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Histone Hyperacetylation Does Not Alter the Positioning or Stability of Phased Nucleosomes on the Mouse Mammary Tumor Virus Long Terminal Repeat

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Received September 26, 1990; Revised Manuscript Received December 7, 1990

ABSTRACT: Activation of mouse mammary tumor virus transcription by the hormone-bound glucocorticoid receptor results in disruption of a nucleosome that is specifically positioned on the promoter. Limited treatment of cells with the histone deacetylase inhibitor sodium butyrate prevents receptor-dependent promoter activation and nucleosome disruption [Bresnick, E. H., John, S., Berard, D. S., LeFebvre, P., & Hager, G. L. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 3977-3981]. On the basis of this observation, we undertook a series of experiments to compare the structure of normal and hyperacetylated mouse mammary tumor virus chromatin. Although butyrate prevents hormone-induced restriction enzyme cutting specifically in the B nucleosome region, chromatin containing hyperacetylated histones does not differ from normal chromatin in general sensitivity to restriction enzymes. Indirect end-labeling analysis of micrococcal nuclease digested chromatin reveals that nucleosomes are identically phased on the mouse mammary tumor virus long terminal repeat in normal and hyperacetylated chromatin. A synthetic DNA fragment spanning the B nucleosome region was reconstituted into a monosome by using core particles containing normal or hyperacetylated histones. Analysis of the structure of reconstituted monosomes by nondenaturing polyacrylamide gel electrophoresis, salt stability, thermal stability, restriction enzyme accessibility, and exonuclease III or DNase I footprinting reveals no effect of histone hyperacetylation on monosome structure. These observations suggest that histone hyperacetylation does not induce a major change in the structure of mouse mammary tumor virus chromatin, such as nucleosome unfolding. We propose that inhibition of receptor-dependent nucleosome disruption by butyrate is not dependent upon such a general change in chromatin structure and subtle chromatin modifications may modulate the interaction of glucocorticoid receptor with chromatin-associated recognition sites.

The hormone-bound glucocorticoid receptor (GR)[1] regulates MMTV transcription by binding to specific DNA regulatory elements (GREs) on the MMTV promoter. A complete understanding of this transactivation mechanism will require a definition of how the GR interacts with soluble transcription factors to form competent transcription complexes and how the organization of MMTV regulatory sequences into chromatin impacts transcription initiation and elongation.

The MMTV LTR exists as a highly reproducible chromatin structure containing six specifically positioned nucleosomes (Richard-Foy & Hager, 1987). Although initial nucleosome mapping studies were performed with LTR-reporter chimeras in BPV-based episomes (Richard-Foy & Hager, 1987), recent studies have shown that identical nucleosome positions exist on single copy integrated MMTV proviruses (H. Richard-Foy and G. L. Hager, unpublished data). We are using the BPV-based episomal system (Ostrowski et al., 1983) to address two issues: (i) what are the differences between transcriptionally inactive and active MMTV chromatin and (ii) does the specific nucleosomal organization have an active regulatory role in hormone-dependent transcription?

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[1] Abbreviations: BPV, bovine papillomavirus; DNase I, deoxyribonuclease I; DTT, dithiothreitol; Exo III, exonuclease III; GR, glucocorticoid receptor; GRE, glucocorticoid regulatory element; LTR, long terminal repeat; MMTV, mouse mammary tumor virus; NF1, nuclear factor 1; nuc-B, nucleosome B; PBS, phosphate-buffered saline; TA (triamcinolone acetonide), 9α-fluoro-11β,16α,17α,21-tetrahydroxypregna-1,4-diene-3,20-dione 16,17-acetonide; TFIID, transcription factor IID; 30S, rat retroviral-associated cellular DNA sequences.

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In response to glucocorticoid hormone, a strong endonuclease hypersensitive site develops in the promoter region spanning one of the nucleosomes in the phased array, nuc-B (Richard-Foy & Hager, 1987; Richard-Foy et al., 1987; Bresnick et al., 1990). We have hypothesized that GR binding is a primary signal to initiate the structural change in this region (Richard-Foy & Hager, 1987; Hager, 1989). A stable complex consisting of at least two transcription factors, NF1 and TFIID, forms on the MMTV promoter in hormone-treated cells (Cordingley & Hager, 1988; Cordingley et al., 1987). Mutagenesis studies have shown that the cis elements recognized by these factors are required for hormone-regulated transcription initiation (Buetti & Kuhnel, 1986), and purified NF1 enhances in vitro transcription of naked MMTV LTR templates in HeLa cell extracts that contain basal transcription factors but lack the hormone-activated GR (M. G. Cordingley and G. L. Hager, unpublished data). These results are consistent with a model in which NF1 is excluded from its cognate recognition site by nuc-B and receptor-dependent nucleosome disruption derepresses the MMTV promoter by facilitating access of factors to their cognate recognition sites in chromatin (Hager, 1989). Although this model presupposes that a major structural change occurs in the nuc-B region, the precise mechanism by which nuc-B is disrupted and, in a more general manner, how transactivating factors target specific chromatin regions for change are not known.

We previously reported that GR-dependent nucleosome disruption does not occur when cells are treated with sodium butyrate prior to hormone treatment (Bresnick et al., 1990). Evidence was provided that butyrate does not significantly affect the amount or hormone-binding affinity of GR and that hormone-induced expression of an MMTV–luciferase reporter in a transient transfection assay is not affected by butyrate. One specific action of butyrate is to induce histone hyperacetylation by inhibiting histone deacetylase (Sealy & Chalkley, 1978; Boffa et al., 1978). We proposed that the inhibitory activity of butyrate on MMTV promoter activation may result from decreased histone deacetylase activity. This could prevent nucleosome disruption either (i) by altering the ability of GR to form a productive complex with GREs in hyperacetylated chromatin or (ii) by causing a change in the structure or stability of MMTV chromatin that prevents the nucleosome-bound GR from initiating nucleosome disruption.

With regard to the second mechanism, there are a number of conflicting reports on the effects of histone acetylation on nucleosome structure and stability [for reviews see Csordas (1990) and Loidl (1988)]. One mechanism by which histone acetylation is thought to influence chromatin structure is that the acetyl group neutralizes the positive charge of highly conserved lysines (e.g., Lys-5, Lys-8, Lys-12, and Lys-16 of histone H4) on the amino-terminal tails of the core histones, decreasing the affinity of histones for the phosphodiester backbone of DNA. Although some studies have proposed that hyperacetylated nucleosomes are partially unfolded (Bode et al., 1983; Oliva et al., 1990) or less stable (Yau et al., 1982; Bode et al., 1983) than normal nucleosomes, several very careful studies show little if any effect of acetylation on the physical state of nucleosomes (Imai et al., 1986; Ausio & van Holde, 1986). It is important to note that all of these studies analyze total cell chromatin, and the possibility exists that intranucleosomal effects of hyperacetylation may be dependent upon the particular DNA sequence in the nucleosome. In addition, as pointed out by Oliva et al. (1990), differences in experimental conditions (e.g., chromatin source, buffer conditions, and specific assays utilized) make it very difficult to compare the results from one study with another. In the work reported here, we use the homogeneous BPV-based episomal system (Ostrowski et al., 1983) and in vitro nucleosome reconstitution on a defined DNA fragment to ask if hyperacetylation alters MMTV chromatin structure. We fail to detect major changes in nucleosome positioning or stability, suggesting that the inability of GR to activate the MMTV promoter in hyperacetylated chromatin does not involve such changes.

Materials and Methods

Cells, Tissue Culture, and Plasmids. The mouse cell line 1361.5 was previously derived from NIH3T3 cells by transformation with the BPV vector pM23 (Cordingley et al., 1987). Plasmid pM23 contains the MMTV LTR driving the *v-Ha-ras* gene with a simian virus 40 (SV-40) polyadenylation signal and small tumor (t) antigen splice site inserted downstream of the *ras* coding sequence. Episomes (pM23) stably replicate in 1361.5 cells at approximately 125 copies. Plasmid pC18 contains a synthetic nuc-B fragment that was constructed by ligating 12 overlapping oligonucleotides spanning the nuc-B region from −255 to −45 and cloning this molecule into pUC18.

Nuclei Isolation and Restriction Enzyme Sensitivity Assay. Nuclei were isolated from 5×10^7 cells that had been treated as described in the figure captions. For cells treated with sodium butyrate, all buffers were supplemented with 10 mM butyrate. Briefly, cells were scraped into ice-cold PBS and were collected by centrifugation for 3 min at 200*g*. Cells were washed once by resuspension in PBS and recentrifugation. The washed cell pellet was resuspended in 10 mM Tris-HCl (pH 7.4)/15 mM NaCl/60 mM KCl/0.15 mM spermidine/0.5 mM spermine/1 mM EDTA/0.1 mM EGTA/0.2% nonidet-P40/5% sucrose/1 mM DTT at 5×10^7 cells/mL, and cells were lysed by 10 strokes of a Dounce homogenizer (B pestle). This material was layered on a cushion of lysis buffer containing 10% sucrose and centrifuged at 1600*g* for 8 min. The supernatant was discarded, and the nuclei were washed by resuspension in 10 mM Tris-HCl (pH 7.4)/15 mM NaCl/60 mM KCl/0.15 mM spermidine/0.5 mM spermine/1 mM DTT (2.5×10^7 nuclei/mL), followed by recentrifugation at 600*g* for 5 min. Washed nuclei were resuspended in the appropriate restriction enzyme buffer (10^7 nuclei/0.1 mL) and were incubated for 8 min at 30 °C with 20–40 units of enzyme. Reactions were terminated by adding 0.5 mL of 10 mM Tris-HCl (pH 8.0)/25 mM EDTA/1% SDS/0.4 mg/mL proteinase K, and samples were incubated for at least 5 h at 37 °C. Genomic DNA was purified by multiple extractions with phenol/chloroform followed by ethanol precipitation. Purified genomic DNA was restricted with *Ava*I or *Pst*I to excise the LTR–*v-Ha-ras* cassette, and 10–20 μg of restricted genomic DNA was resolved on 1.0–1.5% agarose gels. Indirect end-labeling analysis of enzyme cuts in chromatin was carried out by transferring DNA to Nytran (Schleicher and Schuell) membrane, cross-linking DNA to the membrane by ultraviolet irradiation, and hybridizing blots with an *Ava*I–*Cla*I fragment of the LTR (P4A probe).

Micrococcal Nuclease Digestion of Nuclei. Nuclei (10^7) were resuspended in 0.5 mL of nuclei wash buffer containing 1 mM $CaCl_2$ and were incubated for 5 min at 22 °C with 40–80 units of micrococcal nuclease (Worthington). Reactions were terminated, and genomic DNA was prepared as described above for the restriction enzyme sensitivity assay.

Purification of Core Particles for in Vitro Nucleosome Reconstitution. Cores particles were prepared from C127 cells by the method of Ausio and Van Holde (1986). Nuclei were isolated from untreated cells or cells that were treated with

10 mM butyrate for 12 h. All buffers contained 0.1 mM PMSF, and 5 mM butyrate was included in all buffers for purifying hyperacetylated histones. Briefly, nuclei were resuspended in 50 mM Tris-HCl (pH 7.5)/100 mM KCl/1 mM $CaCl_2$ and were digested with 9 units of micrococcal nuclease/mL for 5 min at 37 °C. The digestion was terminated with 10 mM EDTA. After centrifugation at 12000*g* for 5 min in a Ti50 rotor, the supernatant was discarded and the pellet was resuspended in 1.5 mL of 0.25 M EDTA (pH 7.5) and extracted with constant rotation for 1 h at 4 °C. Following centrifugation at 8000*g* for 20 min, the supernatant was collected and adjusted to 350 mM NaCl by dropwise addition of 4M NaCl, and histones H1 and H5 were removed by incubating with CM-25 Sephadex (60 mg/mL) for 2 h at 4 °C with constant rotation. After the Sephadex was removed by centrifugation, the supernatant was dialyzed against 10 mM Tris (pH 7.5)/25 mM NaCl/1 mM $CaCl_2$/0.5 mM DTT/0.1 mM PMSF, with or without 5 mM butyrate for 16 h at 4 °C. Chromatin fragments were digested with micrococcal nuclease to yield predominantly mononucleosomes. Core particles were purified on 5–25% sucrose gradients. Pooled core particles were concentrated and equilibrated in 10 mM Tris-HCl (pH 8.0)/0.1 mM EDTA/1 mM DTT/5% glycerol (with or without 5 mM butyrate) with Centricon 30 microconcentrators to final protein concentrations of 1–4 mg/mL. Core particles purified in this manner were stable for several months at 4 °C. However, due to our concern that hyperacetylated particles might become deacetylated upon prolonged storage despite the presence of butyrate in the storage buffer, particles that were used to reconstitute hyperacetylated monosomes were less than 1 month old. In a control experiment, 1 h prior to nuclei isolation, 2 mCi of [^{3}H]acetic acid (ICN; 27 Ci/mmol) was added to one roller bottle each of untreated and butyrate-treated cells. Sucrose gradient purified cores from butyrate-treated cells contained 2.6-fold more [^{3}H]acetate incorporated into histones than cores from untreated cells.

In Vitro Nucleosome Reconstitution. Plasmid pC18 was linearized with *Xba*I, dephosphorylated with bacteria alkaline phosphatase, and end-labeled with [^{32}P]ATP and T4 polynucleotide kinase. The radioactive B nucleosome fragment (100–200 ng) was isolated by digestion with *Sal*I and purified on a 5% nondenaturing acrylamide gel. The fragment (10–20 ng $\approx$ 100 000–300 000 cpm) was incubated in 50 mM Tris-HCl (pH 8.0), 12 mM $MgCl_2$, 1 mM β-mercaptoethanol, and 800 mM NaCl with a 200-fold mass excess of core particles for 30 min at 37 °C, followed by 16 h at 4 °C to allow exchange of the histone octamer from the donor core particles to the end-labeled B nucleosome fragment. Reconstitution was achieved by diluting the mixture with 50 mM Tris-HCl (pH 8.0)/12 mM $MgCl_2$/10% glycerol to 0.6 M NaCl and incubating for 30 min at 4 °C, followed by dilution to 0.1 M NaCl with the same buffer and incubating for 30 min at 37 °C. The final volume of the reconstitution reaction was 200 μL. For all reconstitutions, a mock reconstitution was performed in which the core particles were omitted. Mock reconstituted samples were used as naked DNA controls for restriction enzyme, Exo III, and DNase I digestions. Reconstituted monosomes either were used immediately or were stored at 4 °C for up to 1 week in which no significant dissociation of the monosomes was observed.

Digestion of Reconstituted Monosomes with Restriction Enzymes, Exo III, and DNase I. Typically, 5000, 10 000, and 30 000 cpm of reconstituted monosomes were used for restriction enzyme, Exo III, and DNase I digestions, respectively. This corresponds to approximately 0.25, 0.50, and 1.5 ng of the *Xba*I–*Sal*I fragment of pC18. For restriction enzyme digestions, the reconstitutes were adjusted to 50, 100, and 100 mM NaCl for *Sac*I, *Hae*III, and *Eco*RI, respectively. Samples were incubated with 5–10 units of enzyme for 15 min at 22 °C. Reactions were terminated by phenol/chloroform extraction, and DNA was purified by ethanol precipitation. For Exo III digestions, reconstitutes were incubated in reconstitution buffer containing 1 mM DTT and 5000 or 10 000 units of Exo III (New England Biolabs) for free DNA and reconstitutes, respectively. For DNase I digestions, reconstitutes were diluted with an equal volume of 10 mM Tris (pH 8.0)/100 mM NaCl/5% glycerol. DNase I (U.S. Biochemical; 10–20 units for reconstitutes or 5–10 units for free DNA) was added, and samples were incubated for 4 min at 22 °C. Reactions were terminated with 10 mM EDTA, followed by phenol/chloroform extraction, and DNA was purified as described above.

Results and Discussion

Normal and Hyperacetylated MMTV LTR Chromatin Are Equally Sensitive to Restriction Enzymes. If histone hyperacetylation causes nucleosome unfolding, or decondensation of higher order chromatin structures, one might expect that these changes would alter the access of restriction enzymes to their DNA recognition sites, similar to the mechanism whereby access of transcriptional regulatory proteins to their specific recognition sites in chromatin is limited. To determine if histone hyperacetylation affects the sensitivity of the MMTV LTR to restriction enzymes, we utilized the 1361.5 cell line, which contains approximately 125 copies of the chimeric LTR–*v-Ha-ras* BPV-based episome pM23 (Cordingley et al., 1987; Bresnick et al., 1990).

As shown in lanes 2–5 of Figure 1, incubation of intact nuclei with *Mbo*I results in cutting of episomal chromatin at three sites: in the 30S rat retroviral-associated sequence linking the LTR with the *v-Ha-ras* open reading frame, in nuc-B, and in the linker between nucleosomes C and D. Cutting on nuc-B is strongly enhanced by hormone treatment (compare lanes 2 and 3), and a weaker enhancement of cutting is seen in the C–D linker. Hormone treatment has no effect on constitutive cutting at the 30S site. These results demonstrate the utility of the restriction enzyme sensitivity assay to monitor conformational states of chromatin that influence the accessibility of chromatin sites to DNA-binding proteins.

Pretreatment of 1361.5 cells with 10 mM butyrate for 4 h prevents hormone-induced enzyme cutting in nuc-B and in the C–D linker without altering constitutive *Mbo*I cutting at the 30S site. Lanes 6–9 of Figure 1 show that *Afl*II cutting in the nuc-B region is enhanced by hormone treatment and that butyrate also prevents hormone-induced *Afl*II cutting. Although induction of histone hyperacetylation with butyrate prevents hormone-induced enzyme cutting, we have not detected any significant differences between the sensitivity of normal and hyperacetylated MMTV chromatin to *Mbo*I or *Afl*II (compare lanes 2 and 6 with lanes 5 and 7). In our previous work (Bresnick et al., 1990), low levels of *constitutive* cutting by *Sst*I in nuc-B and DNase I sensitivity of the LTR were not altered by butyrate treatment.

A similar approach using restriction enzymes as probes for chromatin structure was recently utilized to examine the chromatin organization of the *Saccharomyces cerevisiae* pho5 promoter region (Fascher et al., 1990). In this study, activation of pho5 transcription resulted in greater than 80% cutting on the promoter by *Cla*I, whereas less than 5% cutting was observed in the repressed state. Although our enzyme sensitivity assay does not result in changes of this magnitude, it is ob-

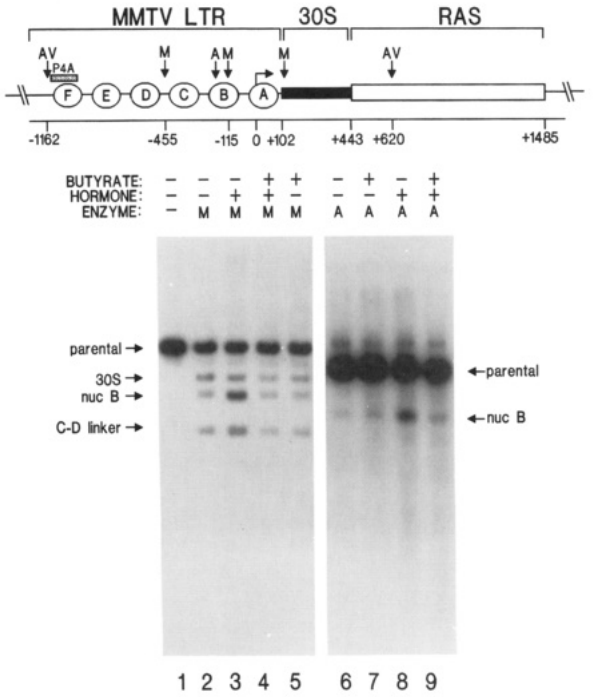

FIGURE 1: Glucocorticoid hormone-induced restriction enzyme sensitivity in the B nucleosome region of MMTV chromatin. Cells (1361.5, episome pM23) were treated with 10 mM butyrate or buffer for 4 h, followed by 100 nM TA or vehicle for 1 h. Nuclei were isolated and were resuspended in restriction enzyme digestion buffer. After an incubation for 8 min at 30 °C with or without 40 units of *Mbo*I (lanes 2–5) or 20 units of *Afl*II (lanes 6–9), nuclei were digested with 0.4 mg/mL proteinase K. Genomic DNA was purified and restricted with *Ava*I, and 20 μg was resolved on a 1.5% agarose gel. Bands were detected by Southern blot analysis with the P4A probe. The bands that result from *Mbo*I and *Afl*II cutting are indicated by arrows. The diagram of the LTR–*ras* fusion gene shows restriction enzyme sites relative to nucleosome positions on the LTR. The sites are numbered relative to the cap site on the A nucleosome that is indicated by the arrow. Specific treatment protocols are diagrammed above the autoradiograms: AV, *Ava*I; M, *Mbo*I; A, *Afl*II.

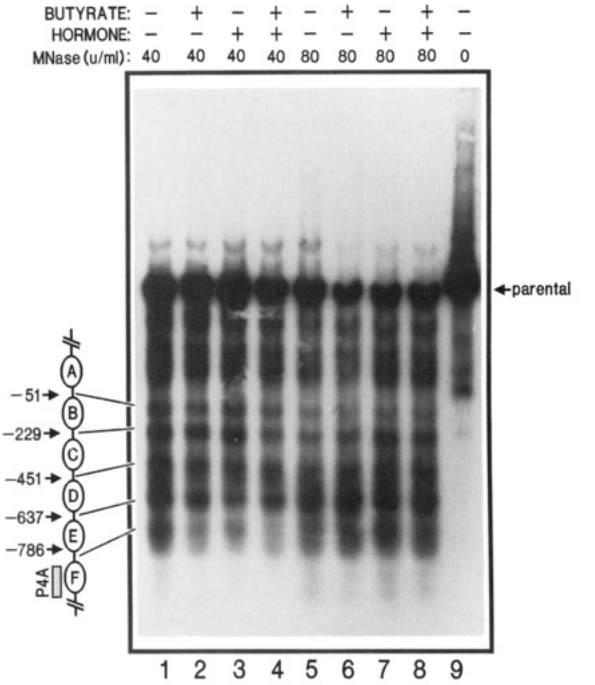

FIGURE 2: Histone hyperacetylation does not alter the specific positions of nucleosomes on the MMTV LTR. Cells (1361.5, episome pM23) were treated with 10 mM butyrate or buffer for 4 h, followed by 100 nM TA or vehicle for 1 h. Nuclei were isolated and were resuspended in micrococcal nuclease digestion buffer. After an incubation for 5 min at 22 °C with 40 or 80 units/mL micrococcal nuclease, nuclei were digested with 0.4 mg/mL proteinase K. Genomic DNA was purified, restricted with *Pst*I, and 20 μg was resolved on a 1.0% agarose gel. Bands were detected by Southern blot analysis with a P4A LTR probe. The arrows, at the left of the figure, indicate the major micrococcal nuclease cutting sites on the LTR. The specific treatment protocols are diagrammed above the autoradiogram.

viously capable of detecting changes in chromatin conformation at the nucleosomal level. If hyperacetylation alters MMTV nucleosome structure, the changes must be beyond the limits of our assay.

Nucleosome Phasing on the MMTV LTR in Normal and Hyperacetylated Chromatin. The possibility exists that neutralizing the positive charge of lysines on the amino-terminal tails of core histones by acetylation could weaken electrostatic interactions between histones and DNA. One consequence of a decreased affinity interaction between histones and DNA would be to relieve constraints on the translational mobility of nucleosomes, allowing a certain degree of sliding of the histone octamer on the DNA. It seems reasonable to suggest that this might generate less stable and less stringently positioned nucleosomes. To determine if hyperacetylation alters nucleosome phasing on the MMTV LTR, we performed indirect end-labeling analysis of micrococcal nuclease digested MMTV chromatin.

Figure 2 shows a representative Southern blot of micrococcal nuclease digested chromatin from 1361.5 cells. Nuclei from untreated, butyrate-treated, and hormone-treated cells were isolated and subjected to a partial digestion with the indicated amounts of micrococcal nuclease. Genomic DNA was purified; after *Pst*I digestion, micrococcal nuclease sensitive sites on the LTR were detected and positioned by indirect end-labeling analysis with a P4A LTR probe (*Ava*I–*Cla*I) as described previously (Richard-Foy & Hager, 1987).

As indicated in the diagram of nucleosome positions on the LTR on the top of Figure 2, the major micrococcal nuclease cuts in LTR chromatin reside in internucleosomal linker regions. The cuts on the LTR at −51, −229, −451, −637, and −786, relative to the cap site, are distinct from cuts on deproteinized genomic DNA (data not shown) and are consistent with previously reported cuts at −60, −249, −444, −651, and −826 (Richard-Foy & Hager, 1987). Deviation of the −786 cut from the previously reported −826 cut can be attributed to anomalous mobility of the resulting small fragment in the lower portion of the 1.0% agarose gel. Under conditions of histone hyperacetylation, there are no qualitative or quantitative changes in the cutting pattern on the LTR or in the 30S and *v-Ha-ras* regions downstream of the LTR. In agreement with our previous work (Richard-Foy & Hager, 1987), the specific hormone-dependent structural change within nuc-B that causes enhanced restriction enzyme cutting and DNase I hypersensitivity does not alter the sensitivity of adjacent linker regions to micrococcal nuclease. We cannot rule out the possibility, however, that subpopulations of nucleosomes from butyrate-treated or hormone-treated cells are selectively destroyed due to extreme hypersensitivity to MNase.

Since hyperacetylation does not affect the enzyme sensitivity of chromatin or the positioning of nucleosomes on the LTR, changes in structural properties of MMTV chromatin induced by hyperacetylation must be very subtle. Detection of such structural changes may require high-resolution genomic footprinting of normal and hyperacetylated nucleosomes at single base pair resolution to map the intimate contacts between core histones and nucleosomal DNA. An alternative experimental approach is to ask if the presence of hyperacetylated histones in a homogeneous reconstituted nuc-B prep-

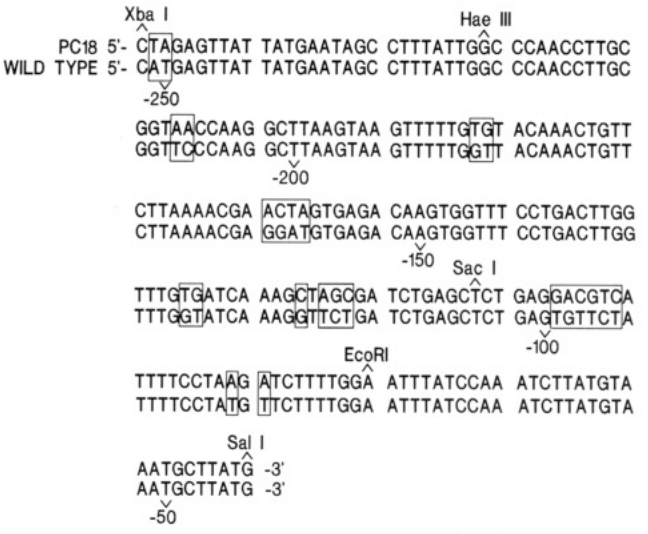

FIGURE 3: Sequence of synthetic pC18 nuc-B DNA fragment used for monosome reconstitution. Twelve overlapping oligonucleotides were ligated to form this synthetic nuc-B fragment. Boxed sequences show deviations from the wild-type LTR sequence, and the sequence is numbered relative to the cap site.

aration alters nucleosome structure or stability.

Reconstitution of Normal and Hyperacetylated B Nucleosomes: No Evidence for Acetylation-Induced Structural Changes. A synthetic DNA fragment spanning the nuc-B region of the MMTV LTR was constructed by ligating 12 overlapping oligonucleotides spanning the B nucleosome region from −255 to −45 and cloning the recombinant molecule into pUC18. The sequence of this synthetic molecule and a comparison to the analogous region of the wild-type LTR is shown in Figure 3. The boxed regions highlight changes that were made in the wild-type LTR to construct the synthetic fragment. We employed this synthetic molecule for reconstitution studies, as the wild-type MMTV LTR lacks appropriately positioned restriction enzyme sites for excising the DNA fragment spanning the B nucleosome region.

Nucleosome core particles were purified from untreated C127 cells or C127 cells treated with 10 mM butyrate for 12 h. Sucrose gradient purified cores were used to donate the histones for reconstitution. In a control experiment, core particles isolated from butyrate-treated cells contained histones with 2.6-fold more acetylated histone than core particles from untreated cells. This degree of hyperacetylation is consistent with that reported by other laboratories (Ausio & van Holde, 1986).

Reconstitution of the nuc-B fragment with a stoichiometric excess of normal and hyperacetylated core particles yields a slow-migrating complex that can be resolved on nondenaturing gels. As shown in Figure 4, all of the free DNA is converted to a slower migrating band, and comparison of lanes 2 and 7 shows that there is no difference in the mobility of normal and hyperacetylated reconstituted monosomes in this gel system. In addition, there is no difference in the sedimentation properties of reconstituted monosomes on 5–25% sucrose gradients (data not shown).

The relative stabilities of normal and hyperacetylated monosomes were tested by incubating monosomes in buffer containing various amounts of NaCl (panel A) or at elevated temperature (panel B) for various periods of time. Panel A shows monosomes that were exposed to increasing concentrations of NaCl and were then resolved on nondenaturing gels. The amount of free DNA increases as the nucleosomes unfold at the higher NaCl concentrations. An intermediate histone–DNA complex (marked by the arrow) forms to the same extent for normal and hyperacetylated monosomes. As we were concerned that the synthetic nuc-B fragment might behave differently from the corresponding wild-type fragment, a wild-type LTR fragment spanning the A and B nucleosomes (Archer et al., 1991) was reconstituted with normal or hyperacetylated core particles into a dinucleosome. No differences were detected in electrophoretic mobility on nondenaturing gels or in salt stability (data not shown). Panel B shows that incubation of monosomes at elevated temperature (80 °C) results in the generation of free DNA, as well as an aggregate that fails to enter the gel. Again, the appearance of free DNA and the aggregate is identical for normal and hyperacetylated monosomes. It is possible, however, that a partially unfolded monosome would not manifest significantly different mobility from a normal monosome in this nondenaturing gel assay. To address the issue of DNA arrangement on the histone octamer, we examined the restriction enzyme sensitivity of reconstituted normal and hyperacetylated monosomes.

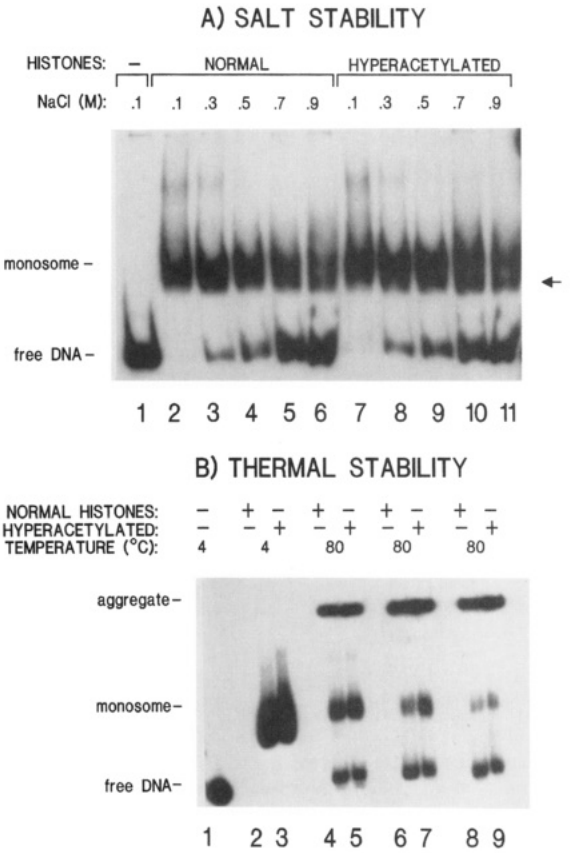

FIGURE 4: Salt and thermal stability of reconstituted normal and hyperacetylated monosomes. Reconstituted normal and hyperacetylated monosomes, labeled at the *Xba*I end, were incubated at 37 °C for 30 min in nucleosome reconstitution buffer containing the indicated concentrations of NaCl (panel A) or were incubated at 4 or 80 °C (panel B) for 1.5 (lanes 4 and 5), 2.5 (lanes 6 and 7), or 4 h (lanes 8 and 9). Samples were applied directly to 5% nondenaturing acrylamide gels, and gels were electrophoresed at room temperature. An intermediate histone–DNA complex that forms at higher NaCl concentrations is indicated by the arrow. Specific treatment protocols are diagrammed above the autoradiograms.

Monosomes or free DNA were subjected to a rapid partial digestion with *Sac*I, *Hae*III, or *Eco*RI. The DNA was purified and analyzed on denaturing urea–acrylamide gels. As shown in Figure 5, the free DNA is cut almost to completion by all three enzymes, whereas the reconstitutes are partially protected from cutting. The reduced level of cutting seen upon reconstitution is not due to nonspecific inhibition of the enzyme by core particles, as addition of core particles to naked DNA does

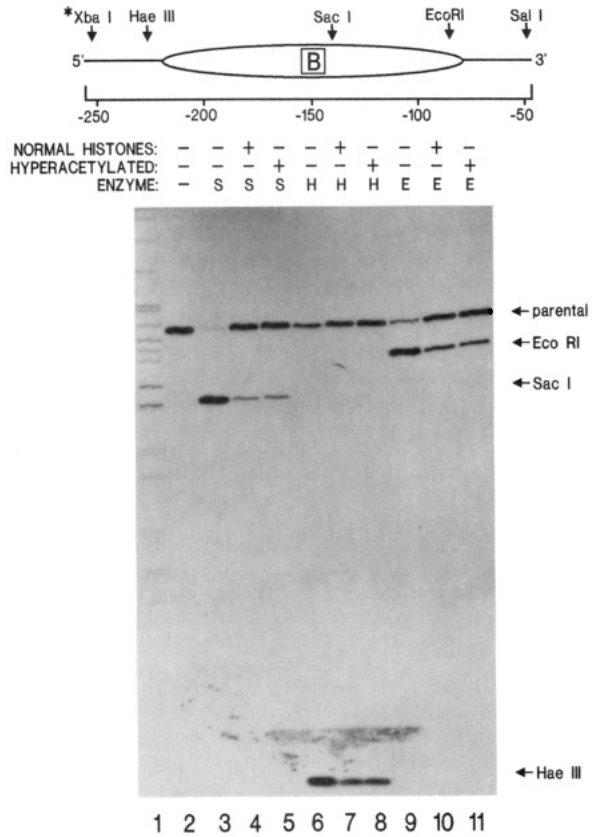

FIGURE 5: Identical restriction enzyme sensitivity of reconstituted normal and hyperacetylated monosomes. Normal and hyperacetylated monosomes, labeled at the *Xba*I end, were adjusted to the appropriate enzyme salt concentration and were incubated with 5–10 units of enzyme for 15 min at 22 °C. Samples were ethanol precipitated and were resolved on an 8% acrylamide–urea gel. Lane 1 shows an *Msp*I digest of pBR322 DNA. The diagram at the top shows the *Xba*I–*Sal*I fragment with relevant restriction sites and the specific treatment protocols. The B nucleosome position is based on indirect end-labeling analysis of chromatin reported previously (Richard-Foy & Hager, 1987) and in this paper. The bands that result from enzyme cutting are indicated by arrows: S, *Sac*I; H, *Hae*III; E, *Eco*RI.

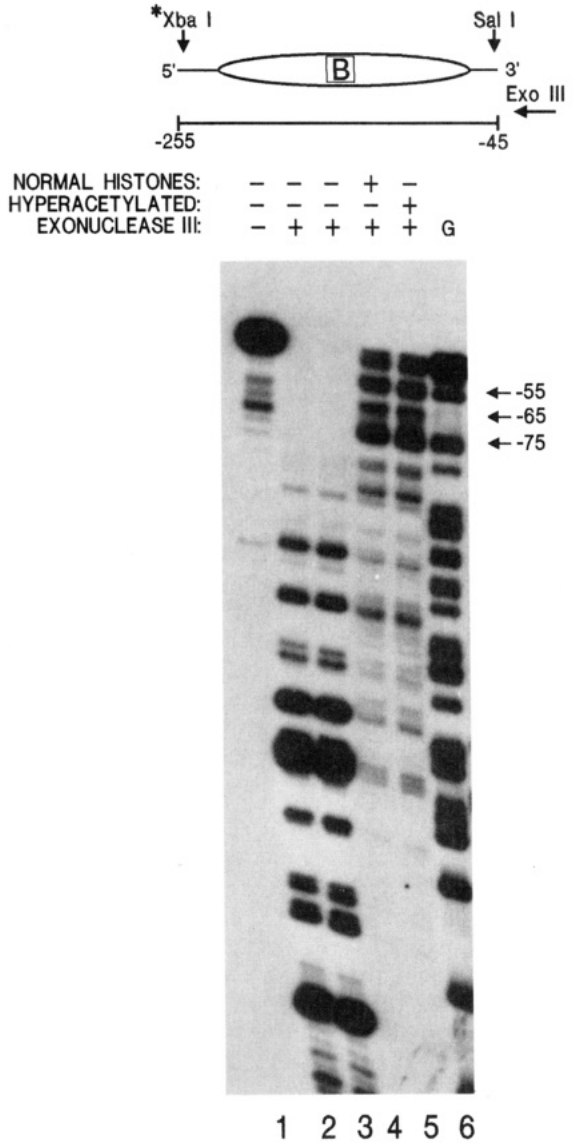

FIGURE 6: Analysis of the 3′ boundary of reconstituted normal and hyperacetylated monosomes by Exo III digestion. Reconstituted normal and hyperacetylated monosomes or free DNA, labeled at the *Xba*I end, were incubated at 22 °C for 30 min in nucleosome reconstitution buffer containing 1 mM DTT and 5000 or 10000 units/mL Exo III for free and reconstituted samples, respectively. Reactions were stopped with 20 mM EDTA and 1% SDS. Samples were extracted with phenol/chloroform, ethanol precipitated, and resolved on an 8% acrylamide–urea gel. The arrows indicate the major Exo III stops for the reconstitutes. Positions on the LTR, relative to the cap site, were assigned from the Maxam–Gilbert G-sequencing ladder (lane G). The diagram at the top shows the *Xba*I–*Sal*I fragment and the direction of Exo III digestion. The nuc-B position is based on indirect end-labeling analysis reported previously (Richard-Foy & Hager, 1987) and in this paper. Specific treatment protocols are diagrammed above the autoradiogram.

not reduce enzyme cutting (data not shown). Protection against cutting is observed at the *Hae*III and the *Eco*RI sites, which are external to the in vivo nuc-B 5′ boundary, as well as the *Sac*I site, which is predicted to be within nuc-B (Richard-Foy & Hager, 1987; Cordingley et al., 1987). One interpretation of these data is that the reconstituted nucleosome can adopt multiple positions on the pC18 fragment. A quantitatively similar degree of protection is observed, however, for normal and hyperacetylated monosomes.

Reconstituted pC18 Monosomes Can Adopt Multiple Translational Positions. To ascertain whether the histone octamer randomly positions on the pC18 fragment or the octamer is subject to stringent constraints on translational or rotational mobility, we used Exo III to map the 3′ boundary of reconstituted normal and hyperacetylated monosomes. Our laboratory has previously used Exo III to map factor interactions with chromatin both in vivo and in vitro (Cordingley & Hager, 1988; Cordingley et al., 1987). The principle behind this technique is that Exo III digests double-stranded DNA processively (3′ to 5′) until a physical barrier is reached that impedes further procession. Figure 6 shows a representative Exo III analysis of the 3′ boundary of sucrose gradient purified reconstituted normal and hyperacetylated monosomes. As shown in lanes 2 and 3, digestion of mock reconstituted DNA with Exo III results in multiple bands, whereas digestion of normal (lane 4) and hyperacetylated (lane 5) monosomes results in predominantly three strong bands that are absolutely specific for the reconstitutes. The positions of these stops relative to the cap site are −55, −65, and −75 as deduced from the G-sequencing ladder (lane 6). The −55 and −75 stops correspond exactly to G residues at these respective positions. Our detection of multiple Exo III stops is consistent with a recent report (Pina et al., 1990) of specific Exo III stops on a reconstituted B nucleosome at −45, −56, −66, and −74. In this case, shorter digestion times resulted in predominantly the −45 stop, suggesting that the upstream stops (−56, −66, and −74) may result from continued procession of Exo III through the nucleosomal DNA. In contrast, Perlmann and

Wrange (1988) reported a single major Exo III stop at −75 on a reconstituted nuc-B, although careful examination of their data reveals an additional stop downstream of the −75 stop. Using a variety of digestion conditions with unpurified and sucrose gradient purified monosomes, we always observe multiple stops.

The presence of three prominent Exo III stops can be explained by distinct nucleosome translational positions that differ by multiples of 10 base pairs, approximately equivalent to a single turn of the DNA helix (for B form DNA). In this regard, it was recently reported that reconstitution of a tandemly repeated or single-copy 5S ribosomal RNA gene into oligonucleosomes and monosomes, respectively, results in multiple translational positions that differ by 10 base pairs (Hansen et al., 1989; Dong et al., 1990). Alternatively, multiple stops may reflect the continued procession of Exo III through nucleosomal DNA. When this data is considered with the substantial protection against *Hae*III and *Eco*RI cutting, it seems likely that multiple translational positions indeed exist. Nevertheless, the specific stops are identical for normal and hyperacetylated monosomes.

DNase I Footprinting of Normal and Hyperacetylated Monosomes. As a final test to compare the structures of reconstituted normal and hyperacetylated monosomes, we examined the DNase I sensitivity of reconstitutes. Digestion of nucleosomes with DNase I results in periodic cutting (approximately every 10 base pairs) of phosphodiester bonds in the minor groove of DNA, which is exposed on the nucleosome surface (Prunell et al., 1979) [for a review see Travers and Klug (1987)]. Figure 7 shows a representative DNase I analysis of the coding strand of normal and hyperacetylated monosomes. Cutting with a periodicity of approximately 10 base pairs is observed beginning at −54 and extending through the nucleosome to −182. Although many of the bands that are generated by DNase I digestion of monosomes (lanes 1 and 2) and free DNA (lane 3) are identical, a number of these bands between −54 and −182 are either enhanced or suppressed in the reconstitutes. The specific pattern of bands resulting from DNase I digestion is identical for normal and hyperacetylated monosomes. DNase I titrations have not revealed any qualitative or quantitative changes in DNase I footprints (data not shown).

Implications of the Data. Several studies have correlated changes in histone acetylation status with chromatin structural transitions at the level of nucleosomes (Bode et al., 1983; Prevelige & Fasman, 1987; Yau et al., 1982) and higher order structure (Annunziato et al., 1988). In one case, total chromatin from 15-day-old chick embryo erythrocytes was fractionated with an antibody specific for tetraacetylated histone H4 (Hebbes et al., 1988). A significant enrichment of heavily acetylated histone H4 was observed in fractions containing an actively transcribed gene (α-D-globin), whereas no enrichment was observed with a nontranscribed gene (ovalbumin). We are unaware of studies that demonstrate specific structural changes in defined genetic loci.

Our results do not reveal differences in nuclease sensitivity of normal and hyperacetylated chromatin or in the structure or stability of reconstituted normal and hyperacetylated monosomes. These results raise the question whether changes in physical properties of bulk chromatin, e.g., increased solubility (Perry & Chalkley, 1982) and nucleosome unfolding (Bode et al., 1983; Oliva et al., 1990), necessarily reflect the specific architecture of individual genes. Only after detailed structural studies are performed with additional inducible genes will it be possible to determine if our results with the MMTV

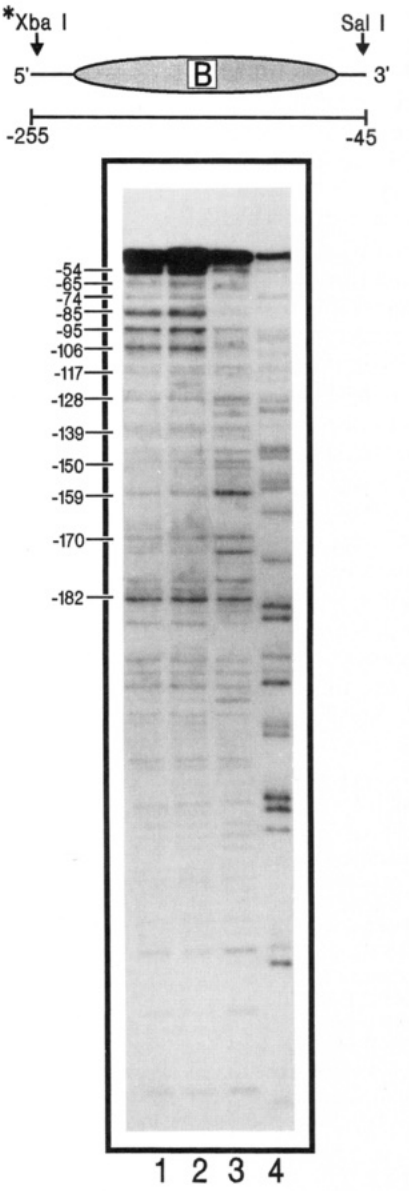

FIGURE 7: Comparison of the DNase I sensitivity of reconstituted normal and hyperacetylated monosomes. Reconstituted normal and hyperacetylated monosomes or free DNA were incubated for 5 min at 22 °C with 0.4 and 0.2 unit of DNase I, respectively. Reactions were stopped with 20 mM EDTA and 1% SDS. Samples were extracted with phenol/chloroform, ethanol precipitated, and resolved on a 10% acrylamide–urea gel. Key: lane 1, normal reconstitute digested with DNase I; lane 2, hyperacetylated reconstitute digested with DNase I; lane 3, free DNA digested with DNase I; lane 4, Maxam–Gilbert G sequencing ladder. The diagram above the autoradiogram shows the *Xba*I–*Sal*I fragment and the nuc-B position based on indirect end-labeling analysis reported previously (Richard-Foy & Hager, 1987) and in this paper.

LTR are unique. Polymerase chain reaction mediated amplification of single-copy genetic loci should prove invaluable for such an analysis.

Despite our inability to detect differences between structural properties of normal and hyperacetylated MMTV chromatin, the MMTV promoter is refractory to activation by the GR after treatment of cells with butyrate. It is theoretically possible that the inhibition is not due to a direct effect on histone acetylation status and that it involves alteration of transcription factor activity, perhaps by influencing the posttranslational processing of factors. Considering that (i) butyrate treatment does not down-regulate the GR (Bresnick et al., 1990), (ii) butyrate does not decrease hormone-de-

pendent expression of luciferase activity from an LTR–luciferase construct in a transient transfection assay (Bresnick et al., 1990), and (iii) perturbation of cell growth with the DNA synthesis inhibitor aphidicolin has absolutely no effect on hormone activation of the MMTV promoter (T. K. Archer and G. L. Hager, unpublished observations), we conclude that the inhibitory effect involves a direct modification of chromatin rather than a nonspecific effect on cells.

In a recent yeast genetic analysis, it was suggested that the SIR3 mating type repressor might directly interact with the amino-terminal tail of histone H4 (Johnson et al., 1990). This provides a mechanism by which histone acetylation could alter gene activity in the absence of major changes in chromatin structure, i.e., by directly modulating the interaction of a DNA regulatory protein with nucleosome-associated DNA recognition sites. As the GR must bind to GREs on nuc-B, it seems reasonable to suggest that direct contacts between histones and GR can occur during initial recognition of GREs on nuc-B. It is easy to imagine how this direct physical interaction could be influenced by histone modifications (e.g., acetylation, ADP-ribosylation, and phosphorylation) that alter charge distribution and, potentially, histone conformation. Consistent with such a mechanism, it has been reported that the GR binds tightly to core histone affinity columns (Ueda et al., 1989). It should be possible to directly test the role of histone modifications in receptor-mediated promoter activation and nucleosome disruption by (i) developing a quantitative binding assay to ask if hyperacetylation affects the binding of GR to reconstituted nuc-B and (ii) establishing a hormone-responsive LTR–reporter system in yeast whereby activation can be performed in a genetic background in which conserved lysine acetylation sites are systematically mutated (Megee et al., 1990).

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