Relationship between Methylation and Acetylation of Arginine-Rich Histones in Cycling and Arrested HeLa Cells†

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ABSTRACT: In the following report the relationship between histone methylation and histone acetylation has been examined in HeLa cells to better define the distribution of these two modifications. By labeling methylated histones in the presence or absence of sodium butyrate, we have found that the methylation of H3 is much more targeted to rapidly acetylated chromatin than is the methylation of H4, which largely involves the unacetylated subtype even in the presence of butyrate. Newly methylated H3 is highly likely to be complexed in nucleosomes that contain acetylated H4, as determined by immunoprecipitating radiolabeled chromatin with antibodies specific for acetylated H4 isoforms. In contrast, dynamically methylated H4 is underrepresented in acetylated chromatin, relative to newly methylated H3. The preferential methylation of acetylated H3 continues after pretreatment of cells with cycloheximide, indicating that not all acetylation-related methylation is associated with histone synthesis. This was confirmed by analyzing histone methylation in cells arrested at the G1/S boundary, in which histone synthesis was sharply lowered (relative to randomly cycling cells): under these conditions H3 methylation declined only ~4-fold, although ongoing methylation of H4 decreased approximately 20-fold. The continuing methylation of H3 in arrested cells included all H3 sequence variants, was selective for acetylated H3, and coincided with methyl group turnover that could not be ascribed to histone replacement synthesis. Most newly methylated H3 in arrested cells was complexed with acetylated H4 in chromatin. These data suggest that a subclass of dynamic H3 methylation exists in HeLa cells that is distinct in site, timing, and pattern from H4 methylation, which is almost entirely associated with the "slow" form of histone acetylation. It is thus unlikely that H4 methylation is linked to transcription in HeLa cells; however, a significant fraction of newly methylated H3 may be associated with metabolically active chromatin.

The fundamental organizational unit of eukaryotic chromatin, the nucleosome, contains two molecules each of the core histones H2A, H2B, H3, and H4. The hydrophilic N-terminal domains (or tails) of the core histones remain enzymatically accessible in chromatin and undergo several posttranslational modifications, including acetylation, phosphorylation, ADP-ribosylation, and methylation [reviewed in Matthews (1988), Bradbury (1992), and Li *et al.* (1993)]. Of these, reversible acetylation is one of the most extensively studied. In organisms from protozoa to mammals, acetylation has been linked to nucleosome assembly, histone replacement, chromatin decondensation, and transcriptional competence [reviewed in van Holde (1988) and Wolffe (1992)]. For example, the acetylation/deacetylation cycle of newly synthesized H4 is a remarkably conserved feature of chromatin metabolism (Louie *et al.*, 1974; Ruiz-Carrillo *et al.*, 1975; Jackson *et al.*, 1976). In more recent experiments, antibodies that specifically recognize acetylated H4 have been used to selectively immunoprecipitate transcriptionally active nucleosomes from both yeast (Braunstein *et al.*, 1993) and human cells (Clayton *et al.*, 1993).

Though first described for histones in 1964 (Murray, 1964), lysine N^{ϵ}-methylation remains among the more enigmatic of histone posttranslational modifications. H3 and H4 are the major, if not exclusive, methylated histones in vertebrates. H3 is typically methylated at lysines 9 and 27, while H4 is methylated solely at lysine 20 (Duerre & Buttz, 1990). Each lysine residue can accept up to three methyl groups, and thus the methyllysines of H3 can be found in mono-, di-, and trimethylated forms; in contrast, the sole site in H4 is maximally dimethylated (Thomas *et al.*, 1975; Paik & Kim, 1980). Methylation begins in S phase, subsequent to histone synthesis and deposition, and increases steadily as cells progress toward M (Allfrey *et al.*, 1964; Shepherd *et al.*, 1971; Borun *et al.*, 1972; Thomas *et al.*, 1975). The methylation of H4 lags slightly behind that of H3, but by the time of mitosis 80−100% of all H3 and H4 molecules are methylated (Shepherd *et al.*, 1971; Borun *et al.*, 1972; Thomas *et al.*, 1975).

The biological function of histone methylation is unknown. Because most (if not all) molecules of H3 and H4 are methylated, it was initially speculated that methylation might be required for proper nucleoprotein assembly and mitotic chromosome condensation, rather than for localized gene activation (Shepherd *et al.*, 1971; Byvoet *et al.*, 1972; Honda *et al.*, 1975). The observation that in many systems there appears to be little or no turnover of methylation is also consistent with this modification being a more generalized phenomenon (Shepherd *et al.*, 1971; Duerre & Lee, 1974; Honda *et al.*, 1975; Waterborg, 1993a). There are, however, several indications that histone methylation is not invariably static. For example, Paik and Kim have described an ϵ-alkyllysinase which has been implicated in histone methyl

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turnover in rat kidney (Paik & Kim, 1974). Also, significant changes in histone methylation patterns follow heat shock in *Drosophila* cells, including a decrease in H3/H4 methylation that correlates with the repression of normal transcription (Desrosiers & Tanguay, 1985, 1988).

Another potentially dynamic characteristic of histone methylation is its close association with acetylation. In plant, avian, and mammalian systems, ongoing methylation is typically targeted to the acetylated isoforms of H3 and H4 (Hendzel & Davie, 1989, 1991; Waterborg, 1990, 1993a; Reneker & Brotherton, 1991). In part on the basis of the close correlation between acetylation and transcription, Hendzel and Davie (1989, 1991) have suggested that methylation helps to maintain active chromatin in a transcriptionally competent state. However, selective methylation of active chromatin was not observed in contact-inhibited human K562 cells, although a general association between acetylation and methylation still obtained (Reneker & Brotherton, 1991). Moreover, in plants (alfalfa), acetylation-coupled methylation has been shown to require histone turnover (Waterborg, 1993a).

To better understand the role of histone methylation, in the following report the relationship between histone methylation and acetylation was examined in cycling and arrested human cells. When deacetylation was inhibited by sodium butyrate, newly methylated H3 became substantially more acetylated than newly methylated H4, indicating that H3 methylation occurs preferentially on chromatin regions engaged in rapid acetylation/deacetylation events. It was also found that newly methylated H3 tended to be complexed in nucleosomes containing acetylated H4; however, newly methylated H4 was underrepresented in acetylated chromatin relative to newly methylated H3. The preferential methylation of acetylated H3 continued when cells were pretreated with cycloheximide or arrested at the G1/S boundary. In contrast, H4 methylation was reduced ~20-fold in arrested cells. Moreover, the continuing methylation of H3 in blocked cells could not be ascribed to histone replacement but instead was due to methyl group turnover. Consistent with the data from cycling cells, H3 methylation in arrested cells occurred on all H3 nonallelic sequence variants and was targeted to nucleosomes containing acetylated H4. These experiments provide evidence for a mode of H3 methylation that (1) is independent of growth-associated histone synthesis, (2) occurs on all major H3 variants, and (3) is selectively associated with rapidly acetylated chromatin. Thus, a subclass of histone methylation, involving primarily histone H3, may have a role not shared by the more general methylation of H3 and H4 following chromatin assembly.

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. To label newly synthesized histones, cells were preincubated in lysine-free MEM[1] ± 50 mM sodium butyrate (Cousens & Alberts, 1979) for 8 min and labeled for 45−60 min with [^{3}H]lysine (80 Ci/mmol, New England Nuclear) at 25−40 μCi/mL in lysine-free MEM (±butyrate) (Perry & Annunziato, 1989). Histone methylation was studied by labeling cells with 50−100 μCi/mL [*methyl*-^{3}H]methionine (100 mCi/mmol, New England Nuclear) in methionine-free medium containing 200 μg/mL cycloheximide, in the presence or absence of 50 mM sodium butyrate; the protein synthesis inhibitor cycloheximide was used to ensure that radiolabeled methyl groups entered proteins solely through posttranslational methylation events [note that cycloheximide *per se* does not inhibit histone acetylation or deacetylation (Annunziato & Seale, 1983; also data presented herein)]. To label acetylated histones, cells were incubated for 60 min with [^{3}H]acetate (4.2 Ci/mmol, New England Nuclear) in the presence of cycloheximide (Annunziato & Seale, 1983).

Cells were arrested using a double-thymidine block (2.7 mM thymidine) (Peterson & Anderson, 1964), based on a HeLa cell cycle of 20−24 h; cells were harvested approximately 17 h after the second thymidine treatment. Arrest was monitored by cytofluorometry. Labeling of methylated histones in arrested cells was performed as described above for cycling cells.

Nuclear Isolation and Chromatin Preparation. Cells were harvested, washed twice in buffer A (10 mM Tris, 5 mM sodium butyrate, 3 mM $MgCl_2$, 2 mM 2-mercaptoethanol, pH 7.6), resuspended in buffer A, and allowed to swell for 15 min. Cells were then lysed with a Dounce homogenizer, and nuclei were pelleted and washed with buffer A (Annunziato & Seale, 1983). Prior to digestion with micrococcal nuclease, nuclei were washed in digestion buffer (10 mM PIPES, 20 mM sodium butyrate, 80 mM NaCl, pH 7.0), resuspended in digestion buffer at 40 A_{260}/mL, and adjusted to 0.5 mM $CaCl_2$. Nuclei were digested with 5 units/mL micrococcal nuclease (Sigma) at 4 °C for 5 min, yielding a soluble chromatin fraction, termed S1 (Perry & Annunziato, 1989). As required, acid-soluble proteins were extracted from either intact nuclei or soluble chromatin with 0.4 N HCl, precipitated with 25% TCA, and then washed with acidified acetone and then with acetone alone (Annunziato & Seale, 1983).

Immunoprecipitation. For all antibody experiments, the penta antiserum first described by Lin *et al.* (1989) was used. We have previously shown that this antiserum exclusively recognizes acetylated, but not unacetylated, isoforms of histone H4 in human cells and that the immobilized antibodies can be used to selectively immunoprecipitate acetylated HeLa cell chromatin (Perry *et al.*, 1993a,b; see also Figure 3A of this paper). For immunoprecipitations, 50−75 μL of undiluted antiserum was preincubated with 15−30 μL of swollen protein A−Sepharose (Pharmacia). In control experiments, preimmune serum was substituted for the antiserum. After washing the Sepharose beads to remove unbound antibody, 75 μL of soluble HeLa S1 chromatin was incubated with the immobilized antiserum for 90 min at 37 °C with constant inversion. Immunoprecipitations were then performed as previously described, yielding unbound (supernatant) and bound (pellet) fractions (Perry *et al.*, 1993a,b). Under these conditions the antiserum was in approximately 2-fold excess over the chromatin to be analyzed, and less than 4% of the input-labeled chromatin was further precipitated by re-treating the unbound supernatant with freshly immobilized antibodies, as measured by scintillation counting.

Gel Electrophoresis and Fluorography. Acid-soluble proteins were subjected to electrophoresis in either SDS

[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; MEM, minimal essential medium.

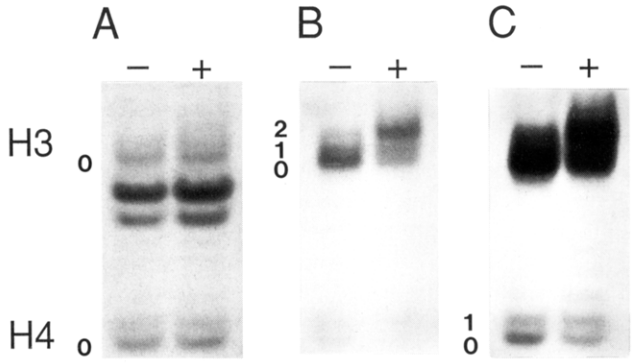

FIGURE 1: Influence of sodium butyrate on the acetylation of dynamically methylated H3 and H4. HeLa cells were labeled for 60 min with [*methyl*-^{3}H]methionine in the presence of cycloheximide, in either the presence (+) or absence (−) of 50 mM sodium butyrate (thus, the plus-butyrate sample was exposed to sodium butyrate for 60 min). Acid-soluble nuclear proteins were subjected to electrophoresis in an acid−urea gel system and analyzed by Coomassie Blue staining (panel A) and by fluorography (panels B and C). Panels B and C represent two different exposures of the gel shown in panel A, in order to resolve both metH3 and metH4. The un-, mono-, and diacetylated histone isoforms are indicated (0, 1, and 2, respectively).

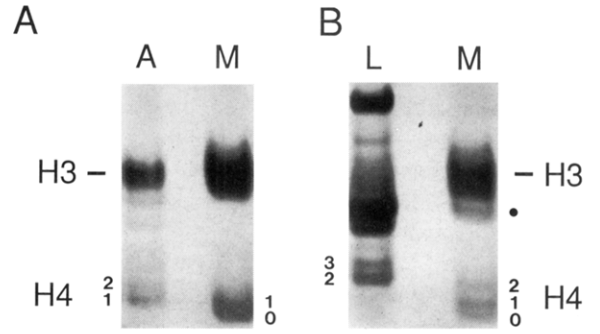

FIGURE 2: Comparison of dynamically methylated H4 with newly acetylated and newly synthesized H4. (Panel A) Cells were labeled with either [^{3}H]acetate (lane A) or [*methyl*-^{3}H]methionine (lane M) in the presence of cycloheximide and butyrate. (Panel B) Cells were labeled with either [^{3}H]lysine (lane L) or [*methyl*-^{3}H]-methionine (lane M) in the presence of butyrate without added cycloheximide. Acid-soluble nuclear proteins were analyzed as in Figure 1; only the fluorographs are shown. The positions of un-, mono-, di-, and triacetylated H4 (0, 1, 2, and 3, respectively, as determined from the stained gel) and of histone H2B (closed circle, lane M) are indicated.

(Thomas & Kornberg, 1975), acetic acid−urea (Panyim & Chalkley), or Triton−acetic acid−urea (6 M) polyacrylamide gel systems (Zweidler, 1978; Bonner *et al*., 1980) and stained with Coomassie Blue. 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

Histone Acetylation and Methylation. As a first approach to the comparison of histone methylation with acetylation, cells were labeled with [*methyl*-^{3}H]methionine in the presence or absence of the deacetylase inhibitor sodium butyrate (Riggs *et al*., 1977); cycloheximide was also added to the medium to prevent the incorporation of radiolabel into nascent polypeptide chains. Following the labeling period (60 min), nuclei were isolated, and acid-soluble nuclear proteins were subjected to electrophoresis in an acid−urea gel system, which separates acetylated histone species from the more rapidly migrating unacetylated forms. Because methylation does not alter the migration of histones in acid−urea gels (Matthews, 1988; Honda *et al*., 1975), acetylation can be monitored by gel position, while methylation is detected through the radiolabel (Figure 1).

As shown by the Coomassie Blue stain (panel A), brief exposure to butyrate (*i.e*., 60 min) did not appreciably affect the acetylation pattern of the bulk histones. Nevertheless, the distribution of newly methylated H3 (metH3) was markedly shifted by butyrate: although some un- and monoacetylated metH3 was still detected, most newly methylated H3 was present in the diacetylated species following butyrate exposure (panel B). In contrast, the migration of metH4 (panel C) was only slightly altered. (Because H3 incorporates substantially more methyl label than H4, a single fluorographic exposure cannot optimally resolve both of these methylated proteins; separate exposures have therefore been presented for H3 and H4). Thus, while the inhibition of histone deacetylation shifts the distribution of both methylated H3 and H4 to more highly acetylated isoforms, the effect of butyrate on H3 is significantly more pronounced. Treatment with calf intestinal phosphatase did not alter the migration of newly methylated H3 or H4 in acid−urea gels (not shown), confirming that butyrate altered the migration of metH3/H4 through acetylation and not through phosphorylation.

Comparison of Dynamically Methylated H4 with Newly Acetylated and Newly Synthesized H4. In light of our inability to detect preferential methylation of multiacetylated forms of H4 in HeLa cells, a direct comparison of H4 acetylation and methylation was made by labeling cells with either [^{3}H]acetate or [*methyl*-^{3}H]methionine in the presence of sodium butyrate (Figure 2A). Following a 60-min labeling period in the presence of butyrate, newly incorporated *acetate* radiolabel was distributed throughout the acetylated forms of H4 (panel A, lane A), while new methyl label was almost totally confined to the un- and monoacetylated species (lane M). Thus, in HeLa cells the ongoing methylation of H4 is clearly not preferentially targeted to rapidly acetylated H4 isoforms. A shorter exposure of the fluorograph (not shown) confirmed the preferential shift of newly methylated H3 to the more highly acetylated species in the presence of butyrate. Further evidence for this will also be presented below.

Newly synthesized H4 is diacetylated when first deposited onto DNA but is quickly deacetylated as nascent chromatin matures (Jackson *et al*., 1976). Because histone methylation begins in S phase (Shepherd *et al*., 1971; Borun *et al*., 1972; Thomas *et al*., 1975), it remained possible that methylation preferentially occurs on newly deposited H4, prior to its deacetylation. Such preferential targeting of methylation to nascent H4 might have been missed by our protocol, because labeling was routinely performed in the presence of cycloheximide. To test this possibility, cells were labeled in the presence of butyrate with either [^{3}H]lysine or [*methyl*-^{3}H]-methionine *minus* cycloheximide. If methylation were to occur preferentially on newly deposited H4 under normal conditions, a sharp increase in the coincidence of methyl label with the nascent diacetylated form of H4 should be observed, especially given the low level of methylation of diacetylated *old* H4 (Figure 1C and 2A). [It should be noted that exposure to sodium butyrate for >18 h arrests cells in G1 (*e.g*., Darzynkiewicz *et al*., 1980); however, the brief

exposures to butyrate used in our experiments do not inhibit DNA replication or nucleosome assembly or induce cell cycle arrest (Annunziato & Seale, 1983; Littlefield *et al.*, 1982)].

As expected (Figure 2B, lane L), 100% of newly synthesized (lysine-labeled) H4 was di- and triacetylated after a 1-h exposure to butyrate [the triacetylated form is a consequence of beginning hyperacetylation (Cousens & Alberts, 1982)]. However, H4 that was methylated during ongoing histone deposition was still predominantly un- and monoacetylated. A comparison of the specific activities of H4 (with one methionine residue) and H2B (with two) indicated that the slight increase in diacetylated metH4 (Figure 2B, lane M) could be accounted for by the direct incorporation of radioactive methionine into the H4 primary sequence [note that H2B is not posttranslationally methylated in vertebrate cells (Duerre & Buttz, 1990; also Figure 1)]. We therefore find no evidence that diacetylated, newly synthesized H4 is preferentially methylated under normal conditions (*i.e.*, minus cycloheximide). Similar conclusions had earlier been reached by Honda *et al.* (1975), working with trout testis; however, in that study labeling was performed for 30 min in the absence of sodium butyrate, conditions that permit the rapid deacetylation of nascent H4.

Immunoprecipitation of Acetylated versus Methylated Chromatin. The experiments presented in Figures 1 and 2 deal with the occurrence of methylated and acetylated lysine residues within the same individual histone protein. Histone octamers contain two molecules each of the four core histones, and although newly methylated H4 was itself not hyperacetylated, it remained possible that H4 methylation involved nucleosomes that contained a second, *acetylated* H4 molecule. To test this, acetylated chromatin was immunoprecipitated with antibodies that selectively recognize acetylated H4. We have previously shown that the "penta" antibodies developed by Lin *et al.* (1989) are highly specific for acetylated H4 in human cells and can be used to immunoprecipitate acetylated HeLa nucleosomes with high efficiency (Perry *et al.*, 1993a,b). HeLa cells were therefore labeled with either [^{3}H]acetate or [*methyl*-^{3}H]methionine in the presence of cycloheximide and sodium butyrate; soluble nucleosomes were prepared by micrococcal nuclease digestion and immunoprecipitated with immobilized penta antibodies. The unbound supernatant and bound immunopellet were then subjected to electrophoresis in the presence of SDS and analyzed by fluorography (Figure 3).

Following a 1-h labeling period, [^{3}H]acetate was incorporated mostly into histones H3 and H4 (panel A, lane I). Greater than 80% of the acetate-labeled chromatin was immunoprecipitated by penta antibodies (Ab, lanes S and P), and no histones were observed in the immunopellet when control serum was substituted for the antiserum (Pre). The stoichiometries of H3 and H4 in the supernatant and pellet fractions were essentially equal, indicating that acetylated H3 is usually (if not always) associated with acetylated H4 in chromatin.

When methyl-labeled chromatin was subjected to the same immunoprecipitation protocol, somewhat different results were obtained (panel B). Although newly methylated H3 was efficiently immunoprecipitated by penta antibodies (indicating a close association between the dynamic methylation of H3 and H4 acetylation), a significantly greater percentage of metH3 was immunoprecipitated relative to metH4. This is consistent with the observation that about half of newly methylated H4 is unacetylated (see Figures 1 and 2). The differential immunoprecipitation of metH3 with penta antibodies has been observed consistently (average difference of 28%, range of 22−33%, for five trials); moreover, such disproportionate immunoprecipitation of acetylated H3 has never been observed [five trials; see also Perry *et al.* (1993a)]. These results were not due to suboptimal immunoprecipitation conditions: reincubating the unbound fraction with a fresh preparation of immobilized antibodies precipitated less than 4% of the input methylated chromatin, still leaving metH4 overrepresented in the supernatant fraction.

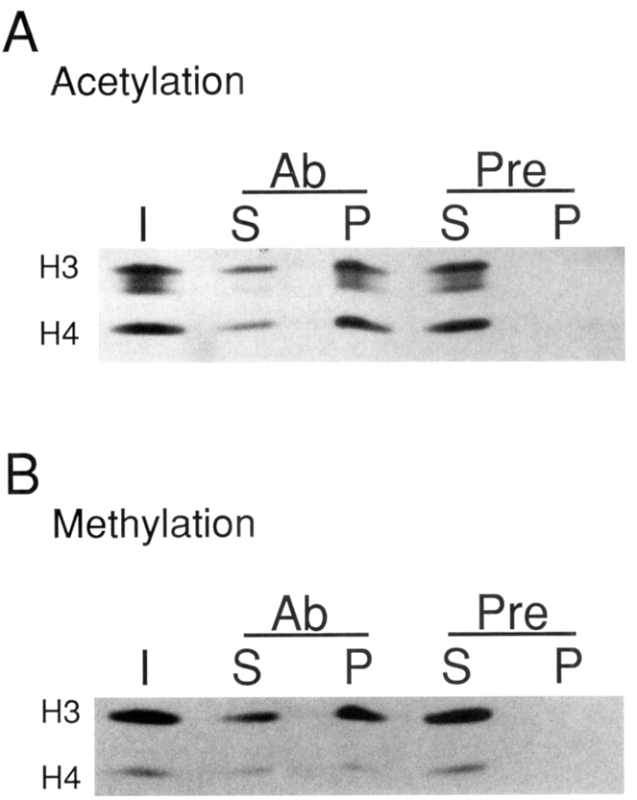

FIGURE 3: Immunoprecipitation of acetylated and methylated nucleosomes with antibodies specific for acetylated H4. Cells were labeled with either [^{3}H]acetate (panel A) or [*methyl*-^{3}H]methionine (Panel B) in the presence of cycloheximide and sodium butyrate. Soluble chromatin was prepared by nuclease digestion and subjected to immunoprecipitation either with the penta antibodies of Lin *et al.* (Ab) or with control serum (Pre). Histones from the starting material (lane I), the immunosupernatant (lane S), and the immunopellet (lane P) were subjected to electrophoresis in the presence of SDS and analyzed by fluorography. The fluorograph in panel B was slightly overexposed to best visualize the H4 band; exposures were optimized prior to scanning to ensure analysis of both H3 and H4 within the linear range.

Both we and others have previously shown that nucleosomes remain intact during immunoprecipitation with antibodies directed against acetylated H4 (Hebbes *et al.*, 1988; Braunstein *et al.*, 1993; Perry *et al.*, 1993b); indeed; because of the antibody's specificity of H4, any nucleosome dissolution should cause the immunopellet to be *enriched* in H4, rather than the reverse. The data therefore provide evidence that ~20−30% of the dynamic methylation of H3 occurs in nucleosomes different from those undergoing methylation of H4 and, further, that these two populations of nucleosomes can be distinguished by their degree of H4 acetylation.

Deacetylation Kinetics of Newly Methylated H3 and H4. Two distinct populations of acetylated histones exist in cultured cells. One population is engaged in rapid acetyla-

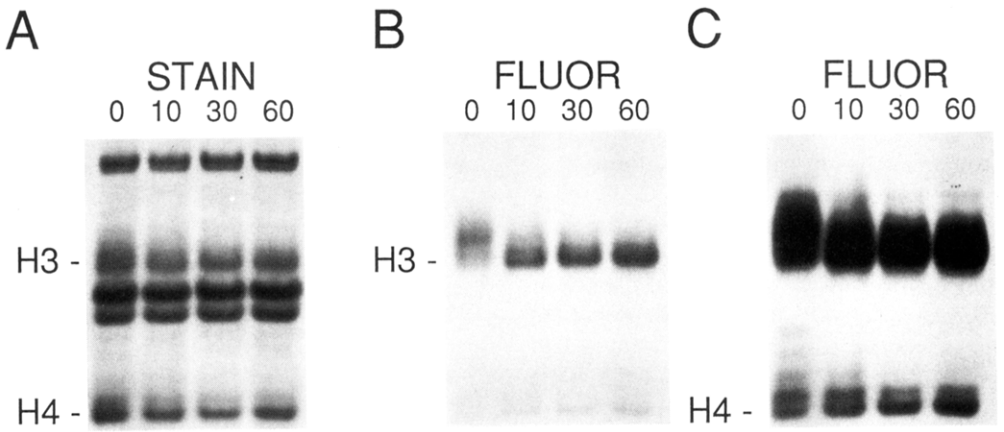

FIGURE 4: Deacetylation of dynamically methylated H3 and H4. Cells were labeled for 60 min with [*methyl*-^{3}H]methionine in the presence of cycloheximide and butyrate (lane 0). Cells were then washed free of sodium butyrate (but maintained in cycloheximide) and chased for 10, 30, and 60 min in the absence of butyrate. Acid-soluble nuclear proteins from each time point were analyzed as in Figure 1. (Panel A) Coomassie stain; (panel B) fluorograph exposed to resolve histone H3; (panel C) fluorograph exposed to resolve H4. The positions of unacetylated H3 and H4 are indicated by dashes.

tion/deacetylation events, with a half-life of ~5−10 min for the loss of the modified isoforms; the other class of acetylated histone is much more slowly modified and concomitantly more slowly converted back to the unacetylated species ($t_{1/2}$ ≈ 30 min) (Covault & Chalkley, 1980). The data of Figure 1 suggest that most newly methylated H3 is associated with the "fast" mode of histone acetylation, while most newly methylated H4 belongs to the population acetylated with "slow" kinetics. To test this, the rates of deacetylation of methylated H3 and H4 were compared. Cells were labeled for 60 min in the presence of cycloheximide and butyrate as before; butyrate was then washed from the medium, and the deacetylation of metH3 and metH4 was monitored in the continuous presence of cycloheximide (Figure 4). (Note that the reverse experiment, pulsing cells briefly with [*methyl*-^{3}H]methionine to determine the initial acetylation rate of metH3/H4, is precluded by the need to sufficiently label methylated histones for detection.)

As seen in panel B of Figure 4, most newly methylated H3 (which is di- and triacetylated) was rapidly deacetylated within the first 10 min following butyrate removal. During the subsequent 50-min chase, monoacetylated metH3 was further deacetylated with slow-type kinetics. The very minor fraction of highly acetylated metH4 was also deacetylated during the first 10 min of the chase (panel C); nevertheless, most metH4 was both acetylated and deacetylated slowly. Therefore, while both metH3 and metH4 are represented in the fast acetylation population, their relative distributions in these histone classes are widely different: metH4 is in general much more slowly acetylated, and is deacetylated with similar kinetics.

Effects of Pretreatment with Cycloheximide and/or Butyrate on Histone Methylation. The differences observed in the methylation patterns of H3 and H4 raised the question as to how well the more highly acetylated species of H4 could serve as substrates for methylation. To study this, cells were *preincubated* for 1 h in the presence or absence of sodium butyrate (the former to accumulate rapidly acetylated H4 isoforms, as described above), plus or minus cycloheximide, prior to labeling with [*methyl*-^{3}H]methionine in the continuous presence of butyrate and cycloheximide as before. Acid-soluble nuclear proteins were subjected to acid−urea gel electrophoresis and analyzed by staining and fluorography (Figure 5A,B).

As seen in the fluorograph (panel B), multiacetylated forms of H4 were readily methylated after preincubation in butyrate, although some methylation still occurred on the un- and monoacetylated isoforms. The increase in multiacetylated metH4 occurred whether or not histone synthesis took place during the butyrate pretreatment (panel B, lanes 2 and 3), consistent with most H4 methylation occurring well after histone deposition and deacetylation (Honda *et al*., 1975; also Figure 2). A comparison of the fluorograph with the stained gel showed that newly methylated H4 was relatively enriched in multiply acetylated species following preincubation in butyrate. Apparently, prior acetylation can potentiate H4 methylation to a certain degree (please see the Discussion). Nevertheless, un- and monoacetylated H4 were strongly methylated when butyrate was omitted during the preincubation period (panel B, lane 1).

Next, the effect of inhibiting protein synthesis on the acetylation-associated methylation of H3 was examined. Cells were preincubated in cycloheximide for 2 h, plus or minus butyrate, and then labeled in the presence of cycloheximide and butyrate as before (Figure 5C,D, lanes 1 and 2); as a control, cells were labeled without preincubation (lane 3). Evan after 2 h in cycloheximide *minus* butyrate, newly methylated H3 was predominantly di- and triacetylated (panel D, lane 1), while bulk H3 was found mainly in the unacetylated form (panel C, lane 1). It is noteworthy that most metH3 is di- and triacetylated after a 2-h pretreatment with cycloheximide, whether or not butyrate is present (compare the first two lanes, panel D), while preincubation with butyrate is needed to shift metH4 to more highly acetylated isoforms (Figure 5B). These experiments therefore point to a class of H3 methylation that is closely linked to rapid histone acetylation but not necessarily to histone synthesis (as judged by insensitivity to cycloheximide pretreatment). To further address this, experiments were performed on growth-arrested cells, as described below.

Histone Methylation during Cell Cycle Arrest. HeLa cells were synchronized by means of a double thymidine block (Peterson & Anderson, 1964). Cytofluorometric analysis confirmed that 80% of the cells were in G1 and the G1/S boundary, with ≤10% of the cells in S (Figure 6C). As expected (Wu & Bonner, 1981), core histone synthesis in arrested cells decreased sharply and was only ~7% that of randomly cycling cells (Figure 6A, fluor, lane 2). Com-

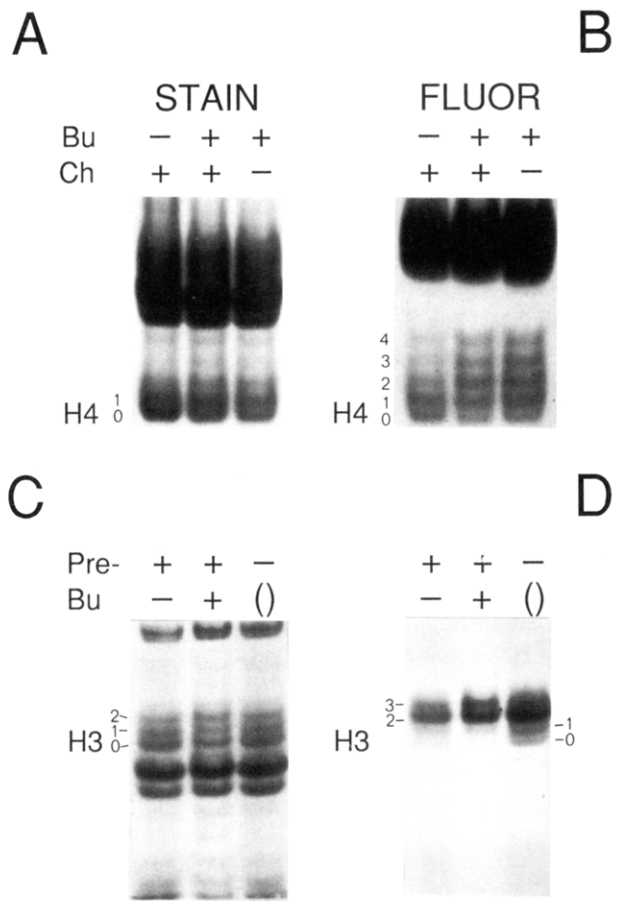

FIGURE 5: Effects of pretreatment with cycloheximide and/or sodium butyrate on histone methylation. (Panels A and B) Cells were preincubated for 60 min either with or without cycloheximide (+/− Ch), plus or minus the simultaneous presence of sodium butyrate (+/− Bu), prior to labeling with [*methyl*-^{3}H]methionine for 60 min in the presence of both cycloheximide and butyrate as usual. The un- (0), mono- (1), and multiacetylated (2−4) isoforms of H4 are indicated. (Panels C and D) Cells were preincubated for 2 h in cycloheximide or not preincubated at all (+/− Pre-); preincubations were also performed plus or minus sodium butyrate (+/− Bu). Cells were then labeled as usual for 60 min with [*methyl*-^{3}H]methionine in the presence of both butyrate and cycloheximide. In all cases, acid-soluble nuclear proteins were subjected to acid−urea gel electrophoresis and analyzed by staining (panels A and C) and by fluorography (panels B and D). The un- (0), mono- (1), and multiacetylated (2, 3) isoforms of H3 are indicated. The gel shown in panels A and B was slightly overloaded to better reveal the multiacetylated forms of H4 by staining.

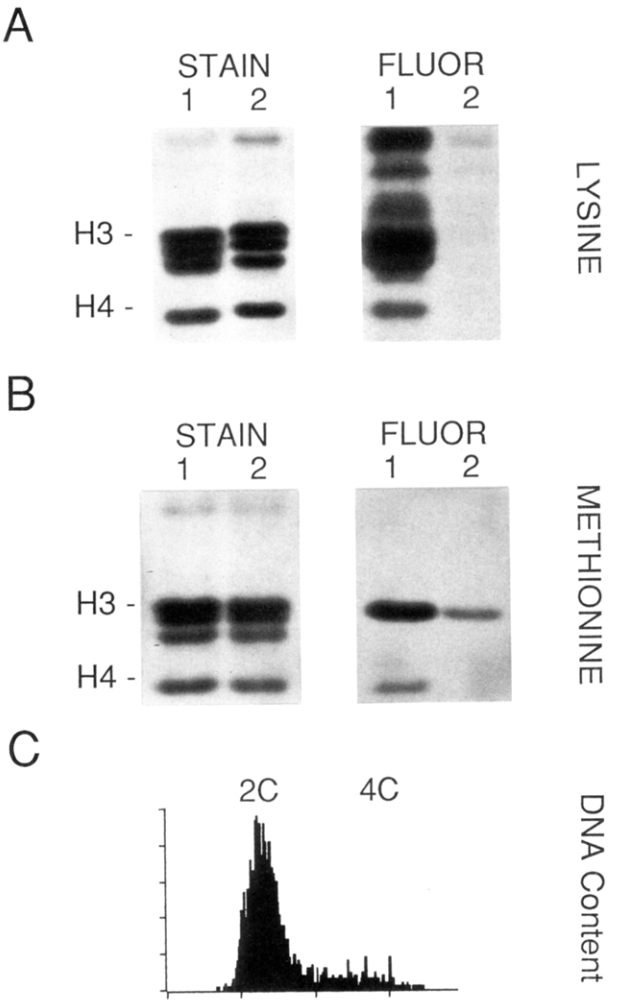

FIGURE 6: Histone synthesis and methylation in arrested cells. Control cells (lanes marked 1) or cells that were arrested through a double thymidine block (lanes marked 2) were labeled either with [^{3}H]lysine (panel A) or with [*methyl*-^{3}H]methionine (panel B), the latter in the presence of cycloheximide; acid-soluble nuclear proteins were subjected to electrophoresis in the presence of SDS and analyzed by Coomassie Blue staining (left-hand panels) or fluorography (right-hand panels); fluorographs were scanned for quantitation. (Panel C) Cytofluorometric analysis of HeLa cells subjected to a double thymidine block.

mensurate with the decrease in histone synthesis, H4 methylation was dramatically reduced, to ≤5% of control levels (Figure 6B, fluor, lane 2). In sharp contrast, the methylation of H3 was partially uncoupled from that of H4 and remained disproportionately high at ~25% of the control level. Moreover, most newly methylated H3 in arrested cells was associated with *acetylated* H4 in nucleosomes, as demonstrated by immunoprecipitating soluble chromatin with antibodies specific for acetylated H4 (Figure 7). As will be shown below, the methylation of H3 in blocked cells is preferentially targeted to the more highly acetylated H3 isoforms. It is therefore concluded that, in HeLa cells, histone H3 can undergo a subtype of methylation that is separable from that of H4, is independent of histone synthesis, and is closely linked to the acetylation of both H3 *and* H4 in chromatin.

Through a series of careful experiments, Waterborg has shown that histone methylation in alfalfa cells is solely due to histone synthesis and turnover and that the correlation between acetylation and methylation is especially strong for the highly labile variant H3.2 (Waterborg, 1990, 1993a,b). We therefore examined histone turnover in synchronized HeLa cells. Newly synthesized histones were labeled with [^{3}H]lysine for 45 min; cells were then arrested by a double thymidine block. At this time (*i.e.*, when arrested cells were labeled with [*methyl*-^{3}H]methionine to study histone methylation, as in Figures 6 and 7) one-half of the lysine-labeled culture was harvested, and acid-soluble nuclear proteins were prepared. The remaining half was maintained in the arrested state for an additional 24 h prior to nuclear isolation and histone preparation. Nuclear proteins were then separated in a Triton−acid−urea (TAU) gel system (which resolves histone primary sequence variants) and analyzed by staining and fluorography. The results are presented in Figure 8A.

No histone protein turnover was observed during cell cycle arrest, as determined by the specific activity of labeled histones prepared from equivalent numbers of cell nuclei (see lanes 1 and 2 in the fluorograph, Figure 8A). It is thus unlikely that the generation of metH3 in blocked cells can be ascribed to methylation following histone replacement synthesis. In addition, by labeling arrested cells with

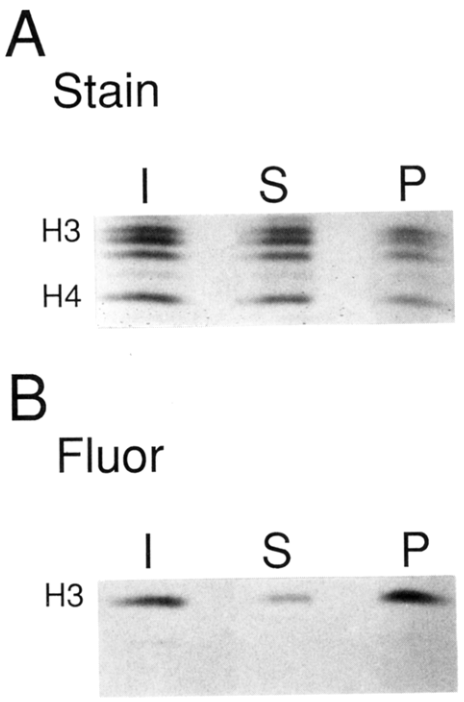

FIGURE 7: Immunoprecipitation of methylated nucleosomes from arrested cells. Cells arrested by the double thymidine block protocol were labeled with [*methyl*-^{3}H]methionine in the presence of cycloheximide and butyrate. Soluble chromatin was immunoprecipitated as in Figure 3. Input (I), supernatant (S), and immunopellet (P) fractions were subjected to electrophoresis in the presence of SDS and analyzed by Coomassie Blue staining (panel A) and fluorography (panel B). No chromatin was precipitated when control nonimmune serum was used (not shown; see also Figure 3).

[*methyl*-^{3}H]methionine (panel B) it was determined that methylation in blocked cells occurred on H3.1, H3.2, and H3.3 and involved the multiacetylated forms of each H3 sequence variant (Figure 8B, fluorograph, lane 2). The distribution of methylated histones in arrested cells was virtually identical to that in randomly cycling cells, except for the previously noted disproportionate undermethylation of H4 (compare lanes 1 and 2, Figure 8B, fluorograph). Electrophoresis in TAU gels also confirmed that methylation shows little or no preference for acetylated versus unacetylated H4 in nonarrested cells, even in the presence of sodium butyrate (panel B, lane 1).

The continued methylation of H3 in the absence of histone synthesis suggests that H3 methyl groups undergo turnover. To test this, arrested cells were labeled with [*methyl*-^{3}H]-methionine in the presence of cycloheximide as usual, then washed free of radiolabel (while maintained in 2.7 mM thymidine), and held in the arrested state for an additional 4 or 24 h. Acid-soluble nuclear proteins from each time point were separated in SDS gels, to best measure total turnover, and analyzed by staining and fluorography (Figure 9).

After an initial increase in labeling observed at the 4-h time point (due to continued incorporation as the labeled amino acid pool equilibrates), ~45% of the methyl label of H3 was lost during the next 20-h period (fluor, lanes 2 and 3). Because the histones themselves are stable during this identical period (Figure 8A), it is concluded that the loss of label is due to methyl group turnover on histone H3.

DISCUSSION

The methylation patterns of H3 and H4 in HeLa cells differ in several important respects. First, although both newly methylated H3 and H4 can be acetylated, the acetylation level of metH3 is more markedly increased by sodium butyrate. Thus, H3 molecules that undergo rapid acetylation/deacetylation are preferentially methylated. In contrast, a considerable fraction of newly methylated H4 remains unacetylated even when sodium butyrate is present during the methylation process, consistent with H4 methylation being predominantly associated with the slow form of histone acetylation.

Second, a greater percentage of newly methylated H3 is complexed in nucleosomes containing acetylated H4. By immunoprecipitating chromatin with antibodies that selectively recognize acetylated H4, it was found that about 20−30% of newly methylated H3 is not associated with newly methylated H4 in chromatin; importantly, such deviation from normal stoichiometry was not observed when [^{3}H]-acetate-labeled chromatin was immunoprecipitated. These values may be an underestimate, because the populations of nucleosomes containing newly methylated H3 versus H4 could be entirely distinct, yet coprecipitate due to independent H4 acetylation events. Thus, our experiments have identified the minimum extent to which the dynamic methylations of H3 and H4 are nonoverlapping in chromatin, as measured by their association with acetylated H4. These two populations of nucleosomes may be separated temporally (through delayed methylation of H4) or spatially (in different chromatin regions). That at least some methylation of H3 is physically isolated from that of H4 is supported by experiments with synchronized cells (see below).

Third, the methylation of H4 is more tightly linked to cell growth and histone synthesis. To avoid problems associated with long-term exposure to cells to cycloheximide, experiments were performed using cells arrested by a double thymidine block. It was found that 20−25% of H3 methylation in arrested cells proceeds independently of the methylation of H4 (involving all H3 sequence variants) and continues when histone synthesis and turnover are minimal. In contrast, H4 methylation declined about 20-fold under the same conditions. Because the methylation of newly synthesized (and deposited) histones is completed by the time of mitosis, cells arrested in G1 will have finished deposition-related methylation events begun in the previous cell cycle (Shepherd *et al.*, 1971; Borun *et al.*, 1972; Thomas *et al.*, 1975). The comparatively low level of H4 methylation in G1, then, is consistent with the targeting of most H4 methylation to newly deposited H4. As suggested by Dixon and colleagues (Honda *et al.*, 1975), the time course of the postsynthetic modifications of new H4 may be acetylation first and then deacetylation, followed by methylation, consistent with the progressive methylation of H3 and H4 until cell division.

The methylation of H3 in S phase typically precedes that of H4 (Shepherd *et al.*, 1971; Borun *et al.*, 1972; Thomas *et al.*, 1975). It was therefore possible that the coupling of H3 methylation to acetylation might be ascribed solely to the methylation of H3 immediately after synthesis and deposition, when new histones are readily hyperacetylated in the presence of butyrate (Cousens & Alberts, 1982). However, our finding that acetylated H3 is preferentially methylated in arrested cells (or in cycling cells following a 2-h preincubation in cycloheximide) argues against this. While the sharp decline in the rate of methylation in arrested cells is consistent with most H3 methylation being linked to histone deposition, the continuing (although depressed) methylation of H3 in blocked cells indicates that a subclass

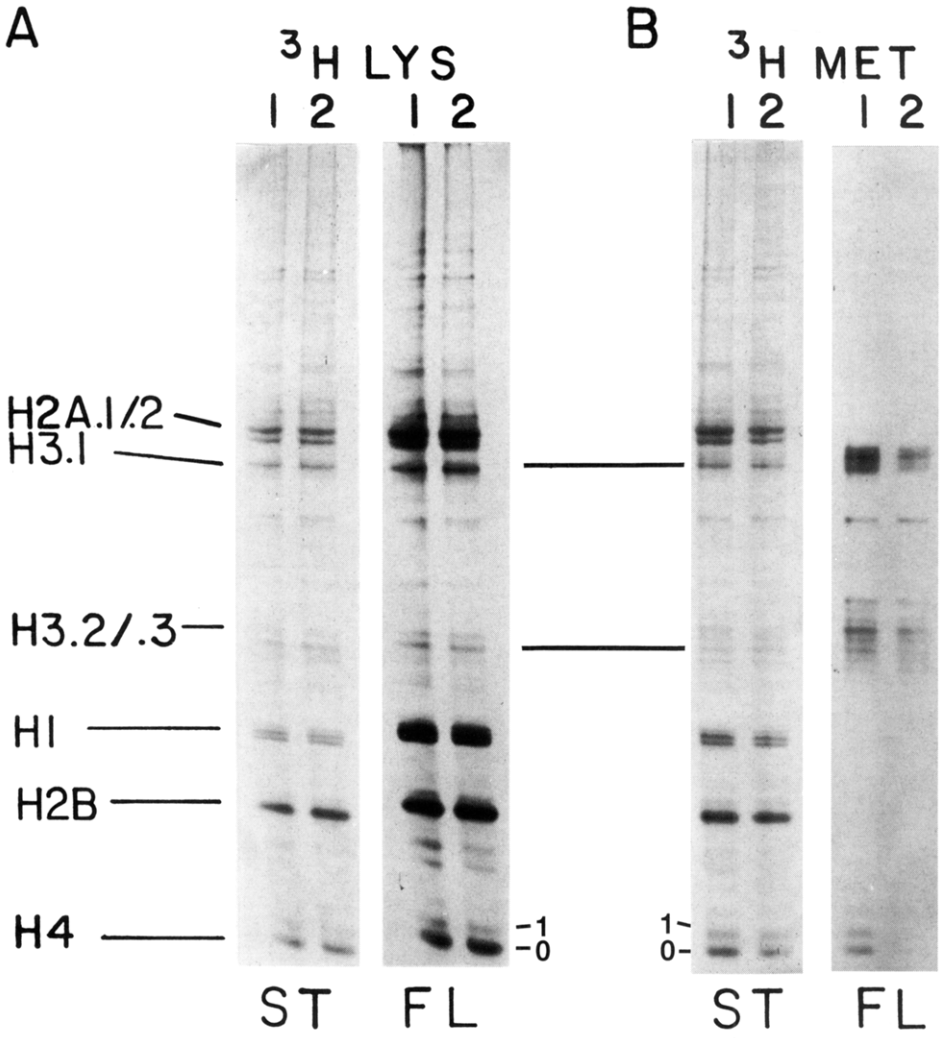

FIGURE 8: Histone turnover and methylation in arrested cells. (Panel A) Cells were labeled for 45 min with [^{3}H]lysine and then arrested by a double thymidine block. Half of the arrested cells were then harvested for nuclear isolation (lane 1); the remaining half was maintained in the arrested state for an additional 24 h prior to nuclear isolation and histone extraction (lane 2). Acid-soluble nuclear proteins were subjected to electrophoresis in a Triton−acid−urea gel system and analyzed by staining (ST, left) or fluorography (FL, right). (Panel B) Control cells (lane 1) or arrested cells (lane 2) were labeled for 60 min with [*methyl*-^{3}H]methionine in the presence of cycloheximide and butyrate; acid-soluble nuclear proteins were analyzed as in panel A.

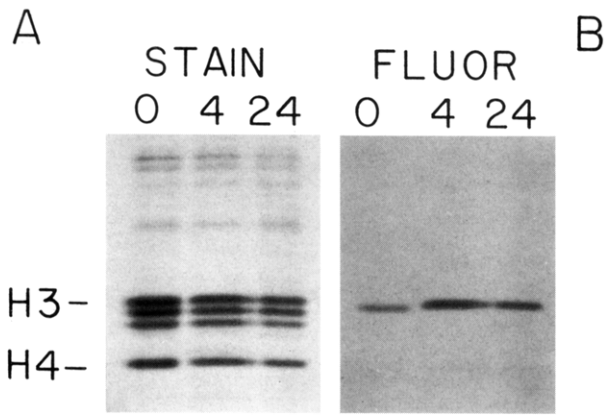

FIGURE 9: Methyl group turnover in arrested cells. Arrested cells were labeled with [*methyl*-^{3}H]methionine in the presence of cycloheximide, harvested, washed, and maintained in the arrested state for an additional 4 h (4) or 24 h (24). For each time point, acid-soluble nuclear proteins were subjected to electrophoresis in the presence of SDS and analyzed by staining (panel A) or fluorography (panel B); the fluorograph was scanned for quantitation.

of H3 methylation may be regulated differently.

The efficient immunoprecipitation of newly methylated H3 with anti-(acetylated) H4 antibodies parallels the immunoprecipitation of transcriptionally active chromatin with the same or comparable antisera (Hebbes *et al.*, 1988, 1992; Clayton *et al.*, 1993; Braunstein *et al.*, 1993). The well-documented association between histone acetylation and transcriptional competence [*e.g.*, Vavra *et al.* (1982), Allegra *et al.* (1987), Ip *et al.* (1988), and Ridsdale *et al.* (1990); reviewed recently in Davie and Hendzel (1994)] had led to the suggestion that acetylation-coupled methylation helps to maintain developing chicken erythrocyte chromatin in a transcriptionally competent state (Hendzel & Davie, 1989, 1991). Conversely, Reneker and Brotherton (1991) found no preferential association between dynamically methylated histones and transcriptionally active chromatin in contact-inhibited K562 cells. As discussed by those authors (Reneker & Brotherton, 1991), differences between the two systems may provide an explanation for this discrepancy. Whereas immature chicken erythrocytes possess only one form of histone acetylation, characteristic of transcriptionally competent/engaged chromatin (and involving only 1−2% of the genome) (Zhang & Nelson, 1988a,b; Hendzel & Davie, 1991), most histone methylation in contact-inhibited K562 cells is associated with the "slow-turnover" form of acetylation (Reneker & Brotherton, 1991), which successively affects up to 80% of total cellular histone (Covault &

Chalkley, 1980). Thus, it may be the class of acetylation itself (and the preponderance thereof) that dictates the degree to which methylation and transcription are interrelated.

Interestingly, methylation in HeLa cells is correlated with both the fast and slow forms of acetylation, in two distinct patterns. H3 methylation is preferentially associated with the rapid form, while newly methylated H4 is largely acetylated with slow kinetics. Apparently, ongoing methylation is relatively indifferent to the acetylation status of H4 in nucleosomes, thereby permitting the unacetylated isoform to be a major substrate. In contrast, H3 methylation appears highly responsive to the accessibility of H3 N-termini in chromatin, as regulated by acetylation. Because virtually all H3 and H4 molecules are eventually methylated, any role for methylation in maintaining chromatin in an "active" state presumably entails methyl group turnover, rather than methylation *per se*. Our results indicate that, in HeLa cells, this form of methylation should mainly involve histone H3. Still, it must be stressed that it is unclear at this time whether the fast acetylation of H3 normally precedes dynamic methylation or vice versa. The fact that the methylation inhibitor adenosine dialdehyde has no effect on histone acetylation (Hendzel & Davie, 1992) suggests that the acetylation of H3 may be the governing modification, rather than the reverse.

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