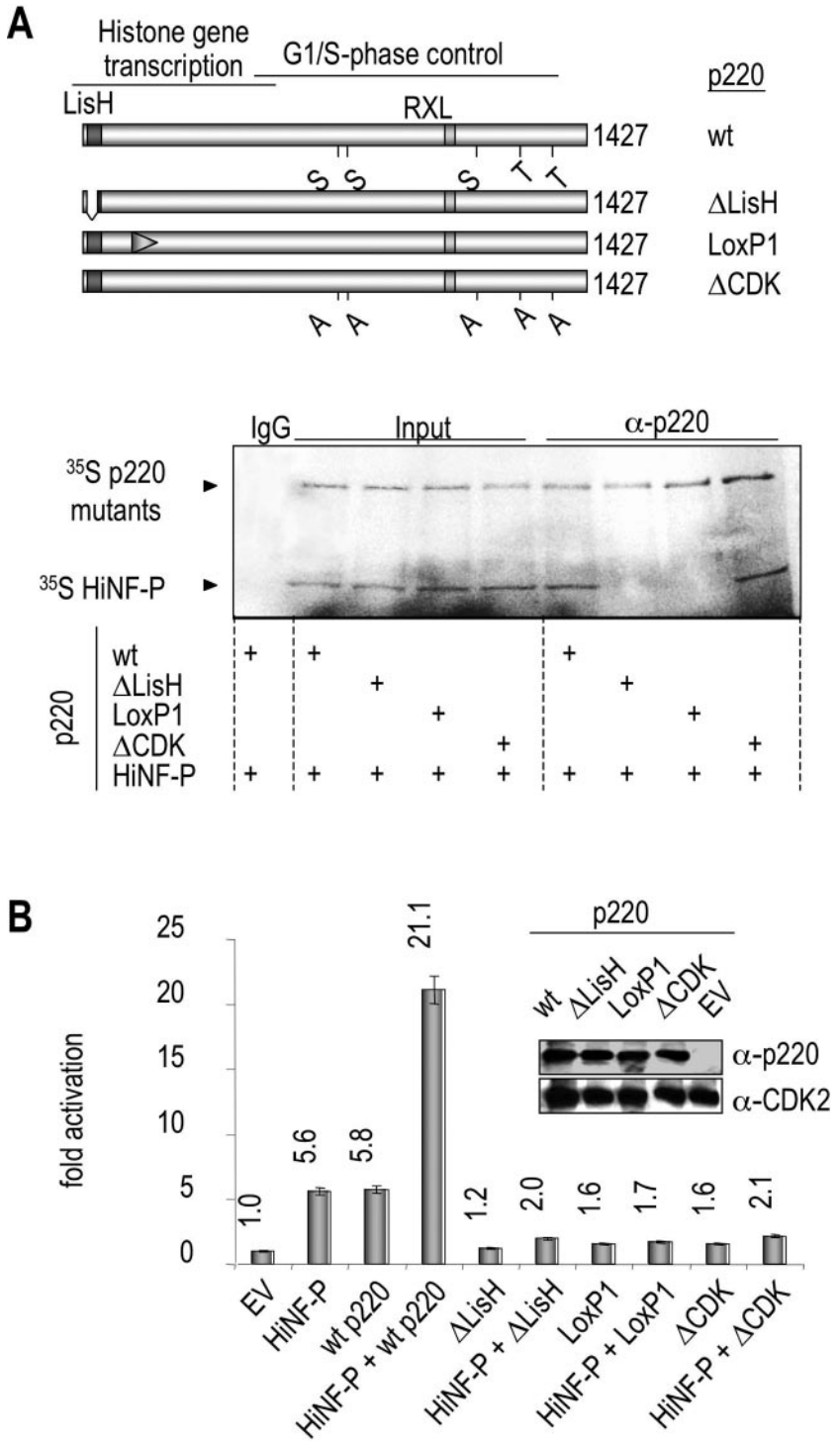

FIG. 6. p220 mutants differ in HiNF-P binding and histone H4 gene activation. The contributions of distinct p220 functions to the interaction with HiNF-P (A) and HiNF-P-dependent activation of the H4 promoter (B) were examined in parallel. (A) N-terminal but not C-terminal substitution mutations of p220 abolish HiNF-P binding. ^{35}S-labeled HiNF-P and normal or mutant p220 proteins were coimmunoprecipitated as described for Fig. 3. The upper band is wild-type (wt) p220 or the indicated p220 mutant, while the lower band is wild-type HiNF-P. IgG, immunoglobulin G. (B) Both N- and C-terminal p220 mutations abrogate synergistic activation of the H4 promoter with HiNF-P. Cos7 cells were transfected with a multimerized HiNF-P recognition site/Luc reporter construct and the indicated expression vectors. Data are presented as fold activation relative to an empty expression vector (EV) control (n = 3 triplicate experiments). The inset shows an immunoblot confirming comparable expression of the p220 proteins relative to the loading control (CDK2). Error bars indicate standard deviations.

reproducible, overlap of HiNF-P with p220 foci. The fidelity of the colocalization was established by confirming nonrandom overlap upon image rotation (Fig. 4A). Furthermore, we do not observe significant overlap with SC-35 mRNA processing domains (Fig. 4B). DNA replication-dependent histone genes are not spliced; therefore, colocalization of HiNF-P and SC-35 is not expected. In addition, using triple-labeling immunofluorescence of HiNF-P, p220, and coilin, we observe pairwise and three-way signal overlap (Fig. 4C). Taken together, these results indicate that a subset of HiNF-P/p220 complexes are associated at or near Cajal bodies, which also associate with histone genes during S phase (7, 22).

The distribution of HiNF-P and p220 during cell cycle progression was examined by in situ immunofluorescence microscopy with synchronized HeLa cells. We find that HiNF-P remains focally distributed throughout the cell cycle but is not detected during mitosis (Fig. 4D). As previously described (14, 34), the number of p220 foci increases by approximately twofold during S phase, and p220 foci are not detected in mitotic cells. Importantly, we observe maximal overlap of immunofluorescence signals in S phase (Fig. 4E and 4F), indicating that the colocalization of HiNF-P and p220 is exquisitely cell cycle dependent. Thus, cell cycle regulation of HiNF-P and p220 subnuclear compartmentalization, perhaps as a component of a larger dynamically organized gene regulatory complex, contributes to control of histone gene transcription during S phase.

Cell cycle-dependent recruitment of HiNF-P, p220, and RNA polymerase II at the endogenous histone H4/n promoter. We addressed whether the interaction of HiNF-P and p220 with histone H4 genes is regulated during the G_1/S phase transition of the cell cycle. ChIP-qPCR analysis was performed with synchronized T98G cells. Our data indicate that actively transcribed histone H4 genes exhibit specific interactions with HiNF-P and p220, as well as with RNAPII, based on peptide competition experiments (Fig. 5A). Serum stimulation of quiescent T98G cells results in synchronous progression toward the G_1/S phase transition (Fig. 5B), concomitant with a 20-fold increase in histone H4 mRNA levels (Fig. 5C) that is mediated in part by a 3- to 5-fold increase in transcription rates (3). The induction of H4 gene expression coincides with a threefold-increased association of RNAPII with the H4/n locus, a representative cell cycle-regulated histone H4 gene. HiNF-P occupancy at the H4/n promoter remains relatively constant during the cell cycle (i.e., less than a 1.8-fold change) (Fig. 5C). Importantly, p220 interaction with the H4/n gene is dramatically increased (up to sevenfold) as cells pass the G_1/S transition. The association of p220 and HiNF-P with the H4/n gene in S phase (Fig. 5C) correlates temporally with our in situ colocalization data (Fig. 4), and with the cell cycle-dependent association of the 1q21 histone cluster containing the H4/n gene with Cajal bodies which we and others previously reported (14, 22, 34). The association of p220 and HiNF-P at the H4 promoter is reduced in G_2 cells (Fig. 5C), yet p220 remains in Cajal bodies during G_2 (14), suggesting that p220 function at the H4 promoter is regulated independently of its localization in Cajal bodies. Importantly, these data indicate that HiNF-P, p220, and RNAPII converge temporally to modulate histone H4 gene transcription during the cell cycle.

The LisH domain and other motifs within the N terminus of p220 are required for interaction with HiNF-P and for HiNF-P/p220-dependent H4 gene activation. The LisH domain (aa 3 to 35) and a functional motif defined by a LoxP linker scan (aa 121 to 145) in the N terminus of p220 are critical for histone H4 gene activation (30). In addition, a p220 mutant that lacks cyclin E/CDK2 phosphorylation sites cannot activate the H4/n promoter (16). We tested whether these p220 mutations affect the physical interaction between HiNF-P and p220 (Fig. 6A) or coactivation of HiNF-P responsive promoters (Fig. 6B). We find that recombinant p220 proteins with mutations in the LisH domain (aa 3 to 35) and LoxP (aa 121 to 145), but not the p220/ΔCDK mutant, have lost the ability to bind wild-type HiNF-P in coimmunoprecipitation assays (Fig. 6A). This result is consistent with the yeast two-hybrid data (Fig. 2) and confirms the importance of the p220 N terminus for HiNF-P binding. However, all three p220 mutants have lost the ability to transactivate basal or HiNF-P-enhanced H4 transcription from a multimerized HiNF-P promoter construct (Fig. 6B). Data obtained with the multimerized promoter monitor intrinsic effects of HiNF-P and eliminate the influence of other histone H4 transcription factors. HiNF-P enhancement of the wild-type histone H4 promoter (data not shown) is slightly lower than that of the multimerized constructs (Fig. 6B) but yields essentially the same results. These data demonstrate that interaction of HiNF-P with the N terminus of p220 is critical for H4 transactivation and that C-terminal CDK2 phosphorylation sites within p220, while not directly required for interaction with HiNF-P, support an essential coactivation function in H4 gene regulation.

HiNF-P supports the cell cycle-dependent recruitment of p220 to coordinately control endogenous histone H4 gene transcription. It remains unclear how p220 is recruited to DNA, because the protein does not contain any discernible DNA binding motifs. The finding that HiNF-P is constitutively associated with the H4/n promoter (Fig. 5) suggests that it is involved in recruiting p220 to the H4/n promoter, and other H4 promoters by inference, to induce replication-dependent H4 transcription. To examine this possibility, we used RNA interference to reduce specifically endogenous HiNF-P mRNA and protein expression and assessed the effect on histone H4/n promoter activity upon p220 coexpression (Fig. 7A). Reduction of endogenous HiNF-P decreases the basal activity of a transfected histone H4/n gene promoter and drastically diminishes p220 stimulation. Because p220 enhancement of H4/n promoter activity depends on the presence of endogenous HiNF-P, this result indicates that p220 functions as a bona fide HiNF-P-dependent coactivator of histone H4 gene transcription.

HiNF-P binds to the conserved H4 subtype-specific element located upstream from the TATA box in the majority of the 15 histone H4 genes (26, 27). To test which histone H4 promoters are occupied by HiNF-P in vivo, we performed ChIP-qPCR assays with multiple H4 loci. We find that HiNF-P occupies 11 of 14 different promoters that we tested (Fig. 7B). Consistent with the critical nature of the HiNF-P/p220 interaction in the regulation of the H4 multigene family, p220 is invariably associated with all HiNF-P-responsive genes, and only one gene (H4/a) interacts weakly with p220 and RNAPII in the absence of detectable HiNF-P. The majority of the HiNF-P/p220-responsive genes are actively transcribed (or poised for transcription), based on the presence of RNAPII.

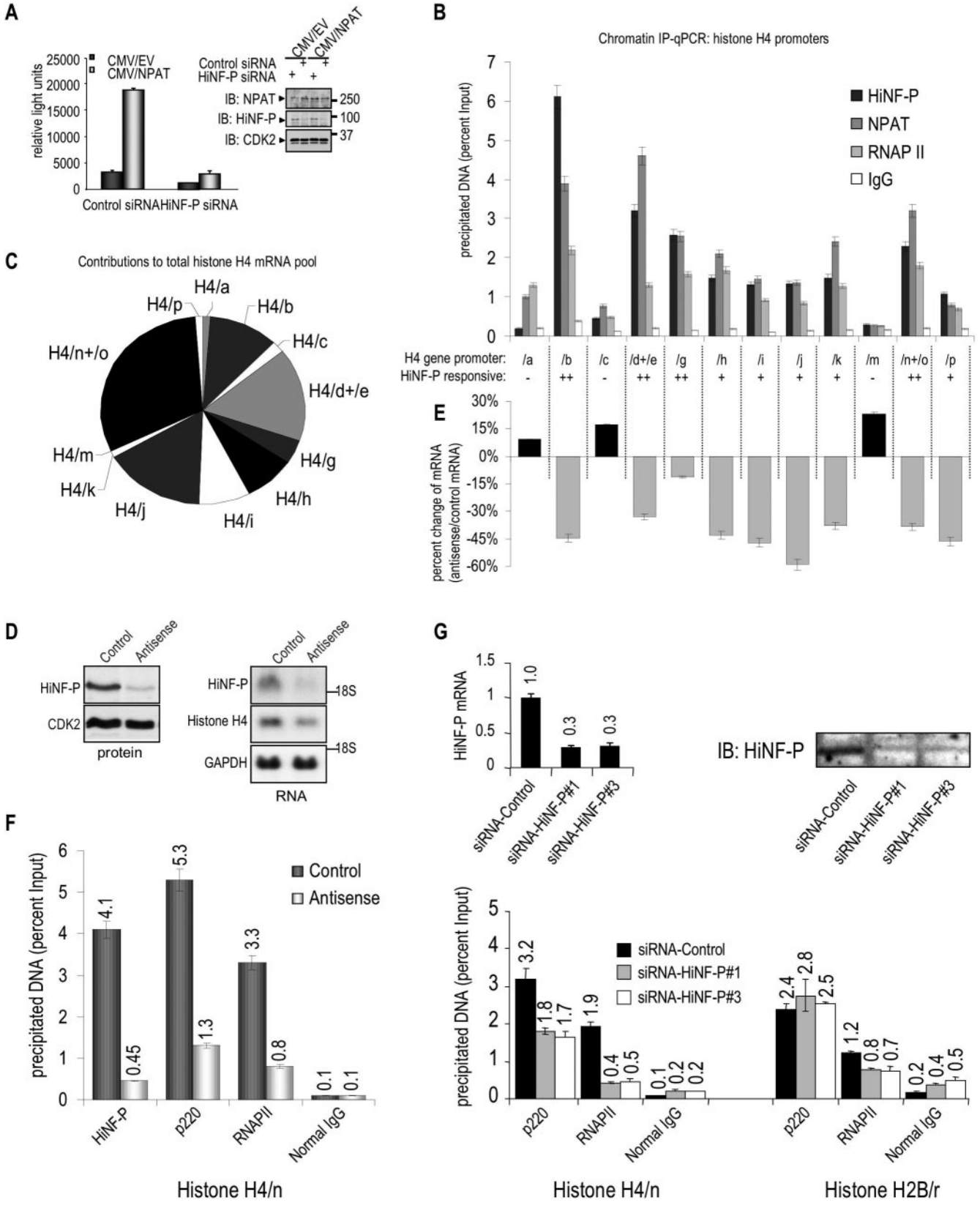

FIG. 7. HiNF-P recruits p220 and RNAPII to coordinate transcription of multiple histone H4 genes. (A) Reduction of endogenous HiNF-P by siRNA impairs p220-dependent transcription of the H4/n gene. Promoter activity was determined in T98G cells treated with control siRNA or HiNF-P siRNA for 48 h and transfected with wild-type H4/n promoter luciferase reporter together with a p220 expression vector (300 ng) or empty vector (EV) (150 ng) for 24 h. Immunoblots (IB) reveal that HiNF-P siRNA treatment significantly reduces the protein levels of HiNF-P but not

To determine the proportion of cell cycle-dependent histone H4 genes that are coregulated by the cyclin E/CDK2/p220/HiNF-P pathway, we quantified the expression of individual H4 mRNAs by quantitative PCR. The data show that the 11 HiNF-P/p220-responsive genes together account for greater than 95% of the total H4 mRNA pool (Fig. 7C). These data are consistent with dramatic reductions in the levels of bulk histone H4 mRNA in p220-depleted cells (31). We definitively established that HiNF-P coordinately controls cell cycle-dependent histone H4 mRNA by HiNF-P knockdown experiments. Antisense oligonucleotide or siRNA directed against HiNF-P (Fig. 7D) reduces expression of the 11 H4 genes that interact with HiNF-P. We observed only limited compensation by HiNF-P independent genes (Fig. 7E and data not shown), as indicated by only a 5 to 20% relative increase in the expression of three H4 genes (i.e., H4/a, H4/c, and H4/m), representing less than 5% of the total H4 mRNA pool. These three genes have mutated site II sequences that we predict will preclude HiNF-P binding. Taken together, our data represent the first direct and comprehensive demonstration that HiNF-P coordinately regulates the multiple functionally expressed human H4 mRNAs.

To assess whether HiNF-P mediates the staged recruitment of p220 and RNA polymerase II to H4 promoters, we analyzed occupancy of these three proteins at the H4/n promoter by ChIP-qPCR assays following HiNF-P knockdown. Reduction of HiNF-P levels significantly decreases HiNF-P interaction with the histone H4/n locus in different cell types (i.e., HeLa and T98G) (Fig. 7F and data not shown). Importantly, in HiNF-P-depleted cells, there is a coordinate reduction in the occupancy of H4/n by RNAPII and p220, as reflected by a concomitant decrease in the amount of immunoprecipitated DNA with antibodies to HiNF-P, p220, and RNAPII (Fig. 7F). However, siRNA-mediated depletion of HiNF-P does not affect the occupancy of p220 and RNAPII at the H2B/r promoter, which is regulated by p220 in an HiNF-P-independent manner (Fig. 7G). Thus, HiNF-P is rate limiting for the recruitment of p220 and RNAPII to transcriptionally competent H4 genes.

DISCUSSION

Here we have shown that the functional interaction between HiNF-P and p220 represents a direct link in a linear pathway that can now be defined as a cyclin E/CDK2/p220/HiNF-P signaling cascade. Our results show that (i) HiNF-P and p220 are capable of directly interacting through defined domains that confer binding specificity, (ii) HiNF-P and p220 are targeted to the same subnuclear compartment, (iii) HiNF-P mediates the ordered recruitment of p220 and RNA polymerase II to histone H4 promoters, and (iv) HiNF-P/p220 interaction is functionally rate limiting for control of cell cycle-dependent histone gene transcription. Thus, the HiNF-P/p220 interaction represents a terminal effector of the cyclin E/CDK2 relay mechanism that functionally couples the onset of DNA synthesis with the coordinate induction of histone H4 gene expression at the G_1/S phase transition.

Transcriptional control at the G_1/S phase transition and cell cycle-dependent interactions of HiNF-P and p220 at H4 gene loci and Cajal bodies. We find that the C terminus of HiNF-P, which is required for histone H4 gene transactivation, interacts with p220. In addition, the 33-amino-acid LisH (6) motif of the N terminus of p220, which is known to be required for histone H4 gene activation (30), represents a critical determinant for the HiNF-P interaction. Although our results show that mutation of the LisH motif abolishes HiNF-P binding, p220 lacking the LisH motif is competent to associate with Cajal bodies and can activate DNA replication (30). The available evidence indicates that the ability of p220 to activate replication is independent of its histone transcriptional activation function (30, 31). The colocalization of HiNF-P and p220 at Cajal bodies is cell cycle dependent and initiates at the G_1/S phase transition. The localization of histone gene loci, HiNF-P, p220, and the histone-specific U7 snRNP with Cajal bodies suggests that this organelle could serve to facilitate assembly of an HiNF-P/p220/RNAPII complex and to couple this transcription complex to the 3′-processing activity that functions in histone mRNA maturation. Thus, the HiNF-P/p220 pathway may be architecturally organized to couple histone gene expression with DNA replication.

The association of p220 with HiNF-P regulated histone H4 loci at the chromatin level is also modulated with respect to the G_1/S phase transition. In cells depleted of HiNF-P mRNA and protein, the resulting disruption of HiNF-P interactions with the H4 cell cycle regulatory sequences precludes recruitment of p220 as well as RNA polymerase II. The cell cycle-dependent interaction of p220 at the onset of S phase may represent a critical step for positioning of RNA polymerase II, as well as the general transcription factors associating with the TATA box, relative to the histone H4 mRNA start site. The catalytic function of p220 in H4 transcription remains to be elucidated. Because HiNF-P is required for the association of p220 with histone H4 genes, HiNF-P may represent the recruiter of p220

p220 or CDK2. Error bars indicate standard deviations. (B) The majority of H4 genes are regulated by HiNF-P. Occupancy of 14 histone H4 gene promoters by HiNF-P (indicated by −, +, or ++), as well as p220 and RNAPII, was assayed by ChIP-qPCR in T98G cells (H4/p = HIST4H4 gene) (1, 15). IgG, immunoglobulin G. (C) Greater than 95% of the total H4 mRNA pool is derived from H4 genes that interact with HiNF-P in vivo. Individual histone H4 mRNA was measured by RT-qPCR in asynchronously growing T98G cells. (D) Antisense oligonucleotides against HiNF-P reduce total histone H4 and HiNF-P mRNA levels (right panel, Northern blot analysis) as well as HiNF-P protein levels (left panel, immunoblot) in T98G cells. CDK2 and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) levels serve as internal controls. (E) Antisense depletion of HiNF-P decreases H4 mRNA levels from genes that interact with HiNF-P based on ChIP data (see panel B). Increased or decreased levels of H4 mRNAs from distinct H4 genes in antisense-treated cells relative to control cells are shown. (F) Antisense depletion of HiNF-P impairs recruitment of p220 and RNAPII to the histone H4/n promoter as determined by ChIPs. Immunoprecipitated DNA quantities from triplicate experiments with antisense-treated and control cells are graphed as percentage of input. (G) siRNA-mediated depletion of HiNF-P has no effect on the recruitment of p220 and RNAPII to the histone H2B/r gene, which is transcribed in an HiNF-P-independent manner. The extent of HiNF-P depletion was determined by qRT-PCR (upper left) and confirmed by Western blotting (upper right).

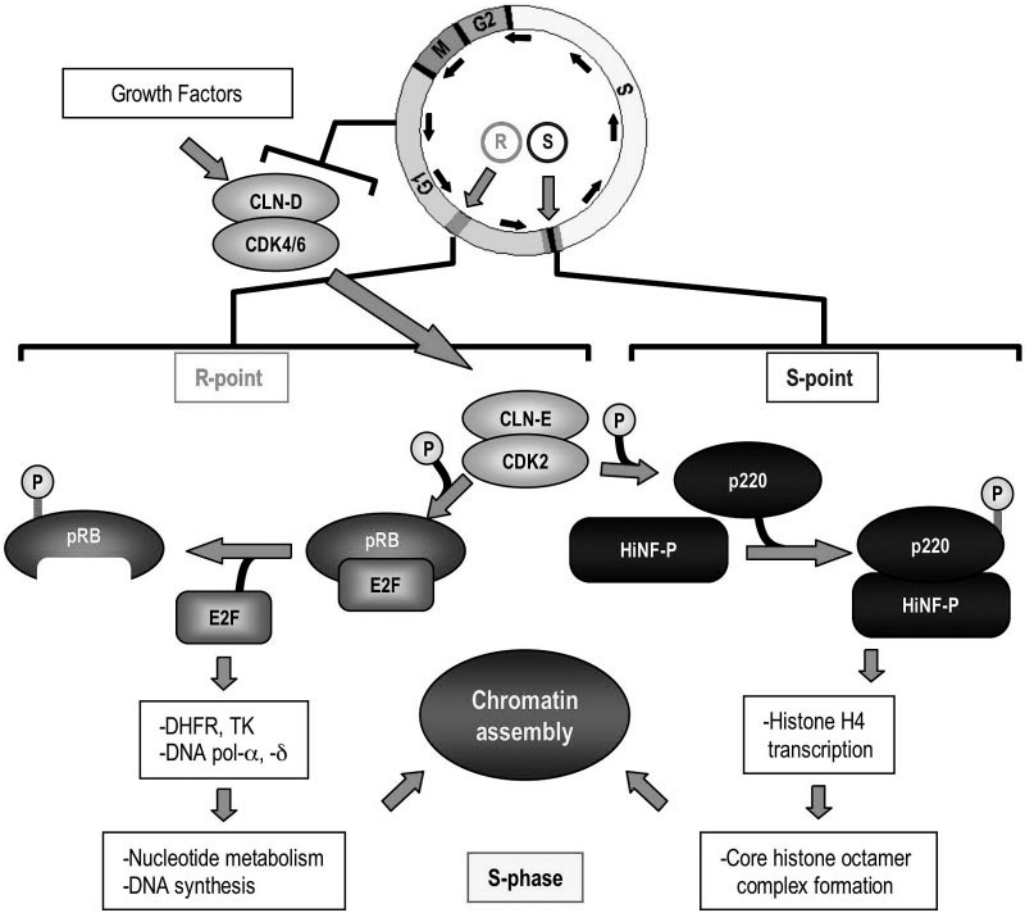

FIG. 8. Model for the regulation of replication-dependent histone H4 genes at the G_1/S phase cell cycle transition via the cyclin E/CDK2/p220/HiNF-P pathway relative to the E2F/pRB pathway. See text for details.

into transcriptional regulatory complexes that assemble at the histone H4 cell cycle promoter element.

Coordinate control of histone gene subtypes to support chromatin assembly of nascent DNA during S phase. Fidelity of chromatin assembly during S phase necessitates the stoichiometric synthesis of the four core histone subtypes to form nucleosome octamers, which contain two copies each of the H4, H3, H2B, and H2A proteins. There is a fundamental requirement for the synchronous synthesis of the histone subtypes, which are each encoded by 10 to 20 distinct mammalian genes. Previous data have shown that p220 depletion reduces expression of all core histone subtypes (31). Moreover, overexpression of p220 activates the expression of reporter constructs containing each of the core promoter subclasses (14, 34). Our data establish that cell cycle-dependent direct interactions between HiNF-P and p220 at Cajal bodies coordinately regulate expression of the 11 distinct human histone H4 genes that account for greater than 95% of the total pool of DNA replication-dependent H4 mRNA. Thus, the HiNF-P/p220 interaction represents a global cyclin E/CDK2-responsive regulatory switch that is obligatory for histone H4 gene expression.

Recent studies have shown that a representative histone H2B gene, which is regulated by the Oct-1 transcription factor, requires the coactivator complex OCA-S, which contains four metabolic enzymes and two chaperonins (35). OCA-S components form a precipitable complex with Oct-1 and support transcription initiation in vitro. p220 associates with OCA-S proteins, but the interaction of p220 with OCA-S or Oct-1 and their localization with Cajal bodies remain to be defined. Because OCA-S associates with an H2B but not the H4 promoter, OCA-S represents an Oct-1-specific adaptor, which modulates H2B transcription (35). Although coordinate regulation of the H4 and H2B subtypes is mechanistically distinct, there is a common dependence on p220 (31).

Global control of core and H1 histone gene expression requires not only coordinate upregulation of transcription at the G_1/S transition but also attenuation to maintain balanced accumulation of each of the histone mRNA subtypes and to suppress expression at the end of S phase. Although posttranscriptional control (i.e., mRNA processing and turnover) contributes to histone gene expression, studies from our laboratory suggest that histone genes are dynamically suppressed during mid- to late S phase. Cell cycle-dependent DNA binding complexes (i.e., HiNF-D) containing pRb, cyclin A, CDK1, and CDP/cut proteins interact with multiple histone gene subtypes, and CDP/cut is known to suppress transcription of all five histone gene subclasses (5, 10, 28). Thus, stoichiometric synthesis may be achieved by the dynamic balance between the

cyclin E/CDK2/p220-dependent activation through HiNF-P, Oct-1, and other subtype-specific regulators and the cyclin A/CDK1/CDP-related suppression of histone gene transcription.

Conclusions. The requirements for genome replication are initially supported by E2F-dependent gene expression that is initiated at the R point in the cell cycle (Fig. 8) and controls deoxynucleotide biosynthesis to supply the building blocks for DNA synthesis. However, competency for genome replication requires histone biosynthesis for immediate packaging of newly replicated DNA as chromatin. Here, we have addressed the integration of signals between the R point late in G_1 and the S point when histone gene expression and DNA replication are initiated. Whereas transcriptional control of the R point is mediated principally by E2F, histone gene expression at the G_1/S phase transition is E2F independent. Our results establish that HiNF-P mediates the coordinate expression of histone H4 genes at the S point by directly complexing with p220 to complete the cyclin E/CDK2 cell cycle signaling cascade.

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