Volume 15 Number 11 1987 Nucleic Acids Research

Cell cycle-dependent changes in conformation and composition of nucleosomes containing human histone gene sequences

Richard Sterner, Lidia C.Boffa[1], Thelma A.Chen and Vincent G.Allfrey*

Rockefeller University, New York, NY 10021, USA

Received March 20, 1987; Revised and Accepted May 11, 1987

ABSTRACT

Unfolding of the nucleosomes in transcriptionally active chromatin uncovers the sulfhydryl groups of histone H3 and permits the selective recovery of the unfolded nucleosomes by mercury-affinity chromatography. This new technique has been used to compare the nucleosomal proteins and their postsynthetic modifications in the unfolded and the compactly beaded nucleosomes of HeLa cells in logarithmic growth, and at different stages of the growth cycle. The Hg-bound nucleosomes are shown to be deficient in replicating DNA sequences, but to remain associated with fragments of nascent RNA chains (or RNP particles) during gradient centrifugations. Both nucleosome fractions contain a full complement of "core" histones but differ with respect to postsynthetic modifications. The Hg-bound nucleosomes contain high levels of the tri- and tetra-acetylated forms of histones H3 and H4. The unbound nucleosomes are deficient in acetylated histones but enriched in phosphorylated H2A. In synchronized HeLa cells, histone H2A and H4 gene sequences occur in the Hg-bound nucleosomes during the S-phase when their transcription takes place, but not in the G_2-phase when the genes are repressed.

INTRODUCTION

A new procedure for the chromatographic separation of nucleosomes from transcriptionally active and inactive DNA sequences has been described (1). It is based on the observation that the nucleosomes of ribosomal genes undergo reversible conformational changes during transcription. This change, an unfolding of the nucleosome "cores" along the transcription unit, uncovers the previously shielded sulfhydryl groups of histone H3 (2). These sulfhydryl groups are not accessible to SH-reagents in the compactly beaded nucleosomes of inactive chromatin (1-7) but they become accessible when transcription begins (1,2,8). The difference in H3-SH accessibility in active and inactive chromatin makes it possible to isolate the unfolded, SH-reactive nucleosomes by mercury-affinity chromatography (1).

The feasibility of this approach has been confirmed by studies which show that liver nucleosomes retained by an organomercurial-agarose column are enriched in DNA sequences expressed specifically in the liver (e.g. the albumin and transferrin genes) but lack DNA sequences expressed in the brain

but not in the liver (e.g. pre-proenkephalin DNA)(1). Hg-affinity chromatography has also been employed to study the rapid and reversible changes in nucleosome conformation that accompany the activation and repression of proto-oncogenic DNA sequences in murine fibroblasts (8). It was shown that the binding of the c-fos and c-myc nucleosomes to the mercury column accurately reflects both the timing and the degree of their expression, as determined by run-off transcription assays in the isolated nuclei.

Unfolding of the nucleosome cores during transcription appears to be a general phenomenon which takes place on genes transcribed by RNA polymerase I (e.g. the ribosomal genes of *Physarum polycephalum* (2)) and on genes that are transcribed by RNA polymerase II (e.g. the albumin and transferrin genes of the rat (1), and murine c-fos and c-myc (8)). The activity of specific genes is readily monitored by recovering the nucleosomes from the mercury column and probing their DNA sequence contents by dot-blot hybridizations to ^{32}P-labeled cDNA probes (1,8).

This procedure has now been applied to the analysis of the differences in structure of active and inactive nucleosomes of cultured human (HeLa) cells. We show that the nucleosomes along the genes for histones H2A and H4 unfold during their expression in the S-phase and revert to the compact conformation when the genes are repressed in the G_2-phase of the cell cycle.

MATERIALS AND METHODS

Cell culture, synchronization, and nucleic acid labelling conditions

HeLa-S3 cells were maintained in suspension culture at 3 - 6 x 10^5 cells/ml in Joklik-modified Eagle's minimal essential medium supplemented with antibiotics, 2 mM glutamine, 1 x nonessential amino acids, and 5 % newborn calf serum (Gibco, Grand Island, NY). Synchronization at the G_1/S boundary was achieved with a 2 mM thymidine block for 12 hr, followed, after 9 hr in standard medium, by a 5 µg/ml aphidicolin block for 12 hr, as described by Heintz et al. (9). The cells were released from the aphidicolin block as described (9), restored to the standard growth medium, and cultured for an additional 5 hr (mid-S phase) or 10 hr (G_2-phase).

Short term RNA labelling experiments were carried out in 200 ml of culture (3 x 10^8 cells in logarithmic growth) containing 25 µCi of uridine-[5-^{3}H] (Sp.Act. 27 Ci/mmole; NEN). After 20 min, the cells were rapidly chilled to 0°C for isolation of the nuclei and preparation of nucleosomes.

DNA pulse-labelling experiments were carried out on synchronized HeLa cell cultures (4 x 10^5 cells/ml) 2 hr after release from the aphidicolin block. The cells were incubated with 5 µCi/ml [methyl-^{3}H]thymidine (Sp.Act. 2.0 Ci/mmole; NEN), rapidly chilled to 0°C, and the nuclei were isolated. After digestion with micrococcal nuclease to release 14 % of the total DNA (average of two experiments), the nucleosomes were chromatographically fractionated and the DNA was extracted from each fraction as described (10).

Isolation of nuclei

Cells were harvested by centrifugation at 600 x *g* for 15 min, washed

extensively with 0.14 M NaCl containing 5 mM Na butyrate, 0.1 mM PMSF and 0.1 mM 1,2-epoxy-3-(p-nitrophenoxy)propane (EPNP), and resuspended at 10^7 cells/ml in 80 mM NaCl, 5 mM Na butyrate, 0.1 mM PMSF, 0.1 mM EPNP, pH 7.2. After 10 min, Triton X-100 was added to a final concentration of 1% (w/v) and the cells were broken by shearing in a Dounce-type glass homogenizer with a tight-fitting pestle (Type B, Kontes, Inc., Vineland NJ). The homogenate was centrifuged at 2,000 x *g* for 10 min and the nuclear pellet was washed free of detergent with appropriate buffers as described below.

Preparation of nucleosomes

Micrococcal nuclease (Cooper Biomedical) was added at a concentration of 10 U/ml to nuclear suspensions containing 1 mg DNA/ml in solution A (25 mM KCl, 25 mM NaCl, 5 mM Na butyrate, 0.1 mM PMSF, 0.1 mM EPNP) containing 1 mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.35 M sucrose, and 15 mM Tris-HCl, pH 7.0. After 5 min at 37°C the reaction was stopped by the addition of EGTA to a final concentration of 3 mM. The digest was rapidly chilled to 0°C and centrifuged at 10,000 x *g* for 20 min. The supernatant, containing the released nucleosomes and representing 10 ± 1% of the total nuclear DNA, was collected for DNA-sizing, protein characterizations, and Hg-affinity chromatography.

Hg-affinity chromatography of nucleosomes

The supernatant fraction (S) was adjusted to 5 mM EDTA, pH 7.5, and applied directly to a 1 x 4 cm column of AffiGel 501 (Bio-Rad Laboratories) pre-equilibrated with solution A containing 5 mM EDTA, 2% sucrose, and 15 mM Tris-HCl, pH 7.8. The column was washed with the EDTA-containing buffer at a flow-rate of 10 ml/hr to remove the unbound nucleosome fraction, and the mercury-bound nucleosomes were subsequently eluted from the column in the same buffer with 10 mM dithiothreitol (DTT; IBI, Inc.) as described (1).

Further purification of nucleosome fractions

The run-off and Hg-bound nucleosome fractions were separated from smaller chromatin fragments, adventitious proteins, and unassociated RNA fragments by gel filtration chromatography on 1.5 x 90 cm columns of Sephacryl S-200 (Pharmacia), as described (1).

In order to compare the association of newly synthesized RNA fragments with Hg-bound and unbound nucleosome fractions, each fraction was prepared from cells pulse-labeled for 20 min with uridine [5-^{3}H] and loaded separately on 5 - 20% sucrose gradients in 0.1 M NaCl, 5 mM Na butyrate, 1 mM EDTA, 0.1 mM PMSF, 0.1 mM EPNP. After centrifugation in a Beckman SW28 rotor at 26,000 rpm for 22 hr, the gradients were collected dropwise into 1 ml fractions, monitoring absorbancy at 254 nm and counting aliquots for tritium activity by scintillation spectrometry.

Carboxymethylation of accessible histone H3-sulfhydryl groups

Isolated nuclei (2 x 10^9/ml) were suspended in 0.14 M NaCl containing 10 mM Tris-HCl, pH 8.2, 5 mM Na butyrate, 0.1 mM PMSF and 0.1 mM EPNP. Five mCi of iodo[^{3}H]acetate (Sp.Act. 230 mCi/mmole; NEN) was added and the reaction allowed to proceed for 45 min at 25°C in the dark. The reaction was terminated by the addition of 2-mercaptoethanol to 0.5%. The labeled

nuclei were washed extensively in 0.1 M NaCl containing 5 mM Na butyrate, 0.1 mM PMSF and 0.1 mM EPNP. Histones were extracted in 4 successive extractions with an equal volume of 0.25 N HCl and precipitated in 5 volumes of cold acetone.

^{32}P-labelling and electrophoretic separation of histones

HeLa cells (2 x 10^9) in logarithmic growth were harvested and resuspended in 50 ml of growth medium containing 20 mCi of carrier-free ^{32}P-orthophosphate (NEN). After 2 hr at 37°C, the cells were collected and washed extensively with cold medium and with 0.14 M NaCl, 5 mM Na butyrate, 0.1 mM PMSF, 0.1 mM EPNP. The nuclei were isolated and digested with micrococcal nuclease as described above. The released nucleosomes were fractionated by Hg-affinity chromatography, as described, and each fraction was dialyzed against 5 mM, followed by 1 mM Na butyrate, and lyophilized. The nucleosomal histones were extracted in 5 % (w/v) guanidium chloride, 0.1 mM PMSF, 0.1 mM EPNP, 100 mM potassium phosphate buffer, pH 6.8, and purified by ion-exchange chromatography on Bio-Rex 70 (Bio-Rad) as described (1).

Histones were separated electrophoretically in 15 % polyacrylamide gels containing 0.1 % SDS (13). For electrophoretic separation of the acetylated forms of the nucleosomal histones, H3 and H4 in particular, the Bio-Rad 70-purified histone fraction was analyzed in gels containing 15 % polyacrylamide, 5.5 % acetic acid, 8 M urea, and 0.3 % Triton X-100 (14), as described (1). Gels were stained either with 0.25 % Coomassie brilliant blue-R, or silver-stained according to Wray et al. (15).

DNA/DNA hybridization procedures

The Hg-bound and unbound nucleosome fractions were each digested with 50 µg/ml RNase A for 1 hr at 37°C, followed by 100 µg proteinase K for 2 hr at 37°C in the presence of 0.1 % SDS. DNA was extracted as described by Maniatis et al. (10). Nucleosomal DNA from each sample was blotted onto a nylon membrane (Zeta-Probe; Bio-Rad) using a slot-blot apparatus (Schleicher and Schuell) in quantities of 1 µg and 5 µg, following the alkaline procedure recommended in Bio-Rad Technical Bulletin #1110 (1986). Membrane-bound DNA was hybridized following the Southern procedure, as described by Maniatis et al. (10). The DNA probes employed were an 866 bp DNA fragment (pHh5G) containing the entire coding region of the human H2A gene and several hundred nucleotides of 5'- and 3'-flanking sequences (16), and a 710 bp DNA fragment (pHu4A) containing a human histone H4 gene and its flanking sequences (16). As a control for a gene not expressed in HeLa cells we employed a 0.3-0.4 kb fragment of human α-globin cDNA cloned in PMB9 (JW101)(17). The probes, nick-translated with [α-^{32}P]dCTP to a specific activity of 1.1-1.8 x 10^8 dpm/µg DNA (18), and purified on an Elutip affinity column (Schleicher and Schuell), were heat-denatured and added to the hybridization fluid. After hybridization at 68°C for 16 hr, the filters were washed (10), dried at 25°C on Whatman 3MM paper, covered with Saran wrap, and applied to Kodak X-O-Mat film. The film was exposed for various times with a Dupont Cronex I-G Plus intensifying screen at -80°C, and developed in Kodak X-O-Mat M4 developer.

RESULTS

Fractionation of HeLa nucleosomes by mercury-affinity chromatography

Nuclei isolated from HeLa S-3 cells in the presence of protease inhibitors (PMSF and EPNP) and 5 mM Na butyrate (to inhibit the deacetylation of histones (19-23) were subjected to limited digestions with micrococcal nuclease to release 10 ± 1 % of the total nuclear DNA. The mixture of nucleosomal particles released into the supernatant fraction (S) was applied to an organomercurial-agarose column (Affi-Gel 501) and separated into a run-off and a mercury-bound fraction as shown in Fig. 1. The basic premise of this fractionation procedure is that unfolding of the nucleosomes in transcriptionally active chromatin makes the sulfhydryl groups of histone H3 accessible for mercury binding. The column employed has been shown to permit the recovery of nucleosomes containing the transcriptionally active DNA sequences of rat hepatocytes (1) and murine fibroblasts (8) by simple displacement of the Hg-bound nucleosomes with 10 mM dithiothreitol (DTT).

The DNA of the Hg-bound and unbound nucleosome fractions was extracted and sized by electrophoresis in 5 % polyacrylamide gels (12). The results show that both fractions contained a mixture of monomeric nucleosomes and multiples of the basic chromatin subunit spaced at ca. 185 bp (Fig. 2). The monomeric and dimeric nucleosomes clearly predominate and represent about 85 % of the total nucleosomal DNA in each fraction. Previous studies of the monomeric nucleosomes of rat liver have shown that the mercury column retains the SH-accessible subunits with over 96% efficiency (1). The present studies indicate that the column will reversibly bind chromatin fragments at least as long as heptamers with DNA lengths in excess of 1.3 kb (Fig. 2).

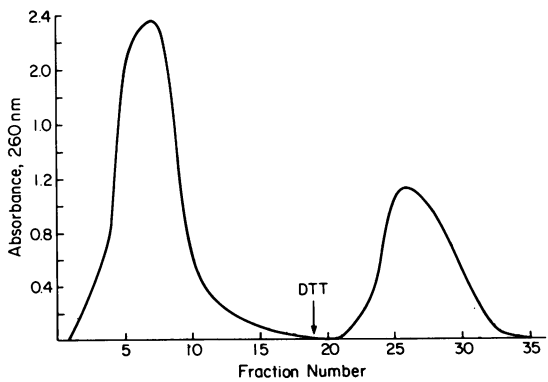

Figure 1. Mercury-affinity fractionation of nucleosomes by organomercurial-agarose column chromatography. Nucleosomes generated by micrococcal nuclease digestion of HeLa cell nuclei eluted as an unbound fraction (first peak) and a mercury-bound fraction displaced from the column by 10 mM dithiothreitol (second peak).

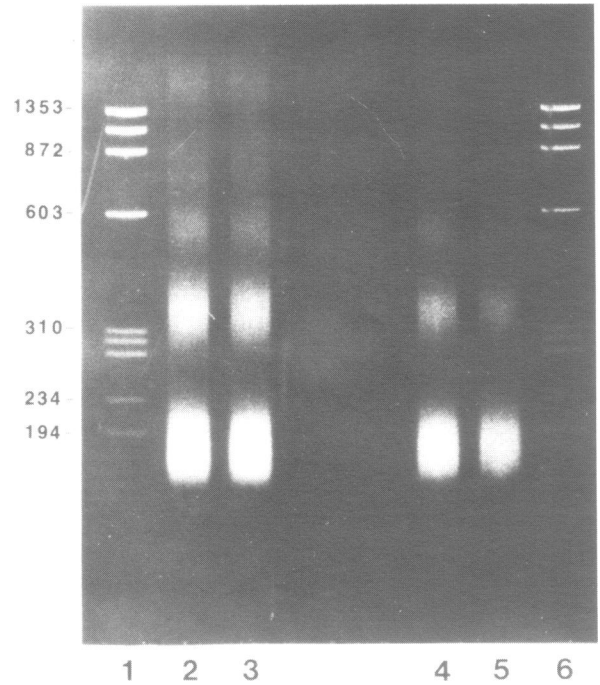

Figure 2. DNA size determination on nucleosome fractions released by micrococcal nuclease digestion and fractionated by mercury-affinity chromatography. Lanes 1 and 6 show the positions of Hae III restriction fragments of ϕX 174 DNA used as size markers. Lanes 2 and 3 show the unbound and Hg-bound nucleosomal DNA, respectively, from cells in the G_2-phase. Lanes 4 and 5 show the unbound and Hg-bound nucleosomal DNA, respectively, from cells in the S-phase. Each sample applied to the gel contained 1 μg of DNA. The numbers to the left indicate the size in base pairs of pertinent size markers.

Hg-bound nucleosomes are deficient in replicating DNA sequences but cosediment with nascent RNA fragments

Previous studies of the composition of mercury-bound nucleosomes were carried out in non-dividing cells, such as adult rat hepatocytes (1) or in quiescent murine fibroblasts entering the G_1-phase of the cell cycle (8). In those experiments the analysis of changes in nucleosome structure due to transcription was not complicated by changes taking place during nucleosome assembly at the replication fork. In the rapidly dividing HeLa cell cultures, however, it is necessary to determine whether the nucleosomes of newly replicating chromatin unfold to a degree that would result in their retention on the mercury column. If so, this would seriously limit the method as a means for the selective isolation of the transcriptionally active chromatin subunits. To test this possibility, HeLa cells were synchronized at the G_1/S boundary

and allowed to proceed into the S-phase when they were pulse-labeled for 10 minutes with ^{3}H-thymidine. The nuclei were isolated and digested with micrococcal nuclease to release ca. 14 % of the total DNA. This led to a substantial enrichment of the radioactively-labeled DNA in the released nucleosome fraction, which contained over 61 % (4.92 x 10^{5} dpm) of the total ^{3}H-activity (8.0 x 10^{5} dpm) incorporated into DNA during the short pulse. A portion of the released nucleosome fraction (S) was applied to the mercury column and the distribution of the radioactively-labeled DNA was compared in the run-off and Hg-bound nucleosomes. Only 10 % (17,500 dpm) of the total radioactivity (173,000 dpm) was retained in the nucleosomes bound to the column. The comparative specific activities of the Hg-bound and unbound nucleosome fractions (after purification on a Sephacryl S-200 column) were 80 dpm/µg DNA and 2,080 dpm/µg DNA, respectively. A repetition of this experiment in which both fractions obtained by Hg-affinity chromatography were passed through Sephacryl S-200 to remove small non-nucleosomal DNA fragments, also showed that the mercury-bound nucleosomes contained only a small fraction (3.8 x 10^{5} dpm) of the total radioactivity (1.7 x 10^{6} dpm) applied to the column. It follows that, despite the preferential release of newly synthesized DNA sequences during limited micrococcal nuclease digestions, there was no preferential binding of the ^{3}H-thymidine-labeled nucleosomes to the mercury column. On the contrary, the Hg-bound nucleosomes, those with accessible H3-sulfhydryl groups, are grossly deficient in their content of replicating DNA. This result is in accord with previous evidence that the SH-groups of the two H3 molecules in the nucleosomes of replicating *Physarum* chromatin do not come apart at the replication fork (4).

A similar pulse-labeling experiment with an RNA precursor gave a contrasting result. HeLa cells in logarithmic growth were incubated in the presence of uridine[5-^{3}H] for 20 minutes prior to isolation of the nuclei and subsequent chromatographic fractionation of the nucleosomes released into the S fraction by micrococcal nuclease digestion. Radioactively-labeled RNA fragments appeared in both the run-off (4.87 x 10^{5} dpm) and mercury-bound (5.01 x 10^{5} dpm) fractions. Each fraction was then subjected to sucrose density gradient centrifugation to determine whether nucleosomes sedimenting through the gradient remain associated with fragments of the newly synthesized RNA chains. No significant labeling of the 11S monomer peak was detected in the unbound nucleosome fraction; all of the labeled RNA in that fraction remained at the top of the gradient. In contrast, 85 % of the radioactive RNA in the Hg-bound nucleosome fraction (4.23 x 10^{5} dpm) co-sedimented with the nucleosomes, leaving only 15 % (7.8 x 10^{4} dpm) of the applied ^{3}H-activity at the top of the gradient. Since only about 20% of the total nucleosomes applied to the column were retained, the presence of approximately equal amounts of radioactive RNA in the bound and unbound fractions indicates a five-fold enrichment of nascent RNA fragments in the Hg-bound fraction. While these results do not prove that the Hg-bound nucleosomes were actively engaged in RNA synthesis (because the RNA may have been associated with

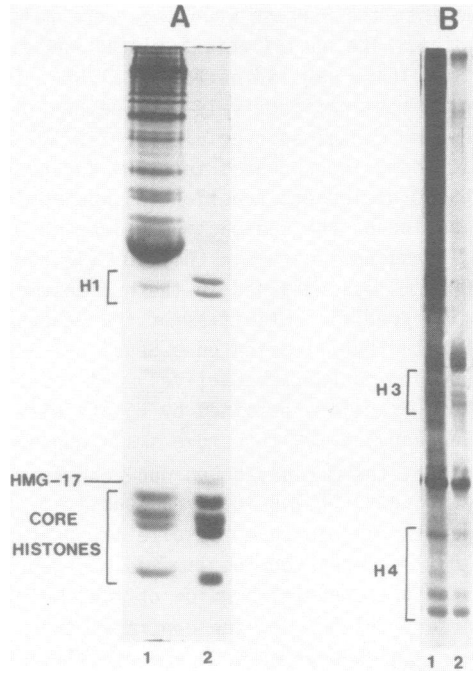

Figure 3. Gel electrophoretic analyses of proteins present in the Hg-bound and unbound nucleosome fractions. A) SDS-polyacrylamide gel electrophoresis of proteins extracted from Hg-bound and unbound nucleosomes (lanes 1 and 2, respectively). The protein bands were visualized by staining with Coomassie brilliant blue-R. B) Triton X-100-acid-urea polyacrylamide gel electrophoresis of proteins from Hg-bound and unbound nucleosomes (lanes 1 and 2, respectively). The protein bands were visualized by silver-staining.

RNP particles which might have co-isolated adventitiously with the nucleosomes during the chromatographic and centrifugal separations), other evidence supports the validity of this conclusion. For example, it has been shown that the corresponding mercury-bound nucleosomes of rat liver contain both RNA and DNA sequences that hybridize to cDNA probes for the albumin and transferrin genes (1). Psoralen cross-linking experiments have also shown the close proximity of nascent RNA chains to histone-containing, nucleosome-like particles *in vivo* (24).

Histones of the Hg-bound and unbound nucleosomes

The protein complements of the run-off and retained nucleosome fractions were compared after each fraction had been further purified by passage through a Sephacryl S-200 column to remove adventitious proteins (of MW less than 200 kD) released during endonuclease digestion of the HeLa nuclei. Electrophoretic analysis of the nucleosomal proteins in SDS-polyacrylamide

gels (13) showed the major core histones, H2A, H2B, H3, and H4, to be present in stoichiometric amounts in both the run-off and mercury-bound nucleosome fractions (Fig. 3A). Histone H1 was also present in the Hg-bound nucleosomes but in lower proportions than observed in the unbound fraction, and in considerably lower amounts than the expected stoichiometry of one H1 per two molecules of each of the core histones (Fig. 3A). The low histone H1 content of the nucleosomes released into the supernatant during a limited digestion with micrococcal nuclease has been noted repeatedly (25 - 28). However, it is also clear that the results of chromatin fractionations following endonuclease digestion vary considerably depending upon the ionic strength and divalent ion concentrations employed (29,30). The ionic conditions we have employed in the isolation and treatment of HeLa cell nuclei would be expected to cause some redistribution of histone H1 (31) and therefore, we cannot conclude that the H1 proportions observed in the isolated nucleosome fractions accurately represent the situation *in vivo*.

Histone modifications in Hg-bound and unbound nucleosomes

Histone acetylation. The modification of nucleosomal "core" histones by the acetylation of one to four lysine residues in their NH_2-terminal domains is a dynamic aspect of histone metabolism which has often been correlated with transcription (reviewed in 32,33). What has been lacking until now is a direct demonstration that the histones of transcriptionally active nucleosomes have high contents of ε-N-acetyllysine while the histones of inactive nucleosomes do not. The separation of active and inactive nucleosomes by Hg-affinity chromatography offers a unique opportunity to compare the histone modifications in the two fractions. The reliability of such measurements, however, depends on the stability and persistance of the hyper-acetylated isoforms of the histones during the isolation of the nuclei and during subsequent steps of nucleosome fractionation. This cannot be achieved unless the deacetylase activities of the cell are inhibited immediately upon lysis and during all further steps of the isolation procedure. This fact is readily demonstrated by experiments comparing the proportions of the various acetylated forms of the core histones isolated from HeLa cells in the presence or absence of the deacetylase inhibitor, Na butyrate (19-23). For this test, HeLa cells were cultured in the presence of 5 mM Na butyrate in order to accumulate high levels of hyperacetylated H3 and H4 (19). To preserve the hyperacetylated forms during isolation of the nucleosomal histones we added 5 mM Na butyrate to the cell lysis buffer and to all other buffers employed. For comparison, an equal aliquot of the HeLa cell suspension was subjected to the same histone isolation procedures, but omitting butyrate from all of the buffers. The levels of histone acetylation in both samples were then compared by electrophoretic separation of the various histone isoforms in acid-urea-Triton gels (14). Because of the positive-charge neutralization due to acetylation of the lysine-ε-amino groups, this procedure resolves the isoforms of histone H4 into 5 bands of decreasing mobility corresponding to the unacetylated form and derivatives containing 1, 2, 3, and 4 ε-N-acetyllysine residues, respectively. Quantitative densitometry of the Coomassie-

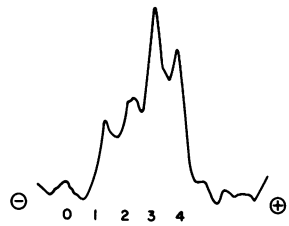

Figure 4. Laser densitometric scanning of an autoradiogram of [^{3}H]carboxymethylated HeLa histone H3. HeLa nuclei were reacted with iodo[^{3}H]acetic acid and the nuclear proteins were subsequently extracted, separated by electrophoresis on Triton-acid-urea gels, and subjected to autoradiography. The numbers 0 - 4 denote the non- through tetra-acetylated H3 isoforms.

stained histone bands gave the following proportions for each isoform when 5 mM Na butyrate was present throughout the isolation: H4-Ac_0=11.8 %; H_4-Ac_1=24.2 %; H4-Ac_2=25.7 %; H4-Ac_3=21.5 %; H4-Ac_4=17.3 %. When HeLa cells from the same culture were lysed in the absence of butyrate and the histones isolated without the inhibitor being present, the corresponding proportions were: H4-Ac_0=80.1 %; H4-Ac_1=17.8 %; H4-Ac_2=1.6 %; H4-Ac_3=0.5 % and H4-Ac_4= not detectable. Consequently, all nucleosome fractions used in Hg-affinity chromatography have been prepared in the constant presence of 5 mM butyrate and, to minimize histone degradation, all buffers contained the protease inhibitors, PMSF and EPNP.

A comparison of the electrophoretic patterns of the histones prepared from the run-off and mercury-bound nucleosomes of HeLa cells in logarithmic growth shows that the histone H4 molecules in the unfolded nucleosomes of the retained fraction have much higher levels of acetylation than do those of the compactly beaded nucleosomes of the run-off fraction (Fig. 3B). The differences in their contents of the tri- and tetra-acetylated forms of histone H4 are particularly striking (Fig. 3B), densitometric analysis showing 40 % of the total H4 to be tri- and tetra-acetylated in the Hg-bound nucleosomes, as compared to only 10.5 % in the unbound fraction.

Similar differences have been found in the levels of acetylation of histone H3. In this case, a more direct assessment of the degree of H3 modification was made possible by exploiting the reactivity of the H3-SH groups in transcriptionally active chromatin *in situ* (1,2). The selective derivatization of histone H3 by the SH-reagent, iodo[^{3}H]acetate, was carried out in intact HeLa nuclei and the histones were then isolated and analyzed by electrophoresis in acid-urea-Triton gels. The proportions of the various acetylated forms of the SH-accessible H3 molecules were determined by autoradiography of the gels and quantitative densitometry of the bands in the H3 region. The results (Fig. 4) clearly establish that the accessible H3 molecules distributed throughout the chromatin are highly acetylated, with 64 % of the radiolabeled H3 present in the tri- and tetra-acetylated

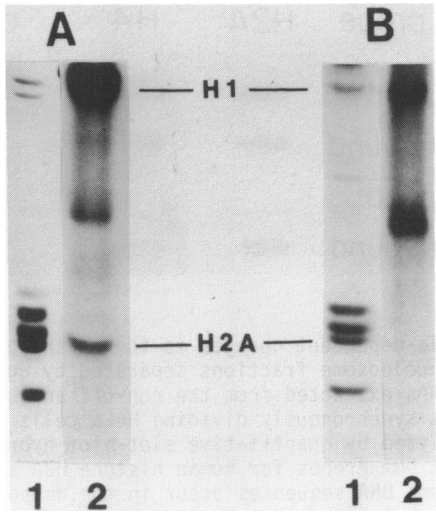

Figure 5. SDS-polyacrylamide gel electrophoretic analysis of protein phosphorylation in unbound and Hg-bound nucleosomes. HeLa cells were incubated in the presence of [^{32}P]orthophosphate and nucleosomes were prepared and fractionated by Hg-affinity chromatography. A) Coomassie blue-stained bands and autoradiography (lanes 1 and 2, respectively) of proteins extracted from unbound nucleosomes. B) Coomassie blue-stained bands and autoradiography (lanes 1 and 2, respectively) of proteins from Hg-bound nucleosomes.

forms and only 21 % present as the non- and mono-acetylated forms. In contrast, no derivatization of H3-sulfhydryl groups takes place in the compactly beaded nucleosomes of inactive chromatin (1). Thus, both procedures confirm that the major histones of the nucleosome core, H3 and H4, are extensively modified in the unfolded nucleosomes to a degree that would be expected to release electrostatic constraints on the associated DNA strand and make it more accessible for transcription.

Phosphorylation of nucleosomal proteins. HeLa cells in logarithmic growth were incubated in the presence of ^{32}P-orthophosphate for 2 hours and the nucleosomes were prepared and fractionated by Hg-affinity chromatography. The histones of the Hg-bound and unbound nucleosomes were extracted, purified by ion-exchange chromatography, and analyzed by SDS-polyacrylamide gel electrophoresis. The distribution of ^{32}P-labeled proteins was determined by autoradiography. A comparison of the histone staining patterns with the corresponding autoradiograms shows that histone H2A in the unbound nucleosomes had incorporated ^{32}P-phosphate (Fig. 5A) while the H2A of the mercury-bound nucleosomes was not ^{32}P-labeled (Fig. 5B). This striking difference in the active and inactive nucleosome fractions suggests that this post-synthetic modification of H2A, unlike the acetylations of histones H3 and H4, may

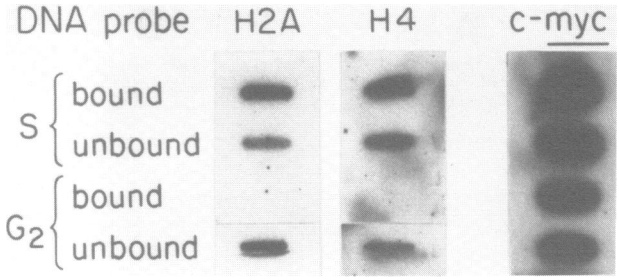

Figure 6. Cell cycle-dependent changes in the distribution of histone gene sequences in nucleosome fractions separated by Hg-affinity chromatography. DNAs extracted from the run-off and Hg-bound nucleosomes prepared from synchronously dividing HeLa cells in the S- and G_2-phases were analyzed by quantitative slot-blot hybridizations to ^{32}P-labeled genomic DNA probes for human histone H2A and H4 genes. Note that the histone DNA sequences occur in the Hg-bound nucleosomes during the S-phase, but not during the G_2-phase. The column on the right compares the hybridization of the nucleosomal DNA fractions to a human c-myc DNA probe (a 9 kb Eco Rl - Hind III restriction fragment isolated from plasmid pHSR-1 (42)). All hybridizations employed 1 µg of the ^{32}P-labeled DNA probes.

correlate with the transcriptional *in*activity of the associated DNA. This conclusion would be in accord with observations showing high levels of H2A phosphorylation in the transcriptionally inert heterochromatin of *Peromyscus* cells (34). Because we have not used phosphatase inhibitors, such as 50 mM Na bisulfite (because of possible changes in the redox state of the histone H3 cysteine residues), the results must be interpreted with caution (cf. 35).

Other phosphorylations of nucleosomal proteins involve histone H1 and an unidentified protein migrating between histones H1 and H2A (Fig. 5). The unidentified protein did not comigrate with HMG-14.

Nucleosome structural transitions on histone genes

The synthesis of the major histones of the nucleosome core particle is coordinated with DNA synthesis in the S-phase of the cell cycle (reviewed in 36,37). This apparent coupling of histone and DNA biosynthesis is achieved by both transcriptional and posttranscriptional control mechanisms which regulate the synthesis of the histone mRNAs (9,38,39) and the stability of the histone messages (9). We have focussed on the relationship between histone gene transcription and chromatin structure by comparing the nucleosomes of the genes for histones H2A and H4 in their transcriptionally active and inactive states, using mercury-affinity chromatography as a probe for nucleosome conformation.

HeLa-S3 cells were synchronized at the G_1/S boundary by successive blocks with thymidine and aphidicolin (9). Following removal of the aphidicolin, the cells were allowed to progress through the cell cycle, taking

aliquots of the suspension at mid-S phase (5 hours) and in the G_2-phase (10 hours). The nuclei were isolated, and the nucleosomes released by a brief micrococcal nuclease digestion were fractionated by Hg-affinity chromatography. The DNA sequence contents of the run-off and Hg-bound nucleosome fractions were probed by slot-blot hybridizations to ^{32}P-labeled cloned genomic probes for human histone H2A and H4 genes (16). The results (Fig. 6) show that the nucleosomes containing these histone gene sequences were retained by the mercury column during the S-phase when transcription was active. Quantitative densitometry of the hybridization signals in the mercury-bound and unbound nucleosomes showed 70 % of the H2A and 60 % of the H4 DNA sequences in the S fraction to be present in the bound nucleosomes. These sequences were not retained by the mercury column during the G_2-phase when transcription of the major histone genes is repressed. At that time, all of the hybridization signal was recovered in the run-off nucleosome fraction (Fig. 6). It follows that the unfolded nucleosomes containing the active H2A and H4 genes must have reverted to the compact beaded state when their transcription had been terminated. This evidence for the reversibility of nucleosome unfolding is in accord with recent findings showing the rapid reversibility of nucleosome conformational changes during the activation and repression of the c-fos and c-myc proto-oncogenes in serum-stimulated murine fibroblasts (8).

In HeLa cells, as in other cell types (40,41), c-myc is transcribed throughout the cell cycle. We have examined the Hg-bound and unbound nucleosome fractions prepared during the S- and G_2-phases for their contents of human c-myc DNA sequences. Slot-blot hybridizations to a ^{32}P-labeled 9 kb human c-myc DNA probe (42), showed the c-myc sequences to be present in the active, Hg-bound nucleosomes at both stages of the cell cycle, in agreement with the known cell cycle-independence of c-myc expression (40,41).

As a control for a gene not expressed in HeLa cells, we examined the Hg-bound and unbound nucleosome fractions for their contents of α-globin DNA sequences. No α-globin DNA could be detected in the nucleosomes retained by the mercury column during either the S- or G_2-phase of the cycle. However, only a weak hybridization to the ^{32}P-labeled α-globin cDNA probe was detected in the run-off nucleosome fractions, and we conclude that much of the inactive α-globin chromatin was not released from the nuclei during the brief digestion with micrococcal nuclease (which released only 10 % of the total nuclear DNA into the S fraction).

DISCUSSION

The selective retention on mercury columns of nucleosomes containing accessible histone H3-sulfhydryl groups provides a new experimental approach to the analysis of nucleosome structural and conformational changes during transcription. Because the unfolding of the nucleosome uncovers the cys-110 sulfhydryl groups of histone H3, which are known to be centrally located in the nucleosome core (3,4,6,43-47), it follows that some DNA-protein

contacts in the middle of the core particle have been altered. And, judging by electron micrographs of the unfolded nucleosomes of *Physarum* ribosomal genes (2), protein/protein contacts within the histone octamer must also have been altered in order to generate the apparent pairs of half-nucleosomes.

Comparisons of the mercury-bound and unbound nucleosomes of HeLa cells show that both fractions have a full complement of core histones H2A, H2B, H3 and H4, but they differ with respect to the postsynthetic modifications of those histones. The Hg-bound nucleosomes have much higher contents of the tri- and tetra-acetylated forms of H3 and H4, histones which occupy the central domain of the nucleosome core (45-47). Because the hyperacetylation of histones H3 and H4 neutralizes the positive charges on the lysine residues clustered in their NH_2-terminal domains, this would be expected to release some of the electrostatic constraints on the enveloping DNA strand.

Several lines of evidence point to acetylation as playing a key role in the mechanism of nucleosome unfolding. For example:

1] comparisons of the thermal denaturation curves of hyperacetylated and hypoacetylated nucleosomes show that the major cooperative transitions in the melting profile (due to melting in the central domain of the nucleosomal DNA) consistently occur at lower temperatures for the hyperacetylated nucleosomes than for the controls (48),

2] the DNA of hyperacetylated nucleosomes is far more accessible to attack by DNase I (23,49,50). And, most significantly, this involves a site at the middle of the nucleosome 60 base-pairs from the ends of the DNA which is DNase I-resistant in unacetylated nucleosomes (49,51). The same site was shown by cross-linking experiments to be a strong binding domain for histone H4 (52),

3] hyperacetylation of the histones in butyrate-treated cells is known to alter nucleosome conformation, as evidenced by an increased reactivity of histone H3 sulfhydryl groups (7), by an enhanced susceptibility of the H3 NH_2-terminal region to phosphorylation (53), and by striking changes in the accessibility of several of the histone epitopes to specific antibodies (54).

The present experiments show that conformational changes associated with histone hyperacetylation can be exploited to permit the isolation of transcriptionally active nucleosomes by selective retention of the unfolded nucleosomes on organomercurial-agarose column. The results provide new and direct evidence in support of the view that hyperacetylation of histones H3 and H4 may control the accessibility for transcription of the associated DNA sequences, as originally proposed (55). The fact that the hyperacetylated Hg-bound nucleosomes contain histone gene DNA sequences when they are being transcribed in the S-phase, and not when their transcription is repressed in the G_2-phase, is strong evidence that nucleosome conformation is dynamically controlled to coordinate with transcription. This is a reversible process which, for some genes, such as c-myc and c-fos, may occur within minutes (8). The rapid kinetics of histone acetylation and deacetylation are compatible with this requirement. How such differential acetylation is achieved in active and inactive chromatin remains a challenging and unsolved problem.

An important question affecting the utility of Hg-affinity chromatography as a means for selecting transcriptionally active nucleosomes is whether the assembly of nucleosomes in replicating chromatin involves sufficient exposure of the histone H3-sulfhydryl groups to allow their retention on the mercury column. Since all of the DNA is replicated while only a fraction is transcribed, this would seriously limit the range of application of Hg-affinity chromatography as a device for the isolation of the chromatin subunits in their transcriptionally active configuration. Fortunately, the pulse-labeling of HeLa cells with ^{3}H-thymidine during the S-phase shows that the Hg-bound nucleosomes are grossly deficient in newly replicated DNA. It follows that the presence of histone H2A and H4 DNA sequences in S-phase nucleosomes is not likely to represent a selective binding to the mercury column of nucleosomes from replicating histone genes.

Finally, it should be noted that changes in nucleosome topography are only a part of a much broader program of chromatin reorganization associated with transcription (see 56-58 for reviews). In the case of the human histone H4 gene, an upstream DNase I-hypersensitive site expands during transcription and narrows again to its original site when the S-phase is completed (59). There is also a disruption of the canonical nucleosome spacing which correlates with transcription in the early S-phase. Further remodelling at the 5'-end of H4 chromatin occurs when transcription and replication are complete (60). How these changes relate to nucleosome unfolding in different regions of the histone genes remains to be determined.

ACKNOWLEDGEMENTS

We thank Dr. Nathaniel Heintz for the generous gift of the human histone H2A and H4 genomic probes, and Dr. Marianne Salditt Georgiev for the human α-globin cDNA probe. This research was supported by NIH Grants GM-17383 and CA-14908 and by NATO Grant RG 83/0943.

[1]Present address: Istituto Nazionale per la Ricerca sul Cancro, Viale Bendetto XV, no. 10, 16132 Genova, Italia
*To whom correspondence should be addressed

REFERENCES

1. Allegra, P., Sterner, R., Clayton, D.F. and Allfrey, V.G. (1987) J. Mol. Biol. 196 (in press).
2. Prior, C.P., Cantor, C.R., Johnson, E.M., Littau, V.C. and Allfrey, V.G. (1983) Cell 34, 1033-1042.
3. Zama, M., Olins, D.E., Wilkinson-Singley, E. and Olins, A. (1978) Biochem. Biophys. Res. Commun. 85, 1446-1452.
4. Prior, C.P., Cantor, C.R., Johnson, E.M. and Allfrey, V.G. (1980) Cell 20, 597-608.
5. Wong, N.T.N. and Candido, E.P.M. (1978) J.Biol.Chem. 253, 8263-8268.
6. Dieterich, A.E., Axel, R., and Cantor, C.R. (1978) Cold Spring Harbor Symp. Quant. Biol. 42, 199-206.

7. Bode, J., Henco, K. and Wingender, E. (1980) Eur. J. Biochem. 110, 143-152.
8. Chen, T.A. and Allfrey, V.G. (1987) Proc. Natl. Acad. Sci. USA (in press).
9. Heintz, N., Sive, H.L. and Roeder, R.G. (1983) Mol. Cell. Biol. 3, 539-550.
10. Maniatis, T., Fritsch, E. and Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY)
11. Burton, K. (1968) Methods Enzymol. 12, 163-169.
12. Maniatis, T., Jeffrey, A. and van deSande, H. (1978) Biochemistry 14, 3787-3794.
13. Laemmli, U. (1970) Nature 222, 680-685.
14. Alfageme, C.R., Zweidler, A., Mahowald, A. and Cohen, L.C. (1974) J. Biol. Chem. 249, 3729-3736.
15. Wray, W., Boulikas, T., Wray,V.P. and Hancock, R. (1981) Anal. Biochem. 118, 197-203.
16. Zhong, R., Roeder, R.G. and Heintz, N. (1983) Nucl. Acids Res. 11, 7409-7425.
17. Wilson, J.T., Wilson, L.B., deRiel, J.K., Villa-Komarov, L., Efstradiadis, A., Forget, B.G. and Weissman, S.M. (1978) Nucl. Acids Res. 5, 563-581.
18. Rigby, P.W.J., Dieckman, M., Rhodes, C. and Berg, P. (1977) J. Mol. Biol. 113, 237-251.
19. Riggs, M.G., Whittaker, R.G., Neumann, J.R. and Ingram, V.M. (1977) Nature 268, 462-464.
20. Boffa, L.C., Vidali, G. and Allfrey, V.G. (1978) J. Biol. Chem. 253, 3664-3666.
21. Candido, E.P.M., Reeves, R. and Davie, J.R. (1978) Cell 14, 105-113.
22. Sealy, L. and Chalkley, R. (1978) Cell 14, 115-121.
23. Vidali, G., Boffa, L.C., Bradbury, E.M. and Allfrey, V.G. (1968) Proc. Natl. Acad. Sci. USA 75, 2239-2243.
24. Sargan, D.R. and Butterworth, P.H.W. (1985) Nucl. Acids Res. 13, 3805-3822.
25. Davie, J.R. and Saunders, C.A. (1981) J. Biol. Chem. 256, 12574-12580.
26. Egan, P.A. and Levy-Wilson, B. (1981) Biochemistry 20, 3695-3702.
27. Rocha, E., Davie, J.R., vanHolde, K.E. and Weintraub, H. (1984) J. Biol. Chem. 259, 8556-8563.
28. Rose, S.M. and Garrard, W.T. (1984) J. Biol. Chem. 259, 8534-8544.
29. Ausio, J., Sasi, R. and Fasman, G.D. (1986) Biochemistry 25, 1981-1988.
30. Walker, P.R. and Sikorska, M. (1986) Biochemistry 25, 2839-2845.
31. Caron, F. and Thomas, J.O. (1981) J. Mol. Biol. 146, 513-537.
32. Allfrey, V.G. in Cell Biology: A Comprehensive Treatise, Goldstein, L. and Prescott, D.M., Eds. Vol. 3, pp. 347-437, Academic Press, New York.
33. Matthews, H.R. and Waterborg, J.H. (1985) in The Enzymology of Post-Translational Modifications of Proteins, Freedman, R. and Hawkins, H.C., Eds., Vol. 2, pp. 125-185, Academic Press, London.
34. Halleck, M.S. and Gurley, L.R. (1980) Exptl. Cell Res. 125, 377-388.
35. Halleck, M.S. and Gurley, L.R. (1982) Exptl. Cell Res. 138, 271-285.
36. Pederson, T. (1976) in Protein Synthesis, McConkey, E.H., Ed., pp. 69-123, Marcel Dekker, New York.
37. Elgin, S.C.R. and Weintraub, H. (1975) Annu. Rev. Biochem. 44, 725-774.
38. Baumbach, L.L., Marashi, F., Plumb, M., Stein, G. and Stein, J. (1984) Biochemistry 23, 1618-1625.
39. Plumb, M., Stein, J. and Stein, G. (1983) Nucl. Acids Res. 11, 2391-2410.
40. Thompson, C.B., Challoner, P.B., Neiman, P.E. and Groudine, M. (1985) Nature 314, 363-366.
41. Rabbitts, P.H., Watson, J.W., Lamond, A., Forster, A., Stinson, M.A., Evan, G., Fischer, W., Atherton, E., Sheppard, R. and Rabbitts, T.H. (1985) EMBO J. 4, 2009-2013.

42. Alitalo, K. Schwab, M., Lin, C.C., Varmus, H.E. and Bishop, J.M. (1983) Proc. Natl. Acad. Sci. USA 80, 1707-1711.
43. Camerini-Otero, R.D. and Felsenfeld, G. (1977) Proc. Natl. Acad. Sci. USA 74, 5519-5523.
44. Eshagpour, H., Dieterich, A.E., Cantor, C.R. and Crothers, D.M. (1980) Biochemistry 19, 1797-1805.
45. Bavykin, S.G., Usachenko, S.L., Lishanskaya, A.I., Schick, V.V., Belyavsky, A.V., Undritsov, I.M., Strokov, A.A., Zalenskaya, I.A. and Mirzabekov, A.D. (1985) Nucl. Acids Res. 13, 3439-3459.
46. Richmond, T.J., Finch, J.T., Rushton, B., Rhodes, D. and Klug, A. (1984) Nature 311, 532-537.
47. Burlingame, R.W., Love, W.E., Wang, B.C., Hamlin, R., Xuong, N.H. and Moudrianakis, E.N. (1985) Science 228, 546-553.
48. Yau, P., Thorne, A.W., Imai, B.S., Matthews, H.R. and Bradbury, E.M. (1982) Eur. J. Biochem. 129, 281-288.
49. Simpson, R.T. (1978) Cell 13, 691-699.
50. Sealy, L. and Chalkley, R. (1978) Nucl. Acids Res. 5, 1863-1876.
51. Ausio, J. and van Holde, K.E. (1986) Biochemistry 25, 1421-1428.
52. Ebralidse, K.K. and Mirzabekov, A.D. (1986) FEBS Lett. 194, 69-72.
53. Whitlock, J.P., Augustine, R. and Schulman, H. (1980) Nature 287, 74-76.
54. Muller, S., Erard, M., Burggraf, E., Coupper, M., Sautiere, P., Champagne, M. and van Regenmortel, M.H.V. (1982) EMBO J. 1, 939-944.
55. Allfrey, V.G., Faulkner, R. and Mirsky, A.E. (1964) Proc. Natl. Acad. Sci. USA 51, 786-794.
56. Cartwright, J.L., Abmayr, S.M., Fleischmann, G., Loewenhaupt, K., Elgin, S.C.R., Keene, M.A. and Howard, C.C. (1982) CRC Crit. Rev. Biochem. 13, 1-86.
57. Igo-Kemenes, T., Horz, W. and Zachau, H.G. (1982) Annu. Rev. Biochem. 51, 89-121.
58. Reeves, R. (1984) Biochim. Biophys. Acta 782, 343-393.
59. Chrysogelos, S., Riley, D.E., Stein, G. and Stein, J. (1985) Proc. Natl. Acad. Sci. USA 82, 7535-7539.
60. Moreno, M.L., Chrysogelos, S.A., Stein, G.S. and Stein, J. (1986) Biochemistry 25, 5364-5370.