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State of Methylation of the Human Osteocalcin Gene in Bone-Derived and Other Types of Cells

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Abstract DNA methylation is a general mechanism of controlling tissue-specific gene expression. Osteocalcin is a bone matrix protein whose expression is limited almost entirely to osteoblasts. We were interested in determining whether the state of methylation of the osteocalcin gene plays a role in its expression by studying human bone-derived (MG-63, U2-Os, SaOs-2) and other types (normal lymphocytes, A-498, Hep G2) of cells. Reverse transcription–polymerase chain reaction (RT-PCR) analysis revealed that osteocalcin mRNA production is stimulated by $1,25(OH)_2D_3$ in MG-63 and induced in SaOs-2 but not in U2-Os osteoblast-like osteosarcoma cells. Genomic analysis of the human osteocalcin gene showed that the local surroundings of this single-copy gene are identical in all cell lines studied. Using an isoschizomeric pair of restriction enzymes and Southern analysis, we found that the osteocalcin gene is identically methylated in all three osteosarcoma cell lines. The same sites are also methylated in human normal lymphocytes and A-498 kidney cells, whereas the degree of methylation is higher in Hep G2 human hepatocellular carcinoma cells. Furthermore, the osteocalcin gene was identically protected against enzymatic digestion at the chromatin level in normal lymphocytes and in all cell lines studied. Induction of hypomethylation of DNA by 5-azacytidine treatment did not cause an induction of osteocalcin synthesis in these cell lines. On the contrary, it attenuated the induction by $1,25(OH)_2D_3$ in MG-63 cells. In gel mobility shift assays, human vitamin D receptor and the AP-1 transcription factor bound to an unmethylated response element oligonucleotide of the osteocalcin gene with greater affinity than to an in vitro methylated response element. These results indicate that the in vivo methylation state of the osteocalcin gene at sites determined in this study does not correlate with the inducibility of this gene. Nevertheless, the in vitro results clearly indicated that hypomethylation of critical regions of the osteocalcin gene promoter is a potential mechanism influencing effective binding of specific nuclear factors and, consequently, gene expression. J. Cell. Biochem. 66:404–412, 1997. © 1997 Wiley-Liss, Inc.

Key words: osteocalcin; osteosarcoma cells; methylation; bone-derived cells; DNA

In higher organisms, control of gene expression is often related to the state of methylation of the cytosine residues in DNA. The methylcytosines mostly occur in CpG doublets and stably alter the local structure of genes affecting protein-DNA interactions, which are required for transcription [Cedar, 1988; Adams, 1990]. The cytidine analog, 5-azacytidine, which does not undergo methylation at the 5-position, acts as a demethylating agent during cell growth. Thus, treatment with 5-azacytidine may selectively activate eukaryotic gene expression [Gellersen and Kempf, 1990; Ferguson et al., 1995; Huynh et al., 1996] and alter the state of differentiation of the cells [Darmon et al., 1984; Nishikawa et al., 1993], although contrasting results have also been reported [Hsiao et al., 1984]. The extent of methylation can be determined—for example, by the use of isoschizomeric pairs of restriction enzymes, such as Hpa II and Msp I. Both enzymes recognize the sequence CCGG, but only Msp I can cleave it when the internal cytosine is methylated. Depending on the cleavage patterns generated after digestion, conclusions can be

Abbreviations: dNTP, deoxynucleoside triphosphate; GAPDH, glyceraldehyde phosphate dehydrogenase; RT-PCR, reverse transcriptase–polymerase chain reaction; SSC, standard saline citrate (150 mM NaCl/15 mM sodium citrate, pH 7.0); SDS, sodium dodecyl sulfate; TBE, 0.1 M Tris-borate, pH 8.3, 2 mM EDTA; VDR, vitamin D receptor; AP-1 + VDRE, AP-1 plus vitamin D response element; $1,25(OH)_2D_3$, 1,25-dihydroxyvitamin D, cholecalciferol; 5-aza, 5-azacytidine.

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drawn on the state of methylation of the gene of interest.

Osteocalcin is a bone-specific calcium-binding protein, which is synthesized almost exclusively by osteoblasts [Hauschka et al., 1975]. A gene for osteocalcin has been localized to human chromosome 1q [Puchacz et al., 1989]. Osteocalcin is a marker of the most differentiated osteoblasts [Owen et al., 1990; Pockwinse et al., 1992]. Previously, it has been found that, of the human osteosarcoma cell lines MG-63, SaOs-2, and U2-Os, only MG-63 cells exhibit $1,25(OH)_2D_3$-inducible synthesis of osteocalcin as studied by Northern blot analysis and osteocalcin radioimmunoassay [Mahonen et al., 1990]. This suggests that these cell lines may represent different stages of osteoblastic differentiation. To ascertain whether the state of methylation of the osteocalcin gene plays a role in its cell- and tissue-specific expression, we studied the state of methylation of the osteocalcin gene in these bone-derived and other types of human cell lines.

MATERIALS AND METHODS

Materials

5-azacytidine was from Sigma Chemical Co. (St. Louis, MO) and $1,25(OH)_2D_3$ from Hoffmann-La Roche Co. (Nutley, NJ). Hpa II methylase and nick-translation kits were purchased from Boehringer-Mannheim (Mannheim, Germany). Restriction endonucleases were from Boehringer-Mannheim or MBI Fermentas (Vilnius, Lithuania). [α-^{32}P]dCTP (>3,000 Ci/mmol) and [γ-^{32}P]ATP (>6,000 Ci/mmol) were from Du Pont de Nemours (Boston, MA). The osteocalcin radioimmunoassay kit was from CIS Bio International (Gif-Sur-Yvette Cedex, France). In lymphocyte isolation Ficoll-Paque from Pharmacia Biotech (Uppsala, Sweden) was used.

Cell Culture

The cell lines (A-498, Hep G2, MG-63, SaOs-2, U2-Os) were obtained from American Type Culture Collection (Rockville, MD). The cells were cultured in Dulbecco's modified Eagle's medium supplemented with 7% irradiated fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin (Gibco, Paisley, UK) at 37°C in a humidified 95% air, 5% CO_2 incubator. The culture medium was replaced 24 h before each experiment by a medium containing 2% charcoal-treated fetal calf serum. 5-azacytidine was added to the cultures when the cells reached 50% confluency to ascertain its incorporation into DNA.

RT-PCR Analysis

Total RNA was isolated from control and 10^{-7} M $1,25(OH)_2D_3$-treated cells by the guanidinium thiosyanate method [Chomczynski and Sacchi, 1987] and further treated by DNAase I. cDNA synthesis and polymerase chain reaction were performed as previously described by Hyttinen et al. [1991]. The synthesized cDNA (2 μl) was used as a template in the PCR procedure initiated with a cycle of 3 min at 96°C. The actual amplification consisted of the following phases: 1 min at 96°C (denaturation), 1 min at 60°C (primer annealing), and 1 min 30 s at 72°C (extension). After completion of 28 cycles, the samples were left at 72°C for 5 min. The primer pairs used in the PCR were 5′-CTCACACTCCTCGCCCTATT-3′ and 5′-GACTGGGGCTCCCAGCCATT-3′ for osteocalcin, resulting in an amplification product of 203 bp [Celeste et al., 1986], and 5′-GGAAAGGTGAAGGTCGGAGTC-3′ and 5′-CAAAGTTGTCATGGATGACC-3′ for glyceraldehyde phosphate dehydrogenase (GAPDH) (used as a control gene), resulting in an amplification product of 501 bp [Ercolani et al., 1988]. The PCR products were run on a 1.5% agarose gel containing ethidium bromide.

DNA Digestion and Southern Analysis

Isolated genomic DNA from the cultured cells [Blin and Stafford, 1976] or lymphocytes from whole blood [Old and Higgs, 1982] was digested with restriction enzymes under conditions recommended by the suppliers. The digested DNA was precipitated, and the fragments were electrophorezed in 0.9% agarose gels and transferred onto nitrocellulose filters. The probe used for methylation analyses was a 1.2 kb Sac I fragment of the human osteocalcin gene (phBGP/SacI) [Celeste et al., 1986]. Hybridization was carried out with the phBGP/SacI probe for 24 h at 65°C in 6× SSC, 10 mM EDTA, 5× Denhardt's solution, 0.5% SDS, and 250 mg/ml denatured salmon sperm DNA. The washes were performed first at room temperature with 2× SSC, then with 2× SSC + 0.1% SDS, and finally at 65°C with 0.1× SSC.

RNA Isolation and Northern Analysis

Total cellular RNA was isolated by the method of Anderson et al. [1974]. Denatured RNA samples were fractionated in 1% agarose formaldehyde gels, transferred onto nitrocellulose filters, and hybridized with the phBGP/SacI probe. Hybridizations and washes were performed as described previously [Pirskanen et al., 1991].

Medium Osteocalcin Concentration

Medium osteocalcin concentrations were measured by radioimmunoassay as recommended by the supplier.

Digestion of Nuclei With Restriction Enzymes

The relationship between DNA methylation and chromatin structure was studied by isolating and digesting nuclei with Msp I prior to isolation of DNA and its digestion with Pst I as previously described [Tazi and Bird, 1990; Antequera et al., 1990]. The DNA fragments recovered from the digests were phenol-chloroform-extracted and ethanol-precipitated. The fragments were separated on 0.9% agarose gels, transferred onto nitrocellulose filters, and hybridized with the phBGP/SacI probe.

In Vitro Methylation of Oligonucleotides and Gel Mobility Shift Assays

5′-end-labelled oligonucleotides were treated with Hpa II methylase as recommended by the supplier. Nuclear extracts were prepared as described in Hurst et al. [1990] without the heat denaturation step. The binding reaction mixture contained 10 μg nuclear protein with 2 μg poly(dI-dC)(dI-dC) in 20 mM Hepes, 4.2% (v/v) glycerol, 70 mM NaCl, 2.25 mM $MgCl_2$, 2.03 mM EDTA, 2.17 mM dithiothreitol, 0.08 mM phenylmethylsulfonyl fluoride, 0.33 μg/ml trasylol, and 0.33 μg/ml leupeptin. The protein-bound DNA complexes were separated from the free probe on a 5% polyacrylamide gel run in 0.25× TBE.

RESULTS

Expression of Osteocalcin mRNA and Protein

The expression of osteocalcin mRNA in MG-63, U2-Os, SaOs-2, A-498, and Hep G2 cell lines was studied using the sensitive reverse transcriptase–polymerase chain reaction (RT-PCR) technique (Fig. 1). According to Northern analysis, none of the cell lines studied showed basal osteocalcin mRNA expression [Mahonen et al., 1990]. However, when using the more sensitive PCR method, trace amounts of osteocalcin mRNA were found in control MG-63 cells. Treatment with 10^{-7} M $1,25(OH)_2D_3$ for 24 h enhanced osteocalcin mRNA production in MG-63 cells and induced SaOs-2 cells to express osteocalcin mRNA. Osteocalcin protein secretion was clearly detectable in $1,25(OH)_2D_3$-treated MG-63 cells, whereas only barely detectable amounts were produced by untreated MG-63 cells or $1,25(OH)_2D_3$-treated SaOs-2 cells. Human osteosarcoma (U2-Os), kidney (A-498), and hepatocellular carcinoma (Hep G2) cells did not express osteocalcin mRNA even after $1,25(OH)_2D_3$ treatment. GAPDH was used as an internal control gene in amplifications to ensure that equal amounts of RNA were used in RT-PCR reactions.

Genomic Analysis of the Osteocalcin Gene

In osteocalcin gene studies, we used normal human lymphocytes as a diploid cell control for the osteosarcoma cells. Southern analyses of DNA from human lymphocytes and the human derived cell lines (MG-63, U2-Os, SaOs-2, A-498, Hep G2) are shown in Figure 2A. After Sac I digestions, the expected 1.2 kb fragment was found in all cells studied. After digestion with Hind III, Kpn I, and Eco RV, one identical restriction fragment of larger size was found in all cell lines. Further, DNA from MG-63 cells was digested with nine randomly chosen restriction enzymes (Fig. 2B) to confirm that there exists only one copy of the osteocalcin gene in the human genome [Puchacz et al., 1989].

State of Methylation of the Hpa II Sites and Chromatin at CpG Islands

Naked DNA from bone-derived and other types of cells was digested with Pst I alone or in

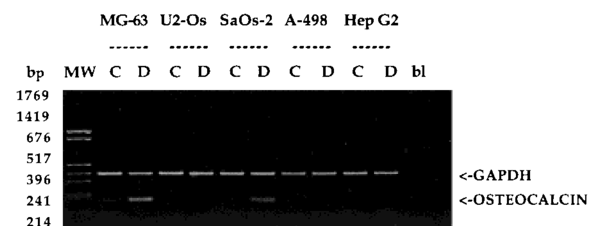

Fig. 1. Identification of osteocalcin mRNA by RT-PCR in control (C) and MG-63, U2-Os, SaOs-2, A-498, and Hep G2 cell lines treated with 10^{-7} M $1,25(OH)_2D_3$ for 24 h (D). Sizes of molecular weight markers are shown but not aligned with their respective bands. bl, blank (no template).

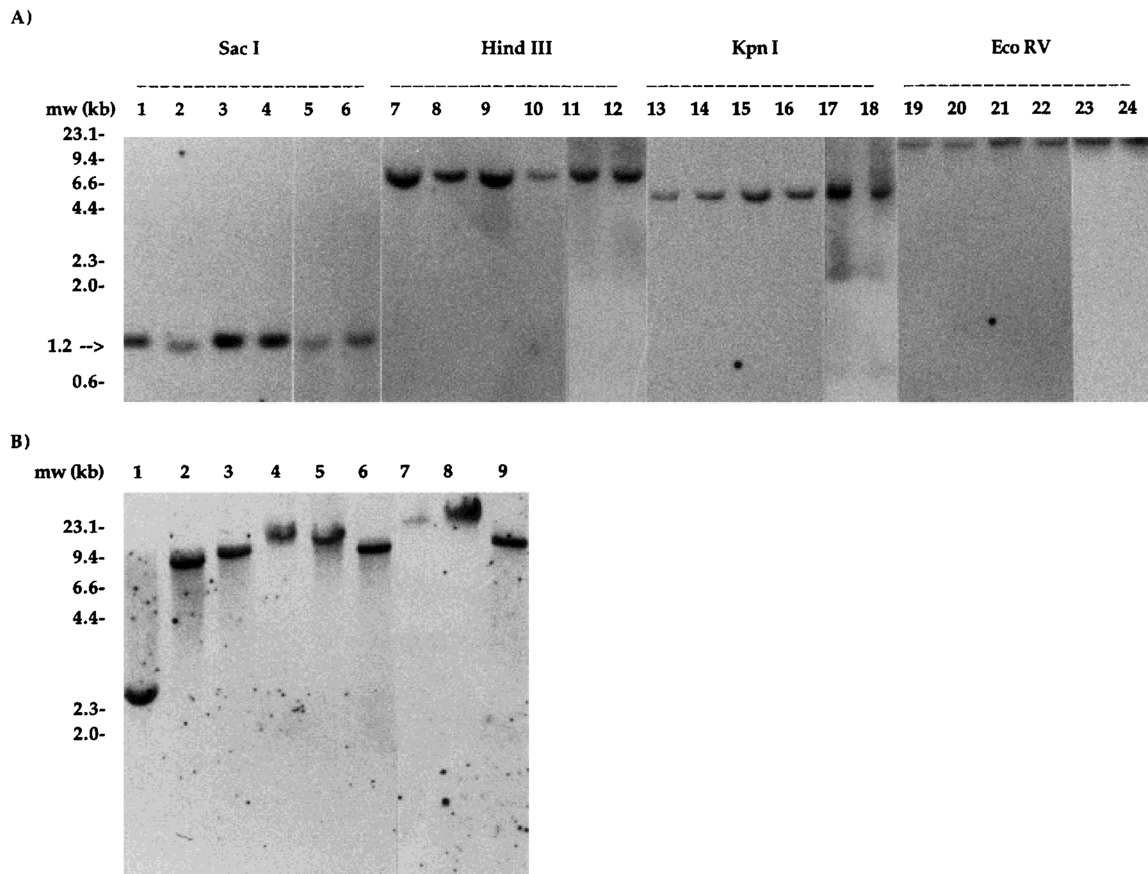

Fig. 2. Southern analysis of genomic DNA. **A:** Ten micrograms of DNA from normal lymphocytes (*lanes 1,7,13,19*), MG-63 (*lanes 2,8,14,20*), U2-Os (*lanes 3,9,15,21*), SaOs-2 (*lanes 4,10,16,22*), A-498 (*lanes 5,11,17,23*), and Hep G2 (*lanes 6,12,18,24*) cells was digested with Sac I, Hind III, Kpn I, and Eco RV. The arrow refers to the location of the 1.2 kb fragment in the Sac I digestions. **B:** DNA from MG-63 cells (10 μg) was analyzed with nine more restriction enzymes: Mva 1269I (*lane 1*), Bgl II (*lane 2*), Nde I (*lane 3*), Not I (*lane 4*), Xho I (*lane 5*), Dra I (*lane 6*), Eco RI (*lane 7*), Sal I (*lane 8*), and Sca I (*lane 9*).

combination with Hpa II or Msp I to determine the methylation state of the osteocalcin gene (Fig. 3). In the cell lines studied, Pst I digestion generated the expected 1.5 kb fragment and another smaller fragment after hybridization with the 1.2 kb Sac I fragment [Celeste et al., 1986]. After codigestion with Pst I plus Msp I, an identical 0.9 kb fragment was observed in all cell lines. After codigestion with Pst I plus Hpa II, however, two fragments of 1.2 and 0.9 kb in size were found in lymphocytes, both in control and $1,25(OH)_2D_3$-treated MG-63 cells, SaOs-2, U2-Os, and A-498 cells, but a larger fragment of about 1.5 kb was found in Hep G2 cells, indicating the presence of methylated CpG.

To further study protection against enzymatic digestion at the chromatin level, whole nuclei from cultured cells were digested with Msp I prior to isolation of DNA and its digestion with Pst I (Fig. 3, lanes 4). The fragments generated (0.9, 1.2, 1.5 kb) were identical in different cell lines studied. Treatment with $1,25(OH)_2D_3$ did not affect the digestion patterns. Presence of nucleosomes blocks access to restriction endonucleases, and, therefore, complete cleavage of the nonmethylated sequence to 0.9 kb fragment by Msp I was not expected [Antequera et al., 1990].

Effect of 5-Azacytidine on Osteocalcin Gene Methylation and Expression

Effects of induced hypomethylation on osteocalcin gene expression were examined by adding 5-azacytidine to the culture medium and preincubating the cells for several generations prior to isolation of DNA. Figure 4 shows the Hpa II/Msp I restriction pattern of the osteocalcin gene in MG-63 cells treated with 5 μM 5-azacytidine alone (lanes 1,2) or in combination with 10^{-7} M $1,25(OH)_2D_3$ (lanes 3,4). A sim-

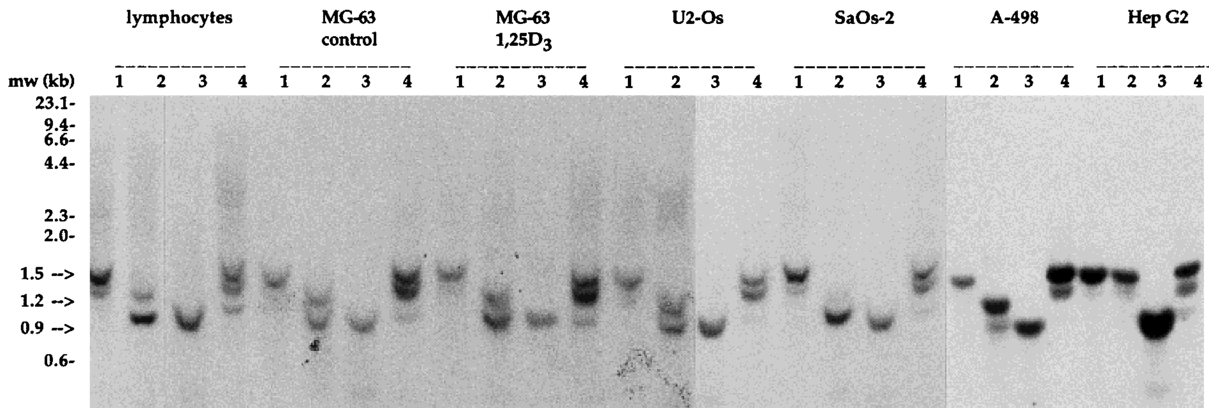

Fig. 3. State of methylation of the Hpa II sites and chromatin protection of the CpG doublets in the human osteocalcin gene. Isolated genomic DNA (10 μg) from normal lymphocytes, both control and $1,25(OH)_2D_3$-treated MG-63 cells, SaOs-2, U2-Os, A-498, and Hep G2 cells was digested with Pst I alone (**lanes 1**) or in combination with Hpa II (**lanes 2**) or Msp I (**lanes 3**). Whole nuclei from the cells were digested with Msp I prior to isolation of DNA and digestion with Pst I (**lanes 4**). The arrows refer to the locations of the 1.5, 1.2, and 0.9 kb fragments.

ilar 0.9 kb fragment was observed in every sample, indicating hypomethylation of the osteocalcin gene. Similar results were obtained when DNA from SaOs-2 and U2-Os cells was studied (data not shown).

In MG-63 cells, different concentrations of 5-azacytidine (0.1, 0.5, 1.0, 2.5, or 5 μM) alone did not affect osteocalcin expression (concentrations 0.5 and 5 μM are shown in Fig. 5, lanes 2,3). Osteocalcin mRNA levels clearly responded to 10^{-7} M $1,25(OH)_2D_3$ treatment (Fig. 5, lane 4). When combined with $1,25(OH)_2D_3$, 5-azacytidine caused a dose-dependent decrease of osteocalcin mRNA (Fig. 5, lanes 5–9) and protein (Table I) levels. Similarly, in SaOs-2 and U2-Os cell lines and in human kidney (A-498) and hepatocellular carcinoma (Hep G2) cells, the 5-azacytidine–induced hypomethylation was not cabable of inducing osteocalcin synthesis alone or in combination with the $1,25(OH)_2D_3$ treatment (data not shown).

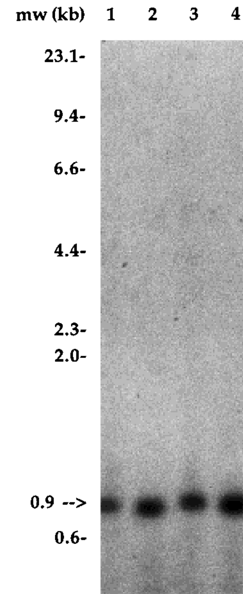

Fig. 4. Effects of induced hypomethylation on osteocalcin gene methylation and expression. Southern analysis of Hpa II (**lanes 1,3**) and Msp I (**lanes 2,4**) digestion patterns of DNA from MG-63 cells after pretreatment with 5 μM 5-azacytidine (**lanes 1,2**) or 5 μM 5-azacytidine + 10^{-7} M $1,25(OH)_2D_3$ (**lanes 3,4**).

Effect of Cytosine Methylation on Protein-DNA Interactions In Vitro

Although we did not see an in vivo correlation between the state of DNA methylation and osteocalcin expression, we wanted to study possible effects of methylation on specific protein-DNA interactions in more detail. The cytosine residues in the center of the AP-1 plus vitamin D response element oligonucleotide (AP-1 + VDRE) of the human osteocalcin gene were methylated in vitro (Fig. 6A) and gel mobility shift assays were performed. As shown in Figure 6B, the methylated and unmethylated oligonucleotides showed binding of one AP-1 and two VDR responsive complexes after incubation with nuclear proteins extracted from untreated MG-63 cells [Jääskeläinen et al., 1994]. Treatment of the cells with 10^{-7} M $1,25(OH)_2D_3$ for 6 h greatly increased vitamin D receptor (VDR) binding to both methylated and unmethylated probes.

The binding affinity to the probes was studied using competition reactions with nonradio-

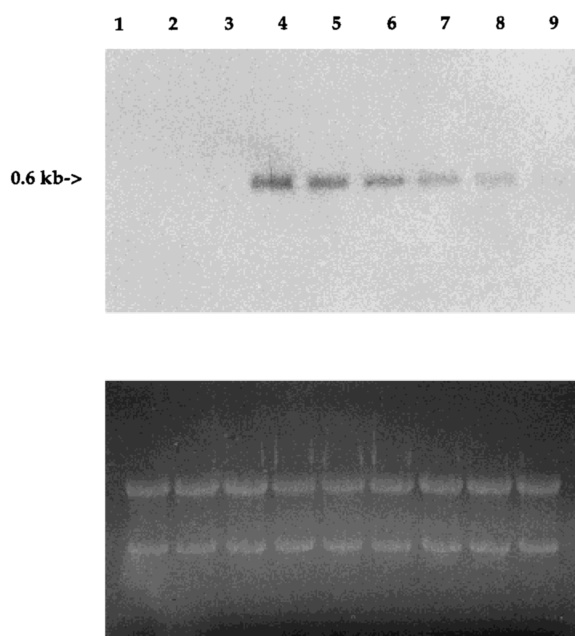

Fig. 5. Northern analysis of osteocalcin mRNA levels in MG-63 cells after treatment with 5-azacytidine and $1,25(OH)_2D_3$. MG-63 cells (**lane 1**) treated with 0.5 μM or 5.0 μM 5-azacytidine (**lanes 2,3**) or with 10^{-7} M $1.25(OH)_2D_3$ (**lane 4**). Cells were also treated with 0.1, 0.5, 1.0, 2.5, or 5.0 μM concentrations of 5-azacytidine in combination with 10^{-7} M $1.25(OH)_2D_3$ (**lanes 5–9**, respectively). **Lower panel:** shows the gel stained with ethidium bromide before transfer.

TABLE I. Effects of 5-Azacytidine on Medium Osteocalcin Concentrations in MG-63 Cells†

	Medium osteocalcin (ng/ml)	
Treatment	Without 1,25	With 10^{-7} M 1,25
Control	0 ± 0	15.5 ± 1.4*[a]
0.1 μM 5-azacytidine	0 ± 0	16.6 ± 1.1
0.5 μM 5-azacytidine	0 ± 0	15.7 ± 1.6
1.0 μM 5-azacytidine	0 ± 0	6.0 ± 1.3*[b]
2.5 μM 5-azacytidine	0 ± 0	5.7 ± 1.0*[b]
5.0 μM 5-azacytidine	0 ± 0	2.9 ± 1.0*[b]

†Cells were pretreated with different concentrations of 5-azacytidine for 72 h and then with or without 10^{-7} M $1,25(OH)_2D_3$ for 48 h. The osteocalcin concentrations were determined as recommended by the supplier. Data are expressed as means ± S.E. (n = 8).
[a]Different from control cells.
[b]Different from $1,25(OH)_2D_3$-treated cells.
$*P < 0.001$.

active unmethylated AP-1 + VDRE oligonucleotide. The binding of the nuclear proteins was already diminished with a 2.5-fold excess of the probe. The competition reaction results revealed that VDR and AP-1 bound to the unmethylated response element oligonucleotide (lanes 5,7,9,11,13) with greater affinity than to the methylated response element oligonucleotide (lanes 6,8,10,12,14). Similar results were obtained with nuclear extracts from SaOs-2 and U2-Os cells (data not shown).

DISCUSSION

Osteocalcin is an abundant, γ-carboxylated protein of bone matrix that functions in the mineralization under direct control of the vitamin D hormone, $1,25(OH)_2D_3$ [Kerner et al., 1989]. Osteocalcin synthesis has been considered to be restricted to osteoblastic bone cells [Owen et al., 1990; Pockwinse et al., 1992] and cells on mineralizing surfaces [Bianco et al., 1985]. Interestingly, nonosteoid tissues [Fleet and Hock, 1994] and platelets [Thiede et al., 1994] were recently shown to contain low levels of osteocalcin mRNA by using RT-PCR and Northern analysis. However, osteocalcin mRNA expression is much lower in nonosteoid than osteoid tissues and not regulated by $1,25(OH)_2D_3$.

In the present study, different human osteosarcoma (MG-63, U2-Os, SaOs-2), kidney (A-498) and hepatocellular carcinoma (Hep G2) cells, and normal lymphocytes were examined to study correlation between the state of methylation of the osteocalcin gene and its inducibility. $1,25(OH)_2D_3$-induced osteocalcin expression has previously been shown in MG-63 osteosarcoma cells [Mahonen et al., 1990]. Now we further studied whether $1,25(OH)_2D_3$ regulates the expression of osteocalcin mRNA in other human bone-derived or other types of cells. Using the highly sensitive RT-PCR method, we found that, in addition to $1,25(OH)_2D_3$-treated MG-63 cells, also untreated MG-63 and $1,25(OH)_2D_3$-induced SaOs-2 cells express small amounts of osteocalcin mRNA.

Genomic analysis of the osteocalcin gene by restriction enzymes revealed single fragments of similar size in all cell lines studied, confirming that the human osteocalcin gene is a single-copy gene [Puchacz et al., 1989] and that the local surroundings of the osteocalcin gene are identical in this respect in the cell lines studied. Interestingly, Rahman et al. [1993] have identified multiple copies of the osteocalcin gene in rat and mouse strains. Desbois et al. [1994] have also provided evidence indicating that the mouse genome contains a cluster of three osteo-

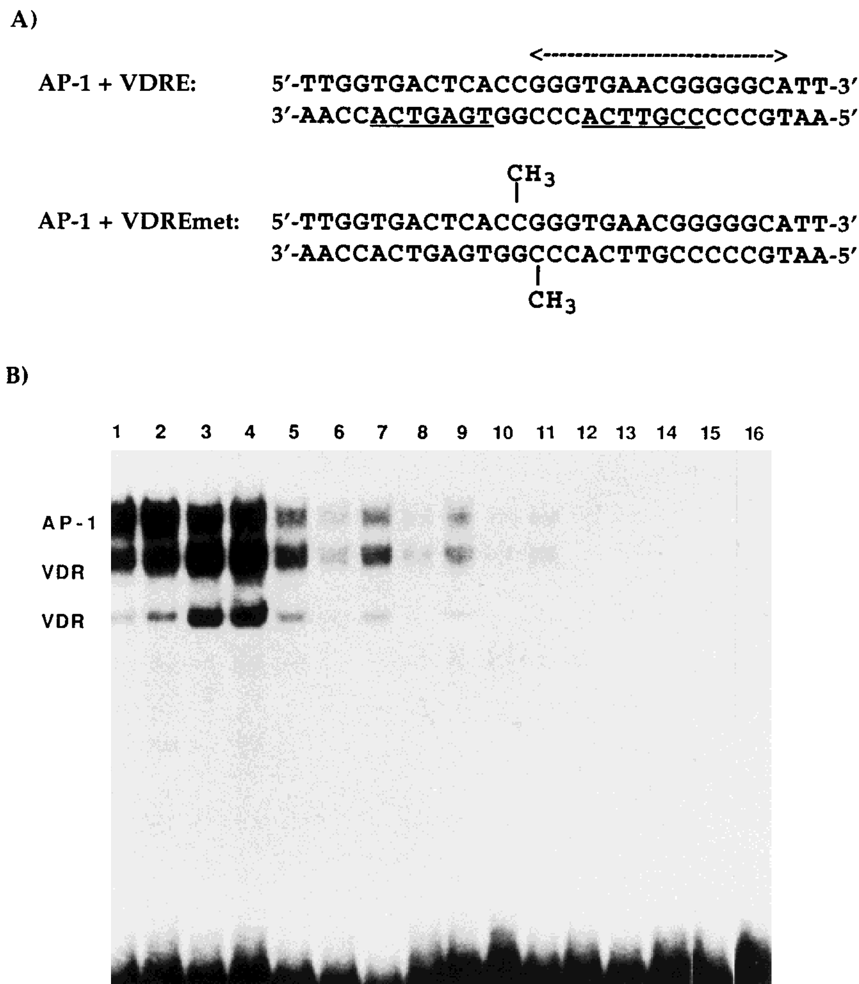

Fig. 6. Gel mobility shift assays of AP-1 and VDR binding. **A:** The two AP-1 binding sites (underlined) and VDRE (GGGTGAnnnGGGGCA) site of the AP-1+VDRE probe are indicated. The methyl group at the specific C residue in the AP-1+VDREmet probe is also indicated. **B:** Band shift analysis of the binding proteins from the MG-63 nuclear extract to the methylated and unmethylated AP-1+VDRE oligonucleotides. The end-labelled AP-1+VDRE (**lanes 1,3,5,7,9,11,13**) and AP-1+VDREmet (**lanes 2,4,6,8,10,12,14**) oligonucleotides were incubated with nuclear extracts from control (**lanes 1,2**) and 10^{-7} M $1,25(OH)_2D_3$-treated (6 h) (**lanes 3,4**) MG-63 cells. Extracts from the cells treated with 10^{-7} M $1,25(OH)_2D_3$ were also incubated in the presence of a 2.5× (**lanes 5,6**), 5× (**lanes 7,8**), 10× (**lanes 9,10**), 20× (**lanes 11,12**), and 40× (**lanes 13,14**) excess of nonlabelled AP-1+VDRE. The probes without additions are also shown (**lanes 15,16**).

calcin genes transcribed in two distinct spatial and temporal patterns.

Our results further indicate that the Hpa II methylation sites are identically methylated in normal lymphocytes and all osteosarcoma and A-498 cell lines. In the Hep G2 cell line, a more extensive methylation pattern was, however, observed. Further, Msp I could not cleave chromatin extensively in these cell lines, suggesting that the osteocalcin gene is protected, possibly by methyl-CpG binding proteins [Meehan et al., 1989; Boyes and Bird, 1992] or due to nucleosome organization [Antequera et al., 1990]. Interestingly, the resistance to the Msp I digestion of chromatin DNA did not correlate with the different methylation state of the naked osteocalcin gene in Hep G2 vs. other cell lines. Also, the basal chromatin structure in MG-63 cells was not changed after the $1,25(OH)_2D_3$ treatment. In contrast, both basal and steroid hormone-induced expression of the murine osteocalcin gene has been shown to be accompanied by modifications of the chromatin structure and nucleosome organization [Montecino et al., 1994; Stein et al., 1996].

To study the effects of hypomethylation on the osteocalcin gene expression, we treated the cell lines with 5-azacytidine, a cytidine analog

that is incorporated into RNA and DNA but cannot be methylated at the 5-position. The cells were cultured for several generations to ensure sufficient 5-azacytidine incorporation. The results indicated that osteocalcin expression was not increased as a result of the treatment. Thus, the degree of methylation of the sites analyzed is most probably not responsible for the regulation of the in vivo expression of the human osteocalcin gene. The clear attenuation of the $1,25(OH)_2D_3$-induced osteocalcin production by 5-azacytidine in these cells was unexpected and might be due to toxic effects of high 5-azacytidine concentrations in MG-63 cells.

DNA methylation may play an important role in the control of DNA-protein interactions and result in activation or repression of specific gene activity [Comb and Coodman, 1990; Doerfler et al., 1990]. Although we did not find an in vivo correlation between the degree of methylation and the expression of osteocalcin, we wanted to determine the effects of specific local in vitro methylation on protein binding. The AP-1 + VDRE oligonucleotide from the promoter region of the human osteocalcin gene was used, taking advantage of the presence of one Hpa II methylation site within the sequence. The results indicated that the in vitro methylation of one cytosine residue in the response element region affects nuclear protein binding. The methyl group at the Hpa II site diminished the binding affinities of VDR and AP-1 to the AP-1 + VDRE element. Our results agree with those by Comb and Goodman [1990], who showed that CpG methylation inhibits proenkephalin gene expression by decreasing AP-2 binding. Also, CpG methylation of the cAMP-responsive element has been reported to abolish specific factor binding as well as transcriptional activation [Iguchi-Ariga and Schaffner, 1989]. These results suggest that DNA methylation may locally prevent essential protein factors from binding to sites containing CpGs possibly by prior binding of methyl-CpG-binding proteins to these sites [Bird, 1992]. It is also possible that the methylated nucleotides alter the steric structure of the DNA double helix [Doerfler et al., 1990; Bird, 1992].

The results revealed an identical state of methylation and protection at the chromatin level of the osteocalcin gene in various bone-derived cells which either expressed or did not express osteocalcin. Furthermore, a similar methylation pattern was found in human lymphocytes and A-498 kidney cells, whereas a more extensive methylation was observed in human hepatocellular Hep G2 cells. Our results suggest that cytosine methylation is not a major determinant of the tissue-specific expression of the osteocalcin gene in the human cell lines studied. On the other hand, the results obtained from the in vitro binding studies clearly indicate that hypomethylation of critical regions of the osteocalcin gene promoter is a potential mechanism capable of influencing effective binding of specific nuclear transcription factors and, consequently, gene expression. The presence of nuclear accessory factors is known to be required for osteocalcin gene transcription. Although the osteosarcoma cell lines, MG-63, SaOs-2, and U2-Os, exhibited $1,25(OH)_2D_3$-stimulated VDR binding to the VDRE of the osteocalcin gene promoter, the binding was not sufficient to induce osteocalcin gene expression in U2-Os cells. The expression of specific nuclear transcription factors may be a further determinant for differential expression of the osteocalcin gene in these cells.

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