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Effect of histone acetylation on structure and in vitro transcription of chromatin

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ABSTRACT

n-Butyrate treatment of growing HeLa cells produces a dramatic increase in the levels of histone acetylation. We have exploited this system to study the effect of histone acetylation on chromatin structure. Chromatin containing highly acetylated histones is more rapidly digested to acid-soluble material by DNase I, but not by micrococcal nuclease. The same pattern of nuclease sensitivity was exhibited by *in vitro*-assembled chromatin consisting of SV40 DNA Form I and the 2 M salt-extracted core histones from butyrate-treated cells. Using this very defined system, it was possible to demonstrate that acetylated nucleosomes do not have a greatly diminished stability. Stability was measured in terms of exchange of histone cores onto competing naked DNA or sliding of histone cores along ligated naked DNA. Finally, it was shown that acetylated nucleosomes are efficient inhibitors of *in vitro* RNA synthesis by the E.coli holoenzyme as well as by the mammalian polymerases A and B.

INTRODUCTION

It is now well established that eukaryotic DNA is packaged within repeating nucleoprotein subunits, the nucleosomes (1-3). However, there is very little information on how the basic nucleosomal structure is modulated to effect the processes of transcription and replication. Post-synthetic histone modifications, e.g. phosphorylation, methylation or acetylation, have been suggested as possible mechanisms (see Ref. 4 for review). By altering DNA/histone binding, these modifications could promote the type of localized variation in chromatin structure that might be expected to occur at times of gene transcription or in preparation for replication. In particular, high rates and/or levels of histone acetylation have been correlated with

increased RNA synthetic capacity in a number of systems (4). Although these studies demonstrate a striking association between the two events, they remain correlative, and are yet to be supported by direct tests of the effect of histone acetylation on chromatin structure.

Efforts directed toward this goal have been frustrated by the difficulty of isolating acetylated histones in bulk. Recently, however, Riggs and coworkers (5, 6) and Hagopian et al. (7) have described a system that greatly enhances the levels of these modified subspecies. They have shown that treating growing cells with n-butyrate results in a dramatic increase in the levels of histone acetylation; for example, approximately 80% of the H4 molecules were acetylated to some degree after such treatments.

We have used the procedure of Riggs and coworkers to prepare acetylated chromatin and histones from HeLa cells in order to more directly test the effect of histone acetylation on chromatin structure. More specifically, we have asked the questions : does highly acetylated chromatin have an altered sensitivity to nuclease digestion? Does the nucleosomal complex formed by DNA and highly acetylated histones exhibit a diminished stability? Do acetylated nucleosomes effectively inhibit transcription by RNA polymerase?

MATERIALS AND METHODS

1) Growth and treatment of cells. Preparation of nuclei. HeLa cells were maintained in suspension culture as previously described (8). "Non-treated" cultures were harvested at concentrations of 4-6 × 10^5 cells/ml. "Treated" cultures, at 2-4 × 10^5 cells/ml, were made 5 or 10 mM in butyric acid and incubated a further 20-24 hours before collection. To obtain labelled cells, 0.850 μCi/ml [^{3}H]-thymidine (90 Ci/mmole, ICN, California) was added to non-treated cultures during the last 20-24 hours growth, and 0.01 μCi/ml [^{14}C]-thymidine (53.6 mCi/mmole, CEA, France) was added to treated cultures 24 hours before the addition of n-butyrate. Nuclei were isolated by a modification of previously published procedures (9). Cells were washed with phosphate-buffered saline and suspended at approximately 2 × 10^7/ml in 0.02%

Triton X-100, 10 mM Tris-HCl pH 7.5, 0.1 mM phenyl methyl sulfonyl fluoride (PMSF). After 15 min at 4°C the swollen cells were homogenized 4-8 strokes with a Dounce B. The homogenate was layered over an equal volume of 10 mM Tris-HCl pH 7.5, 0.1 mM PMSF, 1.4 M sucrose and centrifuged at 650 g for 1 hour at 4°C. The nuclear pellet was overlayered with 30% glycerol, 50 mM Tris-HCl pH 8.0, 0.1 mM EDTA, 0.25 mM dithiothreitol (DTT), was frozen in liquid nitrogen, and was stored at -90°C until use. The freezing of nuclei had no detectable effect on the results of subsequent experiments.

2) Preparation of H1-depleted chromatin. Chromatin from both treated and non-treated cells was depleted of H1 and non-histone proteins by a modification of the procedure of Ilyin et al. (10). Nuclei were suspended at approximately 1 mg DNA/ml in 100 mM NaCl, 10 mM Tris-HCl pH 7.5, 0.1 mM $CaCl_2$ and digested with 100 units micrococcal nuclease (Worthington) per mg DNA for 3 min at 37°C. The reaction was stopped with an equal volume of 0.5 mM EDTA and nuclei were subsequently lysed by dialysis against 0.2 mM EDTA pH 7.5 for 4-6 hours at 4°C. After centrifugation at 4000 g for 5 min, the soluble chromatin was incubated 6-12 hours at 4°C with a 15 fold excess of yeast tRNA (Plenum Scientific Research, Hackensack, N.J.) in 10 mM NaCl, 0.2 mM EDTA, 0.1 m M PMSF, 1 mM Tris-HCl pH 8.0, 1 mM DTT (Buffer A). The resulting H1-depleted chromatin was pelleted through 5-20% sucrose in buffer A onto a 70% sucrose cushion by centrifugation for 10 hours at 23,500 rpm in an SW 25.1 rotor (Beckman) at 4°C. The pelleted material was dialyzed overnight against 0.1 mM PMSF, 1 mM sodium bisulphite, 1 mM EDTA, 20 mM Tris-HCl pH 8.0 (Buffer B) made 500 mM in NaCl, and was concentrated to approximately 100 µg/ml by dialysis against the same buffer under reduced pressure.

3) Histone isolation and characterization. Salt-extracted histones were prepared as described previously (11) with two modifications for both HeLa histone preparations. H1 was extracted with 500 mM NaCl and was removed by centrifugation for 12-15 hours at 4°C in an SW 41 rotor (Beckman) at 39,000 rpm.

The four histones were separated from DNA on hydroxyapatite using a batch procedure. The equilibrated hydroxyapatite was mixed with the salt-dissociated chromatin (1 mg DNA/ml packed hydroxyapatite) and gently stirred for 5 min at 4°C. After pelleting at 4000 g for 2 min, the hydroxyapatite was washed with an equal volume of buffer. The pooled supernatants were clarified by centrifugation for 15 min at 16,000 g.

Acid-extracted histones were prepared from nuclei and H1-depleted chromatin by treatment with 0.4 N H_2SO_4 for 30 min at 4°C, and were precipitated for 12-15 hours with 9 volumes of acetone at -20°C. The precipitate was collected by centrifugation at 16,000 g for 10 min.

Electrophoresis of histones was performed in a 12 cm slab gel apparatus (Hoefer Scientific Instruments, San Francisco), using the method of Panyim and Chalkley (12). Gels were stained for 2 hrs in 25% isopropanol, 10% acetic acid, 0.25% Coomassie Blue and destained in 10% acetic acid at room temperature.

4) Nuclease digestion and kinetic analyses. Nuclei were digested with micrococcal nuclease or DNase I (Sigma) under the conditions specified in the figure legends. Reactions were terminated by the addition of EDTA to 2.5 times excess over the divalent cation concentration. Procedures for the isolation of DNA and electrophoresis of the DNA digestion products have been published (13).

For the kinetic analyses, [^{14}C]-labelled nuclei from butyrate-treated cells and [^{3}H]-labelled nuclei from non-treated cells were digested in the same reaction mixture. At the specified times, aliquots were made 5% in TCA and 100 µg/ml in calf thymus DNA in a final volume of 0.2 ml, were incubated for approximately 2 hours at 4°C, and were centrifuged at 8000 g for 20 min. 0.2 ml of water was added to the pellets and 0.5 ml soluene (Packard) added to both pellets and supernatants. After incubation at room temperature for 30 min, the samples were counted in 5 ml of 0.4% Scintimix-3 (Koch-Light Laboratories, England) in toluene. The kinetic analyses of *in vitro*-assembled chromatin were performed by exactly the same method.

5) Reconstitutions. Exchange experiments. Salt-extracted histones from treated and non-treated HeLa cells and from calf thymus were associated with SV40 DNA Form I or Form III essentially as described by Germond and coworkers (11). Minor modifications were that the reconstitution buffer was buffer B (see above) and that the times of incubation were 1 hour at 2 M NaCl, 15 min at 1.6 M NaCl and 30 min each at 1.4 M, 1.2 M, 0.9 M, 0.85 M, 0.75 M, 0.65 M and 0.5 M NaCl, all at 20°C. For the digestion, exchange and sliding experiments, the histone : DNA ratio was 0.8, while for the transcription experiments, the ratio was 0.6.

The stability of *in vitro*-assembled chromatin and of cellular H1-depleted chromatin was tested by incubation in the presence of competing SV40 DNA Form I at elevated salt concentrations. These exchange experiments were conducted as published (11) except that the exchange buffer was Buffer B (see above) and the nucleoprotein concentration was initially 80 µg/ml. Naked competitor SV40 DNA was added in two fold excess for the experiment using *in vitro*-assembled complexes and at a 1:1 ratio for the experiments using cellular H1-depleted chromatin. In this latter case, treatment with untwistase (UE) was not necessary (see below).

5) Construction of hybrids. Sliding experiments. Hybrids of naked SV40 DNA linked to SV40 DNA packed within nucleosomes were constructed by the following procedure : SV40 DNA Form III (Eco RI cut) was associated with the four core histones as described above and the complexes dialyzed for 12-15 hours against 40 mM Tris-HCl pH 8.0, 1 mM EDTA, 4 mM $MgCl_2$, 10 mM ammonium sulphate, 10 mM β-mercaptoethanol (Buffer C). The nucleosomal reconstitutes were ligated to naked SV40 DNA Form III with T4 ligase (6.67 units/ml, Miles, England) for 2 hours at 12-14°C in buffer C supplemented with 0.1 mM ATP and 50 µg/ml Bovine serum albumin. The concentration of the nucleosomal reconstitute and of the naked DNA was each 80 µg/ml. After ligation, the material was dialyzed for 30 min at 37°C against 10 mM Tris-HCl pH 8.0, 10 mM NaCl and was used immediately thereafter for the sliding experiments.

Sliding was assayed by electron microscopy after incubation of the hybrid molecules at elevated NaCl concentrations for 3 hours at 37°C. During this treatment, the hybrid was present at a concentration of 10 µg/ml, and competing naked SV40 DNA Form I was added at 5 µg/ml.

7) Electron microscopy. Electron microscopy was performed as in earlier papers (11, 14).

8) Transcription experiments. The isolation of E.coli RNA polymerase is described elsewhere (15), and the purifications of calf thymus RNA polymerases A and B (glycerol gradient fractions) were according to previously described methods (16, 17). The methods used for the kinetic analyses of RNA synthesis are detailed in the figure legends.

RESULTS

1) Characterization of histones. n-Butyrate treatment of growing cells has been shown to result in a striking increase in the levels of histone acetylation (5-7). All four core histones are probably affected (5, 7). Fig. 1 (a and b) compares the histone profiles of non-treated HeLa cells and cells treated 24 hours with 10 mM butyrate. The change in the H4 acetylation pattern is most clear; the mono, di, tri and tetra-acetylated forms are all increased in treated cells. The fuzziness in the H2b/H3 region (Fig. 1b) was shown by two-dimensional triton-urea/SDS gels to result from modification of both H2b and H3 (data not shown). There is a change in the relative intensity of the two bands in the H2a region, but whether the slower moving band is actually an acetylated subspecies has not been established. Fig. 1 also demonstrates that the highly acetylated subspecies are retained during the preparation of H1-depleted chromatin (c, d) and of 2 M salt-extracted histones (e, f).

2) Nuclease digestion of chromatin containing highly acetylated histones.

a) Micrococcal nuclease.

When digested with micrococcal nuclease, nuclei from buty-

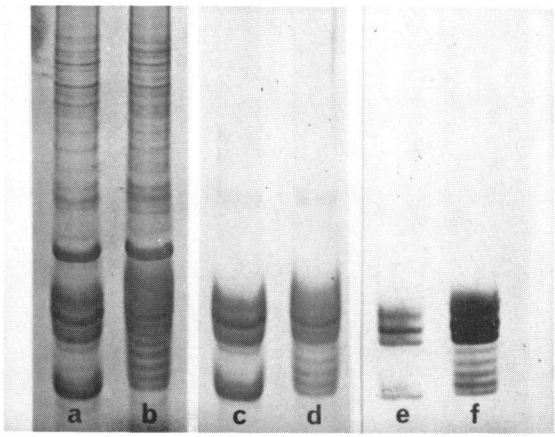

Fig. 1 : Electrophoretic analysis of histones from butyrate-treated and non-treated HeLa cells. Polyacrylamide gel electrophoresis of acid-extracted histones from nuclei of: (a) non-treated and (b) butyrate-treated cells; (c) acid-extracted histones from H1-depleted chromatin deriving from the same nuclear preparation as in (a); (d) acid-extracted histones from H1-depleted chromatin deriving from the same nuclear preparation as in (b); salt-extracted core histones from (e) non-treated and (f) treated cells.

rate-treated cells exhibit the typical repeating pattern of DNA digestion products. Fig. 2 indicates that the repeat size is indistinguishable from that observed after digesting control nuclei (188 base pairs, 18). In addition, the submonomer fragments produced at later times appear identical for the two preparations (Fig. 3).

[^{14}C]-labelled nuclei from butyrate-treated cells and [^{3}H]-labelled nuclei from non-treated cells were digested with micrococcal nuclease in the same reaction mixture in order to monitor the kinetics of digestion. It is clear from Fig. 4a that the rate of reaction is the same for both types of nuclei. Similarly, when 2 M salt-extracted histones from butyrate-treated and control HeLa cells are associated with [^{14}C] and [^{3}H]-labelled SV40 DNA, the digestion of these complexes by micrococcal nuclease proceeds with very similar kinetics (Fig. 4b).

b) DNase I.

DNase I digestion of nuclei from treated and non-treated cells also produces a very similar pattern of DNA digestion

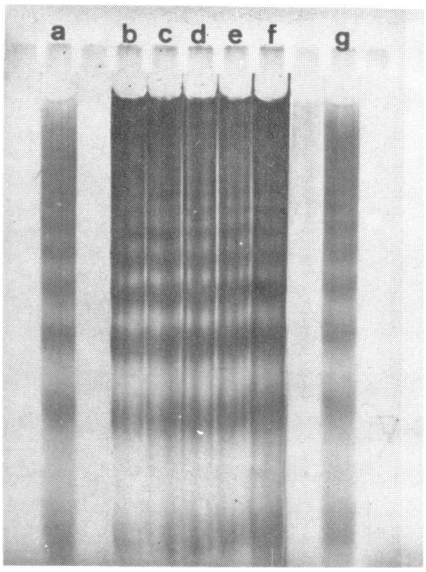

Fig. 2 : Electrophoretic patterns of the DNA fragments produced after mild digestion of nuclei with micrococcal nuclease. Nuclei (at 1 mg/ml DNA in 15 mM Tris-HCl pH 7.4, 60 mM KCl, 15 mM NaCl, 15 mM β-mercaptoethanol, 0.15 mM spermine, 0.5 mM spermidine, 0.34 M sucrose, 1 mM $CaCl_2$) were digested with 1,200 units/ml micrococcal nuclease for 15 min at 37°C. The DNA digestion products were analyzed on 2.5% polyacrylamide/ 0.5% agarose gels. (a) and (g) : micrococcal digests of rat liver nuclei used as markers; (b), (d) and (f) : DNA fragments from non-treated HeLa cells; (c) and (e) : DNA fragments from butyrate-treated cells.

products (Fig. 5). The ten base series (18) is evident in both cases, after times of digestion resulting in as little as 5-7% acid soluble material. As has been observed for other types of nuclei (19), there is an enrichment in the 80 base band and a deficiency in the 60 base band, irrespective of the degree of histone acetylation.

In contrast with the results obtained using micrococcal nuclease, there is a difference in the kinetics of digestion by DNase I (Fig. 4c). The ^{14}C -labelled nuclei from butyrate-treated cells exhibit an enhanced susceptibility to this enzyme; with several preparations of nuclei we have been able to observe a 1.5-2 fold increase in the initial rate of production of acid-soluble material. Fig. 4d demonstrates that it is possible to regenerate the differential sensitivity to DNase I

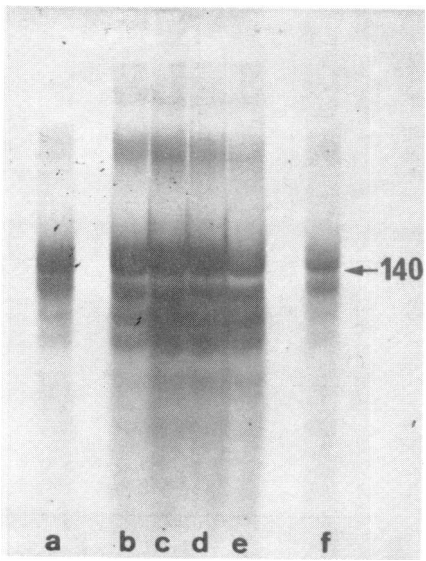

Fig. 3 : Electrophoretic patterns of the DNA products resulting from prolonged digestion of nuclei with micrococcal nuclease. Nuclei were digested under the conditions specified for Fig. 2 except that the time of incubation was 210 min (b and c) or 300 min (d and e). The digestion products were analyzed on 6% acrylamide/0.5% agarose gels. (a) and (f) : micrococcal digests of rat liver nuclei used as markers; (b) and (d) : fragments from non-treated HeLa cells; (c) and (e) : fragments from butyrate-treated cells.

digestion in reconstituted complexes of SV40 DNA and the 2 M salt-extracted core histones.

3) Stability of nucleosomes containing highly acetylated histones.

a) Exchange of histone cores.

Germond and coworkers (11) have described a method to analyze the stability of a nucleosomal complex by monitoring the exchange of histone cores (2 each of the histones H2a, H2b, H3 and H4) onto a competing naked DNA. We have used this method to compare the stability of nucleosomal complexes assembled from SV40 DNA Form I and the 2 M salt-extracted histones from either butyrate-treated or non-treated HeLa cells. The reconstituted chromatin was incubated at elevated salt concentrations (0.25 - 0.85 M NaCl) in the presence of a two-fold excess of

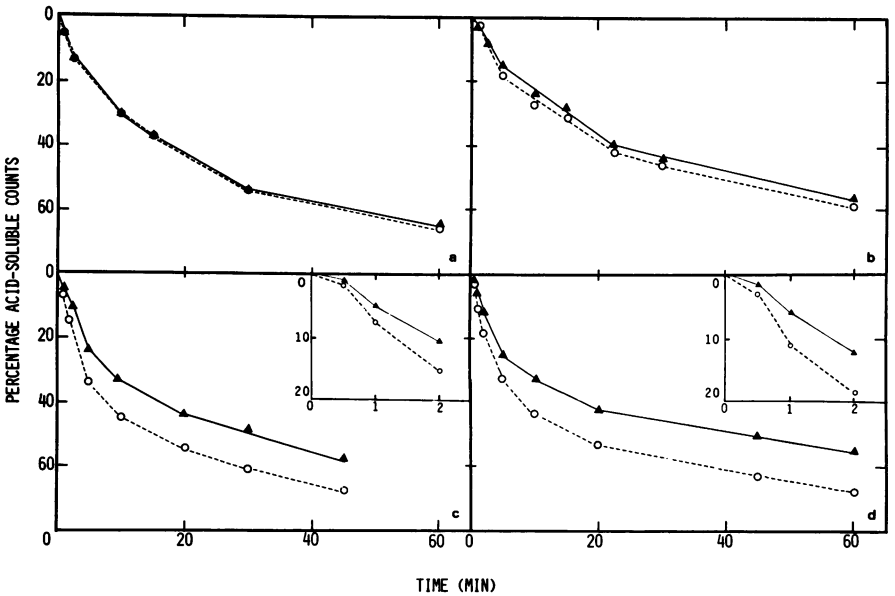

Fig. 4 : Kinetics of digestion by DNase I and micrococcal nuclease. (a) [^{14}C]-labelled nuclei from butyrate-treated HeLa cells (— o —) and [^{3}H]-labelled nuclei from non-treated cells (— ▲ —) were mixed in 10 mM Tris-HCl pH 7.4, 1 mM $CaCl_2$ at a final concentration of 500 µg/ml DNA each. The material was digested with 960 units/ml micrococcal nuclease at 37°C for the specified times and aliquots processed for the percentage TCA-soluble counts. (b) [^{14}C]-labelled SV40 DNA was complexed *in vitro* with the core histones from butyrate-treated HeLa cells (— o —), and [^{3}H]-labelled SV40 DNA associated with the core histones from non-treated cells (— ▲ —). The samples were dialyzed against 10 mM Tris-HCl pH 7.4 for 1 hour at 20°C, and then mixed together and digested as described in (a), except that the enzyme concentration was 250 units/ml; (c) The nuclei described in (a) were mixed in 10 mM Tris-HCl pH 8.0, 10 mM $MgCl_2$ and then digested with 21.6 units/ml DNase I at 37°C; (d) The nucleosomal complexes described in (b) were mixed together in 10 mM Tris-HCl pH 7.4, 2 mM $MgCl_2$ and digested with 100 units/ml DNase I. The inserts in (c) and (d) depict the digestion from 0-2 min in expanded scale.

naked SV40 DNA Form I. After gradually reducing the salt concentration to 0.25 M, an aliquot of the material was processed for electron microscopy. The remainder of the material was divided into two: one half was incubated with untwisting extract (UE) to remove extra-nucleosomal superhelical turns, while the other half was incubated without the addition of untwistase. The samples were treated with sodium dodecyl sulfate to dis-

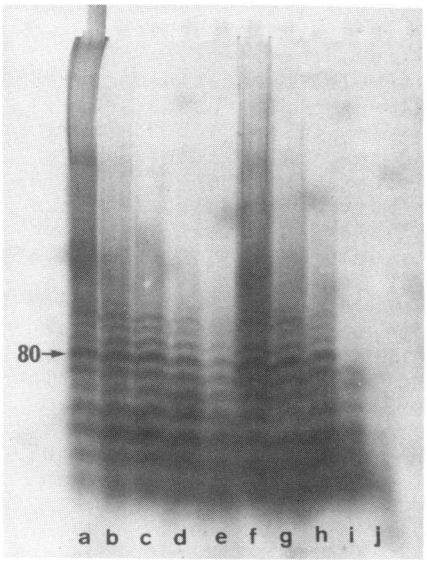

Fig. 5 : Electrophoretic patterns of the DNA fragments produced by digestion of nuclei with DNase I. Nuclei, suspended at 1 mg/ml DNA in the buffer described in Fig. 2 (except that 3 mM $MgCl_2$ replaced 1 mM $CaCl_2$), were digested with 100 units/ml DNase I at 37°C. The DNA digestion products were analyzed on 6% polyacrylamide gels containing 98% formamide (19). (a-e) Fragments produced after 0.5, 1, 2, 5 and 10 min digestion of nuclei from non-treated HeLa cells. In (a), 5,5% of the DNA had been rendered acid-soluble. (f-j) Fragments produced after 0.5, 1, 2, 5 and 10 min digestion of nuclei from butyrate-treated HeLa cells. In (f), 6,7% of the DNA was acid-soluble.

sociate histones and DNA and were subjected to electrophoresis using a gel system which separates SV40 DNA on the basis of number of superhelical turns. In the absence of exchange, the electrophoresed DNA should appear as two populations, one containing relaxed SV40 DNA (FIr, originating from the naked competitor DNA, see Fig. 6) and the other exhibiting a distribution of superhelical turns equal to the number of nucleosomes on the reconstituted chromatin. After complete exchange, a population of molecules should be observed that is characterized by a distribution in number of superhelical turns approximately one third that of the supercoil population before exchange.

Fig. 6 indicates that the histone cores from control (A) and treated (B) cells are exchanged at approximately the same ionic strength. In both cases, the exchange begins at about 0.75 M NaCl and is complete at 0.8 M, although the histones from butyrate-treated cells do exchange to a somewhat greater extent at 0.75 M salt. In several such experiments, we have observed that the more acetylated histone cores exchange at either the same NaCl concentration as control histone cores or at a 0.05 M lower salt concentration. These results were confirmed by electron microscopy (not shown).

A similar protocol allows the determination of the stabi-

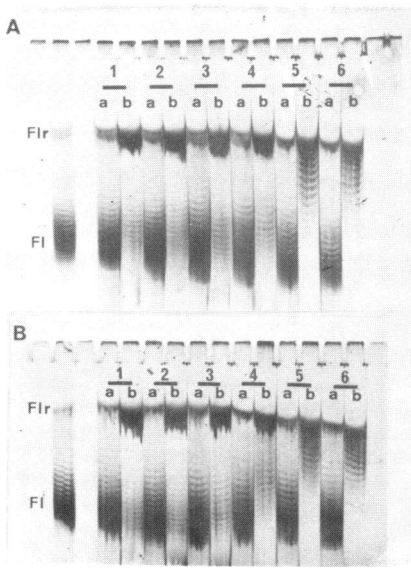

Fig. 6 : Electrophoretic analysis of the exchange of histone cores from *in vitro* assembled chromatin onto naked DNA. Chromatin was assembled *in vitro* from SV40 DNA Form I and the salt-extracted core histones from non-treated (A) or butyrate-treated (B) HeLa cells and was then incubated at various ionic strengths at 37°C for 30 min in the presence of naked SV40 DNA Form I (see text and Materials and Methods). After gradually reducing the salt concentration to 250 mM, the samles were incubated in the absence (a) or in the presence (b) of UE at 37°C for 30 min and then processed as described in Materials and Methods and in Ref. (11). 1, 2, 3, 4, 5 and 6, incubations at 250, 650, 700, 750, 800 and 850 mM NaCl, respectively. The first slot contains naked SV40 DNA Form I marker.

lity of nucleosomes in cellular H1-depleted chromatin. In this case, a complete exchange would be signified by total transfer of histone cores from the linear chromatin fragments to the supercoiled SV40 competitor DNA, because of the higher affinity of histone cores for superhelical DNA (11). Fig. 7 illustrates this transfer of histone cores as observed in the electron microscope. The appearance of clusters of beads and regions of naked DNA in the cellular chromatin before the exchange (Fig. 7a) is a result of the overnight dialysis against 500 mM NaCl (see Discussion). The dialysis was necessary to maintain the solubility of the chromatin fragments. Similar results were obtained using H1-depleted chromatin from butyrate-treated or control cells; in both cases, the exchange was almost complete at 750 mM NaCl.

The results depicted in Fig. 8 also indicate that H1-depleted chromatin from butyrate-treated (A) or control (B) cells is characterized by a very similar stability. In several experiments using this protocol, we observed histone core par-

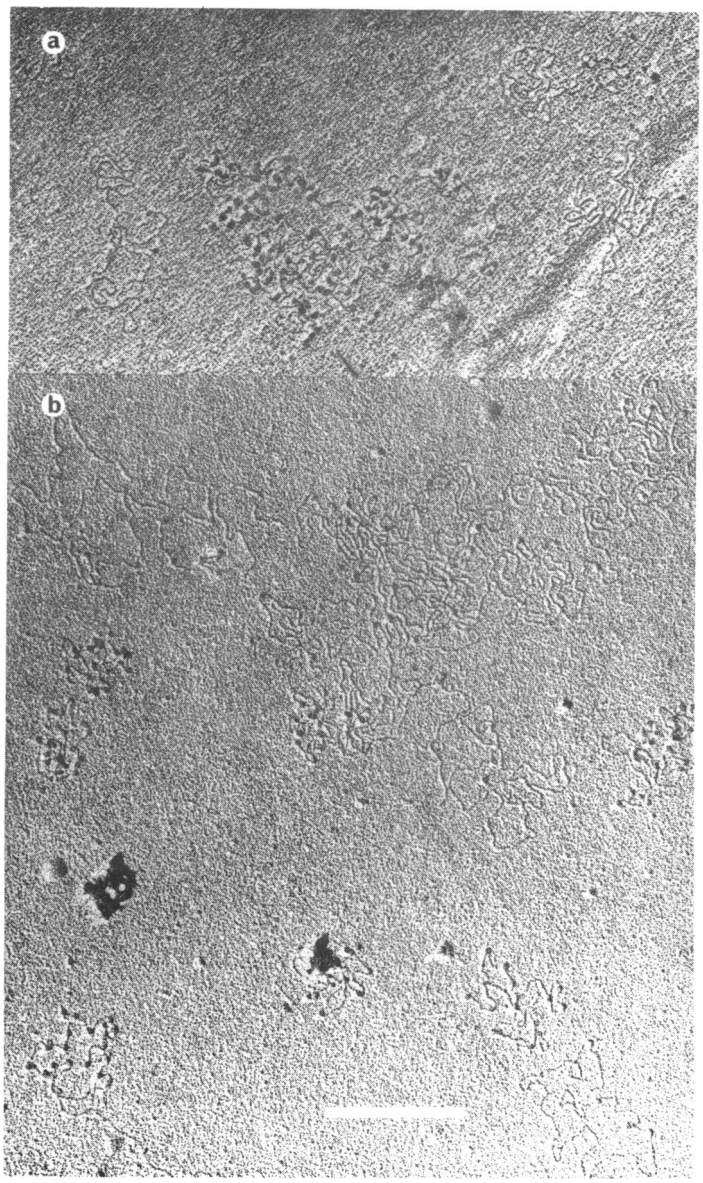

Fig. 7 : Electron microscopic analysis of the exchange of histone cores from H1-depleted chomatin to naked DNA. H1-depleted chromatin was prepared from non-treated HeLa cells and incubated in the presence of naked SV40 DNA Form I at 37°C for 30 min at (a) 250 mM NaCl or (b) 750 mM NaCl. After gradually reducing the salt concentration to 250 mM, aliquots were abruptly diluted to 0.3 µg/ml in 10 mM Tris-HCl pH 7.5 and processed for electron microscopy. The bar represents 0.25 µM.

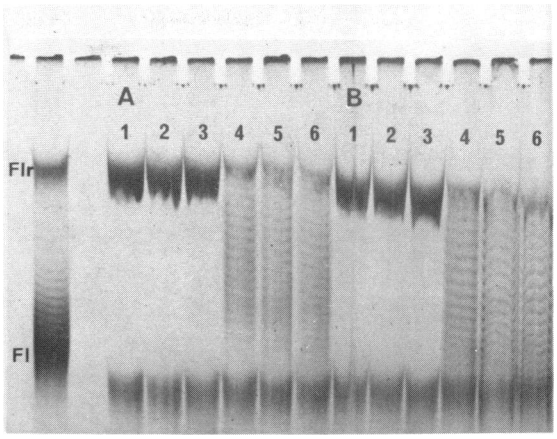

Fig. 8 : Electrophoretic analysis of the exchange of histone cores from H1-depleted chromatin to naked DNA. H1-depleted chromatin was prepared from non-treated (A) or butyrate-treated (B) HeLa cells and then incubated in the presence of naked SV40 DNA Form I at 37°C for 30 min. 1, 2, 3, 4, 5 and 6, incubations at 250, 650, 700, 750, 800 and 850 mM NaCl. The samples were then treated as indicated in Materials and Methods and in Ref. (11). The first slot contains naked SV40 DNA Form I as a marker.

ticle exchange in the range of 0.75-0.85 M NaCl; the histone cores from butyrate-treated cells exchanged onto the competing SV40 DNA at the same ionic strength as those from control cells or at a 0.05 M lower ionic strength (compare Fig. 8a slots 4-6 with Fig. 8b slots 4-6). The addition of UE to remove extranucleosomal supercoils was not necessary for this series of experiments because of contaminating UE in the H1-depleted chromatin preparations.

b) Sliding of histone cores.

It is possible to construct hybrid complexes of naked DNA and *in vitro*-assembled chromatin. Fig. 9a shows an example of such a hybrid complex containing a naked SV40 DNA Form III molecule ligated to another Form III molecule associated with the four calf thymus histones. It should be noted that although confined to only a portion of the ligated complex, the nucleosomes appear well dispersed. It should be possible to observe in the electron microscope any sliding of histone cores which might be induced by treatment with elevated salt concen-

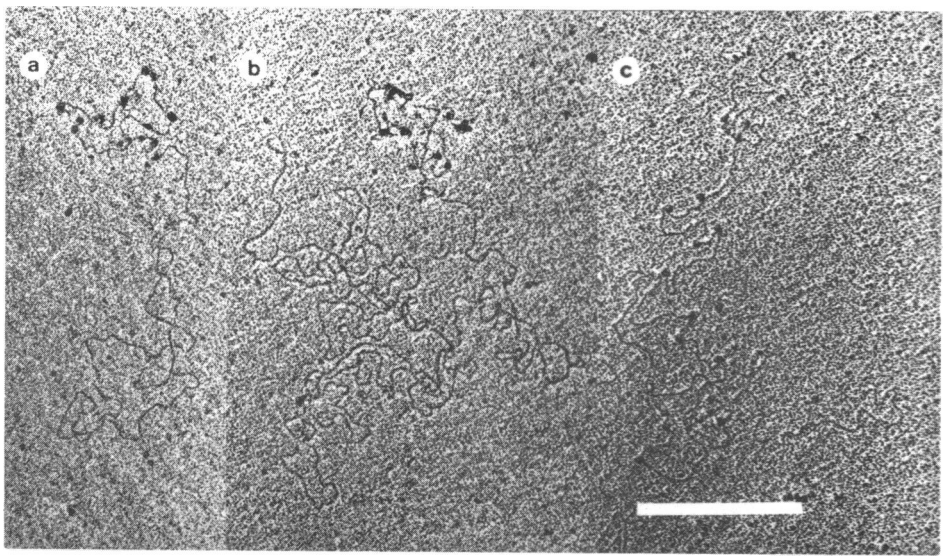

Fig. 9 : Electron microscopic analysis of the effect of salt on hybrid complexes containing naked DNA ligated to nucleosomal reconstitutes. Complexes of naked SV40 DNA Form III ligated to SV40 DNA Form III associated with the four calf thymus histones were constructed as detailed in Materials and Methods. (a) samples were diluted to 0.2 µg/ml in 10 mM Tris-HCl pH 7.5 without prior salt treatment and processed for electron microscopy; (b) and (c) samples were incubated for 3 hours at 37°C in 500 mM NaCl before the dilution for electron microscopy. The bar represents 0.25 µM.

trations. Sliding of histone cores can be distinguished from intermolecular exchange by the addition of naked SV40 DNA Form I as a competitor. When hybrid complexes are treated as long as 3 hours at 37°C with 500 mM NaCl, no evidence of sliding along the naked DNA is observed for the great majority of molecules. Many molecules exhibit clusters of nucleosomes, instead of well dispersed beads, in the reconstituted portion of the hybrid (Fig. 9b) (see discussion). For some ligated complexes (<10%), it appears that some sliding may have occurred (Fig. 9c), but due to the entanglement of the molecules, it is not possible to state this with certainty. At 600 mM NaCl it is possible to observe some beads on the naked SV40 DNA Form I competitor which had been added to distinguish between sliding and intermolecular exchange. It appears, there-

fore, that there is no significant long-distance sliding of histone cores at ionic strengths lower than those which allow exchange.

Hybrid complexes were also constructed using naked SV40 DNA Form III and SV40 DNA associated with the histones from butyrate-treated or non-treated HeLa cells. Both preparations exhibit the same response to elevated salt concentration as described above for hybrid complexes containing calf thymus histones. Thus, we did not find any evidence that acetylation facilitates the sliding of histone cores.

4) Transcription of in vitro-assembled chromatin containing highly acetylated histones.

In order to study the effect of acetylated histones on chromatin transcription, we have tested their ability to inhibit RNA synthesis *in vitro*. Synthesis on complexes of SV40 DNA Form I and the four core histones from butyrate-treated HeLa cells was compared with synthesis on complexes of SV40 DNA Form I and the four calf thymus histones. (In both preparations, 12-15 beads were observed on the SV40 DNA by electron microscopy - see Materials and Methods). Since it was difficult to prepare large amounts of salt-extracted non-treated HeLa cell histones devoid of untwistase activity, calf thymus histones were used as a control because their level of acetylation is very similar to that of non-treated HeLa cell histones. Fig. 10 demonstrates that control and acetylated histones are equally able to inhibit transcription by RNA polymerase. This observation is true for the calf thymus polymerases A and B (panels c and b, respectively), as well as for the E.coli holoenzyme (panel a).

The nucleosomal inhibition of RNA chain elongation during the *in vitro* transcription of chromatin by E.coli RNA polymerase can be partially overcome by increasing the salt concentration during synthesis (15, 21). We have attempted to determine whether the acetylation of histones alters this response to increased ionic strength. The E.coli holoenzyme was allowed to initiate on

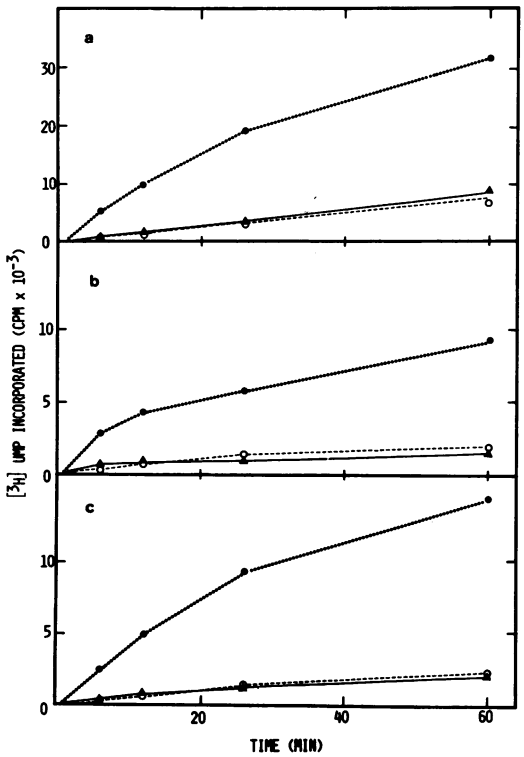

Fig. 10: Transcription of naked SV40 DNA or *in vitro*-assembled chromatin by the E.coli holoenzyme and calf thymus A and B RNA polymerases. 0.2 µg of SV40 DNA Form I (··· ● ···) or an equivalent amount of *in vitro*-assembled chromatin containing histones from calf thymus (— ▲ —) or butyrate treated HeLa cells (-- o --) was incubated under the following conditions: (a) with 0.12 µg E.coli holoenzyme in 125 µl assay mixture containing 12% glycerol, 100 mM Tris-HCl pH 8.0, 0.1 mM EDTA, 0.1 mM DTT, 10 mM $MgCl_2$, 0.12 M KCl, 0.1 mM each ATP, GTP, CTP and 0.125 mM [^{3}H]-UTP (500 cpm/pmole). After various times at 37°C, 10 µl samples were precipitated with 10% TCA-1% sodium pyrophosphate, filtered and counted as described in Ref. (9); (b) with 0.35 µg of purified calf thymus B polymerase in the buffer described in (a), except that 3 mM Mn_2SO_4 replaced $MgCl_2$, 40 mM ammonium sulfate replaced KCl and 40 µl aliquots were precipitated at various times; (c) with 0.3 µg of purified calf thymus A polymerase under the conditions described in (b), but no ammonium sulfate was added.

naked DNA or nucleosomal complexes at approximately 150 mM ionic strength. To study the effect of ionic strength on chain elongation, further initiation was inhibited by the addition of rifampicin, and NaCl was then added to obtain a final ionic strength ranging from 0.1-1 M (for details, see legend to Fig. 11). Fig. 11a plots the incorporation of [^{3}H]-uridine monophosphate (UMP) into acid-precipitable material at increasing NaCl concentrations. RNA synthesis on naked DNA is compared with synthesis on *in vitro*-assembled chromatin containing either the four calf thymus histones or the four histones from butyrate-treated HeLa cells. Fig. 11b expresses the amount of synthesis on the reconstituted complexes as a percentage of that on naked DNA (relative template efficiency). Both plots indicate that acety-

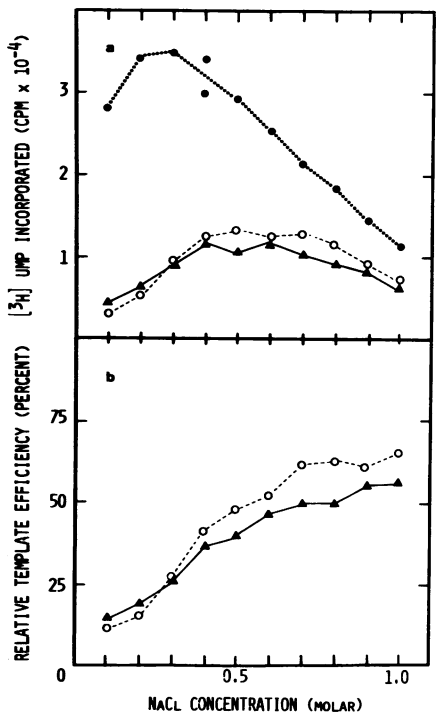

Fig. 11 : Effect of increasing ionic strength on elongation by the E.coli holoenzyme. (a) 0.4 µg SV40 DNA Form I (··· ● ···) or *in vitro*-assembled chromatin (0.4 µg DNA) containing the four histones from calf thymus (— ▲ —) or from butyrate-treated HeLa cells (-- o --) was incubated with 0.24 µg E.coli holoenzyme in 25 µl of the reaction mixture described in the legend to Fig. 10a (except that cold UTP replaced [^{3}H]-UTP). After 10 min at 37°C, an equal volume of the reaction mixture containing in addition [^{3}H]-UTP (final concentration 0.25 mM, 250 cpm/pmole), 20 µg/ml rifampicin, and sufficient NaCl to give the specified final concentrations were added. The incubation was continued for 30 min at 37°C and aliquots were subsequently processed for acid-precipitable counts; (b) the results depicted in (a) are plotted in terms of relative template efficiency; at a given NaCl concentration, the RNA synthesized ([^{3}H]-UMP counts incorporated) on nucleosomal reconstitutes is expressed as a percentage of the RNA synthesis on naked SV40 DNA Form I.

lated and control histones are similar in their ability to inhibit RNA chain elongation at elevated ionic strengths.

DISCUSSION

Histone acetylation has been suggested as the mechanism by which inert chromatin is activated for RNA transcription (22). This hypothesis is an attractive one because a striking correlation has been observed between the enhancement of histone acetylation and the enhancement of RNA synthesis in a number of systems (4). Moreover, Yamamoto and Alberts (23) have proposed that the acetylation-deacetylation reaction can provide the flexibility needed to transduce laterally across an active gene the increased accessibility to RNA polymerase.

n-Butyrate treatment of growing cells produces a dramatic increase in the levels of histone acetylation, providing a system to directly test the effect of this post-synthetic modification on various aspects of chromatin structure and function. Hagopian and coworkers (7) have demonstrated that this heightened degree of acetylation is due to a decrease in the rate of deacetylation rather than to an increase in the rate of acetylation. Thus, it is probable that the sites involved are those which are normally modified *in vivo*.

1) Nuclease digestion studies.

We have digested nuclei from n-butyrate-treated cells in order to study the nuclease sensitivity of chromatin enriched in acetylated histone subspecies. Our data show that the DNA digestion fragments produced by both micrococcal nuclease and DNase I are indistinguishable from those derived from control nuclei. The kinetics of digestion by micrococcal nuclease were the same for both types of nuclei; however, DNase I digested the nuclei from butyrate-treated cells with a 1.5-2 fold more rapid initial rate.

While this work was in progress, Simpson published a study comparing the nuclease sensitivity of nuclei from butyrate-treated HeLa cells (24). He found a ten-fold greater susceptibility to DNase I (measured as initial rate of reaction) for the butyrate-treated samples. We have not been able to reproduce such a large difference under a variety of conditions: 1) cells were treated with 5 mM rather than 10 mM butyrate; 2) cells were labelled with [^{14}C]-thymidine only during the butyrate treatment rather than for 24 additional hours prior to the treatment; 3) nuclei were isolated by Simpson's procedure, which is more rapid and employs divalent cations; 4) nuclei were digested at a 10-fold lower DNase I/chromatin ratio. Thus, we are unable to explain the discrepancy between our results and those of Simpson. Our results are, however, in good agreement with the very recently published report of Vidali and coworkers (25), who observed an approximately 2-fold greater initial rate of digestion for the nuclei of butyrate-treated cells.

Bonner and coworkers (26) have also reported a large increase in the DNase I sensitivity of acetylated chromatin. However,

they studied chromatin which had been chemically acetylated by acetic anhydride. It has been previously demonstrated (27) that this treatment results in the modification of histones which are not modified *in vivo* as well as the acetylation at sites which are not normally acetylated.

Factors other than histone acetylation may be involved in the preferential sensitivity to DNase I characteristic of nuclei from butyrate-treated cells, e.g. non-histone protein differences or H1 modifications. Our experiments comparing the kinetics of digestion of chromatin assembled *in vitro* from SV40 DNA Form I and the 2M salt-extracted core histones indicate that the sensitivity does reside in the core histones and their interaction with DNA. Our experiments also prove that complexes assembled *in vitro* can be used to assay the effect of acetylation on chromatin structure, thus providing a more precisely defined system.

2) Stability studies.

The acetylation of lysines in the amino-terminal portions of histone molecules should loosen their binding to DNA (28). We have sought to determine whether histone acetylation does reduce the stability of nucleosomes by studying the exchange of histone cores onto competing naked DNA. We could observe only a small difference in the salt concentration at which acetylated histone cores versus control histone cores were exchanged (0.05 M NaCl lower for samples from butyrate-treated cells). Similarly, acetylation did not seem to greatly facilitate the sliding of histone cores along naked DNA. In fact, we could observe very little (if any) long-distance sliding even after 500 mM NaCl treatment. Occasionally, molecules displaying some evidence of sliding were observed after salt exposure to 500 mM NaCl; however, it should be noted that this assay would not distinguish sliding from intra-molecular exchange. We have tried without success several different buffers for the sliding assay, including those used for the RNA synthesis studies and for the exchange experiments (see Materials and Methods).

After the ligated complexes were exposed to 500 mM NaCl, it was possible to observe molecules in which the beads of the reconstituted portion of the hybrid appeared as clusters (Fig. 9b).

In contrast, nucleosomes were always well dispersed if the ligated complexes were not salt-treated (Fig. 9a). This finding is consistent with the recent observation (Spadafora, Oudet and Chambon, in preparation) that nucleosomes in H1-depleted chromatin are able to slide together at salt concentrations as low as 150 mM NaCl (see also Fig. 7a for an example at 500 mM NaCl). This clustering response appeared similar whether the *in vitro*-assembled chromatin contained histones from calf thymus, butyrate-treated HeLa cells or control HeLa cells (our unpublished results). Since histone core particles can slide together at 500 mM NaCl, it is somewhat surprising that they do not slide across the ligated naked DNA molecule. One possible explanation is that, if the clustering is a result of hydrophobic interactions between adjacent core particles, there would be nothing to draw core particles across the ligated naked DNA. It is also possible that only one DNA strand was ligated during the preparation of hybrid complexes and that histone core particles cannot slide across a single-stranded nick. However, both strands were ligated under our conditions because the distribution of DNA in monomers versus higher oligomers was the same after electrophoretic analysis in neutral or alkaline conditions (data not shown). Finally, the possibility that ligase is bound to, and thus blocks, the junction between the nucleosomal complex and the naked DNA, is considered unlikely at 500 mM NaCl, because ligase elutes from a phosphocellulose column at approximately 300-400 mM salt.

By two criteria, then, *in vitro*-assembled chromatin enriched in acetylated histones is not characterized by a greatly diminished stability. Experiments were also undertaken to examine the stability of the corresponding cellular chromatins. H1-depleted chromatin was prepared from butyrate-treated and non-treated HeLa cells and subjected to elevated salt concentrations (0.25-0.85 M NaCl) in the presence of competing naked DNA. The exchange of histone cores occurred at approximately the same ionic strength for both preparations. In addition, H1-containing or H1-depleted chromatin was prepared from control and treated cells and was salt-treated (0.3-0.6 M NaCl) in the absence of competitor DNA. Chromatin from the two types of cells showed a

similar tendency to form clusters of beads (as seen in the electron microscope - see above) at a given NaCl concentration (data not shown).

3) Transcription studies.

Although histone acetylation does not seem to greatly affect nucleosome stability, it is still possible that the modified nucleosomes are more accessible to RNA polymerase. However, we have shown that this is not the case for RNA synthesis on chromatin assembled and transcribed *in vitro*. At low ionic strength (0.15 M) the kinetics of transcription by the E.coli holoenzyme and the calf thymus polymerases A and B were not related to the levels of histone acetylation (Fig. 10). Under the conditions employed, the inhibition of polymerase activity is due to a decrease in both initiation and rate of elongation (15); so apparently, acetylated histones are effective inhibitors at both these levels. These results conflict with the finding of Marushige (29) that the acetylation of histones stimulates the rate of chain elongation on chromatin by E.coli RNA polymerase. However, in these studies the histones were chemically acetylated by acetic anhydride and thus the results should be interpreted with caution as discussed above.

The inhibition of RNA chain elongation during the *in vitro* transcription of chromatin by E.coli polymerase can be progressively overcome by increasing the ionic strength during synthesis (120-600 mM NaCl - see Fig. 11). Although no difference was seen at low ionic strength, it is possible that the effect of acetylation on transcription across nucleosomes is revealed only when the ionic interactions between histones and DNA are loosened at the higher salt concentrations. However, it is clear that histone acetylation does not strikingly affect the rate of chain elongation by E.coli RNA polymerase at ionic strengths from 0.1-1.0 M NaCl (Fig. 11). Moreover, the fact that approximately the same plateau level was reached for both acetylated and control chromatin (Fig. 11b) indicates that histone acetylation does not increase the efficiency of RNA chain initiation on chromatin. These results are consitent with the finding that acetylated chromatin does not exhibit a greatly reduced stability at NaCl concentrations within this range.

4) Histone acetylation and active chromatin.

The preferential digestion of acetylated chromatin by DNase I can be correlated with the preferential degradation of actively transcribing sequences by this enzyme (30-33). For example, Garel and coworkers (31) have demonstrated an approximately 5-fold faster digestion of expressed sequences characterized by various rates of transcription. From this correlation it is tempting to conclude that active genes are associated with nucleosomes containing highly acetylated histones. However, our results indicate that chromatin containing these modified histone subspecies is not more accessible to RNA polymerase *in vitro*.

There are at least three ways to interpret these findings. First, it is possible that acetylation is not involved in the mechanism by which nucleosomes are made accessible to RNA polymerase. The fact that both acetylated nucleosomes and active genes are rapidly degraded by DNase I may be just correlative. Second, acetylation may promote RNA synthesis across nucleosomes, but it may not be possible to regenerate this property using our method of reconstitution. Additional factors (H1, non-histone proteins) might be involved in the increased accessibility to RNA polymerase, but not in the heightened susceptibility to DNase I. Third, it is possible that only the most highly acetylated histones are involved in any mechanism to facilitate transcription. Although there is a great increase in the number of acetylated subspecies in butyrate-treated cells, the precentage of the histone cores containing fully acetylated histones may still be very low in our preparation. It may indeed be necessary to have all four core histones in their most acetylated state to obtain the maximum effect. Our assays might not be sensitive enough to detect differences due to such a small number of fully acetylated nucleosomes; in fact, we were able to observe certain small differences in the behavior of nucleosomes from butyrate-treated and control cells (see Figs. 6 and 11). These considerations might also explain why the sensitivity to DNase I digestion exhibited by acetylated nucleosomes was less than that observed for active genes (1.5-2 fold and 5-7 fold). Further exploitation of the butyrate system should allow the isolation of

the most highly acetylated histone species in order to test this hypothesis.

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REFERENCES

1. Kornberg, R.D. (1977) Ann. Rev. Biochem. 40, 931-954.
2. Thomas J.O. (1977) in International Review of Biochemistry, Clark, B.F.C., Ed., Vol. 17 Biochemistry of Nucleic Acids II, University Park Press, Baltimore, Md, USA.
3. Chambon, P. (1978) Cold Spring Harbor Symp. Quant. Biol. 42, 1211-1236.
4. Allfrey, V.G. (1977) in Chromatin and Chromosome Strcuture, Li, H.J. and Eckhardt, R.A., Eds., pp. 167-191, Academic Press, New York.
5. Riggs, M.G., Whittaker, R.G., Neumann, J.R. and Ingram, V.M. (1977) Nature, 268, 463-464.
6. Riggs, M.G., Whittaker, R.G., Neumann, J.R. and Ingram, V.M. (1978) Cold Spring Harbor Symp. Quant. Biol. 42, in press.
7. Hagopian, H.K., Riggs, M.G., Swartz, L.A. and Ingram, V.M. (1977) Cell 12, 855-860.
8. Wilhelm, J., Brison, O., Kédinger, C. and Chambon, P. (1976) J. Virol. 19, 61-81.
9. Brison, O. Kédinger, C. and Wilhelm, J. (1977) J. Virol. 24, 423-435.
10. Ilyin, Y.V., Varshavsky, A.J., Mikelsaav, U.N. and Georgiev, G.P. (1971) Eur. J. Biochem. 22, 235-245.
11. Germond, J.E., Bellard, M., Oudet, P. and Chambon, P. (1976) Nucl. Acids Res. 3, 3173-3192.
12. Panyim, S. and Chalkley, R. (1969) Arch. Biochem. Biophys. 130, 337-346.
13. Bellard, M., Oudet, P., Germond, J.E. and Chambon, P. (1976) Eur. J. Biochem. 70, 543-553.
14. Oudet, P., Gross-Bellard, M. and Chambon, P. (1975) Cell 4, 281-300.
15. Wasylyk, B., Thevenin, G., Oudet, P. and Chambon, P. (1978) J. Mol. Biol., submitted for publication.
16. Gissinger, F. and Chambon, P. (1972) Eur. J. Biochem. 28, 277-282.
17. Kédinger, C. and Chambon, P. (1972) Eur. J. Biochem. 28, 283-290.
18. Compton, J.L., Bellard, M. and Chambon, P. (1976) Proc. Natl. Acad. Sci. USA 73, 4382-4386.
19. Noll, M. (1974) Nucl. Acids Res. 1, 1573-1578.

20. Germond, J.E., Hirt, B., Oudet, P., Gross-Bellard, M. and Chambon, P. (1975) Proc. Natl. Acad. Sci. USA 72, 1843-1847.
21. Camerini-Otero, R.D., Sollner-Webb, B., Simon, R.H., Williamson, P. Zasloff, M. and Felsenfeld, G. (1978) Cold Spring Harbor Symp. Qaunt. Biol. 42, in press.
22. Allfrey, V.G., Faulkner, R. and Mirsky, A.E. (1964) Proc. Natl. Acad. Sci. USA 51, 786-794.
23. Yamamoto, K.R. and Alberts, B.M. (1976) Ann. Rev. Biochem. 43, 721-747.
24. Simpson, R.T. (1978) Cell 13, 691-699.
25. Vidali, G., Boffa, L.C., Bradbury, E.M. and Allfrey, V.G. (1978) Proc. Natl. Acad. Sci. USA 75, 2239-2243.
26. Bonner, J., Wallace, R.B., Sargent, T.D., Murphy, R.F. and Dube, S.K. (1978) Cold Spring Harbor Symp. Quant. Biol. 42, in press.
27. Wong, T.K. and Marushige, K. (1976) Biochemistry 15, 3041-3046.
28. Whitlock, J.P. Jr. and Simpson, R.T. (1977) J. Biol. Chem. 252, 6516-6520.
29. Marushige, K. (1976) Proc. Natl. Acad. Sci. USA; 73, 3937-3941.
30. Weintraub, H. and Groudine, M. (1976) Science 193, 843-856.
31. Garel, A. and Axel, R. (1976) Proc. Natl. Acad. Sci. USA 73, 3966-3970.
32. Bellard, M., Gannon, F. and Chambon, P. (1978) Cold Spring Harbor Symp. Quant. Biol. 42, in press.
33. Garel, A., Zolan, M. and Axel, R. (1977) Proc. Natl. Acad. Sci. USA 74, 4867-4871.