Vol. 89, No. 2, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 27, 1979 Pages 571-579

EFFECT OF DNase I ON HISTONE ACETYLTRANSFERASE AND DEACETYLASE ENZYME ACTIVITIES IN FRIEND CELL NUCLEI: EVIDENCE FOR A PUTATIVE INHIBITOR OF THE DEACETYLASE ENZYMES IN TRANSCRIPTIONALLY ACTIVE CHROMATIN

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Received June 12,1979

SUMMARY: Pancreatic DNase I was used to preferentially destroy the DNA of transcriptionally "active" chromatin in isolated nuclei of normal Friend erythroleukemic mouse cells and in isolated Friend cell nuclei containing high levels of hyperacetylated histones as a result of prior exposure of the cells in culture to sodium butyrate. Histone acetyltransferase and histone deacetylase enzyme assays were performed on these two different populations of treated nuclei and the relative amounts of enzyme activities found were compared with the amounts of similar activities in control cell nuclei not treated with DNase I. The results of such experiments indicate that the relative amount of histone acetyltransferase activity is about the same in all of the tested nuclei regardless of whether they have been exposed to limited digestion with DNase I. On the other hand, the amount of histone deacetylase enzyme activity found in all DNase I-digested nuclei (regardless of their source) is consistently higher (by more than 50%) than the amount of activity found in untreated, control nuclei. These results suggest that limited digestion with the enzyme releases into a soluble supernatant fraction an inhibitor of the histone deacetylase enzymes that is normally present in transcriptionally active chromatin. This conclusion was further reinforced by the results obtained from various supernatant mixing experiments.

INTRODUCTION:

Recent experimental evidence from a variety of sources has lent considerable support to the original suggestion (1) that the post-synthetic modification of chromatin histones, particularly by the acetylation of the ε-N-lysine residues in the amino terminal regions of these proteins, might play an important role in the activation and maintenance of chromatin for transcriptional activity (2-4). For example, various nuclease digestion procedures that are reportedly capable of fractionating chromatin into trans-

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0006-291X/79/140571-09$01.00/0

criptionally active and inactive components (5-7) also result in the selective release of nucleosomes containing highly acetylated levels of histones into the soluble "active" fractions (8,9) that are enriched in transcribed DNA sequences. Also, in this connection, it has been demonstrated that tissue culture cell chromatin containing artificially high concentrations of multiacetylated species of histones as a result of prior treatment of the cells with sodium butyrate (11) – a short-chain fatty acid that has been shown to inhibit the histone deacetylase enzymes both in vivo and in vitro (12,13) – takes on many of the properties characteristic of transcriptionally "active" chromatin. For instance, numerous laboratories have reported that the DNA sequences associated with the butyrate-induced (BA-induced) hyperacetylated histones are much more susceptible than normal chromatin DNA sequences to hydrolysis by pancreatic DNase-I (13-15), an enzyme known to preferentially destroy active genes during short-term digestions (16). These results suggest that BA-treatment of tissue culture cells, with its concommitant induction of nucleosomal histone hyperacetylation, might lead to new gene expression not found in untreated, control tissue culture cells. That this may indeed occur has recently been demonstrated at both the transcriptional and translational levels in BA-treated Friend erythroleukemic mouse cells growing in culture (17,18).

In vivo, the endogenous level of post-synthetic acetylations occurring on chromatin histones seems to be the result primarily of a balance between the activity of groups of nuclear acetylating and deacetylating enzymes (19,20). We therefore decided to assay the levels of these enzyme activities in the chromatin of mouse Friend cells containing varying amounts of transcriptionally active genes and/or varying amounts of highly acetylated nucleosomal histones to determine whether any relationship might exist between the activity of these enzymes and the state of transcriptional activity of the cellular chromatins.

MATERIALS AND METHODS:

Cell Line and Culture Conditions: Friend erythroleukemic cells, clone 745A (21) were maintained and passaged using the techniques in (11). When required, butyric acid (BA), neutralized with concentrated NaOH, was added to a final concentration of 5 mM and the treated cells were grown in the fatty acid containing medium for 24 hr before harvesting for experimental purposes. The (^{3}H)-acetate labeled histones used for the histone deacetylase enzyme assays (see below) were isolated from exponentially growing Friend

cells grown in (^{3}H)-acetate (New England Nuclear) containing medium as previously described (11).

Nuclease Digestion of "Active" Chromatin: Nuclei were isolated from untreated and BA-treated Friend cells as previously described (11) and digested with pancreatic deoxyribonuclease I (E.C.3.1.4.5; Sigma) under conditions known to preferentially destroy the DNA in "active" chromatin (16,19). The untreated, control nuclei were digested with the enzyme to the point of 10-15% solubilization of the chromatin DNA whereas the nuclei from BA-treated cells (which contained high levels of hyperacetylated histones (11)) under the same digestion conditions had 20-30% of their chromatin DNA hydrolyzed by the enzyme (18). In both cases, however, the active genes were preferentially destroyed as shown by appropriate nucleic acid hybridization experiments (16; unpublished data). Non-nuclease digested nuclei from each different population of cells acted as controls for each individual experiment. It is quite important to point out that in all enzyme digested nuclear samples, the DNase I enzyme activity present was completely inhibited by chelation of the divalent cations in the digestion solutions by treatment with ethylenediamine tetraacetate (EDTA) before further enzyme assays were performed.

Assay of in vitro Histone Acetylase and Deacetylase Activity: Assay of histone acetyltransferase activity using (^{3}H)-Acetyl-CoA (New England Nuclear) as a precursor in the reaction mixtures was essentially as described previously (11). For the assay of histone deacetylase activity in various reaction mixtures, (^{3}H)-acetate labeled histones were added to the experimental extracts and assayed as previously described (11) except that the reactions were terminated by boiling prior to extraction of the released (^{3}H)-acetic acid from the mixtures with ethyl acetate.

RESULTS:

Figure 1 shows the effect of DNase I digestion on the endogenous histone acetyltransferase activity found in isolated nuclei from either untreated or BA-treated Friend erythroleukemic cells. From this figure it is evident that the levels of histone acetylase activity remaining in the DNase-I resistant nuclear pellets of both the untreated Friend cells (panel A) and the BA-treated cells (panel B) are similar to those found in the respective control nuclei that have not been exposed to nuclease digestion. Thus, it does not appear that the amount of histone acetylase activity present in the isolated nuclei is markedly affected by either the presence in the nuclei of transcriptionally active DNA sequences or by the absolute amounts of hyperacetylated histones present in the nuclei.

A markedly different result is obtained, however, if histone deacetylase activity is assayed in similar isolated nuclear populations as shown by the data in Table I. This table indicates quite clearly that in isolated nuclei from both untreated Friend cells and from BA-treated Friend cells, the nuclei digested with

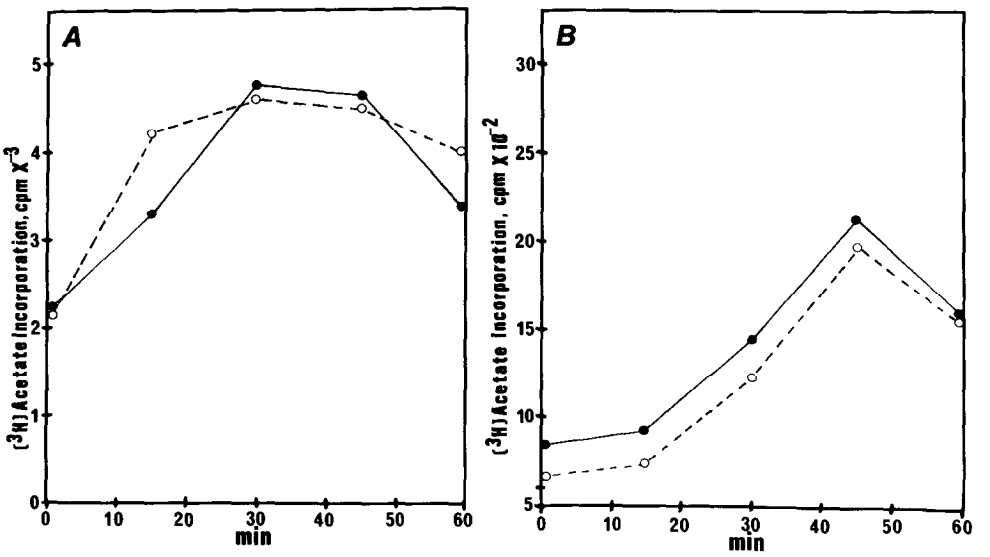

Figure 1. Effect of DNase I digestion of "active" chromatin on histone acetyltransferase activity in Friend cell nuclei. o——o, control nuclei; o---o, DNase I-treated nuclei.

DNase I to the point where most of the "active" DNA has been destroyed contain substantially greater amounts of histone deacetylase activity than comparable control nuclei not exposed to nuclease. This greatly increased amount of deacetylase activity in DNase I-resistant chromatin relative to the amount found in whole, non-enzyme treated, chromatin has been consistently observed in over 20 independent experiments under varying digestion and enzyme assay conditions. Furthermore, the differential results in the levels of enzyme activity cannot be explained by trivial factors such as differences in the degree of chromatin clumping found in control and nuclease-treated samples since shearing of the control chromatin samples to a similar degree of homogeneity as found in the nuclease-treated samples still results in the same relative degree of difference in the two populations as shown in Table I. Therefore, the simplest explanation for these findings would be that destruction of the DNA in transcriptionally active chromatin by DNase I results in the release of an inhibitor of the histone deacetylases from active chromatin.

Other, alternative, explanations are also possible for these results if only the foregoing data are considered. We therefore decided to test the hypothesis that DNase I digestion of chromatin does indeed release deacetylase enzyme inhibitors from active

Table I. Effect of DNase I on histone deacetylase activity in isolated Friend cell nuclei.

Source of Nuclei	cpm of ^{3}H-acetate released, control nuclei	cpm ^{3}H-acetate released, DNase I-treated nuclei	% increase in deacetylase
A) Untreated Cells:			
Expm. #1	2236	3942	76%
Expm. #2	4500	5842	30%
Expm. #3	3681	5853	59%
Expm. #4	5853	9183	57%
Expm. #5	6912	11325	64%
			Ave. 57.2%
B) BA-Treated Cells:			
Expm. #1	564	1119	98%
Expm. #2	967	1459	51%
Expm. #3	2341	3145	34%
Expm. #4	6546	9516	45%
Expm. #5	6901	10643	54%
			Ave. 56.4%

Acetylase activity was assayed as in (11).

chromatin in a more direct manner by conducting a series of supernatant/nuclear pellet mixing experiments followed by enzyme assays.

The overall outline for these mixing experiments is shown in Figure 2. In addition to the various control and calibration deacetylase assays (nos. 1-3 and 6-9) shown in this flow chart, the most important mixing results to note are those shown as deacetylase assays Nos. 4 and 5. For example, deacetylase assay Number 4 is one in which a non-enzyme treated nuclear pellet is mixed with the supernatant taken from a nuclear pellet digested with DNase I. Similarly, deacetylase assay Number 5 is one in which a nuclear pellet that has been digested with DNase I is mixed together with the supernatant fraction from untreated control nuclei and analyzed for activity.

If DNase I destruction of transcriptionally active chromatin DNA does indeed release a soluble inhibitor of the histone deacetylase enzymes, and if this inhibitor can also re-associate with non-enzyme treated chromatin and act to inhibit previously functional deacetylase enzymes, a number of predictions can be made concerning the outcome of such mixing experiments. For example, it would be expected that when a DNase I released soluble

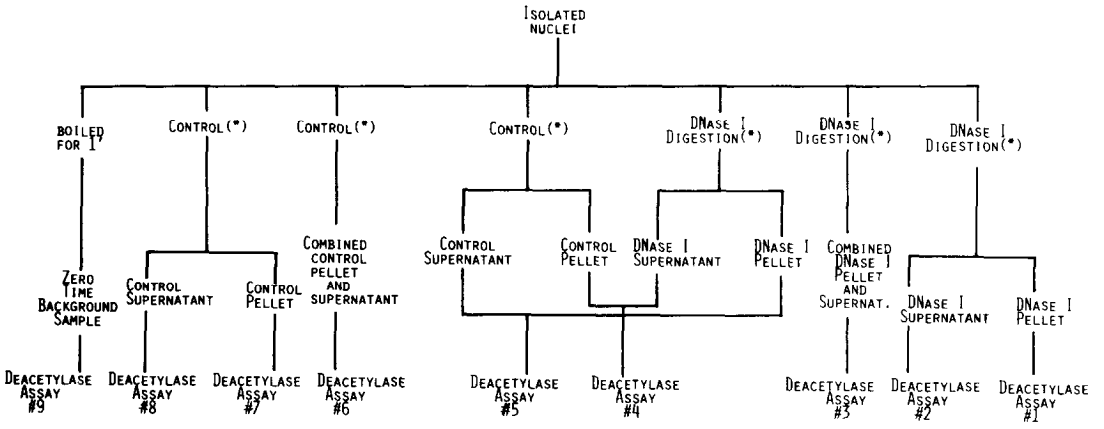

Figure 2. Outline of DNase I released supernatant mixing experiments for histone deacetylase assays. (*) Nuclei (approximately 10^8/ml) were incubated at 37° for 4 min with or without DNase I (100 units/ml) as required. After centrifugation (12,000 x g, 10 min), the supernatants and pellets were used for deacetylase assays as shown.

supernatant fraction is mixed with control, non-treated nuclei that the amount of deacetylase activity in this mixture (assay No. 4) would be considerably less than the amount of enzyme activity found in the reciprocal experiment (assay No. 5) where a DNase I resistant nuclear pellet is mixed with the supernatant fraction from untreated control nuclei. That these predicted results are indeed obtained for both the untreated Friend cell nuclei as well as for nuclei from BA-treated Friend cells is shown clearly in Table II.

Another predicted result from the above mixing experiments is that the amount of histone deacetylase activity found in control nuclear pellets resuspended in the supernatants from DNase I digested nuclei (assay No. 4) should always be less than the amount of enzyme activity found in control nuclear pellets resuspended in their own control supernatants (assay No. 6). Again, as shown in Table II this anticipated result is also found. For example, Table II indicates that the DNase I supernatant from digested Friend cell nuclei inhibits the endogenous histone deacetylase enzyme activity of untreated controls by about 31% when added to the nuclear pellet. Similarly, DNase I supernatant/control nuclei mixing experiments using nuclei isolated from Friend cells exposed to butyrate for 24 hours also show an inhibition of endogen-

Table II. Results of supernatant mixing experiments demonstrating that DNase I-released soluble materials from digested nuclei may contain an inhibitor of the histone deacetylase enzymes.

Assay No.	Assay contents‡	cpm ^{3}H-acetate released, untreated Friend cells	cpm ^{3}H-acetate released, butyrate treated Friend cells
1	DNase I-treated pellet	11,325	10,643
2	DNase I-released super natant	13,042	12,715
3	Combined DNase I pellet and DNase I supernatant	12,211	10,934
4	Control pellet combined with DNase I supernatant	4,736	4,521
5	DNase I pellet combined with control supernatant	11,278	10,283
6	Combined control pellet and control supernatant	6,894	7,006
7	Control pellet	6,912	6,901
8	Control supernatant	6,742	6,967
9	Boiled sample (zero time background activity)	674	572

See Figure 1 for experimental outline.

‡ As outlined in Figure 2

ous deacetylase activity by the DNase I supernatant of about 35%. This inhibition of endogenous histone deacetylase activity by materials released from chromatin by DNase I digestion has been seen in a large number of experiments with an average value of inhibition of about 33% but with a range of inhibitions varying from 27% to about 40% (data not shown). It will be noted that the deacetylase activities of supernatants and pellets are not additive when the two are assayed together (e.g. compare assays 1, 2 and 3 of Table II). It is probable that the added (^{3}H)-acetate labeled histones are bound to chromatin when it is present in the mixture, thus rendering the substrate inaccessible to the soluble deacetylase(s).

All of these mixing experiments are consistent with the hypothesis that transcriptionally active chromatin contains an inhibitor (or inhibitors) of the histone deacetylase enzymes that can be released into a soluble form by digestion of chromatin with pancreatic DNase I.

DISCUSSION:

The experimental results reported here provide considerable support for the existence of an endogenous inhibitor of the histone deacetylases which is selectively localized within the regions of transcriptionally active chromatin in Friend cells. This finding is of special interest because several studies have indicated that there appear to be naturally-occurring, low molecular weight inhibitors of the histone deacetylase enzymes in vivo in cells (22-24). Furthermore, there are numerous indications in the literature that the rates of deacetylation of histones are under physiological control and that these are closely related to changes in the transcriptional activity of cells. For example, during early embryogenesis (25), as well as during later cellular differentiation stages (26,27), the deacetylase activities vary as a function of changes in the rates of RNA synthesis in the differentiating cells. These enzyme rates can also be varied by hormonal stimulation of the appropriate target tissues in rats (28,29) and by exposure of granulocytes to plant lectins (30), and are stimulated in rats by treatment with the hepatocarcinogen, aflatoxin B_1 (31). Finally, it has recently been reported that by artificially inhibiting the histone deacetylase enzymes of cultured Friend cells by treatment of the cells with sodium butyrate, new transcriptional activity can be induced in the cells (17,18). Together these findings suggest that in vivo regulation of the histone deacetylases may play an important role in the regulation of RNA synthetic activity in eukaryotic cells by modulating the levels of acetylation found on the nucleosomal core histones. This possibility warrants further investigation.

Acknowledgements:

This work was supported in part by grants from The National Research Council of Canada (R.R.), by the Medical Research Council of Canada (E.P.M.C.), and by the B.C. Health Care Research Foundation (E.P.M.C.).

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