Histone Deacetylation Is Required for the Maturation of Newly Replicated Chromatin*

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The effects of inhibiting histone deacetylation on the maturation of newly replicated chromatin have been examined. HeLa cells were labeled with [3H]thymidine in the presence or absence of sodium butyrate; control experiments demonstrated that butyrate did not significantly inhibit DNA replication for at least 70 min. Like normal nascent chromatin, chromatin labeled for brief periods (0.5–1 min) in the presence of butyrate was more sensitive to digestion with DNase I and micrococcal nuclease than control bulk chromatin. However, chromatin replicated in butyrate did not mature as in normal replication, but instead retained ~50% of its heightened sensitivity to DNase I. Incubation of mature chromatin in butyrate for 1 h did not induce DNase I sensitivity: therefore, the presence of sodium butyrate was required during replication to preserve the increased digestibility of nascent chromatin DNA. In contrast, sodium butyrate did not inhibit or retard the maturation of newly replicated chromatin when assayed by micrococcal nuclease digestion, as determined by the following criteria: 1) digestion to acid solubility, 2) rate of conversion to mononucleosomes, 3) repeat length, and 4) presence of non-nucleosomal DNA. Consistent with the properties of chromatin replicated in butyrate, micrococcal nuclease also did not preferentially attack the internucleosomal linkers of chromatin regions acetylated in vivo. The observation of a novel chromatin replication intermediate, which is highly sensitive to DNase I but possesses normal resistance to micrococcal nuclease, suggests that nucleosome assembly and histone deacetylation are not obligatorily coordinated. Thus, while deacetylation is required for chromatin maturation, histone acetylation apparently affects chromatin organization at a level distinct from that of core particle or linker, possibly by altering higher order structure.

The heightened sensitivity of newly replicated chromatin to both DNase I and micrococcal nuclease has long been established (1–5). Normally, this increased digestibility persists for approximately 10 min. The bases for this transient alteration in nuclease accessibility are poorly understood; the complex processes involved in chromatin biosynthesis probably involve several structural modifications that contribute to the overall nuclease sensitivity of nascent chromatin. These modifications include conformational transitions of parental monomers (6), a reduction in repeat length (4, 6–9), incompletely assembled (non-nucleosomal) nascent DNA (9–12), and, presumably, a localized unfolding of the 30-nm chromatin fiber (4, 5).

One means of distinguishing among the above possibilities is to use specific inhibitors of cellular metabolism, in order to uncouple a selected process from the many required for chromatin replication. For example, when chromatin DNA is replicated in the presence of puromycin or cycloheximide, both increased nuclease sensitivity (13–15) and non-nucleosomal assembly intermediates persist (9, 16). Thus, concurrent protein synthesis is essential for normal chromatin assembly. Although it is not certain whether this is solely due to a depletion of nascent histones (5, 8, 17–24) or to other protein synthesis-dependent events. Because newly synthesized H4 is acetylated (25–29), and histone acetylation has been correlated with DNase I sensitivity (30–39), we have used the deacetylase inhibitor sodium butyrate (40–44) to determine whether acetylation contributes to the nuclease sensitivity of nascent chromatin.

The experiments in this report demonstrate that acetylation is a factor in the heightened sensitivity of nascent chromatin to DNase I, but not to micrococcal nuclease, and that deacetylation is required for the maturation of chromatin following DNA synthesis. It is further demonstrated that the maintenance of acetylation on newly assembled nucleosomes neither dramatically alters core particle structure, nor inhibits the establishment of normal nucleosomal spacing. Also, in regions of chromatin that have been acetylated to physiological levels, internucleosomal DNA is as resistant to micrococcal nuclease as that in bulk chromatin. Taken together, these findings strongly suggest that during replication histone deacetylation is not required for nucleosome assembly, or for the recovery of normal nucleosomal spacing and linker DNA protection (as measured by micrococcal nuclease). Rather, deacetylation is apparently instrumental in the restoration of some other parameter of chromatin organization, possibly higher order structure.

MATERIALS AND METHODS

Cell Culture and Labeling—HeLa cells were maintained in spinner culture at 37 °C in Eagle’s minimal essential medium supplemented with 5% calf serum.

Long term labeling of cells with [14C]thymidine (60 mCi/mmol, ICN) was performed at 0.02 μCi/ml for one generation (24 h). For pulse labeling, cells (~4 × 10^6/ml) were harvested by centrifugation (250 × g, 1.5 min) and concentrated 20-fold (0.5–1 min pulse) or 5-fold (10–50 min pulse) in prewarmed whole medium, followed by equilibration at 37 °C for 5 min. For 0.5–1 min labeling, cells were incubated with [methyl-3H]thymidine (60 Ci/mmol, New England Nuclear) at 50–60 μCi/ml, and for longer labeling periods, at 0.5–1.0 μCi/ml; labeling in the presence of cycloheximide was performed at 4 μCi/ml.

Labeling with [3H]acetate (2 Ci/mmol, New England Nuclear) was

* This work was supported by National Institutes of Health Grant GM27950. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
performed at 0.3–0.8 mM Cl/m, after concentrating cells 20–30-fold in prewarmed whole medium. Only the core histones incorporated acetate during brief labeling (less than 30 min), as determined by SDS-polyacrylamide gel electrophoresis of total nuclear proteins. Control experiments demonstrated that acetate label was removed from the histones in the absence of butyrate; thus, label was not cycled into amino acids. No incorporation of acetate radiolabel into DNA was detected.

To measure DNA synthesis, cells were prelabeled for 24 h with $[^1]C$ thymidine and incubated with $[^3]H$ thymidine for the times indicated (text). Labeling was halted by diluting an aliquot of cells 5-fold with ice-cold buffer A (10 mM Tris, 3 mM MgCl₂, 2 mM mercaptoethanol, pH 7.6) containing 1% sodium azide. Cells were pelleted in H₂O, and digested with 10 mM trichloroacetic acid. 1 mM sodium butyrate (Baker), adjusted to pH 7.0, was filter-sterilized and added to a final concentration of 50 mM. Cycloheximide was added as required to a final concentration of 200 µg/mL, from a freshly prepared stock solution of 10 mg/mL. For pulse labeling, cells were preincubated for 5–10 min in sodium butyrate or cycloheximide before the addition of radiolabel. Labeling was halted by diluting cells 5-fold in ice-cold C buffer (1 mM Tris, 25 mM KCl, 0.9 mM MgCl₂, 0.9 mM CaCl₂, 0.14 mM spermidine, pH 7.6) containing 20 mM sodium butyrate and 0.1% sodium azide.

Nuclear Isolation; Digestion with DNase I and Micrococcal Nuclease—Cells labeled in the presence of sodium butyrate were washed twice in CB buffer (C buffer plus 20 mM sodium butyrate), and resuspended in CB buffer plus 0.5 mM PMSF. Cells labeled in the absence of butyrate were either treated as above, or washed and resuspended (and nuclei isolated) in C buffer alone, with no difference in the presence of butyrate was highly sensitive to DNase I (Fig. 2). Slab gels were cast and run in TBE buffer containing 10% glycerol, brought to 100 °C for 5 min, and rapidly cooled before application. Electrophoreisis was at 200 V.

DNA was adjusted to 10 mM magnesium acetate, 0.3 M sodium acetate, and ethanol precipitated. Carrier, with 10% trichloroacetic acid, collected on glass fiber filters were precipitated, together with 50 µg each of DNA and albumin carrier, with 10% trichloroacetic acid. Purification DNA was adjusted to 10 mM magnesium acetate, 0.3 mM sodium acetate, and ethanol precipitated.

For digests with micrococcal nuclease (Sigma), nuclei were resuspended at 40 A₂₆₀/µL in 0.2 ml of NCS (Amersham Corp.) containing 5% HzO₂ and autoclaved. Nuclei were then lysed by resuspension in 2 mM EDTA, pH 7.2; insoluble chromatin (pellet) was removed by centrifugation, yielding a supernatant fraction containing 70–80% of bulk DNA as solubilized chromatin.

To measure acid-precipitable radioactivity, nuclei prelabeled with $[^1]C$ thymidine for 24 h in the absence of sodium butyrate were mixed with $[^3]H$ thymidine nuclei and digested with DNase I or micrococcal nuclease. Aliquots of the total digest were removed, brought to 10 mM EDTA, and precipitated with trichloroacetic acid.

Gels Electrophoresis and Histone Isolation—DNase I digestion products were analyzed in 10% polyacrylamide gels according to the procedure of Peacock and Dingman (45). Gels were cast and run in 0.9 M Tris, 0.09 M boric acid, 0.003 M EDTA, pH 8.0 (TBE buffer). The acrylamide:bisacrylamide ratio was 20:1. Purified DNA samples were dissolved in 0.1× TBE buffer containing 10% glycerol, brought to 100 °C for 5 min, and rapidly cooled before application. Electrophoresis was performed at 200 V.

For DNA size analysis of micrococcal nuclease digestion products, chromatin samples were adjusted to 10 mM magnesium acetate, ethanol precipitated, and solubilized in electrophoresis buffer (40 mM Tris, 20 mM sodium acetate, and 1 mM EDTA, adjusted to pH 7.2 with glacial acetic acid) made 5% in glycerol and 1% in SDS. Polyacrylamide slab gels (3%) were prepared according to Loening (46) and run at 90–100 V in the presence of 0.1% SDS; the acrylamide:bisacrylamide ratio was 20:1.

Electrophoretic analyses of nucleoprotein particles were performed in composite 0.5% agarose, 2.5% polyacrylamide gels, according to the method of Todd and Garrard (47). Slab gels were cast and run in 6.4 mM Tris, 3.2 mM sodium acetate, 0.28 mM EDTA, pH 8.0. Electrophoresis was at 110 V at 4 °C, with buffer recirculation.

DNA and nucleoprotein gels were stained with ethidium bromide, photographed, impregnated with scintillant, and exposed to Kodak XAR-5 film. Nucleoprotein gels were prepared for fluorography using Autofluor (National Diagnostics) after ethanol fixation; DNA gels were impregnated with PPO (48, 49).

Histones were extracted from isolated nuclei and chromatin fractions with 0.4 N HCl at 4 °C, precipitated with 25% trichloroacetic acid, washed with acetone, and dissolved in sample buffer. Electrophoresis in SDS-polyacrylamide gels (18%) was performed at 30 mA, according to the method of Thomas and Kornberg (50). Acetylated histones were resolved at 0.9 mM acetic acid, 2.5 µg/mL, 15% polyacrylamide slab gels (51, 52). Protein gels were stained with Coomasie blue in 45% ethanol, 10% acetic acid, and impregnated with PPO for fluorography (48, 49).

Radioactivity Determinations—Aliquots of chromatin fractions were precipitated, together with 50 µg each of DNA and albumin carrier with 10% trichloroacetic acid (Reeve Angel 934-AH, Whatman), and washed twice with trichloroacetic acid and twice with 95% ethanol. Dried filters were incubated with 0.2 ml of NCS (Amersham Corp.) containing 5% H₂O₂ and counted in PPO-toluene (4 g/liter) fluor. The fraction of $^{14}$C counts registered in the $^3$H channel was subtracted by the external standard technique.

RESULTS

Effect of Sodium Butyrate on DNA Replication—Because prolonged incubation of cells in sodium butyrate results in cessation of DNA synthesis and cellular growth (53–57), it was necessary to determine the short term effects of butyrate on replication. HeLa cells were labeled with $[^3]H$ thymidine in the presence or absence of 50 mM sodium butyrate. Control experiments verified that at this concentration butyrate effectively inhibited histone deacetylation (data not presented; see also Ref. 44). No significant reduction in thymidine incorporation was noted for at least 70 min (Fig. 1).

The modest effect of limited butyrate exposure correlates well with the inability of butyrate to inhibit thymidine incorporation in synchronous S phase cells (58) and is consistent with the primary growth block occurring in G1 (58–63). Since butyrate also does not inhibit histone synthesis for at least several hours (28, 64), this agent can be used to examine the involvement of histone deacetylation in the maturation of newly replicated chromatin.

Effects of Sodium Butyrate As Determined by DNase I Digestion—As in normally replicating cells, chromatin DNA labeled with $[^3]H$ thymidine for very brief periods (0.5 min) in the presence of butyrate was highly sensitive to DNase I (Fig. 2A). (Note: in this, as in experiments to follow, the nuclease sensitivity of newly replicated chromatin is compared to that of an internal standard of nuclei prelabeled for one generation with $[^1]C$ thymidine in the absence of butyrate; relative sensitivity is therefore expressed as acid-precipitable $[^3]H$/[^1]C.) With continued replication in the presence of butyrate (10 min), chromatin DNA recovered approximately 50% of the normal resistance to DNase I; however, chromatin labeled for 30 or 50 min in butyrate did not mature further (Fig. 2, A and B), even though DNA synthesis remained vigorous at this time (Fig. 1). In contrast, when replicates in the absence of butyrate nascent chromatin recovered full DNase I resistance within 20–30 min (Fig. 2B), as shown previously (1). Thus,
Deacetylation is required for the full maturation of newly replicated chromatin.

It is unlikely that the continued DNase I sensitivity of butyromatin is due to inhibition of nucleosome assembly. The persistent level of DNase I sensitivity of bu-chromatin was significantly less than that observed for chromatin replicated in cycloheximide (Fig. 2A). The heightened nucleosome sensitivity of chromatin synthesized in cycloheximide has been generally attributed to the inability of de novo nucleosome assembly to proceed without concurrent protein synthesis, apparently due, at least in part, to the absence of newly synthesized histones (6, 13-15, 65). This interpretation has been indirectly supported by observations of non-nucleosomal DNA following replication in the absence of protein synthesis (9, 16, 68). Further evidence that butyrate does not inhibit nucleosome assembly will be presented below in experiments utilizing micrococcal nuclease.

Upon removal of butyrate, bu-chromatin gradually acquired the DNase I resistance of bulk chromatin (Fig. 3A). However, the maturation of bu-chromatin was slower than that occurring normally (cf. Fig. 2B). Conversely, bu-chromatin from cells maintained in butyrate for several hours retained its heightened sensitivity to DNase I and, in fact, became more sensitive (Fig. 3B). This is most likely due to the hyperacetylation of histones during prolonged butyrate exposure. Newly synthesized histones, in particular, showed a marked hyperacetylation after 2-4 h in butyrate (28, 29). For this reason, we have restricted our analyses to chromatin labeled in butyrate for 30 min or less.

To ensure that butyrate was not inducing the preferential sensitivity of bu-chromatin to DNase I, the following experiment was performed. HeLa cells were labeled with [3H]thymidine for 30 min in the absence of butyrate, thereby allowing newly replicated chromatin to mature fully (Fig. 2B). Cells were then washed with culture medium to remove unincorporated radiolabel, preincubated for 10 min at 37°C to "chase" residual radioactive thymidine pools into chromatin, and then further incubated in sodium butyrate for 30 or 60 min. Isolated nuclei were then mixed with nuclei prelabeled with [14C] thymidine, and digested with DNase I as before (Fig. 3C).

Even after 60 min in butyrate, newly matured chromatin remained as resistant to DNase I as normal bulk chromatin (Fig. 3C, circles). Similar results were obtained with chromatin labeled for 24 h with [3H]thymidine and subsequently incubated in butyrate, except that in this case a minor increase in DNase I sensitivity was noted (presumably due to the early

![Fig. 1. Effect of sodium butyrate on DNA replication. HeLa cells were prelabeled for one generation with [3H]thymidine and then incubated for the times indicated with [3H]thymidine. After 7.5 min, the cells were divided into two portions, and sodium butyrate (50 mM) was added to one (arrowhead). At each point, an aliquot of cells was removed, washed, and precipitated with trichloroacetic acid. DNA synthesis in the absence () and the presence (O) of sodium butyrate is expressed as [3H]/[14C].](http://www.jbc.org/)

![Fig. 2. Sodium butyrate inhibits the maturation of newly replicated chromatin. A, cells were labeled with [3H]thymidine in the presence of 50 mM sodium butyrate for 0.5 min (.), 10 min (A), or 30 min (O), or in the presence of cycloheximide (200 μg/ml) for 50 min (C). Isolated nuclei were mixed with nuclei prelabeled with [14C] thymidine for one generation in the absence of butyrate, digested with DNase I at 50 units/ml for the times indicated (final average 90% of [14C] DNA rendered acid-soluble), and acid precipitated. Nuclei labeled in cycloheximide were digested at 25 units/ml (63% of [14C] DNA acid-soluble). The nuclease sensitivity of newly replicated chromatin, relative to normal bulk chromatin, is expressed as [3H]/[14C]; nuclear ratios before digestion have been normalized to 10. B, cells were labeled with [3H]thymidine in the presence of sodium butyrate for 30 min (.), or 50 min (A), or in the presence of cycloheximide (200 μg/ml) for 50 min (O). Isolated nuclei were mixed with nuclei prelabeled with [14C] thymidine and digested with DNase I at 25 units/ml (average 66% of [14C]DNA acid-soluble), except for nuclei labeled for 50 min in butyrate, which were digested at 30 units/ml (78% of [14C]DNA acid-soluble). Relative DNase I sensitivity is expressed as [3H]/[14C].](http://www.jbc.org/)
Deacetylation Is Required for Chromatin Maturation

with \[^{3}H\]thymidine in the presence of DNase I. Isolated nuclei were then digested with DNase I; total DNA was purified, denatured, separated in a 10% polyacrylamide gel, and analyzed by fluorography (Fig. 4). Both bu-chromatin and control chromatin yielded the typical ~10-nucleotide series of fragments (67). There was no obvious increase or decrease in the intensity of any of the bands from the butyrate-treated sample. Similar results have been reported for hyperacetylated bulk chromatin (32). However, since the DNA pattern reflects the sum of all nucleosomes associated with new DNA, including both parental particles and those assembled de novo, subtle pattern variations from newly assembled nucleosomes could be masked by those in more mature particles. Nevertheless, identical results were obtained for bu-chromatin replicated in cycloheximide, in which only parental nucleosomes are represented (data not presented). (Note: Because specific 5’ end-labeling of newly replicated chromatin was not possible, we were unable to determine if the increased intensity of the 60-nucleotide band observed with hyperacetylated chromatin (30) was also present in bu-chromatin.)

Despite similarities in core structure, it was clear that bu-chromatin was digested faster than chromatin replicated under normal conditions, as evidenced by the greater preponderance of smaller fragments in the butyrate-treated sample (compare lanes b and e, Fig. 4). This confirms the data based on acid solubility (Figs. 2 and 3). Since the DNase I ladder is generally considered to be a reflection of core particle organization (see Ref. 67 for review), we conclude that the presence of sodium butyrate during DNA replication does not cause dramatic alterations in the conformation of the nucleosome stages of histone hyperacetylation) (Fig. 3C, triangles). Nevertheless, in neither instance was sodium butyrate capable of inducing the degree of DNase I sensitivity exhibited by bu-chromatin. We therefore attribute the heightened DNase I sensitivity of newly replicated chromatin, at least in part, to the acetylation of histones in nascent nucleosomes.

Core Particle Structure of Bu-chromatin—In order to elucidate the core particle structure of bu-chromatin, cells were labeled either in the presence or the absence of butyrate for 30 min. Isolated nuclei were then digested with DNase I; total DNA was purified, denatured, separated in a 10% polyacrylamide gel, and analyzed by fluorography (Fig. 4). Both bu-chromatin and control chromatin yielded the typical ~10-nucleotide series of fragments (67). There was no obvious increase or decrease in the intensity of any of the bands from the butyrate-treated sample. Similar results have been reported for hyperacetylated bulk chromatin (32). However, since the DNA pattern reflects the sum of all nucleosomes associated with new DNA, including both parental particles and those assembled de novo, subtle pattern variations from newly assembled nucleosomes could be masked by those in more mature particles. Nevertheless, identical results were obtained for bu-chromatin replicated in cycloheximide, in which only parental nucleosomes are represented (data not presented). (Note: Because specific 5’ end-labeling of newly replicated chromatin was not possible, we were unable to determine if the increased intensity of the 60-nucleotide band observed with hyperacetylated chromatin (30) was also present in bu-chromatin.)

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core. However, bu-chromatin retains other immature features that render it highly accessible to DNase I.

Effects of Sodium Butyrate As Determined by Micrococcal Nuclease Digestion—As with DNase I, nascent chromatin is preferentially digested by micrococcal nuclease (4); however, unlike experiments performed with DNase I, sodium butyrate did not inhibit chromatin maturation as measured by micrococcal nuclease digestion: within 10 min, bu-chromatin possessed the same nuclease resistance as bulk chromatin (Fig. 5). Therefore, the rate of maturation of bu-chromatin to micrococcal nuclease digestion was indistinguishable from that of chromatin replicated normally (1–5, 7, 12, 69). This is in contrast to the effects of cycloheximide, which preserves the sensitivity of newly replicated chromatin to both DNase I and micrococcal nuclease (2, 9, 13–15, 68). This again suggests that butyrate, unlike cycloheximide, does not inhibit nucleosome assembly.

In addition to the more rapid conversion of nascent chromatin DNA to acid solubility, micrococcal nuclease can be used to discriminate newly replicated chromatin by the following characteristics: 1) preferential cleavage to mononucleosomes (2–5, 11, 12, 69); 2) a reduced repeat length (4, 6–9); and 3) the presence of non-nucleosomal nascent chromatin DNA (9–12). These diagnostic features of newly replicated chromatin are short-lived, persisting for approximately 10–15 min after passage of the replication fork. Although butyrate did not inhibit chromatin maturation as measured by acid-precipitable DNA, it was possible that butyrate could affect these other features of nascent chromatin. This was tested by micrococcal nuclease digestion and gel electrophoresis.

Cells were labeled with [3H]thymidine in the presence or absence of sodium butyrate for 20 min, sufficient time for normally replicating chromatin to mature completely (Fig. 5). Cells were also labeled with [3H]thymidine for 45 min in the presence of cycloheximide, also with or without butyrate. (Chromatin replicated in cycloheximide remains partially unassembled, and thus possesses an immature structure.) As a control, cells were labeled with thymidine for 45 s. Nuclei were isolated and digested with micrococcal nuclease to the same extent. Soluble chromatin was released by nuclear lysis with 2 mM EDTA; an insoluble pellet was removed by centrifugation (see "Materials and Methods"). DNA in the two fractions was then analyzed by polyacrylamide gel electrophoresis, ethidium bromide staining, and fluorography (Fig. 6).

After mild nuclease digestion, polynucleosomes predominated in the soluble fraction (Fig. 6A); the pellet chromatin consisted of higher molecular weight oligonucleosomes, together with residual monomers, dimers, etc. (Fig. 6B). We have previously shown that newly replicated nucleosomes elute into the EDTA supernatant (Fig. 6C, lane e), while non-nucleosomal nascent DNA (replicated under normal growth conditions or in the presence of cycloheximide) remains in the pellet, appearing as an unstructured smear (Fig. 6D, lanes c' and e') (9, 12).

Sodium butyrate did not inhibit the maturation of newly replicated chromatin by any of the criteria provided by micrococcal nuclease digestion. Unlike nascent chromatin (Fig. 5,

![Fig. 6. Normal micrococcal nuclease resistance, nucleosomal structure, and nucleosomal repeat length of bu-chromatin. Cells were labeled with [3H]thymidine for 20 min in the absence of butyrate (a and a'), for 20 min in the presence of butyrate (b and b'), for 45 min in the presence of cycloheximide (c and c'), for 45 min in the presence of cycloheximide plus butyrate (d and d'), and for 45 min in the absence of butyrate (e and e'). Isolated nuclei were digested with micrococcal nuclease (1 unit/ml) for 1.33 min, and soluble chromatin released with 2 mM EDTA (EDTA SUP'T; A and C) (see "Materials and Methods"). Soluble and insoluble (PELLET; B and D) chromatin DNA was subjected to electrophoresis, stained with ethidium bromide (A and B), and analyzed by fluorography (C and D). Marker fragments (lane f): HaeIII digest of φX174 [3H]DNA (New England Nuclear); fragment sizes are (from bottom) 118, 194, 234, 271/278 (doublet), 310, 603, 872, 1078, and 1353 base pairs long. The positions of mono-, di-, and trinucleosomal bulk DNA are indicated (M, D, and T, respectively). The arrowheads (C) indicate the position of nascent trimer DNA.]
A and C, lane e), bu-chromatin was not preferentially cleaved to mononucleosomes, but instead possessed the same nuclease resistance as bulk chromatin, and as mature chromatin replicated without butyrate (Fig. 6, A and C, lanes a and b). Altering the extent of digestion had no affect on the relative rate of digestion of bu-chromatin (data not presented). Also, butyrate did not interfere with the preferential digestion of chromatin replicated in the presence of cycloheximide (Fig. 6, A and C, lanes c and d). Thus, the maintenance of histone acetylation during replication did not affect the accessibility of linker DNA to micrococcal nuclease: bu-chromatin was cleaved to monomers at the same rate as chromatin replicated in the absence of butyrate for an equal time.

In addition, butyrate did not hinder newly replicated chromatin from acquiring normal nucleosomal spacing: bu-chromatin possessed the same spacing as mature chromatin (~185 base pairs), in contrast to the shortened ~165 base pair repeat of nascent chromatin (compare lanes a, b, and e, Fig. 6C; the position of nascent trimer DNA is indicated by the arrowheads).

Lastly, butyrate did not prevent nucleosome assembly. As with control chromatin, the pellet fraction of bu-chromatin labeled for 20 min possessed typical nucleosomal periodicity, in contrast to chromatin DNA labeled for 45 s, which lacked subunit structure (compare lanes a', b', and c', Fig. 6D). In this regard, butyrate again had a markedly different effect than cycloheximide, which prevented de novo nucleosome assembly (Fig. 6D, lane c', and d'; also see Ref. 9).

**Micrococcal Nuclease Sensitivity of Chromatin Acetylated in Vivo**—In order to ascribe the properties of bu-chromatin to the maintenance of acetylated histones on new DNA, it was important to compare the nuclease sensitivity of bu-chromatin to that of chromatin acetylated in vivo. Our experiments demonstrated that bu-chromatin was preferentially digested by DNase I, but not by micrococcal nuclease. Consistent with this, the heightened DNase I sensitivity of acetylated chromatin has been well established (30–39). In contrast, evidence has been presented both for (30, 70) and against (32, 34, 35, 38) the preferential cleavage of acetylated chromatin to mononucleosomes. In order to resolve this question and to determine if the micrococcal nuclease resistance of bu-chromatin correlated with that of acetylated chromatin, HeLa cells were labeled with [3H]acetate under physiological conditions, and isolated nuclei were digested with micrococcal nuclease.

In the absence of butyrate, acetate label was taken up predominantly by mono- and diacetylated H3 and H4 (Fig. 7, B and D, lane a). In agreement with the data of others (71, 72), acetate incorporation during a brief pulse was mainly into pre-existing histone, since cycloheximide did not significantly suppress the level of histone acetylation (compare lanes a and b, Fig. 7, B and D). This is confirmed by the absence of acetate label in histone H1, since newly synthesized H1 (like H2A and H4) is irreversibly acetylated at the NH2 terminus (see Refs. 73 and 81 for reviews).

The presence of sodium butyrate during brief acetate incorporation had little effect: H3 and H4 remained the predominantly labeled proteins and only a slight increase in the multiacetylated forms of H3 and H4 was noted (lane c, Fig. 7, B and D). (Note: The apparent reduction of acetate uptake in the presence of butyrate (lane c, Fig. 7B) is a reflection of sample load: compare stained gel (Fig. 7A); see also ac-id-urea gel (Fig. 7, C and D).) Clearly, brief exposure to butyrate does not induce large-scale histone hyperacetylation (43, 44, 55, 62), providing further evidence that the DNase I sensitivity of bu-chromatin is not due to the hyperacetylation of histones to artificially high levels.

Micrococcal nuclease digestion of chromatin acetylated in the absence of butyrate showed that acetate label was incorporated into all electrophoretically resolved mononucleosomal classes, including the core, H1-, and HMG protein-containing species (Fig. 8B). This was further confirmed by using the procedure ofSanders (75) to separate nucleosomes possessing H1 from those containing HMG and other nonhistone proteins (data not presented), as described previously for newly replicated chromatin (12, 24). The presence of acetate in all nucleosomal species is consistent with the recent finding that virtually all DNA sequences can become associated with acetylated histones during short term acetate labeling (76).

A comparison of the ethidium bromide-stained gel with the fluorograph showed that acetylated chromatin was cleaved to mononucleosomes at the same rate as bulk chromatin (Fig. 8). We conclude that internucleosomal linkers in acetylated chromatin regions are not more accessible to micrococcal nuclease than the linkers of bulk chromatin (34, 35). Thus, the properties of bu-chromatin are fully consistent with those of chromatin acetylated in vivo.

**DISCUSSION**

By monitoring the maturation of chromatin synthesized in the presence of sodium butyrate, we have observed a novel replication intermediate that possesses normal resistance to micrococcal nuclease, but retains ~50% of the maximum sensitivity of nascent chromatin to DNase I. Butyrate must be present during DNA synthesis for this selective DNase I sensitivity to be maintained, and new chromatin matures following butyrate removal. The simplest explanation for
tyrate has been reported to produce multiple effects, including changes that account for the inhibition of chromatin maturation. It is unlikely that these additional metabolic effects are involved in the inhibition of histone deacetylation, which is strongly inhibited by sodium butyrate (40-44), is required for the maturation of newly replicated chromatin.

In addition to inhibiting histone deacetylation, sodium butyrate has been reported to produce multiple effects, including inhibition of the phosphorylation of histones H1 (36, 62) and H2A (62), enhancement of HMG protein acetylation and/or phosphorylation (77, 78), increase in phosphorylation of H3 (79), and arrest of DNA replication and cell growth (53-63). It is unlikely, however, that these additional metabolic changes account for the inhibition of chromatin maturation. Whereas the inhibition of histone deacetylation is immediate (44), other effects are observed only after prolonged exposure of cells to butyrate, and many appear to correlate with histone hyperacetylation and cell growth arrest (56, 58, 62, 79). In addition, butyrate does not inhibit DNA replication with short term exposure (Fig. 1; see also Refs. 56 and 58). Since the predominant effect of brief exposure to butyrate is the preservation of acetylation, we conclude that histone deacetylation is required for the maturation of newly replicated chromatin. It remains possible, however, that minor chromosomal proteins must also be deacetylated for chromatin to mature. For example, HMG 1, which has been reported to be acetylated in vivo (77), may also be involved in chromatin replication (12).

During the 10-30 min period that replicating chromatin was exposed to butyrate, no hyperacetylation of bulk histone was observed, in agreement with earlier reports (29, 80). Newly synthesized H4, on the other hand, is deposited in a dimodified form (26, 27). While the diacetylated state of nascent histones almost certainly contributes to the DNase I sensitivity of newly replicated chromatin, several lines of evidence indicate that the further hyperacetylation of new histones is not inducing this sensitivity during brief butyrate exposure. After 20-30 min in butyrate, newly synthesized H4 is deposited onto chromatin, but remains predominantly in the nascent, diacetylated form (28, 29). Since butyrate did not increase the DNase I sensitivity of either newly matured or pre-existing chromatin DNA to that of bu-chromatin for at least 1 h, we conclude that butyrate preserves newly replicated chromatin in an immature conformation, but does not artifactsually generate this state.

Our findings are consistent with numerous reports of the increased DNase I sensitivity of acetylated chromatin (30-39). Like control chromatin, bu-chromatin exhibited a typical ~10n-nucleotide ladder of single-stranded DNA fragments after digestion with DNase I. This is, perhaps, not surprising, since even hyperacetylated chromatin yields a 10-nucleotide series after DNase I digestion (30, 32). Similarly, trypsinization of the histone NH2-terminal arms, which contain the sites of histone acetylation, does not disrupt core particle integrity or preclude nucleosome reconstitution (82, 83). Thus, the maintenance of histone acetylation on newly replicated chromatin is not expected, a priori, to induce radical alterations of nucleosome core structure. We assume here that the acetylation sites on nascent histones are a subset of the steady state sites.

Further evidence for the continuation of nucleosome assembly in the presence of butyrate was provided by experiments employing micrococcal nuclease. Within 10 min, bu-chromatin was digested to acid solubility at the same rate as bulk chromatin. This is identical with the rate of maturation of chromatin replicated in the absence of butyrate (1-5, 7, 12, 69). Also, sodium butyrate, in marked contrast to cycloheximide, did not prevent nascent DNA from acquiring subunit structure. Thus, the 50% partial recovery of the DNase I resistance of bu-chromatin may reflect the assembly process, while the balance of remaining sensitivity is apparently due to other factors.

Neither the preferential cleavage of new chromatin to monomers, nor the shortened repeat length of nascent chromatin was exhibited by bu-chromatin. Furthermore, bu-chromatin was digested to mononucleosomes at the same rate as normal, mature chromatin. These mature features of bu-chromatin indicate that typical nearest-neighbor nucleosomal interactions are established in butyrate, and that linker DNA of acetylated nucleosomes is shielded from micrococcal nuclease, most likely by histone H1 (cf. Refs. 84 and 85). As with pulse-labeled nascent mononucleosomes (12), monomers derived from bu-chromatin contained both H1- and HMG protein-containing species (data not presented).

In order to determine if the nucleosomal properties of bu-chromatin correlated with those of acetylated chromatin, the micrococcal nuclease sensitivity of bulk chromatin acetylated

\[ A \]

\[ B \]

\[ C \]

\[ D \]

\[ E \]

\[ F \]

\[ G \]

\[ H \]

\[ I \]

\[ J \]

\[ K \]

\[ L \]

\[ M \]

\[ N \]

\[ O \]

\[ P \]

\[ Q \]

\[ R \]

\[ S \]

\[ T \]

\[ U \]

\[ V \]

\[ W \]

\[ X \]

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to physiological levels was examined. Like bu-chromatin, acetylated chromatin was digested to monomers at the same rate as bulk chromatin, demonstrating that the linker DNA between acetylated nucleosomes possesses normal micrococcal nuclease resistance (34, 35, 38). Thus, if acetylation weakens the binding of the histone NH₂-terminal arms to linker DNA (as proposed by Yau et al. (86)), this alone does not promote a chromatin structure in which the linkers are fully exposed.

Under normal conditions, the maturation of newly replicated chromatin occurs quickly, and histone deposition and deacetylation may be coordinated (26). The acetylated state of newly assembled nucleosomes may in part explain the preferential sensitivity of newly deposited histones to trypsin (87), and the relatively weak binding of nascent histones to DNA (8, 87). Although the hyperacetylation of bulk histones did not promote histone exchange or significantly increase the ability of histones to "slide" along DNA (32), the properties of bulk chromatin may not be strictly applicable to newly assembled nucleosomes in all respects. It is clear, however, that the maintenance of acetylation on nascent nucleosomes does not promote nucleosome sliding, since the repeat lengths of bu-chromatin and bulk chromatin were identical.

The use of sodium butyrate as a probe of chromatin replication permits the operational definition of three distinct stages of the maturation process (Fig. 9). In stage I of chromatin maturation, chromatin in the immediate vicinity of the replication fork is highly sensitive to both DNase I and micrococcal nuclease (this work; see also Refs. 1–5, 11, 69). Stage I chromatin is heterogeneous, consisting of regions of ongoing DNA synthesis, segregated parental nucleosomes, and non-nucleosomal DNA (see Refs. 98 and 99 for reviews). In addition, the histones of stage I chromatin are acetylated, accounting for ~50% of total DNase I sensitivity. The source of these acetylated histones is not entirely certain. To a large degree they will be the acetylated newly synthesized histones, particularly H4, that are deposited preferentially onto new DNA (see Introduction for references). Because the DNase I sensitivity of chromatin replicated in cycloheximide is inherently great, we have been unable to determine if old histones (from segregated parental nucleosomes) are also acetylated (15–15). Given that old histones can also undergo rapid acetylation and deacetylation (76, 80), it is reasonable to propose that parental nucleosomes are also modified during replication (6).

In stage II of chromatin maturation, newly replicated chromatin possesses normal resistance to micrococcal nuclease, non-nucleosomal DNA is completely assembled, and the normal repeat length is established; nevertheless, stage II chromatin remains sensitive to DNase I. The intermediate characteristics of chromatin in stage II are fully consistent with the effects of histone acetylation. If one assumes that chromatin higher order structure is lost during chromatin replication, then this stage of maturation may represent the early steps in chromatin refolding. One current model of chromatin structure which is consistent with the properties of bu-chromatin is the so-called "zig-zag" (or double row) configuration of nucleosomal structure, observed with electron microscopy for chromatin at low to intermediate ionic strengths (68–94). In these structures internucleosomal linkers are masked as a result of interactions between nearest-neighbor nucleosomes, forming a ribbon-like chromatin fiber, yet the fiber remains relatively extended.

Further speculation on the structure of stage II chromatin must await additional experiments, particularly to determine if acetylated chromatin can assume the zig-zag conformation. However, it is worth noting that nucleosomes of trypsinized chromatin (lacking the histone NH₂-terminal arms) can form localized clusters when reconstituted with H1 or H5, although typical higher order structures are absent (95).

In the final stage of chromatin maturation (stage III, Fig. 9), newly replicated chromatin acquires normal resistance to DNase I; this step requires histone deacetylation. It has been suggested that histone acetylation may destabilize chromatin higher order structure (30, 31, 83, 95–97), and recent evidence has implicated the histone NH₂-terminal arms in chromatin condensation (95). In light of this, our results are consistent with a model in which the orchestration of histone acetylation and deacetylation regulates reversible transitions between higher order structures and the extended fiber during DNA replication and nucleosome assembly.

Acknowledgments—We thank Lori Lennon for excellent technical assistance, and Dr. John E. Wiktowicz and Michael G. Riggs for helpful comments during the course of this work.

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Deacetylation Is Required for Chromatin Maturation

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Histone deacetylation is required for the maturation of newly replicated chromatin.

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J. Biol. Chem. 1983, 258:12675-12684.

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