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DNA associated with hyperacetylated histone is preferentially digested by DNase I

Linda Sealy and Roger Chalkley

Department of Biochemistry, School of Medicine, University of Iowa, Iowa City, IA 52242, USA

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ABSTRACT

Butyrate-treated cells give rise to massive hyperacetylation of histones and have been used to test the idea that regions of DNA in association with hyperacetylated histones are preferentially solubilized upon digestion with DNase I. Such hyperacetylated histones can be derived from both pre-existing histones or from histone newly synthesized in the presence of butyrate which leads to extreme modification. The DNA in association with both types of hypermodified histone is equally and selectively digested.

INTRODUCTION

Several workers have described a method for the selective solubilization of active genes by digestion of nuclei with DNase I (2-5). It has been suggested that the active genes are in a different conformation (2,3,6) which provides for preferential attack by DNase I. There is conflicting evidence (7-9) whether actively transcribed genes have gross changes in nucleosome structure, at least as viewed in the electron microscope. However, ribosomal genes and some unique sequences appear to be organized in structures which respond to micrococcal nuclease in a normal manner reflecting at least in general terms a normal nucleosomal organization (2,10-14). It seems likely that the (perhaps subtle) changes in nucleosome organization which lead to selective solubilization of active genes by DNase I also may play a critical role in permitting RNA polymerase to migrate through the compact nucleosome structure.

The mechanism whereby actively transcribed regions in chromatin could become more available to both nucleases and polymerases has long been thought to involve histone modification (15-27). Since histone phosphorylation appears to be primarily concerned with replication processes (28-32), interest now centers upon histone acetylation. Histone acetylation occurs to the level of the accumulation of up to four acetate groups per molecule (24,25,33,34), the modification occuring in the same amino terminal region of the protein

involved in binding DNA (20,21,34). The modification is metabolically very active showing a $t_{1/2}$ for hydrolysis of 3 min (25) an observation which would be consistent with a mechanism involving transient breaking and remaking of the nucleosome structure. Clearly such a process would offer a structural basis for increased nuclease sensitivity as well as providing a means whereby RNA polymerase could migrate through a temporarily loosened nucleosome which nonetheless could rapidly reestablish its structural integrity. Accordingly, we wished to ask whether the regions of the chromatin which were more rapidly solubilized by DNase I contained histones which were modified by acetylation to a greater degree than that of bulk chromatin.

Using nuclei from butyrate treated cells (35,36) we will show that those fractions of chromatin solubilized by digestion with DNase I show a remarkable degree of hyperacetylation at early times. The histones contributing to this degree of hyperacetylation arise from two pools, those histones which were present before butyrate was added and those histones synthesized after the addition of the short chain fatty acid (36). There is no selective hydrolysis of DNA in association with either 'new' or 'old' histone, thus ruling out that the effect we have seen is an artifact of inappropriate deposition of newly synthesized, highly acetylated histone.

MATERIALS AND METHODS

Treatment of Cells. To insure a rapidly dividing population, HTC cells were resuspended in fresh medium (S77) at 2.5 X 10^5 cells/ml approximately 12-24 hrs prior to incubation with 6 mM sodium butyrate for 6 hrs. During this treatment, all cells remained viable as judged by trypan blue exclusion.

Digestion of nuclei with DNase I. Preparation of nuclei and subsequent digestion with nuclease were carried out in the presence of 6 mM butyrate to inhibit deacetylation. Significant deacetylation of hypermodified histone can occur *in vitro* both at 37° and 4°C (unpublished observations). Frozen HTC cells were homogenized in 0.25 M sucrose, 0.01 M tris-HCl, 0.01 M $MgCl_2$, 1% Triton-X 100, 0.05 M $NaHSO_3$, pH 6.5 and nuclei were collected by centrifugation at 1,100 x g for 10 min. Nuclei were washed in this buffer 3 times and once in buffer without $NaHSO_3$ and detergent. The final nuclear pellet was washed in the digestion buffer previously described (2,3) except that 0.25 M sucrose was added. Nuclei were resuspended in this digestion buffer at a concentration of 1 mg/ml and digested at 37° with DNase I (3.36 units/mg of nuclei, Worthington Biochemical Corp). Reactions were terminated by chilling and immediately centrifuging the nuclei at 12,000 x g for 5 min. The supernatant was removed and EDTA added to a final 5 mM concentration. The

pellet was resuspended in 5 mM EDTA, 6 mM butyrate by sonification. The extent of digestion was monitored by measuring the release of material absorbing at 260nm into the supernatant or by determining the amount of acid soluble material as previously described (3).

Histone isolation and analysis. Histones were acid extracted from the supernatant and pellet fractions as previously described (36) except that samples were dialyzed overnight against 0.4 N H_2SO_4, 6 mM butyrate to remove sucrose and EDTA before being precipitated in ethanol. Polyacrylamide gel electrophoresis, scanning, cutting and counting of gels and analysis of densitometer scans were as previously described (36).

RESULTS

DNase I is selective for DNA associated with hyperacetylated histones.

Hyperacetylation of the non-H1 histones was achieved by exposure of HTC cells to 6 mM sodium butyrate for 6 hrs (36). In order to ask if there is preferential digestion of DNA associated with hypermodified histones we have treated nuclei isolated from these cells with DNase I. At various times during the digestion we have pelleted the nuclei and extracted histones both from the solubilized fraction and from the insoluble nucleoprotein which sediments. An analysis on 25 cm urea-polyacrylamide gels of the extent of histone modification in these two fractions at early and late stages in digestion is shown in figure 1. Clearly, at early stages in digestion (approximately 5% of the DNA made acid soluble) a very remarkable difference is seen in the degree of acetylation between histones in the supernatant and those in the pellet. Histones from the solubilized nucleoprotein are enriched in acetylated species, with greater than 95% of H4 histone modified and 85% of this histone appearing in the di-, tri- and tetra-acetylated forms. The ratio of tetra-acetylated H4 to parental H4 increases 10-fold over that found in histone from the insoluble nucleoprotein. This is shown in figure 2 where we have plotted the ratio of H4Ac4/H4Ac0[1] as a function of digestion time for both soluble and insoluble material. Although more extensively modified histones are found in the supernatant at early stages of the digestion, this distinction is in general lost as digestion proceeds. As seen in figure 2, while at early times in the digestion there is a 7-fold enrichment of H4Ac4 in the supernatant, this falls as the digestion proceeds. After a time we also note a decreased yield of histone in the supernatant even though the release of acid soluble nucleotides continues. This is attendant upon the formation of the insoluble limit residue which converts all histone into an insoluble complex. Similar observations to those reported in detail for H4

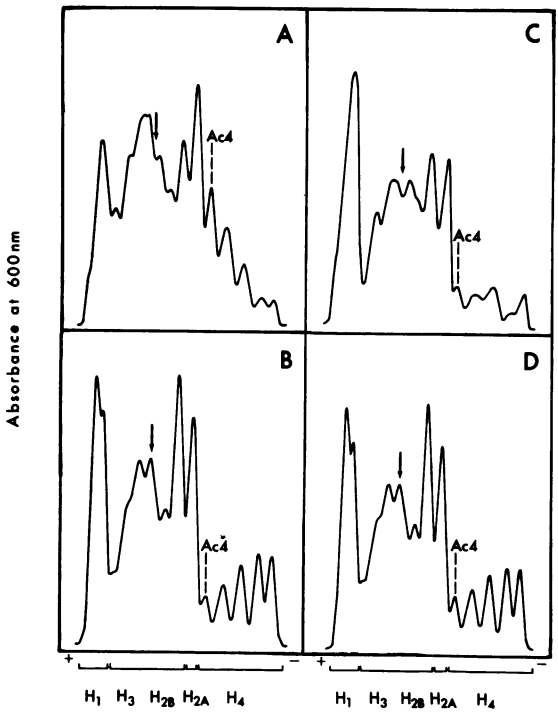

Figure 1. Polyacrylamide gel electrophoresis of histones isolated from DNase I soluble and insoluble fractions.

Nuclei isolated from HTC cells treated with 6 mM butyrate for 6 hrs were digested with DNase I as described in the Materials and Methods. At various times during the digestion histones were isolated from the supernatant and pelleted material and analyzed on 25 cm 2 M urea polyacrylamide gels. Densitometer scans of amido black stained gels are shown for histones extracted from the (A) supernatant, 1 min of digestion, (B) pellet, 1 min of digestion, (C) supernatant, 90 min of digestion and (D) nuclei, 0 min of digestion. Histones isolated from the pellet after 90 min of digestion show a pattern of histone modification identical to that in B. 1 min and 90 min of digestion correspond to 5% and 20% of the initial A_{260} absorbing material rendered acid soluble. The solid arrow represents the mobiltiy of H3Ac0 in a standard calf thymus histone sample co-electrophoresed on a separate gel.

have been made for H3 and H2B with 74% and 55% modified at early times in the solubilized nucleoprotein (compared to 60% and 36% in the insoluble fraction) although these percentages must only be considered approximate due to the overlap of H2B acetylated forms with the H3 region of the gel. As expected the pelleted nucleoprotein displays a pattern of histone modification very similar to that obtained from whole nuclei. Little if any corresponding depletion in acetylated species is observed as the histone from the solubilized material is a small fraction of the total histone at this degree of digestion. actually represent acetylated forms of the histones. HTC cells were labelled

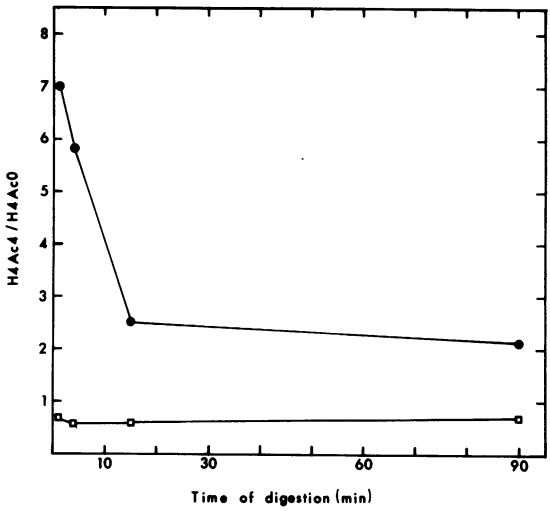

Figure 2. Ratio of tetra-acetylated H4 to parental H4 in nuclease soluble and insoluble fractions as a function of time of digestion.

The experimental procudure was as described in the legend to figure 1. For histone H4 the amount of each modified species on the gel was determined by calculating the area under the corresponding peak in a densitometer scan. The ratio of the amount of H4 tetra-acetylated relative to that in the parental form (H4Ac4/H4Ac0) is plotted as a function of time of digestion for the soluble (● ——●) and insoluble (□——□) fractions. For 1 and 90 min of digestion the amount of acid soluble material is as in figure 1. 4 min and 15 min of digestion correspond to 8% and 10% of the initial A_{260} material rendered acid soluble.

with ^{3}H-acetate in the presence of sodium butyrate. Nuclei were isolated from these cells and digested with DNase I. At various times in the incubation histones were isolated from the soluble and pelleted fractions and analyzed on polyacrylamide gels. Initially we measured the specific activity of all the non-H1 histones in the DNase soluble and insoluble fractions as a function of extent of digestion. The results of such an analysis are shown in figure 3. The greatest difference in specific activity occurs at early stages in digestion when between 5-13% of the DNA has been rendered soluble. The largest specific activity increase in the supernatant amounted to an almost 3-fold increase over that seen in the pellet. The increased specific activity of the 4 non-H1 histones is lost at later stages in the digestion. Since the multiple acetylated bands of H4 are most clearly resolved we have concentrated our analysis on this histone. Such an emphasis upon one histone fraction should not be taken to imply that the effects are any greater in H4 than in any of the other non-H1 histone fractions. In figure 4a is shown the specific activity of H4Ac4 in both DNase supernatant and pellet. At early

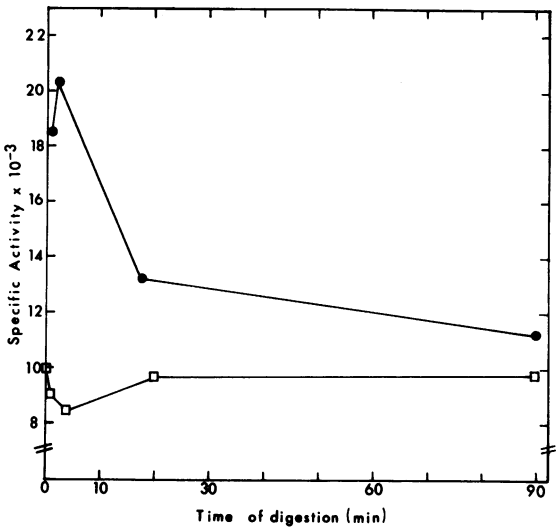

Figure 3. Specific activity of the 4 nucleosomal histones in the soluble and insoluble fractions as a function of time of digestion.

After a 6 hr exposure to sodium butyrate, HTC cells were resuspended in medium (S77) containing 6 mM butyrate at 10X the original cell concentration. ^{3}H acetate (10 mCi, 4.66 Ci/mmol, New England Nuclear) was added for 15 min and the cells collected and frozen. Nuclei were isolated and digested with DNase I and histones extracted from the supernatant and pelleted material at the indicated times during the digestion. Histones were analyzed on polyacrylamide gels and the gels were cut and counted as described in the Materials and Methods. The specific activity of the 4 non H1 histones extracted from either soluble (●——●) or insoluble (□——□) fractions was calculated by dividing the total cpm in the region of the gel containing these histones by the amount of protein on the gel in this region (as determined by measuring the area under the curve in a densitometer scan).

times there is a seven-fold greater specific activity in the supernatant, though this difference is completely lost at late stages in digestion. Similar results are noted for H4Ac3 (data not shown). In contrast the specific activity of H4Ac1 is actually higher in the insoluble nucleoprotein at early stages in the digestion (figure 4b), though once again the specific activity of this H4 fraction is similar in supernatant and pellet at late times in digestion.

Rates of digestion of control and hypermodified nuclei.

The selective digestion of chromatin containing hypermodified histone prompted us to ask whether nuclei from butyrate treated cells would exhibit an increased rate of digestion when compared to nuclei isolated from control cells. Experiments were conducted in triplicate and the release of acid-soluble material from nuclei of 24 hr butyrate treated cells vs control is shown in figure 5. Under these conditions we do not observe any statistical-

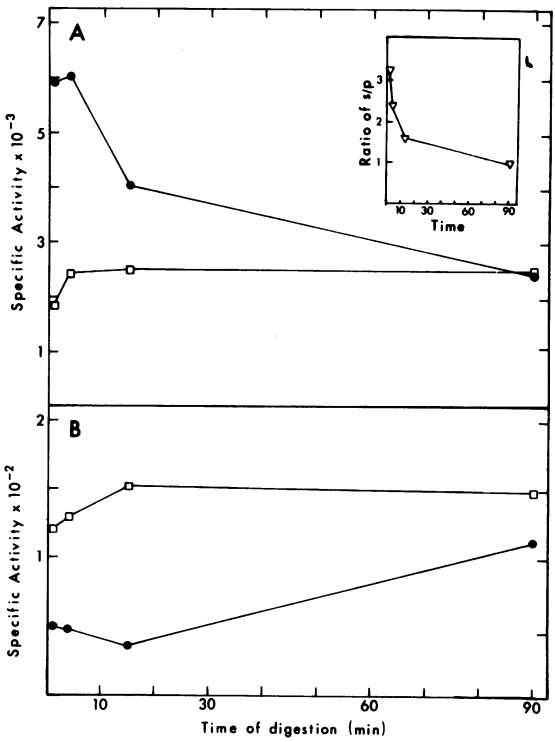

Figure 4. Specific activity of tetra and monoacetylated H4 in the nuclease soluble and insoluble material as a function of time of digestion.

The experimental procedure was the same as given in the legend to figure 3. The specific activity of tetra-acetylated H4 (A) or monoacetylated H4 (B) isolated from either the soluble (●——●) or insoluble (□——□) nucleoprotein was determined by dividing the cpm in the H4Ac4 or H4Ac1 band by the total amount of H4 protein on the gel (area in a densitometer scan.) The insert in 4A shows the ratio of the specific activity of H4Ac4 in the soluble (s) fraction relative to that in the pellet (p) as a function of digest time.

ly significant increased release of acid soluble material in hyperacetylated chromatin. We cannot, however, entirely rule out the possiblity of a difference in initial rate occuring within the first 1-3 min of digestion which would be difficult to observe consistently due to the problems of determining a small amount of acid solubility against a variable endogenous background. This is not particularly surprising since the acid soluble material represents DNA in the final stages of degradation whereas the centrifugation into soluble and insoluble material at pH 7.4 is in part a measure of extent of earlier chain scission in DNA. Accordingly we have also compared the rates of digestion of control and butyrate treated nuclei, monitoring the absorbance at 260nm of the solubilized fraction rather than the acid soluble mater-

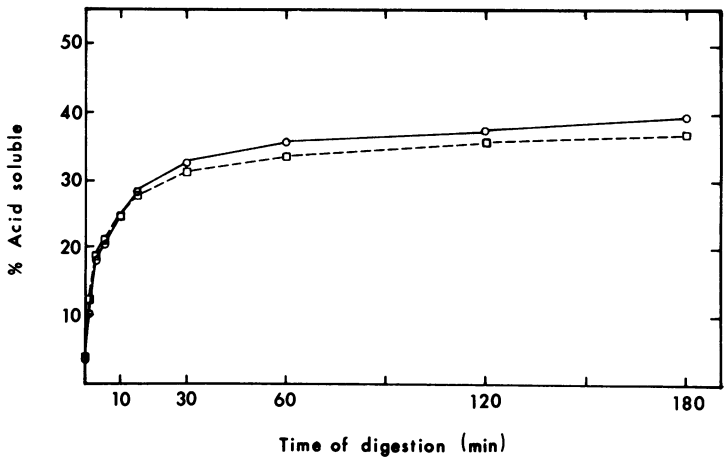

Figure 5. Comparison of rates of digestion by DNase I of hypermodified and control nuclei.

Nuclei were isolated from either control cells (□---□) or HTC cells treated with 6 mM butyrate for 24 hrs (○—○) and digested with Dnase I as described in the Materials and Methods. The percentage of initial A_{260} absorbing material rendered acid soluble was determined in triplicate and the mean value is plotted as a function of time. (Values not corrected for hyperchromicity). The standard deviation (not shown for each individual time point for purposes of clarity) ranged from $\pm$ 0.07% to $\pm$ 3.0%.

ial as an assay. However, results similar to those shown in figure 5 were obtained (data not shown) with only subtle (if any) differences in rate of digestion between the two. These observations may be reconciled with the results of figures 1 and 2 indicating preferential degradation of hyperacetylated regions if hyperacetylation changes the K_m of the nuclease for chromatin without affecting V_{max}. At the concentrations of substrate and enzyme employed in these experiments, there is no increase in early rate of digestion upon comparing 1 mg/ml with 2 mg/ml of nuclei at the same enzyme concentration (data not shown). This indicates that at these conditions of substrate excess the initial rate of digestion is already equal to V_{max} for both hyperacetylated and control nuclei. Thus if hyperacetylation changes the affinity of DNase I for chromatin without changing V_{max}, then no difference in rates of digestion between control and hypermodified nuclei would be observed under these conditions.

Selective digestion of DNA associated with new or old histone.

The selective digestion of chromatin containing the hypermodified histones could be due to the presence of incorrectly deposited histone. With regard to H4, this is that material which has been synthesized in the presence of butyrate, becomes associated with DNA primarily as the H4Ac2 form

Table I

Overall Specific Activity* of H4 Subfractions following Nuclease Solubilization

	(a) Prelabeled (old histone)		(b) Post labeled (primarily newly synthesized histone)	
Histone H4 Band	Pellet	Supernatant	Pellet	Supernatant
H4Ac0	45	10	33	28
H4Ac1	531	334	753	267
H4Ac2	754	965	1929	1095
H4Ac3	860	1861	1619	2300
H4Ac4	1259	4018	1133	3329

*Overall Specific Activity is defined as cpm within given H4 band/total amount of H4 histone on the gel.

To label old (as defined in the text) histones HTC cells were resuspended in medium (S77) at 10X the original concentration and ^{3}H-acetate (10 M Ci) was added for 15 min. Cells were collected and washed once with ice cold medium before being resuspended at normal cell densities (5 x 10^{5} cells/ml) at 37° in medium containing 6 mM butyrate for 6 hr. To label primarily new histone HTC cells that had been treated with 6 mM butyrate for 6 hr were resuspended at 10X the original concentration in medium containing 6 mM butyrate. ^{3}H-acetate (10 mCi) was added for 15 min and the cells collected and frozen. Cells labeled prior to or after butyrate treatment were digested with DNase I and histones isolated and analyzed from the soluble and insoluble nucleoprotein as described in Materials and Methods. Specific activity given for supernatant and pellet are after 1 min of digestion, 5-7% of the initial A_{260} rendered acid soluble.

and is subsequently modified further to H4Ac3 and H4Ac4 (36). It is generally thought that the modification of newly synthesized histone plays a role in deposition (24,27,33) as it is subsequently rapidly deacetylated (33). Accordingly in the absence of significant deacetylation (36) we have considered the possibility that it might bind DNA less effectively and thus lead to increased digestion of the DNA with which it is associated. We have attempted to distinguish between "new" (never deacetylated) and "old" (deposited prior to butyrate treatment) hypermodified histone. To label old histone, HTC cells were pulsed with ^{3}H acetate for 15 min and the radiolabel removed prior to addition of butyrate for six hours. The radioactivity which becomes associated with the histones in the labeling period is not removed because of the butyrate-mediated inhibition of the deacetylase (36). Nuclei were isolated from the labelled, butyrate treated cells and digested with DNase I. Analysis of the specific activity of H4 histone found in the supernatant vs. the

Table II

Specific Activity of Individual H4 Subfractions*

Histone H4 Band	Supernatant	Pellet	Ratio s/p
H4Ac1	3333	3033	1.1
H4Ac2	6172	8446	0.7
H4Ac3	6614	7622	0.9
H4Ac4	8335	7625	1.1

* Specific Activiy of individual subfractions is defined as cpm in a given H4 band/amount of H4 histone in that band.

Experimental procedure was the same as described in the legend to Table I for HTC cells labeled after butyrate treatment for 6 hrs. Specific activities are for 1 min of digestion, 5-7% of the initial A_{260} rendered acid soluble.

pellet fractions as shown in Table Ia indicates that the radiolabelled acetate associated with old histone is selectively found in the more extensively digested supernatant material in agreement with the results presented above. The converse experiment consisted of labelling with ^{3}H-acetate at the end of a 6 hr exposure to sodium butyrate. Under this condition the majority of the radiolabel becomes associated with the histone synthesized in the presence of butyrate ('new' histone). As shown in table Ib the radiolabelled H4 is selectively found in the early DNase supernatant to the same degree as that found for old histone. The data of table I pertain to the specific activity of H4 acetylated bands measured as the cmp/total H4. Selective digestion of acetylated forms is indicated by a higher specific activity in the supernatant relative to that in the pellet. Since similar increases in specific activity of H4 in the supernatant are obtained whether old histone or primarily new histone is labelled, both new and old histone must be contributing to the enrichment of higher levels of acetylation. Further, since both new and old histone are contributing to higher levels of H4 acetylation a specific activity measured as (cpm in a band/histone in a given band) should not show a selective increase in the supernatant if both new and old histone are equally available. That this is the case is shown in table II.

Finally we have confirmed that a 15 min ^{3}H acetate pulse at the end of a 6 hr exposure to butyrate labels primarily new histone. This was demonstrated by pulsing cells with ^{3}H-acetate after treatment with either 6 mM butyrate or 6 mM butyrate and cyclohexamide. As figure 6 shows in the latter case where the incorporation of ^{3}H acetate onto newly synthesized histone is eliminated, radiolabel incorporated into H4 falls to 30% of that observed

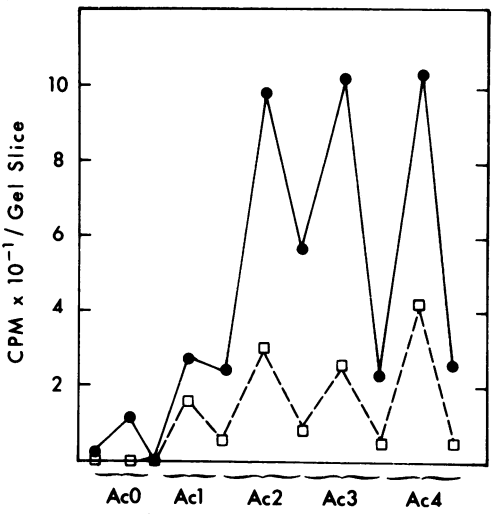

Figure 6. Measurement of ^{3}H-acetate incorporation on both pre-existing and newly synthesized histone in the presence of sodium butyrate.

HTC cells were either treated with 6 mM butyrate for 6 hrs (●—●) or pre-treated with cycloheximide(10ug/ml) for 30 min prior to and during exposure to 6 mM butyrate for 6 hrs (□---□). The cells were resuspended at 10X the original cell concentration in medium containing 6 mM butyrate or 6 mM butyrate and cycloheximide respectively and pulsed with ^{3}H-acetate (1 mCi, 4.66 Ci/mmol, New England Nuclear) for 15 min. Cells were collected and frozen. Histones were isolated as previously described (36), and analyzed on 25 cm 2 M urea polyacrylamide gels. The gels were sliced and counted as described in the Materials and Methods. For 6 mM butyrate treated cells, total incorporation into the H4 on the gel (from both newly synthesized and pre-existing histone) was 470 cpm. For 6 mM butyrate and cycloheximide treated cells, total incorporation into an equal amount of H4 (from only pre-exisiting histone) was 140 cpm.

when both old and newly synthesized histones are acetylated. Thus 70% of the ^{3}H acetate incorporated at the end of a 6 hr exposure to butyrate is associated with new histone.

DISCUSSION

Several previous workers (2-5) have shown that DNase I selectively digests actively transcribing genes. Usually this is observed at early stages in digestion as the effect amounts to a differential digestion and solubilization rather than an absolute difference in susceptibility to lytic action. Using the incubation system previously reported to digest active genes, we have analyzed the types of histone in the soluble and insoluble fractions at the stages of digestion where this differential susceptibility is most apparent (2,3). We find that the initially solubilized chromatin is associated with extensively hyperacetylated histones. This has been shown in two

ways. (1) Direct visualization of the microheterogeneity of the histones of the solubilized fraction reveals a dramatic enrichment in H4Ac3,4 and a decrease in H4Ac 0,1. Corresponding enrichment in H3 and H2b acetylated forms is also observed. (2) Labelling cells for short pulses with ^{3}H acetate and analyzing for specific activity of acetylated histone in DNase soluble and insoluble fractions reveal a 2 to 3-fold enrichment in specific activities of the hypermodified forms H4Ac3,4, in the soluble fraction.

This analysis would not have been possible without the hypermodification of H4 to the level of H3Ac2,3,4 using sodium butyrate. In HTC cells such hypermodification is almost exclusively due to acetylation (36). In addition an important caveat must be exercised since a significant fraction of H4Ac2, 3,4 arises from the massive hyperacetylation of newly synthesized histone (histone synthesis continues vigorously in butyrate-treated cells, at least for the first 6 hours). Since it has been suggested that acetylation and deacetylation of incoming histone may play a role in deposition it is at least conceivable that in butyrate treated cells the new histone is not properly deposited and thus might provide less effective protection against DNA digestion. Acetate labelling prior to addition of the butyrate offers a means for labelling deposited histone. Conversely acetate labelling after 6 hr exposure to butyrate when most of the acetylation sites on deposited histone have been filled, offers a means of labelling what is primarily freshly synthesized histone. When radiolabel added by either of the protocals is assessed for selective release by DNase I, we observed no significant difference between prelabelled or post labelled acetohistone. Thus we conclude that the observation we are reporting is not due to an artifact of anomolous histone deposition during butyrate treatment.

Of course, cells treated with n-butyrate represent a somewhat abnormal system. However, if fatty acid is removed after the six hr exposure the cells rapidly reinititate normal cell replication and viability is not lost. Furthermore, an O'Farrell gel analysis of proteins made during the exposure to butyrate indicates no change in the spectrum of proteins made or their relative rate of synthesis (Rubenstein, Sealy and Chalkley, manuscript in preparation). Thus we expect that in terms of gene regulation these cells have not changed drastically as a result of treatment with sodium butyrate, although we are aware of a number of instances were specific gene products have been induced by exposure of cultured cells to sodium butyrate (38-42).

There appears then to be a positive correlation between increased sensitivity to DNase I and the presence on such chromatin of very highly acety-

lated histones. The evidence is consistent with the preferential solubilization of active genes resulting from these transcribing regions being much more highly acetylated. Since the hyperacetylation occurs in that part of the histone molecules binding DNA, we may expect that the binding to DNA is weaker and thus permits a more ready attack by DNase I. The association of hyperacetylated histones with active genes may reflect a requirement that histones be extensively modified in order to permit transcriptional events to occur. Perhaps a weakened DNA-histone interaction permits this electrostatic bond to be more easily broken by RNA polymerase as it migrates through the nucleosome. The rapid removal of acetate modification *in vivo* would be consistent with a role in which critical structural interactions are briefly interrupted in order to permit transcription to occur.

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