THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 276, No. 41, Issue of October 12, pp. 38307–38319, 2001

Printed in U.S.A.

Regulation of Global Acetylation in Mitosis through Loss of Histone Acetyltransferases and Deacetylases from Chromatin*

Received for publication, January 12, 2001, and in revised form, June 13, 2001
Published, JBC Papers in Press, July 30, 2001, DOI 10.1074/jbc.M100290200

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Histone acetylation, a reversible modification of the core histones, is widely accepted to be involved in remodeling chromatin organization for genetic reprogramming. Histone acetylation is a dynamic process that is regulated by two classes of enzymes, the histone acetyltransferases (HATs) and histone deacetylases (HDACs). Although promoter-specific acetylation and deacetylation has received most of the recent attention, it is superimposed upon a broader acting and dynamic acetylation that profoundly affects many nuclear processes. In this study, we monitored this broader histone acetylation as cells enter and exit mitosis. In contrast to the hypothesis that HATs and HDACs remain bound to mitotic chromosomes to provide an epigenetic imprint for postmitotic reactivation of the genome, we observed that HATs and HDACs are spatially reorganized and displaced from condensing chromosomes as cells progress through mitosis. During mitosis, HATs and HDACs are unable to acetylate or deacetylate chromatin *in situ* despite remaining fully catalytically active when isolated from mitotic cells and assayed *in vitro*. Our results demonstrate that HATs and HDACs do not stably bind to the genome to function as an epigenetic mechanism of selective postmitotic gene activation. Our results, however, do support a role for spatial organization of these enzymes within the cell nucleus and their relationship to euchromatin and heterochromatin postmitotically in the reactivation of the genome.

Chromatin is a dynamic structure that must be reversibly condensed and unfolded, in a regulated manner, to accommodate varying processes such as transcription, replication, and chromosome segregation. Chromatin structure is stabilized by histone-DNA contacts that promote folding of the chromatin fiber into higher order structures (1). The reversible modification of lysines in the amino-terminal tails of core histones by acetylation is widely accepted to be an integral part of gene regulation. Several important transcriptional modulators possess either histone acetyltransferase activity or histone deacetylase activity (2–4). The activities of these two classes of enzymes define a dynamic equilibrium that regulates the transition to highly acetylated/transcriptionally competent chromatin. Gene-specific targeting of these enzymes to upstream activating sequences influences the state of histone acetylation at the local level and is thought to modulate transcriptional activity by reorganizing the local chromatin template (5–11). More recently, genetic studies in yeast demonstrate that at least some HATs[1] and HDACs, characterized primarily as co-activators or co-repressors, act more globally, altering histone acetylation levels over many kilobase pairs of DNA (12, 13). This has resulted in the reemergence of the previously established mode of acetylation action, at the "domain" level rather than at the gene-specific level. The mechanisms leading to selective targeting of this global acetylation are poorly described but may involve aspects of nuclear organization (14).

The most highly acetylated state of the core histones is broadly represented in transcriptionally active or transcriptionally competent euchromatin (15, 16). Hypoacetylated histones, on the other hand, accumulate within transcriptionally silent heterochromatic domains (17, 18). This does not reflect differences in the direct targeting of enzymes, since it is well established that the acetylation state of chromatin turns over continuously in interphase (19–21). The cycles of histone acetylation and deacetylation are not uniform in frequency; for example, they occur slowly in the bulk of chromatin, outside of S-phase, but are rapid in a small proportion of chromatin containing the highest acetylated states of histones. Histone acetylation reduces both intra- and interchromatin fiber associations (22–25) and, therefore, may be part of a process that differentiates euchromatin from heterochromatin domains. Elevated steady-state histone acetylation may define regions of potential gene activity within the genome, whereas dynamic histone acetylation provides a means to regulate higher order chromatin structure and transcriptional competence (12, 16), and may account for a threshold level of histone acetylation being necessary to unfold higher order chromatin structure and facilitate transcriptional elongation (25).

When a cell progresses from interphase to mitosis, dramatic changes in nuclear structure and function occur. For example, transcriptional activity involving all three RNA polymerases is repressed, coincident with the reversible condensation of chromatin (26–31). This may be due to limited access of the tran-

* This project was funded by operating grants from the Canadian Institutes of Health Research (to D. P. B.-J. and X.-J. Y.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Supported by studentships from University Technologies International (University of Calgary), Alberta Heritage Foundation for Medical Research, and the Alberta Cancer Board. Present address: Dept. of Oncology, Cross Cancer Institute, University of Alberta, Edmonton, Alberta T6G 1Z2, Canada.

‖ Supported by the Boehringer Ingelheim Foundation (Germany).

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[1] The abbreviations used are: HAT, histone acetyltransferase; HDAC, histone deacetylase; DAPI, 4′,6-diamidino-2-phenylindole; PBS, phosphate-buffered saline; TSA, trichostatin A.

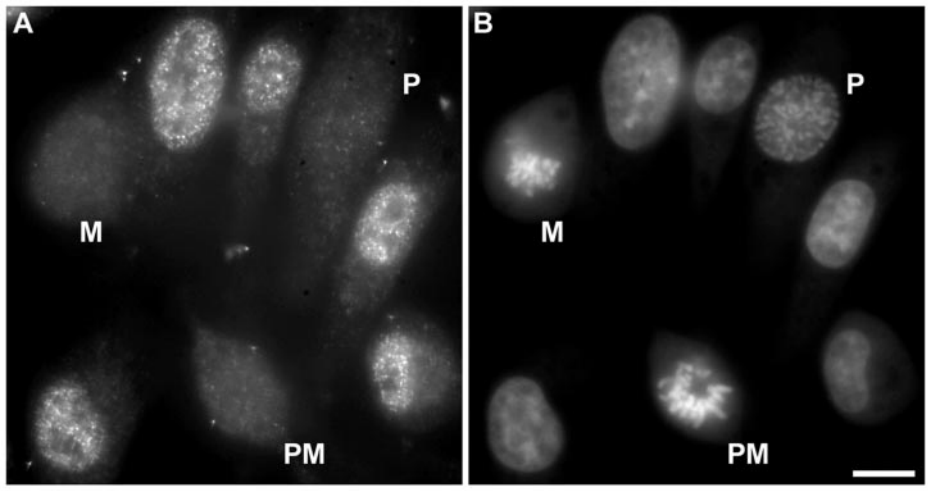

FIG. 1. **Differential levels of highly acetylated chromatin.** A randomly growing population of human fibroblasts (SK-N-SH) were fixed and immunolabeled with an antibody specific to highly acetylated histone H3 (AcH3; *A*) and costained with the DNA-specific dye DAPI (*B*). Three mitotic cells in either prophase (*P*), prometaphase (*PM*), or metaphase (*M*) are distinguishable from the numerous surrounding interphase cells. *Bar*, 10 μm.

scription machinery to the condensed chromosomal DNA (32–34); to inactivation of chromatin-remodeling complexes, such as Swi/Snf (35); or to exclusion of these complexes from mitotic chromatin (36). A number of gene-specific (37–40) and general (27, 41, 42) transcription regulatory factors as well as RNA polymerase II (43) are displaced from mitotic chromatin. How histone acetylation is regulated through mitosis is only poorly understood. Based on the retention of some acetyl-histone epitopes through mitosis and indirect evidence for ongoing acetylation in mitotic cells (44), Jeppesen (45) and Turner (46) have proposed that HATs remain stably bound to chromosomes through mitosis as an epigenetic mechanism for maintaining a lineage-specific program of gene regulation (45, 46).

In this study, we set out to test the hypothesis that chromatin is epigenetically marking chromatin for postmitotic reactivation by examining the mitotic distribution and activity of HATs and HDACs. With quantitative *in situ* imaging and both *in vitro* and *in situ* biochemical assays for HAT and HDAC activity, we examined the histone acetylation state as cells entered, progressed through, and exited mitosis. Biochemical assays for detecting *in situ* HAT and HDAC activities enabled us to establish the temporal sequence of changes in dynamic and steady-state acetylation through mitosis, with high spatial and temporal resolution through the stages of mitosis. The power of these techniques, which combine biochemical and morphological criteria, far exceeds what is possible through conventional biochemical analysis due to the relatively low temporal and stage-specific resolution of the synchronization procedures required for conventional biochemical analysis. Our results demonstrate that *in situ* inactivation of HATs and HDACs occurs although these enzymes possess full catalytic activity when assayed *in vitro*. Instead, the loss of activity in mitosis results from the translocation of the enzymes from a chromatin-associated to a nonchromatin-based compartment. Any epigenetic marker that histone acetylation provides does not require a stable association of HATs and HDACs with the genome.

EXPERIMENTAL PROCEDURES

Immunofluorescence Microscopy—Cells were cultured directly on glass coverslips under conditions recommended by ATCC. Cells were seeded onto coverslips, cultured overnight, and then fixed with 1.0% paraformaldehyde in PBS (pH 7.5) at room temperature for 5 min. Subsequently, cells were washed three times in PBS and then permeabilized in PBS containing 0.1% Triton X-100 for 5 min. Coverslips were inverted onto parafilm containing 25Fl drops of primary antibodies diluted in PBS for 60 min at 22 °C, washed three times in PBS, and then incubated for a further 60 min in the presence of Cy3-anti-rabbit immunoglobin G (IgG) and either fluorescein isothiocyanate-anti-mouse IgG, IgA, and IgM or Alexa 488-anti-mouse IgG (Molecular Probes, Inc., Eugene, OR). Coverslips were washed again in PBS and then mounted on slides with a 90% glycerol-PBS (pH 7.5)-based medium containing 1 mg/ml paraphenylenediamine and 1 fg/ml DAPI.

The antibody specific to histone H3, when both lysines 9 and 14 are acetylated (Upstate Biotechnology, Inc., Lake Placid, NY), has previously been characterized by Boggs *et al.* (47) and shows a strong preference for the most highly acetylated species of histone H3 in mammalian tissue culture cell lines. The antibodies that recognize either acetyl-lysine 9 or acetyl-lysine 14 on H3 or that recognize either acetyl-lysine 5, 12, or 8 on histone H4 were obtained from Upstate Biotechnology. The anti-phosS10AcK14H3 and anti-phosS10AcK9H3 antibodies were obtained from Upstate Biotechnology and Cell Signaling Technology (Beverly, MA), respectively. The $TAF_{II}250$ antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) recognized a single band of ~250 kDa in Western blots, as expected, and has been characterized previously (48). The p300 antibody, obtained from Upstate Biotechnology, recognizes the C-terminal portion of p300, as shown previously (49). The histone deacetylase 1 antibody was obtained from Upstate Biotechnology. Results were verified using the HDAC1 antibody characterized previously (50). The antibodies that recognize either CBP, ACTR, MYST, P/CAF, or HDAC4 were obtained from Upstate Biotechnology. The HDAC2, HDAC3, and HDAC7 antibodies were characterized by Wolfgang Fischle,[2] and the HDAC5 antibody was obtained from Cell Signaling Technology.

Acid Extraction and Immunoblotting of Histones—Mitotic cells were mechanically isolated from randomly growing cell cultures. The mitotic and mitotic-depleted interphase cell populations were concentrated by centrifugation (800 × *g*) and washed twice with 1x PBS. The washed and concentrated cells were resuspended in 5 volumes of ice-cold buffer (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, 1.5 mM phenylmethylsulfonyl fluoride). Both dithiothreitol and phenylmethylsulfonyl fluoride were added just prior to the use of buffer. Concentrated sulfuric acid was added to the suspension to give a concentration of 0.4 N. After incubation on ice for 30 min, the suspension was centrifuged for 15 min at 11,000 × *g* at 4 °C. The resultant supernatant was decanted into another tube and mixed with 1 ml of acetone. After overnight incubation at −20 °C, the precipitated proteins were collected by centrifugation 15 min at 11,000 × *g* at 4 °C. The pellet was washed twice with 1 ml of acetone and air-dried. The pellet was then mixed with 2× SDS-polyacrylamide gel electrophoresis loading buffer.

For Western blot analysis, the sample was boiled for 5 min and loaded onto a 12% SDS-polyacrylamide gel. The electrophoretically separated proteins were either stained with Coomassie Brilliant Blue or transferred to Hybond-C paper (Amersham Pharmacia Biotech). The membrane filters were blocked for 1 h at room temperature with PBS-T (1× PBS, 0.05% Tween 20) containing 5% nonfat milk powder and then washed three times for 15 min each with PBS-T. The primary antibody (anti-H3K9 at 1:500 or anti-H4K12 at 1:750) in 1% bovine serum albumin/TBS-T was applied for 2 h. After three washes with TBS-T, the secondary antibody (goat anti-rabbit horseradish peroxidase conjugate (Sigma) at 1:30,000) in 1% bovine serum albumin/TBS-T was applied

[2] W. Fischle, unpublished observations.

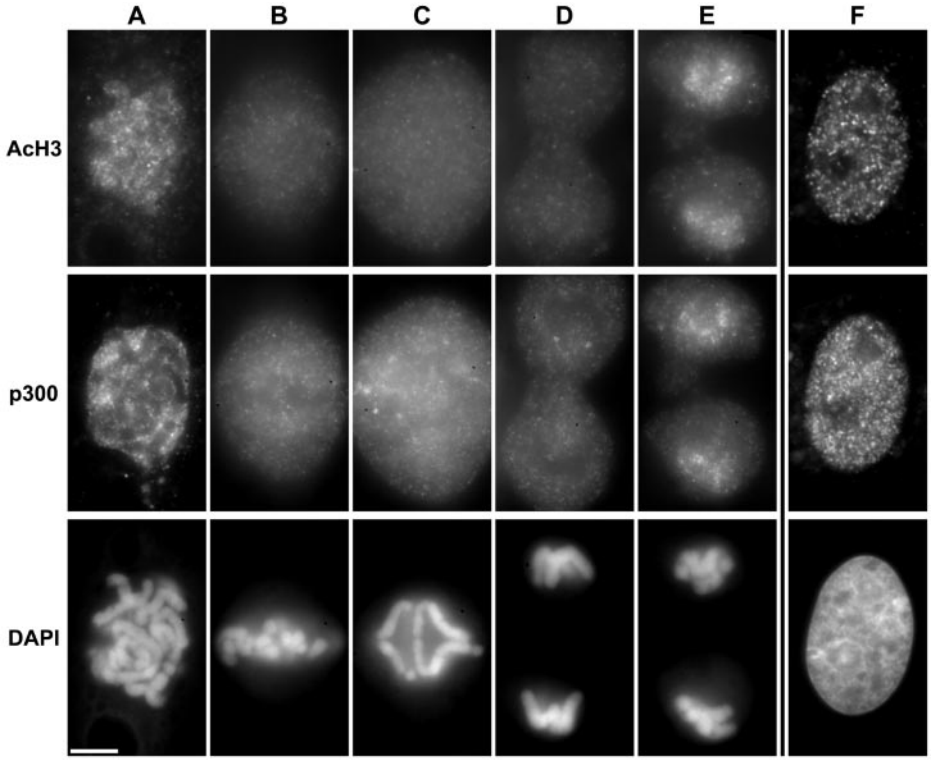

FIG. 2. **Temporal relationship between levels of highly acetylated chromatin, chromatin condensation, and histone acetyltransferase localization during mitosis.** Indirect immunofluorescent images of Indian muntjac fibroblasts at various phases of mitosis: prophase (*column A*), metaphase (*column B*), anaphase (*column C*), early telophase (*column D*), late telophase (*column E*), and interphase (*column F*). Cells were stained with antibodies specific for either highly acetylated histone H3 (*AcH3 row*) or (*p300 row*) and co-stained with DAPI (*DAPI row*). DAPI staining indicates cell cycle stage, anti-AcH3 labeling indicates presence of highly acetylated chromatin, and anti-p300 indicates spatial organization or localization of the HAT-p300. The low amounts of highly acetylated histones in mitosis compared with interphase required a different camera exposure and display look-up table from that used for interphase cells. For this reason, the amount of highly acetylated histone signal in the interphase nucleus (*column F*) is not directly comparable with the remaining cells in the different stages of mitosis (*columns A–E*). Note that the difference in AcH3 signal is directly comparable between the mitotic cells. *Bar*, 10 μm.

for 1 h. The filters were washed again and then developed using the ECL system (Amersham Pharmacia Biotech), as recommended by the manufacturer.

HAT and HDAC Activity Assays—For the *in situ* HAT activity assay, randomly growing cell cultures were incubated with nocodazole (40 ng/ml final concentration) for 3 h before mechanical shake-off. Cells were isolated, concentrated by centrifugation, and plated on coverslips coated with poly-L-lysine. Coverslips were incubated with 1.0 mg/ml poly-L-lysine for 15 min and rinsed briefly, and mitotic cells were applied. Cells were allowed to adhere for 10 min before adding medium containing nocodazole (40 ng/ml final concentration) and the deacetylase inhibitor trichostatin A (TSA) (Wako Pure Chemical Industries, Japan) at a final concentration of 100 ng/ml. Cells were incubated at 37 °C in this media mixture for 15, 30, 60, or 120 min before fixation with 1.0% paraformaldehyde in PBS. For control studies, identically plated cells were incubated with medium containing either TSA only, nocodazole only, or neither TSA nor nocodazole.

For the *in vivo* HDAC activity assay, randomly growing cell cultures were incubated with both nocodazole (40 ng/ml) and TSA (100 ng/ml) for 3 h before mechanical shake-off. Cells were isolated, concentrated, plated on poly-L-lysine coverslips, and allowed to adhere as above. Adherent cells were washed twice with TSA-free medium and then incubated with nocodazole-containing medium for 15, 30, 60, or 120 min and fixed with 1.0% paraformaldehyde in PBS. Control involved incubating plated cells with medium containing neither TSA nor nocodazole.

In the *in situ* HAT and HDAC activity assays, fixed cells were immunolabeled, following the above procedure, with anti-AcH3 (K9 and K14), anti-H4K5, or anti-H4K8.

For *in vitro* HAT and HDAC activity assays, mitotic cells were isolated from randomly growing cell cultures by mechanical shake-off, and the remaining adherent, interphase cells were trypsinized for 5 min and isolated. HAT assays were performed in modification of previously described methods (51, 52). Cells were lysed in buffer B500 (20 mM Tris-HCl, pH 8.0, 10% glycerol, 5 mM $MgCl_2$, 0.1% Nonidet P-40, protease inhibitors, 500 mM KCl). Protein concentrations were normalized using Bradford assays (Bio-Rad). HAT activity was measured by a filter assay using a master mix containing 5× buffer A (50 mM Tris-HCl, pH 8.0, 10% glycerol, 1.0 mM dithiothreitol, 1.0 phenylmethylsulfonyl fluoride, 0.1 mM EDTA, 10 mM sodium butyrate), calf thymus histones type IIa (2 μg/μl) (Sigma), [^{3}H]acetyl-CoA (75 nCi) (4.7 Ci/mmol; Amersham Pharmacia Biotech), and H_2O to a 10-μl final volume. HDAC assays were performed in modification of previously described methods (50). After washing, cells were lysed in the presence of a protease inhibitor mixture (Roche Molecular Biochemicals) either in low stringency lysis buffer (50 mM Tris-HCl, pH 7.5, 120 mM NaCl, 0.5 mM EDTA, 0.5% Nonidet P-40) or in the same buffer containing 50 mM NaF, 1 mM Na_3VO_4, and 1 μM okadaic acid in order to inhibit kinases and phosphatases. Protein concentrations of extracts were normalized using a modified Lowry assay (Bio-Rad). HDAC activity, using different protein concentrations, was measured in a buffer containing 10 mM Tris-HCl, pH 8.0, 10 mM NaCl, and 10% glycerol as end point analysis (2-h reaction time, 200-μl reaction volume). For phosphorylation/dephosphorylation-controlled reactions 50 mM NaF, 1 mM Na_3VO_4, and 1 μM okadaic were included.

TABLE I
Quantitation of DNA and highly acetylated chromatin signal ratios

Cell cycle phase image	Number of areas/image measured (n)	DNA mean signal density $\times 10^6$	AcH3 mean signal density $\times 10^6$	DNA/AcH3 signal ratio
		counts/pixel		
Prophase	3	3.55 ± 0.58	2.69 ± 0.62	1.32
Metaphase	3	2.44 ± 0.16	0.08 ± 0.06	31.32
Anaphase	3	3.36 ± 0.30	0.20 ± 0.02	16.61
Early telophase	2	6.43 ± 0.52	0.27 ± 0.10	23.92
Late telophase	2	6.25 ± 0.48	1.95 ± 0.43	3.20

Digital Image Collection and Quantitation—Cells were examined with a Leica DMRE or Zeiss Universal epifluorescence light microscope. Digital images were collected with a 14-bit CCD camera (Princeton Instruments). Microscope settings and camera detector exposure times were kept constant for each respective channel (Cy3, fluorescein isothiocyanate, or DAPI) but were optimized for individual experiments. Signal intensity from images collected in the Cy3 and fluorescein isothiocyanate channels were directly comparable, when necessary, by *not* subjecting the images to postcapture thresholding.

Raw 16-bit digital image data were used to quantitate the ratio between DNA and highly acetylated chromatin integrated signal densities. Constant digital camera and microscope settings were maintained for the series of images. The signal density was quantified with ErgoVista version 4 (Atlantis). Quantitation was adapted from previously described procedure (53). Line scan profiles drawn across an entire nucleus were generated using Image Tool version 1.28 (University of Texas Health Science Center at San Antonio).

RESULTS

Histone Acetylation States of Mitotic Chromatin—The development of specific acetylated histone antibodies provides the

TABLE II
Characterization of antibodies recognizing different acetylated states of H3 and H4

Antibody	Antibody recognizes acetyl-lysines	Source or reference	Interphase[a]	Mitosis[a]
Anti-AcH3	9 and 14 of H3	Ref. 47	+	−
Anti-H3K14	14 of H3	Upstate Biotechnology	+	+
Anti-H3K9	9 of H3	Upstate Biotechnology	+	−
Anti-H4K5	5 of H4	Upstate Biotechnology; Ref. 60	+	−
Anti-H4K8	8 of H4	Upstate Biotechnology; Ref. 60	+	+/−
Anti-H4K12	12 of H4	Upstate Biotechnology; Ref. 60	+	+
Anti-H4K16	16 of H4	Upstate Biotechnology; Ref. 60	+	+
Anti-tetra-H4	One or a combination of 16, 12, 8, and 5 of H4	Ref. 59	+	+
Anti-penta-H4	One or a combination of 16, 12, 8, 5, and 20 of H4	Ref. 59	+	+
Anti-H2B	5 of H2B	Ref. 80	+	−

[a] Sufficient labeling is denoted with + and a reduction in signal intensity is denoted with −.

means to follow changes in histone acetylation as gene expression is silenced in mitosis. These antibodies include anti-AcH3, which has a strong preference for the most highly acetylated isoform of H3; the lysine-specific acetyl-histone H4 antibodies K16, K12, K8, and K5; and the lysine-specific acetyl-histone H3 antibodies anti-H3K14 and anti-H3K9. Anti-AcH3 recognizes the amino-terminal tail of H3 when lysines 9 and 14 have both been acetylated (47). Lysine 9 is preferentially acetylated in the most highly acetylated isoform of H3 (54). In parallel, lysine 5 of histone H4 is preferentially acetylated in the most highly acetylated isoform of this histone (54, 55). The use of anti-AcH3 and anti-H4K5 allowed us to probe for the most highly acetylated histone species found under physiological conditions. These epitopes are associated with transcriptionally competent and/or active chromatin (15). Indirect immunofluorescence microscopy of SK-N-SH (human neuroblastoma) cells revealed an obvious loss of anti-AcH3 (Fig. 1) and anti-H4K5 (not shown) signal in mitotic cells compared with interphase cells. Similar results were obtained with other mammalian cell lines, including those listed in Table III (bottom). The loss of anti-AcH3 and the anti-H4K5 signal in mitotic cells indicates that mitotic cells are hypoacetylated (do not contain significant amounts of highly acetylated chromatin) compared with interphase cells.

When we examined histone acetylation states through mitosis, a concomitant decrease in the amounts of highly acetylated chromatin was observed as chromatin condensed into metaphase chromosomes, and an increase in amounts of highly acetylated chromatin was observed as mitotic chromatin began to decondense in late mitosis (Fig. 2; Table I). Specifically, prophase cells exhibited lower levels of highly acetylated chromatin than interphase cells (for comparison, see Fig. 1), although with noticeably higher levels than those of metaphase cells (Fig. 2, *columns A* and *B*). As the cells progressed into metaphase and through to telophase, we observed a barely detectable and diffuse signal, indicating that the amounts of highly acetylated histones within mitotic chromosomes are decreased dramatically (Fig. 2, *columns B–D*). We observed a reestablishment of a highly acetylated chromatin state at the late telophase/early interphase boundary when the chromatin is still highly condensed but decondensation has begun (Fig. 2, *column E*). The acetylation continues to increase in a manner that is temporally related to the timing of chromosome decondensation. Significant amounts of acetylation accumulate in the genome prior to the reinitiation of transcription (monitored by probing for fluorouridine incorporation) or the reincorporation of RNA polymerase II into the newly forming nuclei (data not shown). We conclude that amounts of highly acetylated chromatin are substantially reduced during the majority of the mitosis.

TABLE III
Mitotic redistribution of specific HATs and HDACs

Mitotic redistribution[a]	HAT or HDAC
+	p300
+	CBP
+	ACTR
+	MYST
+	TAFII250
+	P/CAF
+	HDAC1
+	HDAC2
+	HDAC3
+	HDAC4
+	HDAC5
+	HDAC7

Mitotic Redistribution of HATs and HDACs[a]	Cell line	Species
+	SK-N-SH	Human
+	293	Human
+	HeLa	Human
+	MRC-5	Human
+	10t1/2	Mouse
+	NIH 3T3	Mouse
+	NRK	Rat
+	CHO	Hamster
+	COS-1	Monkey
+	IM	Indian muntjac

[a] + denotes whether a specific HAT or HDAC undergoes a change in distribution in mitosis (top) and whether this change is observed in a particular cell line (bottom).

The above results support the temporal correlation between levels of histone acetylation and chromatin compaction. Histone acetylation, however, is not completely eliminated, since several antibodies recognizing lower acetylated states of H4 label mitotic chromosomes (Table II) (56–58). Only the most dynamically acetylated isoforms of histones H3 and H4, which correlate with transcriptional activity, are dramatically lost in mitosis. This observed net histone deacetylation of the acetyl-lysine epitopes represented in highly acetylated histones and transcriptionally competent chromatin was confirmed by Western blot analysis. Mechanically isolated SK-N-SH mitotic cells and the remaining mitotic-depleted interphase cells were used to prepare histone-enriched extracts by acid extraction without the use of histone deacetylase inhibitors (see "Experimental Procedures"). Histones from both the mitotic and interphase cell extracts were electrophoretically separated (Fig. 3*A*) and probed for using either anti-H3K9 or anti-H4K12 antibodies using the Western technique. The anti-H3K9 antibody was

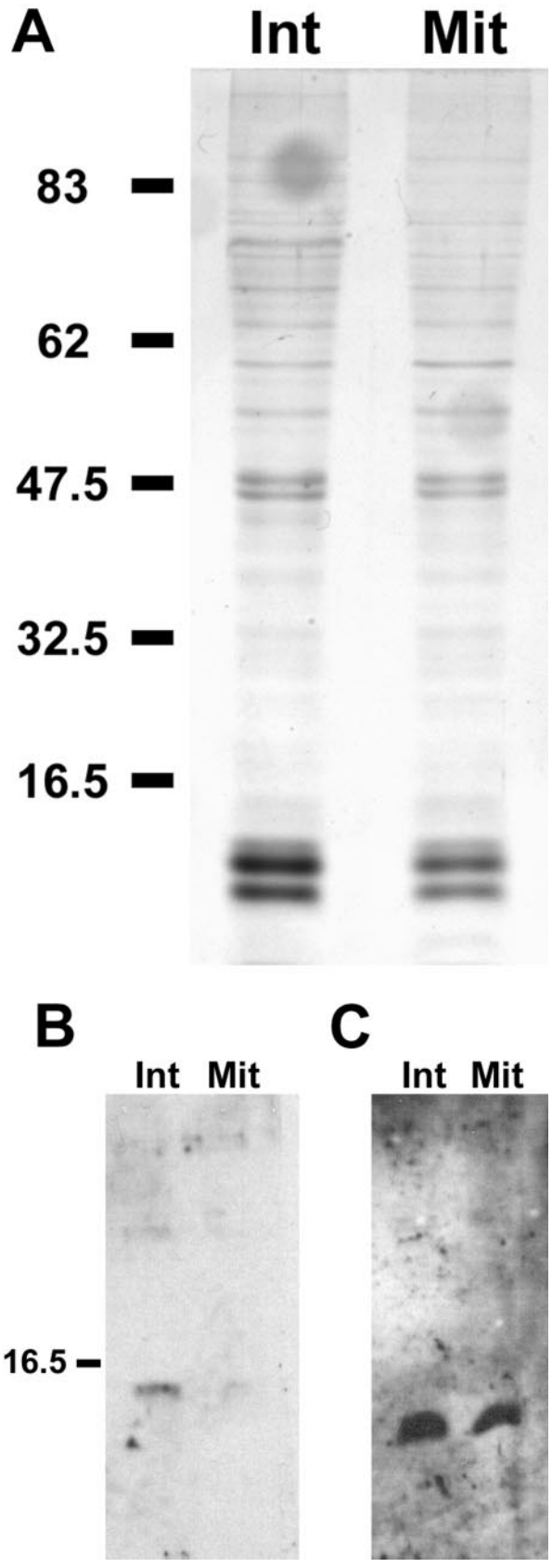

FIG. 3. **Western blot analysis of acetylated histone isoforms.** Histones prepared from SK-N-SH mitotic and interphase cell extracts were electrophoretically separated by SDS-polyacrylamide gel electrophoresis and either stained with Coomassie Blue (*A*), probed for highly acetylated histone H3 using anti-H3K9 (*B*), or probed for low to moderately acetylated histone H4 using anti-H4K12 (*C*). *Int* and *Mit* in *A–C* represent lanes where interphase or mitotic protein extracts, respectively, were electrophoretically separated.

used to probe for the highly acetylated isoform of H3, whereas the anti-H4K12 was used to probe for the low to moderately acetylated isoforms of H4 (54). We observe highly acetylated H3 (Fig. 3*B*) and H4 (not shown) to be dramatically reduced in mitotic cells compared with interphase cells, whereas the low to moderately acetylated histones are retained in both mitotic and interphase cells (Fig. 3*C*). After correcting for differences in gel loading by Coomassie staining and normalizing for background, we measured an ~8-fold decrease in the amount of highly acetylated H3 in mitotic compared with interphase cells. The low to moderately acetylated H4, however, remained approximately equivalent in both mitotic and interphase cells. These results support our observations made by immunofluorescence microscopy (see Figs. 1 and 2 and Table II). Detection of other histone N-terminal tail modifications, such as phosphorylation of H3 (not shown; see Ref. 59), argues against epitope masking of acetyl-lysines in mitotic chromatin. In the remainder of this work, when we refer to a loss in histone acetylation, we are referring to the loss of the highly acetylated H3 and H4, not the lower acetylated (*i.e.* mono- and diacetylated) isoforms.

Phosphoacetylation of Histone H3—Recently, doubly modified phosphoacetylated histone H3 has been found associated with gene activation (60–64). One study raised concern regarding phosphorylation of H3 interfering with antibody recognition of acetyl-lysine-specific epitopes (63). Global phosphorylation of histone H3 occurs at the onset of chromatin condensation in late G_2/early mitosis (59). To determine whether the observed loss of highly acetylated H3 epitopes is due to phosphorylation of H3, we examined the interphase and mitotic labeling patterns of two antibodies that specifically recognize phosphoacetylated isoforms of histone H3. The anti-phosS10AcK9H3 antibody recognizes H3 when both serine 10 is phosphorylated and lysine 9 is acetylated, whereas the anti-phosS10AcK14H3 antibody recognizes the phosphoserine 10 and acetyl-lysine 14 isoform of H3. We found that the labeling patterns from both antibodies were in agreement with our observations using acetyl-lysine-specific antibodies (anti-AcH3K9 and anti-AcH3K14; see Table II) and those published for phosphoserine 10 of H3 (59). Mainly, the anti-phosS10AcK9H3 signal was present in a dispersed punctate pattern in interphase cells (Fig. 4*A*) but was absent in mitotic cells (Fig. 4*C*). The anti-phosS10AcK14H3 signal, on the other hand, was present in both interphase and mitotic cells (Fig. 4, *E* and *G*). Acetyl-lysine 9 on H3 is represented in the highly acetylated state of H3 (54) and is lost during mitosis (Table III), whereas acetyl-lysine 14 on H3 appears first in the lower acetylated isoforms of H3 (54) and is present in mitotic chromatin (Table II). With the dramatic increase in H3 phosphorylation at the onset of mitosis, we would expect the doubly modified phosS10AcK14 epitope on H3 to remain through mitosis, which it does, and a loss of the doubly modified phosS10AcK9 epitope on H3, which also occurs. We interpret the loss of the phosS10AcK9 H3 epitope as an indication that the phosphorylation of H3 does not significantly occlude antibody recognition of acetyl-lysine epitopes on H3. The phosS10AcK9 H3 epitope has been shown to be present in transcriptionally active chromatin (63), and the absence of this epitope in mitosis supports our observations that highly acetylated histones represented in transcriptionally chromatin are lost during mitosis.

Histone-modifying Enzymes (HATs/HDACs), Highly Acetylated Chromatin, and Chromatin Condensation in Mitosis—After establishing that the most highly acetylated histone species were lost during mitosis, we wished to determine the fate of HATs and HDACs during this stage of the cell cycle. It has been proposed that HATs may remain associated with acetylated R bands during mitosis to provide a mechanism of trans-

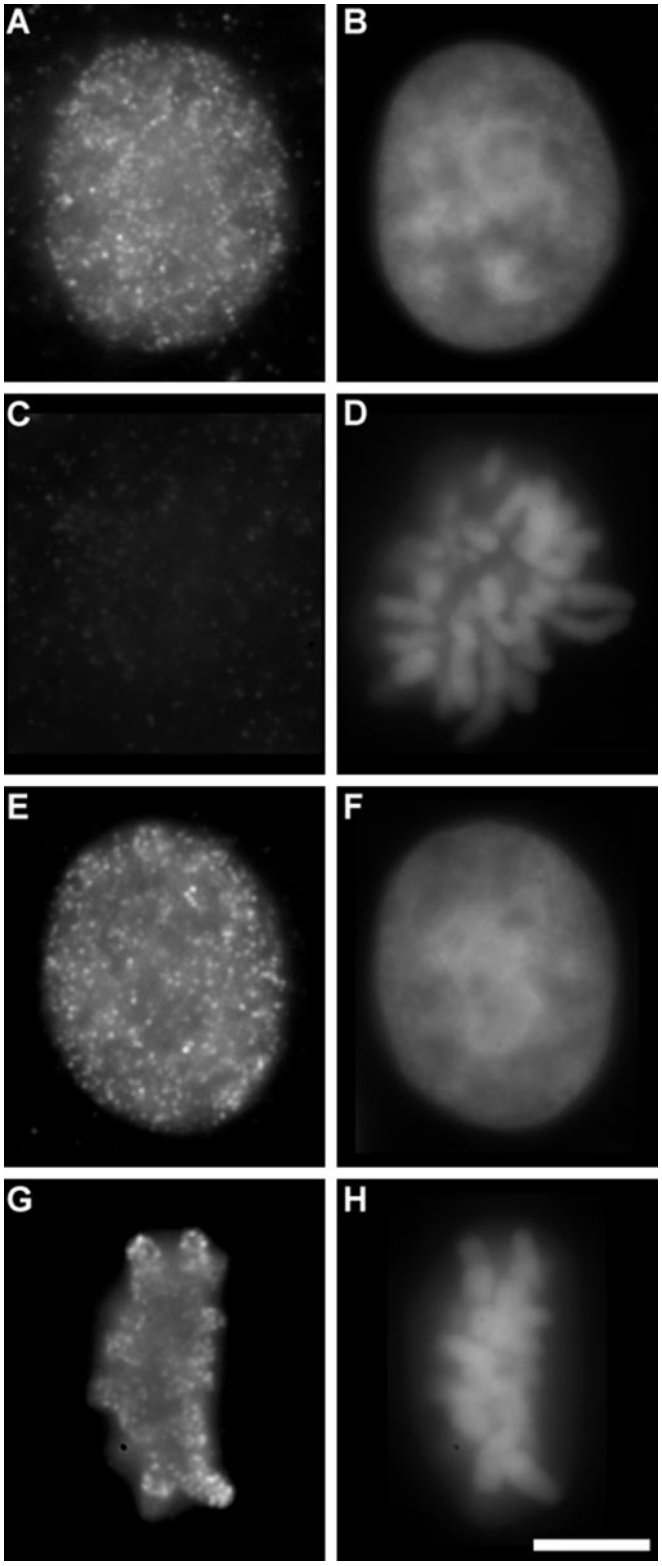

FIG. 4. **Phosphoacetylation of histone H3.** SK-N-SH cells were immunolabeled with antibodies recognizing two isoforms of phosphoacetylated histone H3. Interphase (*A*) and mitotic (*C*) cells immunolabeled with anti-PhosS10AcK9H3, an antibody that recognizes histone H3 when serine 10 is phosphorylated and lysine 9 is acetylated in the N terminus. Interphase (*E*) and mitotic (*G*) cells were immunolabeled with the antibody anti-PhosS10AcK14H3 that recognizes histone H3 when serine 10 is phosphorylated and lysine 14 is acetylated in the N terminus. Corresponding DAPI images are shown (*B*, *D*, *F*, and *H*). *Bar*, 5 μm.

mitting an epigenetic marker for chromatin activation through mitosis (44). Moreover, it has been proposed that bromodomain-containing HATs, which include CBP, $TAF_{II}250$, P300, and P/CAF, may remain associated with specific regions of mitotic chromosomes as a consequence of the demonstrated specific binding of bromodomains to acetyl-lysine (46).

We examined the cellular distribution of a large number of HATs and HDACs using both antibodies and green fluorescent protein-HAT or -HDAC expression constructs (Table III, top, data not shown). Identical results were obtained for all evaluated proteins in several different mammalian cell lines (Table III, bottom). The cellular distribution of the transcriptional adaptor, p300, possesses intrinsic histone acetyltransferase activity (65, 66) and is shown in Fig. 2. In prophase, p300 localizes primarily within foci in the interchromosomal space rather than coincident with the chromosomes (Fig. 2, *column A*). This is in contrast to the punctate focal distribution observed for p300 (Fig. 2, *column F*) and other transcription regulatory proteins observed in interphase nuclei (67). As cells progress to metaphase, p300 is dispersed throughout the cytosol and not associated with the chromosomes. This distribution remains until late telophase/early interphase, when p300 becomes both interchromosomal and accumulates in discrete foci but also begins to associate with the chromosomes. The focal pattern observed at prophase is reestablished at the late telophase/early interphase boundary (Fig. 2, *p300 row*, compare *columns A* and *E*). As a control, cells were labeled with secondary antibodies alone, which produced a negligible signal (data not shown). As might be expected, the timing of p300 redistribution coincides with the timing of chromatin reacetylation, indicating that these two events are linked. In summary, reduced chromatin acetylation correlates with changes in HAT localization, and reacetylation results from the reassociation of HATs with the chromatin.

A similar redistribution of histone deacetylases HDAC1 (Fig. 5) and HDAC3, -4, -5, and -7 (Table III) was observed. In prophase (Fig. 5, *column B*), HDAC1 translocates to the chromatin-depleted interchromosomal domains. The signal becomes diffuse between metaphase and early telophase (Fig. 5, *D–F*). At late telophase/early interphase, HDAC1 accumulates at the interchromosomal domains (Fig. 5*G*), with an increased association with late mitotic chromatin. HDAC1-GFP and HDAC4-GFP are also excluded from the condensed chromatin during mitosis (data not shown). These results were also confirmed by confocal and deconvolution microscopy (data not shown). From combinatorial co-labeling of different HATs and HDACs, we found it difficult to determine the proportion of HATs to HDACs that had translocated away from chromatin in prophase and reassociated with chromatin in late telophase/early interphase (data not shown). This ultrafine temporal ordering is difficult to visualize, but presumably a subset of HDACs are the last enzymes to move off the chromatin in prophase, and the HATs reassociate slightly earlier in late telophase/early interphase. It is clear, however, that the bulk of the enzymes, both HATs and HDACs, follow similar redistribution patterns at similar stages in mitosis.

HAT Activity in Mitosis—The above observations of HAT and HDAC displacement from mitotic chromatin clearly rule out the possibility that HATs and HDACs are stably associated with chromatin during mitosis. It remains to be determined, however, whether their steady-state displacement from mitotic chromosomes results in or reflects a catalytic inactivation of the acetylation/deacetylation process during this part of the cell cycle. Previous indirect observations have indicated that reversible acetylation may be ongoing through mitosis (44). In order to determine directly the fate of HAT and HDAC activi-

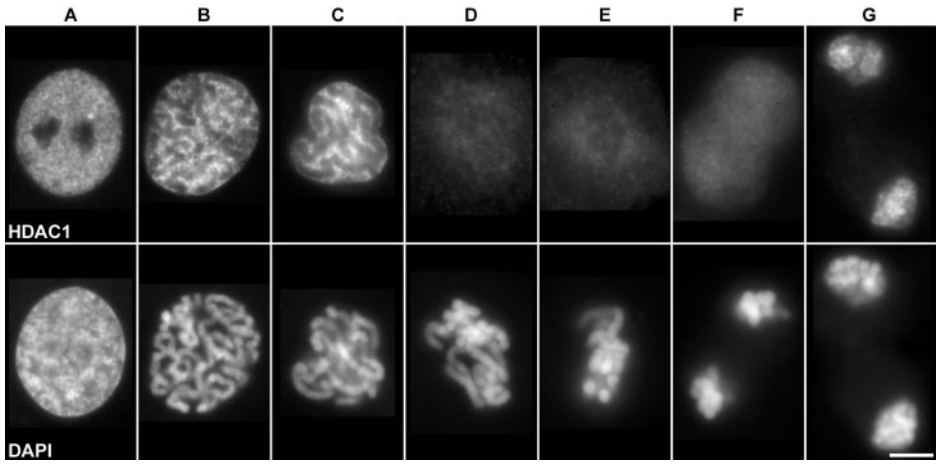

FIG. 5. **Temporal organization of histone deacetylases during mitosis.** Indian muntjac fibroblasts were immunolabeled with an antibody that recognizes the specific histone deacetylase HDAC1 (*HDAC1 row* in *columns A–G*) and co-stained with DAPI. Cells at distinct stages of mitosis were digitally imaged: interphase (*column A*), prophase (*column B*), prometaphase (*column C*), metaphase (*column D*), anaphase (*column E*), early telophase (*column F*), and late telophase (*column G*). *Bar*, 6 μm.

ties during mitosis, it was necessary to develop *in situ* assays for their respective activities. Previously, we have used brief incubations with histone deacetylase inhibitors followed by an analysis of changes in acetylated histone antibody binding to define the locations of dynamic histone acetylation *in situ* (14). This procedure was modified and found to be suitable for the analysis of both HAT and HDAC activity in mitotic cells *in situ* and was used to determine the fate of these activities through mitosis.

Overall nuclear HAT activity can be examined *in situ* through the use of specific biochemical drugs. Deacetylase inhibitors such as TSA (68) are generally used to inhibit HDACs and, therefore, induce increased amounts of highly acetylated histones. Because these levels are experimentally induced, they are usually greater than what is physiologically present and have been termed "hyperacetylated," rather than "highly acetylated." Hyperacetylated histones do contain the physiological epitopes for highly acetylated histones, which allowed us to use the same immunoprobes (anti-AcH3 and anti-H4K5) used to detect transcriptionally competent and highly acetylated chromatin. If a simple shift in the equilibrium between HAT and HDAC activities occurs in mitosis, with HDAC activity presumably maintaining the net deacetylated state, then inhibiting histone deacetylases with TSA will result in increased levels of histone acetylation due to the unrestricted resident HAT activity. We blocked SK-N-SH cells in mitosis with the microtubule-destabilizing drug nocodazole and then treated the cells with TSA over defined time periods in order to determine whether HAT activity persists in mitosis. We did not detect hyperacetylated mitotic chromosomes over the entire time course (Fig. 6, *row A*). Inevitably, a small percentage of interphase cells were co-isolated along with mitotic cells during the mechanical shake-off procedure. These interphase cells conveniently served as a positive control. The TSA treatment blocked HDAC activity, as shown by the net increase in amounts of hyperacetylated chromatin present in these interphase cells over time (Fig. 6, *row C*). We measured a steady increase in highly acetylated histone signal over time in the interphase cells but not in mitotic cells (Fig. 6, *E–H*). This result indicates that HAT activity on the chromatin substrate is significantly decreased during mitosis. Similar results were obtained when we probed for *in situ* HAT activity using the anti-AcH4K8 antibody. This antibody recognizes acetyl-lysine 8 in the N-terminal tail of histone H4. Lysine 8 is preferentially acetylated in the moderately acetylated state of H4 (54). Significant steady-state amounts of this epitope are normally observed in metaphase chromosomes (Table II). We found that the amounts of AcH4K8 increased significantly in interphase cells but did not increase above normal amounts in mitotic cells (data not shown). This indicates that HAT activity in general is decreased in mitosis.

Hyperacetylated Chromatin Does Not Prevent the Mitotic Chromosomal Conformation—Histone acetylation may have multiple roles, including the relaxation of histone-DNA contacts in nucleosomes (69), inhibition of polynucleosome folding (33), and inhibition of fiber-fiber interactions (70). Thus, deacetylation in early mitosis may be a requirement for chromatin condensation into the metaphase chromosomal conformation. We examined whether preventing net histone deacetylation, and thereby maintaining hyperacetylated histones, would inhibit chromosome condensation or progression through mitosis. Chromatin that is still occupied by functional HATs in interphase will become hyperacetylated in the presence of the histone deacetylase drug, and these elevated levels of acetylation should persist if the cells are able to progress from late G_2 or early prophase through to later stages of mitosis. Surprisingly, after this treatment, chromosomes in prophase, metaphase, and anaphase labeled intensely with the AcH3 antibody (Fig. 7) or AcH4K5 (data not shown). Because hyperacetylated chromosomes from prophase, metaphase, and anaphase were observed, any alteration of the chromatin structure by hyperacetylation did not have a significant effect on chromosome condensation or progression through mitosis. This result also indicates that the epitope, if it were present, would be accessible in normally hypoacetylated mitotic chromatin. The effect of high levels of acetylation on chromosome structure and fiber-fiber contacts (25) is being addressed in a comprehensive manner in a separate study.

HDAC Activity in Mitosis—Next, we wished to determine whether HDAC activity is maintained during mitosis. The ability of hyperacetylated chromatin to form mitotic chromosomes (Fig. 8) allowed us to adopt our *in situ* assay to analyze histone deacetylase activity in mitosis. The incubation of randomly growing SK-N-SH cells with both nocodazole and TSA led to the accumulation of metaphase cells with hyperacetylated mitotic chromosomes. While maintaining the metaphase block, we isolated mitotic cells. TSA was then removed, and the cells were fixed *in situ* after 15, 30, 60, or 120 min. We have already shown that HAT activity is decreased during mitosis (see Fig. 6). Therefore, if HDACs are present and functional on these hyperacetylated mitotic chromosomes, then release from the inhibiting drug should cause a net deacetylation to levels normally found in mitotic cells. We observed no measurable (data not shown) decrease in the amount of hyperacetylation when the drug was removed (Fig. 8, *row A* and *panels E–H*). Hyperacetylated chromatin persisted in mitotic chromosomes over the entire time course, whereas the amounts of hyperacetylated chromatin decreased with time in the co-isolated inter-

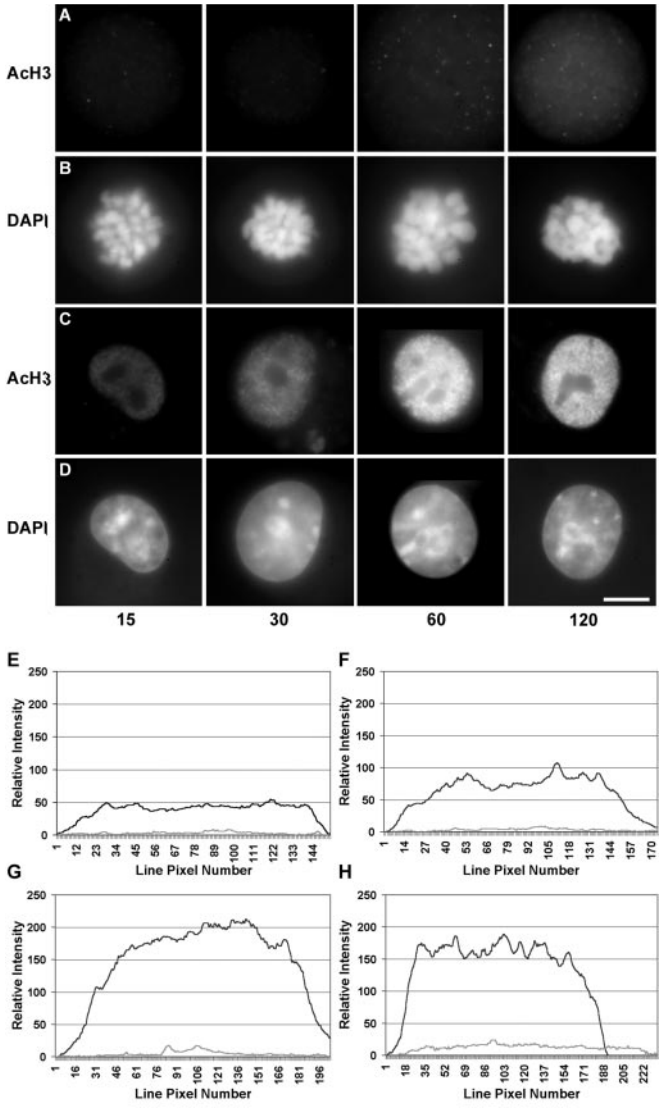

FIG. 6. ***In situ* mitotic histone acetyltransferase activity.** Human fibroblasts were incubated with nocodazole to accumulate mitotic cells. The cells were isolated, the mitotic block was maintained, and the cells were incubated with TSA for 15, 30, 60, or 120 min. Cells were immunolabeled with anti-AcH3 and co-stained with DAPI. Mitotic cells at the defined time points are represented in *rows A* (AcH3) and *B* (DAPI). A small percentage of interphase cells are unavoidably isolated and are represented for the respective time periods in *rows C* (AcH3) and *D* (DAPI). Line scans measuring the highly acetylated histone signal intensity from each of the cells, both mitotic and interphase, at the four different time points in the *in situ* HAT activity assay were plotted. The intensity of AcH3 antibody signal is represented by the *black line* for interphase cells and by the *gray line* for mitotic cells. The 15-, 30-, 60-, and 120-min time points are shown in *panels E*, *F*, *G*, and *H*, respectively. *Bar*, 10 μm.

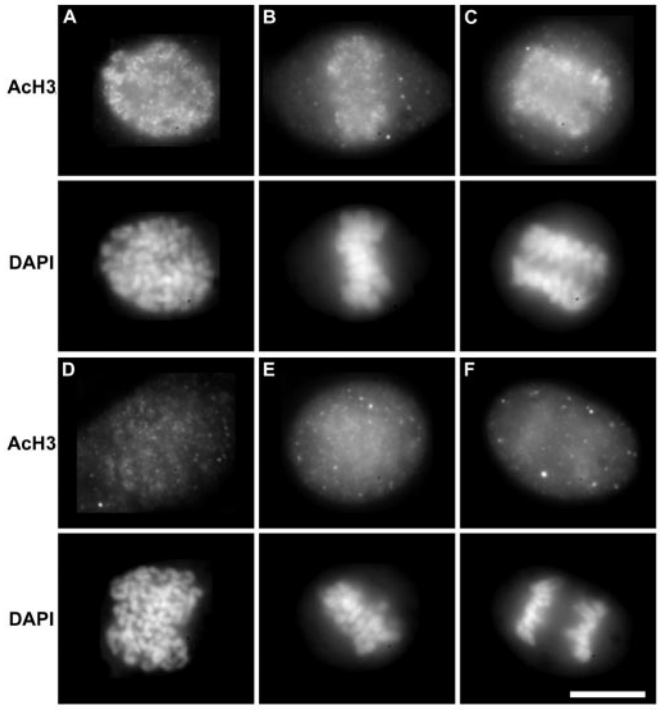

FIG. 7. **Hyperacetylated chromatin condensed into mitotic chromosomes.** A randomly growing culture of human fibroblasts were incubated with TSA (*columns A–C*) or without TSA (*columns D–F*) for 12 h, immunolabeled with anti-AcH3, and co-stained with DAPI. Cells in prophase, metaphase, or anaphase (*columns A* and *D*, *B* and *E*, or *C* and *F*, respectively) were digitally imaged. *Bar*, 10 μm.

phase cells (Fig. 8, *row C* and *panels E–H*), indicating that release from TSA was successful. We conclude that HDACs are neither present within nor active upon mitotic chromatin. These experiments were confirmed with additional acetyl-lysine-specific antibodies to ensure that the absence of deacetylase activity was not restricted to specific acetyl-lysines (data not shown). Our results demonstrate that HDAC activity is generally and dramatically repressed in mitosis.

Loss of HDAC and HAT Activities in Mitosis—Although the *in situ* HAT and HDAC activity assays provide strong functional evidence that the activities of both enzyme classes are decreased during mitosis *in situ*, we wished to determine whether the decreased activity of HATs and HDACs was due to a change in the distribution of the enzymes or a wholesale change in inherent enzymatic activity. Mitotic SK-N-SH cells, isolated mechanically from a randomly growing culture, were separated from the remaining interphase cells. Both mitotic and mitotic-depleted interphase populations were used to prepare cellular extracts, under nondenaturing conditions, to measure the levels of endogenous HDAC and HAT activities. The activity of HDACs varied little between the mitotic and interphase extracts (Table IV, top). In addition, we included known inhibitors of kinases and phosphatases to control for potential HDAC activation or inactivation due to phosphorylation/dephosphorylation events during extract preparation. Phosphorylation of specific HATs during interphase is known to modulate their activity (71, 72), and phosphorylation of both HDAC1 and HDAC3 has been observed *in vivo*.[3] HDAC activity again varied little between mitotic and interphase extracts prepared under these conditions (Table IV, middle). These activities are HDAC-specific, since they were completely inhibited by TSA (data not shown). Moreover, we also observed less than 1.5-fold difference in endogenous HAT activity between mitotic and interphase cell extracts, with mitotic HAT activity being greater than that present in interphase (Table IV, bottom). A small number of interphase cells are inevitably co-isolated with mitotic cells during the mechanical shake-off procedure. Even a large percentage (*e.g.* 20%) of contaminating interphase cells would be insufficient to account for the approximately equivalent levels of both enzymatic activities in mitotic and interphase extracts. These assays clearly show that HDACs and HATs are enzymatically active *in vitro* and thus indicate that a change in substrate association is the basis for their apparent lack of activity *in situ*.

[3] M. J. Kruhlak, M. J. Hendzel, W. Fischle, N. R. Bertos, S. Hameed, X.-J. Yang, E. Verdin, and D. P. Bazett-Jones, unpublished observations.

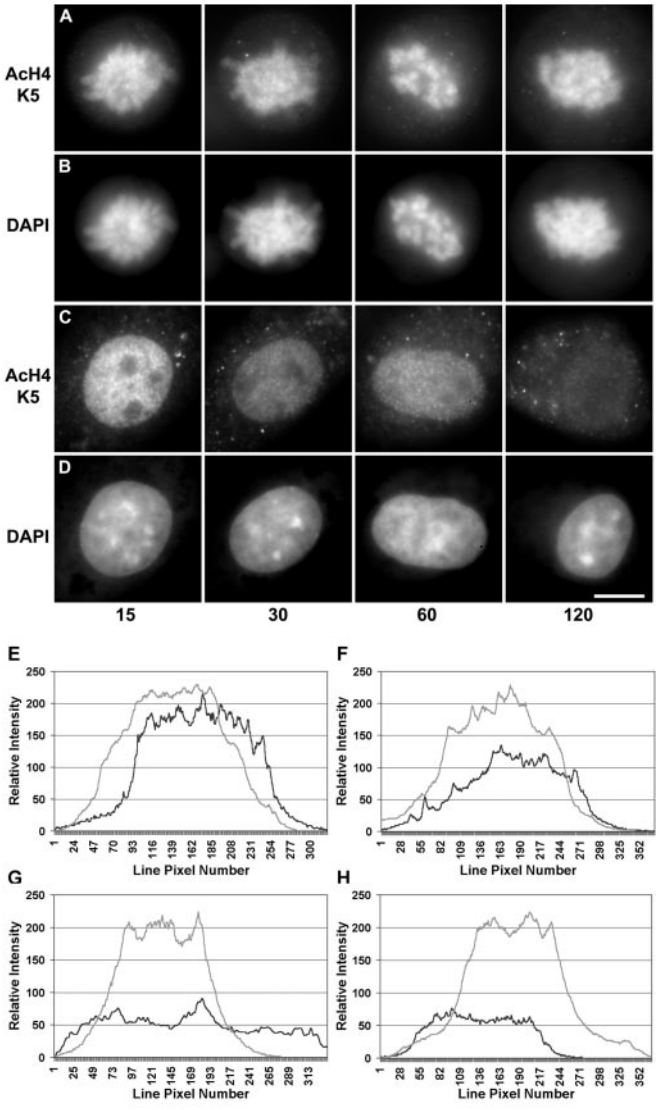

FIG. 8. ***In situ* mitotic deacetylase activity.** Human fibroblasts were simultaneously incubated with both nocodazole and TSA before being isolated and released from TSA for 15, 30, 60, or 120 min. Cells were immunolabeled with an antibody that preferentially recognizes the most highly acetylated isoform of histone H4 (anti-H4K5), shown as AcH4K5 in this figure, and co-stained with DAPI (*DAPI*). Mitotic cells, at the respective time points, are represented in *rows A* (AcH4K5) and *B* (DAPI). A small percentage of interphase cells were also isolated and are represented, at the respective time points, in *rows C* (AcH4K5) and *D* (DAPI). Line scans measuring the highly acetylated histone signal intensity from each of the cells, both mitotic and interphase, at the four different time points in the *in situ* HDAC activity assay were plotted. The intensity of AcH4K5 antibody signal is represented by the *black line* for interphase cells and by the *gray line* for mitotic cells. The 15-, 30-, 60-, and 120-min time points are shown in *panels E*, *F*, *G*, and *H*, respectively. *Bar*, 10 μm.

HDAC Activity Coincides with Reassociation of HATs/HDACs to Late Mitotic Chromatin—Having observed that the spatial and functional relationships of HATs and HDACs change during mitosis, we wished to determine whether the return of HDAC activity coincides temporally with the reassociation of HATs/HDACs with chromatin late in mitosis. A control for the *in situ* HDAC activity experiment was to release cells from both the mitotic block and TSA over a time course before immunoprobing for highly acetylated histones. In this control experiment, cells did progress through mitosis and into interphase. Since both HATs and HDACs dissociate from chromatin in prophase and reassociate with the chromatin in late telophase at approximately the same time, $TAF_{II}250$ was used as a general timing marker for HAT and HDAC relocalization to the cell nucleus. We observe that the relocalization of HATs/HDACs corresponds to the reestablishment of HDAC activity (Fig. 9). Hyperacetylation of mitotic chromosomes was maintained from metaphase through to early telophase. In late telophase, deacetylation of chromatin to normal amounts of highly acetylated chromatin began to occur at the time that $TAF_{II}250$ reestablished focal organization. Since HDACs relocalized in late telophase at approximately the same time point as HATs, we conclude that reestablishment of HDAC activity is concomitant with HDAC reassociation. This supports our previous finding, that HDAC activity is inhibited *in situ* by compartmentalization and not by catalytic inactivation. In addition, it indicates that a common mechanism regulates the compartmentalization of both HATs and HDACs during the cell cycle.

TABLE IV
In vitro mitotic histone deacetylase and histone acetyltransferase activities

Extract (μg/ml)	Mean HDAC activity (n = 4)	Ratio of mitotic to interphase mean activity
	cpm	
Mitotic (25)	3581 ± 123.3	0.92
Interphase (25)	3884 ± 103.4	
Mitotic (50)	4506 ± 71.3	0.93
Interphase (50)	4861 ± 208.7	
Mitotic (100)	5184 ± 26.4	0.93
Interphase (100)	5578 ± 152.5	
Extract (μg/ml)	Mean HDAC activity (n = 4)	Ratio of mitotic to interphase mean activity
	cpm	
Mitotic (25)	838 ± 61.9	1.12
Interphase (25)	746 ± 36.4	
Mitotic (50)	1425 ± 94.2	1.18
Interphase (50)	1207 ± 94.9	
Mitotic (100)	2195 ± 139.1	1.15
Interphase (100)	1903 ± 99.7	
Extract (1 μg/ml)	Mean HAT activity (n = 3)	Ratio of mitotic to interphase mean activity
	cpm	
Mitotic	210.30 ± 16.31	1.41
Interphase	148.80 ± 5.79	

DISCUSSION

Histone Acetylation Profiles in Mitosis—The process of histone acetylation influences gene activity (73). The reversible enzymatic acetylation of core histone N-terminal tail lysines occurs with a high degree of site specificity (54) and characteristic steady-state turnover (21), to provide a sensitive mechanism to efficiently coordinate gene activity while also providing a means to differentiate euchromatin from heterochromatin. A shift in the dynamic equilibrium of HAT and HDAC activities will result in net acetylation or net deacetylation. We observed that this equilibrium shifted dramatically during mitosis. Highly acetylated histones present throughout the interphase nucleus, gradually decrease in amount during the early stages of mitosis, are absent in midmitosis (prometaphase, metaphase, anaphase), and then gradually increase in amount in late mitosis/early interphase (late telophase to early G_1). Thus, an opposite shift in this equilibrium occurs in early G_1 when the amounts of highly acetylated histones indicative of the interphase state are reestablished.

The highest histone acetylation states may change more dramatically from interphase through mitosis for two main reasons. First, the highest acetylated states that represent

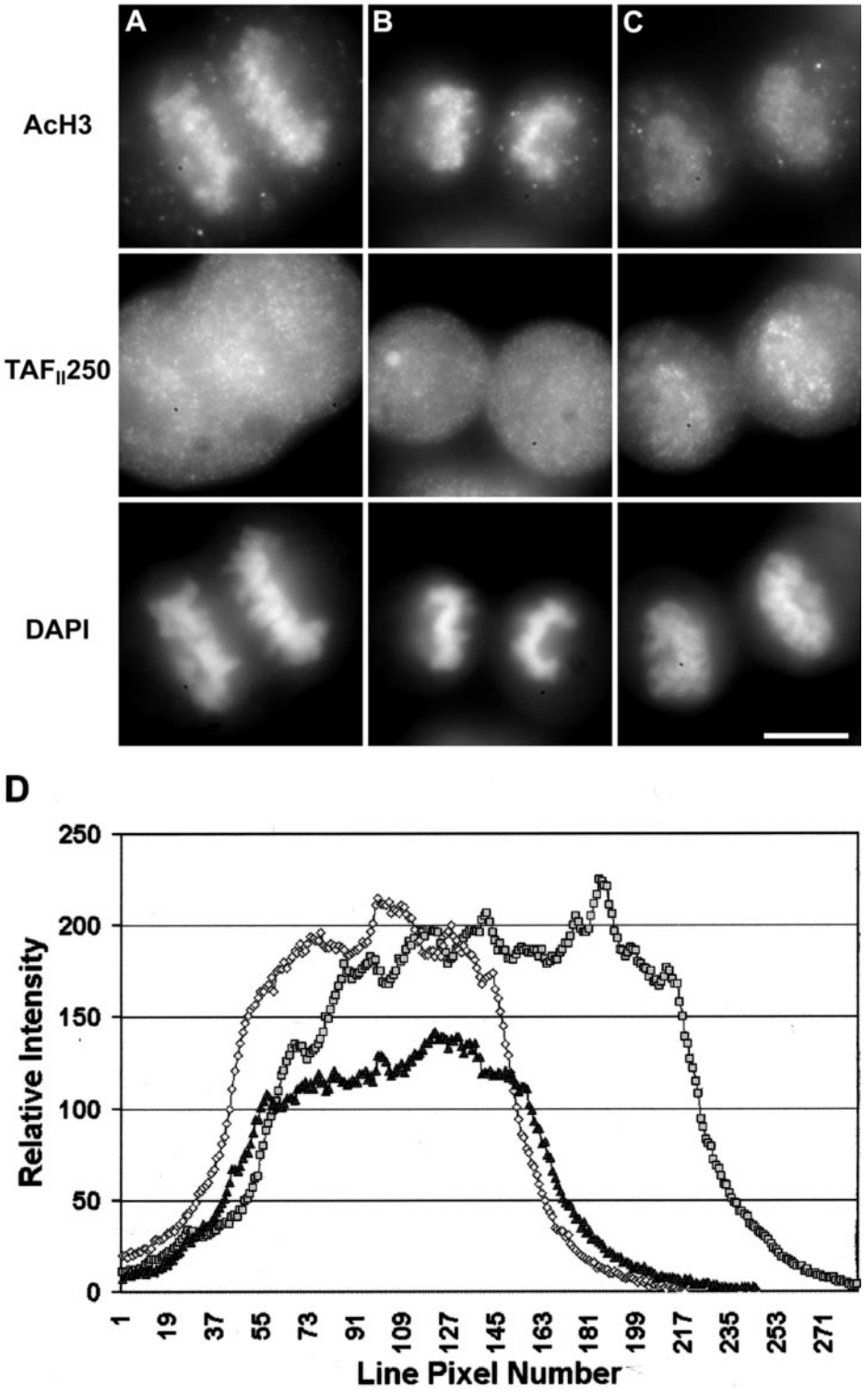

FIG. 9. **Late mitotic temporal relationship between the organization of histone-modifying enzymes (HATs/HDACs) and HDAC activity.** Human fibroblasts were incubated with nocodazole and TSA before being released from both the deacetylase inhibitor and the mitotic block. Cells were co-immunolabeled with anti-AcH3 (*AcH3 row*) and a monoclonal antibody specifically recognizing the HAT-TAF$_{II}$250 (*TAF$_{II}$250 row*) and co-stained with DAPI (*DAPI row*). Cells at either anaphase (*column A*), telophase (*column B*), or early interphase (*column C*) were digitally imaged. Line scans measuring the highly acetylated histone signal intensity from the cells in anaphase, telophase, or early interphase were plotted (*panel D*). The intensity of AcH3 antibody signal is represented by *filled circle* for the anaphase cell, by *filled diamond* for the telophase cell, and by *filled triangle* for the early interphase cell. *Bar*, 10 μm.

transcriptionally competent chromatin are rapidly turned over compared with the low to moderately acetylated states (21, 74). It is well established that transcription is repressed in mitosis, and the changes in acetylation could reflect a direct coupling of these two processes. Second, there is a threshold level of histone acetylation necessary to significantly influence higher order chromatin folding (25). This threshold is reached with the highest acetylated state of the core histones. Thus, compared with the low to moderate acetylated states, a dramatic change in the highest acetylated states will have the most influence on the transcriptional potential and degree of chromatin compaction. This is supported by our observation of low and moderately acetylated histones normally found in mitotic chromatin. Although low to moderately acetylated chromatin may persist through mitosis, the amounts of highly acetylated histone species on a global level are dramatically decreased in mammalian cells from late prophase to late telophase when transcriptional activity is repressed (31). Immunoblotting of the highly acetylated epitopes and the ability to detect experimentally induced hyperacetylated chromatin in mitotic chromosomes demonstrate that our immunofluorescent assays quantitatively reflect the distribution of the examined endogenous epitopes both spatially and temporally. This loss of acetylation is consistently made in a large number of both transformed and nontrans-

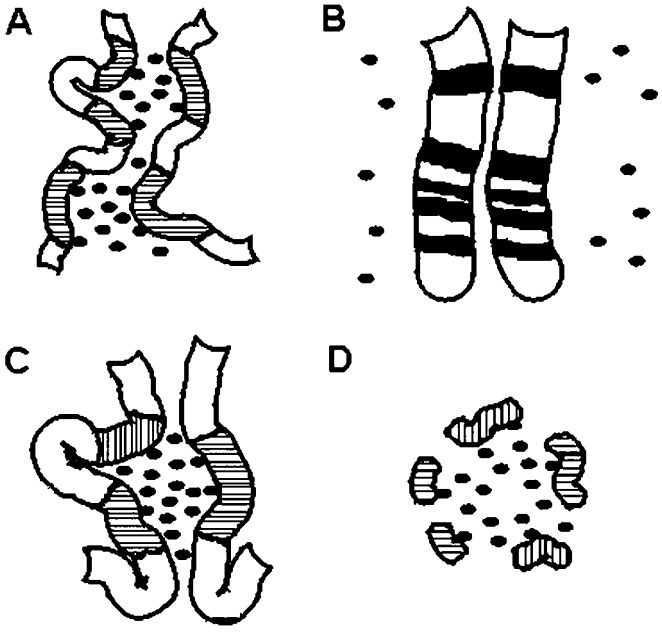

FIG. 10. **A model for regulating chromatin acetylation levels and reestablishing acetylated chromatin domains in late telophase/early interphase.** The *coarsely shaded* areas on the chromosomes represent chromatin containing transcriptionally competent euchromatic regions. R bands (*fine shading* in *B*) contain low to moderate levels of histone acetylation (*e.g.* K12 of H4) during mitosis. The *white segments* of the chromosomes represent heterochromatic regions that do not generally contain elevated levels of acetylated chromatin during the entire cell cycle. The *solid black ovals* represent histone-modifying enzymes, the HATs and HDACs. During prophase (*A*), when chromatin is condensing and levels of highly acetylated chromatin have diminished significantly, HATs/HDACs dissociate from the chromatin and localize in the interchromosomal space. During metaphase (*B*), when chromatin is maximally condensed, HATs/HDACs are localized away from the chromatin, effectively inhibiting their interaction with the chromatin. During late telophase (*C*), when chromatin is decondensing, HATs/HDACs relocalize to the interchromosomal space and reassociate with epigenetically marked regions of the chromosomes to establish potentially transcriptionally active euchromatic regions. During interphase (*D*), euchromatic transcriptionally competent chromatins, containing high levels of acetylation, are organized in territories containing pools of HATs/HDACs that are able to efficiently regulate dynamic levels of highly acetylated transcriptionally active chromatin. These territories represent nuclear architecture such as that associated with interchromatin granule clusters, as previously described (14).

formed cell lines that we have examined. The observed temporal relationship between acetylation and transcription is consistent with the hypothesis that high levels of histone acetylation are involved in establishing transcriptionally competent chromatin domains prior to transcription initiation. Histone acetylation, however, may not be the only chromatin remodeling activity necessary to reestablish a transcriptionally competent state, since ATP-dependent chromatin remodeling factors, such as SWI/SNF, may recruit HATs to mitotic chromatin in late mitosis (75).

Hyperacetylated Chromatin in Mitotic Chromosomes—Multiple mechanistic roles have been proposed for acetylation of core histones in altering local chromatin structure so that transcription initiation and elongation can proceed. At the nucleosomal level, acetylation may disrupt histone/DNA interactions (23), providing access of regulatory factors to the DNA (32–34). Higher order chromatin structure may also be disrupted by histone acetylation, as shown by condensation studies using reconstituted nucleosomal arrays (25, 33).

Consequently, we were surprised to observe that hyperacetylated chromatin can condense into the mitotic chromosomal conformation and that the condensed but hyperacetylated chromosomes did not block a seemingly normal progression through mitosis. Apparently, there is not a strict relationship between histone acetylation and degree of chromatin condensation in mitosis. The ability of hyperacetylated chromatin to condense into mitotic chromosomes may be due to only a small percentage of total chromatin being present in the hyperacetylated state. The treatment of cells with HDAC inhibitors, however, has been shown to increase the acetylation state of slow turnover nonacetylated histones in inactive chromatin along with the dynamic and highly acetylated histones (76–78). In addition, hyperacetylated chromatin appeared to have a relatively uniform presence throughout the mitotic chromosomes. Clearly, there must be other processes that are complementary to deacetylation or that, possibly, act separately from deacetylation, such as histone phosphorylation (59, 79, 80), the loss of chromatin remodeling activities (35, 75), and the establishment of condensin complexes (81) in promoting and/or maintaining the condensed state of chromatin in mitotic chromosomes. Recently, structural maintenance of chromosome proteins, that include the condensin complexes, have been shown to co-localize with condensed and phosphorylated H3 in the early stages of mitotic chromosome condensation (82). Overall, the influence that histone acetylation has modulating accessibility of local DNA templates in interphase is dampened at the global level when chromatin is packaged in mitosis.

HATs and HDACs Do Not Associate with Mitotic Chromosomes—Histone acetylation may function as an epigenetic marker of chromatin, which transmits lineage-specific gene expression profiles from one cell generation to the next (44, 46). This is an intriguing hypothesis that could provide an explanation for the partitioning of euchromatin to intranuclear regions and heterochromatin to perinuclear and perinucleolar regions of the cell nucleus very early during postmitotic nuclear reformation. Furthermore, the binding of acetyl-lysines in specific regions of chromosomes by the bromodomains found in many of the HAT proteins may serve to maintain this association through mitosis and more faithfully transmit the epigenetic information into the next cell cycle (46).

We examined the temporal correlation between amounts of highly acetylated histones and the distribution of HATs/HDACs. Surprisingly, every HAT and HDAC that we tested adopted a similar distribution in mitosis. Both HATs and HDACs translocate to and accumulate at the interchromosomal space, a nonchromatin domain. We observe a gradual translocation of the bulk of the population of HATs and HDACs to the interchromosomal space in the early stages of mitosis. The bulk of HATs and HDACs remain separate from the chromosomes in midmitosis (prometaphase, metaphase, and anaphase), but most of the population relocalizes with chromatin in late mitosis/early interphase (late telophase/early G_1). This coincides temporally with the changing amounts of highly acetylated chromatin at the respective stages of mitosis (see Fig. 2). We have demonstrated previously that HATs and HDACs are present in both chromatin and nonchromatin domains within the interphase nucleus (14). In ongoing experiments, we have observed that these domains are constantly exchanging factors with the nucleoplasm.[3] Overall, this indicates that the distribution of HATs and HDACs is temporally regulated and that the distribution influences the acetylation profile of chromatin. Importantly, our results conclusively demonstrate that HATs do not associate with specific regions of chromosomes during mitosis. Thus, any epigenetic code that is transmitted by histone acetylation must be confined to the low or moderately acetylated states of histones and is independent of HAT and HDAC association during mitosis.

An Altered Association Prevents HAT and HDAC Activity on

the Chromatin Substrate—Although we cannot explicitly determine the proportion of HATs and HDACs able to associate with chromatin during the early and late stage of mitosis when amounts and levels of histone acetylation are gradually changing, we can observe that the bulk of the HATs and HDACs have dissociated from mitotic chromatin in the midstages of mitosis by monitoring the activity of the two classes of enzymes.

The mitotic regulation of transcriptional co-activator or co-repressor activities, including those of HATs and HDACs, could be mediated by protein modifications or differential association with multiprotein complexes (31, 40, 42). Mitosis is a period of the cell cycle when many proteins are phosphorylated and their activity is altered. It is also clear that both HATs and HDACs may be phosphorylated *in vivo* and that this modification has the potential to influence activity. For example, the human homologue of the yeast transcriptional co-activator GCN5 is phosphorylated both *in vitro* and *in vivo*, a modification that affects the enzyme's HAT activity (71). In addition, cell cycle-dependent phosphorylation of the global transcriptional co-activator CBP has been shown to increase its HAT activity during interphase (72). Multiple potential phosphorylation sites present in both HATs and HDACs could be involved in modulating their activity, as observed for other chromatin-remodeling factors (36). Our results, however, clearly argue against a catalytic inactivation due to mitotic phosphorylation. We observe a very small change in *in vitro* HDAC activity and a small increase in *in vitro* HAT activity when comparing mitotic extracts with interphase nuclear extracts.

Another explanation for the low levels of acetylation in mitotic chromatin is that the enzymes translocate from a chromatin domain to a nonchromatin domain. We found that during metaphase, both HAT and HDAC activities on chromatin are diminished *in situ* and that this best correlates with a translocation to a nonchromatin domain. Furthermore, as cells re-enter interphase, the ability of both HATs and HDACs to act on chromatin is correlated temporally with the relocalization of these enzymes to the chromatin. Therefore, we conclude that the levels of acetylation in chromatin are governed by a change in the association of the enzymes with their substrate and not by a modulation of their inherent activities. However, direct modifications of these enzymes and a differential association of HATs and HDACs with multiprotein complexes throughout the cell cycle may be responsible for their translocation between compartments. Because both HATs and HDACs were translocated to the interchromosomal space, we suspect that the two classes of enzymes are regulated by a common mechanism. Thus, the distribution of HATs and HDACs provides a means to regulate the process of histone acetylation in mitosis. The proposed epigenetic mechanism that histone acetylation provides (46) can occur without stable association of HATs and HDACs with chromatin domains in mitosis. The sequestering of these enzymes away from mitotic chromatin may help prevent alterations in established histone acetylation profiles needed to mark chromatin domains for postmitotic reactivation. Thus, our results refute the hypothesis that the stable association of HATs and HDACs mark chromatin domains epigenetically for postmitotic activation and support the hypothesis that HAT and HDAC activity is spatially regulated through assembly into discrete compartments. The concentration of HATs and HDACs into discrete subnuclear compartments would be a particularly effective mechanism if it is maintained in interphase by the nonrandom relative organization of euchromatin, HATs, and HDACs within a spatially ordered interphase nucleus as previously reported (14).

Model for the Establishment of Transcription Domains at Late Telophase/Early Interphase—We propose a model whereby levels of histone acetylation are regulated by translocation of HATs/HDACs to and from nonchromatin domains (Fig. 10). At the early mitotic stage, the transcriptionally competent, highly acetylated chromatin becomes deacetylated to moderate levels, contributing to transcriptional repression. A shift in equilibrium, between chromatin and nonchromatin domains, leads to translocation of the majority of these enzymes to the nonchromatin compartment (Fig. 10*A*). At the global level, the functional activity of the HDACs lags behind that of the HATs to generate a hypoacetylated state. The histone-modifying enzymes remain biochemically active but do not associate with chromatin during metaphase (Fig. 10*B*). During this time, small domains, perhaps R bands, retain histones that are acetylated at low to moderate levels, involving lysine 12 of H4, but are not acetylated at high levels, characterized by the AcH4K5 (acetyl-lysine 5) and AcH3 (acetyl-lysines 9 and 14) antibodies (54, 56–58). This low to moderately acetylated mitotic chromatin may function as an epigenetic marker to preserve chromosome territory organization. Combinatorial relationships between different histone modifications have recently been proposed to form an epigenetic histone code that is read by factors that regulate chromatin structure and activity (46, 83). After the daughter chromosomes segregate and the chromatin begins to reorganize and decondense, a wave of recruitment of histone-modifying enzymes, HATs/HDACs, relocalize or reassociate with the chromatin in a process that may involve the partitioning of euchromatic regions of chromatin adjacent to the small interchromatin space of early G_1 cells, where the HATs and HDACs preferentially enrich (Fig. 10*C*). These newly emerging territories or compartments are maintained by an equilibrium of association of HATs and HDACs between a chromatin-based domain and a nonchromatin domain and may be responsible for organizing chromatin and nonchromatin components observed during interphase (14, 84, 85). Such territories have been observed on the periphery of interchromatin granule clusters (Fig. 10*D*) (14). Biochemical modification and differential localization of both general and gene-specific transcription factors may influence both the distribution and composition of these territories, and thereby regulate gene activity.

Acknowledgments—We thank Maryse Fillion, Wei-Xiang Dong, and Dr. W. Michael Schoel for excellent technical assistance. We thank Dr. C. D. Allis for the gift of acetylated H3 antibody.

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GENES: STRUCTURE AND REGULATION:

Regulation of Global Acetylation in Mitosis through Loss of Histone Acetyltransferases and Deacetylases from Chromatin

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J. Biol. Chem. 2001, 276:38307-38319.
originally published online October 5, 2001