Changes in Chromatin Structure Support Constitutive and Developmentally Regulated Transcription of the Bone-Specific Osteocalcin Gene in Osteoblastic Cells†

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ABSTRACT: Transcription of the osteocalcin gene, which encodes a 10 kDa bone-specific protein, is controlled by modularly organized basal regulatory sequences and hormone-responsive enhancer elements. We have previously shown that in the ROS 17/2.8 rat osteosarcoma cell line, which continuously expresses the osteocalcin gene, key regulatory elements reside in two DNase I hypersensitive sites that are functionally correlated with transcriptional activity. We now report that a specific nucleosomal organization supports this constitutive expression in ROS 17/2.8 cells, and that chromatin remodeling directly correlates with the developmentally regulated transcriptional activation of the osteocalcin gene during differentiation of normal diploid rat osteoblasts. By combining DNase I, micrococcal nuclease, and specific restriction endonuclease digestion analysis, we observed that the presence of DNase I hypersensitive sites (−170 to −70 and −600 to −400) and a selective nucleosome positioning over the OC gene promoter are directly associated with developmental stage-specific transcriptional activation in bone-derived cells.

Among the mechanisms by which eukaryotic cells regulate gene expression, the role of chromatin structure in transcriptional control has been a focus of attention for many years. The differential packaging of principal regulatory sequences in nucleosomes, or at higher orders of compaction, modulates accessibility of specific transcription factors to their cognate binding sequences, thus regulating the level of gene expression. Cells could accomplish this either by maintaining permanent nucleosome-free regions, which appears to be operative for constitutively expressed genes, or by displacing nucleosomes positioned over key regulatory sequences following the interaction of specific DNA binding factors (Adams & Workman, 1993; Fedor, 1992; Felsenfeld, 1992; Gross & Garrard, 1988; Workman & Buchman, 1993). Among the studies that have established a role for nucleosomes in regulating transcriptional activation, Hager and colleagues have shown that in cell lines stably transfected with mouse mammary tumor virus (MMTV) constructs, positioned nucleosomes found at the LTR sequence prevent binding of NF-1 to its cognate site (Archer et al., 1991; Richard-Foy & Hager, 1987). After ligand activation, glucocorticoid receptor is able to bind to a site located proximal to the NF-1 binding domain, generating a hormone-dependent DNase I hypersensitive site (DHS) that renders the NF-1 element available for occupancy and allows consequent interactions of this transcription factor with components of the transcriptional initiation complex (Archer et al., 1992). On the other hand, Wolffe and colleagues have suggested that transcriptional potentiation of the *Xenopus* vitellogenin B1 gene promoter by estrogen-responsive transcription extracts occurs through the formation of a positioned nucleosome (Schild et al., 1993). Such a postulated organization would reduce the distance between distal estrogen binding sequences and proximal promoter elements to facilitate interactions between transacting factors and/or RNA polymerase-associated proteins (Hayes & Wolffe, 1992; Schild et al., 1993). Similarly, Elgin and co-workers have shown that specific positioning of a nucleosome in the promoter region of the *Drosophila* heat shock hsp26 gene allows the interaction of DHS that span two heat shock factor binding sites localized distal and proximal to the transcriptional start site (Elgin, 1988). The same group has demonstrated that $(CT)_n$-rich sequences residing at the boundaries of both DHS are required for formation of hypersensitive sites and heat shock-dependent transcriptional enhancement (Horz & Altenburger, 1981; Lu et al., 1992). On the other hand, it has been demonstrated that nucleosome positioning can be a requirement for tissue-specific expression. McPherson et al. (1993) have reported that the serum albumin enhancer in liver, where this gene is transcribed, is organized as an array of three positioned nucleosomes. This array, which is absent in tissues where the gene is inactive, allows the binding of transcription factor HNF-3 to a site located on the surface of one of the arrayed nucleosomes. This suggests that althought nucleosomes positioned over promoters are usually inhibitory to transcription factor binding, certain factors are capable of organizing nucleosomal structures that define active elements.

The osteocalcin gene (OC) encodes a 10 kDa bone-specific protein. Expression is induced with the onset of mineralization during the differentiation of normal diploid osteoblasts (Aronow et al., 1990; Owen et al., 1990a). Transcription of the OC gene is controlled by modularly organized basal regulatory sequences and hormone-responsive enhancer elements (Markose et al., 1990; Bortell et al., 1992; Owen

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et al., 1990b). The OC box (−99 to −76), which contains binding sequences for members of the MSX homeodomain family of proteins, is a principal element contributing to tissue-specific basal transcription (Hoffmann et al., 1994; Towler et al., 1994a,b). In addition an E-box consensus element (−102 to −97), which can bind Id, a member of the helix−loop−helix family of transcription factors, has been implicated in OC gene transcriptional control (Tamura & Noda, 1994). We have also reported a TGFβ response element (−162 to −134) that includes an AP-1-like consensus sequence, which interacts with Fra proteins (Banerjee et al., 1996). Interestingly, previous reports have established that OC expression is down-regulated when bone-derived cells are treated with TGFβ (Breen et al., 1994a). The vitamin D3 responsive element (VDRE, −466 to −437) represents the key component of steroid hormone-mediated transcriptional enhancement (Bortell et al., 1992; Demay et al., 1990; Markose et al., 1990; Terpening et al., 1991). We have shown that ligand-dependent binding of the vitamin D receptor in vivo is directly correlated with transcriptional up-regulation (Breen et al., 1994b).

ROS 17/2.8 osteosarcoma cells retain some of the principal phenotypic properties exhibited by osteoblasts in vivo, including steroid hormone responsiveness and synthesis of the bone-specific OC protein (Rodan & Noda, 1990). These characteristics have led to the extensive use of this cell line to study the regulatory mechanisms involved in OC gene expression. We have previously shown the presence of DHS at the OC gene promoter, spanning proximal and distal transcriptional elements, in ROS 17/2.8 cells (Montecino et al., 1994). In addition, we found that these DHS are clearly a function of transcriptional activity; they were not detected in non-osseous cells not expressing this gene, and their intensity was either increased with up-regulation of transcription postproliferatively and following vitamin D treatment or decreased after glucocorticoid treatment, which results in down-regulation of OC transcription (Montecino et al., 1994). Another osteosarcoma cell line that has been widely used to study bone-related gene expression is ROS 24/1. These cells express neither OC nor the vitamin D3 receptor (Baran et al., 1991; Dokon et al., 1984; McDonell et al., 1989). Together, these two osteosarcoma-derived cell lines provide an important experimental model to study mechanisms involved in OC gene expression and vitamin D3-dependent transcriptional up-regulation.

Here we report that specific nucleosomal remodeling at the OC gene promoter supports constitutive expression in ROS 17/2.8 cells as well as developmentally regulated transcription of the OC gene in normal diploid rat osteoblasts in culture. Our results indicate that key regulatory elements retain nucleosomal organization in cells not expressing the gene, and that a nucleosomal remodeling accompanies transcriptional activation, as reflected by generation of DHS over these elements.

MATERIALS AND METHODS

Cell culture. Rat osteosarcoma-derived ROS 17/2.8 and ROS 24/1 cells (a gift from S. Rodan and G. Rodan; Merck Sharp & Dohme, West Point, PA) were maintained as reported (Majeska et al., 1980). Primary diploid osteoblasts (ROB) were isolated from 21-day fetal rat calvaria as described by Owen et al. (1990a). Rat hepatoma-derived cells (H4 cells, a gift from S. Grimes) were maintained as previously described (Wolfe et al., 1989). Cells were treated with 10^{-8} M 1,25-dihydroxyvitamin D or vehicle for 24 h as indicated. OC gene and histone H4 gene transcription was determined by nuclear run-on analysis. OC protein levels in the medium were quantitated by radioimmunoassay (Gundberg et al., 1984).

Nuclease Hypersensitivity Studies. DNase I hypersensitivity and micrococcal nuclease digestion analyses were performed according to the indirect end-labeling method (Wu, 1980). Nuclei were isolated from calvarial-derived rat osteoblasts after 3 (proliferating) or 20 (mineralized) days in culture by gentle dounce homogenization (loose pestle) in 8 volumes of lysis buffer [60 mM KCl, 15 mM NaCl, 2 mM EDTA, 0.5 mM EGTA, 15 mM Tris-HCl, pH 7.4, 0.15 mM spermine, 0.5 mM spermidine, 0.5 mM DTT, and 0.2% (v/v) Nonidet NP-40]. The homogenate was then filtered through two layers of cheesecloth. Subsequently, the nuclei were collected by centrifugation and resuspended in digestion buffer (60 mM KCl, 15 mM NaCl, 15 mM Tris-HCl, pH 7.4, 1 mM $CaCl_2$, and 3 mM $MgCl_2$) containing glycerol 20% (v/v). Portions of this suspension were incubated with increasing amounts of DNase I or micrococcal nuclease (MNase, Worthington Biochemicals, Freehold, NJ) in digestion buffer for 10 or 5 min, respectively, at 20 °C with agitation. The digestions were stopped by adding EDTA and EGTA to final concentrations of 25 mM and 10 mM, respectively. Nuclei from ROS 17/2.8 and ROS 24/1 cells were isolated and digested by this same protocol or as previously described with similar results (Montecino et al., 1994). For OC gene analysis, purified DNA was completely digested with the restriction endonucleases *Bam*HI, *Apa*I, or *Ava*I (New England Biolabs, Beverly, MA). For histone H4 gene studies, DNA was digested with *Eco*RI and *Kpn*I. Digested DNA was electrophoresed in 1.2% or 2% agarose gels and transferred to a nylon membrane (Zeta-probe, Bio-Rad Laboratories, Melville, NY) following manufacturers' recommendations. Probes were prepared by restriction endonuclease digestion of the OC gene clone pOC3.4, containing the rat OC gene coding region and flanking sequences, and pPS2, which includes the rat histone H4t gene (Wolfe et al., 1989). These probes were labeled by the random primer method (Feinberg & Volgestein, 1983) using reagents from Stratagene (La Jolla, CA). *Hin*dIII-digested bacteriophage lambda DNA and *Hin*f I-digested pUC19 DNA, labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP, were used as DNA size markers.

Restriction Endonuclease Digestion. Nuclei were isolated from osteosarcoma cells or from normal diploid osteoblasts as described for DNase I and MNase analysis. The final nuclear pellet was resuspended and digested with restriction endonucleases in the specific buffer conditions provided by the supplier (New England Biolabs). *Hin*cII, *Bgl*II, and *Pst*I digestions were performed in NEbuffer 3 [100 mM NaCl, 50 mM Tris-HCl (pH 7.9), 10 mM $MgCl_2$, and 1 mM dithiothreitol (DTT)], *Pvu*II reactions in NEbuffer 2 [50 mM NaCl, 50 mM Tris-HCl (pH 7.9), 10 mM $MgCl_2$, and 1 mM DTT], and *Sst*I (BRL, Grand Island, NY) digestions in REact 2 buffer [50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, and 50 mM NaCl]. The mixture was incubated for 30 min at 37 °C, conditions which gave optimal digestion. The reaction was stopped by the addition of EDTA to a final concentration

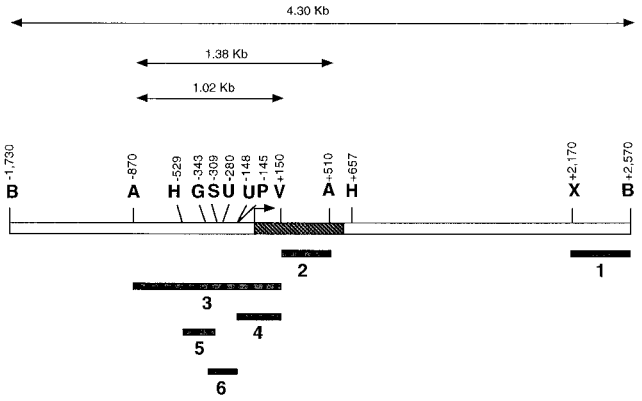

FIGURE 1: Schematic representation of the restriction endonuclease map of the osteocalcin gene and flanking sequences. The diagram shows the DNA fragments used as hybridization probes in the indirect end-labeling experiments (bottom). The hatched box represents the osteocalcin (OC) gene (including exons and introns), and the horizontal arrow over the gene marks the direction of transcription. *A* = *Apa*I, B = *Bam*HI, G = *Bgl*II, H = *Hin*cII, P = *Pst*I, S = *Sst*I/*Sac*I, U = *Pvu*II, V = *Ava*I, X = *Xba*I.

of 10 mM. The purified genomic DNA was then digested to completion with *Apa*I or *Bam*HI, electrophoresed in 1.2% or 2% (w/v) agarose gels, blotted, and hybridized with probes 1, 2, or 5 as indicated (see Figure 1). The intensities of the bands on the autoradiographs of Southern blots were determined using a scanning densitometer (Apple Onescanner). The accessibility to the different restriction endonucleases within the OC gene promoter was quantified as a fraction of the intensity of the band compared to the total intensity of the bands within a given lane on an autoradiograph.

Isolation and Detection of Nucleosomal Particles. Isolation and analysis of nucleoprotein particles were carried out as previously described by Weintraub (1984). Nuclei from ROS 17/2.8 cells were resuspended in a buffer containing 10 mM Tris-HCl (pH 7.4), 10 mM NaCl, 5 mM $MgCl_2$, 0.1 mM $CaCl_2$, and the protease inhibitors PMSF, pepstatin, TPCK, leupeptin, and trypsin inhibitor. MNase (25 units/mL, 13.5 units/mg) was added and the digestion performed at 18 °C for 0, 5, 10, 15, 20, 30, 40, and 50 min. The reaction was stopped by addition of EDTA and EGTA to final concentrations of 10 and 1 mM, respectively. The samples were kept on ice for 30 min to achieve nuclear lysis. Nuclear debris, including nonsolubilized residual chromatin, was separated, and the DNA was purified. Solubilized chromatin nucleoprotein particles were visualized by direct electrophoresis of a small fraction on a 1% agarose gel (not shown). The DNA fragments contained in these solubilized nucleoprotein particles were then purified, electrophoresed in a 2% agarose gel, blotted, and hybridized as indicated (Figure 1).

RESULTS

Nucleosomal Organization of the OC Gene in Rat Osteosarcoma Cell Lines. Specific arrangements of chromatin structure have been associated with regulated expression of eukaryotic genes (Elgin, 1988; Felsenfeld, 1992; Gross & Garrard, 1988). These structural features have been studied principally by analyzing the nuclease accessibility of regulatory regions to DNase I, micrococcal nuclease (MNase), and restriction endonucleases (Gross & Garrard, 1988; Simpson, 1991).

We experimentally addressed the hypothesis that modifications in chromatin structure and nucleosome positioning are functionally linked to OC gene transcription. Indirect end-labeling analyses of DNase I-, MNase-, and restriction endonuclease-digested nuclei isolated from ROS 17/2.8 cells which express the OC gene, and ROS 24/1 cells which do not (Pockwinse et al., 1995), were carried out. The results revealed that the presence of DHS at the OC gene promoter is not only restricted to bone-derived cells (Montecino et al., 1994) but is also directly related to the transcriptional status of the OC gene (Figure 2A,D). ROS 24/1 cells show neither the hypersensitivity nor the vitamin D inducibility that has been previously observed for ROS 17/2.8 cells where the gene is transcriptionally active (Montecino et al., 1994; and see Figure 2A). As an internal control, we confirmed that these two cell types exhibit similar levels of DNase I hypersensitivity of the histone H4 genes (Figure 2B), which are expressed at high levels in both cell lines (Figure 2D). The two hypersensitive domains of the OC gene promoter span regions which contain binding sequences for a series of transcriptional regulators (see Figure 2C) present in both cell lines, with the exception of the VDR, which is absent in ROS 24/1 cells (Hoffmann et al., 1994; and unpublished results).

To define a correlation between DNase I hypersensitivity and modifications in the nucleosomal organization of the OC gene promoter region, we digested nuclei isolated from both osteosarcoma cell lines with increasing concentrations of micrococcal nuclease, which cleaves chromatin primarily between nucleosomes (Simpson, 1991), and analyzed the resulting subfragments by indirect end-labeling. When the partially digested products were revealed by Southern blot analysis using probe 3 (−870 to +150, see Figure 1), we observed the characteristic DNA ladder (Figure 3A), indicating that under our experimental conditions we are detecting the presence of nucleosomal particles spanning the 1.38 kb *Apa*I restriction fragment. This segment includes more than 700 base pairs of the OC gene promoter as well as an important portion of the coding region (see Figure 1).

We then determined the positions of MNase cleavage sites within the promoter by hybridizing Southern blots of nuclease-digested chromatin with radiolabeled probes prepared from the 3′-end of the sequences (probes 2 and 4, Figure 1). Confirmation was by indirect end-labeling from the 5′-end (not shown). Because it has been reported that MNase possesses some degree of sequence preference (Dingwall et al., 1981; Horz & Altenburger, 1981), as a necessary control we compared these digestion patterns with those obtained following incubation of purified genomic DNA with this nuclease. Figure 3B shows cleavage points at −650, −560, −350, −310, −160, −90, and −50 following MNase digestion of chromatin from ROS 17/2.8 cells after restriction digestion with *Apa*I and hybridization with probe 2 (+150 to +510). Comparable nuclease digestion of chromatin from ROS 24/1 cells yielded subbands indicating cleavage only at −560 and −310; cleavage sites at −650 and −350 were minimally detectable. Strikingly, the chromatin cleavage patterns are similar to those obtained after MNase digestion of naked DNA from ROS cells (17/2.8 or 24/1). Subbands representing cleavage sites at −560, −310, and −90 (marked with small circles) were detected, raising the possibility that they represent sequence recognition by

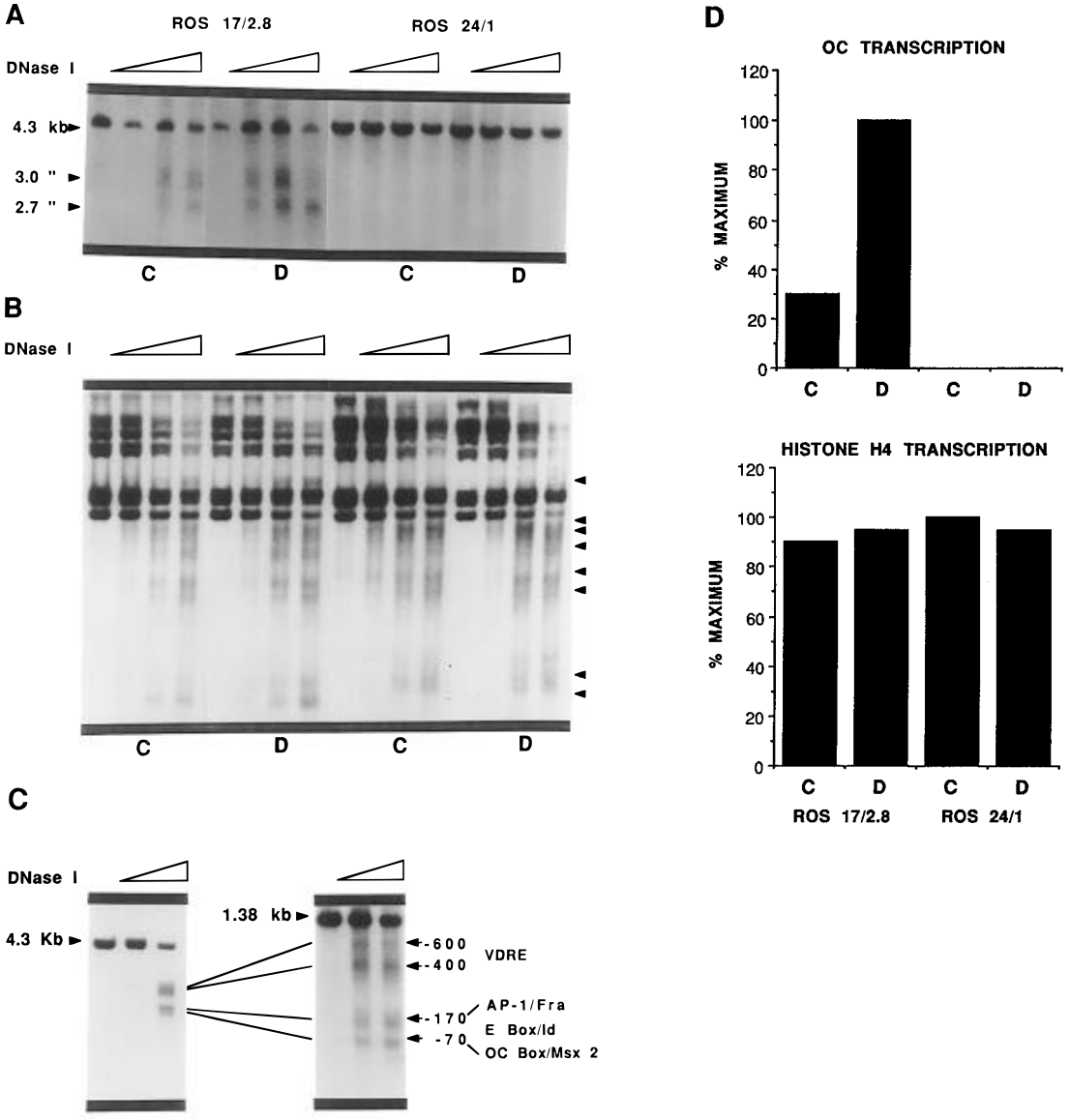

FIGURE 2: DNase I hypersensitivity of the osteocalcin gene is correlated with transcriptional activity in osteosarcoma cell lines. Nuclei isolated from ROS 17/2.8 (which express osteocalcin) or ROS 24/1 cells (which do not express osteocalcin), treated with vehicle (C) or vitamin D (D) for 24 h, were incubated with increasing concentrations of DNase I as described under Materials and Methods, and the purified genomic DNA was then analyzed by the indirect end-labeling method. (A) Samples were digested with *Bam*HI, electrophoresed in a 1.2% agarose gel, blotted, and hybridized with probe 1 (Figure 1). (B) Aliquots of the same samples were digested with *Eco*RI and *Kpn*I, electrophoresed in a 1.5% agarose gel, blotted, and hybridized with a probe directed against the coding region of the rat histone H4t gene (Wolfe et al., 1989). Arrowheads indicate hypersensitive sites. (C) Increased resolution of the DHS in the OC gene promoter was achieved when the samples were digested with *Apa*I, electrophoresed in a 2% agarose gel, blotted, and hybridized with probe 2 (Figure 1) (Montecino et al., 1994). Promoter elements encompassed by DNase I hypersensitive sites are indicated on the right of the blot. (D) Transcriptional activity of the OC (above) and histone H4 (below) genes measured by nuclear run-on assay.

MNase rather than recognition of chromatin structural features.

Multiple studies have established that DNA sequences organized in nucleosomes show markedly reduced accessibility to restriction endonuclease cleavage (Archer et al., 1991; Simpson, 1991). Because of the remarkable similarity between the MNase digestion patterns of chromatin and naked DNA, as well as the possibility of having intranucleosomal MNase cleavage (McGhee & Felsenfeld, 1983; Pfeifer & Riggs, 1991; Zhang & Gralla, 1989), we determined restriction endonuclease accessibility to sites within the OC gene promoter in intact nuclei to further define nucleosome positioning. We compared sensitivity to *Hinc*II, *Bgl*II, and *Pst*I enzymes, which cut in the promoter at functionally strategic positions (see Figure 1), exhibit enzymatic activities that are not affected by methylation, and share similar optimal digestion buffer conditions. The conditions for restriction endonuclease cleavage are compatible with those required for isolation of nuclei and nuclease digestion (DNase I and MNase) in our study (see Materials and Methods).

The results summarized in Figure 4A indicate significant differences in restriction endonuclease accessibility of the OC gene promoter sequences between the ROS 17/2.8 and ROS 24/1 osteosarcoma cell lines (see quantitation below). The most dramatic difference was observed in the distal region of the promoter at the *Hinc*II site (−529). While this sequence was completely protected from cleavage in ROS 24/1 cell chromatin, it was efficiently cleaved in chromatin from ROS 17/2.8 cells and in naked DNA from ROS 24/1 cells (Figure 4A). This result confirms our previous findings based on DNase I hypersensitivity that a major change in

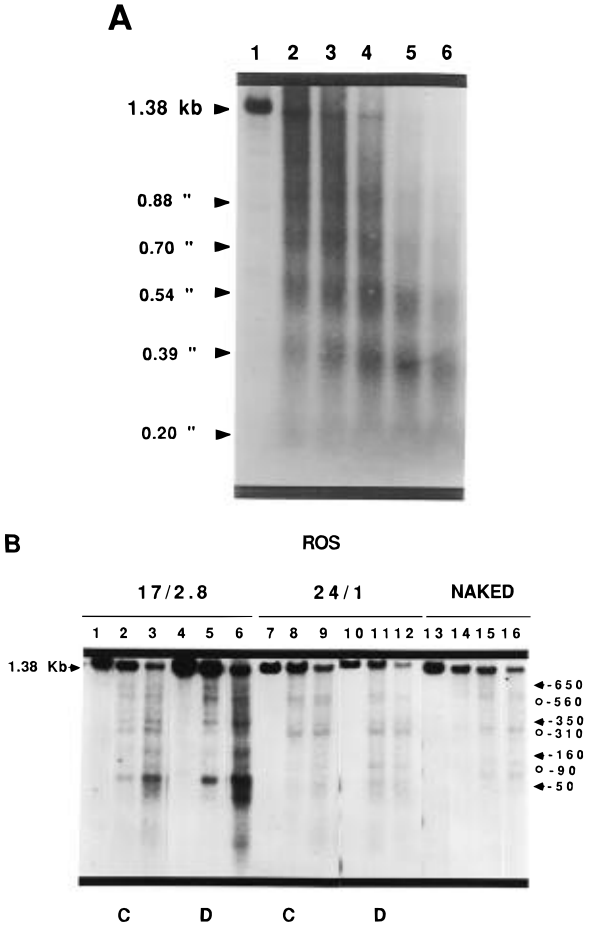

FIGURE 3: Nucleosomal organization of the osteocalcin gene in rat osteosarcoma cell lines. Nuclei isolated from ROS 17/2.8 or ROS 24/1 cells, treated with vehicle (C) and Vitamin D for 24 h (D), were incubated with increasing amounts of micrococcal nuclease (MNase). The purified genomic DNA was then analyzed by the indirect end-labeling method. (A) Samples (25 μg) digested with *Apa*I were electrophoresed in a 2% agarose gel and hybridized with probe 3 (Figure 1). The numbers at the top represent the different concentrations of MNase utilized. Lane 1 = 0, 2 = 5, 3 = 10, 4 = 15, 5 = 25, and 6 = 50 units/mL (13.5 units/mg). (B) Samples from MNase-digested ROS 17/2.8 and 24/1 nuclei and ROS naked DNA were cleaved with *Apa*I, electrophoresed, blotted, and hybridized with probe 2 (Figure 1). Lanes 1, 4, 7, 10, and 13 = 0 unit/mL; lanes 2, 5, 8, and 11 = 5 units/mL; lanes 3, 6, 9, and 12 = 10 units/mL; lane 14 = 0.5 unit/mL, lane 15 = 0.7 unit/mL, and lane 16 = 1.0 unit/mL. Arrowheads mark MNase cleavage sites present exclusively in chromatin, and circles those detected in both chromatin and naked DNA.

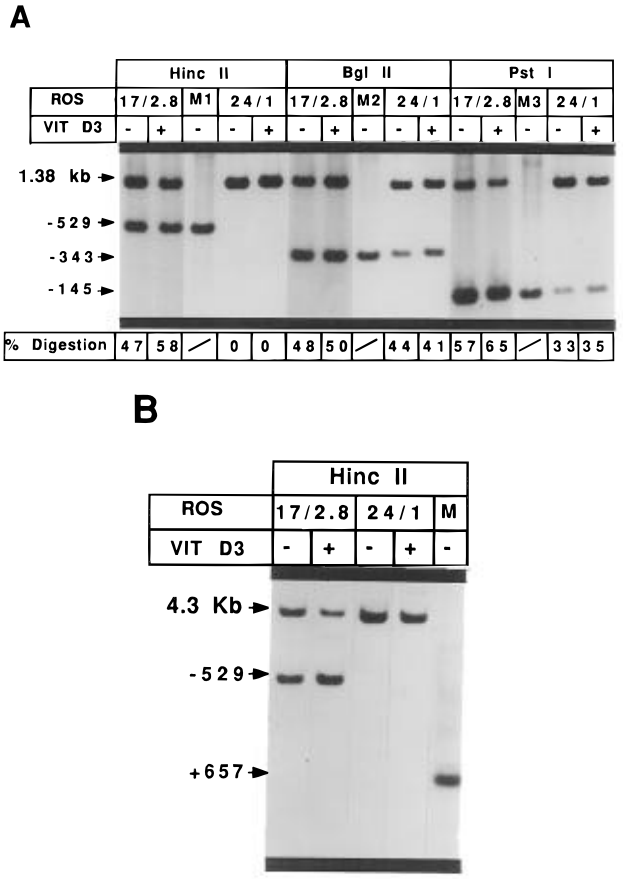

FIGURE 4: Restriction endonuclease accessibility of the osteocalcin gene in osteosarcoma cell lines. Nuclei isolated from ROS 17/2.8 and 24/1 cells, treated with vehicle (−) or vitamin D (+) for 24 h, were incubated with 500 units/mL of the restriction endonucleases *Hinc*II, *Bgl*II, and *Pst*I (indicated at the top) for 30 min at 37 °C, and the products were analyzed by indirect end-labeling. (A) After purification, the genomic DNA was cleaved with *Apa*I, electrophoresed in a 2% agarose gel, blotted, and hybridized with probe 2 (Figure 1). The position of the cleavage is indicated on the left, and the percentage of digestion is shown at the bottom. M1, M2, and M3 correspond to ROS 24/1 genomic DNA cleaved with *Hinc*II−*Apa*I, *Bgl*II−*Apa*I, and *Pst*I−*Apa*I, respectively. (B) DNA samples from ROS 17/2.8 and 24/1 nuclei incubated with *Hinc*II were cleaved with *Bam*HI, electrophoresed in a 1.2% agarose gel, blotted, and hybridized with probe 1 (Figure 1). Lane M corresponds to ROS 24/1 purified genomic DNA digested with *Hinc*II and *Bam*HI. The positions of the two *Hinc*II sites present in the 4.3 kb *Bam*HI restriction fragment (Figure 1) are shown on the left.

chromatin conformation in this region of the OC gene promoter parallels transcriptional activity. As a control, we demonstrated that an additional *Hinc*II site, located in the 3′-end of the OC gene coding region (+657, Figure 1), was not cleaved in ROS 24/1 nor in ROS 17/2.8 cells (Figure 4B). This result is consistent with our observation that this region of the OC gene appears to be organized with randomly positioned nucleosomes in osteosarcoma cells, both in the presence (ROS 17/2.8) and in the absence (ROS 24/1) of OC gene transcription (data not shown).

A similar, although not as dramatic, result was obtained when we analyzed the proximal promoter region of the OC gene. Here cleavage by *Pst*I (−145) was found to be significantly reduced (although still detectable) in ROS 24/1 cells compared to ROS 17/2.8 cells (Figure 4A). We also observed only a minor variation in cleavage efficiency by *Bgl*II (−343) between the two osteosarcoma cell lines. This *Bgl*II restriction enzyme recognition site is located proximal to a chromatin-specific MNase digestion site (−350, see Figure 3A,B), suggesting that this segment may correspond to a linker DNA fragment, which has been shown to be accessible to restriction endonuclease cleavage activity (Archer et al., 1991).

Taken together, results from DNase I, MNase, and restriction endonuclease analyses suggest that nucleosomes are present over specific regions of the OC gene promoter in the bone-derived ROS 24/1 cell line where the gene is transcriptionally inactive (−560 to −350 and downstream of −310). In addition, the results indicate that in ROS 17/2.8 cells, two regions of the promoter, the distal (−600 to −400) and proximal (−170 to −70) domains, exhibit nuclease hypersensitivity. This hypersensitivity appears to reflect alterations or transitions in the nucleosomal organization as a consequence of specific transcription factor interactions which are functionally related to OC gene expression.

Moreover, the strong chromatin-specific MNase cleavage site detected at −50 is located 10 base pairs upstream of the TATA box element. This site, which is only observed in ROS 17/2.8 transcribing the OC gene, may reflect binding of the RNA polymerase transcription complex to this region of the promoter.

Chromatin Organization of the Region between Hypersensitive Sites in the OC Gene Promoter of Transcriptionally Active Cells. In ROS 17/2.8 cells and in differentiated normal diploid osteoblasts (see below), MNase and DNase I digestion patterns suggest that at least part of the segment in the OC gene promoter between the two DNase I hypersensitive sites may be organized as a nucleosome-like structure. We observed that in ROS 17/2.8 chromatin the region between −310 and −150 of the OC gene promoter was protected from cleavage by the restriction endonuclease *Pvu*II (−280). Specificity of nuclease protection at the *Pvu*II site is supported by *Pvu*II cleavage at a proximal nuclease-hypersensitive site (−148) in the OC gene promoter (Figure 5A). In addition, we observed cleavage at an *Sst*I site (−309, Figure 5B) which is proximal to an MNase-sensitive site (−310, Figures 3A and 7A) present in both chromatin and naked DNA. This cleavage by *Sst*I indicated that the putative nucleosome may be positioned downstream of −309.

To further examine the chromatin organization of segment −310 to −150 in the proximal promoter of the OC gene, we used the method initially described by Weintraub (Weintraub, 1984; see Materials and Methods) to verify that this sequence was represented as a nucleosomal particle. ROS 17/2.8 nuclei were extensively digested with MNase. Solubilized nucleoprotein particles were isolated, and their integrity and histone protein composition were confirmed by agarose and polyacrylamide gel electrophoreses, respectively (not shown). Subsequently, the DNA was isolated and analyzed by Southern blot using probe 6 (−343 to −145, Figure 1) for hybridization, which specifically recognizes sequences from the OC gene promoter (Figure 5C, lane 1). This probe principally detected a 145 bp band, which corresponds in size to mononucleosomal DNA (Simpson, 1991). The signal was observed to peak after 30 min, and was still detectable, although significantly reduced, after 50 min of incubation. These results strongly suggest that the DNA segment which remained inaccessible to DNase I and restriction endonuclease activities in the OC gene promoter of transcriptionally active bone-derived cells is organized in a nucleosome-like structure.

Developmentally Regulated Changes in Chromatin Structure of the OC Gene Promoter. Primary cultures of calvarial-derived rat osteoblasts (ROB) develop a mineralized extracellular matrix, with a bone tissue-like organization analogous to osteoblast differentiation in vivo (Aronow et al., 1990; Owen et al., 1990a). 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., 1990a). Because of the marked differences in chromatin structure between ROS 17/2.8 cells which express the OC gene and ROS 24/1 cells which do not, we examined whether specific changes in chromatin organization accompany the developmentally regulated transcriptional activation of the OC gene during osteoblast differentiation. Our experimental approach was to perform limited DNase I, MNase, and restriction endonuclease digestion of nuclei isolated from either proliferating or mineralized rat calvarial-derived osteoblast cultures. DNA fragments were characterized by Southern blot analysis and indirect end-labeling. As shown in Figure 6A, DNase I hypersensitivity in the OC

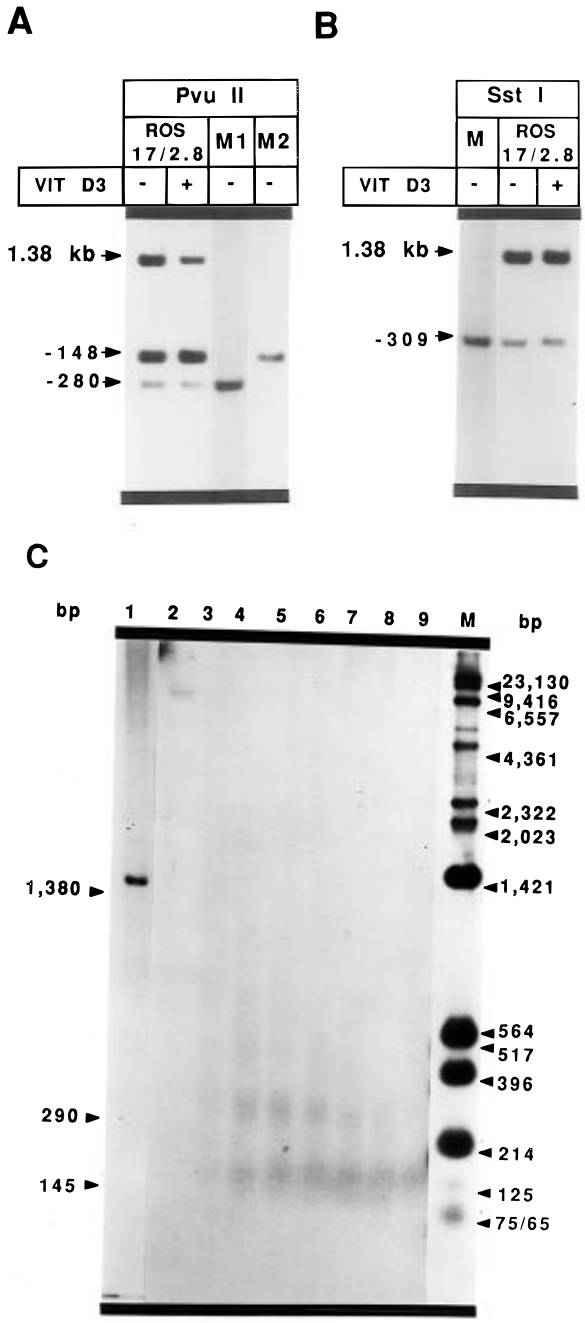

FIGURE 5: Region between the two DNase I hypersensitive sites is organized as a nucleosome. Nuclei isolated from ROS 17/2.8 cells were incubated with restriction endonucleases or MNase and then analyzed by Southern blot. (A) DNA from nuclei digested with *Pvu*II (500 units/mL), then cleaved with *Apa*I, electrophoresed in a 2% agarose gel, blotted, and hybridized with probe 5 (Figure 1). Lanes M1 and M2 correspond to ROS genomic DNA digested with *Pvu*II−*Apa*I and *Pst*I−*Apa*I, respectively. (B) Purified DNA samples from nuclei digested with *Sst*I (500 units/mL) were cleaved with *Apa*I and hybridized with probe 2 (Figure 1). Lane M represents ROS genomic DNA cut with *Sst*I−*Apa*I. (C) Following digestion of ROS 17/2.8 nuclei with MNase, soluble nucleoprotein particles were isolated at different digestion times; the DNA was purified, electrophoresed in a 2% agarose gel, blotted, and hybridized with probe 6 (Figure 1). Lanes 2−9 (shown at the top) correspond to 0, 5, 10, 15, 20, 30, 40, and 50 min of digestion, respectively. Lane 1 represents ROS genomic DNA cleaved with *Apa*I. Lambda DNA digested with *Hin*dIII and pUC19 digested with *Hin*fI were used as DNA size markers (M).

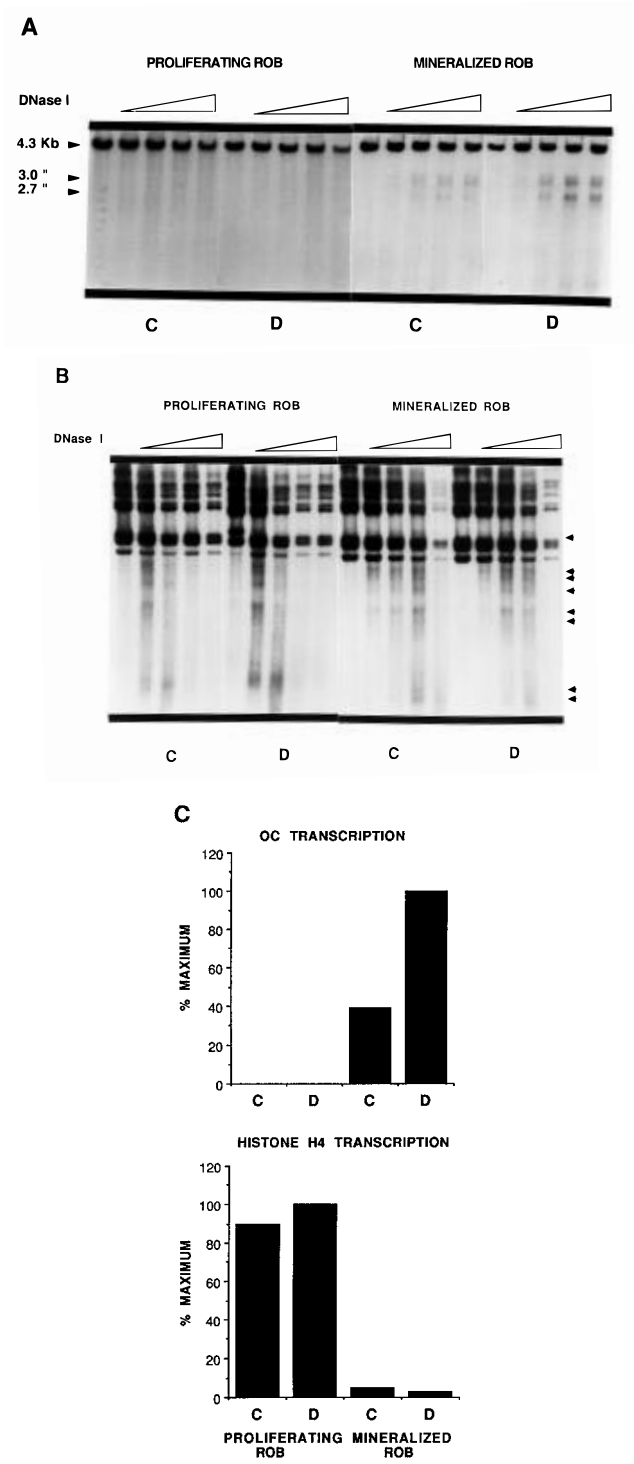

FIGURE 6: Developmentally regulated DNase I hypersensitivity of the osteocalcin gene in normal rat osteoblasts. Nuclei isolated from rat calvarial-derived osteoblast (ROB) cultures on day 3 (proliferating) or day 20 (mineralized) treated with vehicle (C) or with vitamin D (D) for 24 h were incubated with increasing amounts of DNase I. The purified DNA samples were analyzed by the indirect end-labeling method as described in Figure 2. (A) OC gene or (B) histone H4 genes. (C) Transcriptional activity of the OC (above) and histone H4 (below) genes in proliferating and mineralized rat osteoblasts determined by nuclear run-on analysis.

gene promoter is detected only in mineralized cultures, when the gene is actively transcribed (Figure 6C). Two DHS are localized in the same promoter regions as those previously identified in ROS 17/2.8 cells (Figure 2A), and both exhibited increased intensity following vitamin D treatment for 24 h, which results in enhancement of OC gene transcription

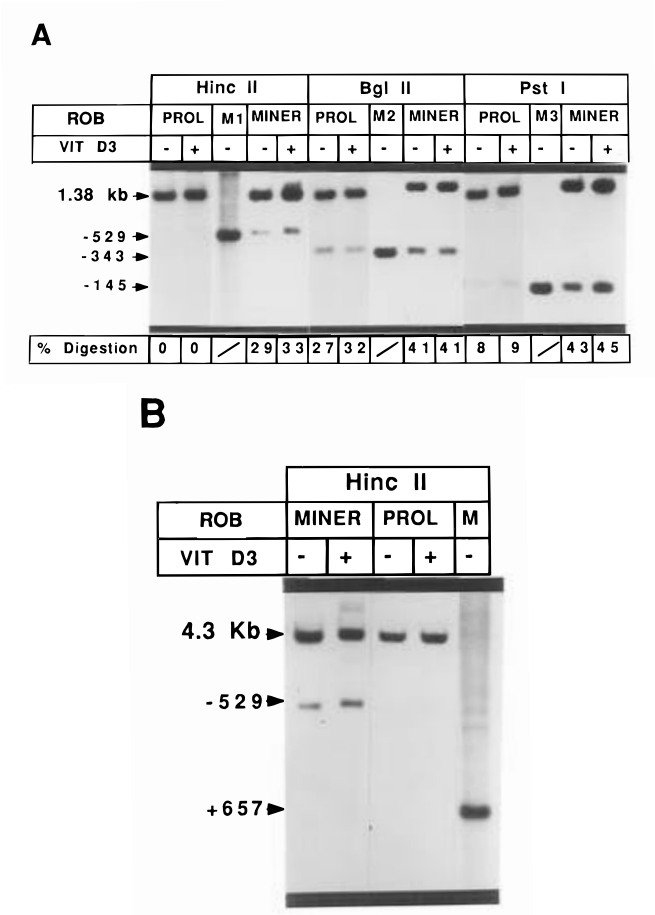

FIGURE 7: Developmentally regulated restriction endonuclease accessibility of the osteocalcin gene in normal diploid osteoblasts. Nuclei isolated from proliferating (day 3) or mineralized (day 20) ROB, treated with vehicle (−) or vitamin D (+) for 24 h, were incubated with the restriction endonucleases *Hinc*II, *Bgl*II, and *Pst*I and analyzed as described in Figure 4. (A) The purified DNA samples were cleaved with *Apa*I and hybridized with probe 2 (Figure 1). (B) Samples from *Hinc*II-digested nuclei, cleaved with *Bam*HI and hybridized with probe 1. See Figure 4 for explanation of symbols.

(Figure 6C). In contrast, DNase I hypersensitivity in the histone H4 genes is detected in both proliferating and mineralized ROB, and vitamin D-responsive modifications are not observed (Figure 6B). These results suggest that the changes in hypersensitivity within the OC gene promoter reflect specific chromatin remodeling associated with transcriptional activation during osteoblast differentiation.

When MNase sensitive sites within the OC gene were mapped in both proliferating and mineralized ROB, we observed a cleavage pattern similar to that previously found in osteosarcoma cells (not shown). Similarly, two of the subbands were also detected in the genomic DNA from ROB cells digested with MNase.

We then examined changes in restriction endonuclease cleavage within the OC gene promoter, which is indicative of modifications in nucleosome positioning during differentiation of normal diploid osteoblasts (Figure 7A). The *Hinc*II site, located in the distal region of the promoter (−529, see Figure 1), was not accessible to the enzyme in early-stage proliferating cells. In mature osteoblasts undergoing extracellular matrix mineralization, this sequence exhibited significant cleavage (see Figure 7A for quantitation). As an internal control, we established that a downstream *Hinc*II site (+657) was not accessible to the restriction

enzyme during either of these two osteoblast developmental stages (Figure 7B), confirming our findings in both ROS 17/2.8 and ROS 24/1 osteosarcoma cells (Figure 4B). We observed that a *Pst*I site, localized in the proximal region of the OC gene promoter (−145), exhibited a 4-fold increase in cleavage in differentiated compared to proliferating osteoblasts. In contrast, we detected only a moderate enhancement in the restriction endonuclease accessibility at the *Bgl*II site (−343) in osteoblasts when the OC gene is transcriptionally active. This result confirms our previous indication that changes in chromatin structure associated with developmentally regulated expression of the OC gene occur primarily at the distal and proximal regions of the promoter. The overall level of digestion by *Hinc*II and *Pst*I in differentiated osteoblasts is reduced when compared to that observed in ROS 17/2.8 cells (Figure 4A). This may reflect expression of OC in approximately 70% of the cells in cultures of normal diploid osteoblasts at the extracellular matrix mineralization stage (Pockwinse et al., 1995), compared to expression of OC in all ROS 17/2.8 cells.

Our results suggest that inaccessibility of the proximal and distal regions of the OC gene promoter to DNase I and restriction endonucleases in proliferating normal diploid osteoblasts, either control or vitamin D treated, is mediated by nucleosomes overlapping these sequences. The digestion pattern exhibited during this early developmental stage, when the OC gene is not transcribed, indicates that nucleosomes can be located between −560 and −350 and downstream −310. However, when these cells initiate OC gene transcription at the onset of extracellular matrix mineralization, the OC gene promoter regions −600 to −400 and −170 to −70 become hypersensitive, suggesting alterations in organization of the nucleosomal array. These changes may be associated with the binding of a series of transcription factors which regulate OC gene transcription (see Discussion).

DISCUSSION

Analysis of chromatin structure indicates that active or potentially active genes are marked by the presence of nuclease-sensitive domains. It has been suggested that these genomic regions reflect alterations in the nucleosomal organization and the binding of specific non-histone chromosomal proteins (Gross & Garrard, 1988). In this study, we have determined that specific changes in chromatin structure accompany transcriptional activity, as well as vitamin D-dependent enhancement, of OC gene transcription both in rat osteosarcoma-derived cells and in normal diploid rat osteoblasts. We have found that most differences in chromatin organization of the OC gene between transcriptionally active and inactive cells occur in two regions of the promoter, between −170 and −70 and between −600 and −400 (Figure 8), where the principal basal and steroid-responsive enhancer elements reside (Bortell et al., 1992; Breen et al., 1994b; Demay et al., 1990; Hoffmann et al., 1994; Markose et al., 1990; Owen et al., 1990b, 1993; Terpening et al., 1991; Towler et al., 1994a,b). DNase I hypersensitivity in these two regions is directly correlated with transcriptional activity of this bone-specific gene. It has been observed in ROS 17/2.8 cells (Montecino et al., 1994) constitutively expressing the OC gene, and in normal diploid osteoblasts only during late stages of differentiation, when the OC gene is transcribed (Aronow et al., 1990; Owen et al., 1990a). Additionally, we have found that in differentiated osteoblasts vitamin D-dependent enhancement of OC gene transcription is accompanied by a marked increase in the intensity of these two DNase I hypersensitive sites.

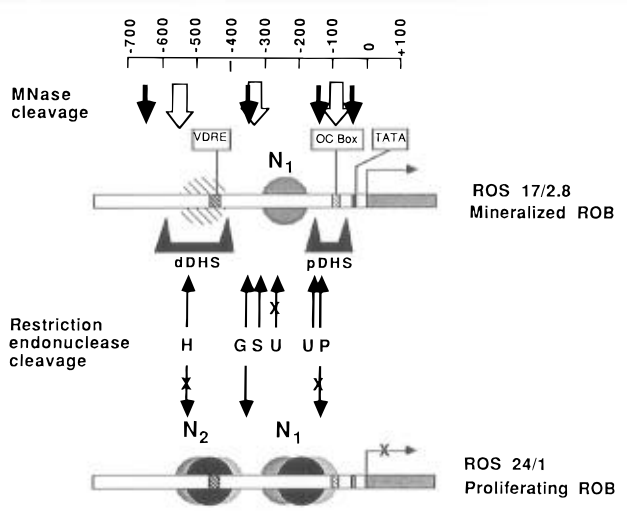

FIGURE 8: Nucleosomal organization of the osteocalcin gene in bone-derived cells. The schematic drawings summarize the results presented in this paper and indicate nucleosome remodeling associated with transcriptional activity of the osteocalcin gene. The filled circles represent putative nucleosomes (N_1 and N_2) spanning the promoter region of the OC gene. The letters H, G, S, U, and P correspond to the restriction sites for *Hinc*II, *Bgl*II, *Sst*I, *Pvu*II, and *Pst*I, respectively.

The absence of DNase I hypersensitivity in cells not expressing OC may be attributed to the presence of nucleosomes spanning the distal (−560 to −350) and proximal (downstream −310) promoter regions of the OC gene. This chromatin organization of the OC gene promoter is supported by the inaccessibility of these domains to restriction endonuclease cleavage in both ROS 24/1 cells and proliferating normal diploid osteoblasts, in which the gene is not transcribed. In proliferating osteoblasts, vitamin D treatment does not modify the inaccessibility to DNase I or restriction enzymes. These results are in direct agreement with the absence of vitamin D-dependent stimulation of OC gene transcription in bone-derived cells not expressing at basal levels (Owen et al., 1990c). Interestingly, most of the transcription factors reported to interact with the OC gene promoter, which have been demonstrated to contribute to transcriptional activity, are present in both ROS 17/2.8 and ROS 24/1 cells as well as in proliferating and mineralizing normal diploid osteoblasts (Hoffmann et al., 1994; and unpublished results). These observations suggest that the inability of these transcription factors to interact with cognate binding sequences in the OC gene promoter may be a principal component for transcriptional repression. We have observed that reporter constructs, driven by OC gene promoter sequences and which lack normal chromatin structure (Archer et al., 1992), are expressed in transiently transfected proliferating normal diploid osteoblasts not transcribing the endogenous gene (Frenkel et al., 1993). In addition, McDonell et al (McDonell et al. (1989) have shown that ROS 24/1 cells, which lack endogenous vitamin D receptor (Baran et al., 1991; Dokon et al., 1984), express transiently transfected OC promoter−reporter constructs when cDNA encoding the vitamin D receptor is cotransfected.

We have observed that the DNA segment between the two DNase I hypersensitive domains in ROS 17/2.8 osteosarcoma

cells, as well as in mineralizing normal diploid osteoblasts, can be organized as a nucleosome (Figure 8). This suggestion is based on the digestion patterns obtained in both cell types, and on the protection to specific restriction endonuclease cleavage exhibited by this segment in chromatin (Figure 5). In addition, we isolated this DNA fragment as part of a nucleoprotein particle containing 145 bp of DNA following extensive MNase digestion (Figure 5C). We are unable to formally exclude the possibility that other proteins, distinct from a histone octamer, could interact with this region of the OC gene promoter, creating a protected DNA segment with characteristics similar to those associated with nucleosomes (Simpson, 1991). However, to date we have not found any protein or protein complex which can specifically interact with sequences located within this region in either soluble nuclear extracts or nuclear matrix protein preparations (unpublished observations). The translational positioning of this nucleosome could reflect protein−DNA interactions occurring in the proximal promoter region of the OC gene which account for the formation of the proximal DNase I hypersensitive site and for OC gene transcriptional activation. Factors which bind to this region and have been shown to influence transcriptional activity include: Msx 2 (−99 to −76), a member of the homeodomain family of regulatory proteins (Hoffmann et al., 1994; Towler et al., 1994a,b); Id (−102 to −97), a component of the helix−loop−helix group of transcription factors (Tamura & Noda, 1994); and a Fra complex (−162 to −134), which is in the family of AP-1 DNA binding proteins (Banerjee et al., 1996). Moreover, we have observed an absolute requirement of sequences residing between −170 and −70 for the formation of the proximal DNase I hypersensitive site, the presence of nucleosomes upstream, and basal transcriptional activity in stably transfected cell lines carrying OC gene promoter deletion constructs driving expression of a reporter gene (manuscript in preparation).

A nucleosome between the two DNase I hypersensitive sites in the OC gene promoter could contribute to the three-dimensional organization of the promoter. A basis can be provided for increasing the proximity of the hypersensitive domains, thus facilitating, through protein−protein interactions, integration of activities at the proximal and distal promoter elements. Such higher order interactions may facilitate the synergistic enhancement of OC gene transcription observed following vitamin D treatment (Bortell et al., 1992; Montecino et al., 1994). Similar three-dimensional models have been proposed for the *Drosophila* heat shock hsp26 gene (Elgin, 1988) and the *Xenopus* vitellogenin B1 gene (Hayes & Wolffe, 1992; Schild et al., 1993). The recent demonstration of interactions between the vitamin D receptor and TATA box binding protein associated factor TFIIB in vivo is consistent with such reasoning (Blanco et al., 1995; MacDonald et al., 1995).

In summary, these results provide a basis for understanding transcriptional control within the context of chromatin structure. The conformational properties of the OC gene within the nucleus of intact cells may modulate transcriptional activity by increasing or reducing the proximity of independent regulatory elements that support responsiveness to physiological mediators of OC gene expression (Stein et al., 1994).

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