Histone postranslational modifications that accompany DNA replication, nucleosome assembly, and H2A/H2B exchange were examined in human tissue culture cells. Through microsequencing analysis and chromatin immunoprecipitation, it was found that a subset of newly synthesized H3.2/H3.3 is modified by acetylation and methylation at sites that correlate with transcriptional competence. Immunoprecipitation experiments suggest that cytosolic predeposition complexes purified from cells expressing FLAG-H4 contain H3/H4 dimers, not tetramers. Studies of the deposition of newly synthesized H2A/H2B onto replicating and nonreplicating chromatin demonstrated that H2A/H2B exchange takes place in chromatin regions that contain acetylated H4; however, there is no single pattern of H4 acetylation that accompanies exchange. H2A/H2B exchange is also largely independent of the deposition of replacement histone variant, H3.3. Finally, immunoprecipitation of nucleosomes replicated in the absence of de novo nucleosome assembly showed that histone modifications do not prevent the transfer of parental histones to newly replicated DNA and thus have the potential to serve as means of epigenetic inheritance. Our experiments provide an in-depth analysis of the “histone code” associated with chromatin replication and dynamic histone exchange in human cells.

The nuclear DNA of eukaryotic cells is complexed with histone proteins to form the nucleoprotein complex termed chromatin. An octamer of the core histones (two each of histones H2A, H2B, H3, and H4) is encircled by approximately two turns of DNA to produce the fundamental repeating unit of chromatin, the nucleosome (1). Although once widely thought to be primarily involved with DNA packaging, it is now known that histones are fundamental participants in the regulation of dynamic chromatin processes. Transcription, replication, repair, recombination, and silencing can be controlled by the precise alignment of nucleosomes on DNA and by the various postranslational modifications that histones undergo (including acetylation, phosphorylation, methylation, ubiquitylation, and poly(ADP-ribosylation)) (2). Most of the postranslational modifications are clustered on the histone N-terminal (and, in the case of H2A, C-terminal) “tail” domains, which project beyond the surrounding DNA (1). The modifications constitute a network of interdependent signals, providing controllable marks for the specific targeting of trans-acting factors to chromatin. It has been suggested that histone modifications can provide an epigenetic language, or “histone code,” that modulates the genetic information transmitted in DNA (3–5).

One of the first demonstrated assignments of a defined histone modification to a specific cellular process involved the acetylation of newly synthesized H4 (6–9). During chromatin replication and assembly, new H4 is acetylated at lysines 5 and 12 prior to deposition onto nascent DNA (10, 11). Deacetylation of new H4 occurs over the next 30–60 min (7) and is required for proper chromatin maturation (12). The “Lys5/Lys12” acetylation pattern of newly synthesized H4 is highly conserved and has been found in organisms as diverse as protozoa (Tetrahymena), Drosophila, and humans (11). (Note that in Tetrahymena, the acetylation sites are at lysines 4 and 11, due to a deletion of the arginine at position 3; also, new H4 in Physarum is predominantly monoaetylated (9)). Despite this widespread occurrence, the function of H4 acetylation during chromatin biosynthesis remains undefined. Notably, in the yeast Saccharomyces cerevisiae, the lysines at positions 5 and 12 of H4 are dispensable for nucleosome assembly, and Lys5, Lys8, and Lys12 act redundantly during histone deposition (13). There is also evidence that the acetylatable lysines of H4 may be required for efficient nuclear import in yeast (14).

In contrast to the conserved acetylation pattern of new H4, the acetylation of nascent H3 varies among species. For example, in Tetrahymena, Lys9 and Lys14 are the predominant sites, whereas in Drosophila, lysine 14 and 23 are preferred (11). In budding yeast, the lysines at positions 9, 14, 23, and 27 all show some acetylation (with Lys9 and Lys27 preferred), but most new H3 is monoaetylated (15). There is also evidence that new H3 in S. cerevisiae is acetylated at lysine 56 (16). The variability of nascent H3 modifications may indicate a nonuniform requirement and/or function for the acetylation of H3 during chromatin assembly.

Human cells contain four distinct histone H3 variants, termed H3.1, H3.2, H3.3, and H3.4, which differ only slightly in amino acid sequence (17–20). The major variant (H3.1) is referred to as replication-dependent, because its synthesis rises sharply in S phase and is linked to DNA replication (21, 22); H3.1 is encoded by 10 genes (19). H3.2 is also replication-dependent but is encoded by only one gene copy (19, 20); it is distinguished from H3.1 by a single amino acid change at position 96 (cysteine in H3.1, serine in H3.2) (20, 23). Synthesis of the replication-independent variant H3.3 is not coupled to DNA replication but continues at a basal level throughout the cell cycle (21, 22). H3.3 can be deposited in a replication-independent manner, often in association with transcription (24–31). Human H3.4 appears to be expressed solely in testis (32).
that report, we also presented evidence that newly synthesized human H3.2 and/or H3.3 showed detectable acetylation at lysines 14 and 18. In light of the observation that bulk H3.3 is enriched in modifications that are indicative of transcriptional competence (33, 34), we have more closely examined the modifications of newly synthesized H3.2 and H3.3 in human cells, in this case including the analysis of histone methylation.

We have also initiated a characterization of the histone modifications that are present in newly replicated nucleosomes and on parental histones that are segregated to newly replicated DNA (35). In conjunction with these studies, we have investigated the exchange of newly synthesized H2A and H2B into replicating and nonreplicating chromatin regions, through the use of antibodies that recognize specific acetylated H4 isoforms. Our results demonstrate that a subset of new H3 carries modifications that are characteristic of transcriptionally active chromatin immediately following their deposition onto DNA. We also show that specific modifications of parental H3 and H4 can persist through the replication process without preventing the transfer of old histones to new DNA and thus may act as effectors of epigenetic inheritance. It is further demonstrated that the exchange of new H2A/H2B dimers into chromatin is concomitant with the acetylation of H4 and can occur independently of the deposition of new H3.3. Finally, we provide evidence that the human cytosolic H3/H4 predeposition complex contains an H3/H4 dimer (not a tetramer) and that cytosolic H2A can be acetylated at lysine 5. Taken together, our results provide a detailed description of histone modifications during chromatin replication, nucleosome assembly, and H2A/H2B exchange in human cells.

EXPERIMENTAL PROCEDURES

Cell Culture and Labeling—HeLa S-3 cells were maintained in spinner culture at 37 °C in minimal essential medium (MEM), supplemented with 5–10% calf serum. Newly replicated DNA was labeled with [3H]thymidine for 5 min in vivo as described previously (36). For microsequencing newly synthesized histones, cells were synchronized using the double thymidine block procedure as described previously (37) and released into S phase for 2–4 h prior to radiolabeling. Cells were then pulse-labeled with L-[4,5-3H]lysine (85 Ci/ml; Amersham) and microsequencing analysis of newly synthesized H3 was performed at 37 °C (42). Histone Modifications in Dynamic Chromatin

Plasmid Construction—An (amino-terminal) FLAG-H4 construct was obtained by PCR using N-FLAG H4/pET3a plasmid as the template (N-FLAG H4/pET3a was a gift from Drs. Christophe Thiriet and Jeffrey J. Hayes). PCR Master Mix (Promega), and the following primers: forward primer, 5'-CCGCCGGGATCAGCGCAAGC-3'; reverse primer, 5'-CACGCCGGATCCTTTAACCCGGAACCGTAC-3'.

The forward primer introduced a XmaI restriction site (underlined) and a Kozak sequence (boldface type); the reverse primer introduced a BamHI restriction site (underlined). Purified PCR products were ligated into pGEM-T vector (Promega) and then excised using Xmal and BamHI. The enzyme digestion products were purified and ligated into pIRE5-hrGFPII (Stratagene), which had been cut using Xmal and BamHI. The constructed plasmid was amplified following transformation into Escherichia coli strain JM109, confirmed by sequencing, and purified using the EndoFree plasmid kit (Qiagen).

Transfection of 293-H Cells—293-H cells were obtained from Invitrogen and maintained according to the supplier’s instructions. Lipofectamine 2000 (Invitrogen) was used for transfection; stable cell lines were obtained by G418 selection after transfection with N-FLAG-H4/pIRE5-hrGFPII. Green fluorescence was observed ~30 h after transfection. Stable cell lines were grown in minimal essential medium supplemented with 10% fetal bovine serum, 4 mM L-glutamine, 50 units/ml penicillin, 50 μg/ml streptomycin sulfate, and 400 μg/ml G418. FLAG-H4 expression was confirmed by immunofluorescence using an anti-FLAG M2 antibody (Sigma) and immunoblotting as described previously (45); at least 95% of the stably transfected cells expressed the FLAG epitope. FLAG-H4 represented <10% of total H4 in the transfected cells, as determined by Western analysis using antibodies recognizing unmodified H4 (the generous gift of Dr. Judith Berman; see Fig. 8).

Nuclear Isolation, Histone, and Chromatin Preparation—Isolated nuclei, S100 cytosolic extracts, and acid-soluble nuclear proteins were prepared as described previously (36); for immunoprecipitation studies, acid-extracted histones were dialyzed overnight in 1 liter of distilled water at 4 °C. Under these experimental conditions, H3 and H4 can associate and co-immunoprecipitate; however, the formation of H3/H4 complexes is less efficient than that observed using salt-extracted histones (46). For the analysis of chromatin-bound radiolabeled histones, soluble chromatin was prepared with micrococcal nuclease (Sigma) at 5 units/ml at 4 °C, in 10 mM PIPES, 20 mM sodium butyrate, and 80 mM NaCl, 0.5 mM CaCl2, pH 7.0 (36). To generate mononucleosomes, nuclei were isolated in Buffer A (10 mM Tris-HCl, 10 mM sodium butyrate, 3 mM MgCl2, 2 mM 2-mercaptoethanol, pH 7.6 (43)), resuspended in Buffer A at 40 A260/ml, adjusted to 0.5 mM CaCl2, and digested with 1.2 units/ml micrococcal nuclease for 1.5–2.0 min at 37 °C; the reaction was stopped by adding EGTA to a final concentration of 5 mM. Digested nuclei were incubated on ice for 15–30 min and then centrifuged for 10 min at ~10,000 × g; the supernatant, containing mononucleosomes, was termed S1 (47). The resulting pellet was resuspended in 2 mM EDTA, pH 7.2, incubated on ice for 20 min, and again centrifuged; this supernatant, containing mono- and poly nucleosomes, was designated S2. The residual pellet was designated P3 (see supplemental Fig. S1) (47). Scintillation counting of trichloroacetic acid-precipitable radioactivity was measured as described previously, using a biodegradable fluid (Ecoscint A; National Diagnostics) (36, 44).

The abbreviations used are: MEM, Eagle’s minimal essential medium; Joklik modification; HU, hydroxyurea; TSA, trichostatin A; PIPES, Piperazine-1,4-bis(2-ethanesulfonic acid); HPLC, high pressure liquid chromatography; TAU, Triton-acid-urea; GFP, green fluorescent protein; acH4, acetylated histone 4.

K. Tong and A. T. Annunziato, unpublished results.

3 The abbreviations used are: MEM, Eagle’s minimal essential medium (Joklik modification); HU, hydroxyurea; TSA, trichostatin A; PIPES, Piperazine-1,4-bis(2-ethanesulfonic acid); HPLC, high pressure liquid chromatography; TAU, Triton-acid-urea; GFP, green fluorescent protein; acH4, acetylated histone 4.

4 K. Tong and A. T. Annunziato, unpublished results.
Histone Modifications in Dynamic Chromatin

Immunoprecipitation—In this study, several different antibodies were used for immunoprecipitation experiments. For immunoprecipitations of newly replicated and newly assembled chromatin, acetylated H4-specific antibodies (generated using a peptide representing the H4 N-terminal domain acetylated at lysines 5 and 12; i.e. the sites acetylated in newly synthesized HeLa H4) were routinely used; these antibodies have been described previously (38, 48). During the course of our experiments, it was necessary to produce additional antibodies to the same Lys/lys—aclaylated H4 peptide. These antibodies (identified in the figure legends) have a minor reaction with denatured acetylated H2A in Western blots (supplemental Fig. S2A); however, they do not immunoprecipitate either acetylated (cytosolic) or newly synthesized H2A (data not presented; also see Fig. 6). Antibodies were also produced using an H4 N-terminal peptide acetylated at lysines 8 and 16, which recognize acetylated H4 primarily at lysine 8 (supplemental Fig. S2B). All other antibodies were purchased from Upstate (Charlottesville, VA).

Chromatin immunoprecipitations were performed essentially as described, with the exception that high salt wash buffer contained 400 mM NaCl (36, 48). All immunoprecipitations used 50–100 μl of antiserum or antibody, and 40–80 μl of packed Protein A-Sepharose beads (GE Healthcare). Antibody/chromatin excess was monitored by sequential immunoprecipitations of the unbound fraction or by doubling the amount of antibody used, as described previously (44). For immunoprecipitations of extracted histones, Protein A-Sepharose beads were blocked with 1 mg/ml acetylated bovine serum albumin (Promega) and 0.1 mg/ml ubiquitin (Sigma). For the analysis of immunoprecipitated histones by gel electrophoresis, washes contained 0.1% SDS; this did not affect the immunoprecipitation of intact newly assembled nucleosomes (see Fig. 4). In preparation for electrophoresis, soluble chromatin in the unbound fraction was adjusted to 10 mM MgCl2 and precipitated with two volumes of ethanol; free histones were precipitated with 25% trichloroacetic acid (12). To separate immunoprecipitated histones in gels containing SDS, immunopellets were resuspended in sample buffer and placed in boiling water for 5 min. To resolve immunoprecipitated histones in Triton-acid-urea gels, proteins were extracted from the immunopellets using the method of Crane-Robinson et al. (49).

Cytoplasmic S100 extracts from 293-H cells were prepared as described previously (36); S1 chromatin fractions were prepared as described above for HeLa cells. Prior to immunoprecipitation, ~20% of each sample was saved as an “input” fraction. For immunoprecipitation, samples were adjusted to 1 mM phenylmethylsulfonyl fluoride, 1 mM EGTA, 0.25% Triton X-100, 0.6 μg/ml leupeptin, 0.8 μg/ml pepstatin, and 0.5 μg/ml microcystin and then incubated with EZview Red Anti-FLAG M2 affinity gel (Sigma), which had been equilibrated with 50 mM Tris-HCl, 150 mM NaCl, pH 7.4. As a control, an equivalent volume of sample was incubated with mouse IgG-agarose affinity gel (Sigma). Immunoprecipitations were performed at 4 °C overnight. Bound and unbound fractions were separated by centrifugation for 2 min at 1200 × g. Input and unbound S100 fractions were precipitated with 25% trichloroacetic acid, washed with acetone, and dried; input and unbound S1 chromatin fractions were adjusted to 10 mM MgCl2 and precipitated with 2 volumes of ethanol. Bound, immunoprecipitated fractions were washed five times with high salt wash buffer (1% Triton X-100, 0.4 M NaCl, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1) and once with 10 mM Tris-HCl, pH 8.1 (36). In some cases, S100 preparations from HeLa or 293-H cells were treated with 5–10 mM MgCl2 and centrifuged at 12,000 × g for 10 min, to remove any possible contaminating chromatin.

Gel Electrophoresis, Fluorography, and Immunoblotting—Radiolabeled DNA was separated in 4% polyacrylamide gels prior to analysis by fluorography (50). Proteins were subjected to SDS-PAGE in 18% polyacrylamide gels (51), as described previously (40). To resolve histone H3 variants, acid-soluble proteins were separated in 0.4% Triton X-100, 0.9 M acetic acid, 6 M urea, 15% polyacrylamide (TAU) gels (52, 53). Fluorography was performed according to published methods (54, 55). For quantitation, films were scanned using a Bio-Rad GS-800 densitometer; the intensity of the bands was determined using Quantity One software. For immunoblotting, proteins were transferred to Immobilon-P membrane according to the methods of Towbin et al. (56) and analyzed as previously described (36). Antibody dilutions are given in the figure legends. Immunoblots of samples containing FLAG-H4 were performed using antiacetyl histone H4 (Lys12) antibodies (Upstate) and goat anti-rabbit IgG secondary antibodies (Western Star System; Applied Biosystems); blots were then stripped and reprobed using anti-FLAG M2 antibodies (Sigma) to determine the efficiency of immunoprecipitation of FLAG-H4 (typically >90%).

RESULTS

Histone Modifications of Newly Replicated Chromatin—Histone modifications present in newly replicated chromatin were analyzed by immunoprecipitation, using antibodies that recognize modifications of H3 and H4. HeLa cells were pulse-labeled with [3H]thymidine for 5 min in the presence of deacetylase inhibitors. Mononucleosomes were prepared by micrococcal nuclease digestion and immunoprecipitated using antibodies specific for histone modifications. As a first approach, we examined acetylation of H3 at lysines 9 and 14 (sites known to be acetylated in newly synthesized H3 in other species), acetylation of H4 at lysine 8 (which is acetylated in the nuclear CAC complex (57)), and methylation of H3 at lysine 4 (a marker for transcriptionally active chromatin (58)). To better compare the results of multiple experiments, we took advantage of the well established “Lys3/ Lys12” deposition acetylation pattern of newly synthesized H4, an acetylation pattern that is present on nucleosomes assembled de novo (8, 11, 48, 57, 59, 60). For each experiment, a control immunoprecipitation was performed using antibodies that recognize H4 acetylated at Lys3 and/or Lys12; we have previously shown that antibodies with this specificity immunoprecipitate newly synthesized with very high efficiency (40). Immunoprecipitations using other antibodies were then compared with this internal standard by dividing the counts/min immunoprecipitated by each antibody by the counts/min immunoprecipitated using anti-acH4-Lys3/Lys12. Because different antibody epitopes may be accessible in chromatin to varying degrees, the immunoprecipitation results do not represent an absolute measure of the prevalence of each modification. Nevertheless, they provide a direct examination of the histone modifications present immediately after DNA synthesis. The results are presented in Fig. 1.

As expected, acetylation of H4 at Lys3 and Lys12 was found in newly replicated nucleosomes. The modification of H3 by acetylation at lysine 14 and by methylation at lysine 4 was also observed in nascent chromatin, along with detectable acetylation at lysine 9 (Fig. 1). Interestingly, a low level of acetylation of new H3.2 and H3.3 at lysine 14 had also been observed in our previous analysis of the modifications of newly synthesized human histones (11). In light of the immunoprecipitation results of Fig. 1, we reexamined the modifications of new H3.2/H3.3, this time including methylation at lysine 4.

HeLa cells were radiolabeled for 8–10 min with [3H]lysine in the presence of deacetylase inhibitors. H3 isoforms were separated by reverse-phase HPLC and microsequenced (because H3.2 and H3.3 coelute during reverse-phase HPLC, these histones were microsequenced together). Lysine residues from cycles 4, 9, 14, 18, 23, and 27 of the
Histone Modifications in Dynamic Chromatin

FIGURE 1. Histone modifications of newly replicated chromatin. HeLa cells were labeled for 5 min with [3H]lysine in the presence of deacetylase inhibitors. Mononucleosomes were prepared by micrococcal nuclease digestion and immunoprecipitated with antibodies that recognize H4 acetylated at Lys5/Lys12 (acH4K5/K12; see supplemental Fig. S2), H4 acetylated at lysine 8 (acH4K8), H3 acetylated at lysine 9 (acH3K9), H3 acetylated at lysine 14 (acH3K14), and H3 dimethylated at lysine 4 (dmH3K4). RNIS control immunoprecipitation using normal rabbit immune serum. Trichloroacetic acid-precipitable counts/min (CPM) in all immunopellets were divided by trichloroacetic acid-precipitable counts/min in the acH4K5/K12 immunopellet, to yield the ratio shown on the y axis. A and B represent duplicate experiments.

sequencing reaction were separated by reverse-phase HPLC under conditions that resolve unmodified lysine from acetylated, monomethylated, and di-/trimethylated lysine. The individual lysine peaks for each modified species were then analyzed by scintillation counting, to determine the distribution of modifications of new H3 at each position. The results are presented in Table 1.

Approximately 10% of the lysine residues at positions 14, 18, and 23 were acetylated (Table 1). In addition, ~10% of lysine 4 of new H3.2/3 was di- or trimethylated, and no acetylation was observed at this site. These results demonstrate that H3 modifications that are most often indicative of transcriptional activity (methylation of Lys4; acetylation of Lys14 and Lys18) are present on a subset of new H3 within minutes of nucleosome assembly. In contrast, modifications that correlate with heterochromatic silencing (di- and trimethylation of K9) are absent.

Because newly synthesized H3.2 and H3.3 were sequenced together in this analysis, we cannot at this time ascribe specific modifications solely to either variant (as was recently shown for bulk H3.2 and H3.3 (34)). To determine if acetylation of H3.2 and H3.3 occurred prior to deposition, cytosolic histones were separated in TAU gels and examined by Western blotting using anti-acetyllysine antibodies (which should not bias the results toward a specific acetylation site). As seen in Fig. 2A, both H3.2 and H3.3 are acetylated in the HeLa cytosolic extract. When cytosolic H3 was detected using an antibody that recognizes the H3 C-terminal domain (Fig. 2B), multiple electrophoretic forms of H3.3 were again identified; overexposure of the blot also revealed a second H3.2 band (data not presented). Acetylation of cytosolic H3.2 and H3.3 at lysine 14 was then directly demonstrated by Western blotting using antibodies specific for acetylation at this site (Fig. 2C). As will be shown below, both newly synthesized H3.2 and H3.3 are also modified in newly assembled chromatin.

To verify the results obtained by microsequencing, radiolabeled newly synthesized histones were extracted from nuclei and subjected to immunoprecipitation using antibodies specific for modified H3 (Fig. 3). Control immunoprecipitations were also performed using antibodies that recognize H4 acetylated at lysine 5 and/or 12 (the acetylation pattern known to be present on newly synthesized H4). Consistent with the microsequencing results, free new H3 could be immunoprecipitated by antibodies that recognize H3 acetylated at Lys14 or methylated at Lys4 but not with antibodies that recognize H3 acetylated at Lys9 (Fig. 3).

Exchange of New H2A and H2B into Acetylated Chromatin—There is increasing evidence that the association of H2A and H2B with chromatin is labile and that H2A/H2B dimer loss and exchange can be driven by transcription and/or chromatin remodeling (36, 61–70). In a previous report, we provided evidence that H2A and H2B exchange occurs at chromatin regions enriched in acetylated H4 (36). However, in those experiments, an “anti-acetylated H4” antiserum was used. In conjunction with our analysis of histone deposition, we reexamined H2A/H2B exchange using more specific antibodies. Newly synthesized histones

### Table 1

| Lysine | Modification | Percentage |
|--------|--------------|------------|
|        | Me2–3        | 10.5       |
|        | Unmodified   | 74.7       |
|        | Monomethyl   | 15.8       |
| 4      | Acetyl       | 1.2        |
| 9      | Acetyl       | 2.8        |
|        | Me2–3        | 1.9        |
|        | Unmodified   | 87.0       |
|        | Monomethyl   | 12.0       |
| 14     | Acetyl       | 11.6       |
|        | Me2–3        | 3.6        |
|        | Unmodified   | 76.1       |
|        | Monomethyl   | 19.2       |
| 18     | Acetyl       | 10.2       |
|        | Me2–3        | 3.4        |
|        | Unmodified   | 78.8       |
|        | Monomethyl   | 8.5        |
| 23     | Acetyl       | 10.9       |
|        | Me2–3        | 6.5        |
|        | Unmodified   | 74.7       |
|        | Monomethyl   | 10.7       |
| 27     | Acetyl       | 6.60       |
|        | Me2–3        | 5.4        |
|        | Unmodified   | 79.4       |
|        | Monomethyl   | 8.5        |
Histone Modifications in Dynamic Chromatin

FIGURE 2. Acetylation of cytosolic H3. 2/H3.3. Histones were extracted from HeLa S100 extracts, resolved by TAU-PAGE, and analyzed by Western blotting using anti-acetyl-lysine antibodies (1:500 dilution) (A), antibodies that recognize the H3 C-terminal domain (1:10,000 dilution) (B), or antibodies that recognize H3 acetylated at lysine 14 (1:700 dilution) (C). A–C represent independent experiments; lanes marked M contain nuclear histones from butyrate-treated HeLa cells. Note that the identification of human histone variants in this and following experiments was based on 1) the assignments of Bonner and co-workers (110), 2) TAU-PAGE following separation of H3 variants by reverse-phase HPLC (C. A. Mizzen and A. T. Annunziato, unpublished observations; see also Ref. 34), and 3) a comparison of stained and radiolabeled histones in SDS and TAU gels.

FIGURE 3. Modifications of newly synthesized H3 extracted from HeLa cell nuclei. HeLa cells were labeled for 10 min with [3H]lysine in the presence of deacetylase inhibitors. Histones were extracted from isolated nuclei using 0.2 M H2SO4, dialyzed against water, and immunoprecipitated with antibodies that recognize H4 acetylated at Lys5 and Lys12 (anti-acH4 (38, 44)), H3 acetylated at lysine 14 (anti-acH3 K14), H3 dimethylated at lysine 4 (anti-methH3 K4), and H3 acetylated at lysine 9 (anti-acH3 K9) (see “Experimental Procedures” for details). Control immunoprecipitations were performed using rabbit normal immune serum (RNS). Supernatant (S) and immunopellet (P) fractions were resolved by SDS-PAGE and analyzed by fluorography.

were labeled for 15 min with [3H]lysine, and soluble chromatin was immunoprecipitated using antibodies that recognize H4 acetylated in two complementary patterns: either at Lys5/Lys12 (38) or at Lys5/Lys16 (supplemental Fig. S2B). Results were analyzed by TAU-PAGE and fluorography (Fig. 4). Anti-acH4-Lys5/Lys12 antibodies immunoprecipitated all four newly synthesized core histones, including H3.1, H3.2, and H3.3 (anti-ac5/12; Fig. 4). Thus, the deposition of all newly synthesized H3 variants, as well as of new H2A/H2B, is associated with “deposition-related” H4 acetylation at lysines 5 and 12. Newly synthesized H3.2 and H3.3 migrated as multiple isoforms, consistent with posttranslational modification by acetylation and/or phosphorylation (brackets, Fig. 4). Anti-acH4-Lys5/Lys16 antibodies also immunoprecipitated new H2A/H2B but significantly less new H3/H4 (the H3.1/H2A ratio was reduced by ~75%, as determined by densitometric scanning). This indicates that anti-acH4-Lys5/Lys16 antibodies are relatively poor at targeting nucleosomes assembled de novo (all of which should contain new, labeled H3/H4). Because the lag in the sequential deposition of H2A/H2B after H3/H4 appears to be on the order of seconds in vivo (71–75), the enrichment for new, labeled H2A/H2B in the anti-acH4-Lys5/Lys16 immunoprecipitate most likely reflects H2A/H2B exchange (64, 65, 76, 77). However, it is not possible in the experiments presented in Fig. 4 to distinguish exchange of new H2A/H2B into nonreplicating chromatin from the association of new H2A/H2B with parental nucleosomes (containing old H3/H4) that are segregated to new DNA at the replication fork (78, 79). To better differentiate replication-coupled chromatin assembly and replication-independent histone exchange, H2A/H2B deposition was examined during the inhibition of DNA synthesis.

HeLa cells were preincubated with hydroxyurea for 10 min and then labeled for 15 min with [3H]lysine in the continuous presence of HU. Because nucleosome assembly is complete within seconds after DNA replication in vivo (71, 73, 74), pretreating cells with HU prior to radio-labeling will highlight the exchange of new H2A/H2B into preexisting chromatin (77, 80, 81). Soluble chromatin was then immunoprecipitated as in Fig. 4 and analyzed by TAU-PAGE and fluorography (Fig. 5).

During the inhibition of DNA synthesis, the proportion of new H3/H4 relative to new H2A/H2B in acetylated chromatin decreased, as shown in the anti-acH4-Lys5/Lys16 immunoprecipitate (cf. Figs. 4 and 5; the H3.1/H2A ratio decreased by >85%), in agreement with previous findings (36). The presence of a low level of new H3/H4 in the anti-acH4-Lys5/Lys13 immunoprecipitate (Fig. 5) suggests that a fraction of the immunoprecipitated nucleosomes was assembled entirely from new histones in the presence of HU (82). Nonstoichiometric deposition of...
Histone Modifications in Dynamic Chromatin

Acetylation of Cytosolic H2A—As we have previously shown, free H2A in HeLa cytosolic extracts is complexed with the nucleosome assembly factor NAP-1 (48). In that report, it was postulated that NAP-1 might be involved in the transcription-mediated exchange of H2A/H2B dimers as well as in replication-coupled chromatin assembly. Newly synthesized H2A is not modified in a unique deposition-related pattern (7, 39, 83). However, if H2A/H2B dimers are exchanged from acetylated transcriptionally active chromatin, displaced H2A/H2B might also be posttranslationally modified. We therefore asked whether cytosolic H2A can be acetylated, by immunoblotting H2A in the S100 extract with an acetylation-specific antibody.

Cytosolic H2A exhibits acetylation at lysine 5 (Fig. 6A). To verify that cytosolic H2A was nonnucleosomal, H4 was immunoprecipitated from the S100 extract; as a control, isolated chromatin was immunoprecipitated under the same conditions. In previous work, we had shown that new H2A, H2B, and H3 remain associated with H4 when intact nucleosomes are immunoprecipitated using antiacetylated H4 antibodies (36). Unlike nucleosomal acetylated H2A, which was coprecipitated together with H4, acetylated H2A in the S100 extract remained in the unbound supernatant (Fig. 6, compare A and B). This verifies that acetylated H2A in the S100 extract is not complexed in nucleosomes. To our knowledge, this is the first demonstration that cytosolic H2A can be specifically modified in vivo. However, a comparison of the positions of acetylated and total cytosolic H2A by immunoblotting following electrophoresis in TAU gels revealed that most cytosolic H2A is not acetylated (data not shown; see also Fig. 2A). This is consistent with the accumulated evidence that newly synthesized H2A (which is present in the S100 fraction (48, 84)) is unmodified and suggests that cytosolic acetylated H2A may have been displaced from nonreplicating acetylated chromatin (perhaps by transcription).

Evidence for a Cytosolic H3/H4 Dimer—Chromatin assembly complexes isolated from somatic cell nuclei (57) contain H3/H4 dimers, not tetramers (85). It has also recently been shown that the chromatin assembly factor Asf1 is associated with an H3/H4 heterodimer in budding yeast (86). Newly synthesized H3 and H4 are found in association with a cytosolic predeposition complex in human cells (36, 48, 60), and it was of interest to determine if this complex also contained an H3/H4 dimer. 293-H cells were transfected with FLAG-H4, and stable transfectants were selected for resistance to the antibiotic G418 (see “Experimental Procedures”). There are 14 H4 genes in human cells (20), and following transfection FLAG-H4 represented <10% of total H4 protein as determined by Western blotting.

A cytosolic S100 extract (containing H3/H4 predeposition complexes) was then prepared from the transfected cells and subjected to immunoprecipitation using immobilized anti-FLAG antibodies. It was reasoned that if H3 and H4 form a tetramer, cytosolic H3/H4 complexes containing FLAG-H4 should contain native H4 as well, due to the vast excess of endogenous H4 being synthesized. H4 in the immunopellet was detected by Western blotting, using either antibodies that recognize H4 acetylated at Lys5/Lys12 or using control rabbit serum (Fig. 7, B and C). Input, unbound (U), and bound (B) fractions were resolved by SDS-PAGE and analyzed by Western blotting using antibodies that recognize H2A acetylated at K5 (acH2A; 1:1000 dilution) or H4 acetylated at Lys3/Lys16 (acH4; 1:5000 dilution; see supplemental Fig. S2).

For cytosolic complexes containing FLAG-H4 did not also possess endogenous H4. Identical results were obtained after cross-linking the S100 with dithiobis(succinimidylpropionate) prior to immunoprecipitation (data not shown). To verify that FLAG-H4 could be assembled into chromatin, mononucleosomes were prepared by micrococcal nuclease digestion (see supplemental Fig. S3) and immunoprecipitated with anti-FLAG antibodies. In this case, the bound mononucleosomes contained FLAG-H4 and native H4 in similar proportions (Fig. 8B).
Thus, FLAG-H4 can readily be assembled into chromatin. Moreover, the results of Fig. 8 provide evidence that the cytosolic FLAG-H4 complex immunoprecipitated in Fig. 7 is not an H3/FLAG-H4 tetramer (containing two molecules of FLAG-H4), because this would result in a preponderance of FLAG-H4 mononucleosomes with only FLAG-H4 in the anti-FLAG immunopreciplet of Fig. 8 (in light of the long term stability of the H3/4 tetramer (35, 87, 88)). Taken together, the results of Figs. 7 and 8 strongly indicate that H3 and H4 form a heterodimer in the cytosolic complex, not a tetramer. This is in agreement with previous conclusions concerning the nuclear H3/H4 preassembly complex (85) and the Asf1 complex in yeast (86).

Modifications of Parental Histones Segregated to Newly Replicated DNA—It has been proposed that histone posttranslational modifications can serve as means of epigenetic inheritance (3, 5, 44, 58). For this to be the case, modifications of “old” parental histones must be preserved during chromatin replication. To examine the modification state(s) of segregated parental nucleosomes, it is necessary to eliminate newly synthesized histones from the analysis. One way to accomplish this is to treat cells with the protein synthesis inhibitor cycloheximide during the radiolabeling of newly replicated DNA. Under these conditions, parental histones segregate to both sides of the replication fork, rapidly forming octamers on new DNA; however, no new histones are available for de novo nucleosome assembly (42, 78, 79).

HeLa cells were preincubated for 10 min with cycloheximide (to allow for the brief lag in histone deposition (77, 89, 90)) and then labeled for 20 min with [3H]thymidine in the continuous presence of cycloheximide and deacetylase inhibitors (see “Discussion”). Mononucleosomes were immunoprecipitated and analyzed as described above by dividing the counts/min immunoprecipitated by each antibody by the counts/min precipitated using anti-acH4-Lys<sup>12</sup>. It has previously been shown that after replication in cycloheximide ~50% of newly replicated DNA lacks histones, consistent with the cessation of de novo nucleosome assembly under these conditions (35, 42, 91, 92).

Along with acetylation at lysine 5 and/or 12 of H4, chromatin replicated in cycloheximide was modified at several additional sites, including acetylation of H4 at Lys<sup>8</sup>, acetylation of H3 at Lys<sup>14</sup>, and methylation of H3 at Lys<sup>9</sup> (Fig. 9, A and B). These results suggest that histone modifications that are correlated with gene activity may be preserved during chromatin replication. To eliminate potential histone pools that might remain after cycloheximide pretreatment, the experiment was repeated using chromatin that had been replicated in vitro in isolated nuclei. During in vitro replication, true DNA synthesis (as opposed to repair) continues within replications initiated in vivo. In addition, no new histones are available for de novo nucleosome assembly, whereas parental histones continue to be transferred to newly replicated DNA (44, 93).

HeLa cells were preincubated for 5 min in cycloheximide, to impo- se a region of unassembled DNA between the last fully assembled nucleosomes (containing newly synthesized H3 and H4) and the DNA to be labeled in vitro. Nuclei were then isolated and incubated in vitro...
Histone Modifications in Dynamic Chromatin

with [3H]TTP in the presence of deacetylase inhibitors. Mononucleosomes were prepared by micrococcal nuclease digestion and immunoprecipitated with antibodies specific for modified histones, and analyzed as in Fig. 1. CPM, counts/min.

In addition to acetylation at lysines 5 and 12, the acetylation of H3 at lysine 14 and methylation of H3 at lysine 4 were also observed on parental nucleosomes replicated in vitro. It should be noted that there is no acetyl-CoA or S-adenosylmethionine in the replication buffer. Thus, the modifications observed must have been present prior to replication. Taken together, the results of Figs. 9 and 10 provide evidence that histone modifications do not prevent the transfer of "old" histones to newly replicated DNA, consistent with the hypothesis that the histone code can act as a means of epigenetic inheritance in dividing cells.

DISCUSSION

Our results demonstrate that a range of posttranslational modifications of H3 and H4 are present in newly replicated chromatin, as determined by the immunoprecipitation of [3H]thymidine-labeled nucleosomes using modification-specific antibodies. Similar experiments performed on chromatin replicated in the absence of concurrent histone synthesis (i.e. in the presence of cycloheximide or in isolated nuclei) further showed that parental histones are able to carry acetylation and methylation marks during replication. Direct microsequencing analysis of [3H]labeled newly synthesized H3, along with immunoprecipitation studies of purified nascent histones, further established that only a subset of new H3 is posttranslationally modified in HeLa cells. Approximately 10% of the lysine residues at positions 14, 18, and 23 of new H3.2/3 were acetylated immediately after deposition; a similar fraction of lysine 4 was modified by methylation.

Triton-acid-urea gel electrophoresis indicated that both new H3.2 and new H3.3 are acetylated, with H3.3 modified to a greater degree. This is consistent with the observation that H3.3 is enriched in modifications that correlate with transcriptional activity (28, 29, 33, 34). Although H3.2 is represented by only one gene copy in the human genome, H3.2 mRNA accounts for ~30% of all replication-dependent H3 transcripts in HeLa cells (19). Moreover, because H3.3 comprises ~20% of total HeLa H3 (53), our microsequencing analysis of H3.2/3 accounts for at least 40% of newly synthesized H3 in this system.

It was previously shown that ~30–40% of H3 associated with the human nuclear CAC complex (which contains the chromatin assembly factor CAF-1) migrates in acid-urea gels as a monomethylated variant; the remaining H3 is unmodified (57). This is consistent with our observation that multiple sites are modified in a subset of new H3, with no one site being 100% acetylated; presumably, any one of the acetylatable N-terminal lysines of new H3, with the apparent exception of lysine 9, can be used to generate the monomethylated histone H3 (57). A similar flexibility in site usage is also found in new H3 in yeast (15). The absence of a definitive pattern of modification for new H3 in human cells, defining a specific "position-related" code, sets H3 deposition in this system apart from chromatin assembly mechanisms in Tetrahymena and Drosophila (11). The very low acetylation at lysine 9 also distinguishes new HeLa H3 from nascent H3 in Tetrahymena and S. cerevisiae (15).

The analysis of cytosolic H3 by TAU-PAGE suggests that H3.2 and H3.3 may be acetylated prior to deposition. This could be a consequence of the selective modification of different H3 variants prior to nucleosome assembly. However, it is also possible that "cytosolic" H3.3 is in part derived from nuclear H3.3 that has been exchanged out of transcriptionally active chromatin (29, 30, 94). Chromatin-bound H3.3 is preferentially modified (28, 29, 33), and it may be that displaced H3.3 retains these modifications. Notably, our analysis of radiolabeled new H3 argues that "transcriptionally active" histone marks can be established immediately following nucleosome assembly, if not beforehand.

Immunoprecipitations of [3H]labeled nascent nucleosomes yielded results in strong agreement with the microsequencing analysis of new H3/H4. Acetylation of H4 at lysines 5 and 12 was readily detected (11), as were the modifications of new H3. In interpreting these results, it is necessary to bear in mind that H3.3 is deposited onto both replicating and nonreplicating chromatin (25). Thus, histone modifications uncovered by microsequencing new H3.2/H3.3 are relevant to the analysis of replication-coupled nucleosome assembly.

Acetylation of nascent nucleosomes at lysine 8 of H4 was detected above background levels. A low level of lysine 8 acetylation was previously observed in H4 associated with the CAC assembly complex prepared from 293 cell nuclei. In those experiments, acetylation of CAC H4 at lysine 8 was found in diacylated as well as triacylated H4 isoforms, and it is possible that this modification occurs subsequent to H4 deposition. This interpretation is consistent with 1) our earlier microsequencing analysis of new H4 (11); 2) the established Lys7/Lys12 diacytation of cytosolic H3/H4 complexes (48); and 3) the properties of the human Hat1 histone acetyltransferase (the enzyme that probably acetylates new H4 and exclusively acetylates lysines 5 and 12 of H4 in vitro) (48, 95).

Our studies of H2A/H2B exchange offer new insights into this process. By focusing on newly synthesized (radiolabeled) H2A/H2B, it has been possible to avoid the use of GFP adducts, which can destabilize histone-DNA interactions (96). It was found that the exchange of new H2A/H2B into nonreplicating chromatin is accompanied by the acetylation of histone H4, consistent with the hypothesis that exchange occurs on transcriptionally competent chromatin (61, 64, 65). However, there does not appear to be a unique site of H4 acetylation involved in H2A/H2B exchange. Our results are consistent with the recent studies of Thiriet and Hayes (69), who showed colocalization of acetylated H4 and exchanging H2A/FLAG-H2B in Physarum. In that report, a more general "antiacetylated" H4 antibody was used, as was the case in our previous analysis of H2A/H2B exchange in HeLa cells (36).

In further agreement with the results of Thiriet and Hayes (69), we observe that the exchange of new H2A/H2B into acetylated chromatin can be independent of the deposition of nascent H3.3. One reason for this may be the elevated ratio of new H2A/H2B to new H3.3 under our experimental conditions. In addition, there is mounting evidence that H3.3 deposition/exchange may be localized to promoters (28) or confined to very highly transcribed genes, such as rDNA loci.
and induced heat shock genes (25, 27) (however, see Ref. 29). In contrast, H2A/H2B exchange appears to result from basal polymerase II transcription and is therefore expected to be more widespread (62, 63, 66, 69, 88). The nucleosome assembly factor NAP-1 may be involved in the exchange process (48, 97, 98).

The analysis of segregated nucleosomes demonstrates that specific histone modifications that are correlated with transcriptional competence (acetylation of H4 at lysine 8; acetylation and Lys4 methylation of H3) and transcriptional silencing (acetylation of H4 at lysine 12) do not prevent the transfer of old histones to new DNA and thus may persist during chromatin replication. Because histone deacetylase inhibitors were included in these experiments (to prevent deacetylation of parental histones during cyclo-

REFERENCES

1. van Holde, K. E. (1988) Chromatin (Rich, A., ed) Springer-Verlag, New York
2. Wolfe, A. P. (1999) Chromatin: Structure and Function, 3rd Ed., Academic Press, Inc., San Diego, CA
3. Strahl, B. D., and Allis, C. D. (2000) Nature 403, 41–45
4. Jenewein, T., and Allis, C. D. (2001) Science 293, 1074–1080
5. Turner, B. M. (2000) BioEssays 22, 836–845
6. Ruiz-Carrillo, A., Wangh, I. I., and Allfrey, V. G. (1975) Science 190, 117–128
7. Jackson, V., Shires, A., Tanphaichit, N., and Chalkley, R. (1976) J. Mol. Biol. 104, 471–483
8. Allis, C. D., Chicoine, L. G., Richman, R., and Schulman, J. G. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 8048–8052
9. Waterborg, J. H., and Matthews, J. H. (1984) J. Biol. Chem. 259, 1237–1241
10. Kumar, S., and Leffak, M. (1986) Biochemistry 25, 1237–1241
11. Baer, B. W., and Rhodes, D. (1983) J. Cell Biol. 95, 1015–1022
12. Kaufman, P. D., Kobayashi, R., and Stillman, B. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 1257–1261
13. Annunziato, A. T., Seale, R. L. (1981) Biochemistry 20, 4183–4189
14. Annunziato, A. T., Seale, R. L. (1982) Biochemistry 21, 5431–5438
15. Seale, R. L. (1977) Biochemistry 16, 2847–2853
16. Perry, C. A., Schneider, C. A., and Annunziato, A. T. (1999) Methods in Enzymology (Wassarman, P. M., and Wolfe, A. P., eds) Vol. 304, pp. 76–99, Aca-
17. Cheung, P., and Lau, P. (2005) Mol. Cell. 13, 13615–13623
18. Lu, M. J., Dadd, C. A., Mizzen, C. A., Perry, C. A., McLachlan, D. R., Annunziato, A. T., and Allis, C. D. (1994) Chromosoma 103, 111–121
19. Isenberg, I. (1979) Annu. Rev. Biochem. 48, 159–191
20. Annunziato, A. T., Schindler, R. K., Riggs, M. G., and Seale, R. L. (1982) J. Biol. Chem. 257, 8057–8151
21. Chang, L., Loranger, S. S., Mizzen, C. E., and Annunziato, A. T. (1997) Biochemistry 36, 469–480
22. Crane-Robinson, C., Myers, F. A., Hebbes, T. R., Clayton, A. L., and Thorne, A. W. (1999) in Methods in Enzymology (Wassarman, P. M., and Wolfe, A. P., eds) Vol. 304, pp. 533–547, Academic Press, Inc., San Diego, CA
23. hardin, J. L., and Reinberg, D. (2003) Mol. Cell 12, 13605–13614
24. Wu, R. S., and Bonner, W. M. (1981) Cell 27, 321–330
25. Wu, R. S., Tsai, S., and Bonner, W. M. (1982) Cell 31, 367–374
26. Frankin, S. G., and Zweidler, A. (1977) Nature 266, 273–275
27. Ray-Gallet, D., Quivy, J. P., Scamps, C., Martini, E. M. D., Lipinski, M., and Al-
28. Thiriet, C., and Hayes, J. J. (2005) Gene Dev. 19, 677–682
