Chromatin Hyperacetylation Abrogates Vitamin D-Mediated Transcriptional Upregulation of the Tissue-Specific Osteocalcin Gene in Vivo†

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ABSTRACT: Cells expressing the bone-specific osteocalcin (OC) gene exhibit two DNase I hypersensitive sites within the proximal (nt −170 to −70) and distal (nt −600 to −400) promoter. These sites overlap elements that independently or in combination contribute to basal and vitamin D-stimulated OC gene transcription. Here we address mechanisms that participate in control of chromatin remodelling at these sites. By applying nuclease digestion and indirect end-labeling or by combining intranuclear footprinting and ligation-mediated PCR, we investigated the effects of nuclear protein hyperacetylation on both chromatin organization and transcriptional activation of the OC gene in bone-derived cells. We report that chromatin hyperacetylation blocks vitamin D stimulation of OC transcription and prevents a key transition in the chromatin structure of the OC gene which is required for formation of the distal DNase I hypersensitive site. This transition involves interaction of sequence-specific nuclear factors and may be required for the ligand-dependent binding of the vitamin D receptor complex, which results in transcriptional enhancement.

The packaging of DNA sequences in nucleosomes and higher-order chromatin structures has been implicated in the regulation of key events in eukaryotic cells such as replication and transcription (*1*). Until recently, it has been widely accepted that the presence of nucleosomes blocks the accessibility of specific transcription factors to their cognate binding sequences. Moreover, gene activation is often accompanied by perturbations of the nucleosomal array, as evidenced by increased nuclease hypersensitivity of specific promoter and enhancer elements (*2*, *3*). Multiple studies, principally in vitro, have shown intrinsic differences in the nucleosome binding capacity of transcription factors. While some factors cannot bind if their sites are assembled into nucleosomes (*4*−*6*), others can specifically recognize and interact with nucleosome-engaged binding sequences although with different degrees of affinity (*4*−*11*). Workman and colleagues have demonstrated that some transcription factors show an inherent cooperativity when binding to adjacent sites on nucleosomal DNA (*10*). Such cooperative binding does not seem to be due to the sliding of the histone octamer after the interaction of the first factor but rather is likely a result of minor alterations in the core particle structure induced by the first binding event. These core particle alterations facilitate the interaction of additional activators to adjacent sites on the nucleosome. Other studies have described the interaction of transcription factors with sequences organized in nucleosomes in vivo. McPherson et al. (*12*) have shown that in liver cells the serum albumin enhancer exists in an array of precisely positioned nucleosomes. This positioning allows the binding of transcription factor HNF-3 to a site on the nucleosomal surface. Taken together, these observations indicate that although nucleosomes positioned over specific promoter sequences may be inhibitory to binding of some transcription factors, other factors are capable of organizing nucleosomal structures that define active elements.

The role of nucleosomes in regulating transcriptional activity is supported by histone acetylation experiments. Hyperacetylation of core histones has been shown to enhance binding of some transcription factors to nucleosomes (*13*−*16*). One possible mechanism is that the histone basic N-terminal domains interact with nucleosomal DNA and prevent binding of transcription factors. Neutralization of the lysine residues within these domains by acetylation can decrease their affinity for DNA and thus alleviate the inhibitory effect. Alternatively, acetylation may directly alter the conformation of the core nucleosomal particle, thus facilitating accessibility to transcription factors (*17*−*20*). The identification of several histone acetyl transferases (*21*−*23*) as well as histone deacetylases (*24*, *25*) has brought major attention to this posttranslational modification in an attempt to determine its physiological role in transcriptional regulation. Recent reports indicate that transcriptional repression by sequence-specific DNA-binding factors can be mediated by the recruitment of a histone deacetylase to the promoter region (*26*−*30*).

The osteocalcin (OC) gene encodes a 10 kDa bone-specific protein that is induced in osteoblasts with the onset of mineralization at late stages of differentiation (*31*, *32*). The

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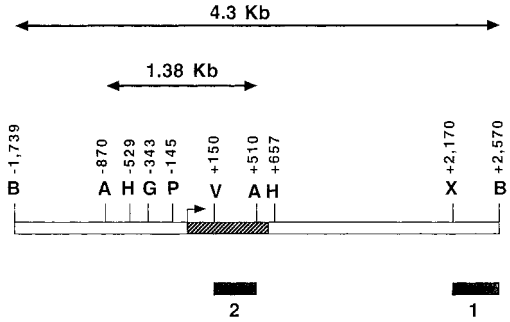

FIGURE 1: Restriction endonuclease map of the OC gene and flanking sequences. The diagram shows the DNA segments used as hybridization probes in the Southern blot analyses (bottom). The hatched box represents the OC gene coding region (including exons and introns), and the horizontal arrow above the gene marks the direction of transcription. A = *Apa*I, B = *Bam*HI, G = *Bgl*II, H = *Hin*cII, P = *Pst*I, V = *Avn*I, X = *Xba*I.

OC gene is expressed constitutively in ROS 17/2.8 osteosarcoma cells. Transcription of the OC gene is controlled by modularly organized basal regulatory sequences and hormone-responsive enhancer elements (*33*−*46*) located within two DNase I hypersensitive sites (DHS) that are present only in bone-derived cells expressing this gene (*47*, *48*). Thus, remodeling of the chromatin structure of the OC gene promoter accompanies the increase in gene expression that occurs during osteoblast differentiation. However, regulatory mechanisms that mediate these conformational modifications in nucleosomal organization of the OC promoter have not been experimentally addressed.

Here we report that butyrate treatment of ROS 17/2.8 osteosarcoma cells, which results in hyperacetylation of nuclear proteins, alters the chromatin organization of the OC gene promoter and prevents vitamin D-mediated transcriptional upregulation. By combining nuclease accessibility, indirect end-labeling, and ligation-mediated PCR analysis, we have determined that protein−DNA and protein−protein interactions that promote the formation of the distal DHS do not occur under conditions of chromatin hyperacetylation. We conclude that the presence of this hypersensitive site reflects and may be functionally related to the ligand-dependent binding of the vitamin D receptor.

MATERIALS AND METHODS

Cell Culture. Rat osteosarcoma-derived ROS 17/2.8 cells (a gift from S. Rodan and G. Rodan; Merck Sharp Dohme, West Point, PA) were maintained as reported (*49*). Cells were treated with 10^{-8} M 1,25-dihydroxy vitamin D_3, 10 mM sodium butyrate, or a combination of both, for various periods of time as indicated. OC gene transcription was determined by nuclear run-on analysis (*48*).

Nuclease Hypersensitivity Analysis. DNase I and micrococcal nuclease digestion analyses of nuclei isolated from confluent cultures of ROS 17/2.8 cells were performed according to the indirect end-labeling method (*50*). Restriction enzyme cleavage of purified genomic DNA, electrophoresis, blotting, and hybridization were performed as previously described (*48*, *50*) using the probes shown in Figure 1.

Restriction Endonuclease Digestion Analysis. Nuclei isolated from ROS 17/2.8 cells were digested for 30 min at 37 °C with the restriction endonucleases *Hin*cII, *Bgl*II, or *Pst*I (New England Biolabs, Beverly, MA) in the specific buffer conditions provided by the supplier, conditions which gave optimal digestion. Purified genomic DNA was subsequently assayed by Southern blot analysis, and the intensities of the autoradiographic bands were determined using a scanning densitometer. The accessibility of the OC gene promoter to the different restriction endonucleases was quantified as a fraction representing the intensity of the selected band relative to the total intensity of bands within a given lane on an autoradiograph (*48*).

Nuclear Extracts and Electrophoretic Mobility Shift Assays. Nuclear extracts were prepared from confluent ROS 17/2.8 cultures as previously described (*51*). Electrophoretic mobility shift assays were performed using probes end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. Binding reactions contained 2.5 mg of nuclear protein and 10 fmol of each oligonucleotide probe: VDRE (*52*), YY-1 (*45*), or AML (*40*). The protein−DNA complexes were electrophoretically fractionated in 4% (80:1) polyacrylamide gels and 0.5× TBE buffer.

Intranuclear Footprinting Analysis. Nuclei were digested with DNase I, and the isolated genomic DNA was analyzed by ligation-mediated PCR using Vent polymerase (New England Biolabs) essentially as described (*53*). First-strand synthesis was accomplished with primer 1 (5′-GACAAACTG-GCTCCAACTCGCATAGC-3′; nt −358 to −383). The DNA was then ligated with the unidirectional linker primer (*53*), and amplification was carried out by PCR using primer 2 (5′-GCATAGCCTGTGATTTTCAGTGTCTGCCGT-3′; nt −377 to −406) and the linker oligonucleotide. Labeling was performed with ^{32}P-end-labeled primer 3 (5′-AGCCTGT-GATTTTCAGTGTCTGCCGTGAGAGCA-3′; nt −381 to −413). Subsequently, the samples were phenol−chloroform-extracted, ethanol-precipitated, resuspended in sequencing load buffer, and separated in 6% polyacrylamide, 7 M urea sequencing gels.

RESULTS

Chromatin Hyperacetylation Affects DNase I Hypersensitivity and Vitamin D-Dependent Transcriptional Upregulation of the OC Gene. Extensive analysis of chromatin structure has indicated that most active genes contain DNase I hypersensitive regions. These domains generally reflect alterations in the classical nucleosomal organization and the binding of specific nuclear factors (*2*, *3*). It has been shown that one of the mechanisms by which cells can regulate chromatin structure is posttranslational modification of the nucleosomal histones (*18*). Among these modifications, core histone acetylation has been the focus of extensive studies. Hyperacetylation can be induced in vivo by treatment of cells with sodium butyrate, an inhibitor of histone deacetylases (*54*, *55*).

We studied the effect of hyperacetylation on the chromatin organization of the osteocalcin (OC) gene in ROS 17/2.8 osteosarcoma-derived cells by analyzing DNase I hypersensitivity detected by the indirect end-labeling method (*47*, *48*). As shown in Figure 2A, we found that very short incubations with butyrate caused a marked and reversible change in the DNase I hypersensitivity pattern normally observed in the promoter of the OC gene in bone-derived cells expressing OC (*47*, *48*). The distal hypersensitive site (−600 to −400),

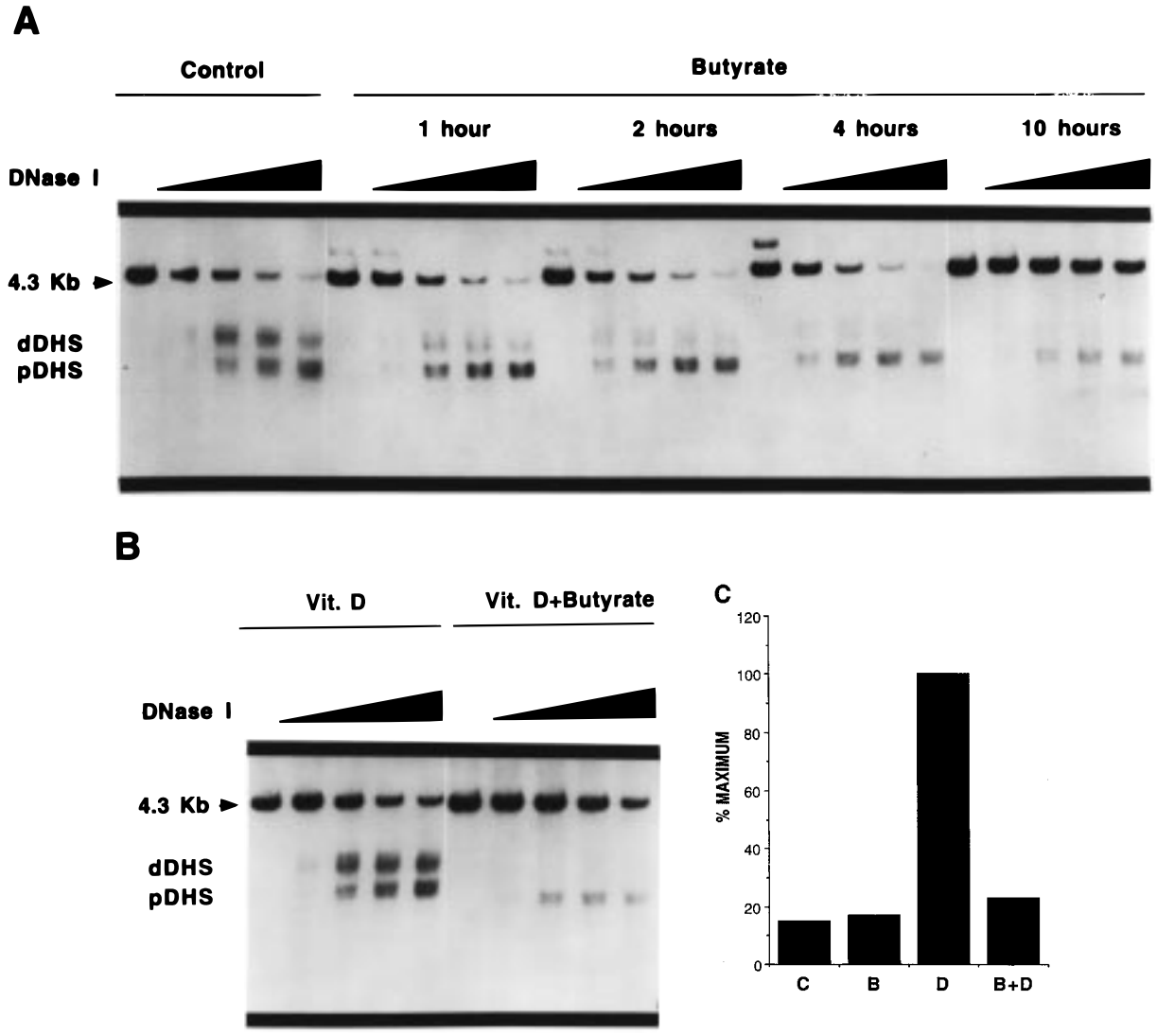

FIGURE 2: Effect of butyrate treatment on DNase I hypersensitivity and OC gene transcription. Confluent ROS 17/2.8 cell cultures were treated with 10 mM butyrate for 1, 2, 4, and 10 h (A), or with butyrate and vitamin D combined for 4 h (B). Nuclei were subsequently isolated and incubated with increasing concentrations of DNase I for 10 min. The purified genomic DNA was then completely cleaved with *Bam*HI, electrophoresed in a 1.2% agarose gel, blotted, and hybridized with probe 1 (see Figure 1). The positions of the proximal (pDHS, −170 to −70) and distal (dDHS, −600 to −400) DNase I hypersensitive sites are marked at the left. (C) OC gene transcriptional activity measured by nuclear run-on assays. C, control; B, 10 mM butyrate; D, 10^{-8} M vitamin D; B+D, 10 mM butyrate and 10^{-8} M vitamin D. All of these incubations were for 4 h.

which includes the VDRE as well as other important regulatory sequences, was markedly decreased after only 1 h and disappeared completely following 4 h of butyrate treatment. Under these conditions, the cells showed a significant increase in the level of histone acetylation (not shown). In contrast, the proximal DNase I hypersensitive site, corresponding to the basal regulatory elements, was not affected by 1 h of butyrate treatment and only slightly reduced after 4 h. Simultaneous addition of vitamin D and butyrate to ROS 17/2.8 cells did not prevent the alteration in the DNase I hypersensitive pattern (Figure 2B). Furthermore, butyrate treatment prevented the vitamin D-dependent enhancement in nuclease hypersensitivity previously reported (*47*, *48*).

When the effect of this short incubation with butyrate on transcriptional activity was measured by nuclear run-on analysis, we found that it did not have a significant impact on basal levels of OC expression. However, vitamin D-dependent upregulation of OC gene transcription was completely inhibited (Figure 2C). Transcription of several other genes was evaluated and found to be either enhanced (e.g., histone H4) or not affected (e.g., osteopontin, alkaline phosphatase, 18S ribosomal RNA) (data not shown).

Chromatin Hyperacetylation Inhibits a Specific Nucleosome Transition in the OC Gene Promoter. We investigated modifications in the nucleosomal organization of the OC gene promoter region that might explain the changes in the DNase I hypersensitivity pattern observed following butyrate treatment. We digested nuclei isolated from control and butyrate-treated confluent ROS 17/2.8 cell cultures with micrococcal nuclease (MNase) and several restriction endonucleases. MNase has been shown to cleave chromatin primarily between nucleosomes, while accessibility to restriction enzymes is markedly reduced when cognate recognition sequences are organized into nucleosomes (*56*).

When MNase cleavage sites within the OC gene promoter were mapped by indirect end-labeling from the 3′ end (also confirmed from the 5′ end but not shown), we observed a

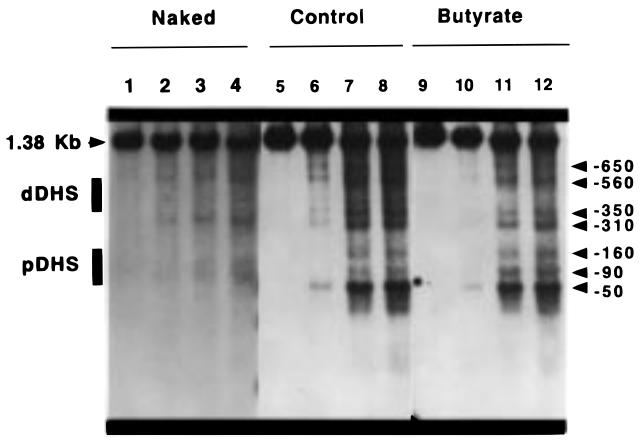

FIGURE 3: Nucleosomal organization of the OC gene promoter in butyrate-treated cells. Nuclei isolated from confluent ROS17/2.8 cell cultures, control or treated with 10 mM butyrate for 4 h, were digested with increasing concentrations of micrococcal nuclease (MNase) for 5 min (lanes 5−12). Then the purified genomic DNA samples were cleaved with *Apa*I and analyzed by Southern blot using probe 2 (see Figure 1). The MNase cleavage sites are indicated at the right. As a control, purified ROS genomic DNA was digested with MNase (lanes 1−4) and analyzed together with the nuclear DNA samples. Lanes 1, 5, and 9, no MNase; lane 2, 0.5 units/mL; lane 3, 0.7 unit/mL; lane 4, 1.0 unit/mL; lanes 6 and 10, 5 unit/mL; lanes 7 and 11, 10 unit/mL; lanes 8 and 12, 15 units/mL.

similar digestion pattern for control and butyrate-treated cells (Figure 3). Cleavages at −50, −90, −160, −310, −360, −560, and −650 were found for both samples, indicating that no significant changes in the previously described (*48*) translational positioning of the nucleosomes over the OC promoter were induced by the butyrate treatment. In contrast, a marked decrease in cleavage by the restriction endonuclease *Hin*cII was detected over the distal region of the OC gene promoter (−529) in the DNA samples from butyrate-treated cells (Figure 4). This cleavage site is located within the distal DNase I hypersensitive site (−600 to −400) which is lost following incubation with butyrate (Figure 2A). Cleavages in other regions of the OC gene promoter by *Bgl*II (−343) and *Pst*I (−145) were not affected by chromatin hyperacetylation. Moreover, no effect on the accessibility of an additional *Hin*cII site (+657, Figure 4B) was observed. This site has been shown not to be cleaved due to the presence of randomly positioned nucleosomes downstream of the OC gene coding region in bone-derived cells [(*48*), and unpublished results].

Consistent with our previous report (*48*), cleavage by *Hin*cII at the distal DNase I hypersensitive site or by *Pst*I at the proximal DNase I hypersensitive site is significantly enhanced following vitamin D treatment (Figure 4). This increased digestion was not observed if ROS 17/2.8 cells were also incubated in the presence of butyrate.

Chromatin Hyperacetylation Does Not Alter the Level of Vitamin D Receptor and Other Transcription Factors That Interact with the Distal OC Promoter. The distal DNase I hypersensitive site in the OC gene promoter includes important transcriptional elements that are recognized by at least three different factors. The VDRE (−466 to −437) interacts with the vitamin D receptor complex in a ligand-dependent manner (*42*−*46*). There are also two binding sequences for NMP-2, a bone-specific protein that was initially identified as a component of the nuclear matrix fraction (*37*, *38*), and which has recently been shown to be an AML/CBFα-related protein (*38*−*40*). Interestingly, these NMP-2/AML binding sites (site A, −604 to −599; and site B, −440 to −435) flank the distal DNase I hypersensitive site (−600 to −400). In addition, we have identified a binding site for NMP-1 (*37*), a ubiquitous transcription factor that can partition between the soluble and the nuclear matrix fractions. NMP-1, which has recently been identified as YY-1 (*45*), binds to a site that partially overlaps one of the VDRE half-elements, providing a potential mutually exclusive binding mechanism (*57*).

Chromatin hyperacetylation has been correlated with changes in the phenotype of several cellular systems. Thus, it has been reported to induce or repress differentiation (*58*, *59*), and to induce cell growth repression, transformation reversion, and apoptosis (*60*−*62*). In most cases, it was suggested that the mechanism would involve changes in the level of expression of key transcription factors or, as recently

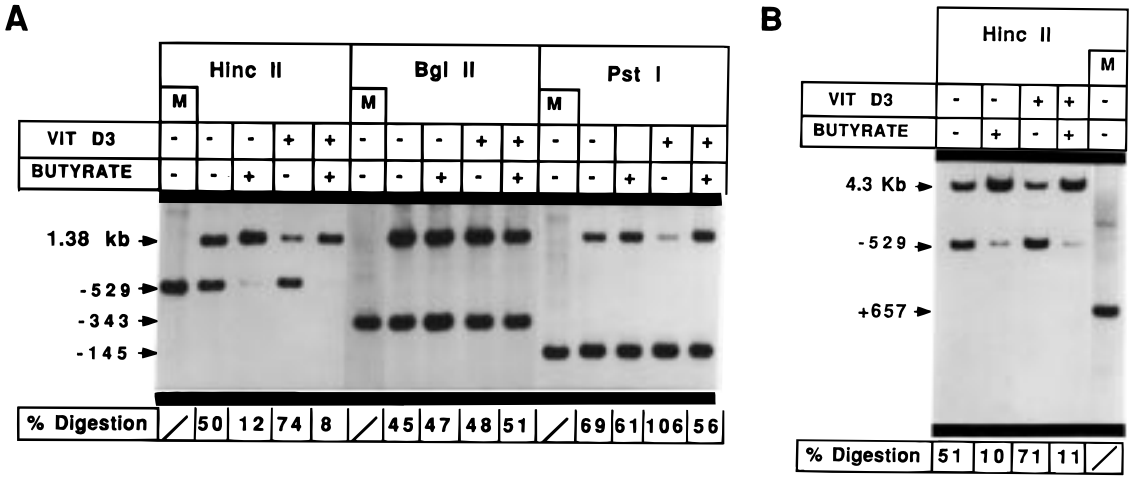

FIGURE 4: Butyrate treatment prevents specific nucleosomal transition. Nuclei from confluent ROS 17/2.8 cells were incubated with 500 units/mL of the restriction endonucleases *Hin*cII, *Bgl*II, and *Pst*I for 30 min at 37 °C. The purified genomic DNA samples were then analyzed by Southern blot. The cleavage positions are shown at the left. The percentage of digestion under the different experimental conditions is shown at the bottom. (A) The purified nuclear DNA samples were cleaved with *Apa*I and hybridized with probe 2 (see Figure 1). M corresponds to purified ROS cell DNA digested with either *Hin*cII, *Bgl*II, or *Pst*I. (B) The samples were completely cleaved with *Bam*HI and hybridized with probe 1 (see Figure 1). M represents ROS cell genomic DNA cleaved with *Hin*cII and *Bam*HI.

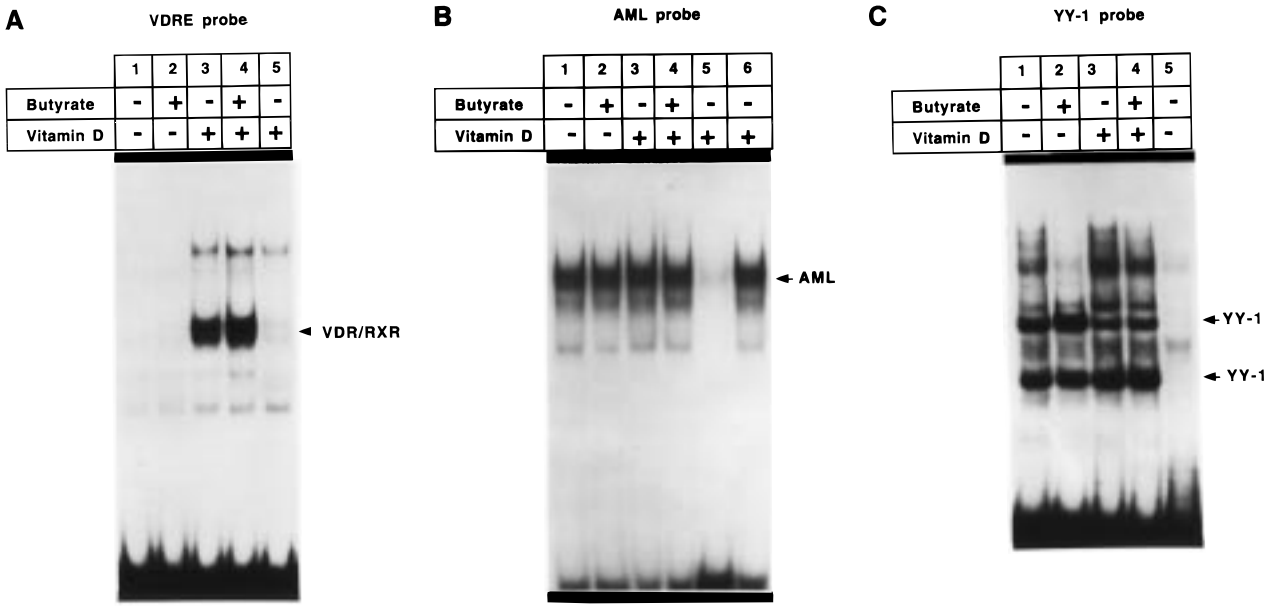

FIGURE 5: Butyrate treatment does not alter transcription factor activity. Nuclear extracts prepared from ROS 17/2.8 cells treated as control (lane 1) or with 10 mM butyrate (lane 2), 10^{-8} M vitamin D (lane 3), or butyrate and vitamin D together (lane 4) were analyzed by gel electromobility shift assay. (A) Rat OC VDRE oligonucleotide. Lane 5, self-competition with a 100-fold molar excess of unlabeled OC VDRE oligonucleotide. (B) AML/CBFα oligonucleotide. Lane 5, self-competition; lane 6, competition with a nonrelated oligonucleotide. (C) YY-1 oligonucleotide. Lane 5, self-competition.

reported, changes in sequence-specific DNA-binding activity due to acetylation of the transcription factor itself (*63*). To evaluate the binding activities of transcription factors that interact with the OC gene distal promoter region, we isolated nuclear extracts from ROS 17/2.8 cells, control and treated with butyrate alone or in combination with vitamin D. Gel electrophoretic mobility shift assays were performed using oligonucleotides which included the OC gene VDRE (*52*), the YY-1 binding element (*45*), and the AML binding sequence (*40*). The results, shown in Figure 5, demonstrate that the levels of these three transcription factors are unaffected by treatment with butyrate. It is important to note that the ligand-dependent formation of the VDR−RXR complex with the VDRE is not inhibited in nuclear extracts from butyrate-treated ROS 17/2.8 cells (Figure 5A). Furthermore, we have found that the activities of these as well as other several transcription factors are not affected even after 10 h of butyrate treatment (data not shown).

Chromatin Hyperacetylation Alters Key Protein−DNA Interactions in Vivo in the Distal Region of the OC Gene Promoter in ROS 17/2.8 Cells. To determine if the loss of distal DNase I hypersensitivity in the OC gene promoter was due to alterations in specific protein−DNA interactions in vivo induced by treatment of ROS 17/2.8 cells with butyrate, we combined intranuclear footprinting and ligation-mediated PCR (LMPCR) analysis. This approach has been successfully utilized to study tissue-specific protein−DNA interactions in vivo, and it has the power to reveal interactions on nucleosomal surfaces (*12*, *64*).

We detected footprinted domains which correspond to the binding sites for the transcription factors NMP-1/YY-1 (*37*, *45*) and NMP-2/AML-site B (*37*, *38*) (Figure 6A, lane 2) in DNase I-digested nuclear DNA samples from ROS 17/2.8 cells expressing basal levels of OC. In the samples from ROS 17/2.8 cells incubated with vitamin D, this pattern is replaced by a larger footprinted area that includes the binding site for the VDR−RXR complex (*42*−*46*) (Figure 6A, lane 4) and represents a hormone-induced change in the protein−DNA interactions that occur in this region. Strikingly, these footprints were not detected in samples from ROS 17/2.8 cells incubated with butyrate alone (Figure 6A, lane 3) or together with vitamin D (Figure 6A, lane 5). Furthermore, these samples revealed a prominent 10 bp periodicity of DNase I cleavage sites (Figure 6, marked with arrowheads) characteristic of rotationally phased nucleosomal DNA (*64*, *65*). A similar 10 bp DNase I cleavage pattern was observed in samples from ROS 24/1 cells (Figure 6B), an osteosarcoma cell line derived from the same tumor as ROS 17/2.8 cells (*66*), but which expresses neither osteocalcin nor the vitamin D receptor.

DISCUSSION

The OC gene promoter provides a paradigm for developmental, steroid hormone responsiveness, and bone tissue specific transcriptional control. Remodeling of the chromatin organization of the OC gene promoter during osteoblast differentiation and in response to vitamin D has been documented (*47*, *48*). However, regulatory mechanisms controlling the conformational modifications in the nucleosomal organization of the OC gene promoter have not been defined.

In this paper we have examined the effect of histone acetylation on the chromatin organization and transcriptional activity of the OC gene. Very short incubations of ROS 17/2.8 cells with sodium butyrate, an inhibitor of histone deacetylase activity (*54*, *55*), blocked the formation of the distal DNase I hypersensitive site in the OC gene promoter. This effect was accompanied by inhibition of vitamin D-dependent OC gene transcriptional upregulation. Although no alterations in the translational positioning of nucleosomes over the promoter region were detected by micrococcal nuclease digestion and indirect end-labeling analyses, a marked reduction in cleavage in the distal promoter by the

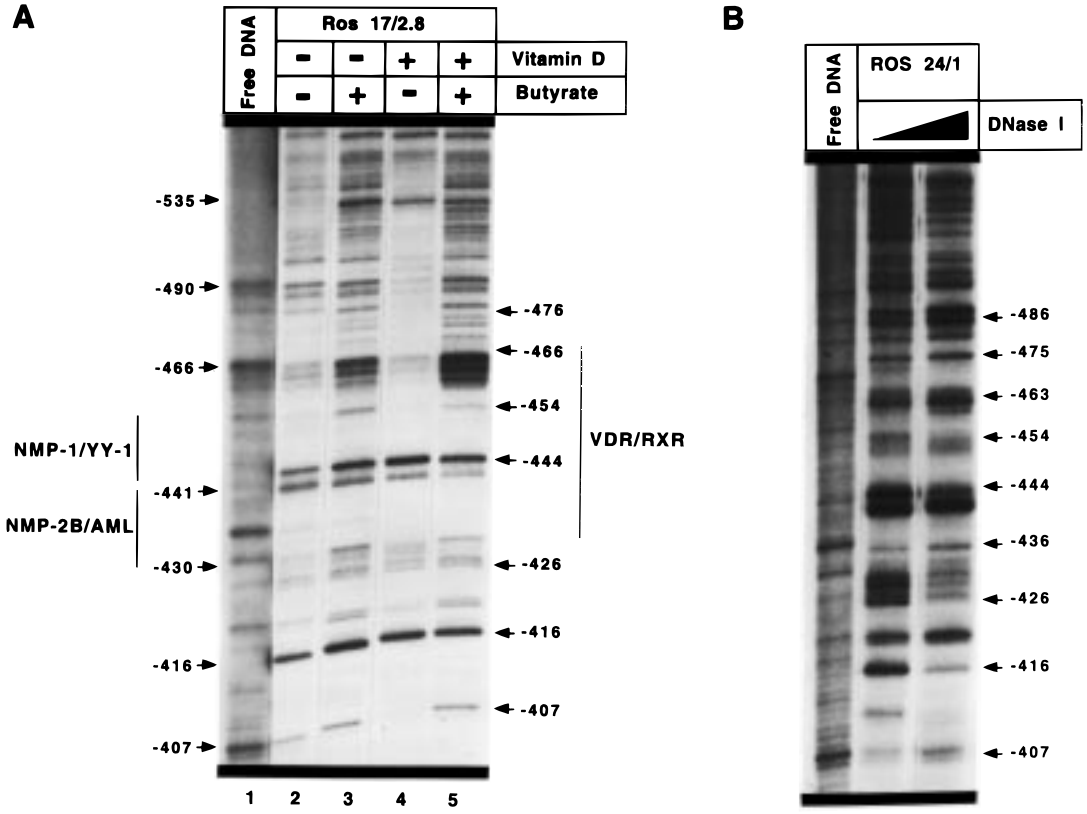

FIGURE 6: Butyrate treatment prevents protein−DNA interactions in the distal region of the OC gene promoter. Nuclei isolated from ROS 17/2.8 cells, which express osteocalcin (A), and from ROS 24/1 cells, which do not (B), were digested with increasing concentrations of DNase I. After purification, the digested nuclear DNA samples were analyzed by LMPCR. (A) Footprints encompassing binding elements for NMP-1/YY-1, NMP-2/AML (site B), and the VDR−RXR heterodimer are shown by vertical bars. Lane 1, protein-free DNA; 2, control; 3, butyrate; 4, vitamin D; and 5, butyrate+vitamin D. The nucleotide positions are indicated at both sides. (B) Lane 1, protein-free DNA; lanes 2 and 3, DNase I-digested samples. The periodic DNase I cleavage every 10 base pairs is indicated on the right.

restriction endonuclease *Hinc*II was observed. This is an indication of nucleosomal organization at this region in the butyrate-treated cells (*56*). By combining intranuclear footprinting and LMPCR analysis, we determined that the footprinted sequences corresponding to binding sites for transcription factors such as NMP-1/YY-1 (*37*, *45*) and NMP-2/AML (site B) (*37*, *38*) were lost following treatment of ROS 17/2.8 cells with butyrate. Similarly, the footprinted regions observed following incubation with vitamin D, which include sequences from −535 to −416, were not detected if the ROS 17/2.8 cells were co-treated with butyrate. These footprints represent protein−DNA interactions that occur in vivo over the distal promoter and which have been implicated in both distal DNase I hypersensitivity and vitamin D-dependent upregulation of OC gene transcription in bone-derived cells (*67*, *68*). Furthermore, the butyrate-treated samples presented a DNase I cleavage periodicity of about 10 bp, which corresponds to the pattern exhibited by DNA in nucleosomes (*64*, *65*). A similar pattern was obtained when DNase I digested samples from ROS 24/1 cells were analyzed. In these cells, where the OC gene is not expressed (*66*), the distal region of the promoter has been found to be organized in nucleosomal structures (*48*).

It is generally assumed that binding of transcription factors to nucleosomal DNA is enhanced by acetylation. This assumption is based on the demonstration that hyperacetylation induces subtle alterations in nucleosomal structure which may increase the accessibility to transcription factors (*13*−*16*). Nevertheless, Hager and colleagues reported that butyrate treatment inhibited glucocorticoid hormone-dependent formation of a nuclease hypersensitive site and blocked transcriptional induction of the mouse mammary tumor virus (MMTV) LTR (*69*). These authors also found that this inhibitory effect was not due to a major change in the chromatin structure such as nucleosome unfolding. Instead they proposed that relatively minor chromatin modifications can modulate the interaction of the glucocorticoid receptor with chromatin-associated recognition sites (*69*, *70*). In contrast, Beato and co-workers (*71*) found that by decreasing the concentrations of histone deacetylase inhibitors, only moderate histone acetylation can be obtained. Moderate acetylation leads to enhanced transcription from the MMTV promoter in the absence of hormone and potentiates transactivation by either glucocorticoids or progestins. Because these inducing inhibitor concentrations lead to a similar type of nucleosomal DNase I hypersensitivity as hormone treatment, it was suggested that moderate acetylation of core histones activates the stably integrated MMTV promoter by mechanisms involving chromatin remodeling similar to that generated by the inducing hormones. The basis for the inconsistency of these results remains unresolved. However, under our experimental conditions, ROS 24/1 cells were not induced to express OC at the butyrate concentrations tested (data not shown). Additionally, no increases above the basal levels of OC gene transcription in ROS 17/2.8 cells were observed. Finally, both the changes in DNase I hypersensi-

tivity and the inhibition of the vitamin D-dependent transcriptional upregulation by butyrate were completely and rapidly reverted by adding butyrate-free fresh media to the ROS 17/2.8 cell cultures.

The absence of protein−DNA interactions over the distal region of the promoter in the butyrate-treated cells was not due to a decrease in the binding activities of the transcription factors which have been shown to interact with these distal sequences. Similar DNA-binding activities were observed for the VDR−RXR complex, AML/CBFα-related proteins, and the transcription factor YY-1 by gel mobility shift assays in extracts from control and butyrate-treated ROS cells.

Taken together, our results suggest that chromatin hyperacetylation inhibits the nucleosomal transition involved in the formation of the distal DNase I hypersensitive site in the OC gene promoter. This transition requires protein−DNA interactions that pre-set this domain for the ligand-dependent interaction of the VDR−RXR complex. The large sizes of the footprinted segments detected by LMPCR in ROS 17/2.8 cells suggest that additional protein−DNA interactions occur within this distal promoter region in vivo. The nature of these DNA binding protein(s) is currently under investigation.

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