Histone Acetylation Increases the Solubility of Chromatin and Occurs Sequentially over Most of the Chromatin

A NOVEL MODEL FOR THE BIOLOGICAL ROLE OF HISTONE ACETYLATION

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The effect of histone acetylation on chromatin solubility has been studied. Nucleosomes are fairly soluble over a range of Mg\(^{2+}\) and Na\(^{+}\) concentrations. Increasing the amount of Na\(^{+}\) relative to a fixed concentration of Mg\(^{2+}\) leads to increased solubility. Although the effect is not large, acetylated cores are more soluble than control cores, all other factors being equal. Acetylated oligonucleosomes are vastly more soluble than control oligonucleosomes with the greatest differential seen in the presence of Mg\(^{2+}\) at low ionic strengths. Solubility of acetylated oligonucleosomes is favored when the majority of the histones in the nucleosomes are all highly acetylated. Contiguous highly acetylated nucleosomes are enriched in the rapid kinetic form of histone acetylation after short term exposure to butyrate. These observations have been exploited in a fractionation scheme for chromatin based on the levels of hyperacetylation attained after varied times of exposure to sodium butyrate. Essentially all DNA sequences in an unsynchronized cell population are associated with rapidly acetylated histones. Since it is highly unlikely that each cell has its own discrete set of sequences bound to acetylated histone and since the amount of rapidly acetylated histone in a single cell is relatively small (~10% of total histone), we surmise that rapid acetylation migrates throughout much of the chromatin, perhaps in a sequential and highly organized manner. We postulate that the biological role of histone acetylation is to provide a means for sequentially exposing the entire chromatin for surveillance for DNA damage and possibly for recognition of different regions of the DNA by specific (regulatory?) proteins.

Histone acetylation is a metabolically active event that has been postulated to provide an enzymatic mechanism for altering the structure of chromatin (Chahal et al., 1980). Although it appears certain that an altered structure exists for transcribed genes (Franke et al., 1977; Weintraub and Groudine, 1976; McKnight et al., 1977; Wu et al., 1979) and for replicative chromatin (Seale, 1975; Klempauer et al., 1980; Jackson and Chalkley, 1981), it is not clear what role histone acetylation might play in the organization of any specific aspect of chromatin structure.

Several groups have described procedures which result in the fractionation of transcriptionally active chromatin (Gottesfeld and Partington, 1977; Bloom and Anderson, 1978; Levy-Wilson and Dixon, 1979). These fractionations seem to be based upon at least two factors. First, certain transcribed genes appear to be selectively excised from the bulk of the chromatin by nucleases used to shear chromatin (Bellard et al., 1977; Bloom and Anderson, 1978; Senear and Palminter, 1981). Selective cleavage of these genes by nucleases probably results from recognition by the nuclease of some aspect of chromatin structure that is altered in transcribed genes. Second, regions most susceptible to nuclease cleavage are preferentially soluble in the presence of mono- and divalent cations. This may be a consequence of rapid digestion of these chromosomal regions to small sizes which are more soluble than larger, more slowly digested fragments (Campbell and Cotter, 1977; Wittig and Wittig, 1977; Sanders, 1978; Seligny and Poon, 1978).

In addition to containing DNA enriched in certain transcribed sequences, some soluble chromatin fractions contain highly acetylated histones (Sealy and Chalkley, 1978; Davie and Candido, 1978; Levy-Wilson et al., 1979; Nelson et al., 1980). Recently, we observed that, after mild digestion with micrococcal nuclease, nuclei containing hyperacetylated histones release a larger fraction of Mg\(^{2+}\)-soluble chromatin compared to nuclei with normal levels of acetylated histones (Perry and Chalkley, 1981). Our data suggested that the increased yield of Mg\(^{2+}\)-soluble chromatin was the result of the recruitment of oligonucleosomes containing hyperacetylated histones that were located within contiguous nucleosomes. In this report, we present data that confirm our previous conclusions and demonstrate that histone hyperacetylation has a greater effect upon the solubility properties of poly nucleosomes than of mononucleosomes. As a result of these studies, we will present a fractionation scheme that is dependent upon the degree of histone acetylation. This procedure has been used to examine the solubility properties of nucleosomes containing distinct kinetic classes of acetylated histones (Cousens et al., 1979; Covault and Chalkley, 1980) and to characterize the DNA sequences associated with nucleosomes containing acetylated histones. We will propose a model for a role of histone acetylation in an ordered surveillance of the chromosomal material.

EXPERIMENTAL PROCEDURES

Treatment of Cells and Nuclear Isolation—Hepatoma tissue cultures cells were grown and labeled with [\(^{3}H\)]thymidine or with [\(^{14}C\)] thymidine as before (Perry and Chalkley, 1981). Cells were pretreated with cycloheximide (20 \(\mu\)g/ml, Sigma) for 30 min prior to labeling with [\(^{3}H\)]acetate (New England Nuclear, 4 Ci/mmol) and then concentrated to about 8 x 10\(^8\) cells/ml. Labeling was with 1 mCi/ml of [\(^{3}H\)]acetate for 5 min and was terminated by dilution with cold (4 °C) medium and centrifugation. Cell pellets were washed once with cold (4 °C) medium containing 50 mM sodium acetate, 50 mM sodium...
butyrate for subsequent 1-h chases in warm (37 °C) medium containing 50 mM sodium butyrate, 20 mg/ml cycloheximide. Nuclei were isolated as previously described (Perry and Chalkley, 1981). “Hyperacetylated” chromatin was prepared from cells grown in the presence of 50 mM sodium butyrate for 18 h.

**Nucleolus Chromatin (Polyribosomes) and Nucleolus Core Preparation—Isolated nuclei were resuspended at 30 A260 units/ml in 50 mM NaCl, 10 mM 1,4-piperazinediethanesulfonic acid, 10 mM sodium butyrate, 5 mM MgCl2, 1 mM CaCl2, pH 6.8. After warming to 37 °C, nuclei were digested for 5 min with 8 units/ml of micrococcal nuclease (Worthington). The digestion was terminated by adjusting to 10 mM EDTA and dialyzed for 12 h against 10 mM 1,4-piperazinediethanesulfonic acid, 10 mM sodium butyrate, 0.1 mM EDTA, pH 6.8 at 4 °C. After dialysis, the nuclear debris was removed by centrifugation at 5000 × g for 5 min. The supernatants routinely contained 80–90% of the input radioabeled DNA.

To facilitate the preparation of nucleosome cores, H1 was removed from the nuclease chromatin with Dowex 50W-X2 resin (Bio-Rad) in the presence of 0.30 M NaCl (Boland and John, 1973). The H1-depleted chromatin was diluted with one volume of distilled water, adjusted to 5 mM CaCl2, and then digested at 37 °C for 10 min with 5 units/A260 of micrococcal nuclease. The digestion was quenched with 50 mM Tris, pH 7.4, and dialyzed as above.

**Solubility Studies—Nucleosomal preparations labeled with [3H] thymidine and containing hyperacetylated histones were mixed with the corresponding control preparation labeled with [3H] thymidine. 5 M NaCl and 1 mM MgCl2 were used to adjust samples to the indicated NaCl and MgCl2 concentrations. Samples were incubated at 4 °C for 30–60 min, centrifuged at 12,000 × g for 10 min. Aliquots from each supernatant were removed and counted in 5 ml of Ready-solvMP.

**Fractionation of Micrococcal Nuclease-digested Nuclei—** Nuclei were resuspended in digestion buffer as above at 40 A260 units/ml, warmed to 37 °C, and digested with 40 units/ml of miccoccal nuclease for 5 min. The digestion was terminated by adjusting to 5 mM ethylene glycol bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid and dialyzed against 10 mM Tris, 10 mM sodium butyrate, 5 mM MgCl2, 0.1 mM ethylene glycol bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid, pH 7.4, at 4 °C for 12 h. After dialysis, the mixture was centrifuged at 12,000 × g for 10 min. Aliquots from each supernatant were removed and counted in 5 ml of Ready-solvMP.

**Gel Electrophoresis—** Histones were electrophoresed on Triton X-100, 20% acrylamide gels, containing 8 M NaCl, 10 mM Tris, pH 7.4, 0.01% bromphenol blue. Gel Electrophoresis—Histones were electrophoresed on Triton acid/urea slab gels containing 8 M NaCl, 10 mM Tris, pH 7.4, 0.01% bromphenol blue.

The poly(A)-mRNA is usually contaminated with rRNA at this point. The ethanol precipitate was resuspended in 10 mM Tris, pH 7.4, 1% SDS and bound once more to oligo(dT)-cellulose as described above. The final yield of material was approximately 500 μg.

All glassware and plastics were baked for 48 h at 110 °C. All solutions were treated with 0.01% diethylpyrocarbonate for 2 h at 60 °C.

**Synthesis of cDNA Complementary toPoly(A)-containing mRNA—** cDNA was synthesized from 20 μg of HTCC poly(A)-containing mRNA using 40 units of avian myeloblastosis virus reverse transcriptase provided by the University of Iowa DNA core facility. The reaction was performed in 50 μl of 50 mM Tris, pH 7.9, 80 mM KCl, 10 mM MgCl2, 30 mM 2-mercaptoethanol, 200 μM deoxynucleotide triphosphates (dNTPs), each containing 10 μM [γ-32p]dATP and [γ-32p]dTTP. The reaction was incubated at 37 °C for 3 h and passed over a Sephadex G-100
column, and the excluded fractions were pooled. [%25P]DNA was treated with 0.5 M NaOH at 60 °C for 1 h to degrade contaminating mRNA sequences, neutralized with Tris, and then precipitated with 4 volumes of 95% ethanol. The cDNA specific radioactivity was 3 × 10^10 cpm/μg.

**Measurement of DNA Reassociation and RNA:DNA Hybridizations—**For DNA reassociation measurements and hybridizations with [%32P]DNA, DNA samples were brought to 0.05 or 0.50 M PB, 5 mM EDTA, 0.3% SDS. Aliquots were sealed in siliconized capillary tubes, heat-denatured at 105 °C for 5 min, and then incubated at 55 or 70 °C (to maintain criterion for 0.05 and 0.50 M PB, respectively) for the length of time required to achieve the desired value of Cot. All values of Cot were corrected so that they corresponded to equivalent values at 0.12 M PB (Britten et al., 1974). After reassociation, samples were quick-frozen in a solid CO2/ethanol bath and stored at −20 °C. Prior to analysis, samples were thawed and expelled into 1 ml of 0.15 M NaCl, 30 mM sodium acetate, 0.5 mM ZnCl2, pH 4.5. Samples were warmed to 37 °C for 15 min and vortexed, and 200 μl of each sample were removed and placed in a separate tube for measurement of the total input of [%14C]DNA. S1 nuclease (Calbiochem) was added to the remaining 800 μl (0.024 unit of S1/100 μg of DNA) and the incubation was continued for 90 min at 37 °C. All samples were precipitated in the presence of 50 μg of salmon sperm DNA by adjusting to 10% trichloroacetic acid. Precipitated DNA was collected by filtration through glass fiber filters (Whatman, GF/A), rinsed with ethanol, and counted in a 5 ml of Ready-solv. In control experiments, between 97-100% of single-stranded DNA and 0-3% of double-stranded DNA were degraded under these conditions. For RNA-DNA hybridization, nonrepetitive [%14C]DNA and a 1000-fold excess of RNA were suspended in 0.5 M PB, 5 mM EDTA, 0.3% SDS. Sealed aliquots were denatured as above and incubated at 70 °C to the desired RNA Cot. All values of RNA Cot were corrected to the standard salt concentration of 0.12 M PB (Britten et al., 1974). Reactions were terminated as above and stored at −20 °C. The amount of [%14C]DNA in the form of RNA:DNA hybrids and DNA duplexes (total hybrids) was measured by S1 nuclease digestion as above. Additional aliquots at each value of RNA Cot were expelled into 1 ml of 10 mM Tris, pH 7.4, to determine the extent of DNA duplex formation. After removing 200 μl from each sample, RNase A ( Worthington) was added to the remaining 800 μl (2 μg of RNase A/100 μg of RNA) and the samples were allowed to incubate for 18 h at 37 °C to degrade all the RNA (Galau et al., 1974). Control experiments performed with [%3H]RNA:DNA hybrids (synthesized with Escherichia coli RNA polymerase and single-stranded M13 DNA) and with [%3H]DNA duplexes resulted in the degradation of 97-99% of the hybridized RNA and 0-5% of the duplex DNA. After adjusting the same sample to 0.15 M NaCl, 30 mM sodium acetate, 1 mM ZnCl2, pH 4.5, 100 μg of denatured salmon sperm DNA and 0.024 unit of S1 nuclease were added and the samples were incubated at 37 °C for 90 min. Samples were precipitated with trichloroacetic acid and collected onto glass fiber filters as described above. The fraction of S1-resistant DNA after digestion with RNase A was used to subtract the amount of DNA duplexes from the total hybrids to yield the amount of RNA:DNA hybrids.

**RESULTS**

**Histone Acetylation Increases the Solubility of Chromatin—**Since previous results have indicated that oligonucleosomes containing hyperacetylated histones are preferentially soluble in buffers containing MgCl2, we wished to compare the effects of histone acetylation on the solubility of polynucleosomes and of nucleosome cores. Polynucleosomes were isolated after light digestion with micrococcal nuclease to mildly shear the chromatin. Nucleosome cores were prepared from a portion of such chromatin preparations by removing H1 with Dowex 50W-X2 resin (Bolund and John, 1973) and digesting the H1-depleted chromatin with micrococcal nuclease.

Agarose gel electrophoresis of DNA isolated from nucleosome chromatin and from nucleosome cores is shown in Fig. 1A. It is apparent that micrococcal nuclease has efficiently digested the spacer regions within polynucleosomes and has generated nucleosome cores. Triton acid-urea gel electrophoresis of histones from control cores and from cores containing hyperacetylated histones is shown in Fig. 1B. It can be seen that significant levels of hyperacetylated histones are associated with nucleosome cores from butyrate-treated cells; furthermore, both nucleosome core preparations are substantially depleted in H1.

In order to accurately assess the effects of histone acetylation on chromatin solubility, nucleosomal preparations from hyperacetylated cells labeled with [%3H]thymidine were mixed with the corresponding material from control cells labeled with [%3H]thymidine. After adjusting to appropriate NaCl and MgCl2 concentrations, samples were centrifuged to remove insoluble material, and aliquots from each supernatant were removed and counted. The results are presented in Fig. 2 which shows the percentage of radiolabeled nucleosomal material that remains soluble as a function of the MgCl2 concentration at various concentrations of NaCl. These data indicate that, in the absence of MgCl2, nucleosomal material remains quite soluble regardless of the NaCl concentration. At low MgCl2 concentrations, millimolar concentrations of MgCl2 result in an almost quantitative precipitation of control polynucleosomes (Fig. 2A). Nucleosome cores, on the other hand, are more soluble than polynucleosomes under comparable conditions and in the absence of hyperacetylation (Fig. 2B). This is not surprising in light of the observations that oligonucleosomes have a greater tendency to self-associate than do mononucleosomes (Campbell and Cotter, 1977; Seligny and Poon, 1978). The MgCl2-induced aggregation observed at low MgCl2 strengths is decreased at higher MgCl2 strengths and results in an increased solubility of polynucleosomes and nucleosome cores.
dynamic acetylation

The solubility of chromosomal material from nuclei digested control nuclei is only sparingly soluble at very low ionic strengths in the presence of MgCl₂. In contrast, a substantial fraction of material is released under these conditions from nuclei containing hyperacetylated histones. In both cases, the majority of the labeled material is released at somewhat elevated ionic strengths (0.2–0.3 mM NaCl).

Agarose gel electrophoresis of the DNA present in fractions eluted from control nuclei (Fig. 4A) confirms that nucleosomes most susceptible to excision by micrococcal nuclease (and hence smaller than the bulk of the material) are more soluble than the larger chromosomal fragments. Fig. 4B shows the distribution of nucleosomal DNA eluted from hyperacetylated nuclei. It is apparent that the additional material solubilized at low ionic strengths in response to histone hyperacetylation is composed largely of nucleosome oligomers.

The extent of acetylation of histones present in fractions eluted from hyperacetylated nuclei was examined by electrophoresis of acid-soluble proteins on Triton acid-urea gels (Fig. 5). As predicted, the material soluble at low ionic strengths is enriched in highly acetylated histones when compared to the remainder of the chromatin. It is intriguing to note, however, that a significant fraction of hyperacetylated histones is associated with regions that are relatively insoluble at low ionic strengths. Accordingly, we suspect that chromatin containing highly acetylated histones interspersed with less acetylated (or nonacetylated) histones would tend to be less soluble. Interspersion of various levels of acetylated histones might occur within individual nucleosomes; additionally, nucleosomes containing highly acetylated histones may be interspersed among nucleosomes containing unacetylated histones.

The Solubility of Rapidly Hyperacetylated Regions of Chromatin—Previous work has shown that there are distinct

![Fig. 2. The solubility of nucleosomes and polyribosomes as a function of MgCl₂ concentration and ionic strength.](image-url)
kinetic classes of acetylated histones in HTC cells (Cousens et al., 1979; Covault and Chalkley, 1980). A small fraction (about 10%) is rapidly acetylated and is also rapidly deacetylated while the remainder is more slowly acetylated and deacetylated. The physical reason for this heterogeneity is not known. Since the majority of histones which become hyperacetylated after prolonged cell exposure to sodium butyrate (as used in the study above) are contributed from the slowly modified class, we were interested in asking whether rapidly hyperacetylated chromatin has solubility properties similar to those after prolonged cell exposure to sodium butyrate (as used in the study above) are contributed from the slowly modified class, we were interested in asking whether rapidly hyperacetylated histones can be identified with chromatin containing slowly hyperacetylated histones. Rapidly hyperacetylated histones can be identified as those histones modified extensively after a short (1-h) exposure to sodium butyrate (Cousens et al., 1979; Covault and Chalkley, 1980).

Cells were prelabeled with [14C]thymidine for one generation prior to treatment with 50 mM sodium butyrate for 1 h. Nuclease-digested nuclei were eluted with increasing concentrations of NaCl as described above. The solubilization of [14C]-labeled chromatin fractions with increasing NaCl is shown in Fig. 6. Nuclei from cells exposed to sodium butyrate for 1 h show a modest increase (about 10%) in the amount of material which is soluble at less than 100 mM ionic strength compared to control cell nuclei. This slight increase is not nearly as substantial as that observed with massively hyperacetylated nuclei (Fig. 3) but it is comparable to the amount of rapidly acetylated histones in these cells. Electrophoretic analysis of the DNA present in these fractions shows that the brief exposure to sodium butyrate resulted in a modest, but significant recruitment of oligonucleosomes into the soluble fraction (Fig. 7, especially in the lanes representing 0-100 mM NaCl).

Acid-soluble proteins from these fractions were examined by Triton acid-urea gel electrophoresis (Fig. 8). It is apparent that a large number of non-histone proteins is solubilized at low ionic strengths. Since some of these proteins migrate with mobilities similar to acetylated forms of the histones, it is difficult to state with certainty whether these fractions are enriched in rapidly acetylated histones as judged by the distribution of stained protein. To avoid this problem, histones were labeled with [3H]acetate during a 5-min pulse and detected by fluorography. The fluorographs are also shown in Fig. 8 with the corresponding stained gels. Fractionated chromatin from control cells pulse labeled with [3H]acetate contains a fairly even distribution of [3H]acetate-labeled histones. There is a slight enrichment of labeled histones in material extracted at low ionic strengths. The label is skewed slightly toward the more highly acetylated forms of the histones which suggests that the enrichment of labeled histones is due to the preferential solubility of mononucleosomes containing acetylated histones. This is seen most clearly with H4 which is resolved better in this gel system than are the other histones.

In marked contrast to the results with nuclei from control pulse-labeled cells, there is a decidedly asymmetric distribution of the [3H]acetate label within fractionated chromatin from cells which were chased for 1 h with sodium butyrate after the 5-min pulse with [3H]acetate. In addition to an increased amount of labeled histones present in the chromatin which is soluble at low ionic strengths, the distribution of the label has been shifted dramatically toward the most highly acetylated forms of histones.

The specific activity of H4 in each chromatin fraction was calculated and the results are presented in Fig. 9. While measurement of the H4 mass present in fractions soluble at low ionic strengths is somewhat imprecise due to the presence of non-histone proteins, it is nonetheless clear that an impressive recruitment of [3H]acetate-labeled histone has resulted from the brief exposure of cells to sodium butyrate. There is a slight increase (about 10%) in the specific activity of whole histone labeled with [3H]acetate and chased with sodium butyrate compared to the specific activity of whole histones immediately following the [3H]acetate-labeling period. While this increase is within the limits of detection, it is insufficient
FIG. 5. Electrophoretic analysis of acid-soluble proteins in chromatin fractions sequentially extracted from hyperacetylated nuclei. Chromatin fractions eluted from hyperacetylated nuclei were extracted with 0.4 N H$_2$SO$_4$, and the soluble proteins were electrophoresed on a Triton acid/urea slab gel.

FIG. 6. Sequential elution of nuclease-digested nuclei from control HTC cells and from HTC cells treated with sodium butyrate for 1 h. The release of [³⁰Cl]thymidine-labeled chromatin from control nuclei (○) and from nuclei containing rapidly hyperacetylated histones (■) was measured at successive NaCl concentrations.

Dynamic Acetylation

H2A

H1

H3

H2 B

H4

Whole 100 200 300 400 Pellet

[NaCl] (mM)

to account for the increased specific activity of acetylated nucleosomes soluble at low ionic strengths after a 1-h butyrate treatment. It is conservatively estimated that there is a 7-fold increase in the specific activity of H4 in the first supernatant fraction compared to unfractionated H4. These results demonstrate that most of the histones which incorporate [³⁰H] acetate during a short pulse without butyrate treatment are associated with chromosomal regions that have solubility properties characteristic of bulk chromatin. Furthermore, these chromosomal regions are not selectively excised by micrococcal nuclease since the fractions that are enriched in mononucleosomes are not substantially enriched in labeled histones. Upon rapid hyperacetylation, these regions of chromatin become significantly more soluble, allowing them to be readily fractionated from the majority of the chromatin.

The Kinetic Complexity of DNA Associated with Rapidly and Slowly Acetylated Regions of Chromatin—Since this fractionation procedure results in the separation of soluble chromatin containing highly acetylated histones from the bulk of the chromatin, it was of great interest to examine the kinetic complexity of the DNA found in the soluble fractions. For example, if rapidly hyperacetylated histones, which constitute 10% of the histone mass, are associated with DNA sequences that contain only 10% of the total DNA sequence complexity (rather than being associated with 10% of the DNA containing 100% of the complexity), then the rate of reassociation of these sequences should be 10 times faster than that of whole HTC DNA. This degree of acceleration in the DNA reassociation kinetics corresponds to a shift in the
Alternatively, if rapidly hyperacetylated histones are associated with random DNA sequences, then the reassociation kinetics of these DNA sequences should be identical with those previously described for whole HTC DNA.

It is important to emphasize the use of cycloheximide to inhibit histone synthesis during these experiments. Newly synthesized histones are acetylated prior to their deposition onto DNA. If the deposition occurs at random with respect to DNA sequences, then it may generate soluble chromatin upon fractionation and thus obscure the kinetic complexity measurements of DNA associated with acetylated, pre-existing histones.

Since we were interested in both classes of acetylated histones (the slowly as well as the rapidly acetylated histones), both sets of DNA sequences were examined as well as those sequences that are present in the soluble chromatin fraction prior to cell exposure to sodium butyrate. HTC cells were prelabeled with [³H]thymidine, incubated with cycloheximide, and then treated with 10 mM sodium butyrate for 0, 1, or 18 h. Nuclei were prepared from these cells, resuspended in buffer A containing 75 mM NaCl, and digested with micrococcal nuclease. This concentration of NaCl was chosen since it results in a substantial yield of highly acetylated chromatin under conditions in which the bulk of the material remains insoluble (Figs. 4 and 7). Nucleosomal material released from nuclei under these conditions was collected as soluble chromatin and the DNA was purified. The yield of DNA from these fractions constituted 5, 13, and 35% of the total DNA present in nuclei from cells treated with sodium butyrate for 0, 1, or 18 h, respectively. Agarose gel electrophoresis of the DNA within each fraction revealed primarily the presence of mononucleosomes in the soluble fraction from control cell nuclei and increasing amounts of oligonucleosomes in the soluble fraction from cells exposed to sodium butyrate for 1 and 18 h (data not shown).

The kinetic complexity of the DNA present in each fraction was measured by observing the rates of reassociation. These data are shown in Fig. 10. The data points represent the average of two determinations at each value of Cot for the three DNA preparations while the solid curve represents the reassociation of whole HTC DNA. This curve also appears to reflect the reassociation of the DNA found in soluble chromatin fractions after varying length of cell exposure to sodium butyrate. A computer-assisted analysis of the kinetics of reassociation of the nonrepetitive sequences in each fraction reveals that the corresponding rate constants vary from each other by no more than a factor of 3. Since the apparent rate of DNA reassociation is proportional to the square root of the DNA length, the rate constant should be corrected to account for the variations in the average DNA size (Wetmur and Davidson, 1968). When the appropriate corrections are made, the range in variation of the reassociation rate constants decreases from 3 to about 2 (Table I). Reassociation rate constant measurements are generally inadequate to reliably quantitate kinetic complexities that differ by less than a factor of 2; therefore, it must be concluded that the kinetic complexities of the DNA sequences associated with highly acetylated histones do not differ significantly from that observed for whole HTC DNA.

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The Association of Transcribed Gene Sequences with Acetylated Histones—Although there are no detectable differences in the overall kinetic complexity of these DNA sequences, it is still possible that they might contain a significant enrichment in transcribed genes since these sequences constitute only a fraction of the total sequence complexity. Accordingly, histone acetylation might take place rapidly on one or a few nucleosomes immediately preceding an RNA polymerase. After passage of the polymerase, the acetylated histones would be rapidly deacetylated, returning the nucleosomes to their normal levels of modification. A prediction of this model is that an inhibition of histone deacetylation should result in
Dynamic Acetylation

Fig. 8. Electrophoretic and fluorographic analysis of acid-soluble proteins present in solubilized chromatin fractions from control nuclei and from nuclei containing rapidly hyperacetylated histones. Acid-soluble proteins were extracted from chromatin fractions eluted in Fig. 6. After electrophoresis on a Triton acid/urea slab gel and staining with Coomassie blue, histones which had been labeled during a 5-min [3H]acetate pulse (control cells) and then exposed to sodium butyrate for 1 h (rapidly hyperacetylated cells) were detected by fluorography. W, whole; P, pellet.

Fig. 9. Specific activity of H4 labeled with [3H]acetate in chromatin fractions from control nuclei and from rapidly hyperacetylated nuclei. The H4 regions of the gels and fluorographs in Fig. 8 were scanned. Area measurements were made to calculate the specific activity of [3H]acetate-labeled H4 in each chromatin fraction and were normalized to the specific activity of H4 in unfracti-onated nuclei. ○, control nuclei; □, rapidly hyperacetylated nuclei. P, pellet.

high levels of histone acetylation within transcribed chromatin. If a sufficiently long period of inhibition of histone deacetylation is allowed, then all transcribed sequences should contain hyperacetylated histones.

This hypothesis was tested by hybridizing 32P-labeled cDNA, complementary to poly(A)-containing mRNA sequences, to a vast excess (8 × 104:1) of DNA isolated from the soluble chromatin fractions described above. In addition, [32P] cDNA was hybridized to a similar excess of total HTC DNA. The results of these hybridizations are shown in Fig. 11. As before, the data points represent the averages of two determinations and the solid curve describes the computer-assisted fit for the hybridization of whole HTC DNA with the [32P] cDNA tracer. Surprisingly, the data obtained with DNA isolated from fractionated chromatin containing rapidly acetylated histones (from cells after 1 h of exposure to sodium
butyrate) fall precisely along the same curve. In fact, the hybridization rates of the cDNA to each of the four DNA preparations are indistinguishable from one another (i.e., they differ from one another by less than a factor of 2; Table I). These results strongly suggest that the sequences which code for poly(A)-containing mRNA are not preferentially associated with acetylated histones.

In those cases for which it has been examined, only a small fraction of the single copy sequences represented in the nuclear RNA of a given cell type or tissue is also represented in its mRNA (Davidson and Britten, 1979). Also, the cDNA population reflects the more abundant mRNA species. In addition, only 20% of the nuclear RNA mass from most mammalian cells contains poly(A) (Greenberg and Perry, 1972; Lewis and Pennman, 1977). The possibility exists, therefore, that the cDNA probe may not accurately reflect the total transcriptional activity of HTC cells. In order to gauge more precisely the concentration of transcribed genes within chromatin fractions containing acetylated histones, it is necessary to perform saturation hybridizations with nuclear RNA. This approach has an additional advantage of increased sensitivity, relative to hybridization kinetics, since it depends upon the final extent of hybridization rather than the rate with which the hybridization occurs. Thus, a 2-fold increase in transcribed sequences within a particular fraction would double the extent of hybridization of [3H]DNA to hnRNA from about 12 to 24%.

In pursuit of this approach, cells containing DNA labeled in vivo to a specific activity of $1 \times 10^7$ cpm/µg were treated with cycloheximide followed by exposure to sodium butyrate for 0, 1, or 22 h. Nuclei were isolated from these cells and digested briefly with micrococcal nuclease, and the nucleosomal material that was soluble in 75 mM NaCl, 5 mM MgCl$_2$ was collected. The DNA in each soluble fraction was isolated and then reassociated as described under "Experimental Procedures." The curve represents the reassociation kinetics of sheared whole HTC cell DNA. , , and * represent the reassociation of DNA present in the soluble chromatin fractions after exposure of HTC cells to sodium butyrate for 0, 1, or 18 h, respectively.

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**Table 1**

| DNA preparation             | Hybridization characteristics of HTC DNA from soluble chromatin fractions |
|----------------------------|--------------------------------------------------------------------------|
|                            | DNA reassociation parameters                                            | Hybridization rates of nonrepetitive DNA to each of the four DNA     |
|                            | Fraction highly repetitive ± S.E.                                         | preparations, it is necessary to perform saturation hybridizations   |
|                            | 0.10 ± 0.02                                                              | with nuclear RNA. This approach has an additional advantage of         |
|                            | 0.12 ± 0.02                                                              | increased sensitivity, relative to hybridization kinetics, since it    |
|                            | 0.14 ± 0.02                                                              | depends upon the final extent of hybridization rather than the rate   |
|                            | 0.13 ± 0.02                                                              | with which the hybridization occurs. Thus, a 2-fold increase           |
|                            | Hybridization parameters                                                  | in transcribed sequences within a particular fraction would          |
|                            | Fraction nonrepetitive ± S.E.                                             | double the extent of hybridization of [3H]DNA to hnRNA                  |
|                            | 0.20 ± 0.11                                                              | from about 12 to 24%.                                                 |
|                            | 0.70 ± 0.02                                                              | In pursuit of this approach, cells containing DNA labeled              |
|                            | 0.71 ± 0.02                                                              | in vivo to a specific activity of $1 \times 10^7$ cpm/µg were treated  |
|                            | 0.02 ± 0.07                                                              | with cycloheximide followed by exposure to sodium butyrate for 0, 1,  |
|                            | 0.72 ± 0.07                                                              | or 22 h. Nuclei were isolated from these cells and digested briefly   |
|                            | 0.74 ± 0.02                                                              | with micrococcal nuclease in buffer A containing 75 mM NaCl, and the  |
|                            | Hybridization with cDNA tracer                                           | Mg$^{2+}$-soluble fractions were collected. The DNA in each soluble   |
|                            | $K_{observed}$ ± S.E. (s$^{-1}$)                                           | fraction was purified and nonrepetitive sequences were isolated by     |
|                            | 6.0 ± 1.4 × 10$^{-4}$                                                    | reassociating the DNA to a Cot of 500 s$^{-1}$ followed by hydroxyapatite |
|                            | 3.8 ± 0.4 × 10$^{-4}$                                                    | chromatography. Since the sequence complexity of nuclear RNA in       |
|                            | 8.0 ± 0.1 × 10$^{-4}$                                                    | HTC cells does not change upon histone hyperacetylation,2 the        |
|                            | 4.0 ± 0.1 × 10$^{-4}$                                                    | nonrepetitive [3H]DNA preparations were hybridized to a 1000-fold     |
|                            | Hybridization of nonrepetitive DNA with excess nuclear RNA                | excess of nuclear RNA isolated from HTC cells containing normal levels   |
|                            | $\%$ nonrepetitive DNA hybridized at saturation ± S.E.                    | of acetylated histones. The formation of RNA:DNA hybrids was           |
|                            | 13.5 ± 0.9                                                               | measured as previously described and plotted as a function of RNA Cot.  |
|                            | 12.4 ± 0.9                                                               | Fig. 12 shows the results of hybridization of nuclear RNA with non     |
|                            | 11.4 ± 0.6                                                               | repetitive [3H]DNA isolated from whole HTC DNA and from soluble        |
|                            | 11.9 ± 1.2                                                               | chromatin fractions after exposure of HTC cells to sodium butyrate for |

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1. J. Couvault, M. Perry, and R. Chalkley, submitted for publication.

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fractionated DNA sequences is presented in Table 1. Taken as a whole, these data clearly demonstrate that the DNA sequences associated with either kinetic class of acetylated histones are not measurably different from the sequences found in whole HTC DNA. This may be taken as strong evidence that the bulk of histone acetylation occurs without regard to either DNA sequence or transcriptional activity.

**DISCUSSION**

Various chromatin fractionation schemes have been used previously to achieve an enrichment for transcribed genes. Such fractionation procedures generally result in a parallel enrichment of acetylated histones as well. In an attempt to understand the basis for these fractionations, we have examined the role that histone acetylation plays in the solubility of chromatin. It was previously demonstrated that digestion by micrococcal nuclease shows no specificity for chromatin containing bulk hyperacetylated histones (Perry and Chalkley, 1981). Therefore, apparent differences in solubility between hyperacetylated and control chromatin cannot be explained by postulating that acetylated nucleosomes have been preferentially excised. The increase in solubility of nucleosome cores upon hyperacetylation of their histones presents a strong argument that histone acetylation, rather than some other effect of sodium butyrate, directly increases the solubility of chromatin.

It is known that cations induce chromatin condensation (Olins and Olins, 1972; Billet and Barry, 1974; Thoma et al., 1979) and may ultimately result in its precipitation. This is thought to result from neutralization of excess negative charges present on DNA in chromatin. Since extensive histone acetylation increases the number of negative charges present in chromatin, it seems reasonable to suggest that this results directly in the increased chromatin solubility that we observe upon histone hyperacetylation. The reduction in the partial charge neutralization of chromatin might increase the persistence length of supercoiled chromatin by disrupting intimate contacts between adjacent turns of a supercoil, resulting in a local unwinding of condensed superhelices (Finch and Klug, 1976; Thoma et al., 1979). This could increase the availability of the DNA within supercoiled nucleosomes without altering the accessibility of internucleosomal DNA and would explain why DNase I, but not micrococcal nuclease, preferentially excises chromatin containing hyperacetylated histones. This last point is also consistent with the observations that, as viewed by solubility effects, the interactions between individual nucleosomes are much less critically affected by histone acetylation than the interactions between intact chains of polynucleosomes.

As a result of the solubility studies, we conclude that fractionation procedures which exploit the solubilization of chromatin with increasing ionic strengths should effect a separation of chromatin containing various levels of acetylated histones. The procedure used was initially described by Sanders (1978) and we find that it yields soluble chromatin fractions enriched in hyperacetylated histones. These histones are present on oligomeric nucleosomes, suggesting that these highly acetylated histones are located on contiguous nucleosomes.

The observation that chromatin containing rapidly hyperacetylated histones becomes increasingly soluble after exposure of cells to sodium butyrate for 1 h is in agreement with previous observations (Nelson et al., 1980). Since rapid hyperacetylation is complete after exposure to butyrate for 1 h (Covault and Chalkley, 1980) and since the vast majority of labeled histones that become rapidly hyperacetylated appears to be recruited into the soluble fraction concomitantly with
oligomeric nucleosomal DNA, it suggests that most, if not all, of the histones available for rapid hyperacetylation in these cells are clustered on nucleosomes that are contiguous to some extent. It is not clear whether these nucleosomes are located within one large domain (comprising about 10% of the genome) or several smaller ones. After a 1-h exposure to butyrate, approximately 50% of all the DNA in the soluble fraction is associated with rapidly hyperacetylated histones and after 18 h of butyrate treatment ~80% of the soluble DNA is associated with hyperacetylated histones (the majority of which are of the slower kinetic form).

Analysis of the kinetic complexities of the DNA sequences associated with rapidly acetylated histones after a short (1-h) term and with both rapidly and slowly acetylated histones after a long (18-22-h) term exposure of cells to sodium butyrate has shown that in both cases they contain most, if not all, of the kinetic complexity of whole HTC cell DNA. Moreover, we have provided evidence that histone acetylation does not occur selectively on DNA sequences that code for either the majority of the mass of poly(A)-containing RNA or the bulk of the complex array of nuclear RNA.

These observations are surprising for two reasons. First, there is the widely held belief that histone acetylation is intimately involved in transcription. However, the data upon which these beliefs were based were primarily correlative in nature; there is no conclusive evidence demonstrating a direct involvement of histone acetylation with transcription. The second reason for surprise lies in the observation that there is no apparent enrichment for transcribed sequences in the soluble fraction, which contains primarily mononucleosomes, from control (i.e. not hyperacetylated) HTC cells. It has been shown previously that the globin gene in chicken reticulocytes and the ovalbumin gene in hen oviducts are preferentially digested with micrococcal nuclease to monomer-sized DNA fragments (Bellard et al., 1978; Bloom and Anderson, 1978; Senear and Palmiter, 1981). However, these represent highly differentiated tissues containing superabundant mRNAs (about 1 x 10^6 copies of each sequence/cell) that direct the synthesis of one or a few proteins (Palmiter, 1972; Hunt, 1974; Axel et al., 1976) and their response to micrococcal nuclease digestion may be atypical. Superabundant mRNA species are clearly specific to particular states of differentiation and, as Davidson and Britten (1979) have pointed out, such mRNA species are not evident in all animal cells and sometimes account for only a small portion of the mRNA mass (although this is clearly not the case with transcripts of the micrococcal nuclease-sensitive genes studied above).

Although there is no electron microscopic evidence relating the structure of the ovalbumin and globin genes in chromatin to their transcriptional activities, such studies on ribosomal gene expression have shown a close correlation between changing patterns of gene expression and alteration in the chromosomal conformation of these genes. These data are consistent with the concept that frequently transcribed genes (ribosomal as well as nonribosomal) lack a particular structure (as viewed with the electron microscope) and are preferentially cleaved from the bulk of the chromatin by micrococcal nuclease.

In order to explain our results, we suggest that HTC cells do not synthesize large quantities of superabundant transcripts that represent a large mass fraction of either the poly(A)-containing mRNA or the nuclear RNA. In this respect, HTC cells would more closely resemble HeLa cells, mouse 3T6 cells, and mouse L cells, as well as a number of other permanent cell lines, in which the concentration of the most abundant mRNA is about 100 times less than that of the superabundant mRNA species found in more highly differentiated cells (Williams and Penman, 1975; Affara et al., 1977; Paterson and Bishop, 1977). In the presumed absence of large quantities of superabundant transcripts, it appears unlikely that the preferential digestion of one or a few very frequently transcribed genes could be detected, except with the aid of a specific gene probe. It would follow, then, that the vast majority of transcribed sequences in HTC cells is neither frequently transcribed nor preferentially excised by micrococcal nuclease.

The weight of the evidence presented in this report leads to the conclusion that histone acetylation is not directly involved in the transcription of chromatin. The data do not exclude the possibility that the transcription of a relatively small number of genes (those transcribed frequently?) might operate through a mechanism directly involving histone acetylation. Therefore, one might ask what role does the vast majority of histone acetylation play, if not in transcription?

The large expenditure of energy (at least 2 x 10^6 equivalents of ATP/min) that is imposed by the amount of rapid histone acetylation and deacetylation that occurs in HTC cells may well be a requirement for cells to constantly "survey" their genomes. Rapid histone acetylation and deacetylation are coupled processes that appear to occur on contiguous nucleosomal arrays throughout the genome. Although only a fraction of the total histone mass is actually acetylated and deacetylated at any one instant, the data demonstrate that in fact most, if not all, of the genome is available for modification. Two possibilities would appear to fit these observations. Either individual cells have distinct and separate sets of acetylated nucleosomes, or all cells have acetylated histones associated with all DNA sequences over a finite time period. The latter explanation seems the more plausible. Thus, we conclude that histone acetylation and deacetylation are not confined to a specific subset of nucleosomes; instead, they appear to be propagated with time over all the chromosomal material. This would explain why the DNA found with acetylated histones contains the entire sequence complexity of whole DNA, rather than a discrete subtraction. Since the major discernible effect of histone acetylation is to reduce nucleosome oligomer interactions (decreased insolubility), we may surmise that the regions which are being acetylated also become less compact. A wave of local extension and subsequent condensation of nucleosome clusters might then ensue from a continuous activity of histone acetylation and deacetylation. This could result in a state of increased accessibility for a given array of acetylated nucleosomes. The possibility of sequentially exposing the entire genome (a dynamic concept of chromosome structure) offers significant advantages to a cell which are not available in the more static concepts of the chromosome developed thus far. In particular, it enables the entire sequence complexity of the genome to be surveyed on a frequent and continuous basis. Such surveillance of the genome might be necessary to expose damaged regions of DNA to proteins (or other factors) involved in DNA repair as well as provide a dynamic state of fluidity that might facilitate, either directly or indirectly, a number of other critical nuclear functions (such as DNA-regulatory protein interaction) and as such might play a role in maintaining transcriptional competence without being directly involved in the transcriptional process itself. Although this model might provide a means for making a given promoter available on a relatively frequent basis, clearly other mechanisms are necessary to maintain the

3 At the time that this manuscript was submitted, we have observed only minimal enrichment (<2.5-fold) of β-globin genes from induced Friend cells and neither enrichment nor depletion of the genetically inactive tandem repeats of the 93-base pair repeat family in HTC cells.
availability of frequently transcribed genes. An appealing aspect of this model is that it does not require pre-existing determinants to mark nucleosomes that need to be acetylated in order to expedite a given nuclear event since all of the nucleosomes should be rapidly acetylated and deacetylated within a relatively short period of time.

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M Perry and R Chalkley

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