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THE GROSS LEVEL OF IN VITRO RNA SYNTHESIS IN HeLa NUCLEI IS UNALTERED BY HISTONE HYPERACETYLATION

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SUMMARY

Nuclei have been prepared from HeLa cells grown in the presence and absence of sodium n-butyrate, which represses histone deacetylase resulting in hyperacetylated chromatin. In vitro transcription using endogenous RNA polymerase activity and also exogenous RNA polymerase II results in no difference in transcriptional activity between the normal and modified species.

INTRODUCTION

Natural templates for eukaryotic RNA polymerases II, III and probably I are DNA with histones attached. Electron micrographs of class II transcription units of Oncopeltus show objects resembling nucleosomes between nascent RNP strands (1), which immunological experiments have demonstrated contain histone (2). The exact nature of these 'beads' is unclear; they may be identical with bulk chromatin nucleosomes or they may be altered in some manner. Active genes are conformationally distinct from silent chromatin in having enhanced accessibility to certain deoxynucleases (3,4), and they may differ in their non-histone composition (5-7) or in their degree of histone modification (8,9).

Histones may be covalently modified by acetylation and phosphorylation at certain specific positions of their primary sequences. Such modifications have been suggested to have a role in gene activation (10) and in chromatin assembly (11).

Recently Ingram and co-workers (12) have shown that growth of cultured cells in the presence of 5-10mM sodium n-butyrate suppresses

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natural histone deacetylation, resulting in specific but reversible hyperacetylation of chromatin bound histone. It had previously been known that sodium butyrate induced changes of morphology, growth rate, enzyme activity and cyclic nucleotide levels in mammalian cell cultures (13), which may be related to levels of histone acetylation. Using this technique several groups have shown (8,14,15) that such hyper-acetylated chromatin has interesting differences from its relatively unmodified counterpart. In particular, hyperacetylated chromatin has increased DNase I sensitivity, strongly reminiscent of that of active genes.

We therefore decided to examine levels of nuclear transcription in HeLa nuclei prepared from butyrate treated and control cells, in an attempt to discover whether or not the gross levels of RNA synthesis are elevated or depressed by the histone acetylation. We have not been able to demonstrate any effect of the modification on *in vitro* nuclear transcription, but the reason for this may lie in an interdependence of factors involved in eukaryotic gene regulation.

EXPERIMENTAL

Growth of HeLa cells. HeLa S3 cells (Flow Laboratories) were grown in Roux bottles in RPMI 1640 medium (Flow Laboratories) containing 10% aseptic calf serum to form sub-confluent monolayers. Histone acetylation (and controls) was accomplished by changing the medium to that containing (or omiting) 5mM sodium n-butyrate, grown for a further 24 hrs., followed by trypsinisation.

Preparation of nuclei. Cells were washed in 150mM NaCl (all solutions for butyrate treated cells also contained 5mM sodium butyrate), followed by nuclear isolation by a method modified from that of Weinmann *et al* (16). Washed cells were resuspended in lysis buffer (10mM HEPES, pH 7.6, 10mM magnesium acetate, 24mM potassium chloride, 0.04% β-mercaptoethanol and 0.5% Triton X-100) and stood on ice for 15 min. to swell osmotically. Cells were lysed by 20 strokes in a Dounce homogeniser (pestle B) and a nuclear pellet collected by centrifugation. This pellet was resuspended in lysis buffer, rehomogenised with 15 strokes (pestle A) and centrifuged. After resuspension in lysis buffer, the crude nuclei were layered over two volumes of suspension buffer (10mM HEPES, pH 7.6, 5mM magnesium acetate, 0.1mM EDTA, 0.04% β-mercaptoethanol and 25% glycerol) with a disturbed interface. Nuclei were pelleted at 3000 rpm for 10 min. and resuspended at 1-2 x 10^8 nuclei/ml in suspension buffer.

These preparations were free of debris, and contained only cell nuclei as judged by phase contrast microscopy.

Nuclear concentration estimation. As these values were of fundamental importance for these experiments they were measured by two methods:

Spectrophotometry: Samples of nuclear suspension were dissolved at 500 fold dilutions by warming in 0.5% SDS. Absorbances of the resulting solutions were measured at 258 and 320nm. Duplicate measurements agreed within an accuracy of 2%.

Nuclear counting: Nuclear suspensions were diluted 100 fold and counted in a Neubauer haemocytometer. Relative nuclear concentrations measured by these methods agreed to within 4%.

Nuclear Transcription. Nuclei were transcribed using endogenous RNA polymerases in 20mM HEPES, pH 7.6, 70mM potassium chloride, 2.5mM magnesium acetate, 0.02% β-mercaptoethanol, 0.4mM adenosine, guanine and cytidine triphosphates and 20μM uridine triphosphate. 300μl incubations contained 20μCi [5' - ^{3}H] uridine triphosphate. Incubations were performed at 25°C, aliquots removed were precipitated by 10% TCA containing 5% sodium pyrophosphate for 30 min at 0°C and filtered on GF/C glass fibre discs (Whatman). Standard liquid scintillation techniques were used for radioactive counting.

Nuclear dilutions were made in suspension buffer. For experiments requiring exogenous RNA polymerase, 2 units of wheat germ RNA polymerase II (Miles) were added to incubations.

Electrophoresis of histones. Histones were acid extracted from HeLa nuclei by 0.1M HCl at 0°C for 30 min and solid material removed by centrifugation. Following dialysis against water the histone samples were lyophilised, dissolved in 4M urea and applied to standard acid-urea polyacrylamide gel slabs (17). Gels were stained in Coomassie Brilliant Blue and scanned on a Joyce-Loebl densitometer.

RESULTS

Histone acetylation. Acid-urea polyacrylamide gel electrophoretic scans of histones acid extracted from normal and 5mM butyrate treated HeLa cell nuclei are shown in figure 1. Examination of the H4 migration shows that there is extensive acetylation of this histone in the butyrate exposed cells, in agreement with the results of several other groups (8,12,14,15). All extraction buffers for the modified nuclei contained sodium butyrate and the acetyl groups are clearly not labile to these procedures.

Nuclear transcription as a function of histone acetylation. Nuclei were prepared from HeLa cells exposed to 0 or 5mM sodium butyrate, and transcribed at identical (0.6×10^{8} nuclei/ml) concentrations. Time courses of RNA synthesis are presented in figure 2. Transcription levels for normal and hyperacetylated nuclei are very similar over

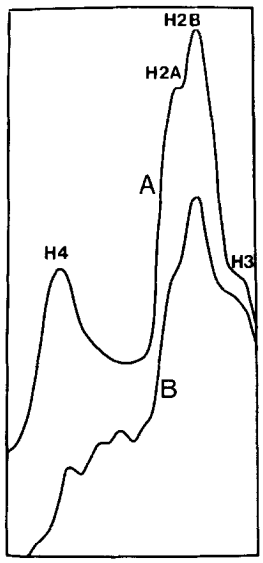


Figure 1. Scans from acid urea polyacrylamide gels of histones extracted from HeLa cells exposed to (A) 0mM and (B) 5mM sodium n-butyrate for 24 hrs.

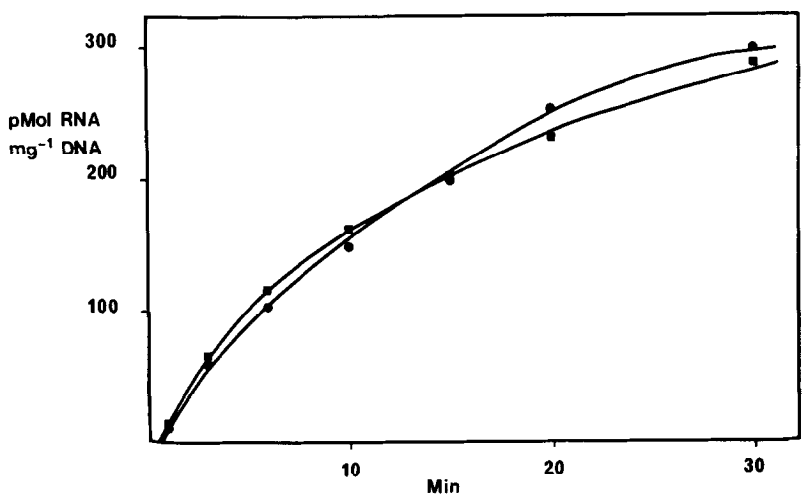
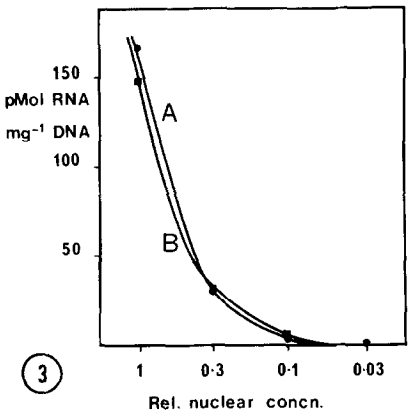


Figure 2. Nuclear transcription time courses for HeLa nuclei with normal (circles) and hyperacetylated (squares) histones.

thirty minute incubations, and may be considered to be identical, given an estimated 4% error in relative nuclear concentrations. Figure 3 shows the effect of nuclear dilution upon the amount of RNA synthesis after 10 minutes incubation for these nuclei. Within experimental error identical transcription is observed for butyrate-treated and untreated



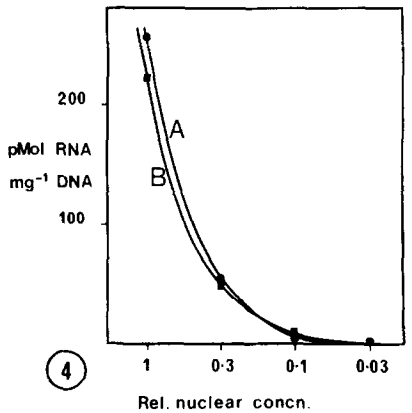


Figure 3. Effect of histone acetylation upon nuclear RNA synthesis by endogenous RNA polymerases as a function of nuclear dilution. (A) Normal nuclei (circles), (B) 5mM butyrate treated nuclei (squares). Absolute nuclear concentrations in this experiment were 2.46mg/ml DNA (0.6×10^8 nuclei/ml) for unit concentration.

Figure 4. Effect of histone acetylation upon nuclear RNA synthesis by endogenous RNA polymerases supplemented by exogenous wheat RNA polymerase II as a function of nuclear dilution. (A) Normal nuclei (circles), (B) 5mM butyrate treated nuclei (squares). Absolute nuclear concentrations were 2.46mg/ml DNA for unit concentration.

nuclei for any given nuclear concentration. However, it is striking that transcription per mg of nuclear DNA is not constant, but shows a pronounced dependence on nuclear concentration. The possible origin of this effect is discussed below.

Nuclear transcription with exogenous RNA polymerase. To study the possible effect of histone aceylation upon transcription by exogenous RNA polymerase, the experiment shown in figure 3 was repeated with the addition of wheat germ RNA polymerase II to the incubations. The results, shown in figure 4, indicate that although overall levels of RNA synthesis are elevated, there is still no measurable effect of histone acetylation. Thus, butyrate treatment appears to have no observable effect upon levels of transcription by either endogenous or exogenous RNA polymerases.

DISCUSSION

From the results presented above it is apparent that hyperacetylation of histones by butyrate treatment of HeLa cells does not affect the

overall level of nuclear RNA synthesis *in vitro*. Similar conclusions have been reached by Chalkley (personal communication) for both hyper- and hypoacetylated nuclei. There are several possible reasons for this.

Firstly, acetylation alone may not affect nuclear transcription processes. Mathis *et al* (18) have been unable to demonstrate differences in *in vitro* transcription of SV40 DNA reconstructed with normal and hyperacetylated histones. Secondly, acetylation may facilitate the passage of RNA polymerases along eukaryotic genes, or be a prerequisite, but active genes may be hyperacetylated even in the untreated nucleus. Under similar experimental conditions, reinitiation of RNA polymerase has not been observed (19), although more sensitive assay systems have detected some reinitiation (20). We may only, therefore, observe the further propagation of polymerase molecules already initiated at highly specific, and thus possibly modified, loci. The results of addition of exogenous polymerase reduce this possibility, but it is still feasible that these enzymes initiate at particular nucleoprotein sites. A third possibility, also diminished by the exogenous polymerase II experiments, is that there is a fortuitous 'balancing' of effects on the multiple eukaryotic polymerase activities. The conditions used in these experiments result in ~60% sensitivity to low levels of α-amanitin, i.e. class II transcription (21).

The effect of nuclear dilution upon transcription levels is intriguing, and suggests the diffusion of an 'activation factor' from HeLa nuclei. Such activators have been observed in several cell types (22-26). Two points are of interest. The effect acts upon transcription by both endogenous polymerase and also exogenous RNA polymerase II. Secondly, the effect does not distinguish between nuclei containing normal and hyperacetylated chromatin.

In summary, we have been unable to observe differences in overall transcriptional activity resulting from histone hyperacetylation. This

does not exclude acetylation as an important effector in eukaryotic transcription. Other factors, such as the presence of specific non-histone proteins, may be required to act in concert. Furthermore, whilst deacetylase suppression changes bulk chromatin structure it may not influence that of already active genes. HeLa cells remain viable in the presence of sodium butyrate for about 36 hours, which does not suggest that a considerable re-organisation of gene-transcription occurs. The exact role of histone acetylation in eukaryotic gene expression is likely to be subtle and will require further elucidation.

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