Influence of histone acetylation on the solubility, H1 content and DNase I sensitivity of newly assembled chromatin

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ABSTRACT

In a previous report [Annunziato, A. T. and Seale, R.L. (1983) *J. Biol. Chem.* **258**:12675] a novel intermediate in chromatin assembly was described (detected by labeling new DNA in the presence of the deacetylase inhibitor sodium butyrate), which retained ~50% of the heightened sensitivity of newly replicated chromatin to DNaseI. It is now reported that nucleosomes replicated in butyrate are considerably more soluble in the presence of magnesium, relative to chromatin replicated under control conditions, and that this heightened magnesium-solubility is reflected in a concomitant increase in the preferential solubility of nucleosomes containing newly synthesized core histones. This differential solubility was accompanied by a 5- to 6-fold depletion of histone H1, and was completely abolished by the selective removal of H1 from isolated nuclei. The removal of H1 also markedly reduced the preferential DNaseI sensitivity of chromatin replicated in butyrate. Further, when mononucleosomes of control and (acetylated) nascent chromatin were compared, no differences in DNaseI sensitivity were detected. These results provide evidence that the interactions between newly assembled nucleosomes and histone H1 are altered when histone deacetylation is inhibited during chromatin replication, and suggest a mechanism for the control of H1 deposition during nucleosome assembly *in vivo*.

INTRODUCTION

Chromatin biosynthesis is a complex, multistep process that involves DNA replication, coordinate histone synthesis and deposition, nucleosome assembly, and the establishment of transcriptionally active and inactive nucleoprotein structures (reviewed in 1). Although these facets of chromatin metabolism have been well studied individually, relatively little is known about their orchestration during *in vivo* chromatin assembly. In particular, the modulation of chromatin higher order structures to accommodate the replication apparatus, and the subsequent reformation of the 30nm fiber, are incompletely understood. One level of control presumably lies in the deposition of H1, given the established role of this histone in regulating chromatin folding (2).

A second (though not mutually exclusive) possibility may reside in the reversible acetylation of the core histones. The universal acetylation of nascent H4 argues strongly for the significance of this modification in chromatin synthesis (3-7), as does the finding that histone hyperacetylation facilitates nucleosome assembly *in vitro* (8). It has also been suggested that histone acetylation may destabilize chromatin higher order structures (9-14), thereby partly explaining the increased DNaseI sensitivity of acetylated chromatin regions (9,10,15-18).

We have previously shown (19) that chromatin replicated in the presence of the deacetylase inhibitor sodium butyrate fails to mature fully; instead, such acetylated, nascent chromatin (bu-chromatin¹) retains ~50% of the heightened nuclease sensitivity of newly replicated chromatin DNA. The effect is detected by DNaseI, but not by micrococcal nuclease, and cannot be ascribed to a block in nucleosome assembly. The observation of this novel replication intermediate, sensitive specifically to DNaseI, indicates that histone deacetylation and nucleosome assembly are not obligatorily coordinated. Thus, the inhibition of deacetylation effects the persistence of structural features inherent to nascent chromatin, at an organizational level distinct from that of core particle formation.

In the following report, the special properties of chromatin replicated in butyrate have been further examined. It was found that mono- and oligonucleosomes of bu-chromatin possess an increased magnesium-solubility, relative to bulk chromatin, in accordance with the properties of acetylated chromatin in general (18, 20). The heightened magnesium-solubility of acetylated, nascent nucleosomes was accompanied by a concomitant increase in the preferential solubility of nucleosomes containing newly synthesized histones. More importantly, this differential solubility was coincident with a several-fold depletion of histone H1, and was completely abolished following the selective removal of H1 from isolated nuclei. The extraction of H1 also considerably reduced the preferential DNaseI sensitivity of chromatin replicated in butyrate. Finally, when purified mononucleosomes of bulk and bu-chromatin were examined, no differences in DNaseI sensitivity were detected, in marked contrast to experiments using whole nuclei. Taken together, these results provide evidence that acetylated, nascent chromatin is relatively H1-deficient, and further indicate that modified H1-mediated interactions (*e.g.*, higher order folding) may provide the basis for the distinctive features of chromatin replicated in butyrate. Our findings suggest a possible mechanism for the regulation of H1 deposition during chromatin assembly.

MATERIALS AND METHODS

Cell Culture and Labeling

HeLa cells were maintained in spinner culture at 37°C in Joklik's minimal essential medium supplemented with 7.5-10% calf serum. For short term labeling of newly replicated chromatin, log phase cells (~5 x 10⁵ cells/ml) were harvested by centrifugation (250 x g; 2 min) and concentrated five-fold in prewarmed whole medium, or whole medium plus 50mM sodium butyrate (as necessary). Following equilibration at 37°C for 10 min, cells were incubated with [*methyl*-³H]thymidine (76 Ci/mmol, New England Nuclear) at 4-8 uCi/ml for 30 min. Cells were harvested by centrifugation, and then washed and resuspended at a three-fold concentration in either prewarmed whole medium or medium plus 50mM butyrate and chased without radioisotope for 15 min at 37°C.

Long-term labeling of cells was performed for approximately one generation (18-24 hr)

in the presence of either 0.02 uCi/ml [^{14}C]thymidine (55mCi/mmol) or 0.1-0.2 uCi/ml [^3H]thymidine. Hyperacetylation of histones was accomplished by incubating cells for 6hr in MEM plus 7mM sodium butyrate. Pre-existing histones were labeled by growing cells for two generations in the presence of 0.3 uCi/ml [^{14}C]lysine (350mCi/mmol) in culture medium depleted 80% in lysine. To label nascent histones, cells were washed and concentrated in prewarmed lysine-free medium as described previously (19), incubated with 25-40 uCi/ml [^3H]lysine (80Ci/mmol) for 30 min in the presence or absence of butyrate, and chased for fifteen minutes in prewarmed medium supplemented with excess (ten-fold) lysine ($\pm$ sodium butyrate).

Nuclear Isolation

Cells were harvested, washed twice in cold Buffer A (10mM Tris, 3mM MgCl_2 , 2mM 2-mercaptoethanol, pH 7.6) and resuspended in Buffer A containing 0.5mM PMSF. Cells were lysed with a Dounce homogenizer, and nuclei were isolated in buffer A, as described previously (19). Cells treated with sodium butyrate were treated as described above with the addition of 20mM sodium butyrate to Buffer A.

Extraction of Histone H1 from Isolated Nuclei

H1 was displaced from nuclei using the method of Lawson and Cole (21); nuclei were resuspended ($0.6\text{--}1.2 \times 10^7/\text{ml}$) in 90mM citric acid-sodium phosphate buffer, pH 3.0-3.25, containing 5mM sodium butyrate, and placed at 4°C for 15 min with occasional agitation. After centrifugation, (400 x g; 2 min), nuclei were treated a second time, and then washed in nuclease Digestion Buffer (see below). Approximately 60% of nuclear H1 is removed by this procedure (as determined by scanning photographic negatives of Coomassie-stained gels). Attempts to alter the extraction conditions to further remove H1 proved unsatisfactory, due to the resulting displacement of core histones (see 21).

Nuclease Digestion and Chromatin Fractionation

For digestion with micrococcal nuclease, nuclei were resuspended at 40 A_{260}/ml (measured in 1% SDS) in Digestion Buffer (10mM PIPES, 80mM NaCl, 20mM sodium butyrate, pH 7.0). For double-label experiments, equal A_{260} units of (^{14}C)- and (^3H)nuclei were mixed prior to washing. Nuclei at 40 A_{260}/ml were brought to 0.5mM CaCl_2 and digested with micrococcal nuclease (Sigma) at 5 U/ml at 4°C for 10 min. Digestions were terminated by the addition of EGTA, pH 7.6 to a final concentration of 5mM, and cooling on ice 30 min. Soluble chromatin was fractionated according to the following procedure: Nuclei were pelleted (12,500 x g) for 8 min to yield the first supernatant fraction (S1); nuclei were then resuspended in an equal volume of 2mM EDTA, 10mM sodium butyrate, pH 7.2, allowed to lyse for 20 min on ice, and pelleted (12,500 x g; 8 min), yielding a second supernatant fraction (S2); the final insoluble chromatin pellet (P) was resuspended in an equal volume of 2mM EDTA, 10mM sodium butyrate, pH 7.2. Approximately 35-50% of total bulk chromatin DNA was routinely solubilized into the S1 fraction under these conditions.

Chromatin solubility in the presence of magnesium was tested by the adjustment of the

S1 to 4mM MgCl₂, and precipitation at 4°C for 30 min. Chromatin fractions were centrifuged (12,500 x g; 10 min) to yield a magnesium-soluble chromatin supernatant (S1S) and a magnesium-insoluble chromatin pellet (S1P). The magnesium-precipitated pellet was then solubilized in Digestion Buffer prior to further analysis.

Digestions with DNaseI (Worthington) were performed at 20 A₂₆₀/ml in Digestion Buffer plus 4mM MgCl₂, at 37°C, for the times indicated in the Figure Legends, until ~45-60% of the input bulk DNA was rendered acid soluble. Reactions were terminated by diluting aliquots into 20mM EDTA, pH 7.2. Isolated nuclei were digested at 50 units/ml; gradient-purified mononucleosomes were digested at 15 units/ml, after the addition of MgCl₂ to 10mM. The S1S chromatin fraction was digested at 200 units/ml. Nuclei remaining in the P chromatin fraction were resuspended in Digestion Buffer, adjusted to 4mM MgCl₂, and digested at 50 units/ml. Chromatin fractions were prepared for liquid scintillation counting as described previously (19).

Isolation of Mononucleosomes

To purify mononucleosomes, nuclei were digested with micrococcal nuclease for 15 min. The reaction was terminated, and nuclei were maintained at 4°C for two hr. A soluble chromatin fraction was obtained by gentle centrifugation (1,000 x g; 3 min). Soluble chromatin was subjected to centrifugation in a 5-40% sucrose gradient, in Digestion Buffer, pH 7.6. Centrifugation was performed for 16 hr at 30,000 rpm in a SW41Ti rotor (Beckman) at 4°C. Fractions corresponding to the monomer peak (detected by absorbance at 260nm) were pooled.

Histone Extraction: Gel Electrophoresis

Histones were extracted and subjected to electrophoresis in either SDS (25) or acid-urea (26) polyacrylamide systems, and quantitated as described previously (19,52). For DNA size analysis, chromatin samples were precipitated and subjected to electrophoresis in 7% polyacrylamide gels in the presence of 0.1% SDS, according to Loening (22), as described previously (19). Gels were stained with ethidium bromide, photographed under ultraviolet illumination, and impregnated with PPO in preparation for fluorography (23, 24).

RESULTS

Effect of Sodium Butyrate on the Solubility of Newly Replicated Chromatin

To determine the effect of inhibiting deacetylation on the solubility of newly replicated chromatin, cells were labeled with [³H]thymidine for 30 min in the presence or absence of 50mM sodium butyrate (sufficient time to allow normal chromatin to mature fully (27)). In most cases, cells were then chased for an additional 15 min without label (plus or minus sodium butyrate), in order to interpose several kilobases of unlabeled DNA between the replication fork and the chromatin to be analyzed. Nuclei were then isolated, mixed with nuclei from control cells (labeled for one generation with [¹⁴C]thymidine), and digested with micrococcal nuclease under isotonic conditions at 4°C. The digested nuclei were then treated to yield a first supernatant fraction (S1), an EDTA-soluble second supernatant (S2), and a residual nuclear Pellet (P) (see "Materials and

Figure 1 Chromatin replicated in sodium butyrate possesses altered solubility properties. In all cases, prelabeling was performed with [¹⁴C]thymidine for one generation. Labeling with [³H]thymidine was performed under various conditions, as discussed below. Isolated (³H)nuclei were mixed with (¹⁴C)nuclei, and digested with micrococcal nuclease; soluble chromatin was prepared as described in "Materials and Methods," yielding a first supernatant (S1), an EDTA-soluble second supernatant (S2), and a residual nuclear pellet (P). Chromatin released into the S1 was further fractionated by adjustment to 4mM MgCl₂, producing both magnesium-soluble (S1S) and magnesium-insoluble (S1P) fractions. A) The enrichment in each fraction for chromatin replicated under various protocols (relative to control (¹⁴C)chromatin) is expressed as %³H/%¹⁴C. Labeling with [³H]thymidine was performed as follows (bars, from left to right): in the presence of sodium butyrate for 30 min, then chased in butyrate 15 min (solid dark); in butyrate 30 min [no chase] (horizontal stripes); without butyrate 30 min, then chased without butyrate 15 min (stippled); without butyrate 24 hr (diagonal stripes). B) Elution patterns of newly replicated and bulk chromatin following replication in butyrate: Cells were labeled with [³H]thymidine in the presence of butyrate 30 min and chased (without isotope) in butyrate for an additional 15 min; isolated (³H)nuclei were mixed with control (¹⁴C)nuclei, and digested with micrococcal nuclease; S1S, S1P, S2, and P chromatin fractions were prepared as described in "Materials and Methods." The distributions of newly replicated (³H, solid dark bars) and bulk (¹⁴C, stippled bars) chromatin in each fraction are expressed as percentages of total radioactivity.

Methods" for details). To assess the solubility of nascent chromatin, nucleosomes released into the S1 were further fractionated into a magnesium-soluble supernatant (S1S) and a magnesium-insoluble pellet (S1P) by adjustment to 4mM MgCl₂. The percentages of new and old chromatin released into each fraction by these methods are presented in Figure 1B.

It is clear that the elution pattern of bu-chromatin¹ differs considerably from that of control chromatin (Fig. 1B): While the majority of bu-chromatin is released into the first supernatant, and remains soluble in the presence of magnesium (S1S), the greater part of bulk chromatin is only solubilized with EDTA (S2). This is reflected in the ³H/¹⁴C ratios of each fraction (Fig. 1A). In separate experiments, we have observed a 6- to 8-fold relative enrichment for new DNA in the S1S, as compared to the EDTA-soluble S2, when chromatin is replicated in the presence of butyrate. In contrast, neither chromatin replicated in the absence of butyrate for 30 min nor chromatin labeled without butyrate for 24 hr (bulk) displays any preferential solubility properties (the ³H/¹⁴C ratios are given in Fig. 1A).

The altered solubility of bu-chromatin was not merely a kinetic effect, attributable to a simple retardation of the rate of replication (or of chromatin maturation): butyrate does not inhibit the rate of DNA synthesis in S phase cells (19, 38). Moreover, both the heightened DNaseI sensitivity and altered solubility of bu-chromatin are retained as long as butyrate is present (19; unpublished observations). Thus, the special characteristics of bu-chromatin persist in conjunction with the maintenance of histone acetylation.

Several control experiments were performed to ensure that butyrate was not artifactually generating the altered solubility of nascent chromatin. When chromatin was replicated in the presence of butyrate, and then chased in the absence of the deacetylase inhibitor, all changes in solubility were reversed. Chromatin replicated normally for 30 min, and subsequently exposed to butyrate, also failed to display the degree of magnesium-solubility of chromatin replicated during butyrate exposure (data not presented). Therefore, as with differential DNaseI sensitivity (19), butyrate is required *during* replication for the full solubility effect to be produced.

Nucleosomal Distribution of Newly Replicated and Bulk Chromatin

It has been previously been shown (19) that bu-chromatin exhibits no preferential sensitivity to micrococcal nuclease, as measured either by digestion to acid solubility or by rate of cleavage to mononucleosomes. To extend these findings, and to determine the nucleosomal distribution of the preferentially soluble material, nuclei from cells labeled for 30 min with [³H]thymidine in the presence of butyrate were digested and fractionated as above; DNA from the chromatin fractions was then analyzed by polyacrylamide gel electrophoresis, ethidium bromide staining and fluorography (Fig. 2).

A comparison of the ethidium stain with the fluorograph shows that both newly replicated and bulk mono- and oligonucleosomes are released into the first supernatant (S1) (Fig. 2A and B; lane a). Further, in the presence of magnesium, the distribution of nucleosomal oligomers in the S1S is the same for both bulk chromatin and chromatin replicated in butyrate

Figure 2 Distribution of nucleosomal DNA in chromatin fractions following replication in butyrate. Cells were labeled with [^3H]thymidine in butyrate for 30 min then chased in butyrate for 15 min. Nuclei were isolated, digested with micrococcal nuclease and fractionated as in Fig. 1. DNA from chromatin fractions S1, S2, P, S1S, and S1P (lanes a-e, respectively) was subjected to electrophoresis, and analyzed by ethidium bromide staining (A) and fluorography (B). Positions of mono- (M) and dinucleosomal (D) DNA are indicated.

(Fig. 2A, B; lane d). The enhanced magnesium-solubility of chromatin replicated in butyrate is therefore not due to the digestion of acetylated chromatin to monomers, nor limited exclusively to mononucleosomes: Nucleosomal oligomers of bu-chromatin are also soluble in magnesium. There was also no evidence that bu-chromatin in the S1S fraction had been extensively cleaved to small fragments, as no DNA smaller than mononucleosomes was observed (Fig. 2B; lane d).

Levels of Histone Acetylation in Chromatin Fractions

The relation of the increased magnesium-solubility of bu-chromatin to histone acetylation was examined by determining the levels of acetylation in the various chromatin fractions. For these experiments, newly synthesized histones were labeled for 30 min with [^3H]lysine in the presence of butyrate, acid extracted, separated in an acid-urea gel system (26), and visualized by fluorography (Fig. 3).

The level of acetylation for new H4 was the same for both the magnesium-soluble and magnesium-insoluble fractions (Fig. 3; lanes d and e), with predominantly diacetylated (and some triacetylated) H4 species in evidence. This is as expected, because during labeling all new histones are continuously exposed to butyrate, and thus no deacetylation can occur. (Note: The protein migrating slightly faster than H2A in lane d is the minor sequence variant H2A.Z [50,51]). The high proportion of diacetylated H4 in Figure 3 further demonstrates that the acetylation level of newly assembled chromatin has been preserved, and that extensive hyperacetylation of nascent histones is minimal.

Figure 3 Levels of H4 acetylation in chromatin fractions. Cells were labeled with [^3H]lysine in the presence of butyrate for 30 min. Isolated nuclei were digested with micrococcal nuclease, and fractionated as in Fig. 1. Acid extracted proteins from chromatin fractions S1, S2, P, S1S, and S1P (lanes a-e, respectively) were subjected to electrophoresis in an acid-urea gel system and analyzed by fluorography. Arrow indicates the position of the parent form of H4, as determined from the Coomassie-stained gel (not shown).

The above data demonstrate that H4 acetylation alone does not alter the solubility of chromatin replicated in butyrate, and that other factors, perhaps in conjunction with histone acetylation, are contributing to the enhanced magnesium-solubility of bu-chromatin. In light of this, it is noteworthy that the S1S fraction was markedly depleted of H1, relative to the S1P (Fig. 3; lanes d and e) suggesting that increased solubility is related to a deficiency of H1. Additional experiments were therefore performed to further analyze the distribution of core and linker histones in the chromatin fractions.

Distribution of Newly Synthesized and Pre-Existing Histones in Fractionated Chromatin

To examine the levels of new and old histones in fractionated chromatin, HeLa cells were incubated with [^3H]lysine for 30 min in the presence of sodium butyrate. Isolated nuclei were digested with micrococcal nuclease and fractionated; acid soluble proteins from the chromatin fractions were analyzed by electrophoresis in SDS, Coomassie Blue staining and fluorography (Fig. 4A, B).

Newly synthesized core histones were represented in all of the chromatin fractions. However, a comparison of the fluorograph (Fig. 4B) with the stained gel (Fig. 4A) revealed that the S1S was clearly enriched for new core histones, relative to the S1P (lanes d and e). This was emphasized by underloading the magnesium-soluble S1S (Fig. 4A, B; lane d). These results were also confirmed in double-label experiments, in which a ~2.5- to 3-fold enrichment in the S1S, relative to the S1P, was observed for new H3 and H4 (*i.e.*, those histones most preferentially associated with new DNA [29, 30, 31, 52]), after replication in butyrate. In contrast, when assembly occurred in the absence of butyrate (Fig. 4C, D), there was little or no enrichment for newly synthesized histones in the S1S relative to the S1P (lanes d and e).

Figure 4 Distribution of newly synthesized and pre-existing histones in chromatin fractions. Cells were labeled with [^3H]lysine for 30 min in the presence (A and B) or absence (C and D) of 50mM sodium butyrate and chased ($\pm$ butyrate) for an additional 15 min. Isolated nuclei were digested with micrococcal nuclease, and fractionated as in Fig. 1. Acid-extracted proteins from chromatin fractions S1, S2, P, S1S, and S1P (lanes a-e, respectively) were analyzed by SDS gel electrophoresis, Coomassie Blue staining (A and C) and fluorography (B and D).

It was also apparent that the S1S was relatively deficient in at least one, and probably both, species of histone H1, while concomitantly enriched in a protein with the mobility of HMG 1 (*i.e.*, migrating slightly faster than H1) (Fig. 4B, D; lane d). When considered along with the solubility data presented in Figure 1, these results indicate that replication in butyrate results in the preferential enrichment of nascent nucleosomes in an H1-deficient chromatin subfraction. Thus, inhibition of deacetylation is coincident to some degree with a depletion of H1 on newly assembled nucleosomes.

Table 1 Histone H1 molar ratios in the S1S and S1P

S1S	<u>H1</u>	<u>H4</u>
a) <i>Plus Butyrate</i>	0.12 0.11	1.0 1.0
b) <i>Minus Butyrate</i>	0.21	1.0
S1P		
a) <i>Plus Butyrate</i>	0.71 0.67	1.0 1.0
b) <i>Minus Butyrate</i>	0.56	1.0

Cells were labeled with [^3H]lysine for 30 min plus or minus sodium butyrate, and chased for 15 min ($\pm$ butyrate). Isolated nuclei were digested with micrococcal nuclease and fractionated as described in Fig. 1. Acid-extracted proteins from chromatin fractions were separated in an SDS gel system and stained with Coomassie Blue. Histone bands were excised and quantitated by liquid scintillation counting. For each histone, the total DPM of (^3H)protein per chromatin fraction was divided by the number of lysine residues per molecule (H1:62; H4:11), and the values were normalized to H4. Data represent the combined molar ratios of the two species of H1 relative to H4.

Quantitation of Histone H1 in Chromatin Fractions

Because the amino acid compositions (and thus the number of lysine residues) of the histones are known, it was possible to use radioactivity profiles of the histones to determine the relative molar ratio of new H1 in the S1S and S1P, according to the method of Albright *et al.* (32). Cells were labeled with [^3H]lysine for 30 min in the presence or absence of sodium butyrate. Following chromatin fractionation, acid-extracted proteins were subjected to electrophoresis in polyacrylamide-SDS gels; stained histone bands (fig. 5A) were excised, and the proteins were solubilized and counted. The results, expressed in relation to new H4, are presented in Table 1.

Magnesium-insoluble, newly assembled nucleosomes in the S1P were found to have typical amounts of H1, *i.e.*, approximately 0.6 relative to H4 (or between 1 and 1.5 molecules per nucleosome), whether or not assembly took place in the presence of butyrate. In contrast, magnesium-soluble nucleosomes in the S1S were 5- to 6-fold depleted of new H1 (relative to the S1P), when assembly occurred in butyrate. Nucleosomes in the S1S were also relatively H1-deficient when assembly took place under control conditions (*Minus Butyrate*, Table 1). Similar results were obtained for bulk histones, as determined qualitatively from stained gels (Fig. 5A), or quantitatively using long-term labeled histones (data not presented). Two conclusions may be drawn from these data. First, it appears that a major, if not primary, basis for enhanced magnesium-solubility is a relative H1-deficiency. Second, and more important, when chromatin assembly occurs without concomitant deacetylation (as with bu-chromatin), nascent nucleosomes are *preferentially* recruited into a magnesium-soluble status, apparently due to a lowered H1

Figure 5 Distribution of H1 in chromatin fractions and depleted nuclei. A) Cells were labeled with [^3H]lysine for 30 min in the presence or absence of sodium butyrate, and chased for 15 min ($\pm$ butyrate). Isolated nuclei were digested with micrococcal nuclease and fractionated as described in Fig. 1. Acid-extracted proteins from the S1S, S1P, S2, and P chromatin fractions (lanes a-d, respectively) were separated in an SDS gel system and stained with Coomassie Blue (the stained gel is shown). [Note: the presence of butyrate did not affect the migration of bulk histones in SDS, and thus only the "plus butyrate" gel is presented]. Bands corresponding to the core histones and histone H1 were excised, solubilized, and quantitated by liquid scintillation counting (see Table 1). B) Histone H1 was extracted from isolated nuclei as described in "Materials and Methods." Proteins from nuclei either before (lane a) or after (lane b) H1 displacement were acid-extracted, subjected to electrophoresis in SDS, and stained with Coomassie Blue.

content. In other words, a substantially greater percentage of nascent nucleosomes fractionate with H1-depleted chromatin (relative to controls) when assembly occurs in butyrate. The resulting enhanced magnesium-solubility is then evidenced by the heightened $^3\text{H}/^{14}\text{C}$ ratio for new DNA in the S1S, as seen in Fig. 1A.

DNaseI Sensitivity of Chromatin Replicated in Sodium Butyrate

Histone deacetylation is required for the maturation of newly replicated chromatin (as measured by the DNaseI sensitivity of isolated nuclei), and it has been suggested that acetylation may exert its influence during assembly by altering chromatin higher order structure (19). If this is the case, then one reasonable prediction is that isolated mononucleosomes should not exhibit heightened sensitivity to DNaseI, relative to monomers from bulk chromatin. A second prediction is that histone H1, which is of fundamental importance to chromatin higher order structure (2), should have a marked influence on preferential DNaseI sensitivity. To test these hypotheses, cells

Figure 6 DNaseI sensitivity of chromatin replicated in butyrate. In all cases, cells were labeled with [^3H]thymidine in the presence of butyrate for 30 min, and then chased in butyrate for 15 min. Isolated (^3H)nuclei were then mixed with control nuclei prelabeled for one generation with [^{14}C]thymidine. The DNaseI sensitivity of newly replicated chromatin, relative to bulk chromatin, is expressed as acid precipitable $^3\text{H}/^{14}\text{C}$; nuclear ratios prior to digestion have been normalized to 10. A) Isolated nuclei were digested with DNaseI either before (●) or after (○) extracting histone H1 as in Figure 5B; Alternatively, mononucleosomes (▲) were purified from H1-containing nuclei, and digested with DNaseI as described in "Materials and Methods." B) Nuclei were either digested with DNaseI immediately after isolation (●), or digested with micrococcal nuclease and fractionated as in Fig. 1; the S1S (▲) and residual P (○) fractions (obtained by micrococcal nuclease digestion, see "Materials and Methods") were digested with DNaseI.

were labeled with [^3H]thymidine for 30 min in the presence of butyrate. Nuclei were isolated and mixed with control nuclei labeled for one generation with [^{14}C]thymidine under normal conditions. The mixed nuclei were either digested immediately with DNaseI, or used to prepare mononucleosomes, which were then subjected to DNaseI digestion. Alternatively, H1 was selectively extracted from nuclei using the procedure of Lawson and Cole (21; Fig. 5B), prior to digestion with DNaseI (see "Materials and Methods"). The results of the DNaseI digests are presented in Figure 6A.

Although whole nuclei replicated in butyrate showed preferential sensitivity to DNaseI, isolated mononucleosomes did not (Fig. 6A). Thus, there is nothing intrinsic in the structure of

individual nucleosomes that confers selective DNaseI sensitivity. Moreover, removal of H1 partially reversed the DNaseI sensitivity of bu-chromatin (by ~50%) (Fig. 6A). This reversal was incomplete, most likely because of the presence of residual H1 remaining after the extraction procedure (Fig. 5B). Control experiments established that the reversal was due to a ~30% increase in the DNaseI sensitivity of bulk chromatin (as compared to only a ~15% increase undergone by bu-chromatin) consistent with bulk chromatin's greater H1 content. Moreover, basic chromatin architecture was not disrupted by the extraction procedure, as normal nucleosomal organization was observed following micrococcal nuclease digestion (data not presented; also 21).

Nascent, acetylated chromatin that is naturally depleted of histone H1 (*i.e.*, magnesium-soluble chromatin in the S1S) also exhibited no heightened DNaseI sensitivity, relative to naturally H1-depleted *bulk* chromatin in the same fraction (Fig. 6B). This demonstrates that when new and old nucleosomes are similarly deficient in H1, acetylation alone cannot induce DNaseI sensitivity. Because chromatin in the S1P was insoluble in magnesium (which is required for DNaseI activity) it was not feasible to measure the nuclease sensitivity of that chromatin fraction. Nevertheless, it was determined that the lack of preferential sensitivity of the magnesium-soluble chromatin was not an artifact due to prior exposure to micrococcal nuclease: Bu-chromatin remaining in the nucleus after micrococcal nuclease digestion is still preferentially cleaved by DNaseI (Fig. 6B).

Effect of H1 on Chromatin Solubility

Taken together, the data presented above suggest that both the altered solubility and increased DNaseI sensitivity of bu-chromatin are mediated by a relative deficiency in linker histone. In order to more directly relate H1 deficiency to altered solubility, the following experiment was performed: Cells were labeled with [³H]thymidine in the presence of butyrate, and chromatin was fractionated as in Figure 1; however, in some cases nuclei were treated to remove H1 prior to digestion with micrococcal nuclease.

Displacement of H1 completely abolished the preferential magnesium-solubility of bu-chromatin, over a wide range of digestion conditions, as both newly replicated and bulk nucleosomes were equally represented in the S1S (Table 2). This was due to the dramatic increase in the relative proportion of bulk chromatin remaining soluble in magnesium. Thus, when depleted of H1, bulk chromatin assumes the solubility properties of bu-chromatin, despite persisting differences in acetylation.

DISCUSSION

Chromatin replicated in the presence of sodium butyrate possesses normal resistance to micrococcal nuclease, but retains ~50% of the maximum sensitivity of nascent chromatin to DNaseI (19). We have now shown that, along with this altered nuclease sensitivity, bu-chromatin acquires an increased magnesium-solubility, which is related to a decreased H1 content. The importance of histone H1 in defining the special features of bu-chromatin was further indicated in experiments using H1-depleted nuclei, in which a marked reduction in both differential solubility

Table 2 Effect of H1 on the distribution of new and old DNA in the S1S

		% ³ H in S1S	% ¹⁴ C in S1S	% ³ H/% ¹⁴ C
<i>H1-Plus</i>				
	i)	44.8	17.1	2.62
	ii)	47.8	19.3	2.48
	iii)	52.8	22.0	2.40
<i>H1-Minus</i>				
	i)	30.2	30.1	1.00
	ii)	57.3	55.7	1.03
	iii)	77.4	67.3	1.15

Cells were labeled with [³H]thymidine for 30 min in the presence of butyrate, and chased for 15 min (+butyrate). Nuclei were isolated and mixed with control nuclei labeled for one generation with [¹⁴C]thymidine. Mixed nuclei were either immediately digested with micrococcal nuclease and fractionated by magnesium precipitation as described in Fig. 1, or digested and fractionated after H1 depletion. Percentages are based on total nuclear (³H)- and (¹⁴C)DNA. (Each horizontal row represents a separate experiment. Enrichment for new DNA is expressed as %³H/%¹⁴C.)

and DNaseI sensitivity was observed. While it is possible that undefined factors, concurrently displaced during H1 extraction, are responsible for the heightened DNaseI sensitivity of chromatin replicated in butyrate, both 1) the native H1-deficiency of bu-chromatin, and 2) the normal digestion kinetics of the S1S (which is intrinsically H1-depleted) argue against this. Therefore, the simplest explanation of the data is that the elevated DNaseI sensitivity of bu-chromatin is at least partly contingent upon a reduced H1 content, resulting from the maintenance of acetylation on newly assembled nucleosomes.

Although the predominant effect of sodium butyrate is the inhibition of histone deacetylation (33), it has been reported that prolonged exposure can have multiple effects (34-37). However, virtually all such secondary changes are induced after several hours of butyrate treatment, and are strongly correlated with histone hyperacetylation and cell cycle arrest (34-38). Because the major effect of brief exposure to butyrate (as in this report) is the maintenance of existing levels of acetylation, it is concluded that the differences we observe are due to the preservation of histone acetylation on newly replicated chromatin.

There have been numerous reports correlating altered nucleosome solubility with H1-deficiency and/or core histone acetylation, particularly in relation to transcriptionally active chromatin (18,19,27,39-45). In these cases, however, it has not been possible to determine if changes in H1 stoichiometry precede or follow histone modification. On the other hand, there is evidence that H1 is deposited subsequent to the core histones during chromatin assembly *in vivo* (29), very close to the time when nascent histones normally become deacetylated (4, 5). It therefore seems likely that our experiments bear upon the first encounter of H1 with the newly assembled chromatin fiber. Relevant to this, Reeves *et. al.* (49) have noted that when DNA

plasmids are transfected into mammalian cells in the presence of sodium butyrate, the assembled plasmid minichromosomes are both H1-depleted, and preferentially sensitive to DNaseI.

The observation that bu-monomers are not preferentially digested by DNaseI argues strongly for the importance of chromatin higher order structures in generating the properties of bu-chromatin. Normal DNaseI resistance was also noted for *hyper*acetylated monomers (9), indicating that acetylation affects chromatin organization at a level above the nucleosome, perhaps by unfolding the 30nm fiber, as has been suggested (9-14,19). A moderate loosening of the 30nm fiber has in fact been observed for hyperacetylated chromatin, as measured by either sedimentation (46), or electron microscopy (14).

It is tempting to speculate that changes in histone acetylation help to control the deposition of H1 during chromatin assembly, thereby regulating transitions in the folding of newly replicated chromatin. A number of points concerning this hypothesis may be raised. First, it is clear that the influence of acetylation on H1 binding is not absolute, since a measurable fraction of bu-chromatin is neither soluble in magnesium nor H1-depleted, and even magnesium-soluble chromatin contains some H1. Thus, acetylation may modulate the deposition (or subsequent anchoring) of H1, but does not in itself comprise an absolute regulatory mechanism. It has also been reported that histone H1 can exchange from smaller to larger chromatin fragments *in vitro*, at physiological ionic strengths (47, 48). However, because bu-chromatin is not preferentially cleaved by micrococcal nuclease (Fig. 3; also 19), it would seem unlikely that such rearrangement occurs under our experimental conditions.

If H1 is exchanging *in vivo* or *in vitro*, it nonetheless appears that the preferential target for deposition is non-acetylated chromatin. This has been further indicated in initial attempts to regenerate the solubility differences of bu-chromatin (by reconstituting H1-depleted nucleosomes), in which new/old ratios intermediate between those presented in Table 2 for H1-plus and H1-minus chromatin have been consistently observed (unpublished observations). Such preliminary studies further suggest that H1 phosphorylation may also influence H1 deposition, consistent with the observation that newly synthesized H1 is typically hypophosphorylated relative to more mature forms (5). We are currently investigating whether the state of H1 itself influences the association of linker histone with acetylated nascent chromatin.

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ABBREVIATIONS

¹ Abbreviations used are: bu-chromatin, chromatin replicated in the presence of sodium butyrate for thirty minutes; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate;

PIPES, Piperazine-N,N'-bis[2-ethane-sulfonic acid]; EGTA, ethylene glycol bis(B-aminoethyl ether)-N,N,N',N'-tetraacetic acid; EDTA, ethylenediamine tetraacetic acid (disodium salt); TCA, trichloroacetic acid; PPO, 2,5-diphenyloxazole.

REFERENCES

1. Annunziato, A.T. and Seale, R.L. (1983) *Molec. and Cell. Biochem.* **55**, 99-112.
2. Thoma, F., Koller, Th., and Klug, A. (1979) *J. Cell Biol.* **83**, 403-427.
3. Louie, A.J., Candido, E.P.M., and Dixon, G.H. (1974) *Cold Spring Harbor Symp. Quant. Biol.* **38**, 803-819.
4. Ruiz-Carrillo, A., Wangh, L.J., and Allfrey, V.G. (1975) *Science* **190**, 117-128.
5. Jackson, V., Shires, A., Tanphaichitr, N., and Chalkley, R. (1976) *J. Mol. Biol.* **104**, 471-483.
6. Waterborg, J.H. and Matthews, H.R. (1984) *Eur. J. Biochem.* **142**, 329-335.
7. Allis, C.D., Chicoine, L.G., Richman, R., and Schulman, I.G. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 8048-8052.
8. Cotten, M. and Chalkley, R. (1985) *Nucleic Acids Res.* **13**, 401-414.
9. Simpson, R.T. (1978) *Cell* **13**, 691-699.
10. Vidali, G., Boffa, L.C., Bradbury, E.M., and Allfrey, V.G. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 2239-2243.
11. Whitlock, J.P., Jr., and Stein, A. (1978) *J. Biol. Chem.* **253**, 3857-3861.
12. Chahal, S.S., Matthews, H.R., and Bradbury, E.M. (1980) *Nature* **287**, 76-79.
13. Allan, J., Harborne, N., Rau, D.C., and Gould, H. (1982) *J. Cell Biol.* **93**, 285-297.
14. Annunziato, A.T., Frado, L.-L.Y., Seale, R.L. and Woodcock, C.L.F. (1988) *Chromosoma* **96**, 132-138.
15. Mathis, D.J., Oudet, P., Waslylyk, B., and Chambon, P. (1978) *Nucleic Acids Res.* **5**, 3523-3547.
16. Nelson, D.A., Perry, M., Sealy, L., and Chalkley, R. (1978) *Biochem. Biophys. Res. Commun.* **82**, 1346-1353.
17. Davie, J.R. and Candido, E.P.M. (1980) *FEBS Lett.* **110**, 164-168.
18. Perry, M. and Chalkley, R. (1981) *J. Biol. Chem.* **256**, 3313-3318.
19. Annunziato, A.T. and Seale, R.L. (1983) *J. Biol. Chem.* **258**, 12675-12684.
20. Perry, M. and Chalkley, R. (1982) *J. Biol. Chem.* **257**, 7336-7747.
21. Lawson, G.M. and Cole, R.D. (1979) *Biochemistry* **18**, 2160-2166.
22. Loening, U.E. (1967) *Biochem. J.* **102**, 251-257.
23. Bonner, W.M. and Laskey, R.A. (1974) *Eur. J. Biochem.* **46**, 83-88.
24. Laskey, R.A. and Mills, A.D. (1975) *Eur. J. Biochem.* **56**, 335-341.
25. Thomas, J.O. and Kornberg, R.D. (1975) *Proc. Natl. Acad. Sci. USA* **72**, 2626-2630.
26. Panyim, S. and Chalkley, R. (1969) *Arch. Biochem. Biophys.* **130**, 337-346.
27. Annunziato, A.T., Schindler, R.K., Thomas, C.A., Jr., and Seale, R.L. (1981) *J. Biol. Chem.* **256**, 11880-11886.
28. Cousens, L.S. and Alberts, B.M. (1982) *J. Biol. Chem.* **257**, 3945-3949.
29. Worcel, A., Han, S., and Wong, M.L. (1978) *Cell* **15**, 969-977.
30. Jackson, V. and Chalkley, R. (1981) *Cell* **23**, 121-134.
31. Jackson, V. and Chalkley, R. (1985) *Biochemistry* **24**, 6921-6930.
32. Albright, S.C., Nelson, P.P. and Garrard, W.T. (1979) *J. Biol. Chem.* **254**, 1065-1073.
33. Riggs, M.G., Whittaker, R.G., Neumann, J.R., and Ingram, V.M. (1977) *Nature* **268**, 462-464.
34. D'Anna, J.A., Tobey, R.A., and Gurley, L.R. (1980) *Biochemistry* **19**, 2656-2671.
35. Boffa, L.C., Gruss, R.J., and Allfrey, V.G. (1981) *J. Biol. Chem.* **256**, 9612-9621.
36. Sterner, R., Vidali, G., and Allfrey, V.G. (1981) *J. Biol. Chem.* **256**, 8892-8895.
37. Whitlock, J.P., Jr., Augustine, R., and Schulman, H. (1980) *Nature* **287**, 74-76.
38. Littlefield, B.A., Cidlowski, N.B., and Cidlowski, J.A. (1982) *Exp. Cell Res.* **141**, 283-291.

39. Sanders, M.M. (1978) *J. Cell Biol.* **79**, 97-109.
40. Davie, J.R. and Saunders, C.A. (1981) *J. Biol. Chem.* **256**, 12574-12580.
41. Rocha, E., Davie, J.R., vanHolde, K.E. and Weintraub, H. (1984) *J. Biol. Chem.* **259**, 8558-8563.
42. Nelson, D.A., Ferris, R.C., Zhang, D. and Ferenz, C.R. (1986) *Nucleic Acids Res.* **14**, 1667-1682.
43. Ridsdale, J.A. and Davie, J.R. (1987) *Biochemistry* **26**, 290-295.
44. Alonso, W.R., Ferris, R.C., Zhang, D., and Nelson, D.A. (1987) *Nucleic Acids Res.* **15**, 9325-9337.
45. Ridsdale, J.A., Rattner, J.B., and Davie, J.R. (1988) *Nucleic Acids Res.* **13**, 5915-5926.
46. McGhee, J.D., Nickol, J.M., Felsenfeld, G. and Rau, D.C. (1983) *Nuc. Acids Res.* **11**, 4065-4075.
47. Caron, F. and Thomas, J.O. (1981) *J. Mol. Biol.* **146**, 513-537.
48. Thomas, J.O., Rees, C., and Pearson, E.C. (1985) *Eur. J. Biochem.* **147**, 143-151.
49. Reeves, R., Gorman, C.M., and Howard, B. (1985) *Nucleic Acids Res.* **13**, 3599-3615.
50. Albright, S.C., Wiseman, J.M., Lange, R.A., and Garrard, W.T. (1980) *J. Biol. Chem.* **255**, 3673-3684.
51. West, M.H.P. and Bonner, W.M. (1980) *Biochemistry* **19**, 3238-3245.
52. Annunziato, A.T., Schindler, R.K., Riggs, M.G. and Seale, R.L. (1982) *J. Biol. Chem.* **257**, 8507-8515.