Faithful inheritance of the chromatin structure is essential for maintaining the gene expression integrity of a cell. Histone modification by acetylation and deacetylation is a critical control of chromatin structure. In this study, we tested the hypothesis that histone deacetylase 1 (HDAC1) is physically associated with a basic component of the DNA replication machinery as a mechanism of coordinating histone deacetylation and DNA synthesis. Proliferating cell nuclear antigen (PCNA) is a sliding clamp that serves as a loading platform for many proteins involved in DNA replication and DNA repair. We show that PCNA interacts with HDAC1 in human cells and in vitro and that a considerable fraction of PCNA and HDAC1 colocalize in the cell nucleus. PCNA associates with histone deacetylase activity that is completely abolished in the presence of the HDAC inhibitor trichostatin A. Trichostatin A treatment arrests cells at the G2-M phase of the cell cycle, which is consistent with the hypothesis that the proper formation of the chromatin after DNA replication may be important in signaling the progression through the cell cycle. Our results strengthen the role of PCNA as a factor coordinating DNA replication and epigenetic inheritance.

Epigenetic markings play an essential role in regulating the gene expression program of vertebrate cells. One of the fundamental challenges of cell division is therefore coordinating the processes of genetic and epigenetic inheritance. The cell must possess multiple mechanisms to coordinate these processes (1). For example, DNA methylation is coordinated with DNA replication (2) by physical association of maintenance DNA methyltransferase 1 with the DNA replication fork protein PCNA1 (3). Inhibition of DNA methyltransferase 1 leads to inhibition of initiation of DNA replication (4).

Similar to DNA methylation, DNA replication-coupled chromatin assembly is essential for the inheritance of the epigenetic code. The specific targeting of nucleosome assembly to the newly synthesized DNA is achieved by direct interaction of histone chaperone CAF-1 with PCNA (5). PCNA is a homotrimeric protein that forms a sliding clamp around DNA and functions as a DNA polymerase processivity factor during replication and nucleotide excision repair. Through its multiple protein–protein interactions, PCNA coordinates events in replication, epigenetic inheritance, repair, and cell cycle control (6). A recent study in Saccharomyces cerevisiae has shown that several mutations in PCNA decrease silencing at telomeres and at the mating-type HMR locus (7). Furthermore, mutations in the Drosophila PCNA gene mus209 suppress repression in the vicinity of heterochromatin (8). The disruption of epigenetic silencing has been attributed to the inability of some of these mutants to associate with CAF-1. However, synergism of several PCNA mutants with CAF-1 mutants suggested that PCNA may participate in silencing through another factor.

During nucleosome assembly, histones H3 and H4 undergo transient acetylation before their deposition onto replicated DNA. In the process of chromatin maturation, newly deposited acetylated histones are generally deacetylated by a mechanism that is poorly understood (9).

In this study, we tested whether HDAC1 could be involved in this process. HDAC1 is a member of a growing family of proteins that currently consists of 16 isoforms divided into three classes (10). HDACs have been found in multiprotein complexes involved in transcriptional regulation, cell cycle, differentiation, and DNA repair. HDAC1 and HDAC2 have been shown to associate with the NuRD nucleosome remodeling complex (11) as well as with the mammalian transcription repressor Sin3 and to be targeted to promoter elements to cause transcriptional repression (12). Recruitment of HDAC1 by retinoblastoma protein (Rb) to E2F1-responsive promoters supports its role in cell cycle regulation (13, 14). In addition, HDAC1 and HDAC2 have been shown to associate with DNA methyltransferase 1 and play a role in transcriptional silencing (15, 16), thereby establishing a link between two major epigenetic modifications: DNA methylation and histone deacetylation.

Similar to DNA replication, DNA repair machinery utilizes chromatin remodeling activities to make the DNA more accessible and to reestablish the proper chromatin structure after DNA repair. In accordance, Hua1 and Rad9, two human checkpoint proteins, were shown to form a complex with HDAC1 (17). In addition, another histone-modifying enzyme, histone acetyltransferase p300, has been shown to form a complex with PCNA and was proposed to play a role in DNA repair after UV-induced DNA damage (18).

We therefore tested the hypothesis that similar to DNA methyltransferase 1 and CAF-1, HDAC1 associates with PCNA, which may serve as a recruiting factor for histone deacetylases to the sites of DNA replication and repair. We also
tested the hypothesis that disruption of HDAC activity would disrupt cell cycle progression.

**MATERIALS AND METHODS**

**Cell Culture and Transfections**—A549 human non-small cell lung carcinoma cells (ATCC CCL 185) were grown in low-glucose Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and 2 mM glutamine. HEK293 human adenovirus type 5-transformed human embryonal kidney cells (ATCC CRL 1573) were grown in high-glucose Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and 2 mM glutamine. MRHF male human foreskin fibroblasts (72-213A, BioWhittaker) were grown in high-glucose Dulbecco’s modified Eagle’s medium supplemented with 2% fetal calf serum and 2 mM glutamine. For transient transfections, HEK293 cells were plated 18 h before transfection at a concentration of 5 x 10⁵ cells/100-mm tissue culture dish. 6 μg of pcDNA3-HDAC1-FLAG plasmid (a kind gift from Dr. T. Kouzarides) (15) were transfected using the calcium-phosphate precipitation method. The medium was replaced 24 h after transfection, and the cells were harvested 48 h after transfection.

**Purification of GST Fusion Proteins**—GST-HDAC1 fusion constructs were a kind gift from Dr. T. Kouzarides (15). GST and GST fusion proteins were expressed in Escherichia coli XA90, induced with 0.1 mM isopropyl-1-thio-D-galactopyranoside for 4 h, and purified following the protocol from Amersham Biosciences, with modifications. To isolate fusion proteins from inclusion bodies, the bacterial pellet was lysed in STE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 150 mM NaCl) on ice, lysozyme was added to a final concentration of 0.1 mg/ml, and the samples were sonicated and centrifuged at 12,000 rpm. The supernatants were bound to glutathione-Sepharose beads, and the concentrations of the different fusion proteins were estimated by subjecting small portions of the samples to SDS-PAGE. Equal amounts of the fusion proteins were used in the binding assays, whereas glutathione-Sepharose beads were added to the binding mixtures to achieve equal amount of beads in the binding reactions.

**GST Pull-down Experiments**—Equal amounts of GST fusion proteins bound to the glutathione-Sepharose beads were incubated in radiommune precipitation buffer (9.1 mM Na₂HPO₄, 1.7 mM NaH₂PO₄, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS) with 2 μg of total cell extracts prepared from either A549 or HEK293 cells and pre-cleared with beads alone. The binding reaction was incubated by rocking overnight at 4 °C. The beads were washed four times with 1 ml of radiommune precipitation buffer, subjected to SDS-PAGE, and analyzed by Western blotting. PCNA was transcribed/translated in vitro using the coupled transcription translation system (Promega) from pHPCNA15 vector (a kind gift from Dr. Edward K. L. Chan) (19). We performed pull-down assays with both in vitro translated PCNA as described previously (15), adding Complete Mini protease inhibitors (Boehringer Mannheim) in all buffers.

**Immunoprecipitations and Western Blot Analysis**—Total cell extract was prepared in radiommune precipitation buffer containing Complete Mini protease inhibitors (Roche Molecular Biochemicals). For immunoprecipitations, 2 μg of cell extract were incubated with 10 μl of agarose-conjugated PCNA antibody or agarose-conjugated mouse IgG by rocking overnight at 4 °C. Bound complexes were washed four times with 1 ml of PBS (0.1 mM Na₂HPO₄, 1.7 mM NaH₂PO₄, and 150 mM NaCl) and resolved on SDS-polyacrylamide gels. After transferring to polyvinylidene difluoride membrane and blocking the nonspecific binding with 5% milk, HDAC1, PCNA, and FLAG-tagged proteins were detected using a 1:1000 dilution of anti-PCNA antibody (PC-10; Santa Cruz Biotechnology) in 5% serum for 1 h at room temperature, washed three times, incubated with a 1:500 dilution of rhodamine Red-X anti-mouse IgG (715-295-100, Jackson Immunoresearch) in 5% serum for 45 min at room temperature, and washed three times. The coverslips were blocked with 10% goat serum for 20 min, incubated with a 1:50 dilution of anti-HDAC1 antibody (H-51; Santa Cruz Biotechnology) in 1.5% serum for 45 min at room temperature, and washed three times. The coverslips were mounted onto slides using Immuno-mount (Shandon). The staining was analyzed using LSM 510 Laser Scanning Microscopy, version 2.5 (Zeiss). The fields were taken with a magnification of x63. The inserts were zoomed three additional times.

**Cell Cycle Analysis by FACS**—A549 cells were plated at a density of 5 x 10⁵ cells/100-mm plate. The next day, cells were treated with 1 μM trichostatin A (Sigma) for 6, 12, and 24 h. Control cells and treated cells were harvested, washed twice in PBS, and fixed in ice-cold 70% ethanol for 30 min. The fixed cells were stained for DNA with a 50 μg/ml solution of propidium iodide in PBS for 30 min at room temperature. The cells were analyzed for DNA content by FACS.

**RESULTS**

**PCNA and HDAC1 Form a Complex in Vivo**—To test the hypothesis that PCNA and HDAC1 are physically associated in human cells, we first tested whether they reside in the same multiprotein complex by coimmunoprecipitation assays. Endogenous HDAC1 protein was coimmunoprecipitated by agarose-conjugated anti-PCNA antibody from two human cancer cell lines (A549 and HEK293) as well as from a non-transformed human fibroblast cell line (MRHF), suggesting that the interaction is not unique to the cancer cells (Fig. 1A). Control immunoprecipitation with an equal amount of agarose-conju-
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To test whether the interaction of PCNA and HDAC1 is direct, we translated PCNA in vitro and carried out GST pull-down assays with purified GST-HDAC1 fusion proteins (Fig. 2D). These experiments confirmed that PCNA interacts directly with region 1 and region 2 of HDAC1 but does not interact with GST alone or with region 3 (amino acids 332–482), which contains a LXXE-like motif previously shown to interact with Rb (14).

PCNA and HDAC1 Colocalize in A549 Cells in Vivo—To confirm the association of PCNA and HDAC1 in vivo, we carried out colocalization studies in A549 cells (Fig. 3). We stained endogenous PCNA protein with anti-PCNA antibody, followed by a rhodamine-conjugated anti-mouse secondary antibody (red). The same slides were subsequently stained for endogenous HDAC1 protein with anti-HDAC1 antibody, followed by a fluorescein-conjugated anti-rabbit secondary antibody (green). Both proteins showed strong nuclear staining, and the merging of confocal images appeared mainly yellow, indicating that PCNA and HDAC1 colocalize in vivo.

Trichostatin A Arrests A549 Cells at G2–M—Because the histone deacetylase activity associated with PCNA is sensitive to TSA, we used TSA to study the functional consequences of inhibition of this activity for the progression of the cell cycle. We treated A549 cells with 1 μM trichostatin A. We followed the cell cycle profile by FACS analysis of control cells and cells treated with TSA for 6, 12, and 24 h (Fig. 5). The normal cell cycle distribution was altered early, after only 6 h, causing the accumulation of cells in G2–M phase. These results are consistent with the hypothesis that the maturation of chromatin through deacetylation may be involved in the timing of the events that drive the cells from replication to cell division.

DISCUSSION

Efficient assembly of nucleosomes onto newly synthesized DNA is essential for maintaining proper genome function. Chromatin organization is influenced by variations introduced at the nucleosomal level (20). One such variation is the deacetylation of newly assembled nucleosomes, a modification involved in the formation of heterochromatin. The pattern of this modification has been faithfully inherited during cell division. Studies in yeast (21, 22) and mammalian cells (23) show that inhibition of deacetylation leads to severe defects in chromosome stability. However, the mechanism of histone deacetylation after DNA replication remains poorly understood.

Here, we present evidence that PCNA associates with histone deacetylase 1. Because PCNA is a resident of the DNA replication fork and plays important roles in recruiting proteins to the fork, this physical linkage between PCNA and HDAC1 can explain the deacetylation of histones immediately after DNA replication. We first show that HDAC1 and PCNA...
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PCNA Associates with histone deacetylase activity. Histone deacetylase activity was precipitated from HEK293 cells using agarose-conjugated PCNA antibody or control mouse IgG as described under “Materials and Methods.” The deacetylase activity associated with the precipitated complexes was assessed by their ability to remove an acetyl group from an acetylated substrate. Once the substrate becomes deacetylated, it reacts with the developer to produce a fluorophore detected on a fluorometric reader. In the presence of 1 μM TSA, the deacetylase activity is inhibited. The error bars represent S.D.s of triplicate experiments.

**Fig. 4.** PCNA associates with histone deacetylase activity. Histone deacetylase activity was precipitated from HEK293 cells using agarose-conjugated PCNA antibody or control mouse IgG as described under “Materials and Methods.” The deacetylase activity associated with the precipitated complexes was assessed by their ability to remove an acetyl group from an acetylated substrate. Once the substrate becomes deacetylated, it reacts with the developer to produce a fluorophore detected on a fluorometric reader. In the presence of 1 μM TSA, the deacetylase activity is inhibited. The error bars represent S.D.s of triplicate experiments.

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coimmunoprecipitate in two cancer cell lines (A549 and HEK293) as well as in a non-transformed cell line (MRHF) (Fig. 1). This indicates that this interaction is universal and is not confined to the cancer cells that might form aberrant complexes. Second, we utilized GST-HDAC1 fusion proteins expressed and purified from bacteria to pull down PCNA from A549 and HEK cells and show that regions 1 and 2, which span the catalytic domain of the HDAC1, both associate with PCNA (Fig. 2, B and C). These regions have previously been shown to interact with the human Hus1 protein involved in DNA repair (17). It has been proposed that Hus1, Rad1, and Rad9, three checkpoint Rad proteins, form a PCNA-like ring structure around the DNA during repair. Therefore, it is likely that this region of HDAC1 recognizes a ring-like structure around DNA and is thus recruited to sites of DNA replication and repair. Third, region 2 of HDAC1 was shown to bind DNA methyltransferase 1 (15), which in turn was shown to bind PCNA (3), thus recruiting to sites of DNA replication and repair.

**Fig. 5.** Trichostatin A arrests A549 cells at the G2-M. A549 cells were treated with 1 μM TSA for 6, 12, and 24 h. Cell cycle profile of control and treated cells was followed by FACS analysis. The percentage of cells in G2-M phase is shown.

Fifth, we demonstrate that PCNA recruits a functional histone deacetylase activity that is inhibited by TSA (Fig. 4). However, because HDAC1 is a member of a large family of histone deacetylases, we do not exclude the possibility that other members of the family also bind to PCNA and contribute to the deacetylase activity in the replication fork. Sixth, to test whether deacetylase activity was necessary for the progression of the replication fork during DNA synthesis, we treated A549 cells with TSA and followed their cell cycle by FACS analysis (Fig. 5). If histone deacetylase activity or the presence of HDAC1 was essential for DNA replication per se or for the progress of the replication fork, then the replication fork should have stalled during the S phase of the cell cycle or at the G1-S boundary after TSA inhibition of HDAC1. In contrast to these predictions, we observed that the cells started to accumulate at the G2-M phase 6 h after TSA treatment (Fig. 5).

Whereas we cannot formally exclude the possibility that TSA has other effects that might have caused G2-M arrest, our data are consistent with the hypothesis that histone deacetylase activity is essential for the formation of proper chromatin structure after the synthesis of DNA. A recent study has shown that after DNA replication, PCNA remains associated with DNA and serves as an imprinting factor for the chromatin assembly (5). This provides a window of opportunity for PCNA to load HDAC1 and allow it to modulate chromatin, leading to its maturation.

Why do cells arrest at G2-M after TSA treatment? It is possible that the disorganized hyperacetylated chromatin interferes either directly with the exit from G2 or indirectly by failing to signal proper progression through the cell cycle to a putative G2-M checkpoint. The G2-M arrest might be induced by a cellular checkpoint that monitors the state of acetylation of the chromatin and guarantees that only cells bearing proper chromatin divide. Such a mechanism might have evolved to guarantee the integrity of the epigenome. This checkpoint might be triggered by the induction of specific genes that are especially sensitive to hyperacetylation of histones and induce G2-M cell arrest. Additional experiments are required to address these questions and define the mechanisms through which the cell coordinates the inheritance of the histone acetylation pattern with cell cycle checkpoints.

In summary, our data are consistent with a role for PCNA as a coordinator of DNA synthesis, epigenetic inheritance, and cell cycle control. The interaction of PCNA with HDAC1 as well as with DNA methyltransferase 1 and CAF-1 establishes its role in coupling two important processes, DNA replication and epigenetic inheritance.

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