Chromatin Structure of the Rat Osteocalcin Gene Promoter in Bone-Derived Cells

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CHROMATIN STRUCTURE OF THE RAT OSTEOCALCIN GENE PROMOTER IN BONE-DERIVED CELLS

By

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CHROMATIN STRUCTURE OF THE RAT OSTEOCALCIN GENE
PROMOTER IN BONE-DERIVED CELLS

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Transcription of the osteocalcin gene, which encodes a bone-specific 10 kDa protein, is controlled by the coordinated utilization of modularly organized basal and hormone-responsive enhancer elements. Activation of these sequences involves the interaction of specific transcription factors to these promoter elements. It is becoming increasingly accepted that nuclear architecture provides a basis for support of tightly regulated modulation of cell growth and tissue-specific transcription which is required for the onset and progression of differentiation. Thus packaging of DNA as chromatin can facilitate the cooperative interaction between activities of independent regulatory elements that contribute to the level of transcription. Here, we show that a specific nucleosomal organization supports the constitutive expression of the osteocalcin gene in ROS 17/2.8 rat osteosarcoma cells and that chromatin remodeling directly correlates with the developmentally regulated transcriptional activation of this gene in normal diploid osteoblasts. By combining DNase I, micrococcal nuclease, and specific restriction endonuclease digestion analysis, we observed that the presence of DNase I hypersensitive sites (proximal: -170 to -70, and distal: -600 to -400) and a selective nucleosome positioning over the osteocalcin gene promoter are directly associated with developmentally stage-specific transcriptional activation in bone-derived cells. In addition, we found that chromatin hyperacetylation prevents a key transition in the chromatin structure which is required for the formation of the distal DNase I hypersensitive site. This transition involves the interaction of specific nuclear factors and is necessary for the subsequent ligand-dependent binding of the vitamin D receptor complex. Finally, we have established a requirement for
sequences residing in the proximal region of the osteocalcin gene promoter for both formation of the proximal hypersensitive site and basal transcriptional activity. Our approach was to assay nuclease accessibility in ROS 17/2.8 cell lines stably transfected with promoter deletion constructs driving expression of a CAT reporter gene.
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<tr>
<td>OC</td>
<td>Osteocalcin</td>
</tr>
<tr>
<td>DHS</td>
<td>DNase I hypersensitive site</td>
</tr>
<tr>
<td>MMTV</td>
<td>Mouse mammary tumor virus</td>
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<tr>
<td>DNase I</td>
<td>Deoxyribonuclease I</td>
</tr>
<tr>
<td>RNase</td>
<td>Ribonuclease</td>
</tr>
<tr>
<td>MNase</td>
<td>Micrococcal nuclease</td>
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<tr>
<td>ROS</td>
<td>Rat osteosarcoma cells</td>
</tr>
<tr>
<td>ROB</td>
<td>Normal diploid rat osteoblast</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>VDRE</td>
<td>Vitamin D responsive element</td>
</tr>
<tr>
<td>NMP-1</td>
<td>Nuclear matrix protein 1</td>
</tr>
<tr>
<td>NMP-2</td>
<td>Nuclear matrix protein 2</td>
</tr>
<tr>
<td>AML</td>
<td>Acute myelogenous leukemia</td>
</tr>
<tr>
<td>Vit D</td>
<td>1,25-(OH)2 vitamin D3</td>
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<tr>
<td>CAT</td>
<td>Cloramphenicol acetyl transferase</td>
</tr>
<tr>
<td>RXR</td>
<td>Retinoic X receptor</td>
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<td>OCBP</td>
<td>Osteocalcin box binding protein</td>
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Chapter 1

Introduction

Development of the osteoblast phenotype.

Osteoblast differentiation is a multi-step series of events modulated by an integrated cascade of gene expression that initially supports proliferation and the sequential expression of genes associated with the biosynthesis, organization, and mineralization of the bone extracellular matrix (Owen et al. 1990a). The ability to culture normal diploid calvarial cells under conditions that support progressive development of the osteoblast phenotype and a bone tissue-like organization has provided a viable in vitro model system for examining the selective expression of genes and physiological signaling mechanisms that mediate osteoblast growth and differentiation (Owen et al. 1990a). The tissue-like organization in vitro is reflected by the progressive development of nodules of multilayered cells in a mineralized extracellular matrix with organized type I collagen fibrils. The ordered deposition of mineral within the collagen fibers initiates and is primarily associated with the nodule area (Bhargava et al. 1988, Pockwinse et al. 1992). A temporal sequence of gene expression defines a developmental sequence in primary cultures of normal diploid osteoblasts that contains four principal periods. Initially, proliferation supports increase of the osteoblast cell population and biosynthesis of type I collagen bone extracellular matrix. At the same time, genes which are required for activation of proliferation (e.g. c-myc, c-fos, c-jun) and cell cycle progression (e.g. histones, cyclins) are expressed together with expression of genes encoding cell adhesion proteins (e.g. fibronectin) and others associated with regulation of extracellular matrix biosynthesis (e.g. TGFβ,
type I collagen). Following the initial proliferation period, expression of genes associated with the maturation and organization of the bone extracellular matrix are up-regulated, contributing to rendering the extracellular matrix competent for mineralization (e.g., alkaline phosphatase). The third developmental period involves gene expression related to the ordered deposition of hydroxyapatite. Genes such as osteocalcin and osteopontin exhibit maximal levels of expression at this stage, when maturation of bone tissue-like organization is ongoing. A fourth developmental period follows in mature osteoblast cultures, during which collagenase and type I collagen gene expression is increased, apoptotic proliferative activity is detectable and compensatory proliferative activity is observed (Lynch et al. 1995). These late events have been suggested to be an indication of remodeling activity which may be sustaining structural and functional properties of the tissue.

The bone-specific osteocalcin gene.

The osteocalcin gene encodes a 10 kDa bone-specific protein, which is expressed only post-proliferatively during differentiation of normal diploid osteoblasts, when mineralization of the extracellular matrix is occurring (Aronow et al. 1990, Owen et al. 1990a). The osteocalcin gene which was initially described as a single copy, has recently been found to be represented as a multigene family (Rahman et al. 1993, Debois et al. 1994). In mouse three genes were identified in all strains examined, while in rat either one or multiple copies were detected dependent on the strains (Rahman et al. 1993). The promoters of the osteocalcin genes expressed in rat, human, and mouse bone have similar overall representation and location of promoter regulatory sequences.
Transcription of the osteocalcin gene is controlled by modularly organized basal regulatory sequences and hormone-responsive enhancer elements (Bortell et al. 1992) which independently and in combination contribute to gene transcription (see diagram on the next page). These include the TATA box (-44 to -31) and the OC box (-99 to -76) which contains sequences that exhibit recognition for members of the MSX homeodomain family of proteins (Hoffman et al. 1994, Towler et al. 1994 a and b) and OCBP, a yet not identified factor which seems to contribute significantly to the tissue-specific expression of the osteocalcin gene (Hoffman et al. Submitted). 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 osteocalcin gene transcriptional control (Tamamura et al. 1994). A TGFβ responsive domain (-162 to -134) which includes an AP-1 like consensus sequence, and interacts with Fra proteins has also been reported (Banerjee et al. Submitted). Interestingly, previous reports have established that osteocalcin expression is down regulated when bone-derived cells are treated with TGFβ (Breen et al. 1994a). Three AML-related sites (site A: -604 to -599, site B: -440 to -435, site C: -138 to -130) have been recently identified by functional and biochemical analysis. These sites appear to be recognized by a bone-specific protein which can be detected as a component of the nuclear matrix fraction (Bidwell et al. 1993, Merriman et al. 1995). The vitamin D responsive element (VDRE, -466 to -437) represents the key component of steroid hormone-mediated transcriptional enhancement (Markose et al. 1990, Demay et al. 1990, Terpening et al. 1991). It has been shown that ligand-dependent binding of the vitamin D receptor in vivo is directly correlated with transcriptional up-regulation (Breen et al. 1994b).
distal promoter

proximal promoter

Site A
VDRE
Site B
NMP-1
-0.6 kB

Site C
TGRE
-0.2 kB

AP-1
AML

RNA pol II
TFII
TAF'S.

E-box
OC-box
TATA
mRNA
The biochemical analysis of eukaryotic transcriptional promoters had suggested that, in general, only relatively small promoter regions were required for accurate initiation in vitro. However, correctly regulated gene expression in vivo is frequently found to require much larger chromosomal regions. The discovery of eukaryotic promoter-specific transcription factors (Engelke et al. 1980, Dynan et al. 1983) which can selectively activate promoters in fractionated cell-free extracts, provided a critical conceptual framework for investigations into mechanisms underlying eukaryotic gene regulation. The subsequent finding that these factors must bind promoter proximal cis-regulatory DNA elements to exert their effects suggested that the control of eukaryotic transcription might occur through an elaborated form of combinatorial control mechanism. Furthermore, the fact that these factors are active in vitro, in the absence of assembled chromatin, indicated that gene activation and repression could occur independently of global changes in chromatin structure. However, even skeptics accept that such in vitro systems include concentrations of both the genes and transcription factors which are considerably higher than those observed in cells and transcription occurs at extremely low rates. In addition, explanations for transcriptional control in an in vivo biological context must address the integration of a broad spectrum of regulatory mechanisms that support interrelationships between intracellular and extracellular signaling pathways which are responsive to both the establishment and maintenance of cell and tissue phenotypic properties. These transcriptional regulatory parameters modulate expression of genes during progressive stages of differentiation as well as those requisite for sustaining the commitment of differentiated cells to defined structural and functional properties (Stein et al. 1994).
Within the nucleus, DNA is packaged into a nucleoprotein complex called chromatin, which consists roughly of a 2:1 mass ratio of protein to DNA. This packaging of the DNA template into chromatin provides compaction and organization for the DNA which is key in processes such as transcription, recombination, replication, and mitosis. When the extended chromatin fiber is observed by electron microscopy, it appears as a filament that resembles "beads on a string". Each of these beads corresponds to the unit repeat of the chromatin, the nucleosome (Kornberg et al. 1992). In a nucleosome the DNA is wrapped twice around a core histone octamer, a structure which consists of two members each of the core histones H2A, H2B, H3, and H4. In addition there is one copy of the linker histone H1. In the octamer, each of the histones has a basic, unstructured N-terminal tail and a globular C-terminal domain that is highly \( \alpha \)-helical (Arens et al. 1991). It has been suggested that the DNA in a nucleosome is wrapped around the globular core of the octamer, while the basic core histone N-terminal tails interact with the DNA.

Kadonaga and colleagues, using in vitro reconstitution systems, have shown additive roles of nucleosome cores and histone H1 on transcription repression (Laybourne et al. 1991). They found that template DNA organized into nucleosome cores was insufficient to produce complete repression of class II promoters. Moreover, they demonstrated that the binding of H1 to these templates completed transcriptional repression. Additional studies have determined that histone H1 also represses transcription by RNA polymerases I and III (Kuhn et al. 1992, Wolffe 1989). In vivo, histone H1 has been found depleted from oligonucleosomes containing unmethylated CpG islands (and thus presumably from many promoters, Tazi et al. 1994) and
Positioned nucleosomes are those located in a precise site relative to DNA sequence in all cells of a given population. In this situation, any particular DNA sequence in the region of the positioned nucleosome would always lie in the same interaction with histones (Simpson 1991). The most widely accepted mechanism to induce nucleosome positioning or regular chromatin organization, involves the binding of non-histone chromosomal proteins. It is assumed that this interaction precludes inclusion of that DNA segment in a nucleosome and creates a boundary which leads to a positioned array of nucleoparticles. The accuracy of this positioning is highest adjacent to the boundary and decays at longer distances from the protein-DNA interaction site (Simpson 1991). GRF 2, a protein that binds to the intergenic control region of the GAL 1 and GAL 10 genes in S. cerevisiae, provided one of the first examples of protein-DNA interactions involved in nucleosome positioning (Fedor et al. 1988). This protein was later shown to bind to sequences upstream in many genes, an rRNA gene enhancer, at centromeres, and telomeres (Chasman et al. 1990). Two additional yeast proteins, α2, produced by α-mating-type cells, and MCM1, act in concert to repress the expression of a-cell mating type-specific genes in α-mating-type cells. The mechanism also involves nucleosome positioning immediately flanking the protein-DNA interaction region (Roth et al. 1990).

Two mechanisms have been postulated to regulate the accessibility of transcription factors to promoters and enhancers in chromatin, nucleosome exclusion and nucleosome displacement (Owen-Hughes et al. 1994). The first involves...
perturbations of chromatin structure during DNA replication, which provides a window of opportunity for transcription factors to access DNA elements prior to chromatin assembly. By this mechanism, binding of the appropriate factors could exclude nucleosomes from subsequently forming over promoters. The second mechanism invokes replication-independent chromatin remodeling. Here alterations in chromatin organization are the direct result of the action of transcription factors. This mechanism implies that some factors, or combinations thereof, are capable of binding nucleosomal DNA to bring about the removal of preexisting nucleosomes from promoter sequences and thereby create regions accessible to additional factors. Multiple studies, most of them using in vitro assays, have shown that there is an inherent difference in the nucleosome-binding capacity of transcription factors. Whereas factors such as HSF (Taylor et al. 1991), NF-1 (Archer et al. 1992), and c-myc homodimers (Wechsler et al. 1994), appear essentially unable to bind their sites assembled into nucleosomes, TBP (Imbalzano et al. 1994), USF (Chen et al. 1994), glucocorticoid receptor (Archer et al. 1992), myc/max and max/max complexes (Wechsler et al. 1994), gal 4 derivates (Taylor et al. 1991), and Sp1 (Chen et al. 1994), can recognize and bind their sites in nucleosomes under at least some conditions, although with varying degrees of reduced affinity.

Histones are subject to extensive post-translational modifications, including phosphorylation, methylation, ubiquitination, ADP-ribosylation, and acetylation, which may affect their function (van Holde 1988). Of these modifications, core histone acetylation has been studied extensively. Histone H4 isoforms acetylated at different lysine residues in their amino terminal regions are found to be distributed to specific chromatin types (Turner 1993), suggesting that histone acetylation may affect chromatin function and transcriptional activity. Removal of the histone amino terminal
tails by trypsin digestion or hyperacetylation of the core histones reduces the ability of H1: both to bind nucleosomes and to repress transcription factor binding (Allan et al. 1982, Ridsdale et al. 1990). Genetic studies in yeast provided clear evidence that the amino terminal regions of the core histones could be involved in the regulation of transcription. Deletion of the N-terminal tails of H4 results in the derepression of expression from diverse yeast loci (Grunstein 1990). However these domains are not essential for viability.

Wolfe and colleagues (Ura et al. 1994) have recently described that core histone hyperacetylation does not significantly alter the preferential and asymmetric binding of linker histones to a mononucleosome core containing a 5S RNA gene. This apparent controversy may be explained if we consider that the acetylation of the core histone tails may subtly alter the conformation of the nucleosome in such a way as to decrease the affinity of linker histones in a manner that would not be evident in the single nucleosome core binding assays utilized by Ura et al. The same group has previously shown that the organization of 5S DNA on histone octamer in a nucleosome core particle is remarkable stable (Bashkin et al. 1993), suggesting that the source of DNA utilized in these studies should be consider carefully.

Nucleosomes can be reconstituted in vitro using hyperacetylated core histones. Although these nucleosomes adopt a distinct conformational state, the nucleosome can be considered essentially folded (Ebralidse et al. 1993). Moreover, changes in the linking number per nucleosome (Norton et al. 1989) as well as alterations in the nuclease sensitivity of the DNA within the nucleosome (Simpson 1978) are consistent with an unwinding or loosening of the DNA from the nucleosome.
It has been demonstrated that core histone hyperacetylation enhances binding of some transcription factors to nucleosomes (Lee et al. 1993, Vettesse-Dadey 1994). Two possible mechanisms to explain this facilitated interaction have been suggested. First, that basic histone N-terminal domains bind to nucleosomal DNA preventing factor binding. Acetylation/neutralization of lysine residues within these domains could simply reduce the affinity of these domains for DNA and thus alleviate their repression of transcription factor binding. An alternative explanation is that acetylation might alter the conformation of the core particle and thereby increase transcription factor access (Ura et al. 1994).

Analysis of chromatin structure indicates that active or potentially active genes are typically marked by the presence of nuclease sensitive domains. These regions have been suggested to reflect the absence of a classical nucleosomal structure and the binding of specific non-histone chromosomal proteins (Elgin 1988, Gross et al. 1988, Felsenfeld, 1992). Nuclease hypersensitivity and nucleosome positioning analysis have been used successfully to show changes in chromatin structure associated with transcriptional activation of heat shock inducible genes (Wu 1980), steroid hormone regulated genes (Zaret et al. 1984), developmentally regulated genes (Mollers et al. 1992), and cell cycle regulated genes (Chrysogelos 1985).

For many years one of the main research interests in our laboratory has been directed toward understanding the molecular mechanisms that relate nuclear architecture and gene expression. Several lines of evidence indicate that the three dimensional organization of the OC gene may have a principal role in the developmentally-regulated and tissue-specific expression of this gene in bone-derived
cells (Bortell et al. 1992). The aim of this thesis is to study the chromatin structure of the rat OC gene promoter, and to determine its role in the regulated expression of this bone-specific gene.
Chapter 2

Materials and methods

Cell culture.

Rat osteosarcoma-derived ROS 17/2.8 and ROS 24/1 cells were maintained as described (Majeska et al. 1980). Cells were subjected to treatment with $10^{-8}$ M 1,25 dihydroxyvitamin D3, 10mM sodium butyrate, or a combination of both as indicated.

Primary diploid osteoblasts (ROB) were isolated from 21-day fetal rat calvaria as previously reported (Owen et al. 1990). Proliferating (day 3) and differentiated (day 20) cultures were treated with vitamin D or vehicle (ethanol) for 4 or 24 hours as indicated.

Rat hepatoma-derived H4 cells and rat-derived fibroblastic R2 cells were maintained as previously described (Wolfe et al. 1989, Topp et al. 1981).

Stably transfected cell lines.

For the stable transfection of ROS 17/2.8 cells, the BBS-calcium phosphate method was employed (Chen et al. 1987). For each construct, four 35 mm wells were transfected, each with 5 µg of the OC-CAT plasmid and 0.42 µg (1/20 the molar amount) of pCEP4 (Invitrogen, San Diego, CA) encoding the hygromycin B-
phosphotransferase gene. Based on preliminary killing curves, selection was performed with 55 units/mL of hygromycin B (Calbiochem, La Jolla, CA). To decrease the sensitivity of the assay to site-of-integration effects, all the resistant colonies (between 10 and 25) from each transfection were pooled; thus the variation among cell pools is expected to be lower than the variation among monoclonal cell lines. To minimize reversal events, cells were routinely maintained in hygromycin B during all the passages preceding the experiment.

OC promoter-chloramphenicol acetyl transferase (CAT) deletion constructs used to stably transfect ROS 17/2.8 cells have been previously described (Frenkel et al. 1993, Aslam et al. 1995). The starting construct was pOCZCAT (-1097OCCAT), which contained a 1097 bp EcoR I-Hind III (Hind III was introduced by mutagenesis) fragment of the 5'-flanking region (-1097 to +24) of the rat OC gene fused upstream of the CAT gene that was previously cloned into the multiple cloning site of pGEM7zf(+). A series of constructs, progressively shortened from the 5'-end, and identical at the 3'-end, were subsequently generated by utilizing Xho I and Sph I sites, present in the multiple cloning site of pGEM and internal restriction sites within the 1097 bp OC sequences. pOCZCAT was digested with Hinc II (-529)/Hind III (+24) to generate a 529 bp fragment that was ligated into the Xbo I (blunt-ended with T4 DNA polymerase)/HindIII fragment of -1097OCCAT to create the -529CAT. The Bgl II (-348) and Aos I (-108) fragments were generated by digesting -1097CAT with Bgl II (-348)/Hind III (+24) and Aos I (-108)/Hind III (+24), respectively. These fragments were ligated into the Xho I (blunt-ended with T4 DNA polymerase)/Hind III fragment of -1097OCCAT to create -34 OCCAT and -108OCCAT.

The average number of integrated CAT genes was confirmed by
Southern blot analysis. Genomic DNA was purified from stably transfected cell lines and digested with Hind III and Xba I to release the CAT gene. The samples were then electrophoresed in agarose gels, blotted, and hybridized with a Hind III / Xba I probe. Equal loading was confirmed by ethidium bromide staining prior to transfer as well as by reprobing the blots with an OC gene probe.

**Nuclei isolation.**

Nuclei were isolated from calvarial-derived rat osteoblasts by gentle dounce homogenization (loose pestle) in eight 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, 0.2%(V/V) nonidet NP-40]. The homogenate was then filtered through two layers of cheesecloth. Subsequently, the nuclei were collected by low speed centrifugation (500xg) and resuspended in digestion buffer [60 mM KCl, 15 mM NaCl, 15 mM Tris-HCl pH 7.4, 1 mM CaCl2, 3 mM MgCl2] containing 20% glycerol. Nuclei from ROS 17/2.8, ROS 24/1, H4, and R2 cells were isolated by this same protocol or as previously described (Montecino et al. 1994) with similar results. When nuclei were isolated from butyrate treated cells, this compound was included at a 5 mM concentration during the entire isolation procedure.

**Nuclease digestion.**

DNase I hypersensitivity and micrococcal nuclease (MNase) digestion analyses were performed according to the indirect end-labeling method (Wu 1980). Isolated nuclei were incubated with increasing amounts of DNase I or MNase (Worthington Biochemicals, Freehold, NJ) in digestion buffer for 10 and 5 minutes,
respectively, at 18 °C with agitation. The digestions were stopped by the addition of EDTA to a final concentration of 25 mM. Purified genomic DNA was then completely digested with several combinations of restriction endonucleases as indicated. Digested DNA was electrophoresed in agarose gels and transferred to a nylon membrane (Zeta-probe, Bio Rad Laboratories, Melville, NY) following the manufacturer's recommendations. Probes were prepared by restriction endonuclease digestion of the osteocalcin 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 et al. 1983) using reagents from Stratagene (La Jolla, CA). Hind III-digested bacteriophage Lambda DNA and Hinf I-digested pUC19 DNA, labeled with T4 polynucleotide kinase and (32P-γ) ATP, were used as DNA size markers.

**Restriction endonuclease digestion studies.**

Isolated nuclei from osteosarcoma cells or from normal diploid osteoblasts were resuspended and digested with restriction endonucleases (500 units/ml) in the specific buffer conditions provided by the supplier (New England Biolabs, Beverly, MA). The mixture was incubated for 30 minutes at 37 °C, conditions that gave optimal digestion. The reaction was stopped by the addition of EDTA to a final concentration of 10 mM. The purified DNA was then digested with a combination of restriction endonuclease, electrophoresed in agarose gels, blotted, and hybridized as indicated. The intensity of the bands on the Southern autoradiogram were determined using a scanning densitometer (Apple onescanner). The accessibility to the different restriction enzymes 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 nucleoparticles was carried out as previously described by Weintraub (Weintraub 1984). Nuclei from ROS 17/2.8 cells were resuspended in a buffer containing 10 mM Tris-HCl pH7.4, 10 mM NaCl, 5 mM MgCl₂, 0.1 mM CaCl₂, 0.2 mM PMSF, 70 µg/ml TPCK, 10 µg/ml trypsin inhibitor, 0.5 µg/ml leupeptin, 1 µg/ml pepstatin. MNase (25 units/ml, 13.5 units/mg) was added and the digestion performed at 18°C for 5, 10, 15, 20, 30, 40, and 50 minutes. The reaction were stopped by addition of EDTA and EGTA to final concentrations of 10 and 1 mM, respectively. The samples were kept on ice for 30 minutes to achieve nuclear lysis. Nuclear debris, including non solubilized residual chromatin, was removed by centrifugation. Solubilized chromatin nucleoparticles were visualized by direct electrophoresis of a small fraction on a 1% agarose gel. The DNA fragments contained in these solubilized nucleoparticles were then purified, electrophoresed, blotted, and hybridized as indicated.

Nuclear extract isolation and gel mobility shift assays.

Nuclear extract preparation from ROS 17/2.8 cells and gel mobility shift assays were performed as previously documented (Shakoori et al. 1994). Probes were end-labeled by using T4 polynucleotide kinase and (³²P-γ)ATP. Binding
reactions contained 2.5 μg of nuclear protein and 10 fmoles of the VDRE (Owen et al. 1990b), YY-1 (Guo et al. in press), or AML (Merriman et al. 1995) double stranded oligonucleotide. The mixtures were fractionated electrophoretically in 4% polyacrylamide gels (80:1) and 0.5 X TBE buffer.

**Intranuclear footprinting analysis.**

Ligation mediated PCR was performed on DNase I cleaved nuclear DNA essentially as described (Muller et al. 1992) using Vent DNA polymerase (New England Biolabs, Beverly, MA). First strand synthesis was accomplished with primer 1 (5'-GACAAACTGGCTCCAAACTCGCATGC-3'; nt -358 to -383) in first strand buffer [40 mM NaCl, 10 mM Tris-HCl pH 8.9, 5 mM MgSO4, 0.01% gelatin, and 200 μM of each dNTPs]. The DNA was then ligated with the unidirectional linker primer (Muller et al. 1992). Amplification was carried out by PCR using primer 2 (5'-GCATAGCCTGTGATTTTCAGTGTCTGCCGT-3'; nt -377 to -406) and the linker oligonucleotide. Eighteen cycles of PCR were performed, each involving denaturation for 1 minute at 96 °C, annealing for 2 minutes at 56 °C, and elongation for 3 minutes at 76 °C. Labeling was carried out with 32P-end-labeled primer 3 (5'-AGCCTGTGATTTTCAGTGTCTGCCGTGAGAGCA-3'; nt -381 to -413) for two more rounds of PCR cycling with an elevated annealing temperature of 58 °C. Subsequently, the samples were phenol/chloroform extracted, ethanol precipitated, resuspended in sequencing load buffer and separated in 6% polyacrylamide, 7 M urea sequencing gels.
Nuclear run on analysis.

Nuclear run on analyses were performed according to previously described protocols (Srivastava et al. 1993).

a) **Nuclear isolation:** Nuclei were isolated from five plates (100 mm) of proliferating cells or three plates of confluent cultures by gentle dounce homogenization (loose pestle) in 10 ml of lysis buffer [10 mM Tris-HCl pH 7.4, 10 mM NaCl, 3 mM MgCl₂, 0.2% Nonidet NP-40]. For mineralized rat-derived normal diploid osteoblast (ROB) cultures, the homogenate was filtered using cheesecloth. After centrifugation at low speed, the nuclear pellet was resuspended in 200 µl of storage buffer [50 mM Tris-HCl pH 8.3, 5 mM MgCl₂, 0.1 mM EDTA, and 40% glycerol]. After counting, the samples were frozen in liquid nitrogen and stored at -70°C for up to two weeks.

b) **Transcription reaction:** The nuclear suspension (200 µl, in storage buffer) was diluted 1:1 with the reaction buffer mix [10 mM Tris-HCl pH 8.0, 300 mM KCl, 5 mM MgCl₂, 5 mM DTT, 1 mM of ATP, 1mM CTP, 1 mM GTP, and 100 µCi (α-³²P) UTP] and then incubated at room temperature for 40 minutes. 50 units of RNase-free DNase I were added and the mixture digested for 5 minutes at 37°C. The reaction was stopped by the addition of 400 µl of 4 M guanidinium thiocyanate, 25 mM sodium citrate pH 7.0. The samples were then phenol-chloroform extracted, and the aqueous phase was ethanol precipitated. After washing with 70% ethanol, the dried radioactive samples were resuspended in 1 ml of hybridization solution [12.5 mM Na₂P₂O₇, 1 M
NaCl, 20 mM Tris-HCl pH 7.4, 2 mM EDTA, 0.1 % SDS, 10X Denhardt's, and 250 μg/ml salmon sperm DNA]. The membranes (slot blots) containing the genes to be analyzed were prehybridized overnight at 65 °C, and then hybridized for 48 hours at the same temperature.
Chapter 3

Changes in chromatin structure support constitutive and developmentally regulated transcription of the bone specific osteocalcin gene in osteoblastic cells


Introduction.

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 regulation 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 et al. 1993, Fedor 1992, Felsenfeld 1992, Gross et al. 1988). 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 et al. 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 et al. 1992, Schild et al. 1993). Similarly, Elgin and coworkers have shown that specific positioning of a nucleosome in the promoter region of the Drosophila heat shock hsp26 gene allows the interaction of DNase I hypersensitive sites that span two heat shock factor binding sites localized distal and proximal to the transcriptional start site (Elgin 1988). The same group has recently demonstrated that (CT)n rich sequences residing at the boundaries of both DNase I hypersensitive sites are required for formation of hypersensitive sites and heat shock-dependent transcriptional enhancement (Lu et al. 1992 and 1993). On the other hand, it has been demonstrated that nucleosome positioning can be a requirement for tissue-specific expression. McPherson et al (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 result suggests that although 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. 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 (Hoffman et al. 1994, Towler et al. 1994 a and 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 (Tamamura et al. 1994). Banerjee et al 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. submitted). 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 D responsive element (VDRE, -466 to -437) represents the key component of steroid hormone-mediated transcriptional enhancement (Markosse et al. 1990, Demay et al. 1990, Terpening et al. 1991). Our laboratory has shown that ligand-dependent binding of the vitamin D receptor in vivo is directly correlated with transcriptional upregulation (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 et al. 1990). These characteristics have led to the extensive use of this cell line to study the regulatory mechanisms involved in OC gene expression. 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 D receptor (Baran et al. 1991, Dokon et al. 1984, McDonell et al. 1989), providing an important experimental model to study mechanisms involved in OC gene expression and vitamin D3-dependent transcriptional upregulation.

Here we determined that specific nucleosomal remodeling at the OC gene promoter supports constitutive expression in ROS17/2.8 cells as well as developmentally-regulated transcription of the OC gene in normal diploid rat osteoblasts in culture. The 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 DNase I hypersensitivity over these elements.

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, Fersenfeld et al. 1992, Gross et al. 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 et al. 1988, Simpson et al. 1991).
Figure 3.1. 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 gene (including exons and introns) and the horizontal arrow over the gene marks the direction of transcription. A=Apa I, B=BamH I, G=Bgl II, H=Hinc II, P=Pst I, S=Sst I/Sac I, U=Pvu II, V=Ava I, X=Xba I.
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 were carried out. The analysis revealed that the presence of DNase I hypersensitive sites at the OC gene promoter is not only restricted to bone-derived cells but is also directly related to the transcriptional status of the OC gene (figures 3.2 and 3.3). 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 (see figure 3.2A). As an internal control, we confirmed that these two cell types exhibit similar levels of DNase I hypersensitivity of the histone H4 genes (figure 3.2B), which are expressed at high levels in both cell lines (figure 3.3). The two hypersensitive domains of the OC gene promoter span regions which contain binding sequences for a series of transcriptional regulators (see figure 3.4) present in both cell lines, with the exception of the vitamin D receptor, which is absent in ROS 24/1 cells (Hoffman et al. 1994 and submitted, Merriman et al. in press). It has been reported that DNase I possesses specificity for AT-rich sequences of DNA (Nelson et al. 1979). Therefore, as a control purified genomic DNA from ROS 17/2.8 cells was partially digested with DNase I and then analyzed by Southern blot using probe 1 (see figure 3.1). As shown in figure 3.5, no specific bands were generated by nuclease digestion. This result indicates that the DNase I hypersensitivity described above for the OC gene promoter in intact nuclei of ROS 17/2.8 cells reflects the chromatin structure of the 5'-regulatory domains and is not an inherent property of the DNA sequence.

To define a correlation between DNase I hypersensitivity and
Figure 3.2. DNase I hypersensitivity at the osteocalcin gene is correlated with transcriptional activity in osteosarcoma cells. Nuclei isolated from ROS 17/2.8 cells (which express osteocalcin) and from ROS 24/1 cells (which do not express osteocalcin), treated with vehicle (C) or vitamin D (D) for 24 hours, were incubated with increasing concentrations of DNase I as described in materials and methods, and the purified genomic DNA was then analyzed by the indirect end-labeling method. A) Samples were digested with BamH I, electrophoresed in a 1.2% agarose gel, blotted and hybridized with probe 1 (see figure 3.1). B) Aliquots of the same samples were digested with EcoR I 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.
Figure 3.3. Transcription of the osteocalcin and histone H4 genes in osteosarcoma cells. Transcriptional activity was measured in nuclei isolated from confluent ROS 17/2.8 and ROS 24/1 cell cultures, control (C) or treated with vitamin D (D) for 24 hours, by nuclear run on analysis. The values shown in these figure correspond to the average basal and vitamin D-dependent transcriptional activity previously observed in these osteosarcoma cell lines (Owen et al. 1990a, c, and 1993).
OC TRANSCRIPTION

% MAXIMUM

C  D  C  D
ROS 17/2.8  ROS 24/1
HISTONE H4 TRANSCRIPTION

% MAXIMUM

C   D   C   D
ROS 17/2.8  ROS 24/1
Figure 3.4. Mapping DNase I hypersensitive sites in the promoter of the osteocalcin gene. Increased resolution of the DNase I hypersensitive sites in the osteocalcin gene promoter was achieved when the samples were digested with Apa I, electrophoresed in 2% agarose gel, blotted, and hybridized with probe 2 (see figure 3.1). The promoter elements encompassed by the nuclease hypersensitive sites are indicated on the right.
DNase I

4.3 Kb

1.38 kb

NMP-2A/AML
NMP-1/YY-1
VDRE
NMP-2B/AML
AP-1/Fra
NMP-2C/AML
E Box/Id
OC Box/Msx 2
Figure 3.5. Partial DNase I digestion of genomic DNA from ROS 17/2.8 cells. 400 μg/ml of purified genomic DNA were incubated with increasing amounts of DNase I (0, 0.1, 0.3, 0.5, 0.7, and 1.0 units/ml, respectively) at 18 °C for 2 minutes. The samples were then completely digested with BamH I, electrophoresed in a 1% agarose gel, blotted and hybridized with probe 1 (see figure 3.1).
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 using probe 3 (-870 to +150, see figure 3.1), we observed the characteristic DNA ladder (figure 3.6A), 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 3.1).

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 3.1). Because it has been reported that MNase possess some degree of sequence preference (Dingwall et al. 1981, Horz et al. 1981), as a necessary control we compared these digestion patterns with those obtained following incubation of purified genomic DNA with this nuclease. Figure 3.6B 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.
Figure 3.6. Nucleosomal organization of the osteocalcin gene in rat osteosarcoma cell lines. Nuclei from ROS 17/2.8 and ROS 24/1 cells, treated with vehicle (C) and vitamin D (D) for 24 hours, were incubated with increasing amounts of micrococcal nuclease (MNase). The purified genomic DNA was then analyzed by indirect end-labeling. Arrowheads mark MNase cleavage sites present exclusively in chromatin and circles those detected in both chromatin and naked DNA. A) Samples (25 μg) digested with Apa I were electrophoresed in a 2% agarose gel and hybridized with probe 3 (figure 3.1). The numbers 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).
Figure 3.6 B (continued). Samples from MNase digested ROS 17/2.8 and ROS 24/1 nuclei, and ROS naked DNA were cleaved with Apa I, electrophoresed, blotted, and hybridized with probe 2 (figure 3.1). Lanes 1, 4, 7, 10, and 13=0; lanes 2, 5, 8, and 11=5; lanes 3, 6, 9, and 12=10; lane 14=0.5; lane 15=0.7; and lane 16=1.0 units/ml.
<table>
<thead>
<tr>
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<th>ROS 17/2.8</th>
<th></th>
<th>ROS 24/1</th>
<th></th>
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<tr>
<td></td>
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<tr>
<td>1.38 Kb</td>
<td>-650</td>
<td>-560</td>
<td>-350</td>
<td>-310</td>
<td>-160</td>
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</table>

- Diagram showing gel electrophoresis with markers at 1.38 Kb.
Figure 3.6 C (continued). Aliquots of some of the same samples described in figure 3.6 B were also cleaved with Apa I and Ava I, and then hybridized with probe 4 (figure 3.1). Lanes 1, 5, and 9=0; lanes 2 and 10=5; lanes 3 and 11=10; lanes 4 and 12=15; lane 6=0.5; lane 7=0.7; and lane 8=1.0 units/ml.
by MNase rather than recognition of chromatin structural features. Interestingly, the last two digestion sites appear to be protected in ROS 24/1 chromatin. The chromatin specific cleavage sites were more accurately confirmed by digesting the samples with Apa I and Ava I, and then hybridizing with probe 4 (-145 to +150, see figure 3.1). This approach facilitated visualization of those sites located in the distal region of the promoter. As shown in figure 3.6C, cleavage sites at -650, -560, -350, -310, and -50 were detected in ROS 17/2.8, while in the ROS 24/1 samples, the MNase cleavage sites at -650 and -350 were now clearly observed.

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 1983, Pfeifer et al. 1991, Zhang et al. 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 have restriction sites in the promoter at functionally strategic positions (see figure 3.1), 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, chapter 2).

The results summarized in figure 3.7A 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
Figure 3.7. Restriction endonuclease accessibility of the osteocalcin gene in osteosarcoma cell lines. Nuclei isolated from ROS 17/2.8 and ROS 24/1 cells, treated with vehicle or vitamin D as indicated, were incubated with 500 units/ml of the restriction endonucleases Hinc II, Bgl II, and Pst I (as indicated at the top) for 30 minutes at 37 °C and the products 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 3.1). The position of the cleavage sites 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, Pst I-Apa I, respectively.
<table>
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<td>17/2.8</td>
<td>M1 24/1</td>
<td>- + - +</td>
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<tr>
<td>M2 24/1</td>
<td>- + - +</td>
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<tr>
<td>17/2.8 M3 24/1</td>
<td>- + - +</td>
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<td>-529</td>
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1.38 kb
Figure 3.7 B (continued). DNA samples from ROS 17/2.8 and ROS 24/1 nuclei incubated with Hinc II were cleaved with BamH I, electrophoresed, blotted, and hybridized with probe 1 (figure 3.1). Lane M corresponds to ROS 24/1 purified genomic DNA digested with Hinc II and BamH I. The position of the two Hinc II sites present in the 4.3 Kb BamH I restriction fragment (figure 3.1) are shown on the left.
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<th>Hinc II</th>
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<tr>
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<tr>
<td>ROS</td>
<td>-</td>
</tr>
<tr>
<td>VIT D3</td>
<td>-</td>
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</table>

B

4.3 Kb

-529

+657

Table:

- ROS: 17/2.8 - 24/1 - M
- VIT D3: - + - + -
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 3.7A). This result confirms our previous findings based on DNase I hypersensitivity, that a major change in 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 3.1) was not cleaved in ROS 24/1 nor in ROS 17/2.8 cells (figure 3.7B). This result suggest that this region of the OC gene may be organized with randomly positioned nucleosomes in osteosarcoma cells, both in the presence (ROS 17/2.8) and absence (ROS 24/1) of OC gene transcription.

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 in ROS 24/1 cells compare to ROS 17/2.8 cells (figure 3.7A). We 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 3.7, A and 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 positioned 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 -310). In addition the
results indicate that in the ROS 17/2.8 cell line, two regions of the promoter, the distal (-600 to -400) and proximal (-170 to -70) domains, exhibit nuclease hypersensitivity. This hypersensitivity appears to reflect 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 ten base pairs upstream of the TATA box element. This site, observed only in ROS 17/2.8 transcribing the OC gene may be an indication of the binding of the RNA polymerase transcription complex to this region of the promoter.

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 3.8A, DNase I hypersensitivity in the OC gene
Figure 3.8. Developmentally-regulated DNase I hypersensitivity of the osteocalcin gene in normal rat osteoblasts. Nuclei isolated from rat calvarial-derived osteoblasts (ROB) cultures on day 3 (proliferating) or 20 (mineralized) treated with vehicle (C) or with vitamin D (D) for 24 hours were incubated with increasing amounts of DNase I. The purified DNA samples were analyzed by the indirect end-labeling method as described in figure 3.2. A) Osteocalcin gene. B) Histone H4 genes.
Figure 3.9. Developmentally-regulated transcription of the osteocalcin gene. Transcriptional activity of the osteocalcin gene and histone H4 genes in proliferating and mineralized rat osteoblasts, incubated with vehicle (C) or vitamin D (D) for 24 hours, was determined by nuclear run on analysis.
OC TRANSCRIPTION

% MAXIMUM

PROLIFERATING ROB

MINERALIZED ROB

C  D  C  D
HISTONE H4 TRANSCRIPTION

PROLIFERATING ROB

MINERALIZED ROB

C

D

C

D

% MAXIMUM
promoter is detected only in mineralized cultures, when the gene is actively transcribed (figure 3.9). Two DNase I hypersensitive sites are localized in the same promoter regions as those previously identified in ROS 17/2.8 cells (figure 3.2A), and both exhibited increased intensity following vitamin D treatment for 24 hours, which results in enhancement of OC gene transcription (figure 3.9). 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 3.8B). 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, although not identical, to the pattern in osteosarcoma cells (figure 3.10A). Defined cleavage sites at -560, -310, -90, and a diffuse band encompassing approximately 60 bp spanning -50 to +10 were detected in ROB chromatin DNA samples digested with Apa I and hybridized with probe 2 (see figure 3.1). Two additional cleavage sites at -650 and -350 were more prominent and clearly defined after digestion with a combination of Apa I and Ava I, and hybridization with probe 4 (figure 3.10B, lane 1). Under these conditions the diffuse band initiated at -50 was minimally detectable in comparison to the prominent band in ROS 17/2.8 samples (figure 3.10B, compare lanes 1 and 2). The cleavage pattern observed in both proliferating and mineralized normal diploid osteoblasts was not influenced by vitamin D treatment. Similar to ROS cells, the bands corresponding to -560 and -310 and a diffuse band centered at -90 were also present in naked ROB DNA digested with MNase (figure 3.10A).
**Figure 3.10. Nucleosomal organization of the osteocalcin gene promoter during normal rat osteoblast differentiation.** Nuclei isolated from proliferating and mineralized rat osteoblast cultures were incubated with increasing concentrations of MNase and the purified DNA samples analyzed as described in figure 3.6. Arrowheads mark MNase cleavage sites present exclusively in chromatin, and circles those detected in both chromatin and naked DNA. A) Apa I digested samples hybridized with probe 2 (figure 3.1). Lanes 1, 5, 9, 13, and 17=0; lanes 6, 10, 14, and 18=5 u/ml; 7, 11, 15, and 19=10 u/ml; lanes 8, 12, 16, and 20=15 u/ml; lane 2=0.5 u/ml; lane 3=0.7 u/ml; and lane 4=1.0 u/ml.
Figure 3.10 B (continued). Samples from ROB (lane 1) and ROS 17/2.8 (lane 2) MNase digested nuclei, cleaved with Apa I and Ava I, and hybridized with probe 4 (figure 3.1).
We then examined changes in restriction endonuclease cleavage within the OC gene promoter as an indication of modifications in nucleosome positioning during differentiation of normal diploid osteoblasts (figure 3.11A). The Hinc II site, located in the distal region of the promoter (-529, see figure 3.1), was not accessible to the enzyme in early stage proliferating cells. In mature osteoblasts undergoing extracellular matrix mineralization this sequence exhibited significant cleavage (29%). 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 3.11B), confirming our findings in both ROS 17/2.8 and ROS 24/1 osteosarcoma cells (figure 3.7B). We observed that a Pst I site, localized in the proximal region of the OC gene promoter (-145), exhibited a four 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 (-600 to -400) and proximal (-170 to -70) 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 3.7A). This may reflect expression of OC in only approximately 70 percent 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
Figure 3.11. 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 (+), were incubated with the restriction endonucleases Hinc II, Bgl II, and Pst I and analyzed as described in figure 3.7. A) The purified DNA samples were cleaved with Apa I and hybridized with probe 2 (figure 3.1).
### Table A

<table>
<thead>
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<th>ROB</th>
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<th>Bgl II</th>
<th>Pst I</th>
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<td>MINER</td>
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<tr>
<td>VIT D3</td>
<td>-</td>
<td>+</td>
<td>-</td>
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</table>

| % Digestion | 0 | 0 | 29 | 33 | 27 | 32 | 41 | 41 | 8 | 9 | 43 | 45 |

- 1.38 kb
- 529
- 343
- 145
Figure 3.11 B (continued). Samples from Hinc II digested nuclei, cleaved with BamH I and hybridized with probe 1 (figure 1).
<table>
<thead>
<tr>
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<td></td>
<td>ROB</td>
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<tr>
<td>VIT D3</td>
<td>-</td>
</tr>
</tbody>
</table>

**Diagram:**
- 4.3 Kb
- -529
- +657
nucleosomes overlapping these sequences. The nuclease digestion pattern exhibited during this early developmental stage, when the OC gene is not transcribed, indicates that nucleosomes can be located between -560 to -350 and between -310 to -90. 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 can be associated with the binding of a series of transcription factors which regulate OC gene transcription (see discussion, chapter 6).

To determine whether this nucleosomal pattern in the OC gene is restricted to bone derived cells, nuclei were isolated from H4 cells, a rat hepatoma cell line, and from R2 cells, a rat fibroblastic cell line. Then MNase digestion was performed under the same conditions used with bone-derived cells. The results are shown in figure 3.12, and they indicate that in both non osseous cell lines, the OC gene appears highly inaccessible to MNase digestion, which is normally an indication of randomly positioned nucleosomes (Simpson 1991). Treatment of these cells with vitamin D did not alter the result. This observation supports functional relationship between tissue-specific chromatin organization and transcriptional activity of the OC gene.

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, 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
Figure 3.12. MNase digestion analysis in non osseous cells. Nuclei isolated from a rat hepatoma cell line (H4 cells) and a rat fibroblastic cell line (R2 cells) treated with vehicle (C) or vitamin D (D) for 24 hours, were incubated with increasing concentrations of MNase (0, 5, 10, 15, and 25 u/ml) for 5 minutes. The purified DNA samples were then digested with Apa I and analyzed by southern blot using probe 2 (figure 3.1).
as a nucleosome. We observed that in ROS 17/2.8 chromatin the region between -310 to -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 the proximal nuclease hypersensitive site (-148) in the OC gene promoter (figure 3.13A). In addition, we observed cleavage at a Sst I site (-309, figure 3.13B) which is proximal to a MNase sensitive site (-310, figures 3.6A and 3.10A) present in both chromatin and naked DNA. This cleavage by Sst I indicated that a nucleosome may be positioned downstream of -309.

To further examine the chromatin organization of the 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, chapter 2) to verify that this sequence was represented as a nucleosomal particle. ROS 17/2.8 nuclei were extensively digested with MNase. Solubilized nucleoparticles were isolated and their integrity was confirmed by agarose and polyacrylamide gel electrophoresis. Subsequently the DNA was isolated and analyzed by Southern blot using probe 6 (-343 to -145, figure 3.1) for hybridization, which specifically recognizes sequences from the OC gene promoter (figure 3.13C, lane 1). This probe principally detected a 145 bp band, which corresponds in size to mono nucleosomal DNA (Simpson 1991). The signal was observed after 10 minutes of digestion, peaked at 30 minutes, and was still detectable, although significantly reduced, after 50 minutes of incubation. These results strongly support our previous observations and together 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.
Figure 3.13. The 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 u/ml), then cleaved with Apa I, electrophoresed in a 2% agarose gel, blotted, and hybridized with probe 5 (figure 3.1). M1 and M2 correspond to ROS genomic DNA digested with Pvu II-Apa I and Pst I-Apa I respectively (figure 3.1). B) Purified samples from nuclei digested with Sst I (500 u/ml) were cleaved with Apa I and hybridized with probe 2 (figure 3.1). Lane M represents ROS genomic DNA cleaved with Sst I-Apa I.
Figure 3.13 C (continued). Following digestion of ROS 17/2.8 nuclei with MNase, soluble nucleoparticles were isolated at different digestion periods, the DNA purified, electrophoresed in a 2% agarose gel, blotted, and hybridized with probe 6 (figure 3.1). Lanes 2-9 (shown at the top) correspond to 0, 5, 10, 15, 20, 30, 40, and 50 minutes of digestion, respectively. Lane 1 represents ROS genomic DNA cleaved with Apa I. Lambda DNA digested with Hind III, and pUC 19 digested with Hinf I were used as DNA size markers (M).
Introduction.

Packaging of DNA sequences in nucleosomes and higher-order chromatin structures has been implicated in the regulation of key events in eukaryotic cells such as replication and transcription. It has been widely accepted that the presence of nucleosomes modulate accessibility of specific transcription factors to their cognate binding sequences. Moreover, gene activation is often accompanied by perturbations of the nucleosomal array which are indicated by increased nuclease hypersensitivity over specific promoter and enhancer elements (Elgin 1988, Gross et al. 1988). Multiple studies, most of them in vitro, have shown that there is an intrinsic difference in the nucleosome binding capacity of transcription factors. While some of them can not bind to their sites if assembled into nucleosomes (Taylor et al. 1991, Archer et al. 1992, Wechsler et al. 1994), others can specifically recognize and interact with their binding sequences although with different degrees of affinity (Taylor et al. 1991, Archer et al. 1992, Wechsler et al. 1994, Chen et al. 1994a and b, Imbalzano et al 1994). Furthermore, Workman and colleagues have recently shown that there is an inherent cooperativity between transcription factors when binding to adjacent sites on nucleosomes (Adams et al. 1995). This cooperative binding does not seem to be due to the sliding of the histone octamer after the interaction of the first factor. Instead it is
likely a result of minor alterations in the core particle structure induced by this first binding which facilitates the interaction of additional activators to adjacent sites on the nucleosome. In addition, recent work has described interaction of transcription factors with nucleosomal sequences in vivo. McPherson et al (1993) have shown that the serum albumin enhancer exists in a liver-specific array of precisely positioned nucleosomes which allow the binding of transcription factor HNF-3 to a site on the surface of these arrayed nucleosomes. Also, Truss et al (1995) have observed simultaneous ligand-dependent interaction of the progesterone receptor, NF-1 and OCT-1 to binding sites on the surface of a rotationally phased nucleosome in the mouse mammary tumor virus (MMTV) promoter stably integrated in the chromosome of mouse cells. The authors indicate that this nucleosome is neither removed nor shifted upon hormone induction. Moreover, they suggest that since these factors are unable to bind simultaneously to free DNA, maintainance of the nucleosome could be a requirement for binding of factors to contiguous sites. These results are in contradiction with previous reports which suggested that this nucleosome is displaced following ligand-dependent interaction of the glucocorticoid receptor (Archer et al. 1992). Taken together these observations indicate that, although nucleosomes positioned over specific promoter sequences can be inhibitory to transcription factor binding, certain factors are capable of organizing nucleosomal structures that define active elements.

Histones are subject to several post translational modifications (van Holde 1988). Among these, core histone acetylation has been studied extensively. Acetylated nucleosomes are known to be concentrated at transcriptionally active loci (Hebbes et al. 1988, Ebralidse et al. 1993). It has been suggested that core histone hyperacetylation enhances transcription factor binding to nucleosomes (Lee et al.
One possible mechanism is that the basic histone n-terminal domain interacts with nucleosomal DNA preventing binding of transcription factors. Neutralization of the lysine residues within this domain by acetylation can decrease their affinity for DNA thus alleviating the inhibitory effect. Alternatively, acetylation may directly alter the conformation of the core particle thus facilitating accessibility to transcription factors (Ura et al. 1994, Turner 1993). Moreover acetylation has been found to induce changes in the linking number per nucleosome (Norton et al. 1989).

The osteocalcin gene (OC) encodes a 10 kDa bone-specific protein. Its expression is induced with the onset of mineralization during osteoblast differentiation (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 which independently or in combination contribute to gene transcription. These include the OC box (-99 to -76) which contains sequences that exhibit recognition by members of the MSX homeodomain family of proteins (Hoffman et al. 1994, Towler et al. 1994 a and 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 the OC gene transcriptional control (Tamamura et al. 1994). Three AML-related sites (site A: -604 to -599, site B: -440 to -435, site C: -138 to -130) have been recently identified by functional and biochemical analysis. These sites appear to be recognized by a bone-specific protein which can be detected principally as a component of the nuclear matrix fraction (Bidwell et al. 1993, Merriman et al. 1995, Banerjee et al. submitted). The vitamin D responsive element (VDRE, -465 to -437) represents the key component of steroid hormone-mediated transcriptional control enhancement (Markosse et al. 1990, Demay et al. 1990, Terpening et al. 1990).
1991). It has been shown that ligand-dependent binding of the vitamin D receptor in vivo is directly correlated with transcriptional upregulation (Breen et al. 1994).

ROS 17/2.8 osteosarcoma cells have retained some of the principal characteristics exhibited by normal diploid osteoblasts in vivo such as steroid hormone responsiveness and synthesis of the bone-specific OC protein. We have shown the presence of DNase I hypersensitive sites at the OC gene promoter, spanning proximal (-170 to -70) and distal (-600 to -400) transcriptional elements in ROS 17/2.8 cells (Figure 3.2 and 3.3, Montecino et al. 1994) as well as in differentiated normal diploid osteoblasts (Figure 3.8, Montecino et al. submitted). This DNase I hypersensitivity is a function of transcriptional activity since they were not detected in non osseous cells or in bone-derived cells not expressing OC, and their intensity was increased with upregulation of transcription post proliferatively and following vitamin D treatment (Montecino et al. 1994). Here we determined that a brief exposure of ROS 17/2.8 cells to sodium butyrate, an inhibitor of histone deacetylases, alters the chromatin organization of the OC gene promoter and affects vitamin D-dependent transcriptional upregulation.

Results.-

Chromatin hyperacetylation affects DNase I hypersensitivity and vitamin D-dependent transcriptional upregulation of the OC gene.

Extensive analysis of chromatin structure has indicated that most active genes contain DNase I hypersensitive regions. These domains generally reflect
Figure 4.1. Effect of butyrate treatment on the DNase I hypersensitivity pattern of the osteocalcin gene promoter. Nuclei isolated from ROS 17/2.8 cells, treated as a control or with 10 mM butyrate for 1, 2, 4, and 10 hours (A), or with butyrate and vitamin D for 4 hours (B), were incubated with increasing concentrations of DNase I. The purified DNA was then completely cleaved with BamH I, electrophoresed in a 1.2% agarose gel, blotted, and hybridized with probe 1 (figure 3.1). The position of the proximal (pDHS, -170 to -70) and distal (pDHS, -600 to -400) DNase I hypersensitive sites are marked on the left.
alterations in the classical nucleosomal organization and the binding of specific nuclear factors (Elgin 1988, Gross et al. 1988). Hyperacetylation can be induced in vivo by treatment of cells with sodium butyrate, an inhibitor of histone deacetylases (Boffa et al. 1978, Sealy et al. 1978). We studied the effect of butyrate treatment on the chromatin organization of the OC gene in ROS 17/2.8 osteosarcoma-derived cells by analyzing DNase I hypersensitivity detected by the indirect end-labeling method (Wu 1980). As shown in figure 4.1, we found that very short incubations with butyrate caused a marked change in the DNase I hypersensitive pattern normally observed in the promoter of the OC gene in bone-derived cells expressing OC. The distal hypersensitive site (-600 to -400) which includes the VDRE as well as other important protein binding sequences, became almost undetectable after only two hours of incubation and disappeared completely following four hours of butyrate treatment. On the other hand, the proximal DNase I hypersensitive site was not significantly affected by butyrate treatment even after ten hours. Simultaneous addition of vitamin D and butyrate to ROS 17/2.8 cells, pretreated or not with butyrate alone, did not prevent the alterations in the DNase I hypersensitivity pattern (figure 4.1B). Furthermore it prevented the vitamin D-dependent enhancement in nuclease hypersensitivity previously reported (Montecino et al. 1994).

When the effect of this short butyrate exposure on the OC transcriptional activity was measured by nuclear run on analysis, we found that it did not have a significant impact on basal levels of OC expression. Instead, vitamin D-dependent upregulation of OC gene transcription was completely inhibited (figure 4.2). Taken together, these results indicated that a short incubation of ROS 17/2.8 cells with butyrate which leads to histone hyperacetylation, induces dramatic changes in the chromatin organization of the rat OC gene promoter and might prevent of vitamin D-
Figure 4.2. Effect of butyrate treatment on the osteocalcin gene transcriptional activity. Confluent ROS 17/2.8 cells were incubated with 10 mM butyrate for 4 hours, in the absence (B) or the presence (B+D) of vitamin D, and the osteocalcin gene transcriptional activity was measured by nuclear run on. These values represent the effect obtained in three independent experiments.
dependent transcriptional upregulation.

**Chromatin hyperacetylation inhibits specific nucleosome transition in the OC gene promoter.**

To determine modifications in the nucleosomal organization of the OC gene promoter region which could explain the changes in the DNase I hypersensitivity pattern observed following butyrate treatment, we digested nuclei isolated from confluent ROS 17/2.8 cultures, treated or not with butyrate, with micrococcal nuclease (MNase) and several restriction endonucleases. MNase has been shown to cleave chromatin primarily between nucleosomes while restriction enzyme activity is markedly reduced when recognition sequences are organized into nucleosomes (Simpson 1991).

When MNase cleavage sites within the OC gene promoter were mapped by indirect end-labeling from 5' and 3' ends, we observed a similar digestion pattern in control and butyrate treated cells (figures 4.3, A and B). Cleavage sites at -650, -560, -360, -310, -160, -90, and -50 were found in both samples, indicating that no significant change in the translational positioning of this nucleosomal array had been induced by butyrate treatment. In contrast, a marked decrease in cleavage by HincII over the distal region of the OC gene promoter (-529) was detected in the DNA samples from butyrate treated cells (figure 4.4). Interestingly, this cleavage site is located within the distal DNase I hypersensitive site (-600 to -400) which is lost following incubation with butyrate (figure 4.1). Cleavage in other regions of the OC gene promoter by Bgl II (-343) and Pst I (-145) was not affected in the treated DNA samples. Moreover, no effect on the inaccessibility to a second Hinc II site (+657, see figure 3.1) was
Figure 4.3. Butyrate treatment does not alter the translational positioning of nucleosomes on the osteocalcin gene promoter. Nuclei isolated from ROS 17/2.8 cells, control or treated with 10 mM butyrate for 4 hours, were digested with MNase for 5 minutes at 18 °C. The position of the distal (dDHS) and proximal (pDHS) hypersensitive sites is indicated on the left. The MNase cleavage positions are indicated on the right. A) Purified DNA samples digested with Apa I and hybridized with probe 2 (figure 3.1). Lanes 1, 5, and 9=0 u/ml; lanes 6 and 10=5 u/ml; lanes 7 and 11=10 u/ml; lanes 8 and 12=15 u/ml; lanes 2, 3, and 4 correspond to 0.5, 0.7, and 1.0 u/ml, respectively.
### Table A

<table>
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<td>9-12</td>
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- **1.38 Kb**
- **dDHS**
- **pDHS**

Markers:
- **-650**
- **-560**
- **-350**
- **-310**
- **-160**
- **-90**
- **-50**
Figure 4.3 B (continued). Samples were digested with Hinc II and hybridized with probe 5 (figure 3.1). Lanes 1, 6, and 11 = 0 u/ml; lanes 2 and 7 = 5 u/ml; lanes 3 and 8 = 10 u/ml; lanes 4 and 9 = 15 u/ml; lanes 5 and 10 = 25 u/ml; lanes 12, 13, 14, and 15 correspond to 0.5, 0.7, 1.0, and 1.3 u/ml, respectively.
Figure 4.4. Effect of butyrate treatment on the restriction endonuclease accessibility at the osteocalcin gene promoter. Nuclei from confluent ROS 17/2.8 cells were incubated with 500 u/ml of the restriction endonucleases Hinc II, Bgl II, and Pst I for 30 minutes at 37 °C. The purified DNA samples were then analyzed by Southern blot. The cleavage positions over the osteocalcin gene promoter are shown on the left. The percentage of accessibility for each enzyme under the different experimental conditions is shown at the bottom. A) The samples were digested with Apa I and hybridized with probe 2 (see figure 3.1). M corresponds to ROS genomic DNA digested with either Hinc II, Bgl II, or Pst I.
<table>
<thead>
<tr>
<th></th>
<th>Hinc II</th>
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<td>-</td>
<td>-</td>
<td>+</td>
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- 1.38 kb
- -529
- -343
- -145

| % Digestion | 50 | 12 | 74 | 8  | 45 | 47 | 48 | 51 | 69 | 61 | 106 | 56 |
**Figure 4.4 B (continued).** The samples were digested with BamH I and hybridized with probe 1 (see figure 3.1). M corresponds to ROS genomic DNA cleaved with Hinc II.
### Table: Hinc II Digestion Results

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<tr>
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<tr>
<td>+</td>
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### Diagram:

- **3.4 Kb**
- **-529**
- **+657**

### % Digestion Table:

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<th>51</th>
<th>10</th>
<th>71</th>
<th>11</th>
<th>/</th>
</tr>
</thead>
</table>
observed (figure 4.4B).

From these experiments we conclude that a key transition in the nucleosome located over the distal portion of the OC gene promoter is required for both formation of the distal DNase I hypersensitive site and subsequent ligand-dependent binding of the vitamin D receptor. Butyrate treatment appears to prevent such a transition, most likely by altering important protein-DNA interactions over this region.

**Butyrate treatment does not alter the level of vitamin D receptor and other important transcription factors that interact with the distal promoter.**

The distal DNase I hypersensitive site includes important transcriptional elements which interact with at least three different factors. The VDRE (-466 to -437) has been shown to be recognized by the vitamin D receptor in a ligand-dependent manner (Breen et al. 1994). There are two binding sequences for NMP-2, a bone-specific protein that was initially identified as a component of the nuclear matrix fraction (Bidwell et al 1993), and which has been recently shown to be an AML-related protein (Merriman et al. 1995, Banerjee et al. Submitted). Interestingly, these binding sites (site A: -604 to -599, and site B: -440 to -435) flank the limits of the distal DNase I hypersensitive sites (-600 to -400). In addition a binding site for NMP-1 (Bidwell et al. 1993), a ubiquitous transcription factor which can partition between the soluble and the nuclear matrix fractions, is located at -450 to -443 (Bidwell et al. 1993). This NMP-1 protein has been recently identified as YY-1 (Guo et al. in press). Interestingly, this binding site partially overlaps with one of the VDRE half elements, providing evidence for a potential mutually exclusive binding mechanism (Guo et al. manuscript in
Figure 4.5. Butyrate treatment does not affect transcription factor levels.

To evaluate the levels of specific DNA binding proteins which interact with the distal region of the osteocalcin (OC) gene promoter nuclear extracts were prepared from ROS 17/2.8 cells, treated with 10 mM sodium butyrate for 4 hours in the presence or absence of vitamin D as indicated on the top of each gel. These extracts were then analyzed by gel mobility shift assay. A) Probe: OC VDRE oligonucleotide (Owen et al. 1993). Lane 5 shows self competition with a 100 fold molar excess of cold OC VDRE oligonucleotide.
A

VDRE probe

<table>
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\[ \text{VDR/RXR} \]
**Figure 4.5 B (continued).** Probe: AML oligonucleotide (Merriman et al. 1995). Lane 5 shows self competition with a 100 fold molar excess of unlabeled AML oligonucleotide. Lane 6 shows no competition by a non-specific unlabeled oligonucleotide.
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B

AML probe

![Image of gel electrophoresis with AML marker]
Figure 4.5 C (continued). Probe: YY-1 oligonucleotide (Guo et al. in press). Lane 5 shows specific competition with a 100 fold molar excess of unlabeled YY-1 oligonucleotide.
### YY-1 probe

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</table>

![Diagram with YY-1 probe results]
Butyrate and its analogs are well established inducers or repressors of differentiation in several systems (Cook et al. 1985, McCue et al. 1984). The mechanism may involve regulation in levels of expression of key transcription factors. To evaluate the level of transcription factors which interact with the OC gene distal promoter region, we isolated nuclear extracts from ROS 17/2.8 cells, control and treated with butyrate alone or in combination with vitamin D, and performed gel electromobility shift assays using oligonucleotides which included the OC gene VDRE (Owen et al. 1993), the YY-1 binding element (Guo et al. in press), and the AML binding sequence (Merriman et al. 1995). The results are shown in figure 4.5, and demonstrate that the levels of these three transcription factors are unaffected by treatment with butyrate. Especially important is that the ligand-dependent formation of the VDR-RXR complex (Shakoori et al. 1994) is not inhibited in nuclear extracts from butyrate treated ROS 17/2.8 cells. These results indicate that both the absence of the distal DNase I hypersensitive site and the lack of vitamin D-dependent transcriptional upregulation of the OC gene are not due to a butyrate-mediated decrease in the level of expression of important transcription factors which interact within this region of the promoter.

Alteration of key protein-DNA interactions in the distal region of the OC gene promoter in ROS 17/2.8 cells incubated with butyrate.

To determine if the loss of distal DNase I hypersensitivity in the OC gene promoter was due to alterations in specific protein-DNA interactions induced by treatment of ROS 17/2.8 cells with butyrate, we combined intranuclear footprinting and
ligation-mediated PCR (LMPCR) analysis. This approach has been successfully utilized to study tissue-specific protein-DNA interactions in vivo, and it has the power to reveal interactions on nucleosomal surfaces (McPherson et al. 1993, Truss et al. 1995).

We detected large footprinted areas among nuclear DNase I-digested DNA samples from both control and vitamin D treated ROS 17/2.8 cells (figure 4.6A). These protected segments include the previously described binding elements for NMP-1 (Bidwell et al. 1993), recently identified as YY-1 (Guo et al. in press), NMP-2B (-440 to -435), a member of the AML family of transcription factors (Merriman et al. 1995), and for the VDR-RXR complex (-465 to -437, Markose et al. 1990, Demay et al. 1990, Terpening et al. 1991, Breen et al. 1994). Strikingly, these footprints were not detected in samples from ROS 17/2.8 cells incubated with butyrate alone (figure 4.6A, lanes 4 and 5) or together with vitamin D (figure 4.6A, lanes 3 and 4). Furthermore, these samples revealed a prominent 10 bp periodicity of DNase I cleavage sites (figure 4.6A, marked with arrowheads) characteristic of rotationally phased nucleosomal DNA (Thomas et al. 1988, Truss et al. 1995). Interestingly, a similar 10 bp DNase I cleavage pattern was observed in samples from ROS 24/1 cells (figure 4.6B), an osteosarcoma cell line derived from the same tumor as ROS 17/2.8 cells (Rodan et al 1990), but which expresses neither osteocalcin nor vitamin D receptor. These results indicate that treatment with butyrate inhibits a key transition of the chromatin organization in the distal domain of the OC gene promoter. This transition may involve the binding of specific transcription factors and the formation of the distal DNase I hypersensitive site which in turn presets the subsequent ligand-dependent binding of the VDR-RXR complex. The enhanced DNase I hypersensitivity observed in the ROS 17/2.8 butyrate treated samples (figure 4.6A, lanes 3 and 4) compared
**Figure 4.6. Butyrate treatment abrogates protein-DNA interactions in vivo in the distal region of the osteocalcin gene promoter.** A) Nuclei isolated from ROS 17/2.8 cells, treated as indicated at the top of the figure, were digested with increasing concentrations of DNase I. The purified genomic DNA samples were then analyzed by LMPCR. The nucleotide positions are indicated at both sides. Footprints encompassing binding elements for NMP-1 (YY-1), NMP-2B (AML) and the VDR-RXR complex are shown. The different experimental conditions are indicated at the top of the figure.
<table>
<thead>
<tr>
<th>Free DNA</th>
<th>Ros 17/2.8</th>
<th>Vitamin D</th>
<th>Butyrate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

**A**

- NMP-1/YY-1
- NMP-2B/AML

**VDR/RXR**

-407
-416
-426
-430
-441
-444
-454
-466
-476
Figure 4.5 B (continued) Nuclei isolated from ROS 24/1 cells, not expressing osteocalcin, were digested with DNase I and the purified DNA samples analyzed by LMPCR. The periodical cleavage by DNase I every 10 base pairs is indicated on the right.
<table>
<thead>
<tr>
<th></th>
<th>Free DNA</th>
<th>ROS 24/1</th>
<th>DNase I</th>
</tr>
</thead>
<tbody>
<tr>
<td>-486</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-475</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-463</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-454</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>-444</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>-436</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-426</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-416</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-407</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
to those from ROS 24/1 cells (figure 4.6B) is consistent with the increased nuclease hypersensitivity shown by hyperacetylated nucleosomal cores (Simpson et al. 1978).
Chapter 5

Requirement of distal and proximal promoter sequences for chromatin organization of the OC gene in bone-derived cells


Introduction.

Chromatin structure remodeling of the osteocalcin (OC) gene promoter is a principal component in the regulation of transcriptional activity of this bone-specific gene. We have previously reported that in ROS 17/2.8 osteosarcoma cells as well as in differentiated normal diploid rat osteoblasts, both expressing OC, key promoter regulatory elements reside in two DNase I hypersensitive sites (see figure 3.2A). These two domains can be further resolved into four subbands, each representing the limits of the hypersensitive regions (figure 3.3). The proximal site (-170 to -70) includes binding sequences that specifically interact with basal transcriptional factors such as MSX (Hoffman et al. Towler et al. 1994a and b), Id (Tamamura et al. 1994), AP-1 (Banerjee et al. submitted), and NMP-2, recently identified as a bone-specific nuclear matrix associated protein (Bidwell et al. 1993, Merriman et al. 1995, Banerjee et al. submitted), and a member of the AML family of transcription factors. On the other hand, the distal hypersensitive domain (-600 to -400) contains the vitamin D-responsive element (VDRE, -465 to -437), which has been shown to interact with the VDR-RXRα complex in a ligand-dependent manner (Markose et al. 1990, Demay et al. 1990,
Terpening et al. 1991, Breen et al. 1994). Two additional NMP-2 sites (site A: -604 to -599, and site B: -440 to -435) have been identified within this domain and suggested to represent specific interaction points between the nuclear matrix and the promoter of the OC gene (Bidwell et al. 1993, Merriman et al. 1995).

In order to begin dissecting the components involved in both chromatin structure and transcriptional regulation of the OC gene, ROS 17/2.8 cells were stably transfected with a series of rat OC gene promoter-reporter constructs, containing progressive 5'-deletions (Frenkel et al. in press). These deletions (shown at the bottom of each figure) sequentially truncate the limits of the distal (-600 and -400) and proximal (-170 and -70) promoter DNase I hypersensitive domains. In addition, they eliminate sequences where key regulatory protein-DNA interactions have been shown to occur.

**Results.**

The first stably transfected cell line to be analyzed contained 15 copies (table 5.1) of the -1097OCAT construct (see materials and methods, chapter 2). These cells expressed high levels of CAT protein (Frenkel et al. in press) and showed a seven fold induction following the addition of vitamin D (table 5.1). Similar to the endogenous OC gene promoter, this transgene promoter exhibits two DNase I hypersensitive domains (figure 5.1A). A strong and broad proximal hypersensitive site spans important promoter sequences implicated in regulation of basal levels of transcription, and a less intense distal hypersensitive site, represented by a single subband is located at -400. This is in contrast to what was previously observed for the endogenous OC gene promoter (see figure 3.2 and 3.3), where the
Figure 5.1. Chromatin structure of the -1.097OCCAT transgene promoter.

Nuclei isolated from the ROS 17/2.8 stable cell line -1,097OCCAT were digested with increasing concentrations of DNase I for 10 minutes at 18 °C or with the restriction endonucleases Hinc II and Pst I (500 u/ml) for 30 minutes at 37 °C. A) The purified DNA samples were completely cleaved with Xba I, electrophoresed in a 1.2% agarose gel, blotted, and hybridized with a CAT gene probe. B) Hinc II and Pst I cleavage activity on the endogenous osteocalcin gene promoter was determined after digesting the samples with Apa I and hybridizing with probe 2 (see figure 3.1). The position of the two DNase I hypersensitive sites (proximal and distal) as well as the restriction cleavage sites are indicated at the sides. Restriction endonuclease accessibility, expressed as a percentage of digestion (see materials and methods, chapter 2) is shown below. In the lower diagram, the most relevant transcriptional elements are indicated. The filled box represents vector sequences and the open box the CAT gene. X=Xba I, H=Hind III, E=EcoR I.
distal hypersensitive site comprises two subbands at -600 and -400. This difference was further confirmed by comparing accessibility to the restriction endonuclease Hinc II, which has been shown to cleave at -529 in the endogenous OC gene (figure 5.1B). We observed that this restriction site was not cleaved in the transgene promoter (figure 5.1A). Because it is well established that DNA sequences packaged into nucleosomes show strong reduction in accessibility to restriction endonuclease activity (Archer et al. 1991, Simpson 1991), we suggest that in the -1097OCCAT transgene the DNA segment located upstream of -400 is organized as a nucleosome. These results also indicate that hypersensitivity at -600 may not be a requirement for vitamin D-dependent transcriptional enhancement (table 5.1).

A similar DNase I hypersensitive pattern was observed in the stable cell line -529OCCAT, carrying 14 copies of the transgene (figure 5.2A and table 5.1). These cells show high levels of basal transcription and a three fold transcriptional enhancement following vitamin D treatment (table 5.1). These results confirm that the hypersensitive site at -600 is not required for vitamin D inducibility, and at the same time suggest that sequences located around or upstream of -529 are important for full vitamin D responsiveness.

When the same analysis was performed in the cell line carrying the -343 OCCAT transgene (figure 5.3A), we observed the broad proximal DNase I hypersensitive domain with a similar intensity to that previously found in the -1097OCCAT and -529OCCAT transgenes (table 5.1). As expected, the transcriptional response to vitamin D is lost completely when the segment -529 to -343, containing the well established rat OC VDRE (Markose et al. 1990, Demay et al. 1990, Terpening 1992) is eliminated. This result indicates that the formation of the proximal nuclease
Figure 5.2. Chromatin organization in the -529OCCAT transgene promoter. Nuclei isolated from the ROS 17/2.8 cell line stably transfected with the -529OCCAT transgene, were digested with DNase I and the restriction endonuclease Pst I as described in figure 5.1. A) The DNA samples were completely cleaved with BamH I and Xmn I, electrophoresed in a 1.2% agarose gel, blotted, and hybridized with a probe directed against the CAT gene coding region. B) Cleavage by Pst I on the endogenous osteocalcin gene promoter. DNA samples were completely digested with Apa I and hybridized with probe 2 (see figure 3.1). See figure 5.1 for an explanation of the symbols.
A
DNase I
3.26 Kb
- - + Vitamin D
pDHS
dDHS

B
1.38 Kb
- + Vitamin D
56 60 % Digestion

40 42 % Digestion

3.26 Kb

24
+1,673

-1,587

529

CAT
TATA box
NMP-2C
NMP-2B
VDR

-145

145
Figure 5.3. Formation of the proximal DNase I hypersensitive domain is independent of the distal promoter region. Nuclei from the ROS 17/2.8 cell line stably transfected with the -343OCCAT transgene were digested with DNase I and Pst I as described in figure 5.2. A) The purified DNA samples were completely digested with Xba I and hybridized with the CAT gene probe. B) Cleavage of the endogenous osteocalcin gene proximal promoter region by Pst I. In the lower diagram, X=Xba I, G=Bgl II, and H=Hind III.
hypersensitive domain in the OC gene promoter is independent of the distal hypersensitive domain, and implies that both basal transcriptional activity and proximal nuclease hypersensitivity, depend on protein-DNA interactions occurring within this region of the promoter. Moreover, when the DNA fragment -343 to -108 was eliminated (-108OCCAT, figure 5.4) no detectable hypersensitivity or significant basal transcriptional activity was observed (table 5.1). Interestingly, this construct contains important basal regulatory elements, such as the OC box (-99 to -76) and the TATA box (-44 to -31) which have been shown to be sufficient to express this transgene at high levels when transiently transfected in ROS 17/2.8 cells (Aslam et al. 1995, Frenkel et al. in press). These results indicate that in the chromatin context, sequences located within the -343 to -108 segment of the OC gene promoter are required for both formation of the proximal nuclease hypersensitive domain and for basal transcriptional activity. Very importantly, the presence of the NMP-2C binding element, which recognizes a tissue-specific protein, member of the AML family of transcription factors (Merriman et al. 1995, Banerjee et al. submitted), and was initially detected as a component of the nuclear matrix fraction (Bidwell et al. 1993), raises the possibility that interactions between the OC gene promoter and the nuclear matrix may be a requirement for tissue-specific expression.
Figure 5.4. The promoter segment -343 to -108 is required for the formation of the proximal DNase I hypersensitive domain. Nuclei isolated from a ROS 17/2.8 cell line carrying the -108OCCAT transgene were digested with increasing amounts of DNase I. After purification, the DNA samples were completely cleaved with BamH I and Apa I, electrophoresed in a 2% agarose gel, blotted, and hybridized with the CAT gene probe. In the lower diagram A=Apa I, O=Aos I, H=Hind III, and B=BamH I.
Table 5.1. Summary of relative expression of the OCCAT transgenes. The relative basal expression of the osteocalcin gene promoter transgenes, as well as the response to vitamin D treatment, were determined by evaluating CAT activity (Frenkel et al. in press). The relative expression values are listed with the activity of -529OC CAT set at 100%. These values represent an average of at least three independent determinations with minimal experimental variation (Frenkel et al. in press).
## Table 5.1
Summary of relative expression of the OC CAT transgenes

<table>
<thead>
<tr>
<th>Construct</th>
<th>Copy number</th>
<th>Relative basal expression</th>
<th>Vitamin D inducibility (fold induction)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-1,097 OCCAT</td>
<td>15</td>
<td>84 %</td>
<td>7</td>
</tr>
<tr>
<td>-529 OCCAT</td>
<td>14</td>
<td>100 %</td>
<td>3</td>
</tr>
<tr>
<td>-343 OCCAT</td>
<td>9</td>
<td>37 %</td>
<td>0</td>
</tr>
<tr>
<td>-108 OC CAT</td>
<td>4</td>
<td>13 %</td>
<td>0</td>
</tr>
<tr>
<td>CAT</td>
<td>3</td>
<td>7 %</td>
<td>0</td>
</tr>
</tbody>
</table>
Chapter 6

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 (Elgin 1988, Gross et al. 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 in both 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 to -70 and -600 to -400 (figure 6.1), where the principal basal and steroid responsive enhancer elements reside (Bortell et al. 1992, Breen et al. 1994, Demay et al. 1990, Markose et al. 1990, Owen et al. 1990b and 1993, Terpening et al. 1991, Hoffman et al. 1994, Towler et al. 1994 a and 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 (figure 3.2), 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. The relationship between increased hypersensitivity and and vitamin D-mediated transcriptional upregulation has been well established (Montecino
Figure 6.1. Nucleosomal organization of the osteocalcin gene in bone-derived cells. The schematic representation summarizes results presented in this thesis and indicate nucleosome remodeling associated with transcriptional activity of the osteocalcin gene. The filled circles represent putative nucleosomes (N1 and N2) spanning the promoter region of this 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.
MNase cleavage

ROS 17/2.8
Mineralized ROB

Restriction endonuclease cleavage

ROS 24/1
Proliferating ROB
et al. 1994). In situ hybridization analysis demonstrated that although a few cells in untreated cultures had high levels of osteocalcin mRNA, all cells in the culture showed some level of osteocalcin mRNA expression. Following vitamin D treatment an increased expression in all cells throughout the culture was observed.

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 of -310) promoter regions of the OC gene (figure 6.1). This chromatin organization of the OC gene promoter is supported by 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 24/1 cells as well as in proliferating and mineralizing normal diploid osteoblasts (Hoffman et al. 1994 and submitted). 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 (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 6.1). This conclusion is based on the digestion patterns obtained in both cell types, and in the protection from specific restriction endonuclease cleavage exhibited by this segment in chromatin (figure 3.13, A and B). In addition, we isolated this DNA fragment as part of a nucleoprotein particle containing 145 bp of DNA following extensive MNase digestion (figure 3.13C). 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 and create 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. 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. Recent reports have suggested that the molecular mechanism of chromatin remodeling may not necessarily involve nucleosome disruption (Truss et al. 1995, Wall et al. 1995). It is possible to describe it as nucleosome sliding. Work from at least two independent laboratories has determined that nucleosomes in principle can move or slide on DNA (Wall et al. 1995, Meersseman et al. 1994), in the absence of any interacting protein. Accordingly, nucleosomes that occupy factor binding sites can be relocated by the interacting factor.
to an adjacent position, sometimes close enough that MNase can not cleave in between the two. Thus transcription factors may take advantage of these nucleosome movements to integrate themselves into chromatin. Once integrated, these proteins function as boundaries that redirect nucleosomes positions. Bradbury and colleagues (Pennings et al. 1994) have reported that nucleosome movements along DNA can be restricted by the presence of histone H1, which binds to the nucleosomal linker DNA. In addition, Kadonaga and coworkers (Laybourne et al. 1991), have observed that the binding of histone H1 enhances transcriptional repression by nucleosomes. Furthermore, in vivo histone H1 is found to be significantly depleted from active genes (Bresnick et al. 1992) and from unmethylated CpG islands, which are normally located in the promoter regions of transcriptionally active genes (Tazi et al. 1990). We and others have identified transcriptionally active elements within the proximal promoter region, which independently or in combination influence OC gene expression. Among these is the NMP-2C site (-138 to -130) which binds to a bone-specific protein complex, initially determined as a component of the nuclear matrix fraction (Bidwell et al. 1993), and recently identified as a member of the AML family of transcription factors (Merriman et al. 1995). This protein-DNA interaction has been shown to be critical for transcriptional activity (Banerjee et al. submitted). Another element, the OC box (-99 to -76) interacts with MSX/Hox proteins, members of the homeodomain family of transcription factors (Hoffman et al. 1994, Towler et al. 1994 a and b). In addition, an E-box (-102 to -97) which binds Id, a helix-loop-helix protein has been implicated in OC gene transcriptional control (Tamamura et al. 1994). The importance of these protein-DNA interactions was partially addressed in chapter 5. We observed that the promoter segment -343 to -108 was critical for inducing both proximal nuclease hypersensitivity (figures 5.3 and 5.4) and basal transcriptional activity (table 5.1). Although it is still premature to conclude that the NMP-2C binding element could be responsible for
such an effect, we can tentatively speculate that specific interactions between the OC gene promoter and the nuclear matrix may be providing the basis for the tissue-specific expression of this gene.

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). The recent demonstration of interactions between the vitamin D receptor and the TATA box binding protein factor TFIIB in vivo is consistent with such reasoning (Blanco et al. 1995, MacDonal et al. 1995). A similar three-dimensional model has been proposed for the Drosophila heat-shock hsp 26 gene (Elgin 1988, Lu et al. 1992 and 1993). Here chromatin organization supports the interaction of two spaced, temperature-induced protein-DNA complexes, each involving DNase I hypersensitive sites. The placement of a nucleosome between the two hypersensitive domains is proposed to provide a conformation that is competent for interactions of cognate promoter binding factors.

We examined the role of histone acetylation in the chromatin organization and transcriptional activity of the OC gene by studying the effect of sodium butyrate, an inhibitor of histone deacetylases (Boffa et al. 1978, Sealy et al. 1978), in ROS 17/2.8 cells (figure 6.2). It was found that very short incubations with this compound reversibly blocked the formation of the distal DNase I hypersensitive site (figure 4.1 and data not shown). This was accompanied by inhibition of vitamin D-
Figure 6.2. Histone hyperacetylation affects nucleosomal remodeling at the osteocalcin gene promoter. This diagram illustrates the alterations in the chromatin structure of the osteocalcin gene promoter induced by treatment of ROS 17/2.8 cells with sodium butyrate. See figure 6.1 for an explanation of the symbols.
MNase cleavage

Control

Restiction endonuclease cleavage

Butyrate
dependent transcriptional upregulation of the OC gene. Although no alterations in the translational positioning of nucleosomes over the OC gene promoter were detected by MNase digestion and indirect end-labeling analysis (figure 4.2), a drastic reduction in cleavage by the restriction endonuclease Hinc II was observed (figure 4.3). Additionally, by combining intranuclear footprinting and LMPCR analyses, we determined that large footprinted areas, present in both control and vitamin D-treated cells, which represent protein-DNA interactions occurring in this region of the promoter, were not detected in samples from ROS 17/2.8 cells incubated with butyrate (figure 4.6A). Furthermore, we determined that the butyrate treated samples presented a DNase I cleavage periodicity of about 10 bp, which corresponds to the pattern exhibited by nucleosomal DNA (Thomas et al. 1988, Truss et al. 1995). Interestingly, a similar pattern is obtained when DNase I-digested samples from ROS 24/1 osteosarcoma cells are analyzed (figure 4.6B).

The absence of protein-DNA interactions over the distal promoter domain of the OC gene in the butyrate treated cells was not due to a decrease in the levels of the transcription factors which normally interact within this region of the promoter. Similar DNA-binding activities for the VDR-RXRα complex, AML-related proteins, and the transcription factor YY-1 were observed by gel mobility shift assays (figure 4.5). Taken together these results suggest that treatment with butyrate inhibits the nucleosomal transition involved in the formation of the distal DNase I hypersensitive site. This transition could be induced by the binding of the transcription factors such as YY-1 (NMP-1, Guo et al. in press) and/or AML (NMP-2B, Merriman et al. 1995), and appears to be a requirement for the subsequent ligand-dependent interaction of the VDR-RXRα complex (Breen et al. 1994).
Nucleosome exclusion has also been proposed as a mechanism to regulate accessibility of transcription factors to promoter and enhancer elements in chromatin (Owen-Hughes et al. 1994). This mechanism involves perturbations of chromatin structure during DNA replication, which provides a window of opportunity for transcription factors to access DNA elements prior to chromatin assembly. Our laboratory has shown that confluent cultures of ROS 17/2.8 cells exhibit minimal levels of proliferative activity (van den Ent et al. 1993). Since all of our studies involved confluent cultures of ROS 17/2.8 cells, the contribution of a replication-dependent mechanism in the osteocalcin gene chromatin remodeling can only be considered as a less likely alternative. However future experiments involving DNA replication inhibitors should address this possibility.

A number of studies have shown that exposure to sodium butyrate enriches cell cultures in G1 cells, and at sufficient butyrate concentration for long periods of time, leads to G1 arrest. In addition, it has been reported that butyrate treatment alters the rate of cell cycle progression from G2 to mitosis and through S-phase by decreasing the rate of DNA synthesis (D'Anna et al. 1980). These pleiotropic effects described for butyrate treated cells should not influence significantly the interpretation of our results. We have observed that the alterations in the chromatin structure of the osteocalcin gene induced by butyrate treatment occur in confluent ROS 17/2.8 cultures after a very short exposure and in a completely reversible manner (Data not shown).

It has generally been assumed that binding of transcription factors to nucleosomal DNA is enhanced by acetylation (Owen-Hughes et al. 1994, Lee et al. 1993). It has been demonstrated that hyperacetylation induces subtle alterations in the
nucleosomal structure which may increase the accessibility to transcription factors (Ura et al. 1994). On the other hand, Hager and colleagues reported that butyrate treatment inhibited glucocorticoid hormone-dependent formation of a nuclease hypersensitive site and blocked transcriptional induction of the mouse mammary tumor virus (MMTV) long terminal repeat (Bresnick et al. 1990 and 1991). They also found that this inhibitory effect was not due to a major change in the structure of the MMTV chromatin, such as nucleosome unfolding. Instead they proposed that only minor chromatin modifications can alter the interaction of the glucocorticoid receptor with chromatin-associated recognition sites (Bresnick et al. 1991). In addition, earlier reports (McKnight et al. 1980) described a reversible inhibitory effect of sodium butyrate on the transcriptional activation of the estrogen receptor in chicken oviduct.

This apparent controversy can be explained if we consider that histone hyperacetylation may be inducing a slightly altered configuration of transcription factor binding elements relative to nucleosomes, which could result in enhancement or prevention of protein-DNA interactions on the hyperacetylated nucleosome. Alternatively, hyperacetylation could be altering the stability of nucleosomes such that the transcription factors can bind to the nucleosomal surface but can not initiate nucleosome disruption (Bresnick et al. 1990). On the other hand, based on genetic and in vitro evidence, Grunstein and coworkers have proposed that the histone H3 and H4 N-termini directly interact with the SIR3 and SIR4 proteins which function as repressors of the silent mating loci (HM loci) and telomeres in yeast (Hecht et al. 1995). Because these are the residues that are differentially acetylated in vivo, a regulatory role for this translational modification in transcriptional repression has been suggested (Grunstein 1990).
NF-1 and OCT-1 to binding sequences located on the surface of a rotationally phased nucleosome in the MMTV long terminal repeat segment stably integrated into mouse-derived cells. The authors indicated that this nucleosome is neither removed nor shifted following hormone induction, which appears to be in direct contradiction with previous reports from other laboratories (Archer et al. 1992).

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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