In vitro transcription of chromatin containing histones hyperacetylated in vivo

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Received 8 August 1980

ABSTRACT

The culture of cells in the presence of sodium *n*-butyrate causes an accumulation of histones that are highly acetylated. When chromatin containing these histones was transcribed with *E. coli* RNA polymerase, an increase in the template activity compared to control chromatin was observed. Titration of chromatin with polymerase under both reinitiating and non-reinitiating conditions showed there was no increase in the number of regions available for transcription. Comparison of the kinetics for single and multiple rounds of transcription indicated that the rate of elongation was increased and probably the rate of reinitiation as well. Comparison of the size of transcripts from control and acetylated chromatin showed a small increase in the average size of transcripts from acetylated chromatin.

When transcription was compared using partially purified HeLa polymerase, an increase was also seen. Studies under various ionic conditions showed that control chromatin required a higher salt concentration for optimum activity than did acetylated chromatin. In addition, at the optimum salt concentration for each chromatin, there was very little difference in the transcriptional activity using exogenous HeLa RNA polymerase.

INTRODUCTION

Acetylation of histones is thought to be involved in a modification of the chromatin structure that leads to increased transcriptional capacity for a given region of chromatin (1,2). Physical studies have shown that acetylated histones have a lower affinity for DNA and that acetylation diminishes their capacity to inhibit RNA synthesis *in vitro* (3,4,5). In addition, there have been a number of *in vivo* studies linking increased RNA synthesis with a prior increase in histone acetylation. After partial hepatectomy, the regenerating liver shows a burst of histone acetylation followed by an increase in RNA synthesis (6). The administration of various hormones causes an increase in histone acetylation followed by an increase in RNA synthesis in target tissues (7,8,9).

In systems in which the chromosomes are either transcriptionally active or inactive, there is a positive correlation between histone acetylation and

transcription. In the fly, Planococcus citrii, the maternal, transcriptionally active, chromosomes of the male incorporate about seven times more radioactive acetate than do the heterochromatic paternal set (10). In the multi-nuclear ciliates Stylonychia mytilus and Tetrahymena pyriformis, the transcriptionally active macronucleus has highly acetylated histones, whereas the histones of the micronucleus of Tetrahymena are not acetylated (11,12,13).

When chromatin is separated into active and inactive fractions, the same results are obtained. The active fraction of calf thymus chromatin has histones with an acetyl content about twice that of the inactive fraction (14). Furthermore, when trout testis chromatin was fractionated by the DNAase II, Mg^{2+} procedure (15,16), the Mg^{2+} soluble, transcriptionally active fraction contained most of the acetylated histones (17,18). Furthermore, the active fraction of Drosophila chromatin incorporates radioactive acetate into histones 40% faster than the inactive fraction and digestion by DNAase II releases acetylated histones faster than nonacetylated histones (19).

Studies in which the histones in chromatin were chemically acetylated have shown an increase in the template capacity of the altered chromatin for exogenous RNA polymerase (20,21) as well as an increase in the ease of displacement of the modified histones from chromatin (22). This chromatin also exhibited an increase in the number of initiation sites for E. coli RNA polymerase (23) and had an increased susceptibility for DNAase I but not micrococcal nuclease (24). However, in these studies one can speculate that the histones were modified at a number of sites in addition to the physiological sites and therefore it is possible that the observed effects are artefactual.

Recently a method has been described that produces cells which accumulate highly acetylated histones after exposure to low concentrations of n-butyrate (25,26). This accumulation is due to a specific inhibition of the histone deacetylases (27,28,29). Using this method, a number of workers have shown that chromatin containing these modified histones is more susceptible to DNAase I but not to micrococcal nuclease (30,31,32). In addition, the spacer DNA adjacent to nucleosomes containing acetylated histones is more susceptible to digestion by DNAase I (33).

The above data strongly suggest that histone acetylation is an important step in the activation of regions of the chromatin involved in transcription. An attempt was made to demonstrate this relationship directly. Mathis and coworkers (34) compared the transcriptional capacity of SV40 Form I DNA reconstituted with calf thymus histones or histones from n-butyrate treated HeLa cells. Using either E. coli RNA polymerase or calf thymus RNA polymerase I or

II, these workers were unable to demonstrate any difference in the transcriptional capacity of the two types of chromatin, although they did show that chromatin reconstituted with the acetylated HeLa histones was more sensitive to DNAase I. However, in view of the uncertainties encountered when using reconstituted chromatin (35) their results are not conclusive. The use of chromatin containing histones hyperacetylated in vivo should provide a more direct test of the hypothesis.

Using HeLa cell chromatin containing histones hyperacetylated in vivo and E. coli or HeLa RNA polymerase we have shown an increased rate of transcription.

MATERIALS AND METHODS

Growth of HeLa Cells

HeLa S3 cells were grown as described previously (25). Cells in logarithmic growth at $3.5\text{--}4 \times 10^5/\text{ml}$ were cultured in medium containing 5 mM n-butyrate for 24 hours. Control cells were harvested at a density of $5\text{--}7 \times 10^5/\text{ml}$.

Preparation of Chromatin

All operations were at 4°C. Cells were collected by centrifugation for 15 minutes at 4000xg and washed two times with 20 volumes of 0.25 M sucrose-TKM (50 mM Tris-HCl, pH 7.6 : 25 mM KCl : 5 mM $\text{Mg}(\text{CH}_3(\text{COO})_2$). The cells ($10^8/5\text{ml}$) were lysed in 0.25 M sucrose-TKM : 0.5% Triton-X 100 : 10 μM phenylmethylsulfonylfluoride with 10 strokes of the B pestle of a Dounce homogenizer. Nuclei were collected at 4000xg and washed twice with 0.25 M sucrose-TKM.

Chromatin was prepared from washed nuclei by a modification of the procedure of dePomerai et al. (36). Nuclei were suspended in 1 mM EDTA : 1 mM Tris-HCl, pH 7.6 : 12.5% glycerol ($2 \times 10^9/5\text{ ml}$) and lysed with three strokes of a Potter-Elvehjem homogenizer and centrifuged for 15 minutes at 15,000xg. The gelatinous pellets were swollen overnight in 1 mM Tris-HCl, pH 7.1 (2ml per 10^9 original nuclei) in an ice bath. The swollen chromatin was sheared for 30 seconds at setting 5 of a Sorvall Omni-mix equipped with a micro-cup attachment. Insoluble material was removed by centrifugation for 10 minutes at 3000xg and the DNA concentration in the supernatant determined by A₂₆₀ after correction for light scattering.

Preparation of E. coli RNA Polymerase using Heparin-Agarose Chromatography

Heparin covalently attached to 4% beaded agarose was prepared as described using Bio-Gel A-15m (Bio-Rad, Inc., Richmond, Ca.) (38). The following two buffers were used in the purification of polymerase: buffer G, 50 mM Tris-HCl, pH 7.9 : 10 mM MgCl_2 : 0.2 M KCl : 0.1 mM EDTA : 0.1 mM DTT : 5% glycerol : 130

$\mu\text{g/ml}$ lysozyme : 23 $\mu\text{g/ml}$ phenylmethylsulfonylflouride; and buffer A, 10 mM Tris-HCl, pH 7.9 : 10 mM MgCl_2 : 0.1 mM EDTA : 5% glycerol. RNA polymerase was prepared by a modification of the procedure of Sternbach *et al.* (39). Unless otherwise specified, all operations were at 4°C. All chemicals were reagent grade except ammonium sulfate which was enzyme grade (Schwarz Mann, Orangeburg, N.J.). Buffers were prepared with twice distilled water.

Cells of *E. coli* B grown to 3/4 log phase in enriched media were obtained as a frozen paste from Grain Processing (Muscatine, Iowa). Solubilization of the enzyme was modified from the procedure of Burgess and Jendrisak (40). A volume of buffer G, in milliliters, equal to the weight of the cells, in grams, was used for grinding. After grinding with sodium deoxycholate as described, the temperature was raised to 8-12°C and kept there for 20 minutes. The mixture was then chilled to 4°C and DNAase I (Type DPFF, Worthington Corp., Freehold, N.J.) was added to a concentration of 5 $\mu\text{g/ml}$ and the solution allowed to stand at 4°C until it was no longer viscous. Cell debris were removed by centrifugation for 90 minutes at 170,000xg.

The supernatant was fractionated with ammonium sulfate as described (41) and the 42% precipitate dissolved in 40 ml of buffer A containing 0.2 M NH_4Cl and dialyzed for 18 hours against 5 liters of this buffer. The dialysate was brought to 75 ml with this buffer and centrifuged for 10 minutes at 10,000xg. The supernatant was loaded onto a 2.5 x 10 cm heparin-agarose column and the enzyme eluted as described (39). The fractions containing the bulk of the enzyme were pooled giving a volume of 150 ml. The enzyme was concentrated to 10 ml by ultrafiltration using an Amicon PM10 membrane (Amicon Corp., Lexington, MA). An equal volume of glycerol was added and the enzyme stored at -40°C at a concentration of 0.23 mg/ml. Enzyme prepared by this method had a specific activity of 2040 units/ml as defined by Burgess (41).

Partial Purification of HeLa RNA Polymerase

RNA polymerase was partially purified by an adaptation of the procedures of Weinmann *et al.* (42) and Jaehning *et al.* (43). All operations were at 4°C. Eight liters of cells cultured as above were collected at 4000xg. They were washed twice in buffer HA (0.05 M Tris-HCl, pH 7.8 : 25% v/v glycerol : 5 mM MgCl_2 : 0.1 mM EDTA : 0.5 mM DTT) and then suspended at a concentration of $0.5\text{--}1 \times 10^8/\text{ml}$ in buffer HA + 0.3 M ammonium sulfate. The resulting viscous solution was sonicated 4-6 times in 10 ml aliquots for 15 seconds in ice with 15 second cooling periods using a Branson Model W-185E Sonifier (Heat Systems Ultrasonics, Inc., Plainview, NY) equipped with the microprobe at 65 watts (setting 5).

The suspension was centrifuged for one hour at 260,000xg and the supernatant, in 60 ml aliquots, loaded onto a 4.6 x 14 cm DEAE-Sephacel column equilibrated with buffer HA + 0.3 M ammonium sulfate and 15% glycerol instead of 25%. The enzyme was washed through the column with this buffer and the peak of enzyme activity pooled and mixed with 1 1/2 volumes of saturated ammonium sulfate solution. The precipitate was collected at 140,000xg for 45 minutes and dissolved in 25 ml buffer HB (buffer HA minus MgCl₂ with 15% glycerol) containing 0.05 M ammonium sulfate. After dialyzing 18 hours against 6 liters of this buffer and clarifying at 30,000xg for 30 minutes, the enzyme was loaded onto a 2.5 x 12 cm heparin-agarose column equilibrated with buffer HB + 0.05 M ammonium sulfate. The non-adsorbed material was washed through the column with 100 ml of this buffer followed by 100 ml of buffer HB + 0.1 M ammonium sulfate. The enzyme was eluted with buffer HB + 0.5 M ammonium sulfate. The fractions of peak activity were pooled and concentrated by precipitation with saturated ammonium sulfate as described above and dissolved in 6 ml buffer HB and stored frozen at -70°C. This enzyme had a specific activity of 17.4 units/mg (one unit equals the incorporation of one nanomole of UTP into RNA in 30 minutes at 37°C).

In Vitro Transcription

1. Using E. coli RNA Polymerase

a. With Reinitiation Transcription of chromatin was carried out using enzyme prepared as described above or purchased from Miles Laboratories (Elkhart, Ind.). Incubations were at 37°C in a volume of 250 µl containing 0.4 mM ATP, CTP, and GTP and ³H-UTP (Schwarz Mann), sp. act. 25 µCi/µmole or α-³²P-UTP (Amersham or New England Nuclear), sp. act. 5 µCi/µmole : 0.05 M Tris-HCl, pH 7.8 : 0.15 M NaCl : 1.6 mM MnCl₂ : 5 mM MgCl₂ : 2.5 mM NaF : 0.1 mM EDTA : 0.5 mM DTT. The reactions were terminated by the addition of 1 ml of 10% trichloroacetic acid containing 10 mM sodium pyrophosphate (TCA-PO₄) and 100 µg yeast RNA (Sigma, Grade VI). After standing on ice for 15 minutes, they were filtered on glass fiber filters (Reeve-Angel 934AH) and washed 5 times with 2.5 ml 10% TCA-PO₄ followed by 2 ml 95% ethanol. Filters containing ³H-UTP were further processed by incubating in tightly capped vials for one hour at 70°C with 0.5 ml of 0.1 M NaOH. After cooling, the filters were neutralized by the addition of 0.5 ml of 0.1 M HCl and counted in 10 ml of ACS (Amersham) with an efficiency of 33-42%. Filters containing ³²P-UTP were dried under an infrared lamp for 10 minutes and counted in 10 ml of ACS.

b. Without Reinitiation In those experiments where it was desired to block reinitiation the enzyme and chromatin were incubated at 37°C in 200 µl

of the reaction mix lacking the triphosphates. After 15 minutes, the triphosphates were added along with 10 μg rifampicin and the radioactive triphosphate, $\alpha\text{-}^{32}\text{P}\text{-UTP}$ (5 $\mu\text{Ci}/\mu\text{mole}$), in a volume of 50 μl . The incubation was continued for the appropriate time and terminated and processed as above with yeast RNA as carrier.

2. Using HeLa RNA Polymerase

Transcription of chromatin was done using the partially purified enzyme prepared as described above. The enzyme and chromatin were combined in 100 μl of 0.4 mM ATP, GTP, and CTP : 0.1 mM $\alpha\text{-}^{32}\text{P}\text{-UTP}$ sp. act. 50 $\mu\text{Ci}/\mu\text{mole}$: 50 mM Tris-HCl, pH 7.8 : 0.1 M $(\text{NH}_4)_2\text{SO}_4$: 2 mM MnCl_2 : 0.1 mM EDTA : 0.5 mM DTT and incubated at 37°C for the appropriate time. The reactions were terminated and processed as above without the addition of yeast RNA. In some experiments it was necessary to remove the ammonium sulfate from the enzyme. This was accomplished by dialyzing the enzyme 18 hours against 100 volumes of buffer HB or buffer HB containing the desired salt.

Extraction of RNA

In vitro transcriptions of 2.5 ml were terminated by the addition of SDS to 0.5%, NaCl to 0.2 M and yeast RNA to 200 $\mu\text{g}/\text{ml}$ and the volumes adjusted to 4.0 ml. They were extracted twice with an equal volume of a 1:1 mixture of phenol and chloroform:isoamyl alcohol (24:1) then extracted twice with an equal volume of chloroform:isoamyl alcohol. The aqueous phase was carefully removed and the RNA precipitated by the addition of 2 1/2 volumes of 70% ethanol at -20°C. The precipitated RNA was kept overnight at -40°C then collected at 30,000xg for 30 minutes. The pellet was dissolved in 1 ml 0.2 M NaCl : 0.1 mM EDTA : 10 mM Tris-HCl, pH 7.1, reprecipitated with ethanol and kept at -40°C until needed.

Electrophoresis of RNA

Isolated RNA was denatured in glyoxal and electrophoresed as described (44). The RNA was collected at 30,000xg for 30 minutes and dissolved in 400 μl of 1 M glyoxal : 50% (v/v) $(\text{CH}_3)_2\text{SO}$: 10 mM $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ pH 7.0 and denatured by heating for one hour at 50°C in capped tubes. Hae III digested colicin E1 DNA (gift of Dr. D. Konkel) was treated identically and used as markers. Just prior to electrophoresis the samples were made 0.025% in bromphenol blue.

Electrophoresis was carried out in a 14 x 22 x 0.3 cm 2.5% acrylamide : 0.065% bis-acrylamide : 0.5% agarose slab gel. Samples of 50 μl were loaded and electrophoresed at 45 milliamps constant current for 7 1/2-8 hours. The gel was stained for 15 minutes with acridine orange, 30 $\mu\text{g}/\text{ml}$ in 10 mM PO_4 , pH 7.0, and destained overnight in the dark at 4°C in 10 mM PO_4 , pH 7.0. The gel was photographed at 254 nm using a red filter then dried and exposed

to Kodak XR-2 x-ray film. After processing, the film was scanned with an Ortec Model 4310 densitometer equipped with a green filter.

General Methods

The concentration of DNA in chromatin was estimated by measuring the optical density at 260 nm using the value 22 l/cm-g (45). The amount of DNA and RNA per A₂₆₀ unit of chromatin was determined by the diphenylamine procedure of Burton (46) and the orcinol procedure of Dische and Schwarz (47), respectively, using chicken blood DNA and yeast RNA as standards.

Chromatin was assayed for protein kinase activity as described (48).

RESULTS

The chromatin for our transcription studies, when prepared from butyrate treated cells, showed the expected high degree of acetylation of histones H4 and a lesser degree of modification of histone H3, while histone H1 was not modified, as expected (25,26). Our technique for isolating chromatin did not alter the acetylation of the histones. The method used does not include extensive washing of the chromatin and uses gentle lysis of the nuclei to retain as many of the chromatin components as possible. The gentle shearing used to solubilize the chromatin produces fragments with an average size of 14,000 bp and with 95% larger than 5200 bp. The characteristics of our chromatin (Table I) were obtained by measuring the amount of DNA per OD unit by the diphenylamine method (46) and the amount of RNA by the orcinol procedure (47). Because of the increased acetylation of histones in chromatin from butyrate-treated cells, it is referred to as acetylated chromatin from here on.

Effect of Treatment with n-Butyrate on Template Activity of Chromatin

Chromatin from control cells or from cells treated for 24 hours in 5 mM n-butyrate was transcribed with *E. coli* polymerase for 20 minutes (Table I). Acetylated chromatin was found to have an average increase in template activity of 55%.

To see if the effect could be enhanced, cells were exposed to varying concentrations of butyrate for 24 hours. The maximum increase in template activity was observed with 5 mM butyrate (data not shown). Incubation for longer times in lower levels of butyrate produced a smaller increase in template activity. Cells cultured for up to 96 hours in 3 mM butyrate only achieved an increase of 33%. These observations link the degree of template activity with the degree of histone acetylation (26). All experiments with acetylated chromatin reported here were done with chromatin obtained from cells treated for 24 hours with 5 mM n-butyrate.

Table I

CHARACTERISTICS OF CONTROL AND ACETYLATED CHROMATIN		
	Control	Acetylated
260/280 ^a	1.74 ± 0.02	1.73 ± 0.03
µg DNA/OD ₂₆₀ ^b	22.3 ± 1.0	21.2 ± 1.4
µg RNA/OD ₂₆₀ ^c	24.2 ± 3.1	25.3 ± 2.8
Kinase Activity ^d pMoles/min/OD ₂₆₀	18.4 ± 0.6	18.2 ± 0.3
EFFECT OF TREATMENT WITH 5 mM BUTYRATE ON CHROMATIN TEMPLATE ACTIVITY ^e		
Sample	nMoles UMP Incorporated/OD ₂₆₀	% Increase
Control	6.7 ± 0.8	--
Acetylated	10.4 ± 0.9	55
REVERSAL OF THE INCREASED TEMPLATE ACTIVITY ^f		
Sample	nMoles UMP Incorporated/OD ₂₆₀	% Increase
Control	7.4	--
Acetylated	10.2	38
Reversed	7.4	0

- a. The OD of a 10:1 dilution was read at 260 and 280 nm. The value shown for the ratio is the average of 12 preparations ± the standard deviation.
- b. The DNA concentration was determined by the diphenylamine procedure (46). Each value is the average of 6 determinations ± the standard deviation.
- c. The RNA concentration was determined by the orcinol procedure (47). Each value is the average of 4 determinations ± the standard deviation.
- d. The amount of cAMP independent protein kinase activity was determined as described (48). Each value is the average of 3 determinations ± the S. D.
- e. 0.11 A₂₆₀ units of chromatin from control or butyrate treated cells were incubated with 5 units of *E. coli* RNA polymerase for 20 minutes. The values listed are the average of seven separate preparations ± the S. D.
- f. Cells were grown for 24 hours in 5 mM n-butyrate, then half were washed free of the drug and incubated for one hour in fresh medium. Chromatin was prepared from control, acetylated and reversed cells and 0.11 A₂₆₀ units transcribed for 20 minutes with 5 units of *E. coli* RNA polymerase.

Riggs and co-workers have shown that the acetylation of histones can be reversed by removing the cells from medium containing n-butyrate and culturing them in fresh medium for as little as 30 minutes (26). This is to be expected from the observations by Cousens *et al.* that the inhibition *in vitro* of histone deacetylases by butyrate is reversible (29). To see if the increase in template activity could also be reversed, cells were grown for 24 hours in 5 mM butyrate. The culture was split into two parts and one part continued in the medium containing butyrate. The other part was washed, then cultured for one hour in fresh medium. Chromatin was prepared from control, butyrate treated and "reversed" cells. The template activity of "reversed" cells was the same as untreated cells, while the cells that remained in butyrate showed a 38% increase in activity (Table I).

Characterization of the Transcription Reaction

The reaction was dependent on all four ribonucleoside triphosphates and in the absence of added enzyme showed very little activity (data not shown). It was also sensitive to the drugs rifampicin (40 μ g/ml gives 93% inhibition) and actinomycin D (4 μ g/ml gives 80% inhibition). After subtraction of the endogenous activity, the residual activity with actinomycin D gives the amount of transcription that was RNA directed (50) which amounts to 10% and 13% for acetylated and control chromatin, respectively.

Nature of the Increase in Template Activity

A number of experiments were conducted to determine the cause for the increased template activity of acetylated chromatin. These experiments showed that the major reason was an increase in the rate of transcription with little or no change in the number of regions available for transcription. In addition, a small increase in the average length of the transcripts was observed. It seemed possible that the increased rate of transcription was due to an increase in the rate of elongation and perhaps the rate of reinitiation as well.

Titration with Polymerase To determine if the number of active regions was the same, equal amounts of chromatin were incubated with increasing amounts of polymerase. Figure 1A shows that the amount of enzyme needed to reach saturation for the two chromatins was approximately equal. When 0.11 A₂₆₀ units of chromatin were used for the study, about 5 units of enzyme were needed to reach saturation. This is equivalent to 9.2 molecules of RNA polymerase per 10,000 bp of DNA.

A similar experiment was done with reinitiation blocked by the addition of 40 μ g/ml of rifampicin. Figure 1B shows that saturation was reached with about 2.5 units of polymerase under these conditions, and that approximately

Figure 1. (A). Control (●—●) or acetylated (○—○) chromatin was incubated with increasing amounts of polymerase for 20 minutes as described in Methods using 2.5 μCi ^3H -UTP. The values indicated are the average of two determinations. (B). Control (●—●) or acetylated (○—○) chromatin was incubated for 15 minutes with increasing polymerase in a reaction mix lacking the triphosphates. The reaction was initiated by the addition of the triphosphates, rifampicin and 1.0 μCi α - ^{32}P -UTP. After 20 minutes the reactions were terminated and the amount of incorporation determined.

equal amounts of enzyme were needed to saturate the two chromatins. In repeats of the above two experiments similar results were seen although the actual amount of enzyme needed for saturation varied by about 5 percent between experiments.

Rate of Transcription The overall rate of transcription was compared by transcribing equal amounts of control and acetylated chromatin with saturating levels of *E. coli* RNA polymerase. When different preparations of chromatin were transcribed, similar results were obtained. Most experiments produced curves like that in Figure 2A. Incorporation of UMP increased linearly for at least the first 20 minutes of the reaction. In most experiments, incorporation continued for at least 90 minutes. Occasionally, as in the figure, the rate of incorporation would decrease after 10 or 20 minutes of incubation. When this occurred, both chromatins behaved in the same manner.

Rate of Elongation The rate of elongation was compared by measuring the incorporation of UMP during a single round of transcription as described in Methods. Figure 2B shows that when equal amounts of control and acetylated chromatin were transcribed under non-reinitiating conditions, the incorporation

Figure 2. (A). 0.11 A₂₆₀ units of control (●—●) or acetylated (○—○) chromatin were incubated with 15 units of enzyme for the indicated times using 2.5 μ Ci ³H-UTP. Each point is the average of two determinations. (B). 0.11 A₂₆₀ units of control (●—●) or acetylated (○—○) chromatin were incubated with 15 units of polymerase under non-reinitiating conditions. After initiation of transcription, the reactions were continued for the indicated times with 0.5 μ Ci α -³²P-UTP. Each point is a single determination.

of UMP was faster with acetylated chromatin as template. In addition, incorporation with control chromatin never reached the level of acetylated chromatin, even after long incubation times. This latter result can only be explained by a longer average length of the transcripts.

Transcript Size RNA labeled with ³²P-UTP was isolated from large scale transcription reactions with control or acetylated chromatin. The RNA was fully denatured in glyoxal (44) and electrophoresed in 2.5% acrylamide : 0.5% agarose gels. Hae III restriction fragments of colicin E1 DNA (gift of Dr. D. Konkel) were similarly treated and used as size markers. Figure 3 shows that the transcripts comprise a broad range of sizes with the bulk of the material running between 400 and 1500 bases. In addition, the transcripts from acetylated chromatin contain a higher percentage of larger molecules compared to those from control chromatin. A similar experiment was done in which the isolated transcripts were denatured by boiling in 98% formamide and electrophoresed in 5% acrylamide gels with essentially identical results (data not shown).

Transcription of Chromatin with HeLa Polymerase

To determine if the increased transcriptional activity of acetylated chro-

Figure 3. 2.2 A₂₆₀ units of control (---) or acetylated (—) chromatin were incubated with 50 units of polymerase as described in Methods using 2.0 μ Ci α -³²P-UTP. The RNA was extracted, denatured, electrophoresed, autoradiographed and scanned as described. Hae III digested colicin E1 DNA in a parallel lane provided size markers. The sizes are from D. Konkel (personal communication). BPB indicates the position of the bromphenol blue dye. B indicates the position of the baseline. (A). RNA profile. (B). Percent increase of acetylated transcripts compared to control.

matin detected by *E. coli* RNA polymerase was also detectable by the homologous enzyme, studies were done using a partially purified enzyme fraction. This enzyme was used because of difficulties encountered in purifying the HeLa RNA polymerase beyond the heparin-agarose step.

Time Course of Transcription Figure 4B shows the results of transcribing equal amounts of control and acetylated chromatin with HeLa polymerase for increasing lengths of time. The increased rate of transcription seen with acetylated chromatin and *E. coli* RNA polymerase was also seen when using the homologous enzyme although the effect was less pronounced, only about a 20% increase. Chromatin transcription with HeLa polymerase began to plateau at 60 minutes, similar to the results obtained with DNA and this enzyme, Figure 4A.

Effect of Different Salts on Transcription of Chromatin In an attempt to maximize the difference in transcriptional activity, the concentration effect of two salts was examined.

Figure 4. (A). 0.14 units of HeLa polymerase were incubated with 20 μ g chick DNA in the standard assay (Methods) for the indicated times. Each point is the average of duplicate assays. (B). 0.11 A260 units of control (●—●) or acetylated (○—○) chromatin were incubated with 0.22 units of HeLa polymerase and 1.0 μ Ci α - 32 P-UTP for the indicated times.

Effect of $(\text{NH}_4)_2\text{SO}_4$ Since HeLa polymerase was normally stored in buffer containing ammonium sulfate, the effect of this salt on the transcription of chromatin was tested. As can be seen in Figure 5A, acetylated chromatin displayed a peak of transcriptional activity at a lower salt concentration than did control chromatin. In addition, at the optimal salt concentration for each chromatin, the transcriptional activity was nearly the same. Both chromatins had the same salt optimum when endogenous RNA polymerase activity was tested, Figure 5A, or when *E. coli* RNA polymerase was used with the same transcription conditions, Figure 5B. No increase was observed when the endogenous activity of acetylated chromatin was compared to that of control chromatin although the actual dpm measured in this experiment were so low that a small increase would not have been detected.

Effect of MgCl_2 All of the above studies with HeLa polymerase were done using 2 mM MnCl_2 , the optimum concentration for the transcription of DNA (51). A recent study has suggested that Mg^{2+} can alter the specificity in a eukaryotic *in vitro* transcription system (52). Therefore, transcription of control and acetylated chromatin was carried out at two different MgCl_2 concentrations using HeLa RNA polymerase that had been dialyzed into buffer containing 0.1 M

Figure 5. (A). 0.22 A₂₆₀ units of control (●—●) or acetylated (○—○) chromatin were incubated with 0.15 units of HeLa polymerase and 1.0 μ Ci α -³²P-UTP for 40 minutes with the indicated concentration of ammonium sulfate. 0.22 A₂₆₀ units of control (■—■) or acetylated (□—□) chromatin were also incubated with no added enzyme. (B). 0.22 A₂₆₀ units of control (●—●) or acetylated (○—○) chromatin were incubated with 0.13 units of *E. coli* RNA polymerase for 40 minutes using the HeLa polymerase conditions (Methods).

NaCl (Table II). The conditions for the transcription were as described in Methods except that 0.15 M NaCl replaced the ammonium sulfate and the divalent cations were either 2 mM MnCl₂, 5 mM MgCl₂ or 10 mM MgCl₂. 0.11 A₂₆₀ units of chromatin were incubated with 0.14 units of HeLa polymerase for 30 minutes.

In the standard assay containing Mn²⁺, acetylated chromatin was 21% more active than control chromatin (Table II). When the manganese was replaced by 5 mM MgCl₂, the activity of the acetylated chromatin was 46% greater than control chromatin but when 10 mM MgCl₂ was used it was only 10% more active. These variations were due mostly to changes in the activity of control chromatin while the transcriptional activity of acetylated chromatin remained almost constant. Thus it is clear that the concentration of Mg²⁺ can alter the transcriptional activity of chromatin in this system as well.

DISCUSSION

When acetylated chromatin was transcribed with *E. coli* RNA polymerase an average increase in template activity of 55% was seen relative to control chro-

Table II
EFFECT OF DIVALENT CATIONS ON TRANSCRIPTION OF CHROMATIN BY HeLa POLYMERASE

[X ²⁺]	Control	Acetylated	% Increase
	(S.D.)	(S.D.)	
2 mM Mn ²⁺	23.6 ± 0.5	28.6 ± 0.3	21%
5 mM Mg ²⁺	21.1 ± 0.7	30.8 ± 0.8	46%
10 mM Mg ²⁺	26.3 ± 0.8	28.8 ± 0.3	10%

matin. This increase was readily reversed as expected from the known reversibility of inhibition of the histone deacetylases (29). After washing the butyrate treated cells with fresh medium and incubating in the medium for 1 hour, the histone pattern was indistinguishable from that of control cells. Treatment of the cells with less than 5 mM butyrate for 24 hours caused a smaller increase in the template activity of acetylated chromatin (M. Dobson, unpublished) that correlated well with less extensive acetylation of histones (26). Thus it is clear that the increase in template activity of acetylated chromatin is linked to increased histone acetylation. No acetylation of non-histone chromosomal proteins was observed in control or butyrate treated cells (M. Riggs, unpublished, 53).

The increase in template activity of chromatin acetylated *in vivo* can be caused by a number of factors including:

1. an increase in the number of regions available for transcription;
2. an increase in the rate of elongation; which causes
3. an increase in the average length of the transcripts; and,
4. an increase in the rate of reinitiation.

Any one of these factors or some combination of them can explain the observed increase in template activity.

An increase in the number of transcriptionally active regions was ruled out by titrating equal amounts of control and acetylated chromatin with increasing amounts of *E. coli* RNA polymerase under reinitiating and non-reinitiating conditions. Both experiments showed that the amount of polymerase needed to saturate control or acetylated chromatin was the same. Therefore, there was no increase in the number of regions available for transcription. A similar conclusion was reached by Rubenstein *et al* who compared the pattern of proteins synthesized by control and butyrate treated HTC cells and saw no difference (54).

The difference in the plateau levels seen after titration under non-reini-

tiating conditions suggests that the rate of elongation is increased. Moreover, an increased rate of transcription was observed when the kinetics of incorporation of UMP were measured during a single round of transcription. Because the number of transcriptionally active regions was the same and reinitiation was blocked by rifampicin, the observed increase in the template activity of acetylated chromatin must be due to an increase in the rate of elongation.

Analysis of the average length of transcripts during multiple rounds of transcription also supports the conclusion that the rate of elongation is faster on acetylated chromatin. When transcripts from control and acetylated chromatin were examined by electrophoresis under denaturing conditions, an increase in the percentage of larger molecules transcribed from acetylated chromatin was seen. Like the increased length seen during a single round of transcription, the increase seen during multiple rounds can best be explained by an increased rate of elongation.

The preceding results suggest that the major contribution to the increased template activity of acetylated chromatin is an increase in the rate of elongation, but do not rule out a contribution from an increase in the rate of reinitiation. A comparison of the rate of incorporation of UMP during a single round of transcription with the incorporation during multiple rounds clearly showed that reinitiation was taking place. When the increase in template activity of acetylated chromatin was compared after 5 minutes of transcription under both conditions, a larger increase was seen for multiple rounds of transcription. This can be explained by a magnification of the increased rate of elongation of acetylated chromatin due to the increased number of chains being synthesized under reinitiating conditions, but a contribution from an increased rate of reinitiation cannot be ruled out.

Transcription of control and acetylated chromatin was also compared using partially purified HeLa RNA polymerase. Like transcription with *E. coli* RNA polymerase, the template activity of acetylated chromatin was greater than that of control chromatin. However, only a 20% increase was seen compared to the average 55% increase seen with the bacterial enzyme. Several factors could contribute to the smaller increase with the homologous enzyme. First, the enzyme-to-template ratio for HeLa polymerase was about 40-fold lower than the ratio used for *E. coli* polymerase based on the number of units of enzyme used in the assays. However, this is unlikely to be the cause. When control and acetylated chromatin were transcribed with the same low levels of homologous and bacterial enzyme using the assay conditions for HeLa polymerase, the increase in the template activity of acetylated chromatin using *E. coli* poly-

merase was about twice that seen with HeLa polymerase (compare the differences in incorporation at 0.15 M ammonium sulfate in Figures 5A and 5B).

A second factor is the different ionic conditions used for the prokaryotic and eukaryotic enzymes. This may have had a more pronounced effect on the reaction than the enzyme-to-template ratio since chromatin is sensitive to the ionic environment. Recent work has shown that the concentration of $MgCl_2$ can affect the percentage of 5s rRNA transcripts relative to the total RNA transcripts when using a mammalian RNA polymerase (52). An experiment was done to test the effect of this divalent cation on our transcription reaction with HeLa polymerase, since the possibility existed that this ion could also affect the transcriptional increase seen with acetylated chromatin. A striking difference was seen in the percent increase of the template activity of acetylated chromatin at the two concentrations of $MgCl_2$ used. In 5 mM $MgCl_2$ a 46% increase was seen, while in 10 mM $MgCl_2$ only a 10% increase was observed. This change was due primarily to a significant increase in the template activity of control chromatin at the higher $MgCl_2$ concentration.

These results are similar to those obtained with ammonium sulfate, where control chromatin showed maximal template activity at a higher salt concentration than did acetylated chromatin. The explanation for these salt effects is probably the lowered affinity of acetylated histones for DNA (3). This causes the normal stimulation of the template activity of chromatin by increasing salt (36,55,56) to occur at lower concentrations when acetylated histones are present. Thus acetylated chromatin displays its maximal activity at a lower salt concentration than control chromatin when transcribed with HeLa RNA polymerase.

There are two conclusions that can be drawn from the work presented. First, acetylation of histones in vivo leads to increased transcription of chromatin by *E. coli* RNA polymerase. Acetylation causes the rate of elongation to increase and possibly the rate of reinitiation without an increase in the number of regions available for transcription. The most likely explanation for these observations is a loosening of the nucleosome structure since acetylated histones have a lower affinity for DNA (3). This loosening could then allow an easier passage of the transcription complex along the DNA and prevent premature termination leading to an increase in the average length of the transcripts. It could also allow the DNA to assume a conformation more favorable to initiation.

Second, these results may have some significance in vivo since the increase was also observed using the homologous enzyme. These results are compatible with the suggestion that histone acetylation plays a key role in the regulation of transcription and contradict those of Mathis et al (34). These workers tran-

scribed chromatin reconstituted with normal or acetylated histones and saw no difference. Their conclusions should be viewed with caution, since all reconstitution techniques employed to date fail to produce the native structure when tested by a number of criteria (35). Our results are consistent with the work of many investigators linking increased RNA synthesis with a prior increase in histone acetylation. They also provide some evidence to support the idea that in vivo acetylation of histones is involved in transcriptional regulation.

ACKNOWLEDGMENTS

We wish to thank Dr. D. A. Konkel for the gift of Hae III restricted colicin E1 DNA. This work was supported by grant AML3945 from the NIH.

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