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Structure of Transcriptionally Active and Inactive Nucleosomes from Butyrate-Treated and Control HeLa Cells†

Patricia A. Egan and Beatriz Levy-Wilson*

ABSTRACT: Nuclei from butyrate-treated or control HeLa cells were separated into micrococcal nuclease sensitive and resistant chromatin. Those regions most sensitive to the nuclease, amounting to some 10% of the chromatin, consisted mainly of mononucleosomes with equimolar amounts of the inner histones H2A, H2B, H3, and H4, very little H1, and equimolar amounts of the two small high-mobility group (HMG) proteins, HMG-14 and -17. Both in butyrate-treated and in control cells, these nuclease sensitive monomers were some 5–7-fold enriched in DNA sequences which are transcribed into cytoplasmic polyadenylated RNA, while resistant monomers are depleted in the same sequences. Electrophoretic analyses of the transcriptionally active mononucleosomes revealed heterogeneity. Several subcomponents were resolved when monomers of butyrate-treated or control cells were electrophoresed at low ionic strength. Active monomer subcomponents differ in their molar content of HMG-14 and -17, in their content of H1 and A24, and in the length of their DNA. Some minor differences between nucleosomes of butyrate-treated and control cells were observed.

Sodium butyrate, at millimolar concentrations, promotes a number of morphological and biochemical changes in a variety of mammalian cell lines without causing cell death. These changes include growth inhibition and morphological alterations in HeLa cells, Chinese hamster ovary (CHO) cells, and neuroblastoma cells (Ginsberg et al., 1973; Wright, 1973; Henneberry & Fishman, 1976), increases in tyrosine hydroxylase (Prasad & Sinha, 1976) and adenylate cyclase activity in neuroblastoma cells, and induction of erythroid differentiation in erythroleukemic cells (Leder & Leder, 1975). Sodium butyrate also induces the hyperacetylation of histones, especially H3 and H4. This effect results from the inhibition of the enzyme histone deacetylase (Candido et al., 1978; Vidali et al., 1978; Sealy & Chalkley, 1978). However, this is reversed upon the removal of sodium butyrate, and the cells regain their normal morphology, and their histones return to the nonacetylated state (Vidali et al., 1978).

Recent work by Reeves & Cserjesi (1979) indicates that sodium butyrate affects the patterns of gene expression in cultured Friend erythroleukemic cells. Thus, 24 h after treatment of these cells with sodium butyrate, not only do the histones become hyperacetylated but also there occurs an induction of the expression of 3800 new unique gene products not found in control cells. In addition, two-dimensional gel electrophoretic analysis of the butyrate-treated cells shows that these cells synthesize many new species of proteins that are undetectable in control cells. These changes are reversed once the fatty acid is removed. These finding suggest that histone hyperacetylation may be involved in the biochemical mechanisms promoting new genomic expression in these cells.

Motivated by these interesting observations, we addressed the question as to whether significant alterations occur in the structure of transcriptionally active and inactive chromatin after exposure of HeLa cells to sodium butyrate. To this end, we chose to investigate the following issues: First, we attempted to fractionate chromatin from HeLa cells into transcriptionally active and inactive moieties. We then characterized in detail the structure and properties of transcriptionally active nucleosomes from control HeLa cells and made further careful comparisons of these characteristics with those of nucleosomes obtained from butyrate-treated cells. Ultimately, we wish to elucidate whether sodium butyrate can alter qualitatively or quantitatively the sets of genes which are found in an active conformation in these human neoplastic cells.

Materials and Methods

Cell Cultures. HeLa cells, strain S3, were grown in minimum Eagle's media, supplemented with 7% bovine calf serum. Penicillin at 100 units/mL and streptomycin sulfate at 100 μg/mL were also added. Growth was at 37 °C under 5% CO_2 (sodium bicarbonate buffer), in roller bottles (Corning), to a density of 5×10^7 cells/bottle.

Preparation of Nuclei. We followed the procedure of Milcarek et al. (1974) with some modifications. In brief, cells were first chilled on ice for 5 min, scraped from the bottles, and spun 10 min at 2000 rpm in a Sorvall GSA rotor. The cell pellet was resuspended in 10 volumes of buffer 1 (10 mM NaCl, 40 mM Tris, pH 8.3, and 1.5 mM $MgCl_2$), supplemented with poly(vinyl sulfate) at 25 μg/mL and spermine at 35 μg/mL. The cells were lysed by the addition of Nonidet P-40 to 0.5% for 2 min on ice. Nuclei were harvested by

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centrifugation for 10 min at 4000 rpm. The nuclear pellet was then washed with a solution of RSB-Ca^{2+} (10 mM NaCl, 10 mM Tris, pH 7.5, 3 mM $MgCl_2$, and 1 mM $CaCl_2$). The cytoplasmic supernatant was adjusted to 0.1 M NaCl, 10 mM EDTA, and 0.5% sodium dodecyl sulfate ($NaDodSO_4$)[1] and used in the isolation of RNA.

Chromatin Fractionation. We followed the procedure of Levy-Wilson & Dixon (1978, 1979) and Levy-Wilson et al. (1979). HeLa cell nuclei were resuspended in RSB-Ca^{2+} at a concentration of 100 A_{260} units/mL and incubated with micrococcal nuclease (0.5 unit/A_{260}) to digest ~5–15% of the input DNA. The reaction was terminated by immediate centrifugation for 5 min at 5000 rpm at 4 °C in a Sorvall HB-4 rotor. This first supernatant was designated "S_1". The pellet was resuspended by gentle homogenization for 20 min on ice in 2 volumes (7 mL) of 1 mM EDTA and 10 mM Tris, pH 7.5, and then centrifuged for 15 min at 10000 rpm. This procedure was repeated twice. This latter supernatant was designated "S_2" and was used as a source of chromatin subunits. The material insoluble in 1 mM EDTA was designated "pellet".

Subunit Fractionation and Isolation. The "S_2" fraction was adjusted to 0.1 M NaCl and left at 4 °C for 2–3 h. A white pellet was recovered by centrifugation at 10000 rpm for 20 min in a Sorvall HB-4 rotor. That pellet, containing nucleosomes insoluble in 0.1 M NaCl, was designated "MN_2". The supernatant, representing chromatin soluble in 0.1 M NaCl (MN_1), was recovered by centrifugation at 10000 rpm for 20 min in a Sorvall HB-4 rotor.

Sucrose Gradients. Samples containing 10–20 A_{260} units of chromatin subunits were analyzed by sedimentation on 5–24% sucrose gradients, in 10 mM Tris, pH 8.0. Centrifugation was for 16 h at 38000 rpm in a Beckman SW41 rotor at 2 °C, or for 40 h at 25000 rpm in the Beckman SW27 rotor at 2 °C (Levy-Wilson et al., 1979).

Extraction of Acid-Soluble Proteins. Acid-soluble proteins were extracted from the various chromatin fractions with 0.4 N H_2SO_4 for 1 h at 4 °C, as described by Levy-Wilson et al. (1977).

Electrophoresis of Proteins. Proteins were analyzed on 20% acid–urea–polyacrylamide slab gels (16.5 cm × 16.5 cm × 1.5 mm), with or without 0.22% Triton X-100 (Levy-Wilson et al., 1979; Kuehl et al., 1980). The running buffer was 5% acetic acid. Protein samples (100 μg) were dissolved in 6 M urea and 10 mM Tris, pH 8.0, just prior to loading. Electrophoresis was for 6 h at 20 mA. Gels were stained with Coomassie blue and destained as described by Kuehl et al. (1980).

Purification of DNA. HeLa nuclei or purified samples of "S_1", "MN_1", "MN_2", and "pellet" were resuspended by homogenization in 5 volumes of NTE buffer (0.1 M NaCl, 10 mM Tris, pH 7.5, and 1 mM EDTA) and 1% $NaDodSO_4$ (Levy-Wilson & Dixon, 1977a). The samples were treated with 20 μg/mL RNase A for 30 min at 37 °C, followed by the addition of 0.5% $NaDodSO_4$, and further treated with 100 μg/mL proteinase-K for 2 h at 37 °C. Then an equal volume of phenol–chloroform (1:1) saturated with NTE buffer was added. After the mixture was shaken for 5 min at room temperature, separation of the phases was accomplished by centrifugation for 10 min at 5000 rpm. The organic phase was removed, and the aqueous phase was reextracted 3 more times with chloroform–isoamyl alcohol (24:1). The aqueous phase was then adjusted to 0.24 M ammonium acetate and precipitated with 3 volumes of cold 95% EtOH at −20 °C. For use in reassociation reactions, DNA from the pellet fraction and from total HeLa cell nuclei was then fragmented to ~400 base pairs by shearing at 10000 psi in a French pressure cell.

Gel Electrophoresis of DNA Fragments. We followed the procedure of Levy-Wilson et al. (1979). Polyacrylamide gels (6%) in TAE buffer (6.4 mM Tris, 3.2 mM sodium acetate, and 0.32 mM EDTA, pH 8.0) were used. The gels were preelectrophoresed for 15 min at 100 V. Electrophoresis was for 5–6 h at 10 mA. A 2–4-μg sample of purified DNA was used per slot. The gels were then stained with ethidium bromide (2 μg/mL for 30 min) and photographed on a UV light box. The size of the DNA fragments was calculated by comparing their mobilities with those of *Taq*I restriction fragments of φX174 DNA.

Electrophoresis of Deoxyribonucleoprotein (DNP) Particles. We followed the procedure of Hutcheon et al. (1980). Samples of MN_1 and MN_2 (1.0 A_{260}) were dissolved in 1 mM EDTA, pH 7.5, and then loaded onto a 4% polyacrylamide slab gel (16.5 cm × 16.5 cm × 3 mm). The gel buffer and the running buffer were TAE. Electrophoresis was for 5 h at 200 V (20 mA) until the bromophenol blue dye had reached the bottom of the gel. Gels were stained with ethidium bromide (2 μg/mL for 20 min) and photographed on a UV light box.

For electrophoresis of proteins, using two-dimensional gels, we followed the procedure of Shaw & Richards (1978), as modified by Hutcheon et al. (1980). Aliquots of 6 A_{260} units of MN_1 or MN_2 were loaded onto a cylindrical (12 × 0.3 cm) 4% polyacrylamide gel (prepared as above). Electrophoresis was for 15 min at 50 V, followed by 4 h at 60 V, or until the bromophenol blue dye had reached the bottom of the tube. The tube gel was removed and soaked in 5% acetic acid, 5% β-mercaptoethanol, and 8 M urea for 30 min at room temperature. The gel was then laid horizontally across a preelectrophoresed 20% acetic acid–urea gel (16.5 cm × 16.5 cm × 3 mm). A solution (200 μL) of 8 M urea, 5% acetic acid, and 1% protamine sulfate was layered over the tube gel. Electrophoresis was for 25 h at 200 V, during which time the protamine migrated through the region occupied by the DNA bands in the tube gel and dissociated the DNA-associated proteins. At the end of the migration, the protamine had reached the bottom of the slab gel. The gel was stained overnight with Coomassie blue and destained as described above. Known amounts of purified standard histones and HMG's were run in a parallel slot of the slab gel.

Quantitation of Proteins. The quantitation of the protein components associated with each of the subfractions of MN_1 and MN_2 was accomplished by scanning the second-dimension acid–urea gels on an *E-C* gel densitometer followed by determination of the areas (by weight) beneath each protein peak with a Mettler analytical balance. The values obtained for the areas were first converted to moles of protein by dividing each area by the corresponding molecular weight of each protein. For calculation of molar ratios, the value for the number of moles of H4 was designated as 1.00, and the number of moles for each of the other proteins was divided by the number of moles for H4 within a given particle, to give the molar ratios for all of the proteins of each particle.

Preparation of Cytoplasmic Polyadenylated RNA. Cytoplasm in TNES buffer was extracted as described by Levy-Wilson & Dixon (1977b). Polyadenylated RNA was obtained by chromatography on oligo(dT)–cellulose, following the method of Aviv & Leder (1972), with some modifications.

[1] Abbreviations used: HMG; high-mobility group; C_0t, product of the initial DNA concentration and time of incubation in M^{-1}; $NaDodSO_4$, sodium dodecyl sulfate; Cl_3CCOOH, trichloroacetic acid.

The RNA was dissolved in ~5 volumes of binding buffer (0.5 M NaCl, 0.01 M Tris, 0.001 M EDTA, pH 7.5, and 0.5% $NaDodSO_4$) and passed through the column 5 times. The unbound material, representing cytoplasmic nonpolyadenylated RNA, was discarded. The column was then rinsed with 100 volumes of washing buffer (0.1 M NaCl, 0.01 M Tris, and 0.001 M EDTA, pH 7.5) to get rid of any remaining poly(A–) RNA. Polyadenylated RNA was eluted with eluting buffer (0.5% $NaDodSO_4$, 0.01 M Tris, and 0.001 M EDTA, pH 7.5). Fractions (2 mL) were collected, the A_{260} value of these was read, and the messenger RNA (mRNA) peak was pooled and precipitated with ammonium acetate and 95% EtOH.

Synthesis of cDNA. The conditions for synthesis of complementary DNA (cDNA) on a template of HeLa mRNA were as described by Levy-Wilson & Dixon (1977a,b).

cDNA/RNA Hybridization Reactions. cDNA/RNA hybridization reactions were carried out in 0.5 M NaCl, 10 mM Tris, pH 7.5, and 1 mM EDTA buffer, containing 0.01% $NaDodSO_4$. Samples, in duplicate, containing 1000 cpm of cDNA and RNA at 200 μg/mL, were sealed in 5-μg capillaries, heated for 5 min at 100 °C, and incubated at 65 °C for various time periods. At the end of each incubation period, samples were diluted in 1 mL of S_1 nuclease buffer (0.3 M NaCl, 0.03 M sodium acetate, and 3 mM $ZnCl_2$, pH 4.5) also containing 40 μg/mL denatured calf thymus DNA. S_1 nuclease (500 units) (Miles) was added for 45 min at 45 °C. Following this incubation, the samples were precipitated at 0 °C by the addition of 20% Cl_3CCOOH, filtered onto glass fiber filters (GF/C), and counted in an Omnifluor–toluene scintillation mixture. A zero time control sample (in duplicate) was included in each experiment, and the counts remaining after S_1 nuclease digestion of this control, which represented self-annealing of the cDNA, were routinely subtracted from all the experimental points. Various samples lacking S_1 nuclease were included in each reaction to monitor cDNA degradation.

cDNA/DNA Reassociation Reactions. Reassociation reactions were carried out essentially as described above, except that the buffer contained NaCl at a concentration of 0.8 M and the samples were sealed in 15-μL capillaries with a DNA concentration of 4 mg/mL (Levy-Wilson & Dixon, 1979). After the capillaries were sealed, the samples were heated for 7 min at 100 °C, and reassociations were at 65 °C as described above for the cDNA/RNA hybridizations.

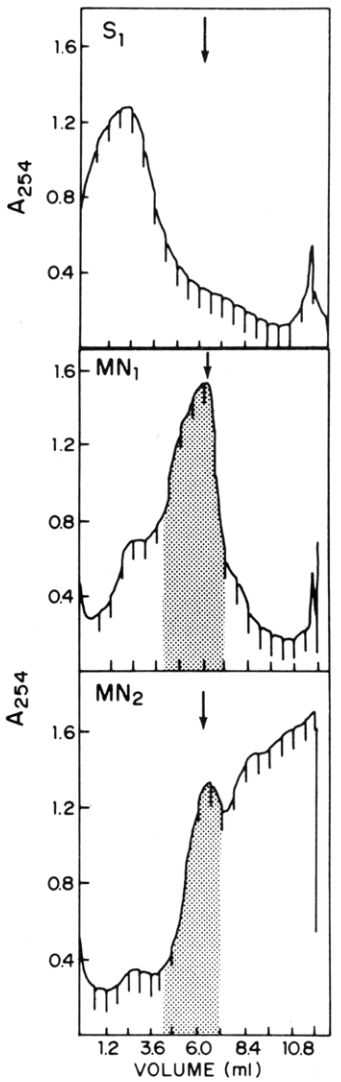

FIGURE 1: Sucrose gradient analysis of fractions S_1, MN_1, and MN_2. Chromatin from control HeLa cells was fractionated as described under Materials and Methods. Aliquots of S_1, MN_1, and MN_2 (12–18 A_{260}) were sedimented on parallel 5–24% sucrose gradients for 16 h at 38 000 rpm in the Beckman SW41 rotor. Sedimentation was from left to right. Aliquots (0.6 mL) were collected, and the A_{254} of each fraction was monitored with an ISCO density gradient fractionator. The arrows show the position of an 11S marker of catalase, cosedimented with the samples.

Results

Chromatin Fractionation. To fractionate chromatin from control and butyrate-treated HeLa cells, we followed the procedure of Levy-Wilson & Dixon (1979). In a typical preparation, among over 70 performed, some 10–20% of the input A_{260}, corresponding to some 5% acid solubility, was recovered in fraction S_1, some 20–40% appeared in fraction S_2 (over 95% of this material was acid insoluble), and some 40–70% of the input A_{260} was found in the acid-insoluble pellet. S_2 was further fractionated to yield fractions MN_1 and MN_2 as described under Materials and Methods. In most preparations, about 30% of the total A_{260} was found in S_2, some half of it in MN_1 and the other half in MN_2. These results were very similar in both control and butyrate-treated HeLa cells.

Analysis of the Various Chromatin Fractions Derived from Control and Butyrate-Treated HeLa Cells. Sedimentation on Sucrose Gradients. Fractions S_1, MN_1, and MN_2, from both control and butyrate-treated cells, were sedimented on 5–24% sucrose gradients as described under Materials and Methods. Figure 1 shows the profile obtained when fraction S_1 is analyzed in this manner. Most of this fraction sediments at about 5–6 S, with very little material in the mononucleosome region (11 S), indicated by the arrow. This profile was the same in samples derived from control or butyrate-treated cells.

The sedimentation profiles of fractions MN_1 and MN_2 from control cells are also illustrated in Figure 1. Fraction MN_1 consists mainly of material sedimenting at 11 S (mononucleosomes), with small amounts of material sedimenting at about 5 S (subnucleosomes). A very similar profile is obtained when fraction MN_1 from butyrate-treated cells is analyzed.

Fraction MN_2 is also very similar in control and butyrate-treated cells. This fraction consists of a monomer peak (11 S) (some 25% of the mass) plus some rapidly sedimenting material corresponding to dimers and trimers.

The pellet fraction, containing mostly higher molecular weight oligonucleosomes, is recovered from the bottom of the tubes upon sedimentation in this manner.

Size of the DNA. The overall size distribution of the DNA from fractions S_1, MN_1, and MN_2 was very similar in control and butyrate-treated HeLa cells. Figure 2 illustrates a polyacrylamide gel of DNA obtained from the various fractions

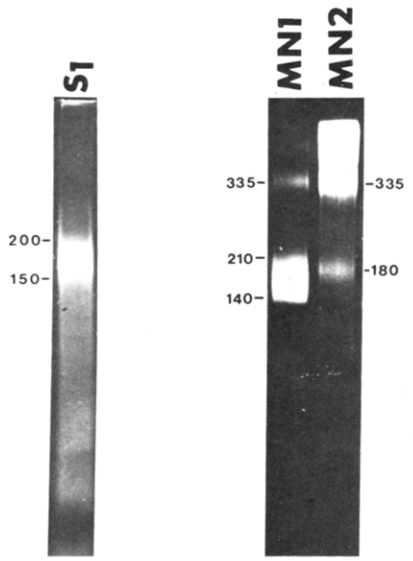

FIGURE 2: Electrophoresis of DNA fragments from fractions of S_1, MN_1, and MN_2 from HeLa cells. S_1, MN_1, and MN_2 were prepared from control HeLa cells. DNA was purified from each of these fractions and then electrophoresed on a 6% polyacrylamide gel as described under Materials and Methods. Electrophoresis was from top to bottom. The sizes were obtained by a comparison with restriction fragments of known sizes coelectrophoresed with the samples.

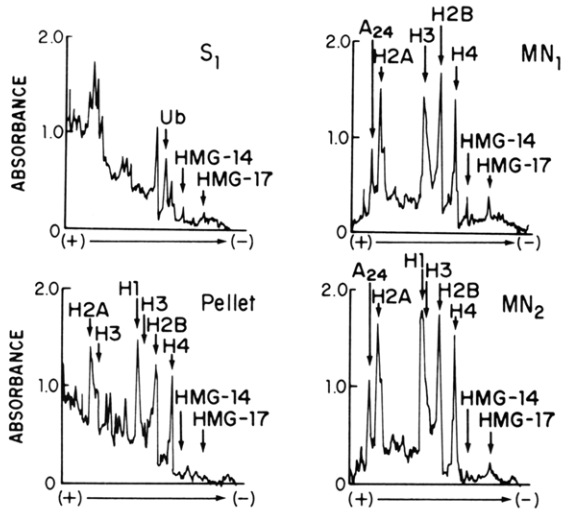

FIGURE 3: Densitometer scans of the acid-soluble proteins from fractions S_1, MN_1, MN_2, and pellet from control HeLa cells.

of control HeLa cells. S_1 contains DNA fragments ranging in size from 150 to 200 base pairs; MN_1 contains DNA ranging in size from 140 to 210 base pairs (monomer), plus a small amount of dimer DNA (335 base pairs). MN_2 contains DNA of 170–190 base pairs (monomer), 335 base pairs (dimer), and 425 base pairs (trimer).

Protein Composition. Acid-soluble proteins from the various fractions were prepared and electrophoresed on acid–urea gels as described under Materials and Methods. The results obtained are illustrated in Figures 3 and 4. Clearly, the overall protein content of the corresponding fractions is very similar for control and butyrate-treated cells. Fraction S_1 contains some H2A and H2B and a series of nonhistone proteins, among which ubiquitin is very prominent. Very small amounts of HMG-14 and HMG-17 are released into this fraction. MN_1, on the other hand, contains the four inner nucleosomal histones H2A, H2B, H3, and H4 together with the two smaller mammalian high mobility group proteins, HMG-14 and HMG-17, and protein A24 (Goldknopf et al., 1977). A major

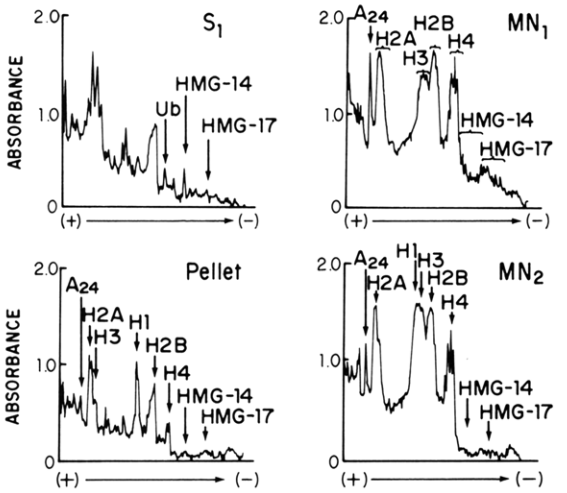

FIGURE 4: Densitometer scans of the acid-soluble proteins from fractions S_1, MN_1, MN_2, and pellet from butyrate-treated HeLa cells.

Table I: Stoichiometries of Proteins of MN_1 from Control and Butyrate-Treated HeLa Cells[a]

protein	MN_1 control		MN_1 butyrate	
	molar ratio	molecules per core	molar ratio	molecules per core
H2A	0.96	2	0.88	2
H2B	0.94	2	1.0	2
H3	0.96	2	0.74	2
H4	1.0	2	1.0	2
HMG-14	0.44	1	0.46	1
HMG-17	0.60	1	0.54	1

[a] The molar ratios were calculated as described under Materials and Methods. The data shown represent the mean value of three determinations.

portion of the HMG-14 and HMG-17 content of HeLa cell chromatin is found in fraction MN_1, corresponding to those salt-soluble monomers most sensitive to micrococcal nuclease (Figure 3). We determined the molar ratios of HMG-14 and -17 in MN_1 monomers. The data indicate that the MN_1 monomer fraction from butyrate-treated cells contains one molecule of HMG-14 and at least one molecule of HMG-17 per octamer of histones (Table I).

In the MN_1 fraction from butyrate-treated cells, a series of bands migrating slightly slower than those of H2B, H3, and H4 can be seen (Figure 4). These slower bands have been shown to correspond to multiacetylated species of these three histones (Vidali et al., 1978; Levy-Wilson, 1981) that accumulate due to an inhibition of the histone deacetylase promoted by sodium butyrate (Candido et al., 1978; Vidali et al., 1978; Sealy & Chalkley, 1978). Furthermore, another series of bands can be seen in the region where HMG-14 and -17 migrate on these gels (Figure 4). Independent studies in our laboratory suggest that this multibanding pattern might be due to hyperphosphorylation of HMG-14 and -17 (Levy-Wilson, 1981).

Fraction MN_2 contains mainly the five histones H1, H2A, H2B, H3, and H4, together with some A24 and traces of HMG-14 and -17 (Figures 6 and 7). The pellet contains the five histones, no HMG's, and smaller amounts of other unidentified nonhistone proteins of slower electrophoretic mobilities (Figures 3 and 4).

Distribution of Transcribed DNA Sequences among the Various Chromatin Fractions. Levy-Wilson & Dixon (1979) demonstrated that fractions MN_1 and MN_2, prepared in a similar manner from trout testis, are 5–8-fold enriched in their

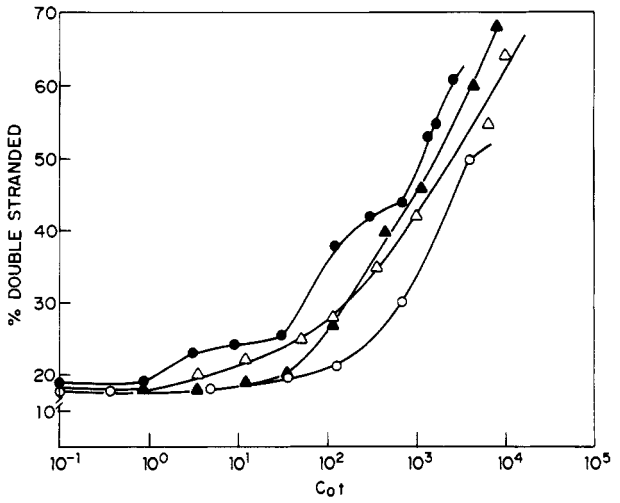

FIGURE 5: Kinetics of reassociation of cDNA complementary to cytoplasmic polyadenylated RNA from control HeLa cells and DNA derived from various chromatin fractions. Reactions were performed as described under Materials and Methods. Each point represents the mean value of four determinations. (●) Reaction driven by MN_1 DNA from control cells; (▲) reaction driven by MN_2 DNA from control cells; (△) reaction driven by total HeLa DNA; (○) reaction driven by pellet DNA from control cells.

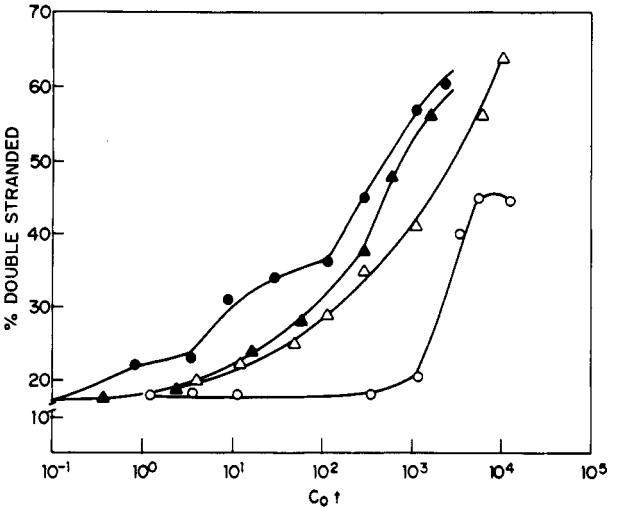

FIGURE 6: Kinetics of reassociation of cDNA complementary to cytoplasmic polyadenylated RNA from butyrate-treated HeLa cells and DNA derived from various chromatin fractions. Reactions were performed as described under Materials and Methods. Each point represents the mean value of four determinations. (●) Reaction driven by MN_1 DNA from butyrate-treated cells; (▲) reaction driven by MN_2 DNA from butyrate-treated cells; (△) reaction driven by total HeLa DNA; (○) reaction driven by pellet DNA from butyrate-treated cells.

content of transcribed DNA sequences, as probed with cytoplasmic polyadenylated RNA. We wished to establish whether, in HeLa cells, transcribed DNA sequences could also be selectively released as mononucleosomes by micrococcal nuclease. Furthermore, we wanted to ask whether the selective recognition of transcribed DNA sequences by this nuclease is altered in any way after exposure of the cells to sodium butyrate.

To answer these questions, we prepared radioactive probes representative of transcribed DNA sequences from control and butyrate-treated HeLa cells. Cytoplasmic polyadenylated RNA was used as template for the synthesis of labeled cDNA probes.

To determine the degree of enrichment in transcribed DNA sequences of the chromatin fractions, we compared the kinetics of reaction of the cDNA probes when driven by DNA from MN_1, MN_2, or pellet DNA with the kinetics obtained when the driver was total HeLa DNA fragmented to a size similar to that of the DNA from the chromatin fractions. For example, Figure 5 shows the reactions of cDNA from control cells when driven by DNA derived from MN_1, MN_2, and pellet from control cells, or by DNA derived from total HeLa nuclei.

At first sight, it is clear that MN_1 and MN_2 DNA drive the cDNA probe faster than does total HeLa DNA, thus indicating that MN_1 and MN_2 are enriched in their content of transcribed sequences as compared to that of total HeLa DNA. Furthermore, DNA from the pellet fraction (nuclease resistant) drives the cDNA at a slower rate than does total DNA, indicating that pellet DNA is depleted in transcribed sequences represented in the cDNA probe. Careful comparison of the $C_0t_{1/2}$ values of each of the curves in Figure 5 shows that fraction MN_1 is 5-fold enriched in transcribed DNA sequences compared to total HeLa DNA as evidenced by a 5 times faster reaction rate.

According to the same reasoning, MN_2 is some 3-fold enriched in active gene sequences, while the pellet fraction is some 4–5-fold impoverished in active genes compared to total HeLa DNA in control cells.

The data illustrated in Figure 6 demonstrate that the micrococcal nuclease fractionation of active and inactive chromatin works just as well in butyrate-treated HeLa cell nuclei. Again, here, MN_1 DNA (butyrate) drives the butyrate cDNA probe some 5–7 times faster than does total HeLa DNA, while the reaction driven by the butyrate pellet is some 5–8-fold slower than that of total DNA.

These results confirm and extend the initial observation of Levy-Wilson & Dixon (1978) in trout testis and show that those nucleosomes most readily released by micrococcal nuclease and with a high content of HMG-14 and -17 are highly enriched in transcribed DNA sequences. The DNA from fraction S_1 (2% of the chromatin) was not assayed for degree of enrichment of active genes during the course of this work due to insufficient material. Nevertheless, S_1 DNA is likely to be highly enriched in active gene sequences since it exhibits the highest sensitivity to micrococcal nuclease.

Heterogeneity of MN_1 and MN_2. Having demonstrated that MN_1 and MN_2 in both control and butyrate-treated HeLa cells correspond to transcriptionally active chromatin, we analyzed the structural organization of these two mononucleosome subsets in further detail.

To this end, we examined the electrophoretic behavior of each of these fractions on 4% polyacrylamide gels at low ionic strength. These gels allow the separation of monomer subfractions of very similar composition. Figure 7 shows gels of MN_1 and MN_2 from control and butyrate-treated HeLa cells. At least four bands can be observed in each of these fractions.

The existence of more than one nucleoprotein subfraction among active nucleosomes poses the question as to whether heterogeneity is due solely to differences in the length of the monomer-associated DNA of each of the subcomponents or is attributable to differences in the protein composition of each of these subfractions.

We, therefore, analyzed the DNA fragments derived from the monomer peaks of many sucrose gradients of MN_1 and MN_2 from control and butyrate-treated cells (see shaded areas in Figure 1). Four major ethidium bromide staining bands were observed in each of these fractions. The sizes of each of these DNA fragments are listed in Table II.

DNA from MN_1 monomer in control cells ranges in size from 94 to 145 base pairs, while that of the same fraction from butyrate-treated cells ranges between 70 and 147 base pairs.

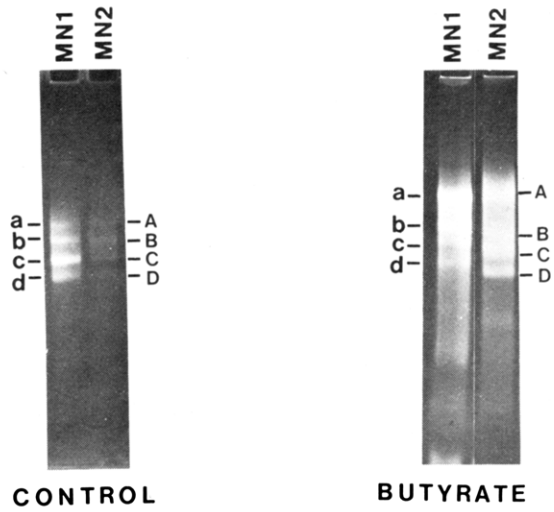

FIGURE 7: Electrophoresis of MN_1 and MN_2 from control and butyrate-treated HeLa cells on 4% polyacrylamide gels. Polyacrylamide gels (4%) at low ionic strength were used. Migration is from top to bottom. Gels were stained with ethidium bromide and photographed under ultraviolet light.

Table II: Size of DNA from Purified Monomers of Control and Butyrate-Treated Cells[a]

	control		butyrate	
band	MN_1	MN_2	MN_1	MN_2
1	145	170	147	170
2	120	140	119	145
3	97	117	97	123
4	94	97	70	95

[a] The data (in base pairs) were obtained from Figure 7.

Table III: Stoichiometries of Proteins of MN_1 and MN_2 Subcomponents from Control HeLa Cells[a]

	MN_1 molar ratios				MN_2 molar ratios			
protein	a	b	c	d	A	B	C	D
H1	0.19	0.12	0.08	0.19	0.10	0.18	0.43	0.31
H2A/H2B	0.79	1.06	0.89	0.83	0.88	1.12	1.26	1.19
H3	0.78	0.72	0.70	0.77	0.61	0.79	0.69	0.69
H4	1.0	1.0	1.00	1.0	1.0	1.0	1.0	1.0
HMG-14	0.55	0.74	0.53					
HMG-17	0.55	0.74	0.45					

[a] The molar ratios were calculated as described under Materials and Methods.

On the other hand, DNA from MN_2 monomers from control cells ranges in size between 97 and 170 base pairs, while in butyrate-treated cells, the size range varies between 95 and 170 base pairs.

To display the proteins from each of the monomer subfractions, we used a novel two-dimensional electrophoresis procedure (Hutcheon et al., 1980) first described by Shaw & Richards (1978). Figures 8 and 9 show the protein composition of the various fractions of control MN_1, control MN_2, butyrate MN_1, and butyrate MN_2, respectively. In control MN_1, we can distinguish four kinds of particles. Subfraction a contains one molecule of HMG-14 and one molecule of HMG-17 bound per histone octamer (Table III). Subfraction b contains one to two molecules of HMG-14 and -17 bound per each histone octamer. Subcomponent c contains one molecule each of HMG-14 and -17 per octamer, while subcomponent d lacks HMG's. Small amounts of H_1 and x can be seen in all MN_1 subfractions. Protein x comigrates with A24 in both Triton and non-Triton-containing acid–urea gel

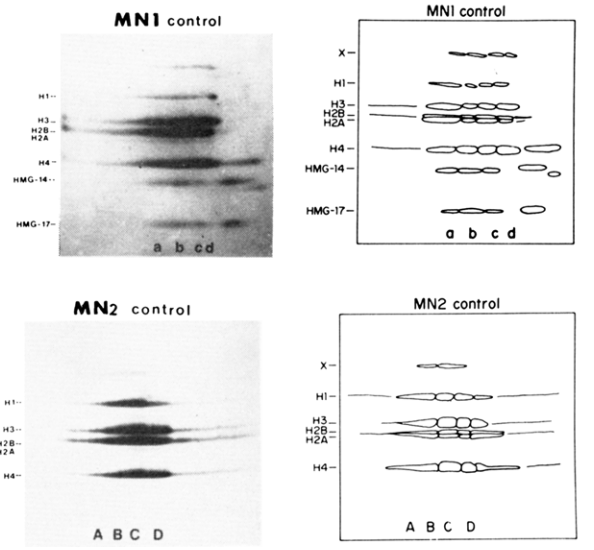

FIGURE 8: Protein components of electrophoretically separated MN_1 and MN_2 monomers from control HeLa cells. Second-dimension acid–urea gels of MN_1 and MN_2 monomers, separated electrophoretically in first-dimension cylindrical gels, were as described under Materials and Methods. Identification of proteins was accomplished by comparison of their mobilities with those of purified calf thymus and HeLa histones, HMG's and A24, run on side slots of the same gels. The left panels show examples of such gels; the right panels show schematic diagrams of the gel patterns.

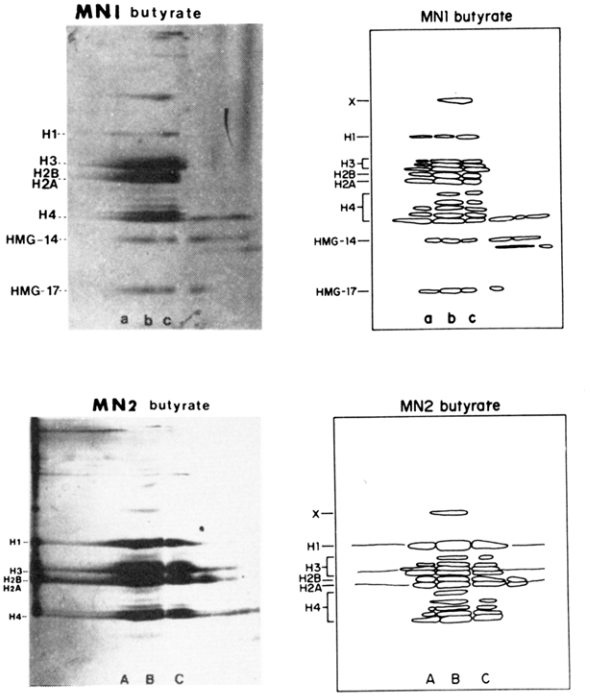

FIGURE 9: Protein components of electrophoretically separated MN_1 and MN_2 monomers from butyrate-treated HeLa cells. All procedures are described in the legend to Figure 8.

and is thus tentatively identified as A24.

In fraction MN_2, we observe three major (B, C, and D) and one minor (A) component. They all lack HMG-14 and -17 and contain variable amounts of H1 (Table III). Subfractions B and C contain traces of x (A24).

Figure 9 shows the protein content of the subcomponents of MN_1 from butyrate-treated cells. In this case, three subfractions, a, b, and c, can clearly be seen. All subcomponents a, b, and c contain varying amounts of HMG-14 and -17 (Table IV) and small amounts of H1. Protein x is present in subfractions b and c.

Table IV: Stoichiometries of Proteins of MN_1 and MN_2 Subcomponents from Butyrate-Treated HeLa Cells[a]

	MN_1 molar ratios			MN_2 molar ratios		
protein	a	b	c	A	B	C
H1	0.06			0.18	0.29	0.37
H2A/H2B	0.85	1.05	1.11	0.86	1.08	1.28
H3	0.77	0.76	0.80	0.90	1.09	1.05
H4	1.0	1.0	1.0	1.0	1.0	1.0
HMG-14	0.6	0.54	0.51			
HMG-17	0.7	1.31	0.75			

[a] The molar ratios were calculated as described under Materials and Methods.

MN_2 from butyrate-treated cells also shows three major subfractions (A, B, and C) (Figure 9), with H1 varying among the subcomponents (Table IV). Subfraction B contains traces of protein x (A24).

In the gels illustrated in Figures 8 and 9, histones H2A and H2B migrated too close together to be completely resolved from one another. To determine the molar ratios of the histones among these various monomer fractions, we also analyzed samples on Triton-containing acid–urea gels (Levy-Wilson et al., 1979). In those gels, H2A and H2B migrate far apart from each other, and their stoichiometries can be determined. The combined results of all our gels indicate that the four inner nucleosomal histones are equimolar in all subcomponents of MN_1 and MN_2, both from control and from butyrate-treated cells.

Discussion

We have applied a novel chromatin fractionation method to HeLa cell nuclei. This method (Levy-Wilson & Dixon, 1978, 1979) yields various chromatin fractions differing in their degree of sensitivity to micrococcal nuclease. When this methodology was applied to trout testis, it was found that those chromatin fractions most sensitive to this nuclease corresponded to the transcriptionally active moieties of the genome (Levy-Wilson et al., 1979; Levy-Wilson & Dixon, 1979). We tested the applicability of the method to human cells and, in particular, neoplastic cells such as HeLa cells. We asked whether the structure and properties of transcriptionally active chromatin were altered after exposure of HeLa cells to sodium butyrate, an agent which promotes a number of morphological and biochemical changes in these cells.

Nuclei, derived from either control or butyrate-treated HeLa cells, were subjected to a limited digestion with micrococcal nuclease. Since it is known that micrococcal nuclease preferentially attacks the DNA of linker regions located between nucleosome cores (Hewish & Burgoyne, 1973; Noll, 1974), one would expect to find in the first supernatant, S_1, both proteins and small DNA fragments derived from those regions. Moreover, the DNA of linker regions should be cut extensively. For instance, in trout testis, fraction S_1 is composed of DNA fragments smaller than 50 nucleotides in length, which are probably complexed with HMG-T and ubiquitin (Levy-Wilson et al., 1979). Thus, it was rather surprising to find that the DNA from the HeLa S_1 fractions was monomeric in size. A reasonable explanation for the lack of further degradation of S_1 DNA by micrococcal nuclease is that potential cutting sites along the linker DNA are protected by proteins and inaccessible to the nuclease. On the other hand, fraction S_1 could also include conformationally altered or structurally incomplete nucleosomes. The sedimentation behavior and the lack of a normal histone complement of this fraction indicate that it is devoid of mononucleosomes.

Fraction P_1 was homogenized gently in 1 mM EDTA to solubilize nucleosomal particles generated by the nuclease. This material, S_2, was separated by centrifugation from the insoluble pellet (P_2). The nucleosomal fraction, S_2, was fractionated into a portion soluble in 0.1 M NaCl (MN_1) and one insoluble portion (MN_2).

The protein compositions of fractions MN_1 and MN_2 and the pellet fraction P_2 were analyzed. The important points are the following: fraction MN_1, in both control and butyrate-treated cells, contains a major portion of the mass of HMG-14 and -17 of HeLa cell chromatin (75–80%) and small amounts of H1. MN_2 and pellet have only traces of HMG-14 and -17 and greater amounts of H1. All three fractions, MN_1, MN_2, and pellet, contain the four inner nucleosomal histones in equimolar amounts, with H2B, H3, and H4 being hyperacetylated, and HMG-14 and -17 being hyperphosphorylated in butyrate-treated cells (Levy-Wilson, 1981).

Prior to further analysis of the structure of the various chromatin fractions, we wished to identify those fractions containing transcribed DNA sequences (active genes). The various hybridization experiments illustrated in Figures 5 and 6 clearly show that, in both control and butyrate-treated cells, fraction MN_1 has the highest content of transcribed DNA sequences; however, MN_2 is also enriched in active chromatin. The pellet fraction, P_2, is in turn depleted of sequences complementary to the cDNA probe. The degree of enrichment of active genes in MN_1 is about 5-fold in control cells and slightly higher in butyrate-treated cells. With the assumption that some 10% of the HeLa cell genome is transcribed, a fraction consisting solely of active genes would be 10-fold enriched in these sequences compared to total DNA. Therefore, a 5–7-fold enrichment in MN_1 means that it is comprised of 50–70% active chromatin.

The observation that a very similar gene enrichment is found in MN_1 and MN_2 monomers from both control and butyrate-treated cells suggests that the presence of high steady-state levels of acetylated histones is not in itself sufficient to establish the "active conformation" preferentially recognized by micrococcal nuclease. Nevertheless, it is still possible that a small subset of histones whose acetyl groups turn over very rapidly (Nelson et al., 1980) might play an important role in delineating the structural features of transcriptionally active chromatin.

Having clearly established that fraction MN_1 and, to a lesser extent, MN_2 correspond to active chromatin, we analyzed in further detail the structure of these fractions and compared nucleosomes derived from control and butyrate-treated HeLa cells. Electrophoretic analysis of fraction MN_1, from both control and butyrate-treated cells on 4% acrylamide gels at low ionic strength, showed the presence of several bands (a, b, c, and d) of different mobilities, each containing DNA fragments of different sizes.

That this heterogeneity is not due solely to differences in the DNA length of the subcomponents of MN_1 and MN_2 was shown by two-dimensional electrophoresis of the protein components of MN_1 and MN_2. The results, illustrated in Figures 8 and 9, show heterogeneity at the protein level in both MN_1 and MN_2 fractions. For instance, MN_1 from control cells contains four different subcomponents differing mainly in their content of HMG-14 and -17 (Table III). In butyrate-treated cells, MN_1 shows three major subfractions differing in protein content. HMG-14 and HMG-17 are present in all three subfractions in major amounts (Table IV). The fourth band seen in the nucleosome gel of Figure 7 probably corresponds to a subnucleosome containing a small DNA

fragment and HMG-14 and -17.

The subfractions of MN_2, when examined at the protein level, vary mainly in the amount of H1 they contain, while maintaining equimolar amounts of the histone octamer (Figures 8 and 9, Tables III and IV). The major difference between control and butyrate-treated cells is the presence of hyperacetylated H2B, H3, and H4 in MN_2 from butyrate-treated cells.

Our finding of equimolar amounts of HMG-14 and -17 in transcriptionally active nucleosomes in HeLa cells is in good agreement with the work of Weisbrod & Weintraub (1978), who showed that these two proteins are responsible for conferring DNase I sensitivity upon the globin gene in erythroid cells.

Moreover, the stoichiometries obtained for HMG-14 and -17 in the various MN_1 subcomponents from control and butyrate-treated cells confirm the observations of Allbright et al. (1980) and Mardian et al. (1980) concerning the number of binding sites for HMG-14 and -17 in mononucleosomes of other cell types.

The heterogeneity found in the MN_1 and MN_2 fractions poses the question as to whether individual gene sequences are exclusively associated with one of these structures or, alternatively, whether a given transcribed sequence might be found in a variety of conformations in chromatin. Future experiments will be directed toward these questions.

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