Analysis of Nucleosome Assembly and Histone Exchange Using Antibodies Specific for Acetylated H4†

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ABSTRACT: Using antibodies that specifically recognize the acetylated forms of histone H4, we show that it is possible to immunoprecipitate newly assembled (acetylated) nucleosomes. Newly replicated HeLa cell chromatin was labeled for 5–30 min with [^{3}H]thymidine in the presence of sodium butyrate (thus inhibiting the deacetylation of newly deposited H4); bulk chromatin DNA was labeled for 24 h with [^{14}C]thymidine. When soluble nucleosomes were incubated with immobilized antibodies, a comparison of the bound and unbound fractions showed up to a 65-fold enrichment for new chromatin DNA in the immunoprecipitate (bound), relative to the supernatant (unbound). No enrichment for new DNA was observed when preimmune control serum was used in a similar fashion. The enrichment for new DNA in the immunopellet was paralleled by a similar enrichment for all four newly synthesized histones. Acetylation was required for antibody recognition: When chromatin was replicated in the absence of butyrate (permitting histone deacetylation and chromatin maturation), equally low levels of new and old chromatin were immunoprecipitated, and no enrichment for new DNA was observed. Competition experiments confirmed these results. Analyses of histone deposition during the inhibition of DNA replication established that acetylated chromatin is the preferential target for H2A/H2B exchange. These experiments provide evidence for the highly selective assembly of newly synthesized H3, H2A, and H2B with acetylated H4, and for the involvement of histone acetylation in dynamic chromatin remodeling. In addition, immunoprecipitations of radiolabeled cytosolic extracts identified a possible somatic chromatin preassembly complex, containing newly synthesized H3 and new (acetylated) H4.

In all eukaryotes so far studied, newly synthesized H4 is reversibly acetylated during chromatin assembly (Ruiz-Carrillo *et al.*, 1975; Jackson *et al.*, 1976; Allis *et al.*, 1985; Waterborg *et al.*, 1984). For organisms such as *Physarum* and *Tetrahymena*, in which deposition- and transcription-related acetylation patterns can be readily distinguished, the specific acetyllysines of nascent H4 have been identified (Pesis *et al.*, 1986; Chicoine *et al.*, 1986). In *Tetrahymena*, newly synthesized H4 is acetylated at two positions, lysine-4 and -11, within the histone N-terminal "tail" (Chicoine *et al.*, 1986). Because the arginine residue at position 3 in vertebrates is deleted in *Tetrahymena*, these sites correspond to lysine-5 and -12 in human H4, sites for which acetylation has been well documented [reviewed in van Holde (1988)]. The conservation of the timing (and, evidently, pattern) of acetylation of newly synthesized H4 argues strongly for a conserved function for acetylation during histone deposition and nucleosome assembly.

Typically, newly synthesized H4 is deacetylated within minutes of its deposition (Jackson *et al.*, 1976), a period that closely corresponds to the maturation of newly replicated chromatin (Worcel *et al.*, 1978; Annunziato *et al.*, 1981). It is somewhat surprising, therefore, that histone deacetylation is not required for proper nucleosome assembly. For example, cellular chromatin replicated and assembled in the presence of the deacetylase inhibitor sodium butyrate possesses normal nucleosomal periodicity and core particle structure (Annunziato & Seale, 1983; Perry & Annunziato, 1989, 1991), and similar results have been obtained for nonreplicating plasmids assembled *in vitro* (Shimamura *et al.*, 1989). However, chromatin replicated in butyrate remains preferentially sensitive to DNase I, suggesting that acetylation may inhibit the complete folding of the nascent chromatin fiber (Annunziato *et al.*, 1988), possibly by altering the affinity of newly replicated chromatin for histone H1 (Perry & Annunziato, 1989, 1991).

The ability to preserve the acetylation of newly synthesized histones, without impeding nucleosome assembly, permits the exploitation of this modification for the analysis of newly assembled chromatin. In a previous study, antibodies were described that recognized the acetylated forms of *Tetrahymena* H4, but which had no affinity for the unacetylated form (Lin *et al.*, 1989). We now report that these antibodies also strongly recognize acetylated, but not unacetylated, human H4 as well. When coupled to protein A–Sepharose, the antibodies can be used to immunoprecipitate newly replicated nucleosomes in an acetylation-dependent manner. In addition to nascent DNA, all four of the new core histones are also immunoprecipitated, providing evidence for the highly selective assembly of newly synthesized histones with acetylated H4 *in vivo*. Parallel studies showed that when DNA replication is inhibited, newly synthesized H2A and H2B preferentially exchange into acetylated chromatin regions. Further, newly synthesized H3 and H4, which fractionate into the cytosol under these conditions, may interact to form a preassembly histone complex.

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EXPERIMENTAL PROCEDURES

Cell Culture and Labeling. HeLa cells were maintained in spinner culture at 37 °C in minimal essential medium (Joklik modified) supplemented with 5–10% calf serum. For long-term labeling of bulk chromatin, cells were incubated with [^{14}C]thymidine (50 mCi/mmol, New England Nuclear) at 0.02 μCi/mL for approximately one generation (18–24 h) under control conditions (minus butyrate). Hyperacetylated bulk chromatin was labeled with [*methyl*-^{3}H]thymidine (90 Ci/mmol, New England Nuclear) at 0.25 μCi/mL for 18–24 h in the presence of 7 mM sodium butyrate. Labeling of newly replicated chromatin with [^{3}H]thymidine for 30 min was performed at 4 μCi/mL in the presence or absence of 50 mM sodium butyrate (following an 8–16-min preincubation in butyrate for the plus-butyrate studies), and then chased for 15 min in the absence of radiolabel (plus or minus butyrate), to allow sufficient time for all of the labeled nascent chromatin to regain normal resistance to micrococcal nuclease, as described previously (Annunziato & Seale, 1983; Perry & Annunziato, 1989). [Note: butyrate does not affect the rate of DNA replication (Annunziato & Seale, 1983; Littlefield *et al.*, 1982) or histone synthesis (Sealy & Chalkley, 1979; Rubenstein *et al.*, 1979) for at least several hours.] To label immature nascent chromatin, cells were pretreated with 50 mM butyrate for 5 min and then incubated with [^{3}H]thymidine (10 μCi/mL) in the continuous presence of butyrate for 5 min (no chase).

To label newly synthesized histones, cells were preincubated in lysine-free MEM (±50 mM sodium butyrate, as required), labeled for 30 min with [^{3}H]lysine (100 Ci/mmol) at 25 μCi/mL in lysine-free MEM (±butyrate), and then chased for 15 min without radiolabel in the presence of excess (10-fold) lysine (Perry & Annunziato, 1989) to allow time for all of the labeled chromatin to regain normal resistance to micrococcal nuclease. For short-term pulses, nascent histones were labeled with 0.5 mCi/mL each of [^{3}H]lysine and [^{3}H]arginine (70 Ci/mmol) for 5 min in the presence of 50 mM sodium butyrate (no chase). To analyze histones synthesized in the absence of ongoing DNA replication, cells were preincubated with 10 mM hydroxyurea for 10 min and then labeled with [^{3}H]lysine for 60 min in the continuous presence of hydroxyurea (plus butyrate). For Western analyses, HeLa histones were moderately acetylated by incubating cells in 7 mM sodium butyrate for 6 h, yielding an even distribution of un-, mono-, di-, tri-, and tetraacetylated forms (Annunziato *et al.*, 1988).

Nuclear Isolation and Chromatin Preparation. Cells were harvested, washed twice in buffer A (10 mM Tris, 5–20 mM sodium butyrate, 3 mM $MgCl_2$, and 2 mM 2-mercaptoethanol, pH 7.6), and resuspended in buffer A. Cells were lysed with a Dounce homogenizer, and nuclei were isolated in buffer A (Annunziato & Seale, 1983). Prior to digestion with micrococcal nuclease, nuclei were washed in digestion buffer [10 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES),[1] 20 mM sodium butyrate, and 80 mM NaCl, pH 7.0], resuspended in digestion buffer at 40 A_{260}/mL, and adjusted to 0.5 mM $CaCl_2$. For double-label experiments, equal aliquots of ^{3}H- and ^{14}C-labeled nuclei were mixed before washing; in this manner, each test of the immunoprecipitation of nascent chromatin contained an internal standard of control bulk chromatin that was never exposed to butyrate. Typically, nuclei were digested with 5 units/mL micrococcal nuclease (Sigma) at 4 °C for 10–15 min, yielding a soluble chromatin fraction, termed S1, containing 35–50% of total nuclear bulk chromatin, as described (Perry & Annunziato, 1989). Nascent chromatin labeled for 5 min was digested with micrococcal nuclease for a shorter period (1–5 min), to compensate for its increased nuclease sensitivity (Annunziato *et al.*, 1981). Hydroxyurea-treated nuclei were digested for 5 min at 4 °C. Nuclease digestions were terminated by adjusting the reaction mixture to 5 mM EGTA.

Preparation of Cytosolic Extracts. Cells were washed with buffer A, and then with HB buffer (20 mM HEPES, 5 mM KCl, 1.5 mM $MgCl_2$, and 5 mM sodium butyrate, adjusted to pH 7.5 with KOH). Cells were resuspended in HB buffer, allowed to swell on ice, and lysed with a Dounce homogenizer. Nuclei were pelleted at 11000*g* for 10 min at 4 °C. The supernatant (cytosolic extract) was then clarified by centrifugation (80000*g*; 90 min) in a Tl-100 ultracentrifuge (Beckman), using a TLA-45 rotor. Extracts were dialyzed overnight against 10 mM PIPES, 80 mM NaCl, 20 mM sodium butyrate, and 1 mM PMSF, pH 7.0, and stored at −70 °C. The presence of acetylated H4 in the cytosolic extract was confirmed by Western analysis, using the "penta" antiserum.

Immunoblotting. Histones were acid-extracted from isolated HeLa nuclei, separated either in the presence of SDS (Thomas & Kornberg, 1975) or in an acid–urea gel system (Panyim & Chalkley, 1969), and transferred electrophoretically to nitrocellulose (Towbin *et al.*, 1979). Acid–urea gels were transferred in the presence of 0.7% acetic acid; SDS gels were transferred in 25 mM Tris–192 mM glycine, 20% methanol, pH 8.3 (Towbin *et al.*, 1979). Routinely, blots were stained with Ponceau Red, quickly destained in water, and photographed before being blocked to provide a photographic record of each blot. To ensure proper alignment of antibody reactions with the total protein profile, the position of each acetylated subspecies of H4 was marked with a pinhole after the acid–urea immunoblots were stained with Ponceau Red. Blots were then blocked with 5% nonfat dried milk and reacted with the "penta" antiserum (Lin *et al.*, 1989) at a 1:300 dilution (during these procedures all of the Ponceau Red stain is removed from the blot). Antibody reactions were then detected in either of two ways: (1) through a secondary antibody color reaction [goat anti-(rabbit IgG) conjugated to horseradish peroxidase], using H_2O_2 and 3′,3′-diaminobenzidine (Harlow & Lane, 1988); or (2) with ^{125}I-protein A (10^6 cpm/mL), followed by autoradiography.

Immunoprecipitation. For all antibody experiments, the "penta" antiserum first described by Lin *et al.* (1989) was used. This rabbit antiserum was raised against an acetylated synthetic peptide that represents the amino terminus of a minor *Tetrahymena* histone variant, hv1. The first eight residues of hv1 are identical to the amino terminus of *Tetrahymena* H4, and closely match the N-terminus of human H4 as well. Moreover, the acetylation pattern of hv1 closely corresponds to those of both ciliate and human H4, with the exception than an additional (fifth) lysine is acetylated in hv1 (Chicoine *et al.*, 1986). For immunoprecipitation reactions, 50–100 μL of undiluted "penta" antiserum was preincubated with up to 25 μL of swollen protein A–Sepharose (Pharmacia). After the Sepharose beads were washed to remove unbound antibody, 75–150 μL of soluble HeLa S1 chromatin in digestion buffer [further adjusted to 0.5% Triton X-100, 0.5 mM dithiothreitol, 1 mM PMSF, and 6 mM EGTA (final concentrations)] was incubated with the immobilized antiserum for 90 min at 37

[1] Abbreviations: PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; PPO, 2,5-diphenyloxazole; DMSO, dimethyl sulfoxide; TCA, trichloroacetic acid.

°C with constant inversion. The beads were then pelleted, yielding an unbound "supernatant" fraction, which was either precipitated with trichloroacetic acid (for scintillation counting) or prepared for electrophoresis (see below). Under these conditions, the antiserum was in approximately 2-fold excess over the chromatin to be analyzed, and less than 4% of the input chromatin was further precipitated by retreating the unbound "supernatant" with freshly immobilized antibodies. The beads were then washed 5 times with wash buffer (20 mM Tris, 0.5 M NaCl, 2 mM EDTA, 1% Triton X-100, 1 mM PMSF, and 20 mM sodium butyrate, pH 8.6), and once with 10 mM Tris, pH 8.6. (The wash buffer releases H1 from chromatin, and thus no conclusions concerning the immunoprecipitation of H1 were possible). Immunopellets were partially dried under vacuum, and either resuspended directly in electrophoresis sample buffer or prepared for scintillation counting as described previously (Annunziato & Seale, 1983). To control for nonspecific sticking of chromatin to the beads, parallel experiments using preimmune serum (instead of the antiserum) were routinely performed. As part of the characterization of the "penta" antiserum for use with HeLa cell chromatin, hyperacetylated bulk chromatin (from cells treated with 7 mM sodium butyrate for 18–24 h) was subjected to immunoprecipitation. Although typically only 5–15% of bulk chromatin is immunoprecipitated (see Results), more than 85% of hyperacetylated chromatin was bound by the antibody (data not shown).

Immunoprecipitations of cytosolic extracts were performed at 21 °C, and were washed with 20 mM Tris, 0.15 M NaCl, 2 mM EDTA, 1% Triton X-100, 20 mM sodium butyrate, and 1 mM PMSF, pH 8.6. In competition experiments, various concentrations either of the penta peptide (originally used to generate the antiserum) or of its unacetylated counterpart with the identical primary sequence were added to the immunoprecipitation reactions (see legends to figures). To determine if nascent proteins in the unbound fraction were bound to chromatin, the immunosupernatant fraction was pelleted through digestion buffer containing 8% sucrose in a TLA-100 rotor (TL-100 tabletop ultracentrifuge, Beckman), at 386000*g* for 16 h; under these conditions, chromatin is pelleted, while 80–100% of "free" protein remains in the sucrose solution, as determined by cosedimenting mixtures of chromatin and BSA.

Gel Electrophoresis and Fluorography. Histones were subjected to electrophoresis in either SDS/or acid–urea/polyacrylamide gel systems and stained with Coomassie Blue, as described previously (Perry & Annunziato, 1989). For DNA size analysis, chromatin samples were ethanol-precipitated and subjected to electrophoresis in 4% polyacrylamide gels in the presence of 0.1% SDS, according to Loening (1967), as described (Annunziato, 1981); DNA gels were stained with ethidium bromide and photographed under ultraviolet illumination. Gels were treated with PPO/DMSO, dried, and exposed to preflashed film in preparation for fluorography (Bonner & Laskey, 1974; Laskey & Mills, 1975). Fluorographs were scanned at 580 nm with a GS 300 densitometer (Hoefer); the peaks were excised and weighed for quantitation.

RESULTS

Antibody Characterization. To test the ability of the "penta" antiserum to detect human H4, HeLa cells were treated with sodium butyrate for 6 h, to yield an even distribution of acetylated and unacetylated H4 subtypes (see Figure 1B, lane S); total acid-soluble nuclear proteins were then subjected to electrophoresis in the presence of SDS,

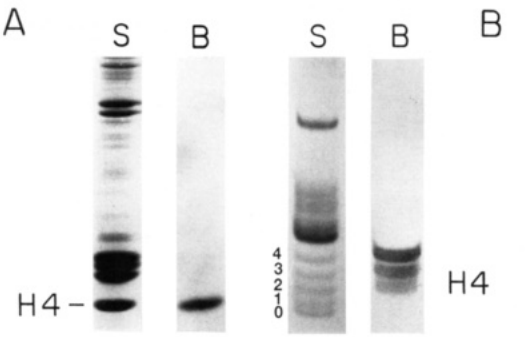

FIGURE 1: "Penta" antibodies specifically recognize acetylated human H4 in Western blots. HeLa cells were pretreated for 6 h with sodium butyrate, to generate an even distribution of acetylated and unacetylated forms of H4 [see Annunziato et al., (1988)]. Acid-soluble nuclear proteins were subjected to electrophoresis in SDS (panel A) or acid–urea gels (panel B). Gels were either stained with Coomassie Blue (lanes marked S) or transferred electrophoretically to nitrocellulose and reacted with "penta" antibodies at a 1:300 dilution (lanes marked B). Antibody reactions were detected either with ^{125}I-protein A (panel A) or with a secondary antibody–peroxidase color reaction (panel B). Only the histone regions of the gels are shown; no other cross-reacting bands were detected. Close inspection of the blotted acid–urea gel (panel B, lane B) reveals pinholes marking the positions of the unacetylated and acetylated forms of H4, detected with Ponceau Red (see Experimental Procedures).

transferred electrophoretically to nitrocellulose, and probed with "penta" antibodies (Figure 1A). On the immunoblot, the antibodies reacted strongly with human H4 (Figure 1A, lane B). No other proteins were recognized by the antiserum. To determine if the "penta" antiserum would specifically recognize the *acetylated* forms of H4, histones from butyrate-treated cells were also separated in an acid–urea gel system. Under these electrophoretic conditions, the acetylated species of H4 migrate more slowly than the unacetylated, or parent, form, yielding a series of well-resolved bands (Figure 1B). On the immunoblot (Figure 1B, lane B), the antibodies strongly recognized the multiacetylated species of H4, and absolutely no reaction with unacetylated H4 was detected. Identical results were obtained when the antibodies were tested against histones from non-butyrate-treated cells (in which the majority of H4 is in the unacetylated form). The "penta" antiserum has also been shown to be specific for acetylated H4 in rat spermatids (Meistrich *et al.*, 1992). It therefore appears that these antibodies provide a widely applicable reagent for the study of histone acetylation.

Immunoprecipitation of Acetylated Nascent Chromatin. For all immunoprecipitation experiments, a soluble chromatin fraction, termed S1, was produced by micrococcal nuclease digestion (see Experimental Procedures). To test the ability of the antiserum to precipitate newly replicated nucleosomes, "penta" antibodies were bound to protein A–Sepharose, and incubated with a mixture of radiolabeled bulk (control) chromatin and acetylated newly replicated chromatin. The latter was generated by replicating chromatin for 30 min in the presence of sodium butyrate, followed by a 15-min chase to allow all of the labeled chromatin to regain full resistance to micrococcal nuclease (Annunziato & Seale, 1983; Perry & Annunziato, 1989). This brief exposure to butyrate prevents the deacetylation of newly synthesized histones, without inducing extensive hyperacetylation; thus, newly synthesized H4 is confined to the di- and triacetylated forms, with no tetraacetylated species in evidence (Cousins & Alberts, 1982; Perry & Annunziato, 1989). The data from two independent experiments are presented in Figure 2.

As seen in Figure 2A, the unbound supernatant (Supt) was dramatically depleted of newly replicated DNA (relative to

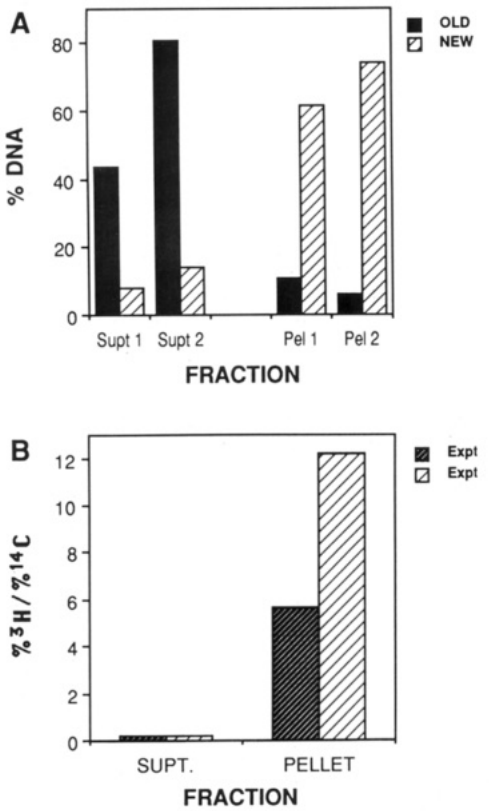

FIGURE 2: Acetylated nascent chromatin is precipitated by "penta" antibodies. (A) HeLa cells were labeled for 30 min with [^{3}H]-thymidine in the presence of sodium butyrate and chased for 15 min without radiolabel in the continuous presence of butyrate (NEW; see right-hand legend). ^{3}H-Labeled nuclei were isolated and mixed with control nuclei from cells prelabeled for one generation with [^{14}C]-thymidine under normal conditions (minus butyrate) (OLD); soluble nucleosomes were then incubated with protein A–Sepharose-conjugated "penta" antiserum. The beads were pelleted, yielding a supernatant fraction (Supt), washed, and recovered by centrifugation, yielding a final Pellet. Data for two independent experiments (numbered 1 and 2) are expressed as the percent of the total acid-precipitable ^{3}H or ^{14}C radioactivity applied to the beads. Radioactivity in the washes is not presented. (B) The ^{3}H/^{14}C ratios in the supernatant and pellet fractions from the data in panel A are given.

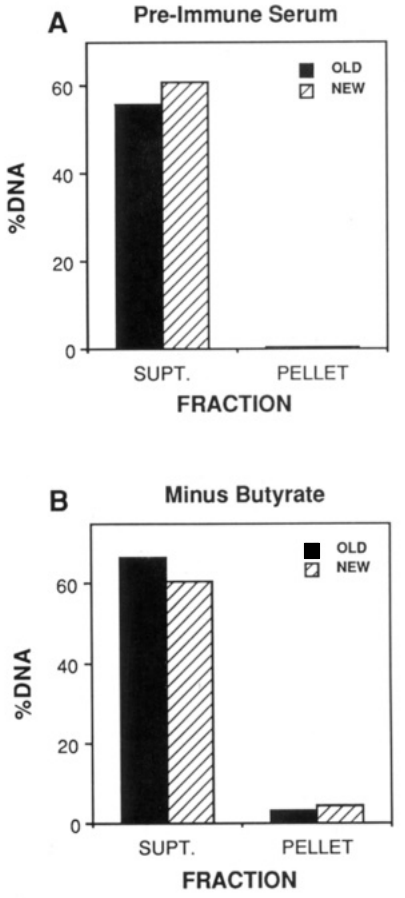

FIGURE 3: Control experiments showing the effects of using the preimmune serum, and of replication in the absence of butyrate. (A) Cells were labeled for 30 min with [^{3}H]thymidine in the presence of sodium butyrate and chased for 15 min without radiolabel in the continuous presence of butyrate (NEW). ^{3}H-Labeled nuclei were isolated and mixed with control nuclei from cells prelabeled for one generation with [^{14}C]thymidine under normal conditions (minus butyrate) (OLD); soluble nucleosomes were then incubated with protein A–Sepharose that had been treated with control preimmune serum, and analyzed as in Figure 2. (B) Cells were incubated with [^{3}H]thymidine for 30 min in the absence of butyrate and chased for 15 min without radiolabel (minus butyrate) (NEW). ^{3}H-Labeled nuclei were isolated and mixed with control nuclei from cells prelabeled for one generation with [^{14}C]thymidine (OLD); soluble nucleosomes were treated with immobilized "penta" antibodies as in Figure 2, yielding supernatant and pellet fractions.

bulk DNA) after incubation with the immobilized "penta" antibody. Moreover, after the beads were washed, only 5–10% of the starting bulk chromatin remained bound, as opposed to 60–80% of the newly replicated chromatin (Figure 2A, Pel). The enrichment for nascent chromatin in the pellet fractions (expressed as % ^{3}H/% ^{14}C) is presented in Figure 2B: When the "penta" antiserum was used, there was a striking 30–65-fold difference in enrichment for new DNA in the pellet, as compared to the supernatant. The immunopellet has consistently displayed a degree of enrichment for newly replicated DNA at the levels shown in Figure 2B, following replication in butyrate.

When protein A–Sepharose was treated with the preimmune serum (instead of the antiserum) prior to incubation with chromatin, less than 0.5% of either new or old DNA appeared in the immunopellet (Figure 3A). Moreover, when replication occurred in the absence of butyrate (permitting the deacetylation of new histones), equally low levels of new and old chromatin remained bound to the immobilized antibodies, and there was no enrichment for new DNA in the pellet (Figure 3B).

The newly replicated chromatin in Figure 2 had been exposed to butyrate for a total of 45 min (including the 15-min chase), and thus it could be argued that our results are dependent upon this length of butyrate treatment. To address this question, cells were labeled with [^{3}H]thymidine for just 5 min in the presence of butyrate, and immediately harvested for nuclear isolation. Following micrococcal nuclease digestion, a mixture of newly replicated and bulk chromatin was subjected to immunoprecipitation with "penta" antibodies. As seen in Figure 4, ~65% of the input nascent chromatin was immunoprecipitated, fully consistent with results obtained with longer butyrate exposure. Because chromatin replicated for 5 min contains new H4 that is exclusively in the diacetylated form [data not presented; also see Ruiz-Carrillo *et al.* (1975) and Jackson *et al.* (1976)], it is concluded that physiological levels of histone acetylation can promote efficient immunoprecipitation of newly assembled chromatin (see also Figure 9).

The above experiments permit several observations. First, it seems clear that the conjugated antiserum can recognize acetylated H4 that is complexed in nucleosomes, with considerable specificity. Similar findings have been obtained by other investigators using antibodies directed against acetylated H4 (Pfeffer *et al.*, 1986; Hebbes *et al.*, 1988; Braunstein *et al.*, 1993). Second, the reaction conditions do

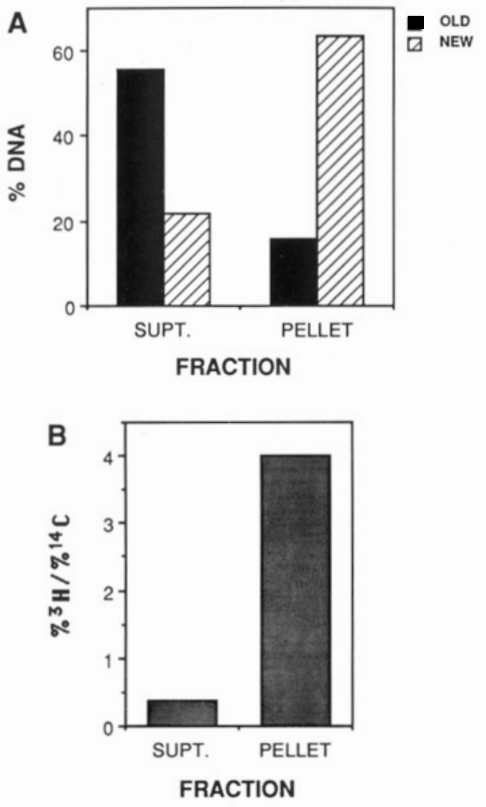

FIGURE 4: Immunoprecipitation of newly replicated chromatin. (A) Cells were labeled with [^{3}H]thymidine for 5 min in the presence of sodium butyrate (no chase), and chromatin was prepared by micrococcal nuclease digestion for 1 min (NEW). Control chromatin was prepared (10-min nuclease digest) from cells prelabeled for one generation with [^{14}C]thymidine (OLD). By separately preparing new and old nucleosomes in this manner, the preferential nuclease sensitivity of newly replicated chromatin was compensated for (Annunziato et al., 1981). A mixture of [^{3}H]- and [^{14}C]chromatin was treated with immobilized "penta" antibodies, yielding supernatant and pellet fractions. Results are analyzed as in Figure 2. (B) The ^{3}H/^{14}C ratios in the supernatant and pellet fractions from the data in panel A are given.

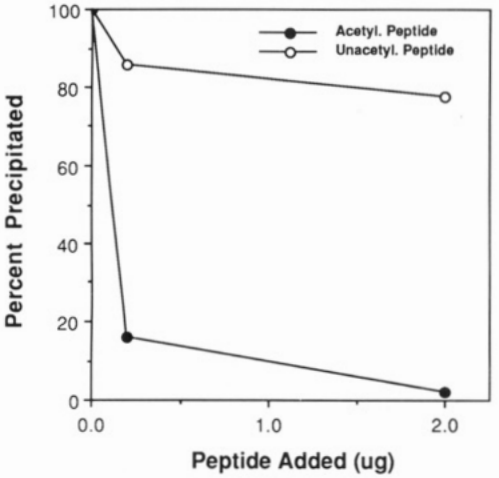

FIGURE 5: Competition of chromatin immunoprecipitation with exogenous peptides. Cells were labeled for 30 min with [^{3}H]thymidine in the presence of sodium butyrate. Soluble nucleosomes were incubated with immobilized "penta" antibodies, to obtain an immunopellet. Prior to incubation with immobilized antibodies, various amounts either of the acetylated "penta" peptide (closed circles) or of its unacetylated counterpart (with the identical primary sequence; open circles) were added to the chromatin sample. Data are plotted by setting the value from the control (no peptide added) as 100% precipitated. Each point represents the average of two independent experiments.

not disrupt histone–DNA interactions within the nucleosome. This is evident because it was the DNA that was labeled, and subsequently used as a tracer for new and old chromatin; had nucleosomes been significantly disassembled during the procedure, new DNA would not have been detected in the pellet fraction. Finally, these results confirm that histone H4 on newly replicated DNA is acetylated, but is rapidly deacetylated under normal conditions (minus butyrate) (Ruiz-Carrillo *et al.*, 1975; Jackson *et al.*, 1976).

Competition of Immunoprecipitation with Exogenous Peptides. One trivial explanation for the immunoprecipitation data is that chromatin replicated in butyrate has a more "open" conformation, simply rendering it more accessible to the antibody. To ensure that the "penta" antiserum was recognizing the acetylated histone tail, competition experiments were performed. Cells were labeled with [^{3}H]thymidine for 30 min in the presence of butyrate (to label acetylated nascent chromatin), and soluble nucleosomes were immunoprecipitated with immobilized antibodies as in Figures 2 and 4. Different amounts either of the original penta peptide (used to generate the antiserum) or of its unacetylated counterpart were added to the reaction. As seen in Figure 5, low levels of the acetylated peptide blocked immunoprecipitation by 85%, and higher levels virtually eliminated the binding of newly replicated chromatin. In contrast, the unacetylated peptide had little effect, even at the highest concentration. Moreover, acetylation *per se* was not inhibitory: treating the immobilized antibodies with acetylated serum albumin did not inhibit immunoprecipitation (not shown). When considered together with the Western analyses and "minus butyrate" experiments presented above, the data provide strong evidence that the "penta" antiserum specifically recognizes the acetylated N-terminus of histone H4 in human chromatin.

Distribution of Newly Replicated DNA in Fractionated Chromatin. The size distribution of new DNA in the supernatant and pellet fractions was then examined. Chromatin was replicated in the presence of [^{3}H]thymidine and sodium butyrate, digested with micrococcal nuclease, and immunoprecipitated with immobilized antibody. The supernatant and pellet fractions, together with the starting S1 chromatin, were then subjected to electrophoresis in a polyacrylamide gel system, and analyzed by ethidium bromide staining (Figure 6A) and fluorography (Figure 6B).

Under our standard nuclease digestion conditions, the S1 containing bulk chromatin ranging in size from monomers to high molecular weight polynucleosomes, in relatively equal proportions (Figure 6A, lane I). Because chromatin replicated in butyrate for 30 min is not preferentially cleaved by micrococcal nuclease (Annunziato & Seale, 1983), the size distribution of nascent chromatin in S1 (Figure 6B, lane I) mirrored the ethidium bromide stain. Thus, chromatin maturation, as measured by resistance to micrococcal nuclease, does not inhibit immunoprecipitation (Figure 2). A comparison of the DNA in the supernatant and pellet fractions revealed that the Sepharose-bound antibody preferentially recognized polynucleosomes, and had little affinity for individual monomers (lanes S and P_1). Treating the supernatant a second time with fresh antiserum did not alter the selective recognition of higher molecular weight chromatin (lane P_2). One possible explanation for this preference is that longer chromatin fibers possess more potential antibody binding sites (*i.e.*, acetylated H4 molecules), thereby permitting more efficient immunoprecipitation. Another factor may be the relative accessibility of the conjugated antibody to acetylated H4 tails within polynucleosomes, as opposed to monomers. Similar observations have been made by others (Hebbes *et*

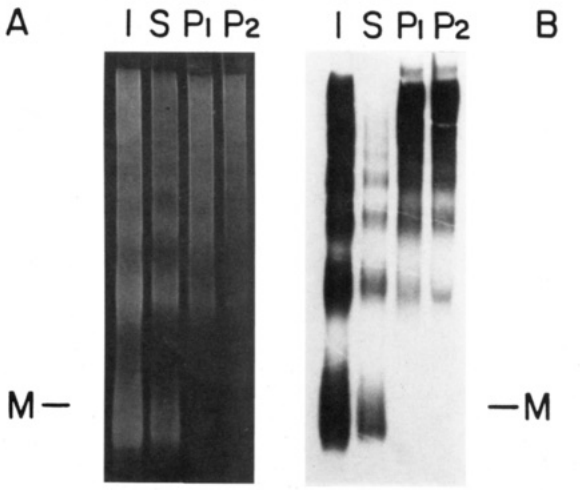

FIGURE 6: Size distribution of newly replicated DNA in fractionated chromatin. Cells were incubated with [^{3}H]thymidine for 30 min in the presence of butyrate and chased for 15 min in the absence of radiolabel (plus butyrate). Soluble input nucleosomes (lane I) were treated with immobilized "penta" antibodies, yielding supernatant (lane S) and pellet (lane P_1) fractions. The supernatant was also treated a second time with fresh Sepharose-conjugated antibodies, yielding a second immunopellet (lane P_2). Chromatin samples were subjected to electrophoresis and analyzed by ethidium bromide staining (panel A) and fluorography (panel B); the position of mononucleosomal DNA is indicated (M). Note: Loads have been adjusted to yield optimum resolution in gels; thus, these data do not relfect the absolute percentage of either newly replicated or bulk DNA present in each fraction (see Experimental Procedures).

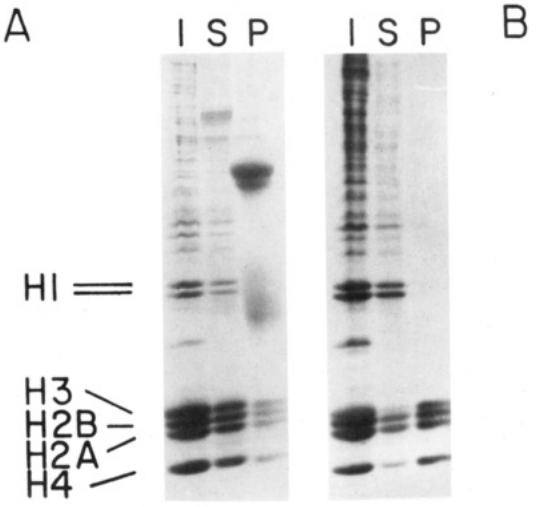

FIGURE 7: Immunoprecipitation of newly synthesized histones. Cells were labeled for 30 min with [^{3}H]lysine in the presence of sodium butyrate and then chased for 15 min in the absence of radiolabel (plus butyrate). Soluble nucleosomes (lane I) were treated with immobilized "penta" antibodies, yielding supernatant (lane S) and pellet (lane P) fractions; during these procedures, histone H1 is lost from the immunopellet (see Experimental Procedures). Chromatin fractions were subjected to electrophoresis in the presence of SDS, stained with Coomassie Blue (panel A), and analyzed by fluorography (panel B). Note: All of the chromatin from the supernatant and pellet fractions was loaded on the gel; proteins in the wash are not shown.

al., 1988), suggesting that this may be a general feature of antibodies directed against acetylated H4.

In agreement with the results obtained from scintillation counting (Figure 3A), no DNA could be detected in the pellet by means of electrophoresis when the preimmune serum was substituted for the "penta" antibody (data not presented).

Immunoprecipitation of Newly Synthesized Histones. To label acetylated newly synthesized histones, cells were incubated for 30 min with [^{3}H]lysine in the presence of sodium butyrate and then chased for 15 min in the absence of radioactive lysine (plus butyrate). An S1 chromatin fraction was prepared by nuclease digestion and treated with immobilized antibodies; the supernatant and pellet fractions were then analyzed by gel electrophoresis, Coomassie Blue staining (Figure 7A), and fluorography (Figure 7B).

Core histones synthesized and assembled in the presence of butyrate were highly preferentially immunoprecipitated, resulting in a very strong labeling of the pellet, despite the low level of "old" histone bound to the beads (compare lanes S and P, panels A and B). The enrichment for all four new core histones complements the enrichment for new DNA (observed in Figures 2 and 4), and provides further evidence that nucleosomes remain intact during the antibody treatment. However, because the antiserum recognizes both new and old acetylated H4, these data provide no evidence either for or against the conservative assembly of newly synthesized histones into the same nucleosomes; rather, the preferential association of new H2A, H2B, and H3 with *acetylated* H4 (either new or old) is indicated.

Inspection of the fluorograph (Figure 7B) suggested that there was a relative excess of H2A/H2B over H3/H4 in the supernatant fraction. This was confirmed by scanning the film. Approximately 78% of the new H3 and H4 was bound to the beads, while only 60–65% of the new H2A/H2B was immunoprecipitated, causing the nascent histone stoichiometries in the supernatant and pellet to be slightly different. After allowing for the 20% of new H4 in the supernatant (which might be complexed with up to 20% of the cofractionating new H2A/H2B), there still remained ~15% of new H2A/H2B in the supernatant that is apparently not associated with new H4. The "extra" new H2A/H2B in the supernatant appeared to be chromatin-bound, as judged by sedimentation through sucrose (data not presented). The disproportionate precipitation of H3/H4 was consistently observed throughout several experiments, in which the efficiency of H4 precipitation varied from 40% to 80% (please see Discussion).

As a control, newly synthesized histones were labeled under conditions that allowed for the normal deacetylation of H4. Cells were incubated with [^{3}H]lysine for 30 min without butyrate and then chased for 15 min in the absence of radiolabel (minus butyrate). Soluble chromatin was then treated with immobilized "penta" antibodies and analyzed by electrophoresis and fluorography (Figure 8). When newly synthesized H4 was permitted to undergo normal deacetylation, ~3% of the labeled histone was detected in the immunopellet (as determined by scanning the fluorograph), and thus the new histones remained almost entirely in the supernatant (lane S, Figure 8B). No histones, either new or old, remained bound to the beads when the preimmune serum was used. The immunoprecipitation of newly synthesized histones (Figure 7), like that of nascent DNA, is therefore strongly correlated with ongoing histone acetylation.

To test the ability of the antiserum to recognize newly assembled nucleosomes containing physiological levels of acetylation, new histones were radiolabeled with [^{3}H]lysine and [^{3}H]arginine for 5 min (plus butyrate), and soluble chromatin was immunoprecipitated. Fully consistent with the results presented above, new nucleosomes containing exclusively the nascent, diacetylated form of H4 [as determined by subjecting nuclear proteins to electrophoresis in an acid–urea gel system (not shown)] were efficiently immunoprecipitated with "penta" antibodies (Figure 9). Approximately 60–70% of the newly synthesized histones appeared in the

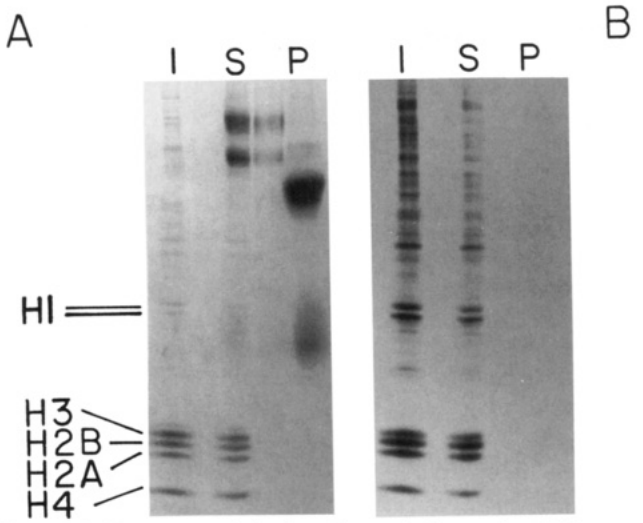

FIGURE 8: Immunoprecipitation of control chromatin. Cells were labeled for 30 min with [^{3}H]lysine without butyrate and then chased for 15 min in the absence of radiolabel (minus butyrate). Soluble nucleosomes (lane I) were treated with immobilized "penta" antibodies, yielding supernatant (lane S) and pellet (lane P) fractions. Chromatin fractions were subjected to electrophoresis in the presence of SDS, stained with Coomassie Blue (panel A), and analyzed by fluorography (panel B). Note: All of the chromatin from the supernatant and pellet fractions was loaded on the gel.

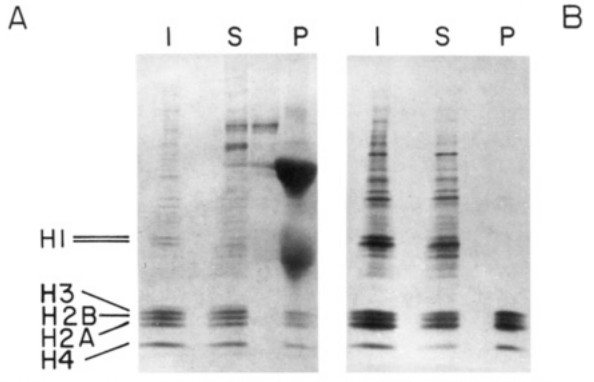

FIGURE 9: Immunoprecipitation of newly assembled nucleosomes. Cells were labeled with [^{3}H]lysine and [^{3}H]arginine for 5 min (no chase). Soluble nucleosomes (lane I) were treated with immobilized "penta" antibodies, yielding supernatant (lane S) and pellet (lane P) fractions. Chromatin fractions were subjected to electrophoresis in the presence of SDS, stained with Coomassie Blue (panel A), and analyzed by fluorography (panel B). Note: All of the chromatin from the supernatant and pellet fractions was loaded on the gel.

pellet fraction (as measured by scanning the film). When considered together with the results of Figure 4 (5-min label with [^{3}H]thymidine), it is evident that histone–DNA interactions stable to immunoprecipitation are formed within minutes of DNA replication and histone deposition and that lengthy exposure to butyrate is not required to immunoprecipitate chromatin replicated and assembled *in vivo*.

Exchange of New H2A and H2B into Acetylated Chromatin. When DNA replication is inhibited, histone synthesis persists at a reduced rate for up to several hours (Nadeau *et al.*, 1978). Under these conditions, newly synthesized H2A and H2B continue to enter chromatin, while new H3 and H4 accumulate in a free pool (Louters & Chalkley, 1985; Bonner *et al.*, 1988; Jackson, 1990), which can be detected in the cytosol following nuclear isolation (Louters & Chalkley, 1985; Bonner *et al.*, 1988). The successful immunoprecipitation of all four newly synthesized histones during normal chromatin synthesis prompted the analysis of the deposition of H2A/H2B in the absence of replication. log-phase cells were

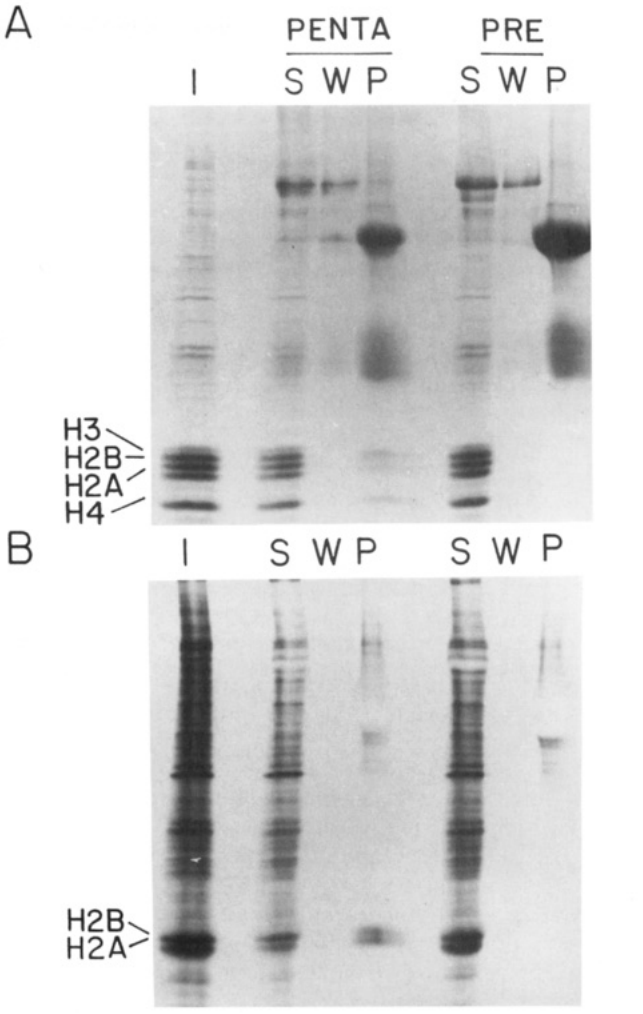

FIGURE 10: Immunoprecipitation of chromatin-bound histones following labeling in the presence of hydroxyurea. Cells were labeled with [^{3}H]lysine for 60 min in the presence of 10 mM hydroxyurea; soluble nucleosomes (lane I) were treated either with immobilized "penta" antibodies (PENTA) or with protein A–Sepharose that had been pretreated with nonimmune serum (PRE), yielding supernatant (lane S), wash (W), and pellet (P) fractions. Chromatin fractions were subjected to electrophoresis in the presence of SDS, stained with Coomassie Blue (panel A), and analyzed by fluorography (panel B). Note that preexisting H3 and H4, as well as old H2A and H2B, can be seen in the Coomassie-stained immunopellet (panel A, PENTA, lane P), consistent with the immunoprecipitation of intact nucleosomes; the identity of labeled H2A/H2B was verified by overlaying the fluorograph upon the dried gel (which retains its Coomassie Blue stain).

preincubated in hydroxyurea and butyrate (to allow time for nucleosome assembly to be completed on recently replicated DNA) and then labeled for 60 min with [^{3}H]lysine in the continuous presence of hydroxyurea (plus butyrate). Soluble nucleosomes were immunoprecipitated with "penta" antibodies, and analyzed by electrophoresis, Coomassie Blue staining, and fluorography (Figure 10).

Consistent with previous findings (Louters & Chalkley, 1985; Jackson, 1990), H2A and H2B were the predominant new core histones associated with chromatin following hydroxyurea treatment (compare Figure 10A,B, lane I). As seen in the stained gel of the immunoprecipitation reaction, typically low levels of bulk histone (~10%) were found in the immunopellet when the "penta" antiserum was used (panel A, PENTA, lane P). In contrast, 40–45% (as determined by scanning) of the chromatin-bound new H2A/H2B was precipitated with antibodies specific for acetylated H4 (panel B, PENTA, lane P), resulting in a marked enrichment for

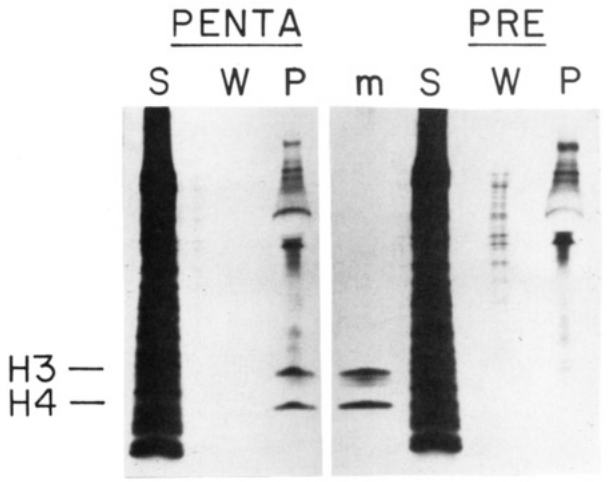

FIGURE 11: Immunoprecipitation of cytosolic H3 and H4. Cells were labeled with [^{3}H]lysine for 60 min in the presence of 10 mM hydroxyurea. A cytosolic extract was prepared and incubated either with immobilized "penta" antibodies (PENTA) or with beads that had been pretreated with nonimmune serum (PRE), yielding supernatant (lane S) and pellet (lane P) fractions. Chromatin fractions were subjected to electrophoresis in the presence of SDS, stained with Coomassie Blue, and analyzed by fluorography; only the fluorograph is shown. Lane m: acid-extracted, acetylated histones labeled with [^{3}H]acetate, used as markers for the positions of nascent H3 and H4.

new H2A/H2B over the coprecipitating bulk histone. No histones appeared in the pellet when control serum was used (PRE, panel B). In separate experiments, we have precipitated up to 60% of input new H2A/H2B with "penta" antibodies. Further, as with histones synthesized under control conditions (Figure 8), only ~5–10% of the new H2A/H2B was immunoprecipitated when butyrate was omitted during hydroxyurea treatment (data not presented). The most likely explanation for these results is that new H2A and H2B preferentially exchange into chromatin that undergoes dynamic acetylation (and deacetylation) when replication is halted.

A Possible Preassembly Histone Complex. We then examined the new H4 that is found in the cytosol following the inhibition of DNA synthesis (Louters & Chalkley, 1985; Bonner *et al.*, 1988). Cells were labeled with [^{3}H]lysine in the presence of hydroxyurea; a cytosolic extract was prepared, and incubated with immobilized "penta" antibodies (or preimmune serum). The unbound supernatants and the washes were TCA-precipitated, and subjected to electrophoresis along with the immunopellets (Figure 11).

Along with new cytosolic H4, a newly synthesized protein with the mobility of H3 was coprecipitated by "penta" antibodies, in apparently equimolar concentration (PENTA, lane P). Given the high specificity of the antiserum (Figures 1 and 5), and the total absence of new histones in the immunopellet when nonimmune serum was used (Figure 11, PRE), it seems likely that new H3 is being precipitated as a complex with newly synthesized H4, at least under the "physiological" ionic conditions used in this study. The coprecipitation of H3/H4 was not affected by treatment with DNase I; however, washing the immunopellet with 0.5 M NaCl removed most of the H3, while leaving H4 bound (data not shown). Thus, "penta" antibodies were not interacting directly with H3. During normal replication *in vivo*, new H3/H4 is targeted to newly synthesized DNA for *de novo* nucleosome assembly (Worcel *et al.*, 1978; Senshu *et al.*, 1978; Jackson & Chalkley, 1981a,b; Annunziato *et al.*, 1982). It therefore seems possible that these nascent proteins are associated in a preassembly histone complex, which accumulates in the absence of ongoing DNA replication.

DISCUSSION

The "penta" antibody generated by Lin *et al.* (1989) is now shown to identify acetylated human H4 with a great deal of specificity, and when bound to protein A–Sepharose to recognize acetylated H4 in chromatin. This latter ability was exploited to immunoprecipitate newly replicated (and newly assembled) nucleosomes, in an acetylation-dependent manner. We are of course well aware that this does not constitute a new method for the specific *purification* of nascent chromatin; we have found that any acetylated chromatin fraction (whether new or old) is capable of being immunoprecipitated with these antibodies [see Perry et al. (1993); also, *cf.* Hebbes *et al.* (1988) and Braunstein *et al.* (1993)]. Nevertheless, through appropriate radiolabeling procedures, it has been possible to investigate the interaction of histone acetylation with chromatin biosynthesis. Several conclusions can be drawn from these studies.

First, our results confirm that newly replicated chromatin is acetylated *in vivo*. A correlation between histone acetylation and chromatin replication had earlier been deduced from the diacetylation of newly synthesized H4 (Ruiz-Carrillo *et al.*, 1975; Jackson *et al.*, 1976) and the preferential association of new H3/H4 with newly replicated DNA (Senshu *et al.*, 1978; Worcel *et al.*, 1978; Jackson & Chalkley, 1981a,b). The strong enrichment for both new DNA and new H3/H4 in the immunopellet is entirely consistent with the preferential targeting of new H4 to nascent chromatin [for a discussion of the acetylation of segregated parental histones, please see Perry *et al.* (1993)].

Second, it is clear that at least 60–65% of newly synthesized H2A/H2B is assembled into nucleosomes containing acetylated H4. Thus, the acetylation of histone H4 is central to the deposition of newly synthesized H2A, H2B, and H3, whether or not these new core histones are conservatively assembled together. It must be stressed that because the "penta" antibody does not uniquely recognize newly synthesized H4, our experiments do not specifically address the question of conservative versus nonconservative assembly mechanisms [reviewed in Annunziato (1990)]. Still, the difference in the immunoprecipitation of new H3/H4 relative to new H2A/H2B, though small, suggests that not all new core histones are coordinately assembled into the same nucleosomes, in agreement with results from several laboratories (Senshu *et al.*, 1978; Jackson & Chalkley, 1981a,b, 1985; Annunziato *et al.*, 1982; Kumar & Leffak, 1986). It is unlikely that these data can be explained by large differences between the pools of new H2A/H2B and new H3/H4: Where examined, pools of the four new core histones have been found to be essentially equivalent, and very small, during ongoing replication (Oliver *et al.*, 1974; Bonner *et al.*, 1988). Moreover, butyrate-treated cells were typically preincubated in butyrate prior to the addition of radiolabel, to ensure the assembly of H2A/H2B with acetylated H4 during stepwise nucleosome assembly (Fotedar & Roberts, 1989; Almouzni *et al.*, 1990; Zucker & Worcel, 1990; Kleinschmidt *et al.*, 1990; Smith & Stillman, 1991). On the other hand, the observation of histone exchange reported herein implies that a limited pool of *old* H2A/H2B may exist in HeLa cells. As suggested by Jackson (1990), nucleosome dissolution during replication and transcription could provide such a pool. It should be noted that under the labeling–chasing protocol used in most of our experiments there is ample time for nucleosome assembly to

be completed, even in the presence of butyrate; this is reflected in the complete recovery of normal resistance to micrococcal nuclease, and the normal stoichiometry of the nascent core histones in unfractionated chromatin (Annunziato & Seale, 1983; Perry & Annunziato, 1989).

Third, the preferential immunoprecipitation of new H2A and H2B during the inhibition of DNA synthesis strongly suggests that acetylated chromatin is the favored substrate for histone exchange, at least under these conditions. Because typically ~45–60% of H2A/H2B was immunoprecipitated following hydroxyurea treatment, it is not possible at this time to conclude if the exclusive sites for exchange are in acetylated chromatin. Similarly, it is not yet known why a minor subclass of nucleosomes containing new H2A/H2B should be relatively resistant to immunoprecipitation following replication in butyrate (Figure 7): factors affecting antibody binding include the degree of antibody accessibility and the overall efficiency of immunoprecipitation. Nevertheless, the strong *preference* (at least) of new H2A/H2B for acetylated chromatin during exchange is indicated. Because acetylated H4 is enriched in transcriptionally active chromatin (Vavra *et al.*, 1982; Allegra *et al.*, 1987; Ip *et al.*, 1988; Hebbes *et al.*, 1988; Ridsdale *et al.*, 1990), our results support proposed models linking gene activity to histone exchange and chromatin remodeling (Jackson & Chalkley, 1985; Jackson, 1987, 1988, 1990; Pfaffle *et al.*, 1990; Hendzel & Davie, 1990).

The coprecipitation of cytosolic new H3 and H4 raises the intriguing possibility that these histones may be associated in a somatic nucleosome assembly complex. It has of course long been known that H3 and H4 can associate to form tetramers under a range of ionic conditions (Kornberg & Thomas, 1974; D'Anna & Isenberg, 1974; Thomas & Kornberg, 1975), and our results may simply reflect the propensity of these histones to interact. It is also possible that the cytosolic H3/H4 is associated with old H2A/H2B in a preformed histone octamer (Stein, 1979; Stein *et al.*, 1985). However, the clear demonstration, in *Xenopus* oocytes, of an assembly complex comprising H3, H4, and the nuclear protein N1 (Kleinschmidt *et al.*, 1985; Dilworth *et al.*, 1987; Sapp & Worcel, 1990; Zucker & Worcel, 1990) suggests that a similar complex may exist in somatic cells, especially in light of the stepwise assembly of replicating DNA [reviewed in Wolffe (1991a,b); also see above references]. Jackson (1990) has presented evidence that when replication is inhibited, at least some new H3 and H4 enter the nucleus, but can only associate very weakly with chromatin in nonnucleosomal structures. Moreover, Louters and Chalkley (1985) have argued that a factor may interact with H3 and H4 that accumulate during hydroxyurea treatment, thereby preventing them from binding tightly (or at all) to chromatin. Similarly, the observation that cytosolic H3/H4 flow through phosphocellulose, while purified H3/H4 remain bound, led Smith and Stillman (1991) to consider the existence of an associated escort polypeptide. Whether such a factor exists, and (if so) whether it is related to other somatic chromatin assembly factors (Smith & Stillman, 1989; Ishimi *et al.*, 1987; Lässle *et al.*, 1992), awaits further study.

In summary, using antibodies specific for acetylated H4, it has been possible to immunoprecipitate newly replicated/newly assembled nucleosomes. When replication accompanies histone synthesis, most (or all) new H3, and a minimum of 60–65% of new H2A/H2B, becomes complexed with acetylated H4. In the absence of DNA synthesis, the preferential deposition of new H2A and H2B onto acetylated chromatin persists, while new H3/H4 form a possible preassembly histone complex. The ability of "penta" antibodies to recognize large macromolecular complexes provides a powerful reagent for the study of histone–DNA interactions during chromatin biosynthesis. In the following paper (Perry *et al.*, 1993), the acetylation of parental histones during chromatin replication is examined.

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