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Reduced CpG Methylation is Associated With Transcriptional Activation of the Bone-Specific Rat Osteocalcin Gene in Osteoblasts

Alejandro Villagra,[1] José Gutiérrez,[1] Roberto Paredes,[1] José Sierra,[1] Marcia Puchi,[1] Maria Imschenetzky,[1] Andre van Wijnen,[2] Jane Lian,[2] Gary Stein,[2] Janet Stein,[2] and Martin Montecino[1]*

[1]Departamento de Biologia Molecular, Facultad de Ciencias Biologicas, Universidad de Concepcion, Casilla 160-C, Concepcion, Chile
[2]Department of Cell Biology, University of Massachusetts Medical School, Worcester, Massachusetts

Abstract Chromatin remodeling of the bone-specific rat osteocalcin (OC) gene accompanies the onset and increase in OC expression during osteoblast differentiation. In osseous cells expressing OC, the promoter region contains two nuclease hypersensitive sites that encompass the elements that regulate basal tissue-specific and vitamin D-enhanced OC transcription. Multiple lines of evidence indicate that DNA methylation is involved in maintaining a stable and condensed chromatin organization that represses eukaryotic transcription. Here we report that DNA methylation at the OC gene locus is associated with the condensed chromatin structure found in cells not expressing OC. In addition, we find that reduced CpG methylation of the OC gene accompanies active transcription in ROS 17/2.8 rat osteosarcoma cells. Interestingly, during differentiation of primary diploid rat osteoblasts in culture, as the OC gene becomes increasingly expressed, CpG methylation of the OC promoter is significantly reduced. Inhibition of OC transcription does not occur by a direct mechanism because in vitro methylated OC promoter DNA is still recognized by the key regulators Runx/Cbfa and the vitamin D receptor complex. Furthermore, CpG methylation affects neither basal nor vitamin D-enhanced OC promoter activity in transient expression experiments. Together, our results indicate that DNA methylation may contribute indirectly to OC transcriptional control in osteoblasts by maintaining a highly condensed and repressed chromatin structure. J. Cell. Biochem. 85: 112–122, 2002.

Key words: CpG methylation; osteocalcin; transcription

Abbreviations used: OC, osteocalcin; kDa, kilo Daltons; Runx/Cbfa, Core Binding Factor Alpha; GST, glutathione-S-transferase; SDS, sodium dodecyl sulphate; PAGE, poly acrylamide gel electrophoresis; RSB, reticulocyte saline buffer; EMSA, electrophoretic mobility shift assay; TBE, tris-borate-EDTA buffer; dDHS, distal DNaseI hypersensitive site; pDHS, proximal DNaseI hypersensitive site; VDR, vitamin D_3 receptor; RXR, 9-cis retinoic acid receptor; ROB, primary rat osteoblast; LUC, luciferase.The contents are solely the responsibility of the authors and do not necessarily represent the official views of the National Institutes of Health.

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*Correspondence to: Dr. Martin Montecino, Departamento de Biologia Molecular, Facultad de Ciencias Biologicas, Universidad de Concepcion, Casilla 160-C, Concepcion, Chile. E-mail: mmonteci@udec.cl

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Transcriptional activity in eukaryotic cells is controlled by multiple regulatory mechanisms. Recent studies have focused on the cellular events that control local gene chromatin structure that modulates accessibility of nuclear regulatory factors to their cognate binding sequences. It has been shown that DNA methylation is one of the principal mechanisms by which cells maintain a stable chromatin configuration that represses transcription [Razin, 1998].

DNA methylation is essential for mouse development and has been shown to play an important role in X-chromosome inactivation and genomic imprinting [Jaenisch, 1997]. Methylation-mediated silencing may also contribute to suppression of transcriptional noise and to the control of tissue-specific gene expression [Walsh and Bestor, 1999]. In eukaryotic cells, DNA methylation occurs on CG or CNG (where N = G, T, A, or C) sequences in a reaction

catalyzed by the enzyme DNA methyltransferase [Laird and Jaenisch, 1996]. The target CpG dinucleotides are found predominantly in regions of the genome known as CpG islands, which are sequences of about 200 bp or larger with a C + G content above 50% [Jones, 1999]. Undermethylated CpG islands are associated with decondensed chromatin structure and principally distributed within the promoter regions of transcriptionally active genes [Laird and Jaenisch, 1996; Cross et al., 1999].

Transcriptional suppression by DNA methylation may involve a direct mechanism in which CpG methylation prevents binding of the basal transcriptional machinery or specific transcription factors that require contact with cytosines in their cognate binding elements [Bird and Wolffe, 1999]. Binding of several factors is indeed blocked by CpG methylation, although some transcription factors are indifferent to methylation status [Holler et al., 1988].

An alternative and non-exclusive mechanism to explain the repressive effect of DNA methylation on gene expression is based on the direct relationship between CpG methylated DNA and a highly condensed chromatin structure. Thus, in vivo hypomethylated CpG islands are located within chromatin domains that contain hyperacetylated core histones and are deficient in linker histones [Tazi and Bird, 1990]. These two properties are strongly associated with an open chromatin conformation and with transcriptionally active genes. In contrast, chromatin assembled on artificially methylated DNA frequently becomes associated with hypoacetylated core histones, refractory to nuclease or restriction enzymes digestion and transcriptionally repressed when introduced into cells [Boyes and Bird, 1991].

The rat 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. Transcription of the OC gene is controlled by modularly organized basal and hormone-responsive elements [Lian et al., 1999] located within two DNaseI hypersensitive sites that are present only in bone-derived cells expressing this gene (Fig. 1) [Montecino et al., 1994, 1996]. Thus, remodeling of the chromatin structure of the OC gene promoter accompanies the onset and increase in gene expression that occurs during osteoblast differentiation.

The distal DNaseI hypersensitive site of the OC promoter contains a recognition site for the vitamin D_3 receptor (VDR) complex. Upon activation by its ligand (1α, 25-dihydroxy vitamin D_3), this vitamin D_3 responsive element (VDRE, -465 to -437) functions as an enhancer: it does not induce transcription, but stimulates basal tissue-specific expression [Lian et al., 2001]. The transcription factor YY1 also binds the VDRE to a recognition sequence overlapping the proximal half-steroid motif. Interaction of YY1 or the VDR/RXR heterodimer with the VDRE is mutually exclusive and mediates YY1-dependent repression of the vitamin D_3-enhanced OC gene promoter activity [Gou et al., 1997].

Another principal regulator of OC gene transcription in bone cells is a member of the Runx/Cbfa family of proteins [Ducy and Karsenty, 1995; Banerjee et al., 1996, 1997; Ducy et al., 1997]. The rat OC promoter contains

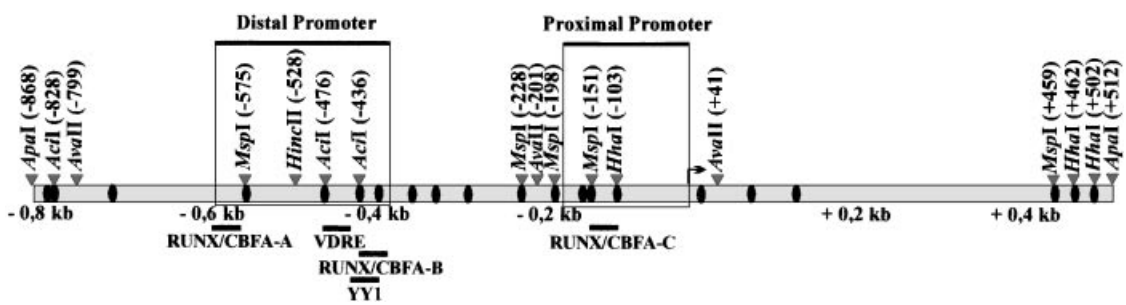

Fig. 1. Schematic representation of the rat OC gene coding and promoter regions. The positions of the putative CpG methylation sites within the *Apa*I/*Apa*I (-868 to $+512$) genomic DNA fragment in the rat OC gene are marked by filled circles. The CpG methylation sites corresponding to the recognition sequences for the restriction enzymes *Msp*I/*Hpa*II, *Aci*I, and *Hha*I, as well as the target sites for *Ava*II cleavage, are also marked at the top. Sequences corresponding to the distal (-600 to -400) and proximal (-170 to -70) OC promoter regions are shown by squares, and the relative locations of key regulatory elements are marked below. The transcription initiation site is indicated by an arrowhead.

three recognition sites for Runx/Cbfa interactions (site A: −605 to −595; site B: −435 to −430; and site C: −138 to −130). Mutation of sites A and B (which flank the VDRE) leads to abrogation of responsiveness to vitamin D_3 [Javed et al., 1999]. Mutation of the three sites results in altered chromatin structure, reflected by loss of the DNaseI hypersensitive sites and by inhibition of OC promoter activity [Javed et al., 1999]. These findings strongly support a role for Runx/Cbfa factors in regulating OC gene expression, not only as transcriptional transactivators, but also by facilitating modifications in promoter architecture and chromatin organization.

The molecular mechanisms by which osteoblasts regulate chromatin structure during differentiation have not been established. We recently found that both YY1 and the VDR/RXR heterodimer are unable to interact with the OC VDRE when it is reconstituted as a nucleosome [Paredes et al., 2002]. In addition, we found that Runx/Cbfa factors can interact with reconstituted nucleosomal DNA only if there is a degree of flexibility in the translational positioning of the histone octamer on the DNA and partial exposure of the Runx/Cbfa site [Gutiérrez et al., 2000]. Together these results indicate that key regulators of the OC gene can not recognize their cognate binding elements in a repressive nucleosomal organization, and therefore, requires previous or concomitant chromatin remodeling events.

To address the role of DNA methylation in establishing a repressive chromatin environment at the OC gene, we determined the methylation status of the rat OC gene promoter in osseous and non-osseous cells and assessed the correlation between CpG methylation and OC expression. Similarly, we evaluated the CpG methylation pattern of the OC gene promoter at different stages of differentiation in primary cultures of rat diploid osteoblasts. In addition, we determined the effect of CpG methylation on the transcriptional activity of an OC promoter-driven luciferase construct transiently transfected in ROS 17/2.8 cells as well as on the ability of the OC transcriptional regulators VDR/RXR, Runx/Cbfa, and YY1 to bind their cognate elements within the distal and proximal regions of the OC gene promoter. Our results suggest that CpG methylation is involved in maintenance of OC transcriptional repression.

MATERIALS AND METHODS

Cell Culture

Rat osteosarcoma-derived ROS 17/2.8 (a gift from G. Rodan: Merck Sharp & Dohme, West Point, PA) and UMR 106 cell lines were cultured and maintained as reported [Majeska et al., 1980; McSheeky and Chamberts, 1986]. Primary diploid osteoblasts (ROB) were isolated from 21-day fetal calvaria and maintained as described [Owen et al., 1990]. Rat hepatoma-derived cells (H4 cells, a gift from S. Grimes, VA Hospital, Shreveport, LA) were maintained as previously described [Wolfe et al., 1989]. OC protein levels in the medium were quantitated by radioimmunoassay [Gundberg et al., 1984].

Transient Cell Transfection Assays

ROS 17/2.8 cell cultures were transiently transfected by the DEAE-Dextran method [Javed et al., 1999]. An OC promoter driven luciferase reporter construct (pOCLUC) was prepared by subcloning the *Eco*RI/*Hind*III promoter fragment from the pOCZCAT plasmid [Frenkel et al., 1993] into the pGL3 vector (Promega, Madison, WI). As internal control in every transfection experiment, the plasmid pβ-gal (Promega), encoding β-galactosidase was used. Cells were treated with 10^{-8} M 1,25-dihydroxyvitamin D_3 (a gift of Dr. M. Uskokovic, Hoffman La Roche, Nutley, NJ) or vehicle for 24 h as indicated. Transfections were repeated four times and each experiment performed in triplicate.

Analysis of the CpG Methylation Pattern at the OC Gene Promoter

Purified genomic DNA was obtained from ROS 17/2.8, UMR-106, H4, and ROB cell cultures and the CpG methylation status determined by combining restriction enzyme cleavage and Southern blot analyses. Genomic DNA was cleaved to completion with either *Hha*I, *Hpa*II, *Ava*II, *Aci*I, or *Msp*I (New England Biolabs, Beverly, MA). The DNA samples were subsequently cleaved with *Apa*I, electrophoresed in a 1.5% agarose gel, transferred to Z-probe membranes (Bio-Rad, Melville, NY) and hybridized with the probe *Hinc*II/*Apa*I (−528/+512), following manufacturer's recommendations. Probes were labeled by the random primer method (GIBCO-BRL, Rockville, MD) using [^{32}P-α]dCTP (New England Nuclear, Boston, MA).

Plasmids and Expression of Recombinant Proteins in *E. coli*

pGEX plasmids (Pharmacia Biotech, Uppsala, Sweden) containing the coding sequences of the human vitamin D_3 receptor (GST-VDR) and retinoid X receptor alpha (RXRα) were generously provided by Dr. Don Chen (University of Massachusetts, Worcester, MA). Plasmids encoding Core Binding Factor (CBF) proteins Cbfa/Runx/p48 and Cbfβ fused to Glutathione-S-transferase (GST) were generously provided by Dr. Scott Hiebert (Vanderbilt University, Nashville, TN). The plasmid encoding GST-YY1 was obtained from Dr. Edward Seto (University of Texas Southwestern, Dallas, TX). The GST fusion proteins were obtained as described [Gou et al., 1997; Gutiérrez et al., 2000; Paredes et al., 2002].

Methylation of the OC Promoter In Vitro

Labeled DNA fragments containing the sequences −547 to −390 and −287 to −57, from the rat OC gene promoter were generated by PCR using specific primers, one of which had been previously end-labeled with polynucleotide kinase (New England Biolabs) and [γ-^{32}P]ATP (New England Nuclear). The plasmid pOC3.4 which contains 1.1 kb of the OC gene promoter was linearized and used as DNA template. The labeled fragments were purified by using G-50 Quick Spin Columns (Boehringer Mannheim) and subsequently methylated using either *Sss*I methylase or *Hha*I methylase (New England Biolabs), according to the manufacturer's instructions. pOCLUC construct was methylated following the same protocol and repurified before use in transient transfection assays. Successful methylation was evaluated by incubation of the modified DNA segments with methylation-sensitive restriction enzymes, fractionation by SDS–PAGE, and autoradiography.

Protein–DNA Interaction Analysis

Binding of the bacterially-expressed proteins to methylated and unmethylated DNA was analyzed by electrophoretic mobility shift assays (EMSA) using the PCR-labeled proximal and distal OC promoter segments described above. Binding reactions contained the indicated amounts of purified proteins, 20 fmol of each labeled promoter segment, and were performed in 20 μl of 10 mM Tris-HCl pH = 7.4, 150 mM KCl, 1 mM EDTA, 0.05% NP40, 0.5 mM β-mercaptoethanol, 0.5 mM PMSF, 50 μg/ml BSA, 10% glycerol, and 2 μg of poly (dI-dC):(dI-dC). 1α,25-dihydroxy vitamin D_3 was added to the binding reactions at a final concentration of 10^{-8} M. Protein–DNA complexes were fractionated in 4% (40:1) native polyacrylamide and 1 × TBE gels, dried, and visualized by autoradiography.

RESULTS

Rat OC Gene Promoter Contains Unmethylated CpG Sites in Bone-Derived Cell Lines Expressing OC

Sequence analysis of the rat OC gene revealed the presence of multiple CpG sites that are potential targets for methylation in vivo by the enzyme, DNA methyltransferase. We focused our study within the region spanning nucleotides from −868 to +661 of the OC gene, which includes all the basal tissue-specific transcriptional regulatory elements as well as the vitamin D_3-responsive element (VDRE) [Lian et al., 2001]. To establish whether there is a significant difference in the CpG methylation pattern of the OC gene associated with its transcriptional status, we compared two bone-derived cell lines that either express (ROS 17/2.8) or do not express (UMR106) OC. As a negative control, we analyzed the CpG methylation pattern of the OC gene in a non-osseous cell line, rat hepatoma-derived H4 [Wolfe et al., 1989], which does not express the OC gene [Montecino et al., 1994]. We had previously found that in both H4 cells and UMR106 cells, the OC gene promoter is organized in a highly condensed chromatin structure with reduced accessibility to DNaseI and micrococcal nuclease [Montecino et al., 1994; unpublished results].

Genomic DNA samples from the three cell lines were analyzed by Southern blot to evaluate the susceptibility of the various CpG sites present in the OC promoter to cleavage by the restriction enzymes *Hha*I, *Hpa*II, *Aci*I, *Ava*II, and *Msp*I. The first three enzymes do not cleave their target sequences when the internal cytosines are methylated. In contrast, cleavage by *Msp*I, which recognizes the same sequence as *Hpa*II, and by *Ava*II is not affected by methylation. As shown in Figure 1, the −868 to +512 region of the OC locus contains five recognition sites for *Msp*I and *Hpa*II (CCGG), and three each for *Hha*I (GCGC), *Ava*II (GGA/TCC), and

*Aci*I (CCGC). The presence of all of these recognition sites in the OC promoter was confirmed by cleaving unmethylated plasmids containing the OC gene coding and flanking sequences with each restriction enzyme and analyzing the digestion products by Southern blot (data not shown).

As shown in Figure 2, cleavage with *Hpa*II and *Msp*I revealed significant differences in the CpG methylation pattern of the OC gene promoter between ROS 17/2.8 and UMR106 cells. The *Hpa*II/*Msp*I sites located at − 575, − 228, − 198, and + 459 were unmethylated in ROS 17/2.8 cells as they were cleaved by both enzymes (Fig. 2, lanes 2 and 3). These same sites were methylated in both UMR106 cells and H4 cells as they could not be cleaved by *Hpa*II (Fig. 2, lanes 4 to 9). Interestingly, the *Hpa*II/*Msp*I site at − 151 was found methylated in each of the three cell lines analyzed.

As shown in Figure 3, cleavage by *Hha*I also indicated changes in the methylation pattern between cell lines that express or do not express the OC gene. Thus, the *Hha*I sites at − 103, + 462, and + 502 were found methylated in both UMR106 and H4 cells (Fig. 3, lanes 3–6), while the − 103 and + 462 sites were unmethylated in ROS 17/2.8 cells (the + 502 site was not analyzed in this cell line) (Fig. 3, lanes 1 and 2). In addition, we found that the recognition sites for *Aci*I at − 850, − 476, and − 436 were methylated in UMR106 and H4 cells (Fig. 4, lanes 3–6) and that the site at − 436 was unmethylated in ROS 17/2.8 cells (Fig. 4, lanes 1 and 2). As a positive control, we determined that the *Ava*II sites located within the region of study (− 799, − 201, and + 41) were readily cleaved in all three cell lines (Fig. 5).

Taken together, our results indicate that decreased CpG methylation at the OC locus accompanies OC transcriptional activity in bone-derived cell lines. Moreover, our findings suggest that osteoblastic cell lines that do not express OC present a methylation pattern similar to that observed in non-osseous cell lines.

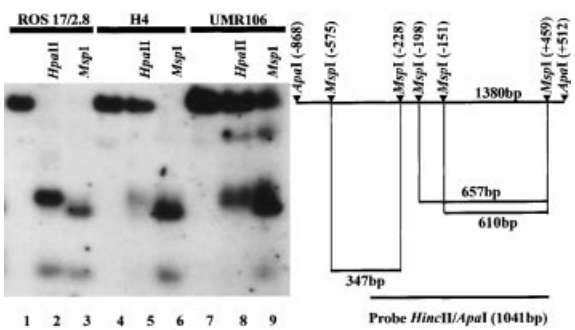

Fig. 2. Comparative analysis of the *Msp*I/*Hpa*II cleavage pattern at the OC promoter in cell lines that either express or do not express OC. Genomic DNA was isolated from ROS 17/2.8 (**lanes 1–3**), H4 (**lanes 4–6**), and UMR 106 (**lanes 7–9**) cell lines, digested to completion with *Apa*I, and then cleaved with either *Msp*I or *Hpa*II (marked at the top of the gel). The samples were then analyzed by Southern blot as described in Materials and Methods. A diagram representing the position of the cleavage sites and the expected restriction fragments (including their sizes) is shown at the right of the gel. The probe used for hybridization is shown below.

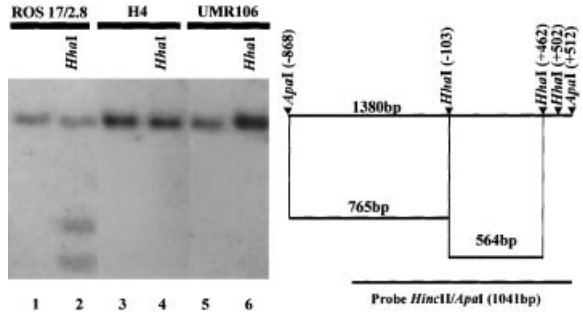

Fig. 3. Cleavage by *Hha*I within the rat OC promoter is prevented in cell lines that do not express OC. Genomic DNA was isolated from ROS 17/2.8 (**lanes 1–3**), H4 (**lanes 4–6**), and UMR 106 (**lanes 7–9**) cell lines, digested to completion with *Apa*I, and then cleaved with *Hha*I (marked at the top of the gel). The samples were then analyzed by Southern blot. See legend in Figure 2 for an explanation of the diagram and symbols.

OC Gene Promoter is Demethylated During Primary Diploid Osteoblast Differentiation in Culture

Primary cultures of calvarial-derived rat osteoblasts develop a mineralized extracellular

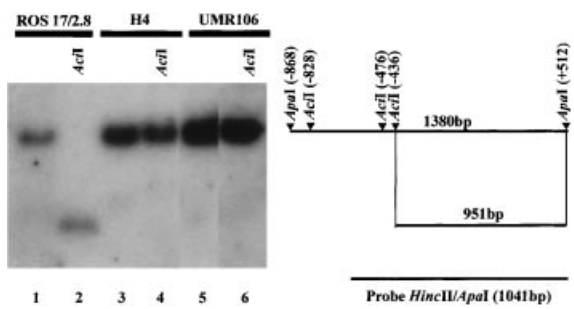

Fig. 4. Cleavage by *Aci*I within the − 868/+512 segment of the rat OC gene promoter is blocked in DNA from cells that do not express OC. Genomic DNA was isolated from ROS 17/2.8 (**lanes 1–3**), H4 (**lanes 4–6**), and UMR 106 (**lanes 7–9**) cell lines, digested to completion with *Apa*I, and then cleaved with *Aci*I (marked at the top of the gel). The samples were then analyzed by Southern blot. See legend in Figure 2 for an explanation of the diagram and symbols.

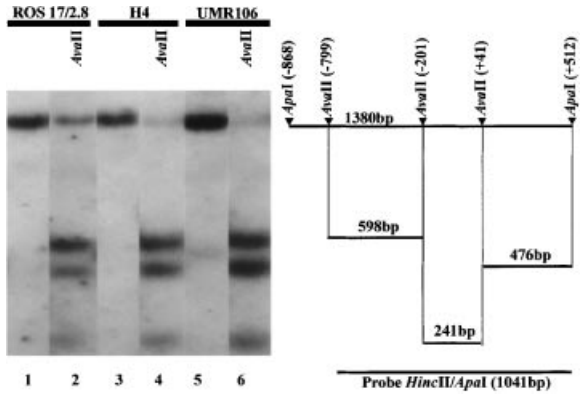

Fig. 5. Cleavage pattern of the rat OC gene with methylation-insensitive restriction enzymes is independent of the OC expression status. Genomic DNA was isolated from ROS 17/2.8 (**lanes 1–3**), H4 (**lanes 4–6**), and UMR 106 (**lanes 7–9**) cell lines, digested to completion with *Apa*I, and then cleaved with *Ava*II (marked at the top of the gel). The samples were then analyzed by Southern blot. See legend in Figure 2 for an explanation of the diagram and symbols.

matrix, with a bone tissue-like organization analogous to osteoblast differentiation in vivo [Aronow et al., 1990; Owen et al., 1990]. The OC gene is expressed in these cells during late stages of differentiation, preceding the initiation of mineral deposition [Aronow et al., 1990; Owen et al., 1990]. This developmentally-regulated transcriptional activation correlates with chromatin remodeling at the OC gene promoter reflected by the presence of two DNaseI hypersensitive sites and a positioned nucleosome [Montecino et al., 1996].

Because of the marked differences in CpG methylation at the OC gene locus between ROS 17/2.8 cells, which express OC and UMR 106 cells, which do not, we examined whether changes in the methylation pattern accompany the developmentally-regulated transcriptional activation of the OC gene during primary osteoblast differentation in culture. Our experimental approach was to isolate genomic DNA from rat calvarial-derived osteoblast (ROB) cultures at various stages of differentiation and to determine the methylation status of the OC gene. The differentiation stage of the cultures was confirmed by assessing the OC biosynthesis rate as a measure of OC expression [Aronow et al., 1990; Owen et al., 1990]. As shown in Table I, proliferating ROB cultures (day 3) did not synthesize OC while mineralized cultures (day 22) expressed the highest levels of the OC protein.

TABLE I. OC Biosynthesis During Primary Diploid Rat Osteoblast (ROB) Differentiation in Culture

Days in culture	OC concentration (ng/dl)
3	0.20 ± 0.14
10	9.789 ± 3.24
16	19.51 ± 3.04
18	56.92 ± 21.3
22	203.95 ± 69.78

By analyzing cleavage with the enzymes *Hpa*II and *Msp*I, we detected changes in the CpG methylation pattern at the OC locus accompanying transcriptional activation of the gene during ROB differentiation in culture. The *Hpa*II/*Msp*I sites at -575 and -228, which were methylated in genomic DNA samples from ROB cultures at day 3 and 10 (Fig. 6, lanes 1 to 6), were unmethylated in samples from mineralized ROB cultures at day 22 (Fig. 6, lanes 10–12). Similar to the ROS 17/2.8 cell line, the *Hpa*II/*Msp*I site at -151 remained methylated throughout the entire ROB developmental sequence. On the other hand, the -198 and $+459$ *Hpa*II/*Msp*I sites were found to be unmethylated at each developmental stage from ROB cells. As it was controlled by gel staining, the different *Hpa*II/*Msp*I cleavage pattern between DNA samples from proliferating and mineralized ROB cells is not due to the loading of uneven concentrations of DNA (not shown).

Together our results indicate that mineralized ROB cultures (day 22) exhibit a CpG methylation pattern at the OC gene comparable

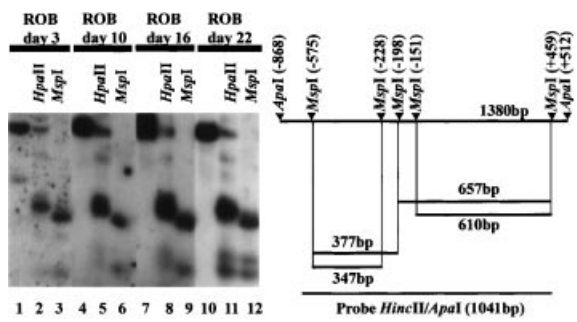

Fig. 6. The rat OC promoter is demethylated during primary diploid osteoblast differentiation in culture. Genomic DNA samples were obtained from primary rat osteoblast (ROB) cultures at different stages of differentiation (marked at the top of the gel). The purified samples were digested to completion with *Apa*I and subsequently cleaved with either *Msp*I or *Hpa*II (marked at the top). The samples were then analyzed by Southern blot. See legend in Figure 2 for an explanation of the diagram and symbols.

to that found in the ROS 17/2.8 cell line. Thus, these data suggest that bone-derived cells expressing OC exhibit a reduced methylation pattern at the OC gene locus.

CpG Methylation Does Not Alter Basal Transcriptional Activity or Vitamin D-Dependent Enhancement of the OC Gene Promoter

To investigate whether CpG methylation prevents OC gene transcriptional activity, we methylated the rat OC gene promoter in vitro and determined its activity by transient transfection assays using a luciferase reporter gene. Methylation of the constructs was carried out using two different DNA methyltransferases, *Hpa*II methylase, which specifically methylates *Hpa*II/*Msp*I sites and *Sss*I methylase, which methylates all CpG dinucleotides. Successful methylation was confirmed by Southern blot to evaluate the sensitivity of the methylated constructs to cleavage by methylation-sensitive restriction enzymes (data not shown). To control for the effect of CpG methylation at the coding region of the luciferase gene on its expression levels, we methylated a luciferase reporter construct driven by the SV-40 promoter (pGL3LUC) and containing the same plasmid backbone as pOCLUC. Because this promoter does not contain *Hpa*II/*Msp*I sites, a significant decrease in the luciferase activity following methylation would be due to modifications at the luciferase coding region.

As shown in Figure 7, transient transfection of the pOCLUC construct into ROS 17/2.8 cells, produced a significant level of luciferase activity within the cell, which was enhanced by the addition of vitamin D_3 to the cultures. Methylation of this construct by *Hpa*II methylase did not cause a significant effect on basal luciferase expression levels nor on vitamin D-dependent enhancement. These findings indicate that methylation at OC gene promoter *Hpa*II/*Msp*I sites does not alter transcriptional activity. On the other hand, methylation of pOCLUC by *Sss*I methylase resulted in decreased luciferase activity (Fig. 7) as has been reported previously [Nourrit et al., 1999]. Because we observed a more dramatic effect on promoter activity when the control construct was methylated by *Sss*I, it was concluded that the reduction in the pOCLUC activity was due primarily to CpG methylation at the luciferase coding region.

Hence, these results indicate that under our experimental conditions, methylation of the *Hpa*II/*Msp*I sites in the OC promoter does not prevent transcriptional activation.

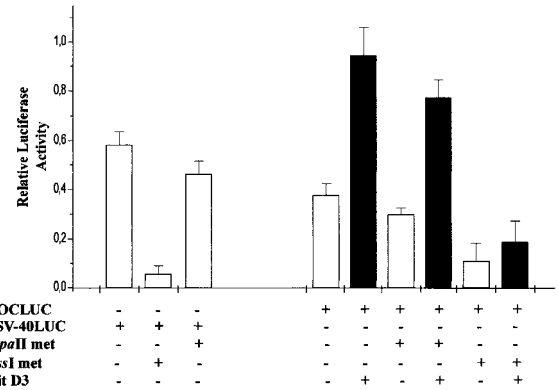

Fig. 7. In vitro methylation does not affect transcriptional activity of the rat OC gene promoter. The plasmids pOCLUC and pSV40LUC were methylated with either *Hpa*II or *Sss*I methylases (marked below) as described in Materials and Methods, and then analyzed for promoter activity by transient transfection assays in ROS 17/2.8 cells cultured in the presence (filled bars) or absence (open bars) of 10^{-8} M vitamin D_3.

CpG Methylation of the OC Gene Promoter Does Not Prevent Binding of Key Transcription Regulatory Factors

Preventing the binding of components of the basal transcriptional machinery or of specific transcription factors that require contact with cytosines in their cognate recognition elements may be a direct mechanism for CpG methylation-dependent inhibition of transcription [Bird and Wolffe, 1999]. To investigate whether CpG methylation affected binding to the OC promoter of the key regulatory factors Runx/Cbfa, vitamin D receptor-9-cis retinoic acid receptor complex (VDR/RXR) and YY1, we performed protein–DNA interaction studies using purified recombinant transcription factors and determined the ability of these factors to recognize their cognate element in a methylated DNA fragment. End-labeled DNA segments containing the proximal (– 287 to – 57) or distal (– 547 to – 390) OC promoter regions were generated by PCR and methylated with either *Hpa*II or *Sss*I methylases. Successful methylation of these fragments was confirmed by cleavage with methylation-sensitive restriction enzymes and Southern blot analysis (data not shown).

As shown in Figure 8, CpG methylation did not prevent binding of the Runx/Cbfa-Cbfb transcription factor complex to its cognate element in the proximal promoter region of the OC gene (Runx/Cbfa site C, – 138 to – 130). Moreover,

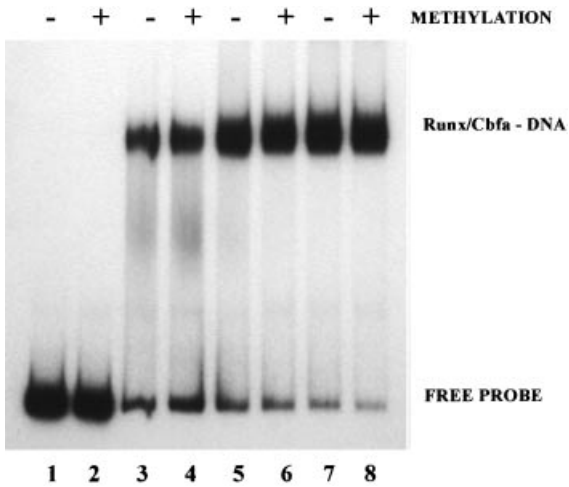

Fig. 8. Binding of Runx/Cbfa transcription factors to the proximal OC promoter is not affected by methylation. Binding of bacterially-produced Runx/Cbfa factors to either methylated (+) or unmethylated (−) proximal rat OC promoter (− 287 to − 57) was assessed by EMSA as described in Materials and Methods. Binding reactions were carried out in the presence of equivalent concentrations of the transcription factor Cbfb [Gutiérrez et al., 2000]. **Lane 1**, unmethylated probe alone; **lane 2**, methylated probe alone; **lanes 3 and 4**, 50 nM GST-Runx/Cbfa/p48; **lanes 5 and 6**, 75 nM GST-Runx/Cbfa/p48; **lanes 7 and 8**, 100 nM GST-Runx/Cbfa/p48. The presence of the Runx/Cbfa-DNA complex is marked at the right of the gel.

increasing concentrations of the factor up to saturating levels significantly reduced the amount of free probe in the binding reaction (Fig. 8, lanes 3–8). Similarly, methylation did not affect interaction of the Runx/Cbfa-Cbfb transcription complex with the Runx/Cbfa site B (− 440 to − 435) in the distal OC promoter region (Fig. 9A). We also found that binding of the VDR/RXR complex to the vitamin D responsive element (VDRE, − 465 to − 437) was not altered by methylation of the distal OC gene promoter region (Fig. 9B). Together, these results indicate that CpG methylation at the OC gene promoter does not block the ability of these two key regulators to activate OC transcription.

It was previously reported that binding of the YY1 transcription factor to its cognate element is reduced by CpG methylation [Satyamoorthy et al., 1993]. Accordingly, we found that the interaction of YY1 with the methylated − 547/ − 390 OC promoter segment, which includes a YY1 binding site (− 450 to − 445), was significantly decreased (Fig. 9C). This result suggests that the role of YY1 as an inhibitor of vitamin D-dependent OC transcriptional enhancement may be prevented by CpG methylation.

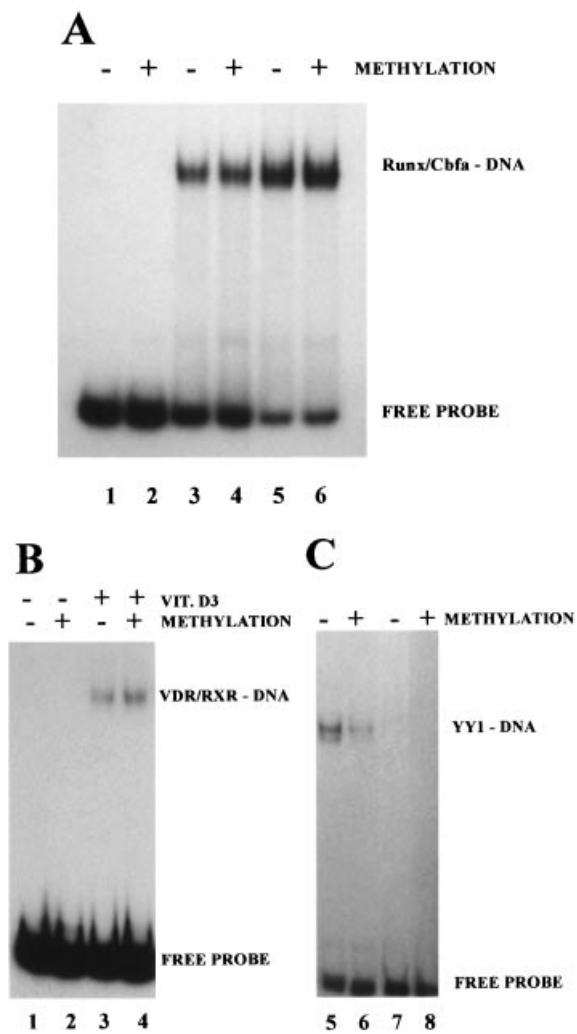

Fig. 9. Runx/Cbfa and VDR/RXR transcription factors bind to a methylated DNA segment containing the distal rat OC gene promoter. Binding of recombinant Runx/Cbfa (**A**), VDR/RXR (**B**), and YY1 (**C**) transcription factors to either methylated (+) or unmethylated (−) distal rat OC promoter (− 557 to − 390) was assessed by EMSA as described in Materials and Methods. Binding reactions for Runx/Cbfa were carried out in the presence of equivalent concentrations of the transcription factor Cbfb [Gutiérrez et al., 2000]. **A**: **Lane 1**, unmethylated probe alone; **lane 2**, methylated probe alone; **lanes 3 and 4**, 50 nM GST-Runx/Cbfa/p48; **lanes 5 and 6**, 75 nM GST-Runx/Cbfa/p48. The position of the Runx/Cbfa-DNA complex is marked at the right of the gel. **B**: **Lane 1**, unmethylated probe alone; **lane 2**, methylated probe alone; **lanes 3 and 4**, 50 nM GST-VDR/RXR. The binding reactions were carried out in the presence of 10^{-8} M vitamin D_3. The position of the VDR/RXR-DNA complex is marked at the right of the gel. **C**: **Lanes 1 and 2**, 50 nM GST-YY1; **lane 3**, unmethylated probe alone; **lane 4**, methylated probe alone. The position of the YY1-DNA complex is marked at the right of the gel.

DISCUSSION

The OC gene encodes a bone-specific protein that is expressed at late stages of osteoblast

differentiation, preceding mineralization of the extracellular matrix [Aronow et al., 1990; Owen et al., 1990]. Transcriptional activation of the OC gene is accompanied by chromatin remodeling at the promoter region, reflected by the presence of two DNaseI hypersensitive sites spanning the basal tissue-specific and steroid hormone-dependent OC regulatory elements

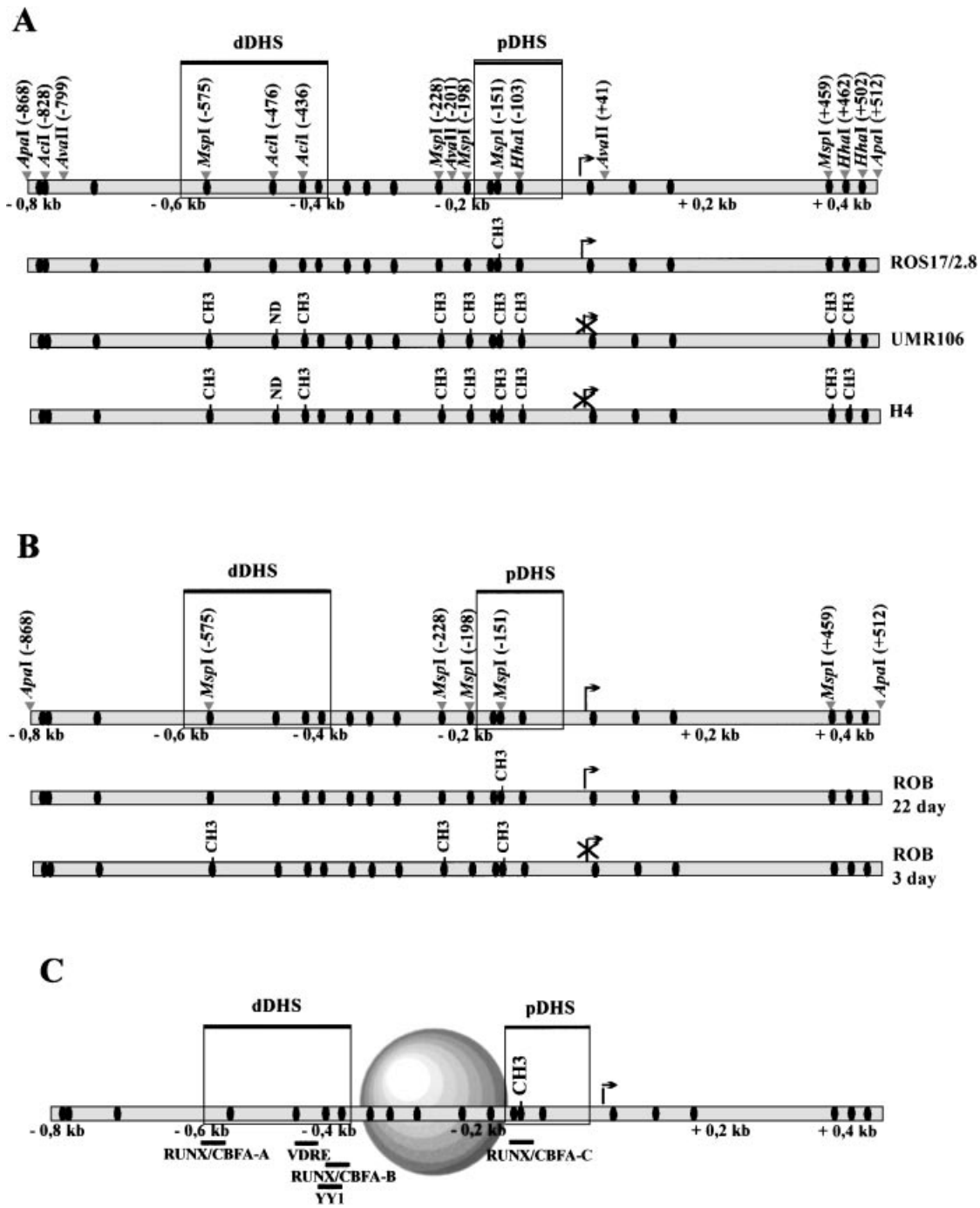

Fig. 10. Summary diagrams representing the methylation status of the CpG sequences within the OC gene in rat-derived cell lines (**A**) and primary rat diploid osteoblasts in culture (**B**). **C**: The postulated model for the remodeled chromatin structure of the OC promoter in bone-derived cells expressing OC [Montecino et al., 1996], including a nucleosome (●) positioned between the distal (dDHS) and proximal (pDHS) DNaseI hypersensitive sites. See legend in Figure 1 for an explanation of the symbols.

[Montecino et al., 1996]. We are interested in understanding the molecular mechanisms that mediate this transition at the OC promoter between a highly condensed chromatin structure in bone-derived cells that do not express this gene and the chromatin remodeled state that accompanies OC transcriptional activation. DNA methylation is one of the main mechanisms by which cells maintain a stable chromatin configuration that represses transcription [Razin, 1998]. Hence, we analyzed whether there are differences in the CpG methylation pattern at the rat OC gene locus between cells that either express or do not express this gene. We determined that a significant reduction in methylation at the OC gene accompanies OC transcriptional activity in osteoblastic cell lines (Fig. 10A). Moreover, we found that the OC promoter is sequentially demethylated during differentiation of normal diploid osteoblasts, correlating with OC expression (Fig. 10B).

It has been proposed that CpG methylation may directly inhibit transcriptional activity by preventing the interaction of transcription factors that require contact with cytosines in their cognate binding element [Bird and Wolffe, 1999]. However, we did not find a significant effect of DNA methylation at the OC promoter on either basal or vitamin D-enhanced transcriptional activity as measured by transient transfection assays. Furthermore, we determined that binding of the key transcriptional activators Runx/Cbfa and VDR/RXR, to both distal and proximal OC promoter regions is not affected by CpG methylation. Hence, our findings indicate that DNA methylation does not result in a direct inhibitory mechanism of OC gene expression in osteoblastic cells.

An alternative mechanism to explain the repressive effect of DNA methylation on gene expression relates to the direct relationship between CpG methylation and highly condensed chromatin structure [Bird and Wolffe, 1999]. Methylated promoters are often organized as tightly packaged nucleosomes including histone H1 and hypoacetylated core histones [Razin, 1998]. Recently, several proteins containing domains that bind methylated DNA have been described to be involved in transcriptional repression. These proteins are thought to modify chromatin structure by recruiting histone deacetylase activities to methylated DNA, resulting in a highly condensed, repressive chromatin organization [Razin, 1998; Bird and Wolffe, 1999]. We have reported that the OC promoter region in bone-derived cells that do not express OC, presents a highly condensed chromatin structure, with decreased accessibility to the nucleases DNaseI, micrococcal nuclease, and restriction enzymes [Montecino et al., 1994, 1996].

Interaction of Runx/Cbfa is required for both basal bone-specific and vitamin D-enhanced transcription of the rat OC gene [Javed et al., 1999]. Similarly, Runx/Cbfa binding sites are necessary for chromatin remodeling at the OC gene promoter [Javed et al., 1999], indicating that Runx/Cbfa factors regulate OC gene expression not only as transcriptional activators, but also by facilitating modifications in promoter architecture and chromatin organization. Interestingly, we have recently demonstrated that Runx/Cbfa factors can interact with nucleosomal DNA only if there is a degree of flexibility in the translational positioning of the histone octamer on the DNA fragment and partial exposure of the Runx/Cbfa site [Gutiérrez et al., 2000]. Thus, Runx/Cbfa proteins are unable to interact with their binding sites within a repressive nucleosomal enviroment [Gutiérrez et al., 2000; Paredes et al., 2002], and therefore, require a previous or concomitant chromatin remodeling process to promote transcriptional activity of the OC gene.

Taken together, our findings indicate that CpG methylation may support the condensed chromatin organization of the OC gene in osteoblastic cells that do not express OC. This methylated pattern is reduced during differentiation of nomal diploid rat osteoblasts in culture, correlating with the chromatin remodeling events that accompany OC transcriptional activation.

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