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Table of Contents

Cover.....	1
SF 298.....	2
Table of Contents.....	3
Introduction.....	4
Body.....	4-7
Key Research Accomplishments.....	7
Reportable Outcomes.....	7
Conclusions.....	7
References.....	7
Appendices.....	8-23

INTRODUCTION

Cancer is fundamentally a disease of inappropriate cell division. Progression through the mammalian cell division cycle is controlled by cyclin dependent kinases (cdks) and their regulatory subunits, the cyclins. Entry of cells into S-phase depends upon the activities of cyclin D/cdk4 and cyclin E/cdk2 complexes. During the last few years, it has become clear that deregulation of the cyclin E pathway is associated with breast cancer (1).

Although much is known about how cyclin E/cdk2 is regulated, relatively little is known about how it controls S-phase events, especially when its activity is deregulated. My work is focused on understanding how cyclin E controls S-phase events through its interaction with a novel protein, p220^{NPAT}.

The available data indicate that p220 links cyclin E to important S-phase events that are required for DNA replication, including induction of histone gene transcription (2, 3, 4). Our previous data showed that overexpression of p220 in U2OS cells by transient transfection could activate both histone gene promoters in reporter constructs and S-phase entry (3, 4). p220 also has the ability to activate S-phase, probably independently of histone transcription, and understanding how this is accomplished is an important goal of my research.

BODY

My central hypothesis is that the p220/cyclin E signaling pathway promotes cell division by establishing a transcriptional program responsible for expression of genes required for S-phase. Understanding how this pathway operates and the genes it controls could have important ramifications for understanding how cyclin E/Cdk2 promotes proliferation normally and in transformed cells.

In the second funding year of this grant, the focus was put to dissect the role of p220 in G1-S transition of cell cycle in a loss-of-function system that uses HCT116 cells engineered to contain a conditional p220 allele. The role of p220 in multiple aspects of G1-S transition, including S-phase entry, histone gene expression, and Cajal body maintenance, was addressed.

Task 2 in my proposal, which is intended to examine the transcription regulation of global genes regulated by p220 was performed in the first funding year. Since according to the analysis, no specific gene was activated when p220 was overexpressed, tasks in this aim were not continued.

1. Development and initial characterization of HCT116 cell lines conditionally-expressing p220

To generate the "loss-of function" system, we developed human somatic HCT116 cells that conditionally express p220 (HCT116p220^{flox/-} and HCT116p220^{flox/-}CreER). Infecting the HCT116p220^{flox/-} cells with Ad-Cre or treating the HCT116p220^{flox/-}CreER cells with hydroxyl-tamoxifen (OHT) leads to the rapid removal of the floxed allele.

In the first funding year of this grant, with the p220 conditional cells described above as the "loss-of-function" system, we found that p220 is required for the cell proliferation, S-phase entry, and histone gene transcription in HCT116 cells (details seen in the first year annual report of this grant). In the second funding year, we characterize the requirement of p220 for these S-phase entry events further in a more detailed way.

2. p220 is required for expression of replication dependent histone genes in S-phase as well as outside S-phase

In experiments examining histone gene expression in serum starved cells, we noticed that histone H4 mRNA levels are also reduced in p220^{-/-} cells, when compared to p220⁻-expressing cells (see first year annual report). This, coupled with the fact that previous studies had seen a low, yet quantifiable, level of histone mRNA in G0 and G1 cells (5, 6), led us to ask whether p220 might contribute to low level histone transcription outside S-phase. In principle, the use of highly quantitative PCR techniques that were not available when early studies on histone transcription were performed might allow a more precise determination of whether "replication-dependent" histone expression can occur in the absence of DNA replication. To examine histone expression outside S-phase, we sought to further reduce the S-phase content of our synchronized population by depleting S-phase cells from the population using aphidicolin, an inhibitor of DNA replication. Previous studies have demonstrated that inhibition of replication by aphidicolin leads to a rapid depletion (<60 min) of histone mRNA. Using a highly sensitive BrdU incorporation method, we were unable to detect S-phase cells after 3 hours in aphidicolin, consistent with a 50% drop in the total levels of histone H2A and H4 mRNAs in both p220^{fllox/-}/LacZ and p220^{fllox/-}/Cre cultures, as determined by quantitative RT-PCR (data not shown). However, the levels of H4 mRNAs are 5-fold lower in the p220^{fllox/-}/Cre cultures than in control cultures (Figure 1), indicating that p220 contributes to "basal" histone H4 transcription independent of DNA replication. In contrast, we were unable to detect significant differences in histone H3, H2A, and H2B levels under these conditions (Figure 1). The simplest interpretation of this data is that HCT116 cells express what we refer to as "basal" levels of histone genes outside S-phase and that p220 contributes substantially to histone H4 expression independent on ongoing DNA synthesis S-phase.

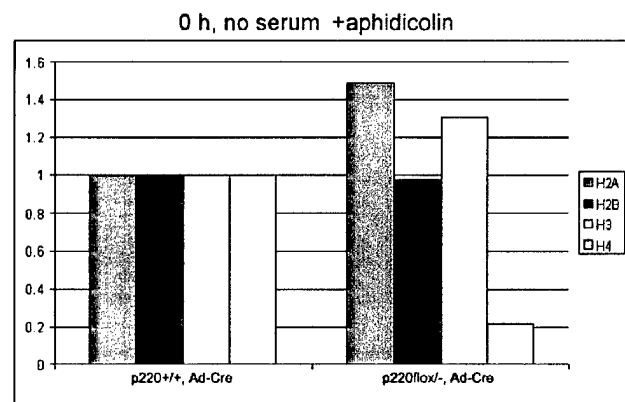


Fig. 1 p220 is required for histone H4 expression outside S-phase. p220^{+/+} or p220^{fllox/-} cells were treated with aphidicolin to block cells at G0/ G1.

3. p220 deficiency leads to dissociation of p80^{coilin} from Cajal bodies

Cajal bodies are complex organelles made up of a large number of proteins, many of which have not been identified (7, 8). A well characterized CB component is p80^{coilin}. Although this protein is not essential for mouse development, its absence reduces association of the SMN protein with CBs (9). We considered the possibility that p220 was required for proper localization of p80^{coilin}.

First, we examined p80^{coilin} localization in asynchronous p220^{fllox/-} cells 5 days after infection with either Ad-Cre or Ad-lacZ (Figure 2A). The vast majority of cells treated with Ad-LacZ maintained focal anti- p220 and anti-coilin staining (Figure 2A). In contrast, cells treated with Ad-Cre demonstrated a dramatic increase in the number of cells displaying altered patterns of coilin staining. In particular, we noted cells that had lost focal coilin staining and exhibited either a diffuse pattern, or a "dissociative" pattern containing a large number of small punctuate foci (Figure 2A). In this asynchronous population, 38% of p220 negative cells displayed dissociative or dispersed patterns of p220 staining. Similar results were obtained using p220^{fllox/-}; ER-Cre cells treated with OHT (Figure 2B and data not shown). We next examined coilin staining in synchronized cells lacking p220. p220^{+/+}

or p220^{flox/-} cells were treated with Ad-Cre or Ad-lacZ, and arrested in G0 by serum withdrawal. Under these conditions, greater than 68% of cells displayed normal patterns of coilin foci, independent of the presence of p220 (Figure 2C). Previous studies have demonstrated that the number of coilin-positive CBs increases as cells enter S-phase. When p220^{flox/-} cells expressing Ad-LacZ were stimulated to re-enter the cell cycle with serum, the percentage of cells with focal anti-coilin staining increased to 79-92% and, as expected, a subset of coilin-positive foci in these cells were p220 positive. In contrast, with p220^{flox/-} cells treated with Ad-Cre, less than 42% of these cells displayed focal coilin staining (Figure 2C). These data indicate that p220 is not required for localization of coilin in Cajal bodies during G0 but is important for efficient retention of coilin in Cajal bodies as cells traverse the G1/S transition.

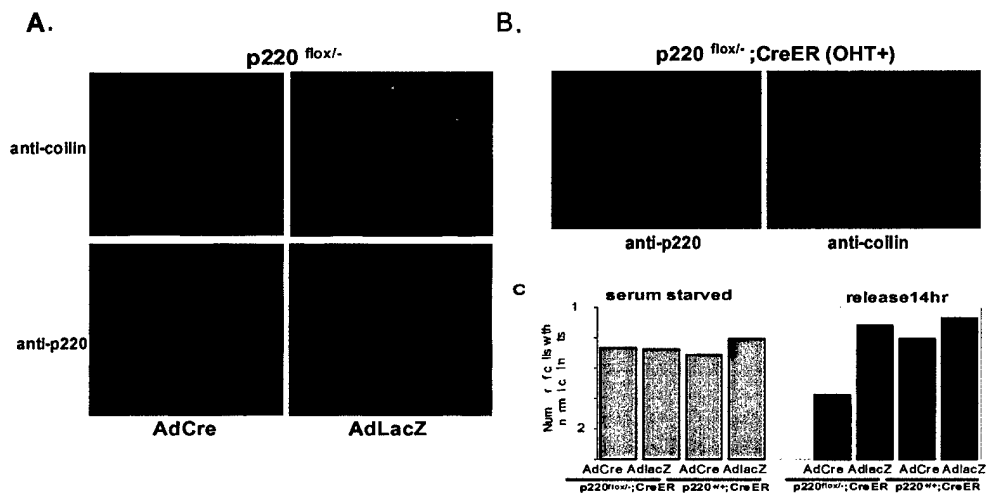


Fig. 2 p220 is required for maintenance of p80coilin localization during the G1/S transition. (A) Some Ad-Cre treated cells lost p80coilin staining or displayed altered p80coilin localization typified by a "dissociative" staining pattern; (B) OHT treated p220^{flox/-}; ER-Cre cells displayed altered p80coilin localization typified by a "dissociative" staining pattern; (C) Disruption of p80coilin localization during the G1/S transition.

When p220^{flox/-} cells expressing Ad-LacZ were stimulated to re-enter the cell cycle with serum, the percentage of cells with focal anti-coilin staining increased to 79-92% and, as expected, a subset of coilin-positive foci in these cells were p220 positive. In contrast, with p220^{flox/-} cells treated with Ad-Cre, less than 42% of these cells displayed focal coilin staining (Figure 2C). These data indicate that p220 is not required for localization of coilin in Cajal bodies during G0 but is important for efficient retention of coilin in Cajal bodies as cells traverse the G1/S transition.

4. p220 phosphorylation status in G0/G1

The finding that p220 contributes to "basal" histone H4 expression outside S-phase led us to examine the phosphorylation status of p220 in G0/G1 cells. Cyclin E/Cdk2 activity is frequently deregulated in tissue culture cells, and could, in principle, lead to inappropriate p220 phosphorylation in G0/G1. This phosphorylation could, in turn, lead to increased histone expression outside S-phase. HCT116 cells synchronized in G0 by serum deprivation contain 2 p220 foci. However, these foci do not react with antibodies directed against phospho-T1350 (Figure 3). In contrast, HCT116 cells in S-phase (13 h after release from serum deprivation) contain the expected 4 p220 foci and these foci react with the anti-phospho-T1350 antibodies (Figure 3). Similar results were obtained using antibodies directed against phospho-T1270 (data not shown). These data indicate that basal p220-dependent histone gene expression occurs without detectable phosphorylation of p220 on two prominent Cdk2 phosphorylation sites and suggest a phosphorylation independent component to p220 function outside S-phase.

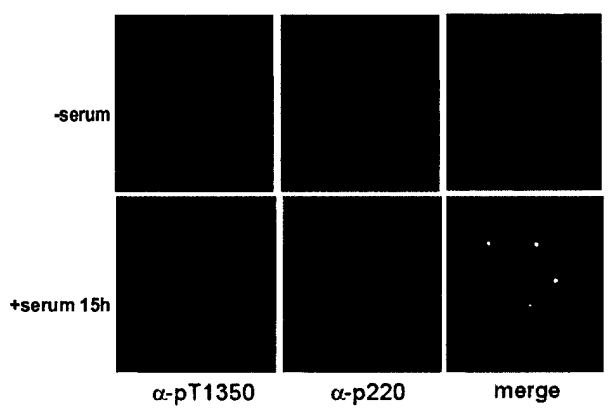


Fig. 3 p220NPAT is not phosphorylated on Thr-1350 in G0 HCT116 cells

KEY RESEARCH ACCOMPLISHMENTS

Generation and characterization of p220 conditional expressing HCT116 cells, with which demonstrated p220 is required for cell proliferation, S-phase entry, histone gene transcription in and outside of S-phase, as well as Cajal body components localization.

REPORTABLE OUTCOMES

Xin Ye, Yue Wei, Grzegorz Nalepa, and J. Wade Harper.

“The cyclin E/Cdk2 substrate p220NPAT is required for S-phase entry, histone gene expression, and Cajal body maintenance in human somatic cells”

Molecular and Cellular Biology 2003 23:8586-8600.

CONCLUSIONS

According to the results from experiments using the “gain-of –function” and the “loss-of –function” cells, we found that p220 is an essential downstream component of the cyclin E/Cdk2 signaling pathway and functions to coordinate multiple elements of the G1/S transition.

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APPENDICES

Xin Ye, Yue Wei, Grzegorz Nalepa, and J. Wade Harper. "The cyclin E/Cdk2 substrate p220NPAT is required for S-phase entry, histone gene expression, and Cajal body maintenance in human somatic cells", *Molecular and Cellular Biology* 2003 23:8586-8600.

The Cyclin E/Cdk2 Substrate p220^{NPAT} Is Required for S-Phase Entry, Histone Gene Expression, and Cajal Body Maintenance in Human Somatic Cells

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Cyclin E/Cdk2, a central regulator of the G₁/S transition, coordinates multiple cell cycle events, including DNA replication, centrosome duplication, and activation of the E2F transcriptional program. Recent studies suggest a role for cyclin E/Cdk2 in activation of histone transcription during S phase via the Cajal body-associated protein p220^{NPAT}, and in addition, p220 can promote S-phase entry independently of histone transcriptional activation when overexpressed. Here we have examined the requirement for p220 in histone transcription, cell cycle progression, and Cajal body function through analysis of human somatic HCT116 cells engineered to contain a conditional p220 allele. p220 is required for proliferation of HCT116 cells, as assessed after expression of Cre recombinase in p220^{lox/-} cells. This defect was due to an inability of these cells to transit from G₀/G₁ into S phase, and cell cycle arrest occurred in the presence of elevated Cdk2 kinase activity. Expression of human papillomavirus E7, but not E6, eliminated cell cycle arrest in response to p220 depletion. Optimal expression of all four core histone genes required p220, as did optimal transcription of a histone H4 promoter-luciferase construct. Basal histone H4 expression in G₀/G₁, although p220 dependent, occurs in the absence of detectable phosphorylation of p220 on Cdk2 sites. Cells lacking p220 displayed defects in the localization of the Cajal body component p80^{collin} as cells progressed from G₀ to S phase in response to mitogenic signals. These findings indicate that p220 is an essential downstream component of the cyclin E/Cdk2 signaling pathway and functions to coordinate multiple elements of the G₁/S transition.

The cell division cycle is a complex series of events that culminates in the duplication of the genome and segregation of replicated chromosomes into daughter cells. The decision to enter into the cell cycle in eukaryotes is made during G₁, a time when cells are poised to transduce growth factor signals that ultimately interface with the basic cell division machinery composed of cyclin-dependent kinases (Cdks). A central feature of the G₁ phase in normal diploid fibroblasts is the conversion of a cell from a growth factor-dependent proliferative cycle to growth factor-independent proliferation. In biochemical terms, this is thought to reflect the conversion of type D Cdk activity to type E Cdk activity (41, 42). Type D cyclins in complexes with Cdk4 serve as sensors of growth factor signaling and activate cyclin E/Cdk2 complexes through multiple mechanisms, including sequestration of the Cdk2 inhibitor p27 and activation of the E2F transcriptional program (reviewed in reference 42). Once cyclin E/Cdk2 is fully activated, cells are no longer dependent on stringent growth factor signals for further cell cycle progression. Indeed, previous experiments have demonstrated that activated cyclin E/Cdk2 complexes are sufficient to drive quiescent cells into S phase (8) and to promote S phase independently of the Rb/E2F pathway (22, 24).

Precisely how cyclin E/Cdk2 catalyzes S-phase entry is not

clear, but its ability to coordinate multiple S-phase events is likely to be critical for genome integrity. S phase requires that multiple events occur concurrently, including activation of the DNA replication process, activation of centrosome duplication, and production of DNA synthesis precursors. Cyclin E has been implicated in each of these functions. However, relevant substrates for these activities are only beginning to be understood. Through inactivation of Rb and derepression of E2F1-3, cyclin E promotes the production of enzymes required for DNA replication and nucleotide synthesis (14, 35, 45). The critical nature of the cyclin E program is indicated by the fact that cells that persistently overexpress cyclin E display genomic instability (44, 33).

Proper DNA replication also requires that the core components of nucleosome, histones H2A, H2B, H3, and H4, are produced concurrently with S phase. Recent studies (34) have reinforced the established concept that DNA replication and histone gene expression synthesis are both functionally and temporally linked (21, 29). Loss of histone production leads to inhibition of DNA synthesis, independently of Cdk2 activity. Likewise, inhibition of DNA synthesis leads to repression of histone synthesis. Thus, it is well established that, during S phase, histone mRNA levels dramatically increase relative to those in other cell cycle phases, through both transcriptional and posttranscriptional mechanisms (11, 19; reviewed in reference 29). During S phase, histone mRNA is stabilized (about sevenfold) and processed via the action of SLBP1, which binds the 3' stem-loop in histone mRNAs and facilitates processing via endonucleolytic cleavage, as well as histone translation (13, 40). SLBP1 accumulation is cell cycle regulated and increases

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during S phase through largely translational mechanisms (13, 51). Histone transcription is activated (about fivefold) concurrently with S phase, and this involves distinct transcriptional activators that function through subtype specific *cis*-acting elements in histone promoters (2, 3, 9, 10, 21, 28, 38, 47, 48, 49). Although activation of histone mRNA synthesis is clearly activated during S phase, it is not clear to what extent the expression of what are thought to be replication-dependent histone genes can occur outside S phase. However, previous studies have indicated detectable levels of histone mRNA in serum-deprived cell populations that contained 2 to 3% S-phase cells (11, 19). Whether this reflects incomplete cell cycle synchrony is an open issue.

Precisely how cell cycle positional information is relayed to the histone transcriptional apparatus is unknown. However, recent data implicate the cyclin E/Cdk2 substrate p220^{NPAT} as being involved in this process. p220 was identified as a cyclin E/Cdk2 binding protein (25, 52), and initial studies implicated p220 in control of the G₁/S transition and in histone gene transcription (26, 52, 53). p220 is localized in small nuclear organelles called Cajal bodies (CBs) (26, 53). CBs were first identified in 1903 (7), but their components and functions have only begun to emerge in the last decade (18, 31, 36). In normal diploid fibroblasts in G₁, two p220-containing CBs are associated with replication-dependent histone gene clusters on chromosome 6p21. As these cells enter S phase, two additional p220-positive CBs become associated with replication-dependent histone gene clusters on chromosome 1q21. p220-positive CBs persist until mitosis, when CB components become dispersed. By using phosphorylation-specific antibodies against Cdk2 sites in p220, we demonstrated that CB-associated p220 is phosphorylated during the cell cycle (26) and the timing of phosphorylation correlated with accumulation of cyclin E in CBs during late G₁ (23, 26). Consistent with a role in histone transcription, overexpression of p220 in tissue culture cells leads to activation of histone H4 and H2B promoter-reporter constructs through cell cycle regulatory elements in these promoters, and in this context, p220 is activated by cyclin E/Cdk2 (26, 53). It is not clear whether p220 is also required for activation of endogenous histone transcription.

Enforced expression of p220 drives cells from G₁ into S phase, apparently without affecting the ability of cells to progress through S phase (52). Recent work with p220 mutants that are unable to activate H4 transcription indicated that its ability to promote cell cycle entry is not dependent on its ability to activate histone transcription (50). Moreover, it is not clear whether p220 normally plays a direct role in allowing cells to enter S phase. To delineate the roles of p220 in cell cycle control, we have examined the phenotype of human somatic cells engineered to conditionally express p220. We found that p220 is essential for cell proliferation and that its absence during the G₁ phase of the cell cycle leads to cell cycle arrest prior to S-phase entry. This arrest occurs in the presence of high Cdk2 activity, suggesting that p220 activity is required downstream of Cdk2 activation. This arrest occurs concurrently with dissociation of the CB component p80^{coilin} from CBs, suggesting a role for p220 in maintaining proper CB assembly as cells attempt to enter the cell cycle. Interestingly, expression of human papillomavirus (HPV) E7—which inactivates pRb—overrides cell cycle arrest resulting from p220 de-

pletion. In contrast, E6—which blocks p53 function—has no effect on S-phase entry in p220-null cells. p220-null cells entering S phase as a result of E7 expression display reduced levels of mRNAs for all four core histones, demonstrating a role for p220 in replication-dependent histone gene expression. p220 was also found to be critical for “basal” expression of histone H4 outside S phase. These data indicate that p220 is a central component in the cyclin E/Cdk2 signaling pathway responsible for orchestrating the G₁/S transition.

MATERIALS AND METHODS

Generation of targeting constructs. A human genomic library in λ phage was screened with the p220 cDNA, and one candidate clone (p220-22) containing exons 2 and 3 on a 10-kb genomic insert was identified. To generate a conditional knockout construct, the open reading frame of a neomycin (Neo) resistance gene linked with polyadenylation sequences (5) was inserted in frame into exon 2, and flanking *loxP* sites were inserted 613 nucleotides upstream and 734 nucleotides downstream of the Neo cassette. This was followed by insertion of an additional exon 2 and flanking intronic sequences, as well as an additional *loxP* site located 734 nucleotides 3' to the duplicated exon 2. A conventional targeting construct contained a promoterless Neo cassette inserted in frame within exon 2, followed by 1.4 kb of exon 5 and flanking intronic sequences, which was produced by PCR from BAC clone RP11-56d3.

Generation of p220-deficient cells. HCT116 cells were transfected with a p220 conditional knockout construct with Lipofectamine, and selection was performed with medium containing G418. Screening for homologous recombination events at both 5' and 3' sequences was accomplished by PCR with Extended High Fidelity polymerase (Roche) and oligonucleotide pairs specific to the conditional allele and sequences outside the homology used for recombination (Fig. 1). One clone identified in this manner was infected with adenovirus (Ad)-Cre, and colonies were screened for loss of the exon 2-Neo fusion by PCR. These p220^{lox/+} cells were then transfected with a promoterless Neo vector containing Neo in exon 2 and with exons 3 and 4 deleted. Recombination at the existing wild-type allele was verified by PCR analysis. PCR products representing the junctions of recombination sites were directly sequenced to verify their structures. To delete the conditional allele, p220^{lox/-} cells were treated with Ad-Cre or Ad-LacZ as a control and cells were assayed as described below. Equal numbers of infectious particles of Ad-Cre and Ad-LacZ were used, as determined by immunostaining of infected cells with anti-Cre and by *in situ* β -galactosidase assays. To generate cells expressing an estrogen receptor-Cre fusion (ER-Cre), whose localization is under the control of hydroxyl-tamoxifen (OHT), p220^{lox/-} cells or p220^{+/+} cells as controls were transfected with a plasmid expressing ER-Cre recombinase linked with a puromycin resistance marker (32). Two days after transfection, cells were placed on selection medium containing 1 μ g of puromycin per ml and three independent colonies were isolated for each genotype (p220^{lox/-} ER-Cre and p220^{+/+} ER-Cre). Immunoblotting of cell extracts from these cell lines indicated indistinguishable levels of ER-Cre, and immunofluorescence assay indicated cytoplasmic localization of ER-Cre in the absence of OHT and nuclear accumulation in the presence of OHT (data not shown). To generate E6 and E7 p220^{lox/-} cells, p220^{lox/-} cells were infected with retroviruses expressing either HPV E6 or E7. Twenty-four hours after infection, cells were placed on selection medium containing 1 μ g of puromycin per ml and pooled puromycin-resistant cells were used in cell cycle and transcription experiments.

Cell synchronization and proliferation assays. For colony formation assays, p220^{+/+}, p220^{lox/+}, or p220^{lox/-} cells were plated at a density of 100/100-mm-diameter culture dish prior to infection with either Ad-LacZ or Ad-Cre. Cells were incubated for 14 days, and the medium was replaced on alternate days. Colonies were visualized with methylene blue and prior to quantification. To examine colony formation in p220^{lox/-} ER-Cre or p220^{+/+} ER-Cre control cells, 200 cells were plated and cultured in the presence or absence of OHT and the number of surviving colonies was determined after 14 days with methylene blue. To examine cell proliferation, p220^{lox/-} ER-Cre cells were cultured in the presence or absence of OHT. After 3 days, cells were trypsinized and counted. Ten thousand cells per 35-mm-diameter dish were plated for each group in triplicates in the presence or absence of OHT. At the indicated times, cells were released into 0.1% EDTA-phosphate-buffered saline and counted by trypan blue exclusion. For synchronization, p220^{lox/-} or p220^{+/+} control cells were infected with Ad-Cre or Ad-LacZ. Three days after infection, cells were starved with serum-free medium for 48 h and then stimulated to re-enter the cell cycle by

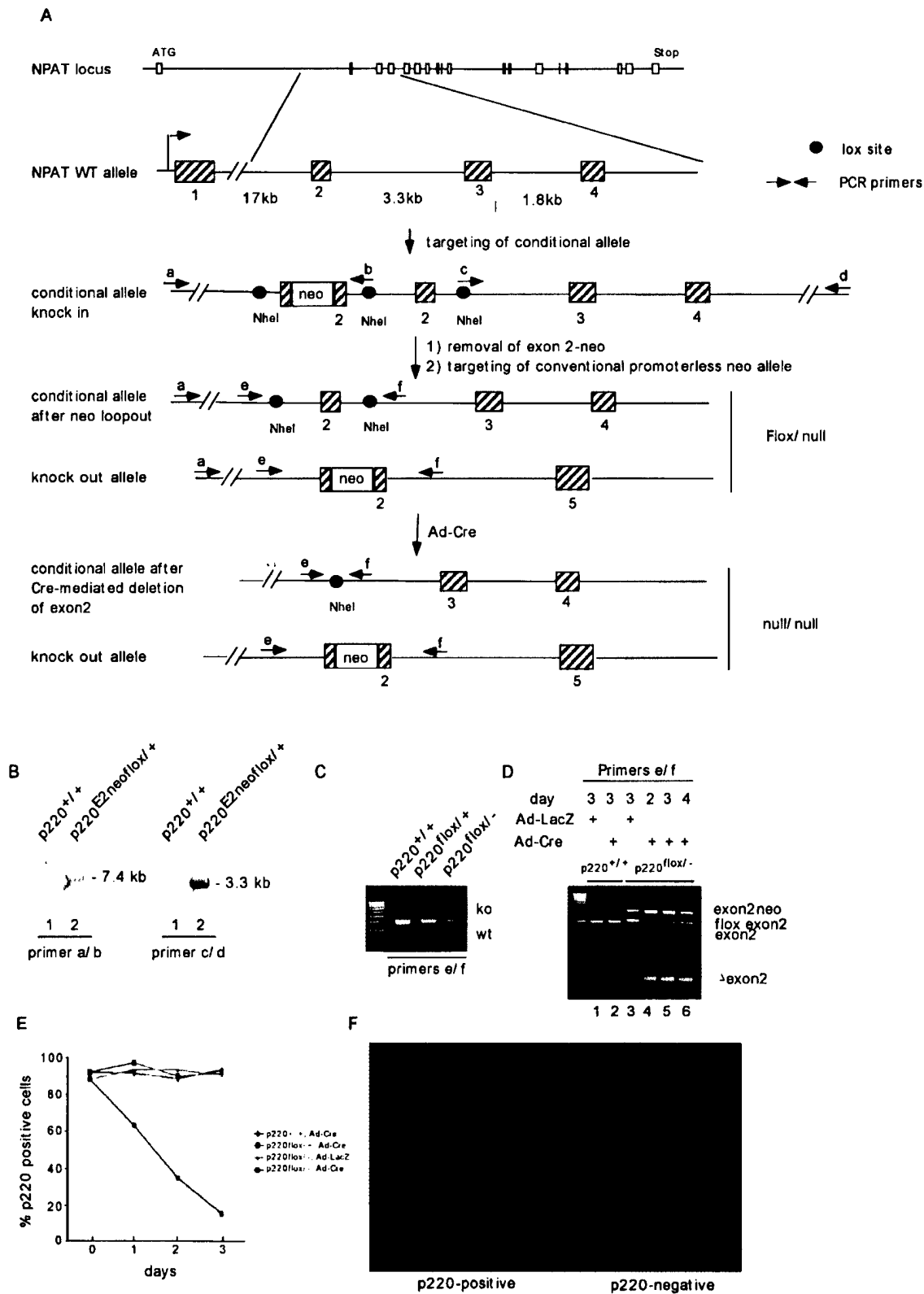


FIG. 1. Creation of HCT116 cells that conditionally express p220. (A) Structure of the p220 locus and approach used to generate p220^{flox/-} cells. See the text for details of construction. Primers used for PCR are indicated. Primers b and c specifically recognize the fusion of loxP and p220 sequences and are therefore specific to the recombined allele. Primers a and d are outside the regions used for targeting. WT, wild type. (B) Homologous recombination of the conditional promoterless-neo targeting construct. Primers specific to the loxP-p220 junction (primers b and c) and primers outside the recombination boundary (primers a and d) were used in extended PCRs. Clone N118 contained the expected 7.4- and

addition of medium containing 10% serum for the indicated time. Cells were prepared either for immunofluorescence assay or for flow cytometry. In some experiments, aphidicolin (1 μ g/ml) was used to block cells in S phase for 3 h.

Immunofluorescence assay and flow cytometry. For immunofluorescence assays, cells were grown on ultrathin cover slides (Fisher), fixed in 95% ethanol–5% glacial acetic acid, and permeabilized with 0.2% Triton X-100 for 10 min. Cells were then blocked with 5% goat serum (Sigma) for 30 min, incubated with primary antibodies (affinity-purified anti-p220 at 1:100) for 1 h, washed five times with phosphate-buffered saline, incubated with fluorochrome-conjugated secondary antibody for 1 h, and generously washed. For double immunostaining, cells at this point were fixed again and the immunostaining procedure was repeated with other primary and secondary antibodies. The following primary antibodies were used: anti-coilin monoclonal antibody (provided by M. Carmo-Fonseca, University of Portugal), anti-p220 DH4 monoclonal antibody (provided by J. Zhao, University of Rochester), and anti-phospho Pol II (Santa Cruz Biotechnology). To measure the relative levels of phosphorylated and unphosphorylated p220, cells were stained first with monoclonal antibody DH4 and subsequently stained with antibodies directed against phospho-T1350 (26). Nuclei were then counterstained with 4',6'-diamidino-2-phenylindole (DAPI), and cells were mounted in SlowFade Light Antifade reagent (Molecular Probes). Images were taken on a Nikon/DeltaVision deconvolution microscope (Applied Precision) as a series of 0.2- μ m-thick z sections, processed with a Softworx image workstation, and presented as a projection of transnuclear z sections, or on an Olympus BX60 microscope fitted with a Hamamatsu charge-coupled device camera.

For bromodeoxyuridine (BrdU) incorporation, cells were pulse-labeled with BrdU, incubated in 3 N HCl for 10 min, stained with AlexaFluor594-conjugated mouse anti-BrdU antibody (Molecular Probes), and then stained for p220 as described above. For bromouridine (BrU) incorporation, cells were pulse-labeled with BrU for 15 min, fixed, and stained with an anti-BrdU antibody. For flow cytometry, cells were harvested, fixed in ethanol, and stained with propidium iodide. Flow cytometry analysis was performed with a FACScan instrument.

Transcription analysis. For luciferase assays, p220^{lox/-} ER-Cre or p220^{+/+} ER-Cre cells at 70% confluence were rendered quiescent in the presence or absence of OHT and then transiently transfected in triplicate with pGL2-histone H4 promoter luciferase (53) and CMV-LacZ plasmids (0.1 μ g) with Eugene 6 (Roche). Thirty-six hours after transfection, cells were collected and luciferase assays were performed with a luciferase assay kit (Promega) as previously described (50). β -Galactosidase assay were used to normalize the luciferase activities. To examine the expression of endogenous histone genes, mRNA was isolated from cells after synchronization, Ad infection, and release with RNAzol. mRNA was subjected to Northern blot analysis with histone H4 or β -actin as a probe or used for quantitative reverse transcription (RT)-PCR. mRNA was subjected to RT with oligonucleotides specific for each of the four core histones, as well as glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a control. Quantitative PCR (in triplicate) was accomplished with SYBR-green fluorescence (Applied Biosystems) and an ABI Prism 7700 sequence detection system. The following oligonucleotides were used: H2A, 5' primer GACGAGGCTCAACAAGCTG and 3' primer TGTTGGGCAGGACACCG; H2B, 5' primer CCGACACCGGCATTCAT and 3' primer GCGCTCGAAGATATCGTTGAC; H3, 5' primer TACCAGAAGTCCACCGAGCTG and 3' primer GATT TCTCGACACAGGCG; H4, 5' primer AATCCGCGATGCAGTTACCT and 3' primer CCACGTCCATGGCTGTGA. For Northern blot assays, mRNA levels were quantified by phosphorimager analysis. The fold difference in histone transcripts versus histone transcript levels in p220^{+/+} cells infected with Ad-LacZ was calculated by the $\Delta\Delta$ CT method, with GAPDH signals as an internal control.

Kinase assays. To examine Cdk2 kinase activity, the indicated cells were lysed in 50 mM Tris-HCl–2 mM EDTA–150 mM NaCl–0.1% Triton X-100–10 mM β -glycerol phosphate–5 mM NaF–10 mM *p*-nitrophenylphosphate–protease inhibitors (Roche) and cleared by centrifugation (14,000 \times g, 15 min). Extracts (0.5 mg) were subjected to immunoprecipitation with anti-Cdk2 or control immuno-

globulin G antibodies (Santa Cruz Biotechnology) in combination with protein A-agarose. Twenty percent of the washed immune complexes was used for histone H1 kinase activity as previously described (26). Reaction products were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and visualized by autoradiography. The remaining immune complexes were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and probed with anti-Cdk2 antibodies to demonstrate equal Cdk2 levels.

RESULTS

Construction of human somatic cells that conditionally express p220^{NPAT}. In order to examine the role of p220 in cell cycle control, we used promoterless-*neo* technology (5) to generate human HCT116 cells that conditionally express p220. HCT116 cells are a well-characterized colorectal cancer cell line that has a stable karyotype and intact DNA damage and mitotic spindle checkpoints (6). Previous studies with random retroviral integration into the mouse genome had suggested that p220 is required for embryogenesis (12), and we therefore took into consideration the possibility that p220 might be required for cell proliferation and sought to make a conditional allele. The targeting construct for the conditional allele contained a duplication of exon 2 with the first exon 2 containing a promoterless neomycin resistance polyadenylation cassette (Fig. 1A). Both the E2-*neo* cassette and the duplicated exon 2 were flanked by *loxP* sites. Homologous targeting was established by PCR with primers that were specific to p220 sequences outside the targeting construct and primers that were composed of *loxP* and p220 sequences and were therefore specific to the conditional allele (Fig. 1B). Direct sequence analysis of these PCR products verified their identity (see Materials and Methods). The extent of homologous targeting was 1%. Following homologous targeting, the *neo*-disrupted exon was removed by Cre-mediated excision, leaving two *loxP* sites flanking an otherwise wild-type exon 2 sequence (p220^{lox/+}). A conventional targeting construct lacking exons 3 and 4 and containing a promoterless-*neo* cassette in exon 2 was then used to replace the remaining wild-type allele, generating p220^{lox/-} cells (Fig. 1A and C).

To verify Cre-dependent excision of exon 2, p220^{lox/-} cells were treated with Ad expressing Cre recombinase (Ad-Cre) or β -galactosidase (Ad-LacZ) and genomic DNA from these cells was examined for loss of the conditional allele by PCR (Fig. 1D). With Ad-Cre treatment, loss of the 1.5-kb PCR product derived from the floxed allele (but not the corresponding 2.6-kb product derived from the conventional null allele) was observed with concomitant appearance of the 200-bp product produced from the excised allele. In contrast, infection with Ad-LacZ did not alter either p220 allele (Fig. 1D). We verified loss of p220 expression with a highly sensitive immunofluorescence assay and deconvolution microscopy, which allows visu-

3.3-kb bands representing homologous recombination in 5' (left side, lane 2) and 3' (right side, lane 2) ends, respectively. These PCR products were absent in control HCT116 cells (left side, lane 1, and right side, lane 1). (C) PCR analysis of p220^{lox/-} cells. Primers e and f (see panel A) were used for PCR with p220^{+/+}, p220^{lox/+}, and p220^{lox/-} cells. The band at 1.5 kb represents either the wild-type (wt) or floxed allele, while the band at 2.6 kb represents the conventional promoterless-*neo* allele (ko). (D) Cre-mediated removal of floxed exon 2. p220^{+/+} or p220^{lox/-} cells were infected with Ad-LacZ or Ad-Cre for the indicated times, and genomic DNA was subjected to PCR analysis with primers e and f. Treatment of p220^{lox/-} cells led to the formation of a 200-bp PCR product (lanes 4 to 6) representing deletion of exon 2 (Δ exon 2). (E, F) Loss of p220 foci in p220^{lox/-} cells expressing Cre recombinase. Asynchronous p220^{+/+} or p220^{lox/-} cells were treated with Ad-LacZ or Ad-Cre for the indicated time, and cells were subjected to immunostaining with anti-p220 antibodies (F). p220^{lox/-} cells expressing Cre but not LacZ or p220^{+/+} cells demonstrated progressive loss of p220 expression (E).

alization of p220 as brightly staining nuclear foci (26) (Fig. 1E and F). As early as 24 h after addition of Ad-Cre, ~40% of the cells lacked p220 staining (Fig. 1E and F). During the next 3 days, the percentage of p220-negative cells increased to ~85%. In contrast, Ad-LacZ-expressing cells or p220^{+/+} cells expressing Cre retained p220 expression over the time course (Fig. 1E).

To facilitate analysis of p220^{lox/-} cells, we also generated p220^{lox/-} and corresponding p220^{+/+} cells that constitutively express an ER-Cre fusion protein (32). ER-Cre is cytoplasmic in the absence of OHT but translocates to the nucleus upon its addition (32; data not shown). Relocation of ER-Cre to the nucleus leads to rapid removal of the conditional allele, as determined by genomic PCR analysis, and immunofluorescence assay of these cells demonstrated the expected loss of p220 protein (data not shown; see below).

p220^{NPAT} is required for cell viability. We next examined whether p220 is required for viability in HCT116 cells. p220^{lox/-} cells were treated with Ad-Cre (or Ad-LacZ as a negative control) for 2 days, which corresponded to the time when >60% of the cells displayed levels of p220 undetectable by immunofluorescence assay. Cells were then plated at low density for 12 days to allow colony visualization. p220^{lox/-} and p220^{lox/+} cells expressing LacZ formed colonies with high efficiency, while the colony formation seen with Ad-Cre was reduced by more than 10-fold (Fig. 2A and B). Given that a small fraction of the cells still expressed p220 on the basis of immunofluorescence assay results, we examined whether residual colonies contained an intact conditional p220 allele by genomic PCR. Of the more than 60 colonies examined in two independent experiments, none were found to have undergone excision of exon 2 (Fig. 2C and data not shown). Similar results were obtained with p220^{lox/-} ER-Cre cells in the presence of OHT, which displayed an 80-fold reduction in colony number relative to that of cells in the absence of OHT (Fig. 2D and data not shown). Again, surviving colonies (*n* = 10) had not undergone excision of exon 2 (data not shown). In addition, p220^{lox/-} ER-Cre cells grow poorly in the presence of OHT, relative to control p220^{+/+} ER-Cre cells in the presence or absence of OHT (Fig. 2E). These data indicate that p220 is required for HCT116 cell viability and/or proliferation.

p220^{NPAT} is required for cell cycle entry from G₀. The inability of p220^{-/-} cells to proliferate led us to ask whether cell cycle arrest resulting from p220 depletion occurred at a particular stage. p220^{lox/-} and control p220^{+/+} cells were infected with either Ad-Cre or Ad-lacZ, and after 3 days, cells were deprived of serum for 48 h. Flow cytometry revealed substantial G₁ arrest under serum starvation conditions (11 to 28% of cells in S or G₂/M phase) (Fig. 3A and B). Serum was then added back to release the arrested cells into the cell cycle, and the ability of cells to enter S phase was assessed. At 13 h after serum addition, the percentage of S- and G₂/M-phase cells increased to >51% in p220^{lox/-} cells expressing LacZ and p220^{+/+} cells expressing Cre while only 21% of p220^{lox/-} cells expressing Cre were in S or G₂/M phase (Fig. 3A and B). These data suggested that cells lacking p220 were defective in S-phase entry.

Because the efficiency of excision of the conditional p220 allele is less than 100%, we hypothesized that the small increase in S- and G₂/M-phase cells seen by flow cytometry in the

presence of Cre reflected cells that had not undergone conversion to the p220^{-/-} state. To examine this possibility, we labeled cells 13 h after release from quiescence with BrdU and then used an immunofluorescence assay to simultaneously score cells for BrdU incorporation and p220 expression (Fig. 3C). Overall, there was a 65% reduction in the number of BrdU-positive nuclei in p220^{lox/-} cells treated with Ad-Cre, relative to cells treated with Ad-LacZ. The majority of Ad-LacZ-treated cells contained p220-positive foci, and of these p220-positive cells, 79% were BrdU positive (Fig. 3C), indicating efficient S-phase entry. In contrast, greater than 95% of Ad-Cre-treated cells lacking p220 foci were BrdU negative while 96% of the residual p220-positive cells in this culture were BrdU positive (Fig. 3C). These results indicate that p220 is required for S-phase entry from quiescence.

G₁ arrest in p220^{-/-} cells occurs with high Cdk2 kinase activity. The inability of p220^{-/-} cells to enter S phase could reflect a requirement for p220 downstream of cyclin E/Cdk2, or alternatively, loss of p220 could lead to activation of a checkpoint signal that leads to the inhibition of Cdk2 activity. Such inhibition could occur through induction of Cdk inhibitors, inhibitory phosphorylation of Cdk2, or degradation of cyclin A or E. To examine this question, we examined Cdk2 kinase activity in quiescent p220^{lox/-} and p220^{-/-} cells and in cells 13 h after serum-stimulated cell cycle re-entry. This corresponded to the time when more than 50% of LacZ-expressing p220^{lox/-} cells have entered S phase (Fig. 3). As expected, Cdk2 kinase activity was low in quiescent cells and was increased at 13 h, reflecting cell cycle entry. Importantly, Cdk2 activity and protein levels were equivalent in p220^{lox/-} cells expressing LacZ or Cre (Fig. 4, lanes 7 and 8) and were comparable to those seen in p220^{+/+} cells treated similarly (Fig. 4, lanes 5 and 6). These data indicate that p220 is required for S-phase entry independently of cyclin E/Cdk2 and cyclin A/Cdk2 activity.

HPV E7 eliminates G₁ arrest in p220-null cells. Previous studies have highlighted a role for pRb and/or p53 in cell cycle arrest during the G₁ phase of the cell cycle. p53 is required for cell cycle arrest in G₁ in response to DNA damage via induction of the p21 Cdk inhibitor, while pRb is required for cell cycle arrest upon p21 induction (reviewed in references 41 and 42). pRb is also important for cell cycle arrest in response to differentiation and developmental signals. To examine the potential contribution of these G₁ checkpoint mechanisms to cell cycle arrest due to loss of p220, we used E6 and E7 proteins derived from HPV (4, 27, 39). E6 is known to functionally inhibit p53, while E7 blocks the activity of pRb. Pools of p220^{lox/-} and p220^{+/+} cells expressing either E6 or E7 were generated as described in Materials and Methods. Both p220^{lox/-} E6 and p220^{lox/-} E7 cells were found to undergo cell cycle arrest during serum deprivation in the presence of Ad-Cre. Cells were then stimulated to enter the cell cycle by serum addition and examined 13 h later by flow cytometry and BrdU incorporation (Fig. 5A and B). Upon serum stimulation, p220^{lox/-} E6 cells displayed a 13.6% increase in S/G₂-M-phase cells, similar to that displayed by p220^{lox/-} Ad-Cre-treated cells (Fig. 3A). In contrast, p220^{lox/-} E7 cells displayed a larger increase in S/G₂-M-phase cells (33.2%), slightly smaller than that seen with p220^{+/+} E7 cells (42.3%). When we examined them at the single-cell level by assaying BrdU incorpora-

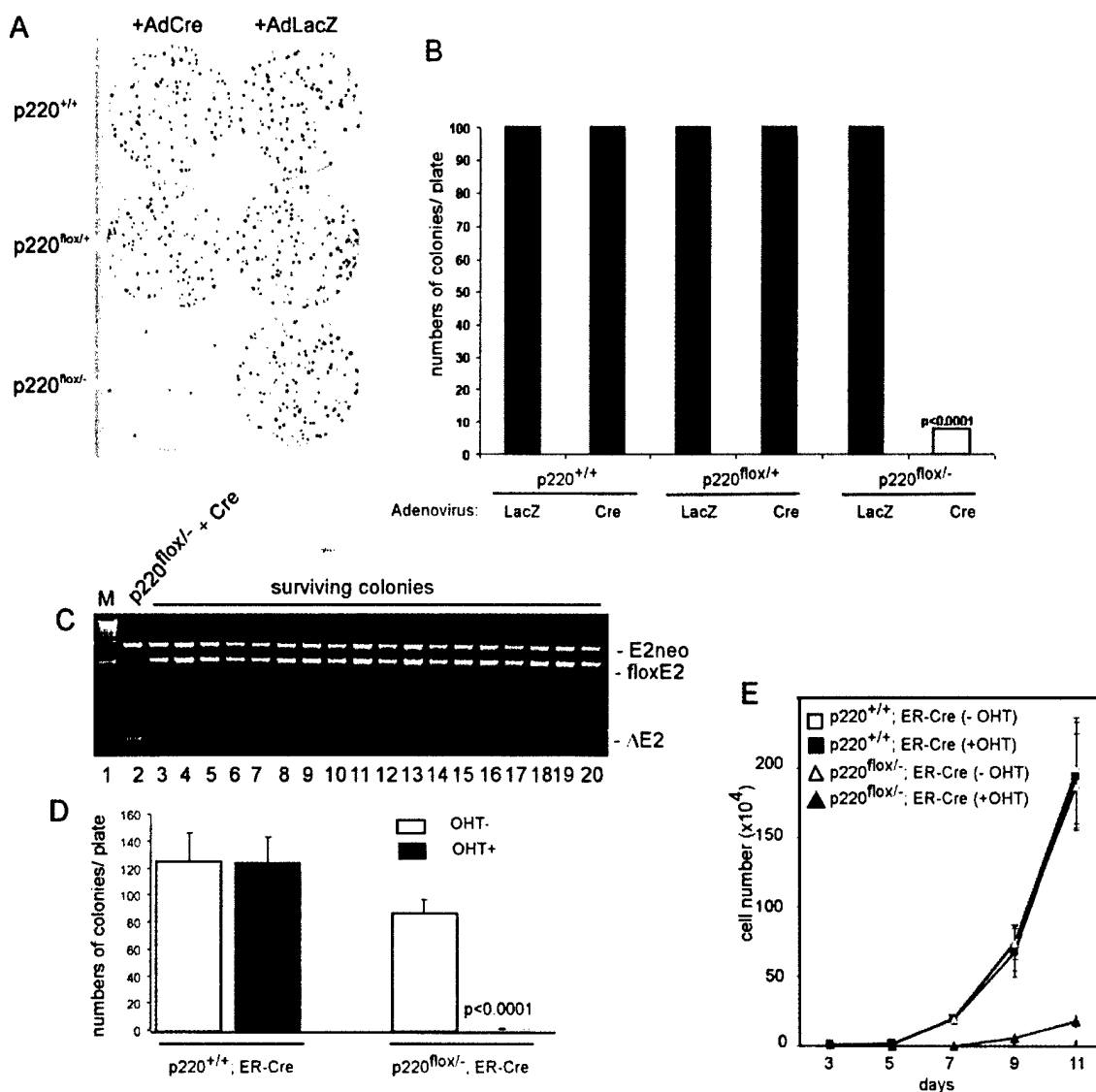
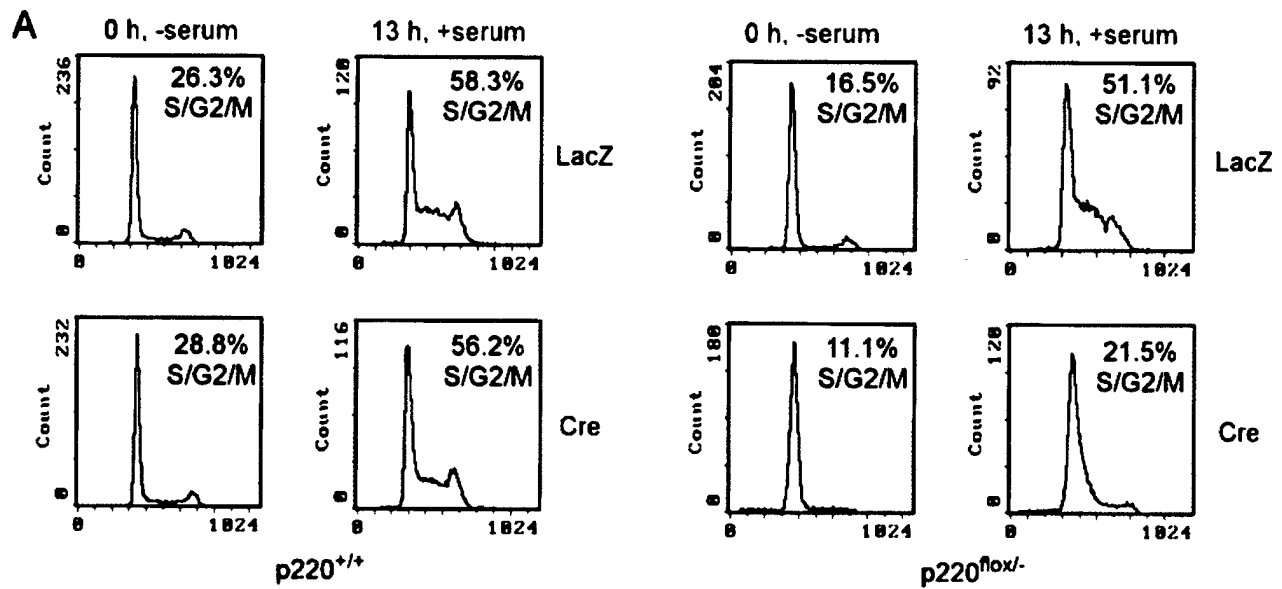


FIG. 2. p220 is required for cell viability. (A, B) Absence of colony formation in cells lacking p220. Cells of the indicated genotypes were infected with either Ad-LacZ or Ad-Cre for 2 days prior to plating at low density, in triplicate. Colonies were scored 2 weeks after plating. The average numbers of colonies formed are indicated in panel B. (C) Colonies surviving Cre recombinase contain an intact p220^{lox} allele. Genomic DNA was isolated from 18 residual surviving colonies and subjected to PCR with primers e and f (Fig. 1A) (lanes 3 to 20) along with DNA from p220^{lox/-} cells transiently expressing Cre recombinase (lane 2). A total of 40 surviving colonies were tested and found to retain the conditional p220 allele. (D, E) p220^{lox/-} ER-Cre cells are inviable in the presence of OHT. p220^{lox/-} ER-Cre or p220^{+/+} ER-Cre cells were grown for 2 days in the presence or absence of OHT, and 200 cells were plated in triplicate. After 2 weeks, surviving colonies were quantitated (D). To examine cell proliferation, cells were grown as in described for panel D and cells were plated equivalently at low density for each time point. At the indicated times, the number of viable cells was determined by trypan blue exclusion.

tion, we found that Ad-Cre-treated p220^{lox/-} E6 cells displayed 15% BrdU labeling while p220^{lox/-} E7 cells displayed 44% S-phase cells, comparable to the BrdU incorporation observed with Ad-Cre-treated p220^{+/+} E6 (40%) and p220^{+/+} E7 (64%) cells. As shown in Fig. 5B, p220-negative, BrdU-positive cells are readily seen in cultures of p220^{lox/-} E7 cells after Ad-Cre treatment. These data indicate that E7 blocks a pathway responsible for mediating cell cycle arrest in response to p220 depletion. It is conceivable that pRb is a relevant target of E7 in this context.

p220^{NPAT} promotes histone gene expression. The abundance of core histone mRNA increases dramatically as cells enter S phase (10- to 35-fold in various experiments) (11, 19). This increase is due to both transcriptional and posttranscriptional mechanisms, although the transcriptional component is thought to represent only a three- to fivefold increase. The posttranscriptional accumulation of histone mRNA largely reflects the activity of SLBP1, which promotes histone mRNA processing during S phase, the cell cycle stage when it is most efficiently translated (reviewed in reference 29). It has been



B

genotype	virus	time (h)	Exp #1		Exp #2	
			G1	S/G2/M	G1	S/G2/M
p220 ^{+/+}	LacZ	0	72.6	26.3	74.8	22.6
	Cre	0	69.9	28.8	74.2	22.6
p220 ^{lox/-}	LacZ	0	81.5	16.5	77.4	19.3
	Cre	0	82.8	11.1	75.6	14.4
p220 ^{+/+}	LacZ	13	40.4	58.3	47.4	51.2
	Cre	13	41.9	56.2	47.9	50.6
p220 ^{lox/-}	LacZ	13	47.4	51.1	44.7	53.4
	Cre	13	78.5	21.5	72.8	23.5



FIG. 3. p220 is required for S-phase entry. (A, B) Defective S-phase entry in cells lacking p220. Cells of the indicated genotypes were infected with Ad-LacZ or Ad-Cre for 3 days, and cells were arrested in G₀ by serum deprivation for 2 days. Cells were either subjected to flow cytometry directly (-serum) or induced to enter the cell cycle by serum addition (13 h, +serum) prior to flow cytometry (A). The results of two independent experiments are shown in panel B. Experiment 1 corresponds to the data in panel A. (C) Absence of DNA replication in cells lacking p220. p220^{lox/-} cells were synchronized in G₁ after infection with Ad-LacZ or Ad-Cre as described for panel A, and BrdU incorporation was examined 13 h after serum addition. Cells were subjected to a double-immunofluorescence assay with anti-BrdU and anti-p220 antibodies. Arrows indicate p220-deficient cells that are also BrdU negative. The asterisk indicates a residual p220-positive cell that is also BrdU positive.

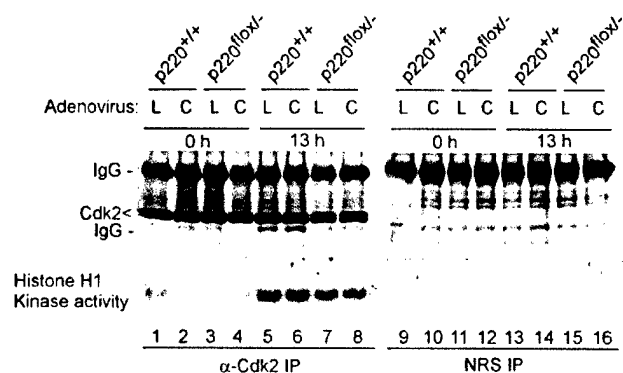


FIG. 4. Cell cycle arrest in p220^{-/-} cells occurs in the presence of Cdk2 activity. Cells of the indicated genotypes were subjected to the synchronization procedure used for Fig. 3A in the presence of Ad-LacZ (L) or Ad-Cre (C). At the indicated times, cells were lysed and subjected to immunoprecipitation (IP) with normal rabbit immunoglobulin G (IgG) (NRS) or anti-Cdk2 antibodies. Washed immune complexes were subjected to immunoblotting with anti-Cdk2 antibodies or used in histone H1 kinase assays.

previously reported that p220 activates histone H4 and H2B reporter constructs in transient cotransfection experiments, implicating p220 in histone gene transcription (26, 53). This led us to ask whether cells deficient in p220 are defective in endogenous histone gene expression.

Initially, we examined histone H4 expression in cells that had been stimulated to re-enter the cell cycle from G₀ in the presence or absence of p220. As shown in Fig. 6A, p220^{+/+} cells treated with Ad-Cre or Ad-LacZ and p220^{lox/-} cells treated with Ad-LacZ displayed cell cycle-regulated expression of an ~400-bp histone H4 mRNA, as determined by Northern blot analysis of cells released from G₀. The level of histone H4 mRNA was low in G₀ and increased about sixfold as cells entered S phase (8 to 16 h) (Fig. 6A, lanes 1 to 12). In contrast, p220^{lox/-} cells treated with Ad-Cre displayed substantially lower levels of histone H4 mRNA throughout the time course, increasing to a maximum of 1.7-fold at 13 h (Fig. 6A, lanes 13 to 16). The small increase in histone mRNA may reflect the absence of complete removal of the p220-floxed allele (Fig. 1). In order to quantify differences in expression and to expand the analysis to other histone subtypes, we used real-time RT-PCR in both G₀ cells and in cells released from quiescence by addition of serum (Fig. 6B and C). Subtype-specific primer sets were developed and found to give a single-peak dissociation curve, demonstrating PCR specificity (Fig. 6D and data not shown). In cells synchronized by serum deprivation, loss of p220 led to a 10-fold decrease in the abundance of histones H2A and H4, a 3-fold reduction in H3, and a 2-fold reduction in histone H2B mRNA, relative to controls with p220^{+/+} cells infected with Ad-LacZ (Fig. 6B). Similar results were observed at 13 h (Fig. 6C), a time when histone H4 mRNA levels in p220-expressing cells was maximal (Fig. 6A).

We also used transient transfection of histone H4 promoter-reporter constructs to verify loss of histone transcriptional activity in cells lacking p220. Initially, we examined transcriptional activity in cycling p220^{+/+} ER-Cre and p220^{lox/-} ER-Cre cells. Cells were cultured for 36 h in the presence or absence of OHT and then transfected with a pGL-histone

H4-luciferase reporter plasmid. Thirty-six hours later, cell extracts were assayed for luciferase activity. All three control groups (p220^{+/+} ER-Cre cells with or without OHT and p220^{lox/-} ER-Cre cells in the absence of OHT) displayed similar levels of luciferase activity. However, the level of activity observed in p220^{lox/-} ER-Cre cells in the presence of OHT was substantially reduced in cycling cells (Fig. 6H). Similar experiments were performed with these cells after serum deprivation, which leads to substantial accumulation of G₁ cells. While the overall activity was reduced significantly, again loss of p220 expression (plus OHT) led to reduced levels of luciferase activity (Fig. 6H).

A major limitation of this approach for studying p220 function is that p220-null cells arrest in G₁ and therefore, we would anticipate that expression of replication-dependent histone genes would not occur in the absence of p220. In order to examine histone gene expression during S phase in the absence of p220, we took advantage of p220^{lox/-} E7 cells, which enter S phase in the apparent absence of p220. With quantitative RT-PCR assays and cells released from serum starvation for 13 h, we found that the levels of expression for all four core histones were substantially reduced. The levels of H2A and H2B were reduced by 50% relative to those in p220^{lox/-} E7 cells treated with Ad-LacZ, while the levels of H3 and H4 were reduced by more than fourfold (Fig. 5C). Again, the incomplete removal of p220 upon Cre treatment likely contributes to the overall level of histone transcription seen, and the actual contribution of p220 to histone transcription is likely to be larger. Taken together, these data indicate that p220 is critical for optimal levels of core histone expression as cells enter S phase.

Expression of replication-dependent histone genes outside S phase. In experiments examining histone gene expression in serum-starved cells, we noticed that histone H4 mRNA levels were also reduced in p220^{-/-} cells, compared to those in p220-expressing cells (Fig. 6A and B). This, coupled with the fact that previous studies had seen a low, yet quantifiable, level of histone mRNA in G₀ and G₁ cells (11, 19), led us to ask whether p220 might contribute to low-level histone transcription outside S phase. In principle, the use of highly quantitative PCR techniques that were not available when early studies on histone transcription were performed might allow a more precise determination of whether "replication-dependent" histone expression can occur in the absence of DNA replication.

We first examined the possibility that the differences in the number of residual S-phase cells in these experiments due to p220 depletion is responsible for the differential histone expression observed in G₀/G₁ cell populations. However, we found that the percentage of residual BrdU-positive cells 48 h after serum deprivation was the same in both p220^{lox/-} Ad-LacZ and p220^{lox/-} Ad-Cre cells (~15%; data not shown). Thus, the difference in histone expression seen in p220^{lox/-} and p220^{-/-} cells is unlikely to reflect the absence of complete synchrony. To examine histone expression outside S phase, we sought to further reduce the S-phase content of our synchronized population by depleting S-phase cells from the population with aphidicolin, an inhibitor of DNA replication. Previous studies have demonstrated that inhibition of replication by aphidicolin leads to a rapid depletion (<60 min) of histone mRNA. With a highly sensitive BrdU incorporation method,

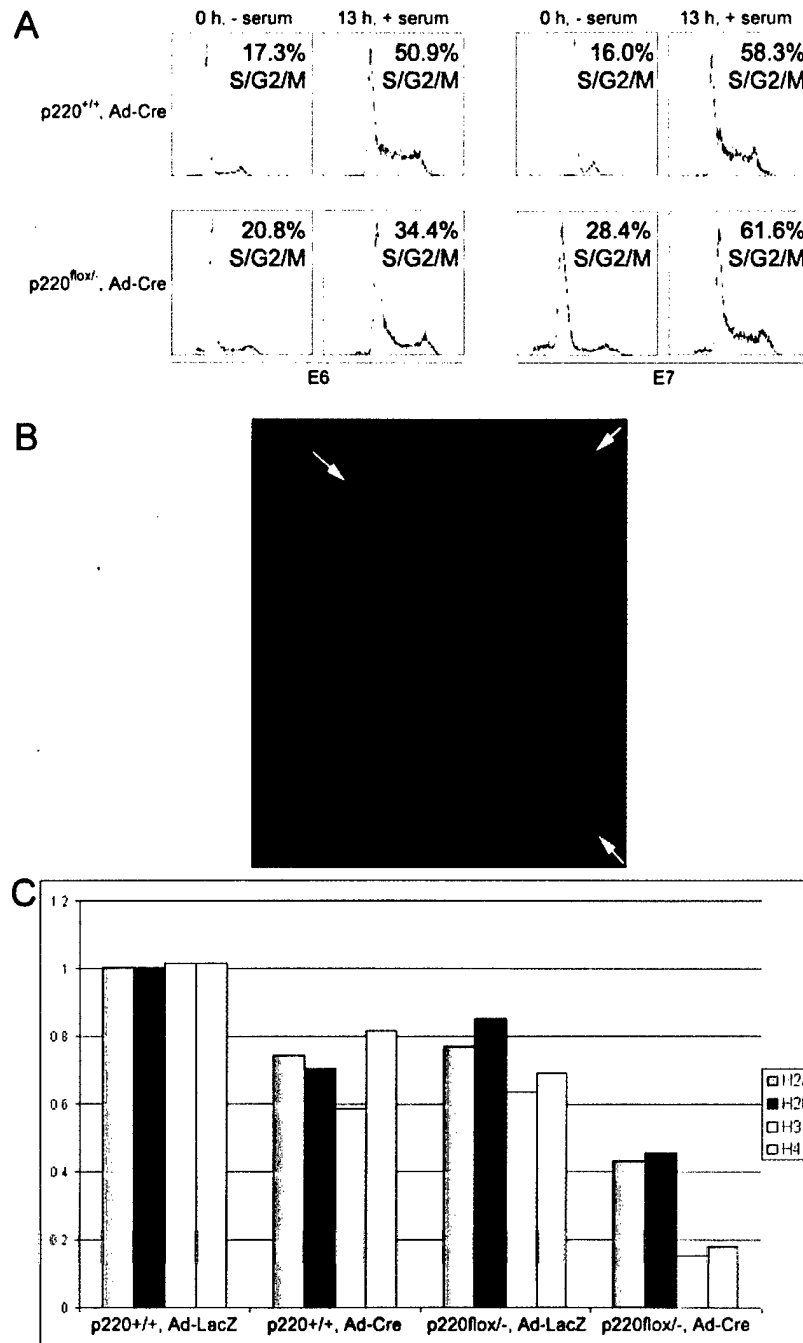


FIG. 5. HPV E7 eliminates G₁ cell cycle arrest in response to depletion of p220. (A) Expression of HPV E7, but not HPV E6, eliminates cell cycle arrest in G₁ in response to p220 depletion. Pools of p220^{lox/-} or p220^{+/+} cells expressing either E6 or E7 were arrested by serum starvation in the presence of Ad-Cre, and flow cytometry was performed before and after serum stimulation (13 h). (B) BrdU incorporation in p220-negative cells expressing E7. p220^{lox/-} E7 cells were treated with Ad-Cre and subjected to serum starvation as described for Fig. 3. Cells were serum stimulated for 13 h, and pulse BrdU incorporation was examined by immunofluorescence assay together with p220 staining to identify cells that had not yet depleted p220. A BrdU cell still maintaining p220 expression is indicated by the green arrow, while cells undergoing DNA replication (red) while lacking detectable p220 staining (green) are indicated by the white arrows. (C) Reduced levels of replication-dependent histone gene expression in p220-null cells expressing E7. p220^{+/+} E7 or p220^{lox/-} E7 cells were treated with the indicated Ad, subjected to serum starvation for 48 h, and then stimulated to re-enter the cell cycle by serum addition. Thirteen hours later, the levels of histone mRNAs were determined by RT-PCR.

we were unable to detect any S-phase cells ($n = 1,000$) after 3 h in aphidicolin. Under these conditions, the levels of H2B and H4 mRNAs were reduced to 25 and 45%, respectively, compared with those in untreated cells (Fig. 6E). The BrdU con-

ditions used coupled with deconvolution microscopy are sufficient to allow even a single replication focus to be visualized. Thus, there is clearly detectable H2B and H4 expression in the absence of detectable DNA replication. We then examined

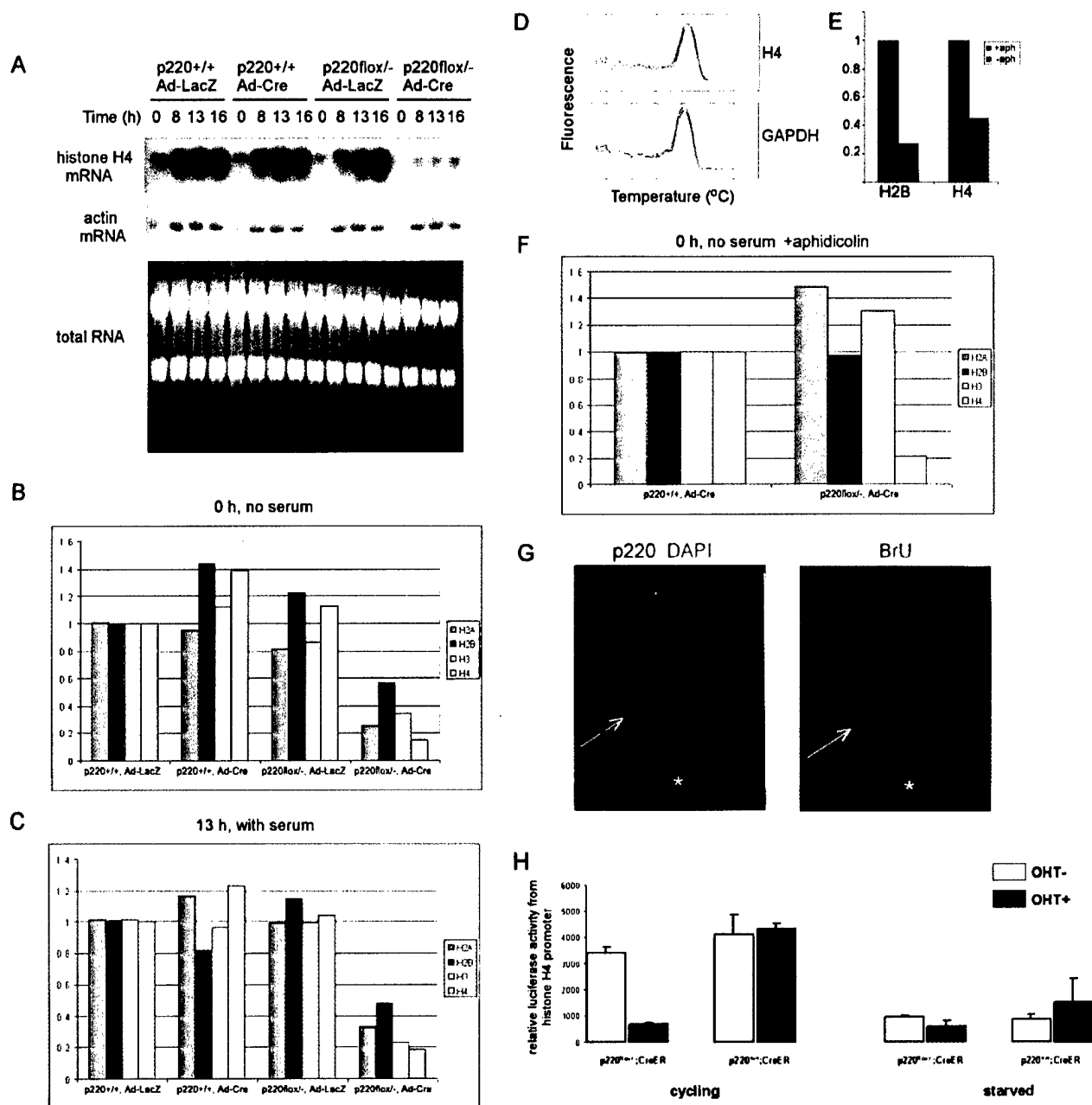


FIG. 6. p220 is required for histone gene expression. (A) Induction of histone H4 expression during S-phase entry is p220 dependent. Cells of the indicated genotypes were synchronized as described in the legend to Fig. 3A after treatment with Ad-Cre or Ad-LacZ. At the indicated times after serum addition, mRNA was isolated and used for Northern analysis with either a histone H4 or an actin probe. (B, C) p220 is required for optimal expression of all four core histone genes in G₀/G₁. mRNA from panel A was used for quantitative RT-PCRs with primers specific for histones H2A, H2B, H3, and H4, and GAPDH was used as a control (see Materials and Methods). Assays were performed in triplicate. (B) RNA from the 0-h time point was used. (C) RNA from the 13-h time point was used. (D) Dissociation curve analysis revealed a single peak for GAPDH and histone H4, indicating a unique RT-PCR product. Similar profiles were found for H2A, H2B, and H3 (data not shown). (E) Real-time quantitative PCR analysis reveals decreased but detectable levels of histone transcripts in HCT cells arrested in G₀/G₁ by serum deprivation and treated with aphidicolin (aph). Reactions were performed in triplicate. Black bars, RNA isolated from serum-starved p220^{+/+} cells; red bars, RNA isolated from serum-starved p220^{+/+} cells cultured in the presence of aphidicolin. (F) Loss of p220 results in decreased expression of histone H4 in serum-deprived, aphidicolin-treated cells. Real-time PCR assays were performed in triplicate. (G) p220-deficient cells are not defective in global transcription as determined by BrU incorporation. Asynchronous p220^{+/+} ER-Cre or p220^{lox/-} ER-Cre cells were treated with Ad-LacZ or Ad-Cre for 3 days, and cells were labeled with BrU for 0.25 h. Cells were subjected to p220 immunostaining, and BrU was then detected with an immunofluorescence assay. Representative p220-positive and -negative (white arrows) cells are shown. p220, red; BrU, green; DAPI, blue. (H) p220 is required for optimal histone H4 promoter reporter activity. p220^{+/+} ER-Cre or p220^{lox/-} ER-Cre cells were either allowed to cycle continuously or subjected to serum starvation in the presence or absence of OHT (as indicated) and then transfected with a pGL-histone H4-luciferase construct along with a plasmid expressing LacZ. After 36 h, cells were harvested and assayed for β -galactosidase and luciferase activities. Normalized luciferase activities from triplicate transfections, each assayed in triplicate, are shown.

whether this expression was dependent on p220. p220^{lox/-} or p220^{+/+} cells were serum starved and treated with Ad-Cre, and aphidicolin was added prior to mRNA isolation and quantitative PCR to examine the expression of histone genes. We found that the levels of histone H4 were dramatically reduced (fivefold) in p220^{lox/-} cells treated with Ad-Cre relative to those in p220^{+/+} cells treated similarly (Fig. 6F), indicating that p220 contributes to "basal" histone H4 transcription independently of DNA replication. In contrast, we were unable to detect significant differences in histone H3, H2A, and H2B levels under these conditions (Fig. 6F). The simplest interpretation of this finding is that HCT116 cells express what we refer to as basal levels of histone mRNAs outside S phase and that p220 contributes substantially to histone H4 expression independently of DNA synthesis in S phase.

Three lines of evidence indicate that the loss of histone mRNA is not a reflection of a general loss of transcription due to depletion of p220. First, β -actin levels accumulated in cells upon cell cycle re-entry independently of p220 status (Fig. 6A). Second, we examined global transcription by monitoring incorporation of BrU into newly transcribed mRNA with an immunofluorescence assay. The extent of BrU incorporation into p220^{lox/-} cells transiently expressing Cre recombinase was indistinguishable in the presence or absence of p220, as determined by immunofluorescence assay (Fig. 6G). Third, we examined the intranuclear distribution of phosphorylated RNA polymerase II, which is an additional marker for ongoing transcription. Again, the punctate patterns of phosphorylated RNA polymerase staining were indistinguishable in p220^{lox/-} and p220^{-/-} cells (data not shown).

p220 phosphorylation status in G₀/G₁. The finding that p220 contributes to basal histone H4 expression outside S phase led us to examine the phosphorylation status of p220 in G₀/G₁ cells. Cyclin E/Cdk2 activity is frequently deregulated in tissue culture cells and could, in principle, lead to inappropriate p220 phosphorylation in G₀/G₁. This phosphorylation could, in turn, lead to increased histone expression outside S phase. HCT116 cells synchronized in G₀ by serum deprivation contain two p220 foci. However, these foci do not react with antibodies directed against phospho-T1350 (Fig. 7A). In contrast, HCT116 cells in S phase (13 h after release from serum deprivation) contain the expected four p220 foci and these foci react with the anti-phospho-T1350 antibodies (Fig. 7A). Similar results were obtained with antibodies directed against phospho-T1270 (data not shown). These data indicate that basal p220-dependent histone gene expression occurs without detectable phosphorylation of p220 on two prominent Cdk2 phosphorylation sites and suggest a phosphorylation-independent component to p220 function outside S phase.

p220^{NFAT} deficiency leads to dissociation of p80^{coilin} from CBs. CBs are complex organelles made up of a large number of proteins, many of which have not been identified (17, 18, 20, 36). A well-characterized CB component is p80^{coilin}. Although this protein is not essential for mouse development, its absence reduces association of the SMN protein with CBs (20, 46). We considered the possibility that p220 is required for proper localization of p80^{coilin}.

First, we examined p80^{coilin} localization in asynchronous p220^{lox/-} cells 5 days after infection with either Ad-Cre or Ad-lacZ (Fig. 7B). The vast majority of cells treated with

Ad-LacZ maintained focal anti-p220 and anti-coilin staining (Fig. 7B). In contrast, cells treated with Ad-Cre demonstrated a dramatic increase in the number of cells displaying altered patterns of coilin staining. In particular, we noted cells that had lost focal coilin staining and exhibited either a diffuse pattern or a "dissociative" pattern containing a large number of small punctate foci (Fig. 7B). In this asynchronous population, 38% of p220-negative cells displayed dissociative or dispersed patterns of p220 staining. Similar results were obtained with p220^{lox/-} ER-Cre cells treated with OHT (Fig. 7C and data not shown). We next examined coilin staining in synchronized cells lacking p220. p220^{+/+} or p220^{lox/-} cells were treated with Ad-Cre or Ad-lacZ and arrested in G₀ by serum withdrawal. Under these conditions, greater than 68% of the cells displayed normal patterns of coilin foci, independently of the presence of p220 (Fig. 7D). Previous studies have demonstrated that the number of coilin-positive CBs increases as cells enter S phase. When p220^{lox/-} cells expressing Ad-LacZ were stimulated to re-enter the cell cycle with serum, the percentage of cells with focal anti-coilin staining increased to 79 to 92% and, as expected, a subset of coilin-positive foci in these cells were p220 positive. In contrast, with p220^{lox/-} cells treated with Ad-Cre, less than 42% of these cells displayed focal coilin staining (Fig. 7D). These data indicate that p220 is not required for localization of coilin in CBs during G₀ but is important for efficient retention of coilin in CBs as cells traverse the G₁/S transition.

DISCUSSION

A central question in cell cycle control concerns the identity and functions of critical targets of cyclin-dependent kinases. Much of our understanding of the role of G₁ Cdk2 in cell cycle entry has come from analysis of the Rb family members. In contrast, relatively little is known about cyclin E targets downstream of Rb. In this work, we have explored the function of p220, a cyclin E target implicated in G₁/S control. The notion that p220 is subject to regulation by cyclin E/Cdk2 is supported by several facts. First, cyclin E physically associates with p220 in vitro and in vivo (25, 52). Second, p220 is phosphorylated on at least five sites by cyclin E/Cdk2 in vitro and four of these have been detected in p220 isolated from mammalian cells with either mass spectrometry or phosphospecific antibodies (26). Third, coexpression of cyclin E/Cdk2 with p220 leads to stimulation of p220's ability to promote S-phase entry and histone gene transcription, while mutations in particular phosphorylation sites reduce histone reporter activation, suggesting a functional connection (26, 52, 53).

In this work, we sought to examine the extent to which cells use p220 to promote events linked with S-phase entry. With promoterless-*neo* technology, we generated HCT116 cells carrying a conditional allele of p220, allowing removal of p220 in response to expression of Cre recombinase. Several facts were established. First, p220 is required for HCT116 cell proliferation. Second, this defect in cell proliferation appears to manifest itself primarily in a block to DNA replication. G₀/G₁ cells lacking p220 are unable to enter S phase in response to serum, as determined either by flow cytometry or by BrdU incorporation assay. Third, this arrest occurs in the presence of S-phase levels of cyclin E/Cdk2 activity, confirming that p220

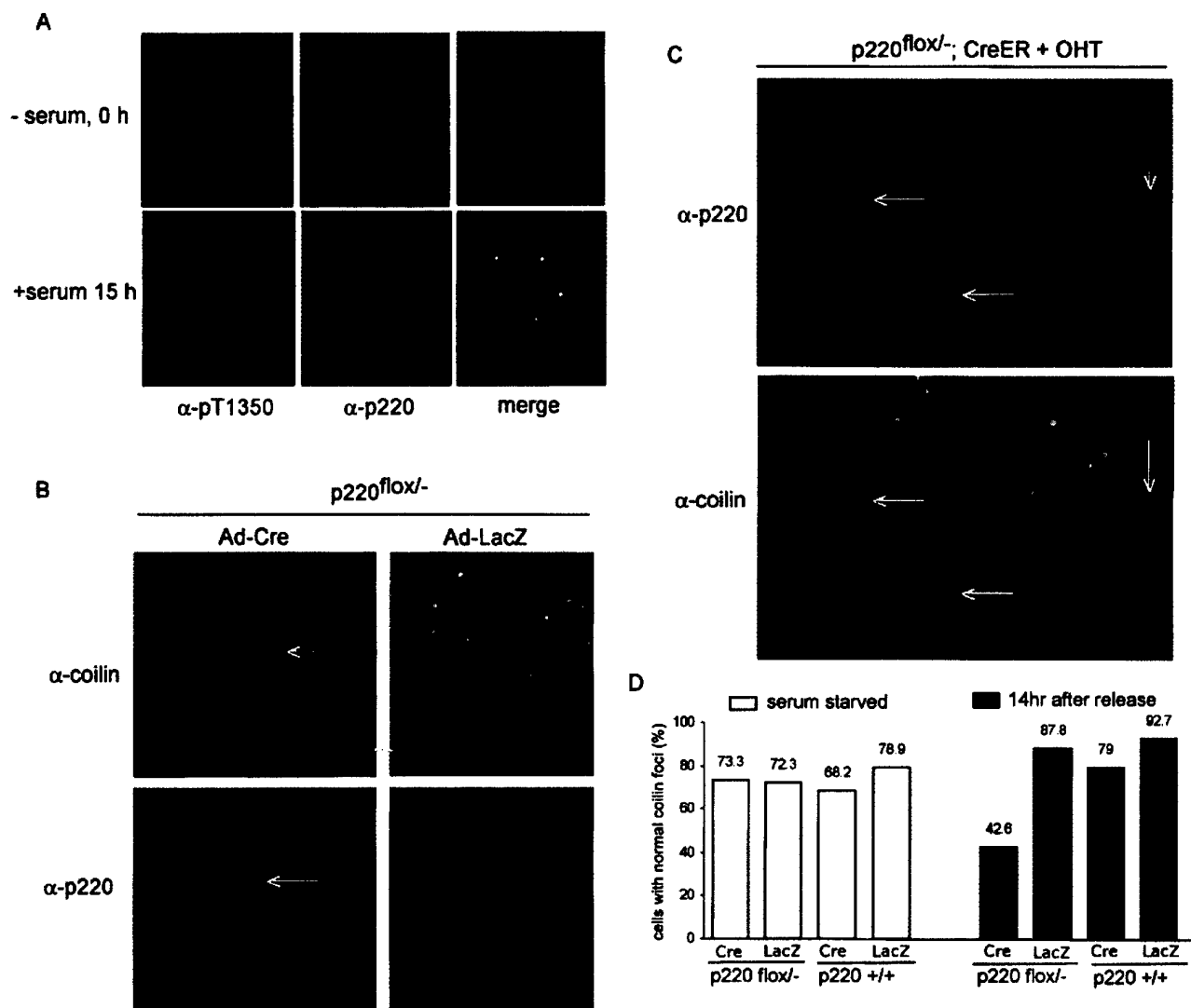


FIG. 7. p220 is unphosphorylated in G₀ HCT116 cells and is required for maintenance of p80^{coilin} localization during the G₁/S transition. (A) p220 is not phosphorylated on Thr-1350 in G₀ HCT116 cells. HCT116 cells were rendered quiescent by serum deprivation (2 days) and either maintained in the absence of serum (0 h) or induced to enter the cell cycle by serum addition (15 h). Cells were subjected to immunofluorescence assays with rabbit antibodies directed against either phospho-T1350 in p220 (green) or a monoclonal antibody directed against p220 (53) (red). Nuclei were stained with DAPI (blue). Cells in G₀ (no serum) contain two p220-positive foci, while cells in S phase (15 h) contain four p220 foci. p220-positive foci react with anti-T1350 in S-phase cells but not in G₀ cells. (B to D) Altered p80^{coilin} localization in cells lacking p220. (B) Asynchronous p220^{lox/-} cells were treated with Ad-LacZ or Ad-Cre, and after 5 days, cells were subjected to immunofluorescence assays with anti-p80^{coilin} and anti-p220 antibodies: green, p80^{coilin}; red, p220; blue, DAPI. Some cells displayed altered p80^{coilin} localization typified by a dissociative staining pattern (arrow). (C) Asynchronous p220^{lox/-} ER-Cre cells were grown in the presence or absence of OHT (4 days). p80^{coilin} and p220 proteins were detected by immunofluorescence assay as for panel B. (D) Disruption of p80^{coilin} localization during the G₁/S transition. Cells of the indicated genotypes were infected with Ad-Cre or Ad-LacZ for 3 days, serum was removed for 2 days, and then cells were either maintained in low-serum medium (0 h) or induced to reenter the cell cycle by serum addition (13 h). Cells were subjected to immunofluorescence assay as described for panel B, and cells with focal, dissociative, and dispersed p80^{coilin} were determined along with the status of p220 staining. At least 100 cells per group were counted.

functions downstream of cyclin E/Cdk2 activation. Fourth, expression of HPV E7, but not E6, allows S-phase entry in the absence of p220, implying a role for pRb in maintaining a G₁ block when p220 levels fall below a crucial level. Fifth, optimal expression of histone genes during S phase requires p220. Sixth, low levels of histone gene expression were detected outside S phase and expression of histone H4 in this setting was dependent on p220 but apparently independent of p220 phos-

phorylation by Cdk2. Finally, p220 is required to maintain focal localization of p80^{coilin} in CBs as cells traverse G₁/S.

The available data suggest that p220 plays multiple independent roles in cell cycle progression (Fig. 8A). We previously showed that mutations in p220 that block its ability to activate transcription of histone reporter constructs have little effect on the ability of p220 to promote S-phase entry (50). Thus, it seems likely that p220's histone transcriptional activation func-

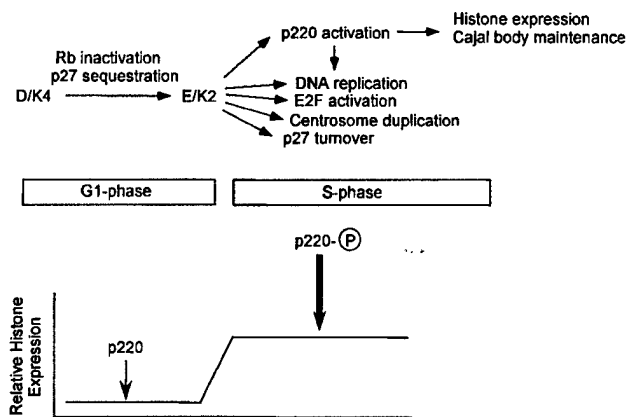


FIG. 8. p220 functions downstream of cyclin E/Cdk2 to control DNA replication and histone gene transcription. (Top) The cyclin E/Cdk2 signaling pathway. See text for details. (Bottom) Model for control of histone transcription by p220. During the G_0 and G_1 cell cycle phases, histone transcription is low but detectable. In this cell cycle phase, expression of histone H4 appears to be under the control of p220, although p220 is not phosphorylated by Cdk2. As cells enter S phase, p220 is phosphorylated by Cdk2 and this leads to an increase in p220's ability to promote expression of all four core histone genes.

tion is not critical to its S-phase-promoting function. This hypothesis is corroborated by our finding that inactivation of pRb by E7 rescues S-phase entry but not core histone expression in p220-deficient cells. E7 promotes S-phase entry by multiple mechanisms that include (27), but are not limited to (39), derepression of Rb-suppressed transactivation of cyclin E. Thus, expression of E7 shifts the cell cycle balance from Rb-mediated G_1 arrest toward constitutive activation of cyclin E/Cdk and accelerates progression through the G_1/S transition. This reduction of the S-phase entry threshold might be sufficient to promote DNA replication but not full histone transcription in the absence of p220.

Since core histones are essential for cell survival and their evolutionary conservation is extremely high, we believe that replication of DNA in the absence of proper synthesis of core histones might lead to aberrant chromatin assembly and organization. This, in the long term, would possibly result in cellular lethality. More importantly, the cell cycle-related phenotype of E7 p220^{-/-} cells provides another compelling piece of evidence that p220^{NPAT}—in concordance with previous research (50)—controls multiple cellular pathways at the G_1/S boundary. As shown here, these pathways can be functionally separated because only DNA replication, but not histone transcription, is restored by inactivation of Rb in a p220^{-/-} background. However, the mechanistic details of how p220 promotes DNA replication and histone transcription remain to be elucidated.

The majority of studies on histone transcription have focused on S-phase-dependent activation, and little is known about the pathways controlling basal histone transcription that occurs in G_0 or G_1 . There are both common and specific *cis*-acting regulatory elements in replication-dependent histone genes (21). Both subtype-specific consensus elements and the common YY1 element located in the structural gene contribute to cell cycle-regulated transcription, and in some cases,

trans-acting factors that interact with subtype-specific consensus elements have been identified (Oct1 in the case of H2B and IFR2 in the case of histone H4) (3, 9, 10, 15, 28, 47–49). Our work has uncovered what appears to be a phosphorylation-independent function of p220 in histone H4 expression, but not expression of other core histones, outside S phase. We propose (Fig. 7B) that during G_0/G_1 , unphosphorylated p220 functions to support low levels of histone H4 expression. As cells approach the G_1/S transition and p220 becomes phosphorylated by Cdk2 (26), p220 activity is enhanced, leading to an approximately fivefold increase in core histone gene expression in HCT116 cells. Replication-dependent histone genes are relatively unique in that they are organized in large clusters of genes on chromosomes 1 (~13 genes) and 6 (~55 genes) (1, 30). Presumably, this organization is important for the coordinate expression of the four core histone subtypes, such that a balance of histone precursors is generated during S phase. The tethering of p220-containing CBs with these gene clusters is interesting in this regard and presumably indicates local action of p220 in promoting coordinate expression of these genes. It remains to be determined whether the pathways used by p220 to activate histone H4 transcription during G_0/G_1 are analogous to those used to achieve higher levels of transcription during S phase and precisely how p220 activates transcription of all for classes of histone genes. One possibility is that p220 functions through elements common to all core histone genes, thereby providing a mechanism for coordinate activation of these genes. It is also possible that p220 plays additional roles in histone mRNA accumulation, possibly through effects on posttranscriptional histone mRNA processing. The identification of proteins that interact with p220 is required to more fully understand how p220 functions to control histone transcription and S-phase entry.

Our work has also revealed a role for p220 in maintaining focal p80^{coilin} localization as cells enter S phase. CBs are heterogeneous in nature (reviewed in reference 36). In the HCT116 cells used here, p220-positive foci always contain p80^{coilin} but not all p80^{coilin}-containing foci, CBs, contain p220. The basis of this heterogeneity is unknown, but it likely reflects the fact that p220-positive CBs are tethered to histone gene clusters while other CBs are bound to other gene loci, including snRNA genes (16, 43). Previous studies have demonstrated that the number of CBs and the intensity of p80^{coilin} immunostaining increase as cells progress from G_1 into S phase. This is due, in part, to the generation of two p220-containing CBs tethered to chromosome 1 histone gene clusters during S phase (26, 53). In HCT116 cells arrested by serum deprivation, the focal nature of localization is maintained in the absence of p220. When p220^{lox/-} cells are induced to enter the cell cycle from G_0 , focal p80^{coilin} staining is maintained, as expected. In contrast, cells lacking p220 display aberrant patterns of coilin staining. In particular, a significant fraction of cells display what we refer to as dissociative p80^{coilin} localization. This pattern features punctate p80^{coilin} staining throughout the nucleus. We found that a subset of p220-deficient cells maintained p80^{coilin} staining. Given the finding that a substantial fraction of quiescent HCT116 cells failed to enter S phase by 13 h after serum addition (Fig. 3A), it is possible that p220-negative cells that retain focal p80^{coilin} staining represent those that did not exit G_0 . We postulate that the presence of p220 in

CBs is required to maintain focal p80^{coilin} staining. This suggests a dynamic interplay among p220, p80^{coilin}, and CBs. These organelles are known to be highly dynamic and to undergo ATP-dependent reorganization (37). The mechanism by which p220 promotes p80^{coilin} localization remains to be determined.

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