Articles

Histones in Transit: Cytosolic Histone Complexes and Diacetylation of H4 During Nucleosome Assembly in Human Cells†

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ABSTRACT: The organization and acetylation of nascent histones prior to their stable incorporation into chromatin were examined. Through sedimentation and immunoprecipitation analyses of HeLa cytosolic extracts, two somatic non-nucleosomal histone complexes were detected: one containing nascent H3 and H4, and a second containing H2A (and probably H2B) in association with the nonhistone protein NAP-1. The H3/H4 complex has a sedimentation coefficient of 5−6S, consistent with the presence of one or more escort proteins. H4 in the cytosolic H3/H4 complex is diacetylated, fully in accord with the acetylation state of newly synthesized H4 in chromatin. The diacetylation of nascent human H4 is therefore completed prior to nucleosome assembly. As part of our studies of the nascent H3/H4 complex, the cytoplasmic histone acetyltransferase most likely responsible for acetylating newly synthesized H4 was also investigated. HeLa histone acetyltransferase B (HAT B) acetylates H4 but not H3 *in vitro*, and maximally diacetylates H4 even in the presence of sodium butyrate. Human HAT B acetylates H4 exclusively on the lysine residues at positions 5 and 12, in complete agreement with the highly conserved acetylation pattern of nascent nucleosomal H4 (Sobel *et al.*, 1995), and has a native molecular weight of ∼100 kDa. Based on our findings a model is presented for the involvement of histone acetylation and NAP-1 in H2A/H2B deposition and exchange, during nucleosome assembly and chromatin remodeling *in vivo*.

Histone post-translational modifications have long been correlated with the spatial and temporal modulation of chromatin function [see van Holde (1988) and Wolffe (1995)]. One of the more firmly established examples of this is the reversible acetylation of newly synthesized histone H4 during chromatin biosynthesis: in organisms as diverse as protozoa, insects, echinoderms, and vertebrates, the ϵ-amino group of specific lysine residues in the N-terminal "tail" domain of newly synthesized H4 is first acetylated, then deacetylated as chromatin assembly proceeds [Allis *et al.*, 1985; Giancotti *et al.*, 1984; Chambers & Shaw, 1984; Ruiz-Carrillo *et al.*, 1975; Jackson *et al.*, 1976; Shimamura & Worcel, 1989; Dimitrov *et al.*, 1993; reviewed in Annunziato (1995)]. Although histone H4 is potentially acetylated on each of four lysine residues (those at positions 5, 8, 12, and 16), only a specific subset of these sites are modified in new H4. It has recently been shown that in such evolutionarily distant organisms as *Drosophila* and humans, new H4 is exclusively acetylated on lysines 5 and 12 (K5/K12), in complete conformity with the analogous K4/K11 deposition-related pattern exhibited by *Tetrahymena* H4 (in which the arginine residue at position 3 has been deleted) (Chicoine *et al.*, 1986; Sobel *et al.*, 1995). That the acetylation pattern of new H4 has been so rigorously conserved—at least since the divergence of multicellular organisms from the Protista—strongly suggests a unique role for this modification during nucleosome assembly.

Likely candidates for the enzyme(s) responsible for acetylating newly synthesized H4 have been described. These are the "type B" (*i.e.*, histone acetyltransferase B or HAT B) class of enzymes, which appear in the cytosol following the isolation of nuclei. Among the hallmark features of canonical HAT B enzymes are that they are generally specific for H4, acetylate free H4, and appear to be cytoplasmic (Garcea & Alberts, 1980; Sures & Gallwitz, 1980; Weigand & Brutlag, 1981; Salvador *et al.*, 1985; Richman *et al.*, 1988; Lopez-Rodas *et al.*, 1991a,b; Parthun *et al.*, 1996). If HAT B enzymes act to acetylate nascent H4 during chromatin assembly, then among their properties should be the ability to generate the deposition-specific K5/K12 acetylation pattern (or its equivalent). In the case of *Tetrahymena* this prediction is realized, and microsequence analysis has shown that *Tetrahymena* HAT B acetylates H4 *in vitro* at lysines 4 and 11 (Richman *et al.*, 1988). However, despite numerous studies of HAT B activities from a variety of species, no other native acetyltransferase capable of acetylating H4 completely and exclusively in the deposition-related pattern has thus far been described. For example, a cytoplasmic HAT B activity prepared from *Drosophila* embryos solely acetylates lysine 12 (Sobel *et al.*, 1994), even though chromatin-bound nascent H4 in this system is

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diacetylated in the conserved K5/K12 pattern (Sobel *et al.*, 1995). Similarly, a HAT B-type activity isolated from budding yeast was also specific for K12 when a peptide representing the amino-terminus of yeast H4 was used as a substrate (Kleff *et al.*, 1995; Parthun *et al.*, 1996), although the recombinant yeast enzyme exhibited a broader specificity (Parthun *et al.*, 1996). In contrast, HAT B prepared from pea embryos acetylates lysines 5, 12, and 16 equally well, with no clear preference for any of these sites (Mingarro *et al.*, 1993).

It is now well established that nucleosome assembly occurs in a stepwise fashion, with the deposition of H3/H4 preceding that of H2A/H2B (Dilworth *et al.*, 1987; Fotedar & Roberts, 1989; Almouzni *et al.*, 1990; Sapp & Worcel, 1990; Zucker & Worcel, 1990; Kleinschmidt *et al.*, 1990; Smith & Stillman, 1991). The sequential nature of chromatin biosynthesis is reflected in the manner in which histones are sequestered in cell types that maintain large histone pools. For example, in *Xenopus laevis* oocytes, which possess approximately 15 000 nuclear equivalents of core histone (Woodland & Adamson, 1977), H3/H4 are associated with the 110 kDa protein N1, while H2A/H2B are complexed with a pentamer of nucleoplasmin; notably, H4 in the H3/H4·N1 complex is diacetylated [reviewed in Laskey *et al.* (1993)]. In non-embryonic cells, however, histone pools are extremely small, and the *in vivo* organization of nascent histones prior to nucleosome assembly is only partially understood.

Stillman and colleagues, utilizing a cell-free system derived from human cultured cells, have described key elements of the pathway by which somatic histones are targeted to newly replicated DNA (Stillman, 1986; Smith & Stillman, 1989, 1991; Kaufman *et al.*, 1995; Verreault *et al.*, 1996). During replication-coupled nucleosome assembly, the nuclear factor CAF-1 (a three-subunit protein complex) appears to function as a molecular escort, bringing new H3/H4 to nascent DNA (Smith & Stillman, 1989, 1991; Kaufman *et al.*, 1995). The *in vitro* assembly reaction also relies on the small pool of soluble histones found in the cytosol (Smith & Stillman, 1989; Fotedar & Roberts, 1989); these are very likely newly synthesized proteins in transit to the nucleus (Smith & Stillman, 1991; Perry *et al.*, 1993b). Although both the p150 and p60 subunits of CAF-1 are needed to recruit H3/H4, most cytosolic histones are not associated with either of these proteins (Kaufman *et al.*, 1995; Verreault *et al.*, 1996).

A thorough explanation of *in vivo* nucleosome assembly will require an understanding of the disposition of nascent H3 and H4 prior to their interaction with CAF-1, of how H2A/H2B are deposited onto replicating chromatin, and of the acetylation dynamics of newly synthesized H4. We have therefore examined the complement of "cytosolic" histones that are required for replication-coupled nucleosome assembly *in vitro*. In cytosolic extracts prepared from control HeLa cells newly synthesized H3/H4 form a complex prior to their association with the CAF-1 complex, in agreement with our previous findings for cells treated with the DNA synthesis inhibitor hydroxyurea (Perry *et al.*, 1993b). The H3/H4 complex sediments in sucrose gradients at ~5−6S (greater than expected for a simple H3/H4 tetramer) and contains diacetylated H4. Cytosolic H2A and H2B also appear to be assembled into a fast-sedimenting complex, and evidence is now presented that in HeLa cells cytosolic H2A is associated with the putative "assembly protein" NAP-1, as was recently shown for H2A and H2B in *Drosophila* embryo extracts (Ito *et al.*, 1996). Thus, as with embryonic systems, stepwise nucleosome assembly in somatic cells may involve two distinct histone/escort−factor complexes.

We have also investigated the human cytoplasmic acetyltransferase which in all likelihood acetylates newly synthesized H4. Unlike all metazoan HAT B enzymes thus far described, the native HeLa activity diacetylates H4 precisely and exclusively in the K5/K12 deposition pattern. Sucrose gradient analysis indicates that the molecular weight of native HAT B is approximately 100 kDa, similar to HAT B enzymes from calf thymus (Sures & Gallwitz, 1980) and maize embryos (Eberharter *et al.*, 1996).

EXPERIMENTAL PROCEDURES

Cell Culture and Labeling, Histone and Cytoplasmic Extract Preparation. HeLa cells were maintained in spinner culture at 37 °C in minimal essential medium (Joklik modification) supplemented with 5−10% calf serum. Newly synthesized histones were labeled with 1.0 mCi/mL [^{3}H]-lysine (100 Ci/mmol; New England Nuclear) for 5 min in the presence of 50 mM sodium butyrate, as described previously (Annunziato *et al.*, 1982). In some cases, cells were treated with 10 mM hydroxyurea for 1−2 h (in the presence of 50 mM sodium butyrate). Marker acetylated histones were radiolabeled with [^{3}H]acetyl-CoA in isolated nuclei (Perry *et al.*, 1993a).

HeLa S100 cytoplasmic extracts were prepared essentially according to the procedure of Stillman (1986), as described previously (Perry *et al.*, 1993b). Briefly, cells were washed in Buffer A (10 mM Tris-HCl, 5 mM sodium butyrate, 3 mM $MgCl_2$, 2 mM 2-mercaptoethanol, pH 7.6) and then with HB buffer (20 mM HEPES,[1] 5 mM KCL, 1.5 mM $MgCl_2$, 0.5 mM dithiothreitol, 5 mM sodium butyrate, adjusted to pH 7.5 with KOH). Cells were lysed with a Dounce homogenizer, and nuclei were pelleted at 11000*g* for 10 min at 4 °C. The supernatant was then clarified by centrifugation (80000−100000*g*; 90 min) at 4 °C in a Tl-100 ultracentrifuge (Beckman). Extracts were either used immediately for HAT B assays or immunoprecipitation reactions, or stored at −70 °C.

To chemically crosslink cytosolic histone complexes, an S100 (prepared in the absence of DTT) was adjusted to 1.5 mg/mL DSP using a stock solution of 100 mg/mL in DMSO. Crosslinking was performed for 1 h at 4 °C and was halted by the addition of ammonium acetate to 30 mM (De Gunzberg *et al.*, 1989). Crosslinks were reversed by boiling samples in the presence of 0.5 M 2-mercaptoethanol. The efficiency of histone crosslinking was routinely monitored by one- and two-dimensional gel electrophoresis and western analysis of the untreated, crosslinked, and reversed samples.

Total nuclear histones were acid-extracted from nuclei prepared in Buffer A, precipitated with 25% TCA, washed with acidified acetone and then acetone alone (Annunziato & Seale, 1983). Histone pairs H3/H4 were separated from

[1] Abbreviations: HEPES, *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid]; PIPES, piperazine-*N*,*N*′-bis[2-ethanesulfonic acid]; EGTA, ethylene glycol bis(*β*-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; BSA, bovine serum albumin; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; PPO, 2,5-diphenyloxazol; DMSO, dimethyl sulfoxide; TCA, trichloroacetic acid; DTT, dithiothreitol; HU, hydroxyurea; DSP, dithiobis[succinimidyl propionate]; BCIP/NBT, 5-bromo-4-chloro-3-indolyl phosphate/Nitro blue tetrazolium.

H2A/H2B using the salt extraction method of Simon and Felsenfeld (1979). For some experiments, all four core histones were co-purified by the salt elution procedure of Stein and Mitchell (1990). Salt-purified histones were dialyzed to either water or PB buffer (10 mM PIPES, 80 mM NaCl, 20 mM sodium butyrate, 1.0 mM EGTA, 0.1 mM PMSF, pH 7.0).

Synthesis of Acetylated and Unacetylated Peptides. Peptides corresponding to the first 18 amino-terminal residues of human H4 (Hayashi *et al.*, 1982) were synthesized as previously described (Lin *et al.*, 1989) using either acetylated or unacetylated lysine residues at specific positions during the synthesis of each peptide. Peptides used in this report contained a carboxy terminal glycine (residue 19) and cysteine (residue 20), for coupling to carrier protein for later antibody production. As with naturally occurring histone H4, peptides were also acetylated on the N-terminal serine residue. Peptides were purified by preparative HPLC chromatography, and evaluated by analytical HPLC, amino acid composition, and mass spectroscopy. Purified peptides were solubilized in water.

Antibody Production, Immunoprecipitation. Polyclonal antibodies that recognize acetylated human H4 were generated by Charles River/East Acres Biologicals (Southbridge, MA); the antigen was a synthetic peptide representing a diacetylated N-terminus of human histone H4 [acetylated solely on the lysine residues at positions 5 and 12, *i.e.*, those residues known to be acetylated in newly synthesized H4 (Sobel *et al.*, 1995)]. Synthetic peptides coupled to keyhole limpet hemocyanin (~1.0 mg/injection) were dissolved in water, emulsified with an equal volume of Freund's adjuvant, and injected subcutaneously into rabbits. Approximately 3 weeks later, the injection was repeated using 0.5 mg of antigen and incomplete Freund's adjuvant. Sera were stored in frozen aliquots. Antibodies generated in this manner ("anti-acH4") were specific for acetylated isoforms of histone H4 (Figure 1); however, as expected they were not specific for newly synthesized H4, presumably due to transcription-related acetylation (Turner *et al.*, 1989; Brownell & Allis, 1996). In agreement with our previous results using the "anti-penta" antibodies described by Lin *et al.* (1989), the anti-acH4 antibodies efficiently immunoprecipitated cytoplasmic complexes containing nascent H3 and H4 (see Results), and could be blocked by excess tetraacetylated or K5/K12-diacetylated (but not unacetylated) H4 peptides. Other anti-histone antibodies used in this study are presented in Figure 1.

Immunoprecipitations were performed essentially as described, using antibodies immobilized with Protein A-Sepharose beads (Pharmacia) (Perry *et al.*, 1993b). In parallel control experiments, non-immune serum was substituted for the antiserum. Prior to the adding the S100, the beads were treated with 1.0 mg/ml BSA and 100 μg/mL ubiquitin (Sigma), to reduce non-specific binding. The S100 (dialyzed to PB buffer) was treated with DNase I and RNase, and then pre-cleared using beads that had been treated with non-immune serum. The S100 was then incubated with immobilized antibodies for 2.5 h at room temperature. Immunopellets were washed five times with 20 mM Tris-HCl (pH 8.6), 0.15 M NaCl, 20 mM sodium butyrate, 1.0 mM EDTA, 1% Triton X-100. In some experiments H3/H4 complexes were crosslinked with DSP prior to immunoprecipitation; however, crosslinking precluded the immunoprecipitation of H2A with anti-H2A antibodies.

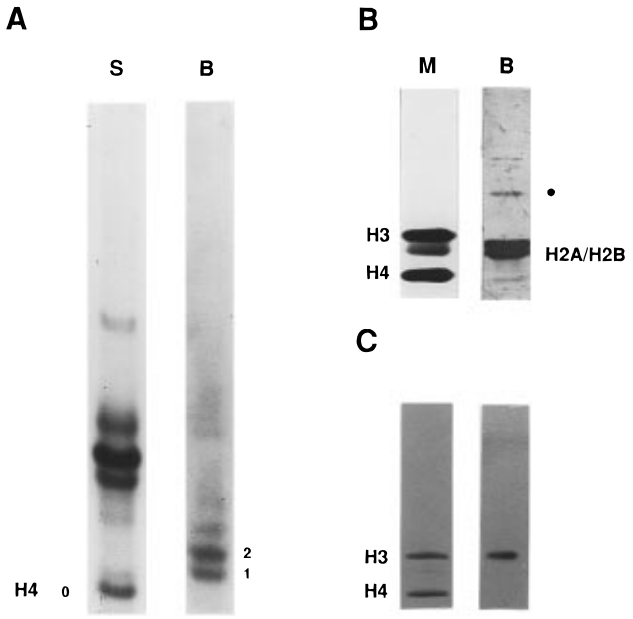

FIGURE 1: Antibody characterization. (A) Acid-soluble proteins from HeLa cell nuclei were separated in an acid-urea gel, and either stained with Coomassie Blue (lane S) or transferred to Immobilon-P membrane, stained with ponceau S, and probed with antibodies generated against the 5/12 diacetylated N-terminus of human histone H4 (lane B). Antibodies were detected by a secondary color reaction; mono- and diacetylated H4 isoforms (labeled 1 and 2) were aligned with reference to the ponceau stain. (B) HeLa nuclear proteins were resolved by SDS−PAGE and subjected to western analysis using polyclonal antibodies generated against sea urchin histone H2A; antibodies were visualized as in panel A. Note that denatured H2B is also recognized by the anti-H2A antibodies (lane B); the solid circle (lane B) indicates a protein with the mobility of ubiquitinated H2A; because this band is also recognized by anti-ubiquitin antibodies, it is provisionally identified as uH2A. Lane M contains radiolabeled acetylated marker histones, co-electrophoresed and transferred to membrane with the histones in lane B, and detected by fluorography. (C) HeLa nuclear proteins were resolved by SDS−PAGE and subjected to western analysis using antibodies specific for histone H3 (Braunstein, 1996). Antibodies were detected by a light reaction. Marker histones (lane M) are as in panel B. For the blots in panels A, B, and C, all of the proteins detected by western analysis are shown.

Gel Electrophoresis and Immunoblotting. Proteins were subjected to electrophoresis in either SDS (Thomas & Kornberg, 1975) or acid-urea (Panyim & Chalkley, 1969) polyacrylamide gel systems as described previously (Perry & Annunziato, 1989). In preparation for fluorography, gels were treated with PPO/DMSO, dried, and exposed to preflashed Kodak X-Omat AR film (Bonner & Laskey, 1974; Laskey & Mills, 1975). Immunoblotting and western analyses were performed as described previously (Perry *et al.*, 1993b). Antibody reactions were detected either one of two ways: (1) through a secondary color reaction (secondary antibody conjugated to alkaline phosphatase), using premeasured BCIP/NBT tablets (Sigma); or (2) using a chemiluminescent alkaline phosphatase substrate (Tropix). Detection of acetylated histone H4 was routinely performed using the "anti-penta" antibody first described by Lin *et al.* (1989), which in HeLa cells is specific for acetylated H4 (Perry *et al.*, 1993b). Antibodies that recognize histones H2A, H2B, and H3 are shown in Figure 1; H3 was detected using an antibody that specifically recognizes the H3 N-terminus (Braunstein, 1996) (Figure 1). A monoclonal antibody that recognizes human NAP-1 was the gift Dr. Yukio Ishimi

(Ishimi *et al.*, 1985). For western analyses, anti-histone antisera were diluted as follows: anti-H3, 1:50; anti-H2A and anti-penta, 1:200. Anti-NAP-1 was typically diluted 10-fold. To detect radiolabeled marker proteins on western blots, membranes were sprayed with a 20% solution of PPO in toluene and allowed to dry before placing against film.

Sucrose Gradient Analysis. Cytosolic S100 extracts (0.5−1.0 mL) were adjusted to 1.0 mM $MgCl_2$ and treated with DNase I (50 units/mL) and RNase (4 units/mL) for 1 h at room temperature, and then adjusted to 2 mM EDTA. The S100 was then layered onto a 5−20% linear sucrose gradient containing either HB buffer minus DTT, or PB buffer, and sedimented at 160000*g* for 18.5 h at 4 °C in a Beckman SW41 Ti swinging-bucket rotor. Omitting the DNase/RNase treatment or varying the gradient buffer conditions did not alter the sedimentation characteristics of cytosolic histone complexes. Fractions (300 μL) were collected from the top by displacing the gradient with 70% sucrose, and either precipitated with TCA [after the addition of 5 μg purified ubiquitin (Sigma) as carrier] in preparation for PAGE and immunoblotting, or used in acetyltransferase assays. Typically, about forty 300 μL fractions were obtained from each gradient; in most experiments adjacent fractions (1 and 2, 3 and 4, *etc.*) were pooled, yielding 20 samples per gradient. For all sucrose gradient experiments a parallel gradient was also run, containing the following protein molecular weight markers: lactalbumin (14.2 kDa), carbonic anhydrase (29 kDa), chicken egg albumin (49 kDa), bovine serum albumin (66 kDa; 4.3S), and catalase (240 kDa; ∼11S).

In Vitro Histone Protein Acetylation. Salt-purified histones (typically at 1−2 μg of histone H4 per ml) were labeled in 400−600 μL of S100 cytosolic extract, using an acetyl-CoA generating system (Vu *et al.*, 1987) containing 10 μM CoA, 0.05−0.015 unit/mL acetyl-CoA synthetase, and 1−4 mCi/mL [^{3}H]acetate; to provide energy for acetyl-CoA synthesis an ATP regenerating system was also included, comprising 0.1 mg/mL creatine phosphokinase, 3 mM ATP, 40 mM phosphocreatine, and 5 mM $MgCl_2$ (final concentrations from a 10× stock solution). To directly compare the cytosolic HAT B activity to our previous studies of HeLa nuclear histone acetyltransferase, in some experiments the S100 extract was adjusted to the *in vitro* acetylation/replication buffer conditions of Perry *et al.* (1993b), using a 5× stock solution; both buffer conditions yielded the same results. Labeling was performed for 1−1.5 h at 37 °C. For microsequence analysis acid-extracted total histones (sufficient to provide 15 μg of histone H4) were labeled in a 400 μL volume. After cooling each reaction on ice, acid-soluble proteins were extracted from the reaction mixture, and prepared as described above. The use of an acetyl-CoA regenerating system permits the efficient radiolabeling of H4 in volumes greater than 100 μL at reasonable cost.

In Vitro Acetylation of H4 N-Terminal Peptides. In vitro acetylation of a synthetic peptide representing the unacetylated N-terminus of histone H4 (2.5 μg in a 25 μL reaction) was performed in sucrose gradient fractions adjusted to an additional 60 mM HEPES (pH 8.0). Peptides were labeled with 8.0 μCi/mL [^{3}H]acetyl-coenzyme A (1−3 Ci/mmol; New England Nuclear) for 20 min at 37 °C. Incorporation was determined by collecting ^{3}H-labeled peptides on Whatman P-81 phosphocellulose paper (Horiuchi & Fujimoto, 1975), as described previously (Sobel *et al.*, 1994). Dried filters were counted in a biodegradable scintillation cocktail.

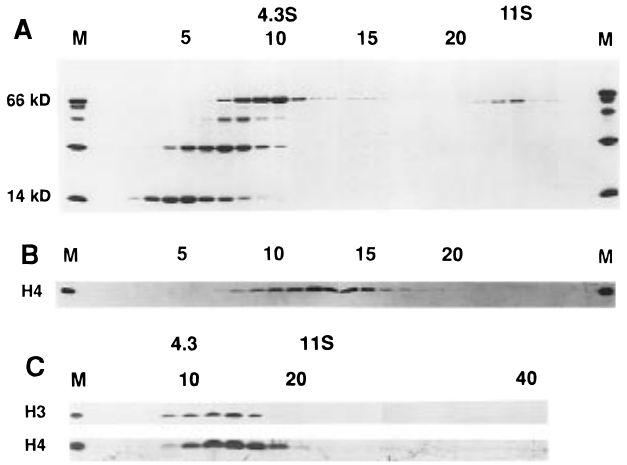

FIGURE 2: Cytosolic H3 and H4 sediment as a 5−6S complex. S100 preparations and parallel marker gradients were subjected to sedimentation analysis as described in Experimental Procedures; fractions were analyzed by SDS−PAGE. (A) Marker sucrose gradient stained with Coomassie Blue, showing the positions of BSA (4.3S) and catalase (11.3S). Note that catalase (240 kDa) comprises four identical noncovalently associated subunits of 60 kDa that dissociate in SDS. Marker proteins (resolved in lane M) are lactalbumin (14.2 kDa), carbonic anhydrase (29 kDa), chicken egg albumin (45 kDa), catalase monomeric subunit (60 kDa), and BSA (66 kDa). (B) Western analysis of a sucrose gradient containing a HeLa S100 prepared from cells treated with hydroxyurea (for 1 h). The blot was probed with "anti-penta" antibodies, specific for acetylated H4 (Lin *et al.*, 1989); only the H4 region is shown; no other cytosolic proteins were detected in the blot. (C) Western analysis of a sucrose gradient containing an S100 from control HeLa cells; the membrane was sliced and the resulting strips probed with either anti-H3 or anti-penta antibodies. Note that for panels A and B, only the first twenty-six fractions are presented (no histones appeared in the remaining fractions). For panel C adjacent paired fractions were pooled, so that the entire gradient could be displayed. The numbers above each gradient thus refer to gradient fractions (not gel lanes) to simplify comparison between experiments. In panels B and C, lane M contains isolated nuclear histone markers.

Microsequencing. For protein microsequencing, acid-soluble proteins from the *in vitro* acetylation reaction were separated in the presence of SDS, and transferred electrophoretically to Immobilon-P membrane (Millipore) in 10 mM CAPS, 10% methanol, pH 11.0 (adjusted with NaOH). The membrane was then rinsed with distilled water, stained with Coomassie Blue R, and destained with 50% methanol/10% acetic acid. To identify the H4 band the membrane was sliced to remove ∼2 mm of the sample lane's edge; this was then analyzed by fluorography (overnight exposure). Only one radiolabeled band was detected, which had the same mobility as radiolabeled marker H4 in an adjacent lane. The membrane-bound protein was then deblocked and sequenced as previously described (Sobel *et al.*, 1994), yielding the predicted human H4 sequence (see text). At each cycle a portion of the sample was analyzed by scintillation counting to determine which of the four acetylatable lysines of H4 had been radiolabeled through *in vitro* acetylation (Sobel *et al.*, 1994).

Activity Gel Assays. The catalytic subunit of HeLa HAT B was studied by means of an activity gel assay, as previously described (Brownell & Allis, 1995; Brownell *et al.*, 1996). Briefly, samples containing HAT B activity were subjected to SDS−PAGE in a polyacrylamide gel into which histones (1 mg/mL; Sigma H9250) had been polymerized; the upper reservoir buffer also contained histones at 0.1 mg/

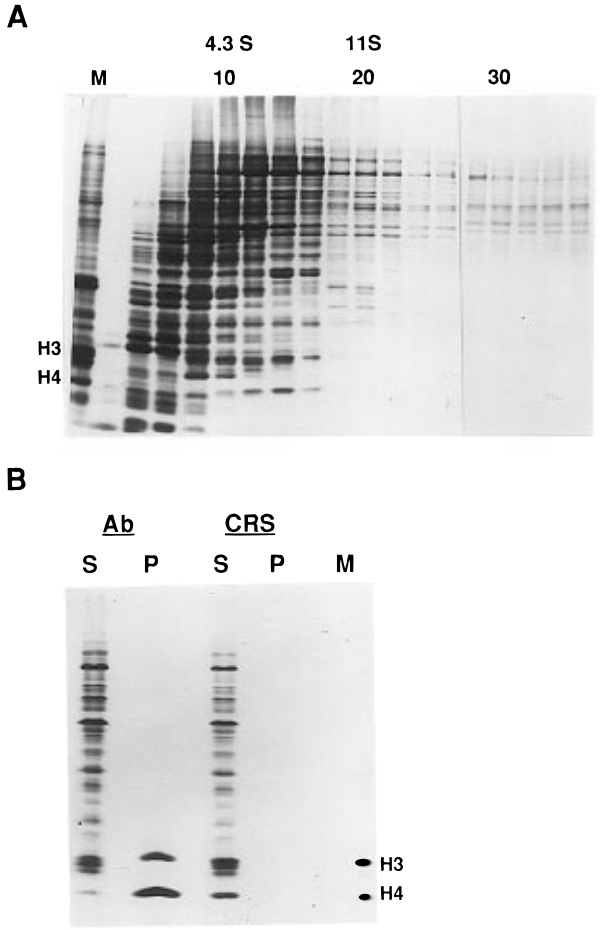

FIGURE 3: Nascent soluble H3 and H4 form a native 5−6S complex. (A) Cells were labeled with [^{3}H]lysine for 5 min in the presence of sodium butyrate. An S100 was prepared and separated in a sucrose gradient; fractions were analyzed by SDS−PAGE and fluorography; numbers above the gradient refer to gradient fractions. Lane M contains acid-extracted radiolabeled nuclear proteins. S values were determined from a parallel marker gradient. (B) Nascent histones were labeled and separated in a sucrose gradient as in panel A. Fractions 10 through 17 were pooled and immunoprecipitated using either anti-acH4 antibodies (Ab; antibodies described in Figure 1), or control rabbit serum (CRS); the unbound supernatant (S) and the immunopellet (P) were analyzed by SDS−PAGE and fluorography. Lane M contains [^{3}H]acetate-labeled histone markers. To better resolve histones in the unbound fraction, the immunosupernatant was acid-extracted and half the total sample loaded on the gel.

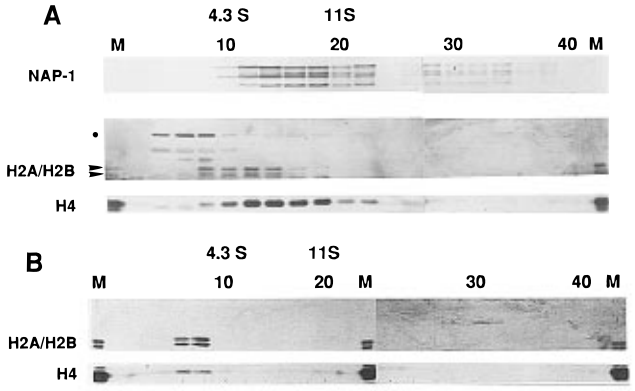

FIGURE 4: Sedimentation of cytosolic H2A, H2B and NAP-1. (A) An S100 from control cells was dialyzed to PB buffer and separated in a sucrose gradient (containing the same buffer); gradient fractions were resolved by SDS−PAGE and transferred to membrane for immunoblotting. Prior to incubation with the primary antibodies, the blot was cut into strips, thereby separating the regions containing H4, H2A/H2B, and NAP-1; the blot was then probed with antibodies that recognize either acetylated H4, H2A/H2B, or NAP-1. The position of a protein with the mobility of ubiquitinated H2A (uH2A) is indicated by the solid circle; numbers above the gradient refer to gradient fractions. (B) Total salt-purified core histones in PB (∼5 μg of each histone in 500 μL, to yield approximately the same degree of immunostaining as native H2A/H2B in gradients containing the S100) were separated in a sucrose gradient in PB buffer. Adjacent fractions were pooled and one-half of each sample was subjected to SDS−PAGE and analyzed by western blotting, using anti-penta or anti-H2A antibodies; the remaining half of each sample was similarly analyzed by immunoblotting to detect the position of H3, which precisely cosedimented with the other core histones (not shown). (Note: Because only ∼30% of total nuclear H4 is acetylated, the immunostaining of H4 is an underestimate of total H4 concentration.) Lane M: histone markers.

mL. Proteins in the gel were then slowly renatured, and the gel was incubated with [^{3}H]acetyl-CoA, allowing active acetyltransferases to radiolabel histones in the surrounding gel matrix. The position of the acetyltransferase activity was then detected by fluorography.

RESULTS

A Cytosolic Histone Complex Containing Newly Synthesized H3 and H4

When DNA replication is inhibited by hydroxyurea (HU), histone synthesis persists at a reduced rate for up to several hours (Nadeau *et al.*, 1978). Under these conditions histones accumulate in a free pool, which can be detected in the cytosol following the isolation of nuclei (Louters & Chalkley, 1985; Bonner *et al.*, 1988). Nascent "cytosolic" H4 from HU-inhibited cells is acetylated, and as we have previously shown can be immunoprecipitated in a complex containing histone H3 (Perry *et al.*, 1993b). To determine the size of this complex, a cytosolic extract from HU-treated HeLa cells was separated in a 5−20% sucrose gradient. Fractions from the gradient were then subjected to electrophoresis in the presence of SDS, and analyzed by immunoblotting using antibodies that are specific for acetylated histone H4 (Lin *et al.*, 1989; Perry *et al.*, 1993b) (Figure 2). (Note: for a description of other anti-histone antibodies used in this study, please refer to Figure 1.)

As seen in the immunoblot (panel B), acetylated cytosolic H4 appeared in the ∼5−6S gradient region [*cf.* the position of BSA (4.3S), panel *A*]. Because we have previously shown that cytosolic H4 from HU-treated cells is associated with H3 (Perry *et al.*, 1993b), the results in Figure 2B provide evidence that H3 and H4 are complexed in a structure that sediments faster than either the H3/H4 dimer (∼27 kDa) or the canonical H3/H4 tetramer [53 kDa, or ∼3S (Kornberg & Thomas, 1974)], yet more slowly than an 11S mononucleosome (Noll, 1974). Treating the S100 with DNase I and RNase did not alter the sedimentation of acetylated H4 in gradients, nor did varying the ionic strength from "low" (*i.e.*, 3 mM Mg^{2+}) to physiological (110 mM Na^+). Importantly, H4 from *control* cytosol (prepared with no hydroxyurea pretreatment) also sedimented at 5−6S, as did cytosolic histone H3 as determined by western blotting (Figure 2C). Thus there appears to be a native soluble H3/H4 complex in somatic cells that contains acetylated H4, with sedimentation properties identical to the 5−6S H3/H4·N1 complex in *Xenopus* oocytes (Kleinschmidt *et al.*, 1985).

The rapid sedimentation of H3/H4 can not be ascribed to either the p60 or the p150 subunits of CAF-1. As demon-

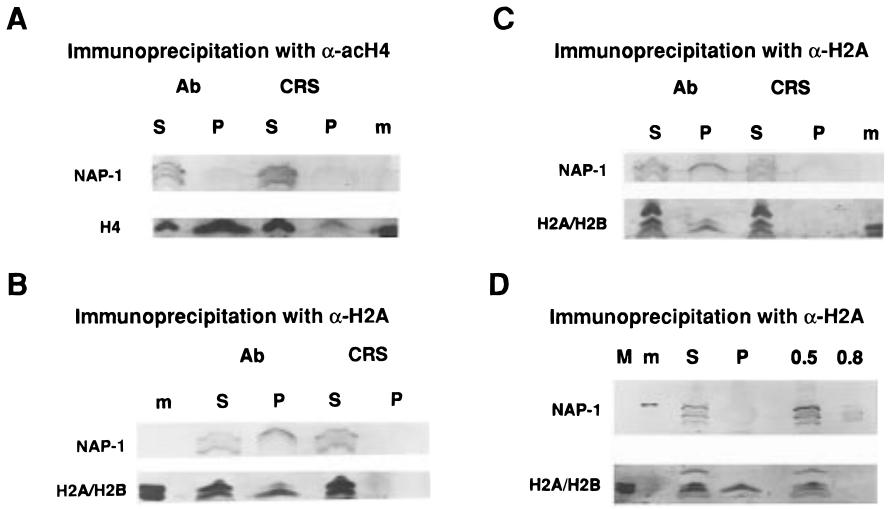

FIGURE 5: Cytosolic HeLa H2A is associated with NAP-1. (A) An S100 was subjected to immunoprecipitation using either anti-acH4 antibodies (to precipitate the H3/H4 complex), or control rabbit serum (CRS); supernatant and pellet fractions were subjected to SDS−PAGE and transferred to Immobilon-P membrane. The membrane was sliced, and the strips probed with either anti-penta antibodies (to determine H4 immunoprecipitation efficiency), or anti-NAP-1. Lane m contains histone markers. (B) An S100 from cells pre-treated for 1.5 h with hydroxyurea was subjected to immunoprecipitation using either anti-H2A or control rabbit serum; supernatant and pellet fractions were analyzed by western blotting, using either anti-H2A or anti-NAP-1 antibodies. Lane m contains histone markers. (C) An S100 from control cells was immunoprecipitated using anti-H2A, and analyzed as in panel B. Lane m contains histone markers. (D) An S100 from control cells was immunoprecipitated using anti-H2A, and analyzed as in panels B and C; following immunoprecipitation the beads were washed sequentially with buffer containing 0.5 M NaCl (lane 0.5) and 0.8 M NaCl (lane 0.8); no 0.15 M NaCl wash was performed. Lane M contains histone markers; lane m contains recombinant yeast NAP-1. For all immunoprecipitations, 100% of the unbound and bound samples were loaded on the gel.

strated by Kaufman *et al.* (1995) p150 is required for p60 to interact with cytosolic H3 and H4. Because the S100 contains p60 but no p150, most cytosolic p60 is unassociated with H3/H4 (and *vice versa*) (Kaufman *et al.*, 1995). This was confirmed by western analyses of sucrose gradient fractions using antibodies directed against p60 (the gift of Dr. Bruce Stillman): as expected, most p60 sedimented as a free protein in fractions 8−10, not with the H3/H4 complex in fractions 10−17 (data not shown). The basis for the sedimentation behavior of the H3/H4 complex must therefore lie elsewhere (please see Discussion).

Our next experiments were performed to assess whether *newly synthesized* histones were present in the native H3/H4 complex. Control HeLa cells were incubated for 5 min with [^{3}H]lysine to label nascent histones; a cytosolic S100 was then prepared and subjected to sucrose gradient sedimentation as in Figure 2. Gradient fractions were analyzed by SDS−PAGE and fluorography, to visualize all of the cytosolic proteins that had been radiolabeled during the 5 min pulse (Figure 3A). To determine if nascent H3 and H4 were indeed present in the 5−6S region, fractions 10 through 17 from a second radiolabeled gradient were pooled and immunoprecipitated with anti-acH4 antibodies. Of the hundreds of radiolabeled proteins present in these fractions, only two major [^{3}H]lysine-labeled proteins were immunoprecipitated (Figure 3B). These had the mobilities of histones H3 and H4 (as measured by labeled histone markers). Thus, from precisely the region of the gradient where H3 and acetylated H4 had been positively identified by immunoblotting (Figure 2), newly synthesized H3/H4 were present in a co-precipitating complex. Because only cytosolic H3 and H4 can be uniquely targeted to newly replicated DNA by CAF-1 *in vitro* (Smith & Stillman, 1991), it is not unreasonable to propose that this complex is a naturally occurring intermediate in nucleosome assembly.

It can be seen in Figure 3B that while the unbound immunosupernatant contains newly synthesized histones H2A and H2B (labeled during the 5 min pulse) the immunopellet is completely devoid of these histones. This is in sharp contrast to the immunoprecipitation of *bona fide* nascent nucleosomes, which yields all four newly synthesized histones in the immunopellet (Perry *et al.*, 1993b). Because identical results were obtained when the distribution of H2A/H2B after immunoprecipitation was monitored by immunoblotting, or when proteins in the S100 were crosslinked with DSP prior to immunoprecipitation (data not shown), it is concluded that the cytosolic H3/H4 complex contains no H2A/H2B. This is as expected, since the histone octamer (∼108 kDa) is unstable under physiological ionic conditions in the absence of DNA, dissociating instead into the $H3_2$-$H4_2$ tetramer and two H2A/H2B dimers (Eickbush & Moudrianakis, 1978; Ruiz-Carrillo & Jorcano, 1979; Stein & Page, 1980). It is also consistent with the chromatographic behavior of the cytosolic histone complement (Smith & Stillman, 1991). As will be shown, cytosolic H2A/H2B exhibit sedimentation behavior indicative of an independent histone complex.

NAP-1 Is Associated with Cytosolic H2A

The sedimentation characteristics of H2A and H2B were then examined. In sucrose gradients most H2A and H2B sedimented at ∼4−5S, only partially overlapping the H3/H4 region (Figure 4A) yet faster than purified histones fractionated under identical gradient conditions (Figure 4B). When considered together with the established propensity of H2A and H2B to dimerize at this ionic strength (Eickbush & Moudrianakis, 1978; Karantza *et al.*, 1996), our results suggest that some cytosolic H2A/H2B dimers may be part of a larger complex (or complexes). Interestingly, a protein with the mobility of uH2A (indicated by the solid circle) was observed at the top of the gradient. Because this protein

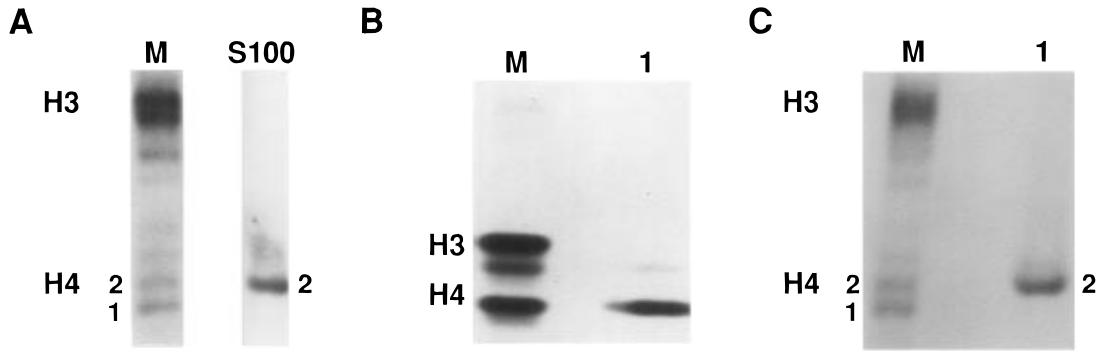

FIGURE 6: Diacetylation of histone H4 in vivo and in vitro. (A) Acid-soluble proteins in the cytosolic S100 were subjected to electrophoresis in an acid−urea gel system, transferred to membrane, and probed with antibodies that recognize all of the acetylated species of human H4 (Lin *et al.*, 1989; Perry *et al.*, 1993b); lane M contains radiolabeled acetylated histone markers, co-transferred along with the sample in lane 1 and detected by fluorography. (B, C) Total acid-soluble nuclear proteins (panel B) or salt-purified H3 and H4 (Simon & Felsenfeld, 1979) (panel C) were acetylated *in vitro* by human HAT B in a HeLa S100, in the presence of a [^{3}H]acetyl-CoA generating system (lane 1). Labeled proteins were subjected to electrophoresis in SDS (B) or acid−urea gels (C), and analyzed by fluorography; lane M, acetylated histone markers. For the acid−urea gels the positions of mono- and diacetylated H4 are indicated.

has the same mobility as nuclear uH2A, and also comigrates with a protein recognized by anti-ubiquitin antibodies, this band has been tentatively identified as uH2A. If this identification is correct, then it is striking that uH2A sediments as a free protein, largely independent of non-unbiquitinated H2A/H2B. The relatively high level of uH2A in the cytosol is consistent with the results of Jackson (Jackson, 1990), which suggest that H2A is ubiquitinated while in a soluble nuclear pool.

The human protein NAP-1, first described by Ishimi *et al.* (1983, 1984), has been described as a potential somatic nucleosome assembly factor. NAP-1 (∼55−58 kDa) can bind all four core histones *in vitro* and is able to transfer histones to non-replicating plasmid DNA. Although NAP-1 can be detected immunologically in HeLa cell nuclei (Ishimi *et al.*, 1985), it is also found in the cytosolic S100 following nuclear isolation [this report; also Ishimi *et al.* (1985)]. Recently, the *Drosophila* homologue of NAP-1 was shown to be associated with H2A and H2B in fly embryo extracts (Ito *et al.*, 1996). We therefore examined the sedimentation characteristics of NAP-1 in sucrose gradients, using a monoclonal antibody provided by Dr. Yukio Ishimi (Ishimi *et al.*, 1985).

As is typical, human NAP-1 appeared in western blots as a multiple series of bands (Figure 4A). Multiple species of NAP-1 have also been noted in yeast (Kellogg *et al.*, 1995; Yoon *et al.*, 1995), *Xenopus* (Ishimi & Kikuchi, 1991), and soybean (Yoon *et al.*, 1995), and were still observed following the inclusion of leupeptin, pepstatin, and PMSF during S100 preparation. NAP-1 sedimented considerably faster in sucrose gradients than predicted on the basis of its 58 kDa molecular weight (*cf.* the 4.3S/66 kDa position of BSA), with the greatest concentration of NAP-1 partly overlapping the histone region. Crosslinking proteins in the S100 with DSP did not alter the sedimentation of either H2A/H2B, H3/H4, or NAP-1.

To determine if any cytosolic histones are associated with NAP-1, HeLa S100 preparations were subjected to immunoprecipitation using anti-histone antibodies; the bound and unbound fractions were then analyzed by SDS−PAGE and by western blotting (Figure 5). When immunoprecipitations were performed using anti-acH4 antibodies (to precipitate the H3/H4 complex) no NAP-1 was found in the immunopellet (Figure 5A); identical results were obtained when the H3/H4 complex was crosslinked with DSP prior to immunoprecipitation (not shown). In contrast, when immunoprecipitations were performed using anti-H2A, a protein recognized by anti-NAP-1 was co-precipitated. This was the case when cells were pre-treated with hydroxyurea to accumulate soluble histones (Figure 5B) or when control cells were analyzed (Figure 5C). No NAP-1 was observed in the immunopellet when the beads were washed with 0.5 M NaCl (Figure 5D), as predicted if NAP-1 associates with H2A through ionic interactions (Ishimi *et al.*, 1984). Although it has not been possible to perform the complementary experiment of immunoprecipitating NAP-1 to co-precipitate H2A (since all attempts to immunoprecipitate NAP-1 with the monoclonal antibody proved negative), our results strongly suggest that at least some cytosolic H2A is associated with NAP-1. Because we had previously shown that the deposition of H2A and H2B *in vivo* is selectively targeted to nucleosomes containing acetylated H4 (Perry *et al.*, 1993b), and because nascent H4 is universally diacetylated, we next examined the acetylation state of H4 in the cytosolic H3/H4 complex.

Acetylation State of Cytosolic H4

Newly synthesized chromatin-bound H4 is diacetylated in virtually all systems that have been examined (reviewed in Annunziato, 1995). The diacetylation of *cytosolic* H4 was previously indicated by electrophoretic analyses of pulse-labeled histones (Bonner *et al.*, 1988). This was verified by western analysis of a HeLa S100, using antibodies that recognize all of the acetylated species of human H4: As shown in Figure 6A, virtually all of the acetylated cytosolic H4 was in the diacetylated isoform. Because newly synthesized H4 is diacetylated at positions 5 and 12 in HeLa cells even when chromatin assembly occurs in the presence of the deacetylase inhibitor sodium butyrate (Sobel *et al.*, 1995), the diacetylation of cytosolic H4 strongly argues for the completion of deposition-related diacetylation prior to nucleosome assembly in somatic cells, as formerly observed for the stored H4 in *Xenopus* oocytes (Woodland, 1979). However, with the sole exception of *Tetrahymena* HAT B (Chicoine *et al.*, 1986; Sobel *et al.*, 1995), no native histone acetyltransferase has been described that will completely and exclusively acetylate H4 in the complete deposition-related pattern *in vitro*. We therefore tested the HeLa S100 for an

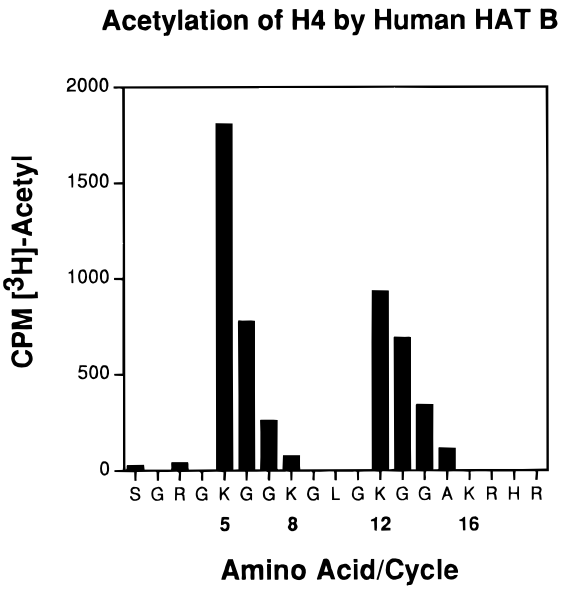

FIGURE 7: K5 and K12 are the sole sites of H4 diacetylation by human HAT B. Total acid-soluble nuclear proteins were reacted *in vitro* with HeLa HAT B as in Figure 6B,C. ^{3}H-acetylated H4 was subjected to deblocking and microsequence analysis. The one-letter amino acid sequence (*x*-axis) shows that the correct amino acid sequence was obtained. A portion of the sample at each cycle was reserved for determining the ^{3}H cpm at each position; background (165 cpm), calculated by averaging the counts for the first four cycles (which contain no lysine), was subtracted from each cycle. The positions of the four acetylatable lysines of HeLa H4 at positions 5, 8, 12, and 16 are indicated.

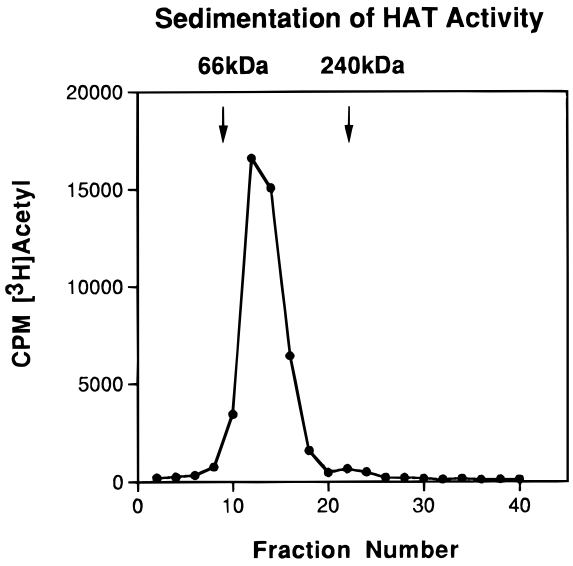

FIGURE 8: Size analysis of HeLa HAT B. An S100 was separated in a 5−20% sucrose gradient. Fractions (300 μL) were collected, and adjacent fractions were pooled for HAT B assays using a peptide representing the unacetylated N-terminus of histone H4 as a substrate (Sobel *et al.*, 1995; Kleff *et al.*, 1995); numbers on the *x*-axis refer to gradient fractions. Incorporation of [^{3}H]acetyl-CoA was measured by the filter binding assay and scintillation counting. Molecular weight markers were separated in a parallel gradient.

enzyme with the ability to diacetylate H4 on lysines 5 and 12.

Human HAT B Diacetylates Histone H4 Exclusively on Lysines 5 and 12

As a first approach to the analysis of human HAT B, total HeLa histones were incubated with an S100 in the presence of [^{3}H]acetyl-CoA; the products were then analyzed by SDS−PAGE and fluorography (Figure 6B). As reported for HAT B enzymes in other systems (Garcea & Alberts, 1980; Sures & Gallwitz, 1980; Weigand & Brutlag, 1981; Salvador *et al.*, 1985; Richman *et al.*, 1988; Lopez-Rodas *et al.*, 1991a,b), H4 was virtually the only acetylated product, with only trace incorporation observed into H2A, and none into H3 or H2B. The degree of H4 acetylation was then examined by separating the reaction products in an acid-urea gel system, which can resolve the acetylated histone isoforms (Figure 6C). A comparison with acetylated histone markers revealed that H4 had been diacetylated *in vitro*, as expected if the cytoplasmic enzyme is generating the complete deposition-related, diacetylated pattern. Additional experiments (not shown) verified that the acetyltransferase activity was inactive when chromatin was used as the substrate, rather than free histones, and that treatment with alkaline phosphatase did not change the "diacetylated" migration of the radiolabeled H4 in acid-urea gels. The cytoplasmic location and substrate specificity of the HeLa activity are hallmark features of typical HAT B enzymes.

To directly and unequivocally determine the sites acetylated by HeLa HAT B *in vitro*, another acetylation reaction was performed. The radiolabeled H4 was then microsequenced, and counts/minute at each amino acid position measured. As shown in Figure 7, the predicted amino acid sequence of human H4 was obtained, demonstrating that the proper protein had been analyzed. Significantly, [^{3}H]acetate label was incorporated exclusively into the lysine residues at positions 5 and 12, demonstrating that the HeLa HAT B activity diacetylates H4 precisely at the residues known to be acetylated in newly synthesized H4 *in vivo* (Sobel *et al.*, 1995). It is therefore highly likely that this enzyme is acetylating H4 in the cytosolic nascent H3/H4 complex, thereby accounting for its efficient immunoprecipitation by anti-acH4 antibodies (Figures 3 and 5).

Size Analysis of Human HAT B

The native size of HeLa HAT B was then determined by sucrose gradient analysis. To unambiguously locate the H4 acetyltransferase, standard HAT B assays were performed with each of the fractions, using a synthetic peptide representing the unacetylated N-terminus of H4 as a substrate (Figure 8). A major peak of activity was found sedimenting at ~100 kDa. That this was indeed the HAT B activity was verified by using the 100 kDa peak from a second gradient to acetylate histone proteins *in vitro*, and then analyzing the radiolabeled acetylated proteins by gel electrophoresis and fluorography (Figure 9). In agreement with results obtained using the unfractionated cytosol (Figure 6), the 100 kDa activity vigorously acetylated histone H4 *in vitro* (Figure 9A, B). As with HAT B enzymes from calf thymus (Sures & Gallwitz, 1980) and *Zea mays* (Lopez-Rodas *et al.*, 1991a), a low level of activity towards H2A was also detected. The value of ~100 kDa corresponds closely to the size estimates of native HAT B from calf thymus [98 kDa, (Sures & Gallwitz, 1980)] and maize embryos [90−95 kDa (Eberharter *et al.*, 1996)].

The catalytic subunit(s) of histone acetyltransferases can be identified by means of an "activity gel assay", in which core histone substrates are polymerized into an SDS−polyacrylamide separating gel (Brownell & Allis, 1995;

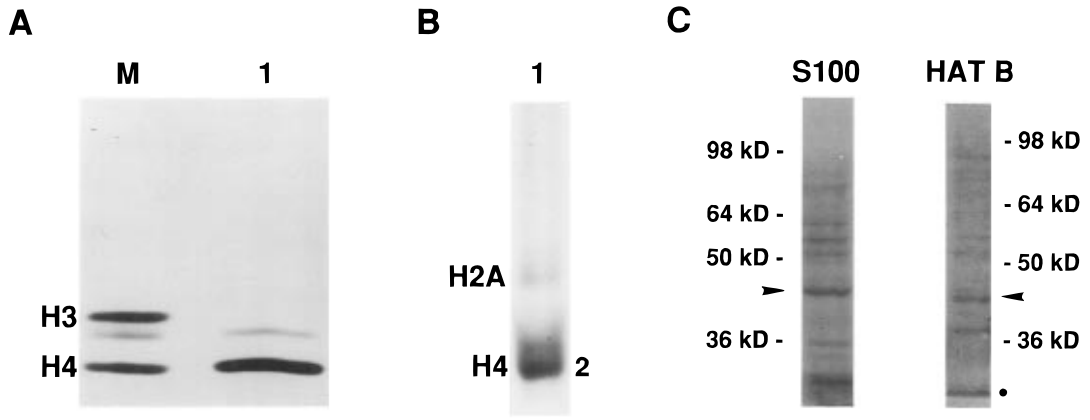

FIGURE 9: Substrate specificity and activity gel analysis of HeLa HAT B. (A) An S100 was separated in a sucrose gradient and fractionated as in Figure 9. Fractions 11 through 14 were pooled and used for an *in vitro* HAT B assay in the presence of all four HeLa core histones and a [^{3}H]acetyl-CoA generating system. Reaction products were analyzed by SDS−PAGE and fluorography (lane 1). Lane M, radiolabeled acetylated histone markers. (B) Histones were reacted with the 100 kDa HAT B activity as in panel A, separated in an acid−urea gel, and analyzed by fluorography. The fluorographs in panels A and B were slightly overexposed to better reveal the faint H2A band. (C) A total S100 preparation (lane S100) and the 100 kDa HAT B activity collected from a sucrose gradient (lane HAT B) were subjected to SDS−PAGE in a histone-containing polyacrylamide gel; histone acetyltransferase was then detected by an activity gel assay using [^{3}H]acetyl-CoA. The gel fluorograph is shown; a prominent band at 44 kDa in both lanes is indicated by the arrowhead. [Note: the signal at the ion front of the HAT B lane (solid circle) appeared across the bottom of the entire gel, independently of added sample.]

Brownell *et al.*, 1996). To determine the size of the HeLa HAT B catalytic subunit, an activity gel assay was performed using either the 100 kDa HAT B peak from a sucrose gradient, or the total unfractionated S100 (Figure 9C). Although several weaker bands were also observed in the fluorograph, a prominent band in both samples (and the strongest band common to both lanes) had a molecular weight of ~44 kDa. Identical results were obtained when purified H4 was polymerized into the gel matrix instead of total histone; in contrast, no bands were seen when BSA was used as a substrate (data not shown). The presence of several bands may be related to the need to denature and renature the applied sample (and substrate) in this SDS gel-based assay, causing a decrease in the specificity of other cytoplasmic acetyltransferases. Because of this, it is not certain at this time that the 44 kDa band represents the catalytic subunit of human HAT B. Nevertheless, the value of 44 kDa is intriguing, since this is equivalent in size to the yeast *HAT1* gene product [*i.e.*, the catalytic subunit of a HAT B-type enzyme (Kleff *et al.*, 1995; Parthun *et al.*, 1996)], and to the small subunit of maize HAT B (Eberharter *et al.*, 1996).

DISCUSSION

The results presented in this report permit several conclusions concerning the organization and modification of histones prior to their assembly into chromatin. It has been demonstrated that nascent *cytosolic* H3 and H4 form a pre-assembly 5−6S complex under normal conditions in somatic cells, extending earlier studies on histones in cells treated with the DNA synthesis inhibitor hydroxyurea (Senshu & Ohashi, 1979; Perry *et al.*, 1993b). Our findings also complement the work of Kaufman *et al.* (1995), who demonstrated that the p150 subunit of CAF-1 is needed for the p60 subunit to interact with cytosolic H3/H4. Because p150 is essentially absent from the cytosol, little if any cytosolic H3/H4 is associated with p60 (Kaufman *et al.*, 1995). Nevertheless, close to 100% of cytosolic H3/H4 exhibits sedimentation behavior consistent with a ~100 kDa complex (*i.e.*, larger than a simple H3/H4 tetramer) (Figures 2 and 3), while our own unpublished results confirm the absence of both p60 and p150 from this fast-sedimenting structure. Moreover, neither H2A nor H2B are complexed with H3/H4 in the HeLa S100.

After the results presented in this report were submitted for publication, evidence was presented that human cytosolic H4 is associated with the small subunit of CAF-1 (p48) (Verreault *et al.*, 1996). When considered together with our own observation that cytosolic H3 is complexed with acetylated H4 as part of a 100 kDa complex, the added presence of p48 provides an independent explanation for the anomalous sedimentation of nascent H3/H4: for example, a tetramer of H3/H4 (~53 kDa) in association with one molecule of p48 (48 kDa) would yield a complex of the appropriate molecular weight. However, p48 does not appear to be synthesized at the same rate as H3/H4, as judged by fluorography of the immunoprecipitated complex (Figure 3B).

A further conclusion permitted by our results is that a NAP-1·H2A complex is present in HeLa cell cytosol. This is the first observation of a naturally occurring association of mammalian NAP-1 with native cellular histones (as distinct from reconstituted complexes), and also the first demonstrated interaction of NAP-1 with any histone in somatic cells. Although it is not yet clear if NAP-1 is associated with H2B in the S100 (since little H2B was detected in the immunopellets), genetic experiments in yeast have suggested that H2A and H2B are co-transported into the nucleus as a heterodimer (Moreland *et al.*, 1987); it thus may be that anti-H2A antibodies disrupt H2A/H2B dimers that form under these ionic conditions (Eickbush & Moudrianakis, 1978). In light of the observations that *Drosophila* NAP-1 (dNAP-1) facilitates the assembly of regularly spaced nucleosome arrays *in vitro* (Bulger *et al.*, 1995), and that embryonic dNAP-1 is associated with both H2A and H2B (Ito *et al.*, 1996), our results may reflect an evolutionarily conserved escort function for NAP-1 in the deposition of H2A/H2B dimers. If H3 and H4 are solely escorted by CAF-1 or subunits thereof *in vivo*, then two independent histone/escort-factor complexes may function during nucleo-

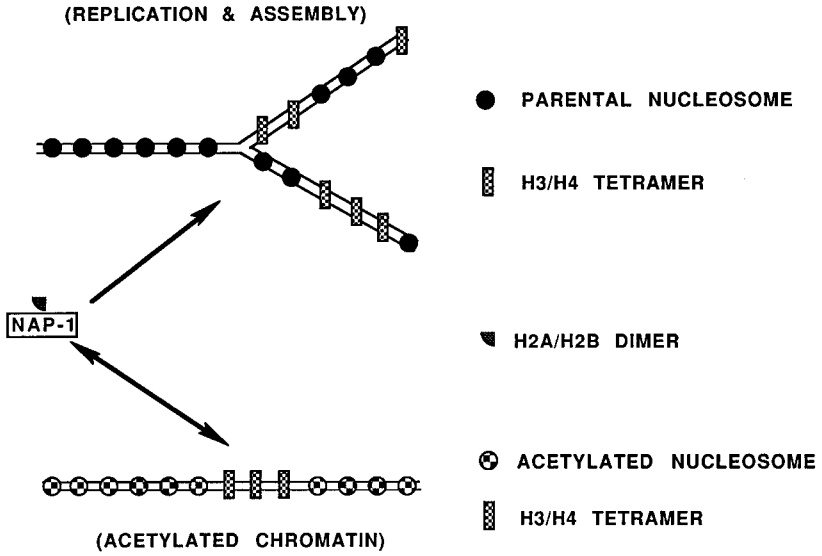

FIGURE 10: Proposed relationships linking histone acetylation, NAP-1, and H2A/H2B deposition and exchange. The association of NAP-1 with H2A (and possibly H2B) [Figure 5; see also Ito *et al.* (1996)], together with the nuclear localization of NAP-1 in HeLa cells (Ishimi *et al.*, 1985), is consistent with NAP-1 acting as an *intranuclear* chaperone for H2A and H2B as well as a possible escort during nuclear import [as suggested by Ito *et al.* (1996)]. The demonstrated targeting of H2A/H2B deposition and exchange to acetylated chromatin *in vivo* (Perry *et al.*, 1993b) may thus be facilitated by NAP-1. Note that acetylation of the nascent $H3_2H4_2$ tetramer on replicating chromatin is ensured by the diacetylation of new H4, as catalyzed by HAT B; the dynamic acetylation of non-replicative chromatin may be associated with transcription (Brownell *et al.*, 1996). See Jackson (1990) for a further discussion of intranuclear histone pools and histone exchange in somatic cells.

some assembly in somatic cells, as is the case in extracts prepared from *Xenopus* and *Drosophila* embryos (Kleinschmidt *et al.*, 1985; Dilworth *et al.*, 1987; Bulger *et al.*, 1995). It should be noted, however, that our sucrose gradient analyses indicate that only a fraction of cytosolic H2A/H2B is associated with NAP-1 (Figure 4A). Because H2B itself contains a conserved nuclear localization sequence (Moreland *et al.*, 1987) it is possible that other components of the nuclear import apparatus are interacting with H2A/H2B, in part accounting for their rapid sedimentation in sucrose gradients.

The S phase-coupled movement of cytoplasmic dNAP-1 into the nucleus has led to the proposal that NAP-1 is a histone import factor (Ito *et al.*, 1996). Interestingly, Stillman and colleagues, using a cell-free system derived from human 293 cells, have found that purified nuclear H2A/H2B can substitute for cytosolic H2A/H2B during replication-coupled nucleosome assembly *in vitro* but that only cytosolic H3/H4 (present in a crude extract) can be specifically directed to replicating DNA by human CAF-1 (Smith & Stillman, 1991). It therefore remains to be seen if NAP-1 is operating in the human cell-free system (perhaps supplied by the "H3/H4" extract), or if mammalian NAP-1 is dispensable *in vitro*. If the latter is the case, NAP-1 may act predominantly during nuclear import. On the other hand, the evidence that NAP-1 in conjunction with transcription factors can displace nucleosomal histones *in vitro* is consistent with NAP-1 also serving as an intranuclear histone chaperone (Walter *et al.*, 1995). In this regard, it should be noted that not all "cytosolic" histones present in the S100 need be located in the cytoplasm in living cells, but may in fact reside in a soluble nuclear pool (Jackson, 1990).

Cytosolic H4 in the S100 is diacetylated, and we have now identified a human HAT B activity that acetylates free H4 completely and exclusively in the K5/K12 deposition-type pattern exhibited by newly synthesized H4 (Sobel *et al.*, 1995). To date there have been four other HAT B enzymes for which the acetylation patterns have been precisely determined: those of *Tetrahymena*, *Drosophila*, *Saccharomyces*, and pea. Of these, only the *Tetrahymena* (Richman *et al.*, 1988) and human (this report) native HAT B enzymes generate the complete diacetylated pattern *in vitro*. Interestingly, although the naturally occurring yeast activity solely acetylates H4 on lysine-12 *in vitro*, the recombinant catalytic subunit (the *HAT1* gene product, HAT1p) also acetylates lysine-5, which has led to the suggestion that the native enzyme is negatively regulated (Parthun *et al.*, 1996). Such negative regulation has thus far not been observed with either the HeLa or the *Tetrahymena* activities. Moreover, the recent detection of a nuclear chromatin assembly complex (CAC) containing CAF-1 and diacetylated H4 directly links the diacetylated state to the chromatin assembly apparatus (Verreault *et al.*, 1996). In that report it was also noted that ~1% of CAC-associated nuclear H4 is triacetylated, with the additional acetylation located on lysine-8. As has been suggested, the K8-acetylation of some CAC-associated H4 may be related to the dynamic acetylation/deacetylation of newly assembled nucleosomes within a subset of chromatin domains (Verreault *et al.*, 1996; Roth *et al.*, 1996).

The role of the reversible acetylation of nascent H4 is still unknown. Possible functions for this apparently universal modification include simple charge neutralization (to suppress the promiscuous binding of new H4 to chromatin), or a role in escort factor recognition. However, genetic analyses of H4 in yeast— including studies involving deletion of the H4 N-terminus—have argued against a specific requirement for the acetylation of new H4 [reviewed in Annunziato (1995)]. For example, mutating lysines 5 and 12 of H4 to unacetylatable arginine residues only slightly increases cell doubling time (Megee *et al.*, 1990; Durrin *et al.*, 1991; Park & Szostak, 1990). Although the amino-terminal tails of H3 and H4 are redundant in supporting cell viability and nucleosome assembly (Kayne *et al.*, 1988; Morgan *et al.*,

1991; Ling *et al.*, 1996), this effect has thus far not been ascribed to compensating histone acetylation. If compensating H3-H4 acetylation is not required for nucleosome assembly, then the reversible acetylation of new H4 may be a non-essential adaptation that nevertheless increases assembly efficiency, perhaps by facilitating H2A/H2B deposition as assisted by NAP-1. Because (1) H2A/H2B deposition and exchange are targeted to acetylated chromatin *in vivo* (Perry *et al.*, 1993b) and (2) both histone acetylation (Lee *et al.*, 1993; Vettese-Dadey *et al.*, 1996) and NAP-1 (Walter *et al.*, 1995) increase transcription factor access to nucleosomal DNA, our present findings point to possible relationships among histone acetylation, NAP-1, nucleosome assembly, and chromatin remodeling (Figure 10). This of course would not preclude additional functions for acetylation during chromatin assembly, as facilitated by HAT enzymes with distinct histone and/or site specificities (Brownell & Allis, 1996; Verreault *et al.*, 1996). It also remains to be seen how dispensable the acetylation of nascent H4 is in HeLa cells, where most new H3 appears to be unacetylated (Sobel *et al.*, 1995).

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