Mechanisms Contributing to Transcriptional Regulation and Chromatin Remodeling of the Bone Specific Osteocalcin Gene

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MECHANISMS CONTRIBUTING TO TRANSCRIPTIONAL REGULATION AND CHROMATIN REMODELING OF THE BONE SPECIFIC OSTEOCALCIN GENE

By

Soraya Gutiérrez Gallegos

Submitted to the Faculty of the University of Massachusetts Graduate School of Biomedical Sciences, Worcester in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

November 20, 2002
Department of Cell Biology
MECHANISMS CONTRIBUTING TO TRANSCRIPTIONAL REGULATION AND CHROMATIN REMODELING OF THE BONE SPECIFIC OSTEOCALCIN GENE

A Dissertation Presented

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Department of Cell Biology

November 20, 2002
Dedication

To my parents,

Blanca Gallegos & Jorge Gutiérrez
ACKNOLEDGEMENTS

I would like to thank Drs. Janet Stein, Gary Stein and Jane Lian for giving me the opportunity to become part of their research group. I am especially thankful for their guidance, support and encouragement through these years. Their commitment and dedication to science and their efforts to make each one of us feel at home are inspiring. In this regard I would also like to thank my mentors in Chile Drs. Maria Imschenetzky and Marcia Puchi; their friendship and unconditional support have been a constant since I met them during my undergraduate years.

I would also like to thank the members of my thesis committee for taking the time to evaluating and correcting this dissertation and for their very helpful suggestions during the development of this project.

During my stay at the Stein/Lian laboratories, I had the opportunity to meet and work with several people who in many ways have influence my life and my scientific career. Amjad Javed, my dearest friend, comes to my mind first. I would like to thank him for his support, scientific input and thorough discussions that helped me to think “out of the box”. I am also deeply thankful for various collaborations, especially in the generation and analysis of the stable cell lines that were a fundamental piece of my research work. I deeply appreciate the friendship of Kaleem Zaidi his sense of humor and his scientific input made my work a lot easier.

My deepest appreciation to Shirwin Pockwise not only for her superb scientific knowledge, but also for taking care of every single detail in the lab. always with a big
smile. Special thanks to Rosa Mastrototaro for her kindness and dedication that make our work much more pleasant.

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Last but not least, my heartiest thanks to Elizabeth Bronstein and Judy Rask for all their help. I am also in debt to Karen Concaugh for her help with the references and to Harold Hill for color prints.

All the dedication of this work goes to my parents, siblings, brother in law, nice and nephew for their love, care and encouragement.
ABSTRACT

Activation of tissue-specific genes is a tightly controlled process that normally involves the combined action of several transcription factors and transcriptional co-regulators. The bone-specific osteocalcin gene (OC) has been used as a prototype to study both tissue-specific and hormonal responsiveness. In this study we have examined the role of Runx2, VDR and C/EBP factors in the regulation of OC gene transcription. Contributions of the Runx and VDRE motifs to OC promoter activity were addressed by introducing point mutations within the context of the rat (-1.1 kb) osteocalcin promoter fused to a CAT-reporter gene. The functional significance of these mutations was assayed following transient transfection and after genomic integration in ROS 17/2.8 osteoblastic cell lines. Furthermore, we tested the effect of these mutations on the chromatin organization of the OC promoter. Our data show that all three Runx sites are required for maximal activation of the OC promoter and that the distal sites contribute significantly to the basal activity. Strikingly, mutation of the three Runx sites abrogates responsiveness of the OC promoter to vitamin D; this loss is also observed when only the Runx sites flanking the VDRE are mutated. Chromatin changes that result in the appearance of DNase I hypersensitive sites during activation of the OC gene are well documented. Mutation of the three Runx sites results in altered chromatin structure as reflected by absence of DNase I hypersensitive sites at the vitamin D response element and over the proximal, tissue-specific basal promoter. These data are consistent with the critical role of Runx2 in osteoblast maturation and bone development.

Mutation of the VDRE resulted in a complete loss of vitamin D responsiveness; however, this mutant promoter exhibited increased basal activity. The two DNase I
hypersensitive sites characteristic of the transcriptionally active OC gene in osteoblastics cells were not altered upon mutation of the VDRE element, although restriction enzyme accessibility in the proximal promoter region was decreased. We also found an increased level of histone H3 acetylation at the VDRE mutant promoter in comparison to the endogenous gene. Thus binding of VDR to OC promoter is required to achieve a normal transcriptional regulation and chromatin structure of the OC gene.

Although Runx2 is considered a master gene for bone development and osteoblast differentiation, it is noteworthy that osteoblast-specific transcription of the rat OC promoter occurs even in the absence of Runx sites. Therefore, other transcription factor(s) should be able to drive OC expression. We characterized a C/EBP enhancer element in the proximal promoter of the rat osteocalcin gene that resides in close proximity to a Runx element, essential for tissue-specific activation. We find that C/EBPβ or δ and Runx2 factors interact together in a synergistic manner to enhance OC transcription in cell culture systems. Mutational analysis demonstrated that this synergism is mediated through the C/EBP responsive element in the OC promoter and requires a direct interaction between Runx2 and C/EBPβ or δ.

Taken together, our findings strongly support a mechanism in which combinatorial interaction of Runx2, VDR, C/EBPβ or δ and probably other transcription factors are needed for regulating OC expression. In this process Runx factors not only act as simple transcriptional transactivators but also by facilitating modifications in promoter architecture and maintaining an active conformation of the target gene promoter.
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## INTRODUCTION

## RESULTS

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## DISCUSSION

## CHAPTER 5

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## INTRODUCTION

## RESULTS

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- C/EBP Proteins Activate The Osteocalcin Gene Through A C/EBP Responsive Element In The Proximal Promoter
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<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALP:</td>
<td>Alkaline Phosphatase</td>
</tr>
<tr>
<td>AML:</td>
<td>Acute Myeloid Leukemia</td>
</tr>
<tr>
<td>ANOVA:</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>AP-1:</td>
<td>Activating Protein 1</td>
</tr>
<tr>
<td>AR:</td>
<td>Androgen Receptor</td>
</tr>
<tr>
<td>ARC:</td>
<td>Activator Recruited Cofactor</td>
</tr>
<tr>
<td>BMP:</td>
<td>Bone Morphogenetic Proteins</td>
</tr>
<tr>
<td>BSP:</td>
<td>Bone Sialoprotein</td>
</tr>
<tr>
<td>C/EBP:</td>
<td>CCAAT/Enhancer Binding Protein</td>
</tr>
<tr>
<td>CAT:</td>
<td>Chloramphenicol Acetyltransferase</td>
</tr>
<tr>
<td>Cbfa:</td>
<td>Core Binding Factor alpha</td>
</tr>
<tr>
<td>CBP:</td>
<td>CREB Binding Protein</td>
</tr>
<tr>
<td>CDP:</td>
<td>CCAAT Displacement Protein</td>
</tr>
<tr>
<td>ChIP:</td>
<td>Chromatin Immuno-Precipitation</td>
</tr>
<tr>
<td>CMV:</td>
<td>Cytomegalovirus</td>
</tr>
<tr>
<td>COX-2:</td>
<td>Cyclooxygenase-2</td>
</tr>
<tr>
<td>CREB:</td>
<td>camp Response Element Binding Protein</td>
</tr>
<tr>
<td>CTD:</td>
<td>Carboxi-Terminal Domain</td>
</tr>
<tr>
<td>DEPC:</td>
<td>Diethylpyrocarbonate</td>
</tr>
<tr>
<td>DHS:</td>
<td>DNaseI Hypersensitive Sites</td>
</tr>
<tr>
<td>Dlx5:</td>
<td>Distal-less homeobox protein-5</td>
</tr>
<tr>
<td>DNA:</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>DNaseI:</td>
<td>Deoxyribonuclease I</td>
</tr>
<tr>
<td>DR:</td>
<td>Direct Repeat</td>
</tr>
<tr>
<td>DRIP:</td>
<td>Vitamin D-Receptor Interacting Proteins</td>
</tr>
<tr>
<td>ECM:</td>
<td>Extracellular Matrix</td>
</tr>
<tr>
<td>Gla:</td>
<td>γ-CarboxyGlutamic Acid</td>
</tr>
<tr>
<td>GR:</td>
<td>Glucocorticoid Receptor</td>
</tr>
<tr>
<td>HAT:</td>
<td>Histone Acetyl Transferase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>HDAC</td>
<td>Histone Deacetylase</td>
</tr>
<tr>
<td>HES-1</td>
<td>Hairy and Enhancer of Split 1</td>
</tr>
<tr>
<td>IFN β</td>
<td>Interferon β</td>
</tr>
<tr>
<td>IGF-1</td>
<td>Insulin-Like Growth Factor-1</td>
</tr>
<tr>
<td>Ip</td>
<td>Immunoprecipitation</td>
</tr>
<tr>
<td>IQR</td>
<td>Interquartile Range</td>
</tr>
<tr>
<td>LS</td>
<td>Least Square</td>
</tr>
<tr>
<td>MGP</td>
<td>Matrix Gla Protein</td>
</tr>
<tr>
<td>MMTV</td>
<td>Mouse Mammary Tumor Virus</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>NCoA62</td>
<td>Nuclear Receptor Coactivator, 62,000 Da</td>
</tr>
<tr>
<td>NF1</td>
<td>Nuclear Factor 1</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear Factor kappa B</td>
</tr>
<tr>
<td>NMTS</td>
<td>Nuclear Matrix Targeting Sequence</td>
</tr>
<tr>
<td>OC</td>
<td>Osteocalcin</td>
</tr>
<tr>
<td>OG</td>
<td>Osteocalcin Gene</td>
</tr>
<tr>
<td>OP</td>
<td>Osteopontin</td>
</tr>
<tr>
<td>ORG</td>
<td>Osteocalcin Related Gene</td>
</tr>
<tr>
<td>OSE</td>
<td>Osteoblast Specific Element</td>
</tr>
<tr>
<td>OSF</td>
<td>Osteoblast Specific Factor</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffer Saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PEBP</td>
<td>Polyoma Enhancer Binding Protein</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein Kinase A</td>
</tr>
<tr>
<td>PPARγ2</td>
<td>Peroxisome Proliferator-Activated Receptor γ</td>
</tr>
<tr>
<td>REML</td>
<td>Restricted Estimation by Maximum Likelihood</td>
</tr>
<tr>
<td>RHD</td>
<td>Runt Homology Domain</td>
</tr>
<tr>
<td>ROB</td>
<td>Rat Osteoblasts</td>
</tr>
<tr>
<td>ROS</td>
<td>Rat Osteosarcoma</td>
</tr>
<tr>
<td>RSB</td>
<td>Reticulocyte Saline Buffer</td>
</tr>
<tr>
<td>RSV</td>
<td>Raus Sarcoma Virus</td>
</tr>
</tbody>
</table>
RUNX: Runt Related Proteins
RXR: Retinoid X Receptor
SAGA: Spt-Ada-Gen5-Acetyltransferase Complex
SDS: Sodium Dodecyl Sulfate
SHE: Steroid Half Element
SRC: Steroid Receptor Co-activators
SSC: Sodium Chloride/Sodium Citrate Buffer
SWI/SNF: Switching Mating Type (SWI) or Sucrose Nonfermenting (SNF)
TF: Transcription Factor
TGF: Transforming Growth Factor
TLE: Transducin Like Enhancer
TR: Thyroid Hormone Receptor
TRAP: TR-Associated Protein
VDR: Vitamin D Receptor
VDRE: Vitamin D Response Element
WT: Wild Type
YY1: Yin Yang 1
CHAPTER 1

General Introduction

Proliferation
Day 6

Matrix Maturation
Day 12

Mineralization
Day 20

Osteocalcin
I. BONE BIOLOGY

1. Bone Tissue

Bone is a dynamic connective tissue that not only provides protection from external injury to the brain and spinal cord, but also houses the bone marrow, supports hematopoiesis, provides an ample source of calcium and phosphate and is essential for the respiratory process.

There are three major divisions of the vertebrate skeleton: craniofacial, axial and appendicular and each one of them is derived from distinct embryonic origins. The neural crest gives rise to the craniofacial skeleton, the sclerotome forms most of the axial skeleton, and the lateral plate mesoderm generates the appendicular skeleton. In regions of the craniofacial skeleton and the clavicle, differentiation into osteoblasts produces bone directly by a process known as intramembranous bone formation. In the remaining skeleton, differentiation into chondrocytes produces a framework of cartilage models (anlagen) of the future bones; these cartilage models are subsequently replaced by bone by the process of endochondral ossification [Mundlos and Olsen, 1997].

During skeletogenesis, four primary phases can be distinguished: (1) the migration of precursors cells to the location in the embryo where skeletal elements will develop, (2) the epithelial-mesenchymal interaction that leads to (3) characteristic mesenchymal condensations of high cell density and (4) the differentiation into osteoblasts or chondrocytes [Hall and Miyake, 2000].

Progressing from condensation to differentiation of cells identifiable as chondroblasts or osteoblasts requires down-regulation of genes controlling proliferation
and up-regulation of genes associated with differentiation. The patterns and molecular
regulation of mesenchymal cell differentiation are only superficially known. Cells that
participate in bone formation originate from pluripotent mesenchymal stem cells that
can differentiate to other phenotypes like myoblasts, adipocytes and fibroblasts (Fig.
1.1). Commitment of the mesenchymal stem cell to the different cell lineages is
believed to be determined by transcription factors that act as “master switches”
inducing the expression of a set of specific genes that characterize the muscle,
adipocyte, chondrocyte or osteoblast cell (Fig. 1.1).

In addition to osteoblasts (the bone forming cells), and chondroblasts (the
cartilage forming cells), there is a third specific cell type of the skeleton: the osteoclast
or bone-resorbing cell. Osteoclasts are derived from the hematopoietic stem cells, and
have a common differentiation pathway with the monocyte/macrophage lineage until
the final differentiation steps. Osteoclast progenitors are recruited to bone via blood
circulation. The activity and differentiation of osteoblasts and osteoclasts are closely
coordinated during embryonic development of the bone and during growth and adult
life as bone undergoes continuous remodeling.

2. The Osteoblast Phenotype

A) In Vivo Differentiation Of Osteoblasts

Based on morphological and histological studies, osteoblastic cells are
categorized in a presumed linear sequence progressing from osteoprogenitor to lining
cells or osteocytes. The progression of osteoblast maturation requires the sequential
activation and suppression of genes that encode phenotypic and regulatory proteins.
Figure 1.1 Cell lineages: Pluripotent mesenchymal stem cell can differentiate to originate different cell lineages triggered by the expression of “master genes”.
(Adapted from Borfmen H. and Czerniak B. “Molecular Biology of Bone Tumors” in Bone Tumors, 1998).
Signaling molecules like Bone-Morphogenetic Proteins (BMPs) and Transforming Growth Factors (TGFs) indirectly mediate a cascade of gene expression. Transcription factors and regulatory proteins that directly engage in protein-DNA and in protein-protein interactions are also important for the development of bone tissue and osteoblast differentiation (Fig. 1.2). The molecular and cellular processes involve in the transformation of the most primitive pluripotent cell to the undifferentiated multipotential cell and presumed osteoprogenitor are not understood. The stem cells that give rise to progeny for bone formation and tissue renewal can be identified only by biologic consequences.

The osteoprogenitor appears to have limited cell renewal capacity compared to the stem cell. The determined osteoprogenitor is recognizable in bone as a preosteoblast, a key feature of the preosteoblast population is its capacity to divide and increase the size of bone. Preosteoblasts are usually observed as one or two layers of cells behind the osteoblast near bone-forming surfaces; i.e., they are usually present where active mature osteoblasts are laying down a bone matrix (Fig. 1.3). These cells appear elongated, fibroblastic, or spindle shaped with an oval or elongated nucleus and with notable glycogen content. Preosteoblasts may express a few phenotypic markers of the osteoblast, e.g., alkaline phosphatase, but less than the mature osteoblast. Once the preosteoblast ceases to proliferate it will differentiate into a mature osteoblast. In vivo, several structural features characterize this osteoblast, including its size and cuboidal morphology, a distinguishing round nucleus, a strongly basophilic cytoplasm and a prominent Golgi complex. The osteoblast synthesizes and vectorially secretes most of the proteins that form the bone extracellular matrix (ECM). As the mature...
Figure 1.2 Growth and differentiation of osteoblast lineage cells: Progression through the osteoblast lineage from a pluripotent mesenchymal stem cell is regulated by numerous physiologic mediators, some of which are indicated in the diagram.
Figure 1.3 Differentiation of osteoblastic cells: A schematic representation of the mammalian growth plate is shown. The right side of the figure represents different stages of osteoblastic cells during differentiation and their relative location in the growth plate. (Adapted from Karaplis AC. "Embryonic Development of Bone and the Molecular Regulation of Intramembranous and Endochondral Bone Formation" in Bilezikian JP, Raisz LG, Rodan GA “Principles of Bone Biology” Second Edition, 2002 and Borfimen H. and Czerniak B. “Molecular Biology of Bone Tumors” in Bone Tumors, 1998)
osteoblast becomes surrounded by the mineralized matrix, the cell differentiates further into an osteocyte, which is considered the most mature or terminally differentiated cell of the osteoblast lineage.

B) In Vitro Differentiation Of Osteoblasts

A large number of cell culture models are currently available to study the expression of osteoblast-related genes in vitro. These include primary culture from calvaria or trabecular bone from several mammalian species, osteosarcoma-derived cell lines, and experimentally immortalized cells. Some of these in vitro models, especially the calvaria-derived cultures, undergo changes that mimic osteoblastic differentiation in vivo [Rodan and Noda, 1991].

Primary cultures of calvarial-derived rat osteoblasts develop a mineralized extracellular matrix with a bone tissue-like organization. A temporal pattern of gene expression reflecting progressive differentiation of the osteoblast has been mapped from the proliferating cell to the fully mature osteocyte in vitro [Bellows et al., 1986; Owen et al., 1990a]. The general profile of cell growth and bone phenotypic gene expression characterizes three major periods of cell and tissue development: proliferation, matrix maturation and mineralization (Fig. 1.4). Genes supporting cell cycle progression (e.g., histones), cell growth control (e.g., c-fos, c-jun) and ECM biosynthesis (e.g., collagen, fibronectin, TGF β) are expressed during the initial proliferation period. At the first key transition point, genes associated with the proliferation process are down regulated while genes related to maturation and organization of the bone ECM are induced (e.g., alkaline phosphatase, matrix Gla protein). At a second transition point, expression of genes associated with ECM
Figure 1.4 In vitro developmental stages of rat osteoblasts: Top panel: Fetal calvarial derived rat primary osteoblast cultures were grown for the indicated days and stained with toluidine blue to show actively proliferating cells (d6). Matrix maturation shows the alkaline phosphatase histochemistry indicating a post proliferative osteoblast culture with every cell stained positively for the enzyme. Mineralization of bone-like nodules is demonstrated by the von Kossa silver and alakaline phosphatase double stain. The large black masses indicate the mineral deposition and failure of red staining demonstrates the absence of alkaline phosphatase enzyme. Bottom panel shows Northern blot analysis of the temporal expression of osteoblast phenotype related genes during development of the osteoblast phenotype. Cessation of proliferation (day 1-6) leads to induction of phenotypic markers that characterize the subsequent postproliferative stages. The second stage (matrix maturation) is characterized by maximal levels of alkaline phosphatase, considered an early marker of the differentiated osteoblast. The maturation of osteoblast development (mineralization) is achieved concomitant with maximal levels of OC expression.
mineralization are upregulated (e.g., osteocalcin and osteopontin), as well as genes associated with the establishment and maintenance of the fully differentiated bone cell (e.g., collagenase).

3. The Osteocalcin Gene

Osteocalcin (OC) is a major noncollagenous protein product of the differentiated osteoblast, which forms the ECM. The protein is synthesized in a pre-pro form of 10 kDa; but it is secreted as a 5.6 kDa protein that accumulates in the bone ECM bound to mineral. It contains three residues of a calcium binding amino acid γ-carboxy glutamic acid (Gla), that are formed after protein translation by carboxylation of glutamic acid residues by a vitamin K and CO₂ dependent carboxylase enzyme complex. In the presence of calcium, the Gla residues allow specific conformational changes and promote osteocalcin binding to hydroxyapatite and subsequent accumulation in bone matrix [Hauschka et al., 1989].

The physiological role of osteocalcin is still unknown. It has been proposed that osteocalcin may act as inhibitor of hydroxyapatite deposition [Boskey et al., 1985] or it may have a role in mediating bone resorption [Lian et al., 1986; Glowacki and Lian, 1987; Lian et al., 1984]. Defective osteocalcin production has been observed in some humans and animals with osteopetrosis, a severely deforming disease characterized by the failure to remodel bone and calcified cartilage [Lian and Marks, 1990; Shalhoub et al., 1994; Bollerslev et al., 1994].

Both the human and the rat osteocalcin genes exhibit similar overall organization. The human gene is a single copy gene localized on chromosome 1 [Puchacz et al., 1989]. It has a size of ~1.2 kb with four exons that predict a protein of
125 amino acids, the signal peptide contains 26 amino acids in exon 1, a propeptide of 49 amino acids in exon 2 along with the γ-carboxylation recognition sequence, two stretches that become carboxylated in exon 3, and the remainder of the molecule and untranslated region in exon 4. Interestingly, in selected strains of rats and in mouse three osteocalcin related genes have been found, two of which (OG1 and OG2) are activated in bone and one (osteocalcin related gene ORG) in kidney [Desbois et al., 1994; Rahman et al., 1993]. Osteocalcin deficient mice are normal at birth, viable, fertile, have no skeleton patterning defects and no ectopic bone formation. The expression of noncollagenous proteins such as osteopontin (OP), matrix gla protein (MGP) and bone sialoprotein (BSP) is not significantly affected by the absence of osteocalcin. Over time the mutant mice develop a phenotype marked by higher bone mass and bones of improved functional quality [Ducy et al., 1996].

Extensive screening of protein and mRNA extracts [Fraser and Price, 1988] and of tissue sections by immunohistochemistry [Bronckers et al., 1985; Camarda et al., 1987] from virtually all tissues has failed to detect osteocalcin in any tissue other than bone and dentin. Consequently, osteocalcin has been extensively used both as a marker of osteoblast differentiation and to study tissue-specific gene expression. Identification of promoter regulatory elements (Fig. 1.5) that are responsive to basal and tissue specific transactivation factors, steroid hormones, and other physiologic signals provides a basis for our understanding of regulatory mechanisms contributing to tissue-specificity, developmental expression and biological activity of osteocalcin [Lian et al., 1999b; Lian et al., 1998].
Figure 1.5 Osteocalcin Promoter: Organization of the bone-specific rat osteocalcin promoter is schematically illustrated, indicating regulatory domains within the initial 700 nucleotides 5' to the transcription start site. Cognate binding factors are shown above the designated regulatory elements. DNase I hypersensitive sites are indicated by solid triangles, the positioned nucleosome found in the actively transcribing gene is also indicated (Montecino et al, 1994).
The promoter of the rat osteocalcin gene has a modular organization consisting of positive and negative regulatory elements with consensus sequences for numerous physiologic modulators that have been shown to affect synthesis of OC in vitro. These include vitamin D [Bortell et al., 1992; Markose et al., 1990; Kerner et al., 1989; Demay et al., 1990; Morrison et al., 1989; Lian et al., 1989b], estrogen, glucocorticoids, retinoic acid, TGF β, cyclic nucleotides and γ-interferon. A series of elements contributing to basal and tissue specific expression include a TATA sequence (located at -42 to -39), and binding elements for AP-1, OC box for homedomain protein binding (Dlx5, Msx2 and CDP/cut helix-loop-helix proteins) and an osteoblast specific complex [Hoffmann et al., 1994; Tamura and Noda, 1994; Towler et al., 1994a]. Interestingly, the minimal OC promoter containing the OC box is sufficient to support osteoblast-specific expression in vitro [Heinrichs et al., 1993].

In the characterization of bone-specific transcription factor complexes associated with the rat osteocalcin gene sequences, three Runx regulatory elements were identified and designated as sites A, B and C [Banerjee et al., 1996a; Merriman et al., 1995; Bidwell et al., 1993]. Site C (-135 to -130) resides in the proximal promoter and sites A and B (-604 to -599 and -440 to -435), which flank the vitamin D response element (VDRE -461 to -445), are located in the distal region.

Osteocalcin is the best studied example of a bone-specific and vitamin D responsive protein. The vitamin D responsive element (VDRE) discovered in the osteocalcin gene promoter [Morrison et al., 1989; Markose et al., 1990; Lian et al., 1999b; Sone et al., 1991] represents the key component of steroid mediated transcriptional enhancement. It has been shown that ligand-dependent binding of the
vitamin D receptor in intact cells is directly correlated with transcriptional up-regulation [Breen et al., 1994; Demay et al., 1990; Terpening et al., 1991; Lian et al., 1989b]. However, vitamin D is unable to induce osteocalcin gene expression prior to its basal expression during the osteoblast differentiation sequence [Owen et al., 1991; Owen et al., 1993; Shalhoub et al., 1992].

II. BONE RELATED TRANSCRIPTION FACTORS

1. Runx Proteins

Runt related factors (Runx) belong to the Runt domain gene family. In *Drosophila melanogaster*, the pair rule gene runt plays an important role in the formation of the segmented body pattern, in sex determination and in the development of the nervous system [Canon and Banerjee, 2000]. The α subunit of these heterodimeric transcription factors binds to DNA weakly by recognizing the sequence Pu/TACCPuCA [Meyers et al., 1993; Kamachi et al., 1990]. There are three mammalian genes which encode the α subunit: RUNX1 (CBFA2/PEBP2αB/AML1), RUNX2 (CBFA1/PEBP2αA/AML3) and RUNX3 (CBFA3/PEBP2αC/AML2). The β subunit, called Cbfβ/Pebp2b, does not directly interact with DNA, but associates with the α subunit and increases the affinity of the α subunit to DNA. Multiple isoforms for each mammalian gene product exist, which are generated by alternative splicing; however, all the Runx proteins share a functional domain, referred to as the runt homology domain (RHD) because it is 70% identical to a region in the *Drosophila* protein Runt (Fig. 1.6). The three mammalian Runx proteins are more than 50%
Figure 1.6 Nomenclature and structural comparison of Runx proteins:
Schematic comparison of a transcriptionally active protein isoform from each of the mammalian RUNX genes and the *Drosophila* runt gene. The C-terminus pentapeptide (VWRPY) is 100% conserved. Amino acid numbers for the full-length proteins and of various regions are indicated below. The presence of two unique domains in the amino and carboxy regions of the Runx2 proteins is shown with their respective amino acid numbers. Different nomenclatures used for runt domain proteins are shown in the table. The nomenclature committee of the Human Genome Organization has recently adopted the RUNX designation for the runt related transcription factors.
identical at the amino-acid level, and greater than 93% identical within the RHD, which is responsible for the DNA binding activity and Cbfβ interaction.

Mice with null mutation of Runx1 are embryonic lethal at midgestation due to hemorrhage in the central nervous system and disruption of fetal liver hematopoiesis [Okuda et al., 1996]. A similar phenotype was found in the homozygous mutant mice for CBFβ/PEBP2b [Wang et al., 1996]. Runx2 nullizygous mice present a complete absence of bone, smaller size and neonatal death due possibly to respiratory failure [Komori et al., 1997; Otto et al., 1997]. Runx 3 knock out mice are born in mendelian ratios; however they display hyperplasia of the gastric epithelium and die within 24 h of birth due to low glucose levels in the blood stream [Li et al., 2002].

Runx proteins can act as activators or repressors of transcription. In their capacities as transcriptional activators, the mammalian Runx proteins interact with several other transcriptional regulatory proteins e.g.: p300, Androgen Receptor (AR), Glucocorticoid Receptor (GR), PU.1, Smad 1, Hairy and Enhancer of Split 1 homologue (HES-1) [Kitabayashi et al., 1998; Ning and Robins, 1999]. As repressors, Runx proteins can interact with the Groucho/TLE class of co-repressors through a conserved VWRPY C-terminal sequence motif [Levanon et al., 1998], or can alternatively repress transcription by a Groucho-independent mechanism [Javed et al., 2000; Javed et al., 2001]. The current interpretation of numerous studies is that Runx proteins function as context dependent regulators, which activate or repress transcription depending upon the organization of a particular promoter/enhancer in a specific cell type at a certain time.
An interesting property of Runx proteins is their association with the nuclear matrix, an anastomosing network of fibers and filaments first identified by Berezney and Coffey [Berezney and Coffey, 1975]. In fact, a conserved 31-35 aminoacid intranuclear targeting signal in the C-terminus of Runx 1, 2 and 3 has been identified [Zeng et al., 1997; Zaidi et al., 2001]. Association of Runx factors with the nuclear matrix increases their transactivation potential for various promoters [Zeng et al., 1998].

2. Vitamin D And Vitamin D Receptor

The active form of vitamin D, 1,25-(OH)₂D₃, exerts its effects by binding to the vitamin D receptor (VDR), which is a member of the nuclear receptor superfamily. VDR is a phosphoprotein that undergoes a hormone-dependent phosphorylation in intact cells [Pike and Sleator, 1985]. Phosphorylation of VDR by protein kinase A (PKA) results in a reduction in the transactivation capacity of the receptor in response to vitamin D [Jurutka et al., 1993]. In vivo, over-expression of casein kinase II, which phosphorylates VDR at Ser 208 in response to vitamin D treatment, causes an increase in 1,25(OH)₂D₃-induced transcriptional activity [Jurutka et al., 1996]. Phosphorylation of Ser 51 by protein kinase C diminishes VDR binding and nuclear localization of the VDR [Hsieh et al., 1991; Hsieh et al., ]; this evidence strongly suggests that differential phosphorylation plays a role in determining VDR activity.

Binding of the ligand to VDR triggers a cascade of events, resulting in the transcriptional regulation, activation or suppression, of specific target genes (Fig. 1.7). A key event is the ligand-induced change in receptor conformation [Allan et al., 1992; van den Bemd et al., 96 A.D.] that exposes sites for interaction with specific cofactors.
Figure 1.7 Proposed mechanism of action of 1,25(OH)$_2$D$_3$ in target cells: The free form of vitamin D (D$_3$) enters the target cell and interacts with the vitamin D receptor (VDR), which is phosphorylated (P). The ligand-VDR complex interacts with the retinoid acid X receptor (RXR) to form a heterodimer, which will bind to the vitamin D responsive element (VDRE), causing an enhancement or inhibition of transcription of vitamin D responsive genes. (Taken from Holick MF “Vitamin D: Photobiology, Metabolism, Mechanism of Action, and Clinical Applications” in Favus MJ “Primer on the Metabolic Bone Diseases and Disorders of Mineral Metabolism” Fourth Edition, 1999)
In addition to heterodimerizing with the retinoid X receptor (RXR), VDR has been shown to interact with SRC-1, a member of the p160 class of co-modulators that possesses intrinsic HAT activity. VDR also interact with TFIIB (a protein associated with the basal transcription machinery), NCoA 62 (nuclear receptor coactivator, 62,000 Da), DRIPs (vitamin D-receptor-interacting proteins) and Smad 3 (a member of the Smad protein family of intracellular transducers of the TGF β-BMP superfamily). The particular co-regulatory proteins recruited to VDR may contribute to the specificity of transcriptional regulation.

For several VDR target genes a functional VDRE has been defined. Generally it consists of two imperfect direct repeats of a hexameric nucleotide sequence spaced by three base pairs (DR3-type VDRE), and although the consensus sequence AGGTCA is still recognizable, considerable heterogeneity in half-site sequences exists. This heterogeneity is the basis for positive and negative VDREs [Van Leeuwen et al., 2001]. In most cases the proximal (3') half-site is the binding site for the VDR; whereas the distal site (5') half site is the binding site for its dimer partner RXR [Staal et al., 1996; Jin and Pike, 1996; Lemon and Freedman, 1996]. Besides the DR3-type VDREs, DR4-type VDREs and inverted palindromics motifs spaced by nine nucleotides (IP9) have been characterized as functional VDREs. Furthermore, VDR binding to direct repeats with a spacing of six nucleotides, a palindrome without spacing and an inverted palindrome with a 12 nucleotides spacing has been reported [Carlberg et al., 1993].

The VDR knock out mice present hypocalcaemia, hypophosphatemia, hyperparathyroidism and severely impaired bone formation [Li et al., 1997]. A recent study using mice transgenic for VDR under control of the osteocalcin promoter...
indicated bone anabolic effects of vitamin D. In mice expressing the transgene, a 20% increased trabecular bone volume, increased bone strength and reduced bone resorption surface was observed [Thomas et al., 2000].

3. C/EBP Transcription Factors

The CCAAT/ enhancer-binding proteins (C/EBPs) comprise a family of transcription factors that are critical for normal cellular differentiation and function in a variety of tissues. There are currently six known members of the C/EBP family designated as C/EBPα, β, δ, γ, ε and ζ [Cao et al., 1991]. C/EBP, like many transcription factors, is a modular protein, consisting of an activation domain, a DNA binding basic region and a leucine-rich homo and/or heterodimerization domain [Agre et al., 1989; Vinson et al., 1993]. C/EBP dimerization is a prerequisite to DNA binding [Birkenmeier et al., 1989].

C/EBPα was the first member cloned [Landschulz et al., 1988] [Birkenmeier et al., 1989]. There are two isoforms of this protein that are generated from the same mRNA by a ribosomal scanning mechanism [Descombes and Schibler, 1991]. Targeted disruption of C/EBPα results in perinatal lethality due to hypoglycemia [Wang et al., 1995].

C/EBPβ is constitutively expressed at high levels in liver, intestine, lung and adipose tissues [Cao et al., 1991]. C/EBPβ, like the α family member, has two isoforms generated by ribosomal scanning. The truncated protein translated from the third AUG exhibits a dominant negative mechanism of transcriptional regulation [Descombes and Schibler, 1991]. Protein kinase-dependent phosphorylation of
C/EBPβ appears to activate transcription [Trautwein et al., 1993]. C/EBPβ knockout mice show severe defects in carbohydrate metabolism, lipid storage, macrophage maturation and female sterility [Tanaka et al., 1995].

C/EBPγ and δ are both intronless genes which are expressed constitutively. C/EBPγ is highly expressed in non-differentiated progenitor cells [Roman et al., 1990], whereas C/EBPδ is detected in intestine, adipose tissue and lung and is highly stimulated by Lipopolysaccharide (LPS) [Alam et al., 1992; Cao et al., 1991]. C/EBPγ is missing the N-terminus transactivating element but can heterodimerize with C/EBPα and β, thereby interfering with transcriptional regulation of target genes by these proteins [Cooper et al., 1995]. In contrast the ability of C/EBPδ to transactivate target genes is comparable to C/EBPα and β [Cao et al., 1991]. Homozygous disruption of C/EBPδ shows neurologic and lipid storage defects. Mice deficient for both C/EBPβ and C/EBPδ expire perinatally and lack lipid accumulation in brown adipose tissues [Tanaka et al., 1997].

C/EBPε is represented by four isoforms, arising from alternative promoters and/or splicing [Yamanaka et al., 1997]. C/EBPε promotes granulocytic differentiation of the promyelocytic cell line NB4 and its expression can be induced by retinoids [Morosetti et al., 1997]. C/EBPε null mice die within a few months after birth due to immature granulocyte and myeloid proliferation [Yamanaka et al., 1997].

C/EBPζ is ubiquitously expressed and is induced by DNA damage [Fornace, Jr. et al., 1989]. The presence of two prolines in the DNA binding domain of C/EBPζ
results in an altered conformation and consequently the heterodimers are unable to interact with the C/EBP motifs [Ron and Habener, 1992].

C/EBP proteins are known to interact both physically and functionally with other transcriptional activators or co-activators such as NF-κB, Stat3 and PPARγ. Both C/EBPβ and Runx factors have been shown to cooperate with chromatin remodeling factors (p300, SWI/SNF) and other enhancer-binding proteins [Kitabayashi et al., 1998; Kowenz-Leutz and Leutz, 1999] and Runx1/AML-1 [Stein et al., 1993; Zhang et al., 1996a].

III. CHROMATIN STRUCTURE AND TRANSCRIPTION

The ability of each cell to program its genome and determine which genes are to be expressed at a given time and under certain stimuli is central to tissue differentiation. A fundamental mechanism controlling the selectivity of gene expression is the limited ability of many transcription factors to access the genome. This is achieved by packaging genes into chromatin, which greatly impairs the binding of many proteins to their target DNA sequences.

The basic unit of chromatin, the nucleosome, consists of 146 base pairs of DNA wrapped around two subunits each of the highly conserved core histones H2A, H2B, H3 and H4 [Kornberg, 1974; Luger et al., 1997]. In addition, the linker histone H1 binds nucleosomes, facilitating chromosome condensation and serving regulatory functions [Crane-Robinson, 1999; Bouvet et al., 1994]. Nucleosomes assemble into increasingly condensed structures termed the 10nm nucleosom al filament, the 30nm fiber, and ultimately chromosomes (Fig. 1.8).
Figure 1.8 Levels of Chromatin Packing: DNA associates with a histone octamer to form the nucleosome (beads on string). Each nucleosome associates with histone H1, and the fiber coils into a solenoid structure (30-nm fiber). In interphase chromosomes, long stretches of 30-nm chromatin loop out from extended scaffolds. In metaphase chromosomes, the scaffold is folded into a helix and further packed into a highly compacted structure. (Taken from Alberts, Bray, Johnson, Lewis, Raff, Roberts, Walter http://www.essentialcellbiology.com)
Early studies of gene regulation in eukaryotes identified activator proteins (transcription factors) that bind to short DNA motifs found in the promoter and enhancer regions of genes. The regulatory sequences of most eukaryotic genes contain binding sites for multiple transcription factors, allowing each gene to respond to multiple signaling pathways and facilitating the fine-tuning of transcript levels. The activities of many transcription factors are context dependent and can be modulated by other regulators bound nearby. It was first thought that eukaryotic activators, like the corresponding proteins of prokaryotic cells, exert their effects directly, through contacts with components of the transcription machinery. Then genetic and biochemical studies in yeast revealed the existence of global transcription regulators that go between activators (or repressors) and the transcription machinery [Bjorklund et al., 1999].

Two broad classes of global regulators can be distinguished, those that affect the chromatin template and those that function through RNA polymerase and associated proteins. Members of the first class include SWI/SNF and ISWI chromatin-remodeling complexes, histone acetyltransferase (HAT) complexes, and histone deacetylase (HDAC) complexes. The second class is represented by the Mediator complex in yeast and by one or several closely related complexes in mammalian cells [DiRenzo et al., 2000; Rachez and Freedman, 2001]. It is generally believed that these regulators function in distinct stages, with alteration of the chromatin template preceding the stimulation of RNA polymerase activity.

Co-regulators that act on chromatin can be divided into two general subgroups: ATP-dependent nucleosome remodeling complexes and activities that catalyze post-translational modifications of histones. While both ATP-dependent remodeling and
histone modifying complexes can bind nonspecifically to DNA, several independent observations imply that the activities of these complexes are regulated both spatially and temporally. For example, human SWI/SNF participates in nuclear receptor-mediated transcriptional regulation, and both the glucocorticoid receptor and the estrogen receptor have been shown to recruit SWI/SNF to responsive promoters [Deroo and Archer, 2001; Hassan et al., 2001]. Nuclear HAT complexes and transcription coactivators with intrinsic HAT activities have also been found to interact with transcriptional activators [Roth et al., 2001]. Direct interactions between activators and these complexes can have three functional consequences that may affect the overall rate of chromatin remodeling: First, these interactions can specifically increase the affinity of the remodeling complex for a given DNA region due to the contacts between the activator and the complex resulting in an increased local concentration of the remodeling factor. Second, assuming that the binding of the remodeling complex to the chromatin is the rate-limiting event, targeting would increase the rate at which the remodeling complex binds to chromatin. Finally, it is possible that interaction of the remodeling complex with the specific binding factor may directly affect the remodeling activity.

Alterations in the chromatin organization of the osteocalcin gene promoter during osteoblast differentiation provide a paradigm for remodeling chromatin structure and nucleosome organization that is linked to phenotype-specific gene expression. Basal expression and vitamin D enhancement of OC are accompanied by increased accessibility to DNase I. In proliferating osteoblasts, where OC is not being expressed, the promoter of the gene is uniformly packed in nucleosomes. This nucleosomal array
on the OC promoter contributes to maintaining the repression of gene transcription (Fig. 1.9). As osteoblast development progresses and OC expression starts, two DNase I hypersensitive (DHS) regions are formed encompassing the VDRE and the Runx sequences in the proximal and distal promoter (Fig. 1.9). Moreover, vitamin D up-regulation of OC expression results in an increased hypersensitivity in both DHS regions [Montecino et al., 1994b]. These changes in nuclease accessibility are accompanied by changes in nucleosome placement [Montecino et al., 1994a]; in fact, when the gene is being actively transcribed the proximal region and upstream steroid hormone responsive enhancer sequence appear to become nucleosome free, allowing an increased accessibility to transcription factors. The same pattern of nuclease accessibility is observed in established cell lines. Osteoblastic cell lines that express OC and that are responsive to vitamin D (e.g., ROS 17/2.8) exhibit the two DHS in the OC promoter; meanwhile in osteoblastic cell lines that do not express OC (e.g., ROS 24/1), the OC promoter region is resistant to DNase I digestion [Montecino et al., 1996b].

Activation of tissue-specific genes is a tightly controlled process that normally involves the combined action of several transcription factors and transcriptional co-regulators [Graves, 1998; Thanos and Maniatis, 1995; Maniatis et al., 1998]. Runx2 is essential for osteoblast differentiation and regulates the expression of numerous bone-related genes. VDR belongs to the steroid and thyroid hormone receptor superfamily and mediates the biological effects of vitamin D, a physiologic regulator of bone formation. Runx2, and VDR factors have been shown to cooperate with chromatin remodeling factors (p300, SWI/SNF) and other enhancer-binding proteins [Kitabayashi
Figure 1.9 Chromatin modifications at the Osteocalcin locus during osteoblast development influence OC transcription: During in vitro differentiation of primary rat calvarial cells the activation of OC expression is noted only in mature osteoblasts. No DNaseI hypersensitivity is detected when the gene is silent (d0-d10) indicating a close conformation of the chromatin. Concomitant with OC activation in post-proliferative, alkaline phosphatase positive mature osteoblast the appearance of the two DNaseI hypersensitive sites are evident. Vitamin D treatment results in increased OC transcription which is accompanied by enhanced nuclease accessibility.
Transcription of the rat and human OC genes is strongly influenced by 1,25-dihydroxyvitamin D. The rat OC promoter contains three recognition motifs for Runx interaction (Sites A, B and C), as well as a vitamin D responsive element (VDRE). Because both Runx and VDR can recruit other transcription factors and cofactors into regulatory complexes [Aronson et al., 1997; Kitabayashi et al., 1998b; Lemon and Freedman, 1999] [Rachez et al., 1999], and VDR can also interact with the basal transcription machinery [MacDonald et al., 1995a; Blanco et al., 1995; Lemon et al., 1997], their binding sites may impose structural constraints on the OC promoter to facilitate interactions and activities of the proximal and distal elements. Our working hypothesis is that Runx2 and VDR factors are required not only to regulate the transcription of OC but also for the chromatin remodeling events that accompany OC gene expression. We addressed this by introducing into the OC promoter point mutations that abolish binding of Runx or VDR and generating a series of osteoblastic ROS 17/2.8 stable cell lines with integrated wild type or mutant promoters. The effects of these mutations on transcriptional activity and chromatin organization of the OC promoter were analyzed. Our results establish an essential role for Runx2 not only in transcriptional regulation but also in hormonal responsiveness and in the chromatin re-arrangements required for maximal OC expression (Chapter 3).

The data presented in Chapter 4 support the requirement of the VDRE for both basal and vitamin D enhancement of OC transcription. Mutational analysis indicates that chromatin changes in the OC promoter upon hormone treatment require an intact VDRE.
Although Runx2 is considered a master gene for bone development and osteoblast differentiation, it is noteworthy that osteoblast-specific transcription of OC occurs even in the absence of Runx sites in the rat OC promoter [Javed et al., 1999; Hoffmann et al., 1996]. Therefore, transcription factor(s) other than Runx2 may drive OC expression. In this regard, the role of C/EBP transcription factors in regulation of the OC promoter activity is presented in Chapter 5. C/EBP is a family of transcription factors that are critical for normal cellular differentiation and metabolic functions in a variety of tissues. Our studies demonstrate that C/EBP factors are potent activators of OC expression. We observed functional synergism between C/EBP and Runx2 that may account for the maximal induction of the OC gene in mature osteoblasts. Consistent with this role peak expression of Runx2, C/EBPβ and δ occurs concomitant with maximal levels of OC in differentiated osteoblasts.
CHAPTER 2

Materials and Methods
MATERIAL AND METHODS

Site Directed Mutagenesis And Expression Constructs

Constructs containing the rat OC (-1,097/+23 or -208/+23) promoter fused to the chloramphenicol acetyl transferase (CAT) gene have been described previously [Banerjee et al., 1996b]. Site directed mutagenesis was performed to incorporate two-nucleotide substitutions into the core-binding motif (ACC) of each individual Runx site (RACCRCW) in the 1.1 kb rat osteocalcin (OC) promoter fragment (Fig. 3.1). Mutations were generated by a PCR based approach [Ausubel et al., 1997] using the following synthetic oligonucleotides (Integrated DNA Technologies, Inc., Coralville, IA): Site A: 5’CCATCAAAAgACTAAATAAGAAATGCC 3’; Site B: 5’CATTACTGAttGCTCCTTCTGGGG 3’; Site C: 5’GTCACCAAgACAGCATCCTTTG 3’ and pUC/M13 Reverse primer: 5’TACACAGGAAACAGCTATGAC3’. The mutations (indicated by lower case letters) were selected such that no other DNA-protein binding sites would be generated. Plasmids bearing mutations in two of the three Runx sites were generated by replacing the wild type sequence with the mutant by restriction digestion of the -1.1 kb rOC promoter. The mABC plasmid bearing mutations in all three Runx sites was obtained by digestion of mAB with Bgl II-Hind III to release the wild type site C, which was replaced with the mutant site C plasmid fragment.

The same approach was used to generate the -208 OC-CAT C/EBPm plasmid, containing mutation of the C/EBP binding site in the -208 OC promoter (shown in lower case), in this case the oligonucleotides used were: 5’GGTTTGACCTAgactagtCATGACCCCCCA 3’, pUC/M13 Reverse primer:
5'TCACACAGGAAACAGCTATGAC3' (PCR 1),
5'TTGGGGGTCATGactagtcTAGGTCAAACC 3', pUC/M13 forward primer:
5'CGCCAGGGTTTTCCCCAGTCACGAC 3' (PCR 2) and -208 OC-CAT as template;
this mutation introduced a unique site for the SpeI restriction enzyme. The PCR
products were digested with BamHI-SpeI (PCR1) and ApaI-SpeI (PCR2). A three way
ligation reaction was set using ApaI-BamHI digested -208 OC-CAT as backbone.

The -208 OC-CAT Runx mutant plasmid was generated by digestion of the mC
CAT plasmid [Javed et al., 1999] with SphI-PpMU, followed by blunt ending and self-
ligation. To generate the plasmid with mutations in both C/EBP and Runx (Site C)
binding sites, two PCR reactions were set as above but in this case -208 OC-CAT Runx
mt was used as template. All plasmids were sequenced using the pUC/M13 forward
primer. The expression constructs encoding the wild type Runx2 and Runx2 Δ361 are
as reported earlier [Banerjee et al., 1997; Javed et al., 2001]. Runx2 Δ230 was prepared
by PCR amplification of the coding sequences using the forward primer
5'CGGGATCCATGCGTATTCC3' and a reverse primer with an engineered stop
codon 5' GGCTCGAGTCTATTAGATCATCAGGC 3'. Non-encoded nucleotides
are underlined. The PCR fragment was digested with BamHI-XhoI and ligated to
similarly digested pcDNA 3.1 His C vector (Invitrogen Inc., San Diego, CA). In-frame
ligation of these constructs was confirmed by DNA sequencing. Expression constructs
of C/EBP α, β and δ were obtained from Dr Alan Friedman (Johns Hopkins Hospital,
Baltimore MD).

Generation of the construct carrying the VDRE mutation (SHE) has been
previously described [Aslam et al., 1999].
Cell Culture, Transient Transfection and CAT Reporter Assays

Normal rat diploid osteoblasts (ROB) obtained from 21 day fetal rat calvariae were isolated and maintained as described [Owen et al., 1990a]. HeLa cells were maintained in Dulbecco modified Eagle medium (Gibco Life Technology, Grand Island, NY). Rat Osteosarcoma (ROS 17/2.8) cells were grown in F12 supplemented with 5% (v/v) fetal calf serum. HeLa and ROS 17/2.8 cells were plated at a density of 8x10^4 cells/well in six well plates and transfected 24 h later with 1-2.5 μg of either wild type or mutant reporter plasmids, 100 ng of RSV luciferase and 0.1-0.75 μg of either CMV empty vector, C/EBP β, δ or Runx expression constructs. The total amount of exogenous DNA was maintained at 3 μg/well with salmon sperm DNA. Cells were transfected with DNA in the presence of 7 μl/well of SuperFect (Qiagen Inc., Valencia, CA) and incubated at 37°C for 2.5 h with occasional swirling. The transfection mix was aspirated and cells were washed twice with phosphate buffered saline (PBS) and then incubated at 37°C in F12 medium supplemented with 5% (v/v) fetal calf serum (ROS 17/2.8 cells) or DMEM supplemented with 10% (v/v) fetal calf serum (HeLa cells) for 24-48 h. Cells were washed twice with ice cold PBS and lysed with 300 μl of Reporter Lysis Buffer (Promega Corp., Madison, WI) at room temperature for 30 min. Cell lysates were collected and stored at -70°C or used immediately for CAT assay as described previously [Frenkel et al., 1993]. Luciferase activity was determined in the same lysate with luciferase assay reagents from Promega Corp. (Madison, WI). Luminescence was quantitated using a Monolite TM 2010 instrument (Analytical Luminescence Laboratory, San Diego, CA).
Construction Of Stable Cell Lines

ROS 17/2.8 cells with genically integrated wild type, Runx and VDRE mutant constructs were generated by the calcium phosphate method [Sambrook and Russell, 2001]. For each construct, four 100 mm plates were transfected with 15 μg of the -1.1 OC-CAT plasmid and 5 μg of pCEP-4 (Invitrogen, San Diego, California) encoding the Hygromycin B phosphotransferase gene. Cells were harvested at 95% confluency and replated for selection in media containing 55 U/ml Hygromycin B (Calbiochem, La Jolla, California) based upon preliminary killing curves. Resistant colonies (60-75) from each plate were pooled and propagated as polyclonal cell lines. Each pool was expanded until 20 x 10^6 cells were available for preparation of frozen stocks. Monoclonal cell lines were generated by limited dilution of different polyclonal parental cell lines. Cells were routinely maintained in media containing Hygromycin B for measuring CAT activity and responsiveness to steroid hormones and growth factors.

Stable cells were plated at 2 x 10^5 cells/well in a six well plate and treated with 10^{-8} M vitamin D for 24 h. Cells were washed twice with phosphate buffered saline and lysed by adding 300 μl of Reporter Lysis Buffer (Promega Corp., Madison, WI) at room temperature for 30 min. CAT activity assays were performed as described above.

Nuclear Extracts And Electrophoretic Mobility Shift Assay

Nuclear extracts from ROS 17/2.8, HeLa and primary rat osteoblast cells at different stages of culture (d7, d14, and d20) were prepared by a modified Dignam method [Dignam et al., 1983] using 0.42M KCl for extraction. The whole isolation procedure was carried out on ice. Cells were collected at 95% confluency, washed
twice with ice-cold PBS and pelleted by centrifugation at 70 X g (1,600 rpm in IEC) for 5 min at 4°C. Cells were gently resuspended in 5-10 volumes of NP-40 lysis buffer [10mM Tris pH 7.4, 3mM MgCl₂, 10mM NaCl, 0.5% (v/v) Nonidet P-40 (NP-40)] supplemented with 1X Complete™ protease inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, IN), incubated on ice for 20 min and pelleted at 70 X g for 5 min. This pellet was washed once with 3 volumes of hypotonic buffer (10mM HEPES pH7.9, 1.5mM MgCl₂, 10mM KCl, 1X Complete™) and extracted for 1h at 4°C in 250-300 µl of extraction buffer (20mM HEPES pH7.9, 1.5mM MgCl₂, 420mMKCl, 0.2mM EDTA, 20% (v/v) glycerol, 1X Complete); insoluble material was pelleted at 16,000 X g for 30 min. Aliquots (25 µl) of the nuclear extracts were snap-frozen in liquid nitrogen and stored at -80°C until further use. Protein concentration of nuclear extracts was determined by Bradford assay. For the electrophoretic mobility shift assay, the desired amount of nuclear extract was brought up to 10µl with KN 100 buffer (10mM HEPES pH7.9, 0.2mM EDTA and 100mM KCl), combined with 10 µl of a DNA mixture containing 20 fmol of probe DNA and 1 µg of poly (dI-dC)-(dI-dC) as non-specific competitor, and incubated at room temperature for 30 min. For competition experiments, unlabelled double-stranded oligonucleotide (240 to 2000 fmol) was added to the binding reaction with the other components. Samples were loaded without tracking dye onto a 4% acrylamide: bisacrylamide (30:1) gel in 0.5X Tris borate/EDTA. Electrophoresis was performed for 2 to 2.5 h at 200v. Gels were dried and subjected to autoradiography.
Studies Of DNase I Hypersensitive Sites And Restriction Enzyme Accessibility

DNase I digestion and restriction enzyme analysis was performed according to the indirect end labeling method [Wu, 1980]. ROS 17/2.8 cells were plated at a density of 1 x 10^6 cells per 100 mm plate and nuclei were isolated on day 9 by dounce homogenization (loose pestle) in eight volumes of RSB buffer (10mM Tris HCl pH 7.4; 10mM NaCl; 3mM MgCl₂) with 0.5% (v/v) NP-40. To evaluate cell lysis, an aliquot of nuclei was stained with 0.4% (w/v) Trypan blue 1:1 (v/v).

The nuclear suspension was diluted by adding an equal volume of RSB buffer and nuclei were collected by centrifugation. The pelleted nuclei were resuspended in RSB buffer and the DNA concentration was estimated by absorption at 260 nm. Aliquots of 20 A260 units were digested with increasing concentrations of DNase I (0 to 5 units) (Worthington Biochemicals, Freehold, NJ) in 1 ml final volume for 10 min at room temperature, or with 500U/ml of restriction enzyme, in their corresponding buffer, for 30 min at 37°C. The reaction was stopped by adding EDTA, SDS, RNase One (Promega, Madison, WI) and Proteinase K (Fisher Biotech, Fairlawn, NJ) to a final concentration of 25mM, 0.5% (v/v), 1U/ml and 200μg/ml, respectively, and incubated at 37°C overnight. The samples were extracted once with phenol:chloroform:isoamyl alcohol (24:24:1) and twice with chloroform:isoamyl alcohol (24:1). Nucleic acids were precipitated with 2.5 volumes of ethanol at -70°C for at least 4 h and then resuspended in 25mM Tris pH 7.8. DNA was digested with BamHI or Xbal (4 units/μg of DNA) to release a 4.3 Kb fragment from the OC gene [Montecino et al., 1996a] or a 2.7 Kb fragment from the chromosomally integrated pOCZCAT fusion gene, respectively [Frenkel et al., 1996]. The digested DNA was extracted with
phenol:chloroform, precipitated with ethanol and resuspended in 25mM Tris pH 7.8. DNA samples (10 μg) were electrophoresed in a 1.2% agarose gel (Biorad, Hercules, CA) and then transferred to a nylon membrane (Hybond N+, Amersham Pharmacia Biotech, Arlington Heights, IL) according to the manufacturer's instructions.

Hybridization probes were prepared by restriction digestion of pOC 3.4 [Lian et al., 1989a], containing the rat OC gene and flanking sequences, with XbaI-BamHI and pOCZCAT with XbaI-NcoI. The probes were labelled by the random primer method using α³²P-dCTP and the Stratagene Prime-It II kit (Stratagene, La Jolla, CA). Hybridization was carried out at 65°C with 1 ng of probe (10⁹ cpm/μg specific activity) per 10cm² membrane. The blots were analyzed by autoradiography or by using a STORM Phosphorimager (Molecular Dynamics, Sunnyvale, CA).

Statistical Representation And Analyses Of The Data

The data for the Runx mutants are displayed as box and whisker plots [Hoaglin et al., 1983] where the length of the bar represents the range of observations between the first and third quartile (i.e., the interquartile range or IQR). The length of the whiskers represents the interval between the first or third quartile and the most extreme observation that does not meet the definition for outlier, i.e., a value more than 1½ IQR’s from either the first or third quartile depending upon whether the observation is above or below the median.

The distributional characteristics of promoter activities were evaluated graphically by using histograms and the Kolmogorov-Smirnov one-sample test for Normality [Siegel, 1956]. If data were not normally distributed, monotonic transformations were applied (i.e., natural logarithms) to achieve normality. The
significance of Runx sites to promoter activity was evaluated using Analysis of Variance (ANOVA) for mixed model using restricted estimation by maximum likelihood (REML) [McLean et al., 1991].

**Isolation Of Total Cellular RNA And Northern Blot Analysis**

Total cellular RNA was isolated from normal rat fetal calvarial osteoblasts or adult rat tissues as described previously [Chomczynski and Sacchi, 1987]. Cells at different stages of differentiation were washed and scraped in PBS. Cell pellets were frozen in liquid nitrogen and kept at -150°C, once all the time point were collected, samples were thawed and processed together. RNA pellets were briefly air dried and dissolved in 400 μl of DEPC (Diethylpyrocarbonate) treated water and stored at -70°C until further usage. RNA samples were electrophoresed in 1% formaldehyde-agarose gels and transferred to Hybond-N+ membrane (Amersham Pharmacia Biotech, Arlington Heights, IL) in 20X SSC. Blots were hybridized with random primed (Prime-It kit; Stratagene, La Jolla, CA), [³²P] labeled cDNA probes for C/EBPα (NcoI fragment), C/EBPβ (EcoRI-XhoI fragment), C/EBPδ (BamHI-EcoRI fragment), human GAPDH (EcoRI-HindIII fragment), human histone H4 (HindIII-XbaI fragment of FO108), alkaline phosphatase (EcoRI fragment), Runx2 (BamHI-XbaI fragment) and rat OC (EcoRI-BamHI fragment) at 42°C overnight. The blots were washed and subjected to autoradiography.

**Immunoprecipitation**

HeLa cells were transfected with Runx2 and C/EBPβ expression constructs; approximately 1 x 10⁷ cells per immunoprecipitation (Ip) were lysed in 800 μl of NP-40
buffer [150mM NaCl, 50mM Tris pH 8.0, 1% (v/v) NP-40, 1X Complete (Roche Molecular Biochemicals, Indianapolis, IN), 25μM MG132 (Sigma-Aldrich, St. Louis, MO)] and extracted at 4°C for 15 min, followed by centrifugation at 16,000 X g for 15 min. The proteosome inhibitor MG 132 was added to all buffers to avoid degradation of Runx proteins [Huang et al., 2001]. The supernatant was transferred to a clean microcentrifuge tube and precleared with 20 μl of protein A/G PLUS agarose beads (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at 4°C for 30 min. The beads were collected by centrifugation at 1,000 X g for 5 min at 4°C. Xpress antibody (3 μg, Catalog #: R910-25 Invitrogen Corp., Carlsbad, CA) was added to the precleared cell lysate followed by incubation at 4°C for 1 hour. To precipitate the immuno-complexes, 50 μl of protein A/G PLUS agarose beads were added and further incubated at 4°C with agitation for 1 hour. The beads were washed twice with 1 ml of washing buffer (20mM Tris pH 8.3, 0.5% (v/v) sodium deoxycholate, 0.5%(v/v) Nonidet P-40, 50mM NaCl, 2mM EDTA, 1X Complete, 25μM MG 132), suspended in 2X SDS sample buffer and analyzed by Western blotting.

Western Blot Analysis

Transfected or un-transfected HeLa and ROS 17/2.8 cells cultured on 100 mm dishes were lysed on the plate by adding 300 μl of SDS Lysis buffer (2% (v/v) SDS, 10mM dithiothreitol, 10% (v/v) glycerol, 2M urea, 10mMTris-HCl pH 6.8, 0.002% (v/v) bromophenol blue, 1X Ccomplete, 25μM MG132). Proteins (30-40 μg) were resolved in 10% SDS-PAGE and transferred to Immobilon™-P (Millipore Corp, Bedford, MA). Antibodies against C/EBPβ (sc-150) and Lamin B (sc-6217) were
purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Epitope tagged Runx proteins were detected with horseradish peroxidase-conjugated mouse monoclonal Xpress (R911-25) antibody (Invitrogen Corp., Carlsbad, CA). Monoclonal antiserum for tubulin (clone DM 1A Product No T9026) was purchased from Sigma-Aldrich (St. Louis, MO).

**Chromatin Immunoprecipitation Assay (ChIP)**

Chromatin Immunoprecipitation (ChIP) assay was performed as described elsewhere [Soutoglou and Talianidis, 2002] with some modifications. Cells were treated with 1% (v/v) formaldehyde at 37°C for 10 min. Crosslinking was stopped by adding glycine to a final concentration of 0.125M. Cells were washed with ice cold PBS, collected by centrifugation at 165 x g for 5 min at 4°C. The cell pellet was resuspended in lysis buffer (25mM HEPES pH 7.8, 1.5mM MgCl₂, 10mM KCl, 0.1% (v/v) NP-40, 1X Complete, 25μM MG-132) and incubated on ice for 10 min. Following dounce homogenization (20 strokes, pestle A), the nuclei were collected by centrifugation at 750 x g for 5 min, resuspended in sonication buffer (50mM HEPES pH 7.9, 140mM NaCl, 1mM EDTA, 1% (v/v) Triton X-100, 0.1% (v/v) Na-deoxycholate, 0.1% (v/v) SDS, 1X Complete, 25μM MG-132) and sonicated on ice to a DNA size of 200-800 bp. The samples were centrifuged at 16,000 x g for 15 min and precleared with A/G plus- agarose beads precoated with 2μg sonicated salmon sperm DNA and 1mg/ml BSA for at least one hour at 4°C. 30 A₂₆₀ units of the precleared chromatin were immunoprecipitated with 5μg of antibody and the immunocomplexes were collected by binding to A/G plus- agars beads. The beads were washed twice with...
each of the following buffers: sonication buffer, sonication buffer containing 500mM NaCl, LiCl buffer (20mM Tris pH 8, 1mM EDTA, 250mM LiCl, 0.5% (v/v) NP-40, 0.5% (v/v) Na-deoxycholate) and 10mM Tris pH 8. The immuno complexes were eluted in 50mM Tris pH 8, 1mM EDTA and 1% (v/v) SDS at 65°C for 15 min, adjusted to 200mM NaCl and incubated overnight at 65°C to reverse the crosslinking.

Following treatment with RNase One (1U/ml) and Proteinase K (20μg/ml), the samples were purified using PCR purification kit from Qiagen Inc. One tenth of the immunoprecipitated DNA and input DNA were analyzed by PCR using oligos described in Table 4.2. PCR amplifications (26 cycles) were performed in the presence of 0.1μCi α³²P-dCTP per reaction and the products were resolved in 4% polyacrylamide gels.

The antibodies used for ChIPs were anti-acetyl-Histone H3 (Catalog # 06-599), anti-acetyl Histone H4 (Catalog # 06-866) from Upstate Biotechnology and anti phosphorylated RNA Pol II CTD-Ser-2P (Clone H5, Catalog # MMS-129R) from Covance Inc.
Multiple Runx/Cbfa/AML Sites in the Rat Osteocalcin Promoter Are Required for Basal and Vitamin D-responsive Transcription and Contribute to Chromatin Organization.


INTRODUCTION

Bone tissue-restricted expression of the osteocalcin (OC) gene during development of the osteoblast phenotype requires a multiplicity of transactivating factors. Among the key regulators of osteocalcin expression are transcription factors that play essential roles in embryonic formation of the skeleton and osteoblast differentiation. These include the Runx (Runt related proteins)/Cbfa (core binding factors α)/AML (acute myelogenous leukemia) family of runt homology domain (RHD) DNA binding proteins (reviewed in [Komori, 1998]), the Msx and Dlx homeodomain proteins [Hoffmann et al., 1994; Towler et al., 1994b; Ryoo et al., 1997], AP-1 proteins [McCabe et al., 1996] and steroid hormone receptors (reviewed in [Lian et al., 1999a]).

The Runx family of transcriptional activators is critical for the development of hematopoietic and skeletal tissues. Three known genes, Runx3 (Cbfal/AML-3/mPebp2a), Runx1 (Cbfα2/AML-1/mPebp2b), and Runx3 (Cbfα3/AML-2/mPebp2c), each encode several mRNA splice variants [Speck and Stacy, 1995; Ahn et al., 1996; Levanon et al., 1994]. The tissue-specific transcriptional properties of the Runx proteins are in part accounted for by their selective representation in distinct cellular phenotypes. Runx1 primarily regulates expression of genes related to the development of thymus and hematopoietic tissues, and a null mutant of this gene results in embryonic lethality due to absence of definitive hematopoiesis. Several isoforms of Runx2 have been described; one is expressed in hematopoietic tissues [Zhang et al., 1997b] and another is highly expressed in osteoblast lineage cells of bone [Stewart et
al., 1997; Xiao et al., 1998; Thirunavukkarasu et al., 1998; Ducy et al., 1997; Banerjee et al., 1997] and in hypertrophic chondrocytes [Komori et al., 1997]. Ablation of the Runx2 gene in mice reveals the importance of this factor in development of the skeleton with a consequent absence of mineralized connective tissues [Komori et al., 1997; Otto et al., 1997; Mundlos et al., 1997]. The Runx class of RHD proteins was initially identified in bone as an osseous cell-specific DNA binding complex extracted from the nuclear matrix [Bidwell et al., 1993; Merriman et al., 1995]. Subsequently, Runx proteins were shown to regulate tissue-specific expression of the osteocalcin promoter [Banerjee et al., 1996a; Ducy and Karsenty, 1995]. Although overexpression of the Runx1, Runx2 or Runx3 factors in non-osseous cells can confer expression of the bone-specific osteocalcin gene [Banerjee et al., 1996a], the DNA binding activity present in mature osteoblasts consists primarily of the Runx2 gene product [Banerjee et al., 1997; Ducy et al., 1997].

The bone-specific rat osteocalcin promoter contains three recognition sites for Runx interactions (sites A, B and C [Merriman et al., 1995]). Notably, all three motifs bind a similar osteoblast-specific DNA binding complex, first designated NMP-2 [Merriman et al., 1995; Bidwell et al., 1993]. While only one Runx site fused to a minimal OC promoter is sufficient to confer enhancer activity in osseous and non-osseous cells [Banerjee et al., 1996a], the presence and positioning of multiple Runx sites suggest that spatial organization of the native OC promoter may be important for interaction of Runx proteins with other OC promoter regulatory factors. For example, transcription of the rat and human osteocalcin genes is strongly influenced by 1,25-dihydroxyvitamin D. Runx sites A and B flank the vitamin D response element which
mediates 3-10 fold enhancer activity of the rat and human promoters. A third Runx site C (also designated OSE2 in mouse [Ducy and Karsenty, 1995]) is located in the proximal promoter (nt -136 to -130). A positioned nucleosome resides between Runx sites B and C in the transcriptionally active rat osteocalcin promoter [Montecino et al., 1996b]. Because Runx factors associate with the nuclear matrix [Stein et al., 1997] and can recruit other factors into complexes [Palaparti et al., 1997; Aronson et al., 1997; Levanon et al., 1998; Speck and Stacy, 1995; Ito, 1997], Runx binding sites may impose structural constraints on the osteocalcin promoter to facilitate interaction and activities of the proximal and distal regulatory elements (e.g., the VDRE and TATA domains). The function of the Runx motifs within the context of the native rat osteocalcin promoter has yet to be examined.

In these studies, we establish that all three Runx elements within the rat OC promoter contribute to basal transcriptional activity, suggesting a functional interaction among the three sites. In contrast to recent studies of the mouse OC promoter [Frendo et al., 1998], the distal site A (nt -605/-599) in the rat OC promoter contributes far more to Runx dependent promoter activity than the proximal site C/OSE2. Furthermore, the presence of the upstream Runx sites is critical for vitamin D induction of osteocalcin promoter activity. Mutation of all three Runx sites results in altered chromatin organization as reflected by loss of DNase I hypersensitive sites in the OC promoter. These findings suggest that transcriptional activity of the OC gene, which requires interactions of proximal and distal regulatory elements, may be facilitated through spatial constraints of the promoter imposed by the binding of the nuclear matrix associated Runx factors to critically positioned recognition sequences.
RESULTS

Full Basal Activity Of The Osteocalcin Promoter Requires The Distal Runx Sites

A single Runx element is sufficient to confer tissue-specific transactivation of the proximal osteocalcin promoter [Banerjee et al., 1996a; Ducy et al., 1997]. To address the contributions of the multiple Runx sites in the native rat OC promoter to osteocalcin transcription, we carried out site-directed mutagenesis of each of the three Runx elements, designated sites A, B and C (Fig. 3.1). Initially, we established two-nucleotide substitution mutations in the core of each OC Runx site, which abrogate bone-specific Runx binding activity (Fig. 3.2). The appropriate WT and mutant oligonucleotide sequences for each site (shown in the Methods section) were examined in gel mobility shift assays using nuclear extracts from ROS 17/2.8 rat osteosarcoma cell lines. These extracts contain abundant levels of Runx2, which forms an osteoblast-specific complex that can be supershifted by Runx2-specific antibody [Banerjee et al., 1996a; Banerjee et al., 1997]. Figure 3.2A demonstrates that each mutation results in loss of the Runx binding complex. Because competition of binding to the WT sequence is not observed in the presence of 80 fold excess (1nM) of mutant oligonucleotide representing sites A, B or C (Fig. 3.2B, lanes 5-8, respectively), the mutations have completely abrogated Runx binding.

The locations of the Runx sites within the rat OC promoter [Merriman et al., 1995] suggest different activities, while promoter deletion analysis [Banerjee et al., 1996a; Hoffmann et al., 1996] indicates redundant function for the multiple Runx sites. The functionality of each site was therefore tested following transient transfection of
Figure 3.1 Wild type and mutated Runx motifs in the rat osteocalcin gene promoter: The positions and nucleotide numbers of the three Runx sites (A, B, C) relative to the vitamin D response element (VDRE), glucocorticoid response element (GRE), a TGFβ responsive AP-1 site, and two primary transcriptional elements requisite for basal transcription, the OC Box, and the TATA box are indicated. The positioned nucleosome and DNase I hypersensitive sites that are present when the gene is transcribed are indicated. The Runx core recognition sequence at each site is indicated with mutant nucleotides (mt) designated below. The lower panel shows the WT oligonucleotide probes, used in gel mobility shift assays, containing the site A, B and C Runx motifs within the context of flanking sequences of the rat OC promoter.
Figure 3.2 Mutations of the three rat osteocalcin Runx sites result in loss of Runx binding: (A) Panel compares formation of the osteoblast specific complex from nuclear extracts of ROS 17/2.8 cells with oligonucleotides containing wild type (WT) sequences representing Runx site A (lane 1), site B (lane 3), and site C (lane 5) and mutated (M) Runx sequences of sites A (lane 2), B (lane 4), and C (lane 6) in gel mobility shift assays. Runx complexes are indicated by solid arrowheads. A non-specific complex (site B) is indicated by an open arrowhead. (B) Panels site A, site B, and site C show corresponding competition assays for each Runx site using the wild type sequence as probe with increasing amounts (0, 20, 60, 80X) of either wild type (WT) oligonucleotide (lanes 1-4, respectively) or Runx site mutated oligonucleotides (lanes 5-8, respectively) as competitor.
the WT and mutant OC promoter (-1.1 kb) constructs into ROS 17/2.8 cells (Fig. 3.3B, and Table 3.1). Activity of the OC WT promoter was compared to activity of promoters having single or multiple Runx site mutations. While mutation of each of the sites reduced promoter activity compared to control, mutation of the proximal site C had the least effect (83% of WT, p value of 0.206 is non-significant). Mutation of sites A and B independently or of the two sites AB and AC, reduced promoter activity to approximately 50% of the WT, but high statistical significance of the effect was found in these transient assays only for mAB (p<0.001). Transcriptional activity of the Runx three-site mutation, mABC, was decreased to 40% of the control level (p<0.001).

To determine the contribution of the Runx sites to OC gene promoter transcription within a genomic context, we established a series of ROS 17/2.8 cell lines which contain stably integrated OC-CAT reporter gene constructs with single, paired or triple Runx mutations in the -1.1 kb rat OC promoter (Fig. 3.3A). As described in Materials and Methods, we examined four independent pools of cell lines for each construct to compensate for positional effects on promoter activity. Due to site-of-integration effects and copy number, basal activity for each cell pool varied among the cell lines over a 2-fold range. Figure 3.3C shows that mutation of each of the individual Runx sites resulted in significantly decreased promoter activity, with reductions in transcription greater than those observed following transient transfection (Fig. 3.3B). Again, mutation of the proximal site C minimally affected transcription; even as a stable integrant in osteoblasts (p<0.05). Mutations involving site A had a more pronounced effect in decreasing promoter activity in the stable cell lines (from 20-30% of control, p<0.001). This conclusion is further supported by the finding that
Figure 3.3 Requirement for multiple Runx sites for maximal transcriptional activity of the rat osteocalcin gene promoter: (A) 1.1 kb of rat OC 5' sequences containing either single or multiple site mutations are schematically illustrated. Single site mutations are designated mA, mB, or mC, two site mutations mAB, mAC or mBC and mABC is the triple mutation.
Figure 3.3 Requirement for multiple Runx sites for maximal transcriptional activity of the rat osteocalcin gene promoter: (B) Following transient transfections in ROS 17/2.8 cells, normalized CAT-reporter activity of the WT promoter and promoters with Runx site mutations are compared. Reporter activity was assayed 24 hr after transfection of ROS 17/2.8 cells. The $^{14}$C CAT activity was quantitated by a Betoscope analyzer (Betagen, Waltham, MA) and normalized to luciferase. (Aliquots of the lysate were assayed for luciferase activity to normalize CAT activity.) Each bar represents the LS mean ± SEM (n=12). Single asterisk indicates statistically less than WT p<0.001; double asterisk, p<0.01.
Figure 3.3 Requirement for multiple Runx sites for maximal transcriptional activity of the rat osteocalcin gene promoter: (C) Activity of WT and Runx sites mutated promoters stably integrated into ROS 17/2.8 cells. Each group represents promoter activity in 4 independent cell lines, with each cell line assayed in 4 separate experiments in triplicate. Cells were harvested 3 days after plating as cells reached monolayer confluency for quantitation of CAT activity normalized to total protein of the cell lysate. Each bar represents the LS mean ± SEM (n=18). The pGEM control represents a promoterless CAT containing stable cell line. Single asterisk indicates statistically less than WT p<0.001; double asterisk, p<0.01.
### TABLE 3.1

**CONTRIBUTION OF DISTAL Runx SITE A AND SITE B TO OC PROMOTER ACTIVITY**

**p Values for activity of Runx sites tested against all groups**

<table>
<thead>
<tr>
<th>Effect of Each WT Site</th>
<th>Transient Assays</th>
<th>Stable Cell Lines</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.0001</td>
<td>0.0001</td>
</tr>
<tr>
<td>B</td>
<td>0.0001</td>
<td>0.0001</td>
</tr>
<tr>
<td>C</td>
<td>n.s.</td>
<td>0.0001</td>
</tr>
<tr>
<td><strong>Interactions Between Sites</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A with B †</td>
<td>0.0013</td>
<td>0.0001</td>
</tr>
<tr>
<td>A with C †</td>
<td>n.s.</td>
<td>0.0062</td>
</tr>
<tr>
<td>B with C †</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>A with B with C †</td>
<td>0.0001</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

n.s. Not significant (i.e., >0.05).

† The interactions between the indicated sites were tested for significance. When sites A and B or A, B and C are present together in transient assay or stable cells and sites A and C are present together in a genomic context, the presence of the other site(s) influences the contribution to transcriptional activity of the promoter.
transcription of mBC is only reduced to 80% of control, indicating that site A contributes a significant level of Runx dependent activity to the OC promoter (Fig. 3.3C). Because all possible mutation groups were examined and sample sizes within a group were large, the statistical significance of the contribution of each Runx site to OC promoter activity could be compared (Table 3.1). On the basis of whether each of the sites is present or absent in a construct, the effect of site A or B, but not C, was always significant. Furthermore, two way ANOVA of the LS mean of each group revealed a significant interaction between sites A and B. There is a 2 to 3 fold greater effect contributed by site A when site B is present and by site B when site A is present. Taken together, these findings indicate that all of the Runx sites in the rat OC promoter contribute to basal transcription and that the distal sites A and B play predominant roles in supporting promoter activity within a chromosomal context.

**Functional Compensatory Activity Of The Three Runx Sites In Osteocalcin Transcription**

The mutagenesis studies suggest that the distal sites A and B provide a significant level of functional activity and that the proximal site C does not contribute equally to basal promoter activity. To further assess involvement of each of the three Runx sites in transcription of the rat osteocalcin gene, we examined the consequences of forced expression of Runx2 on activity of wild type and mutant promoter constructs in non-osseous cells, which do not express osteocalcin. In HeLa cells, for example, OC promoter activity is very low, and transcription factors necessary for bone tissue-specific expression are unlikely to be present. Runx forced expression in HeLa cells results in detectable wild type OC promoter activity (3.3 fold induction, Fig. 3.4); as
Figure 3.4 Runx2 mediated transactivation of OC promoters containing mutated Runx sites in HeLa cells: 0.5 µg of Runx2 expression plasmid and indicated OC-CAT plasmids (2.5 µg) were co-transfected into HeLa cells and assayed 36 hours following transfection. The CAT activity was quantitated by direct counting using a Betascope analyzer (Betagen, Waltham, MA). CAT activity was calculated as percent conversion and normalized for luciferase values used as internal control. A statistically significant difference (p<0.001) of the mABC group to WT is indicated by asterisk.
expected, mutation of all three Runx sites dramatically reduces Runx dependent OC promoter activity. In contrast, mutation of the single sites A, B or C resulted in induction of OC promoter activity to approximately the same extent, but somewhat less than wild type. Together these findings suggest that any one of the three sites can support induction of the OC promoter when cellular levels of Runx2 are available. However, compensatory effects are partial, indicating that all three sites are necessary for maximal promoter activity. Thus, these findings indicate that Runx-dependent induction of promoter activity at each Runx site is similar, suggesting functional compensatory activity. Notably, the mutational analyses support a selective contribution of the distal sites versus the proximal site C to basal promoter activity (see Fig. 3.3).

Steroid Hormone Modification Of Transcription Is Dependent On Runx2 Regulatory Elements

Our mutational studies suggest that the two distal Runx sites, which flank the vitamin D response element (VDRE), are essential to basal expression of the osteocalcin gene (Fig. 3.3C). The VDRE is a strong enhancer of OC transcription and vitamin D responsiveness necessitates interaction of the VDR/RXR heterodimer complex at the distal VDRE (nucleotides -461/-441) with TFIIB and other general transcription factors at the proximal TATA binding element (nucleotides -32/-29). We therefore addressed the potential contribution of Runx sites in mediating vitamin D responsiveness within the context of chromosomal integration of the OC promoter in stable cell lines. The importance of examining vitamin D responsiveness as influenced by the Runx motifs within a chromosomal context is supported by our previous studies.
demonstrating the presence of a positioned nucleosome between Runx sites B and C on the osteocalcin promoter and increased DNase I hypersensitivity in response to vitamin D [Montecino et al., 1996a; Montecino et al., 1996b].

Expression of the stably integrated WT promoter was enhanced by vitamin D from 4 to 7 fold in the four stable cell line pools, independent of basal promoter activity (Fig. 3.5). In striking contrast, the four stable cell lines in which all three Runx sites of the integrated OC promoter were mutated (mABC cell line, designated T1 to T4, Fig. 3.5) exhibited nearly complete inhibition of vitamin D stimulation of transcription (summarized in Fig. 3.6A). This abrogation of vitamin D responsiveness of the mABC promoter was also observed following transient transfection of the WT and mABC promoters into ROS 17/2.8 cells (Fig. 3.6B). In three different experiments (total n=18), the mean folds induction of the WT OC promoter by 10^-9 M 1,25 (OH)_2D_3 in 24 h is 7.4 (±0.2 standard deviation [SD]) but only 1.6 (±0.3 SD) for the mABC construct.

We then proceeded to examine the contribution of each Runx site to vitamin D dependent promoter activity. Mutation of any one of the single Runx sites (mA, mB, or mC) decreased the vitamin D response from 5 fold induction (WT) to 2.5 and 2.8 fold induction for mutant sites A and C, respectively, and 1.9 fold stimulation with mutant site B (mB) (Fig. 3.6). When vitamin D responsiveness of the paired mutations was examined (Fig. 3.6), the contribution of the distal sites to functional activity of the VDRE was further defined. Notably, the mAB two-site mutation nearly eliminated vitamin D stimulatory activity, similar to the effect of the triple mutant. In contrast, two-site mutations involving the proximal site C (mAC and mBC) had less effect, reducing vitamin D responsiveness to 2.5 and 3 fold induction respectively, similar to
Figure 3.5 Loss of vitamin D responsiveness of the rat osteocalcin promoter with three mutated Runx sites (mABC): (A) Four independent ROS 17/2.8 cell lines with stably integrated WT promoters (WT₁, WT₂, WT₃, WT₄) and the triple Runx site mutation (mABC) cell lines, designated as T₁, T₂, T₃, T₄, were treated 3 days after plating for 24 hours with $10^{-8}$ M $1,25(OH)_2D_3$ and assayed for CAT activity normalized to total protein in the cell lysate. Each bar represents mean value of n=3 determinations.
Figure 3.6 Distal Runx sites A and B in the osteocalcin promoter are required for vitamin D enhancer activity: (A) ROS 17/2.8 cell lines containing stably integrated wild type and indicated Runx mutant promoter-CAT reporter constructs were examined for responsiveness to vitamin D (10^{-8} M, 1,25(OH)_{2}D_{3}, 24 hrs). pGEM control is a promoterless-CAT stable cell line. Enhancer activity of vitamin D (vitamin D-treated/control non-treated cells) is reported as fold induction. Each bar represents pooled data from n=3 or 4 separate cell lines, each assayed in triplicate in 2 to 4 independent experiments. Single asterisk designates p<0.001 statistical significance of mutant cell line versus WT.
Figure 3.6 Distal Runx sites A and B in the osteocalcin promoter are required for vitamin D enhancer activity: (B) ROS 17/2.8 cell transiently transfected with wild type and indicated Runx mutant promoter-CAT reporter constructs were examined for responsiveness to vitamin D (10⁻⁸ M, 1,25(OH)₂D₃, 24 hrs). Enhancer activity of vitamin D (vitamin D-treated/control non-treated cells) is reported as fold induction. Each bar represents pooled data from three different experiments (n=18).
the consequences of the single site C mutation. Thus, these results confirm a critical role for sites A and B, not only in basal expression of the OC promoter, but also in vitamin D regulation of osteocalcin promoter activity. These findings suggest that the Runx sites support structural organization of the osteocalcin promoter that is permissive for interaction of the distal VDRE and proximal TATA binding factors required for enhancer activity of the VDR/RXR complex [Haussler et al., 1997; Stein et al., 1997; Guo et al., 1997].

**Runx Binding Factors Contribute To Chromatin Organization Of The OC Promoter**

Previous studies carried out by our laboratory have shown the presence of DNase I hypersensitive sites in the native OC promoter as well as in the transgene of stable cell lines with an integrated OC promoter-CAT gene (pOCZCAT) [Montecino et al., 1994b; Montecino et al., 1996a; Montecino et al., 1996b]. These promoters exhibit two DNase I hypersensitive sites, designated DHS I in the basal promoter region and DHS II in the distal promoter encompassing the VDRE. The nuclease accessibility of both regions is functionally related to the extent to which the OC gene or the transgene (pOCZCAT) is expressed. Based on these findings and the observation that the triple Runx mutant (mABC) does not respond to vitamin D, we examined the effect of this mutation on nuclease hypersensitivity of the OC promoter. Figure 3.7 shows the consequences of DNase I digestion of nuclei from the ROS 17/2.8 cell line carrying the wild type 1.1 kb rat OC promoter transgene. Two DNase I hypersensitive sites are present both in the control cells and in cells treated with vitamin D (Fig. 3.7C). An increase in the DNase I hypersensitivity is observed following vitamin D treatment. In
Figure 3.7 DNase I hypersensitive profile of WT OC promoter: (A) Diagrammatic illustration of the OC promoter-CAT transgene showing the regions used as probes. (B) Schematic representation of the endogenous OC gene showing the Bam HI-Xba I fragment used as probe.
Figure 3.7 DNase I hypersensitive profile of WT OC promoter: Nuclei were isolated from untreated (left panels) and vitamin D-treated (10^8 M 1,25(OH)_2D_3) for 24 hours (right panels) ROS 17/2.8 stable cell lines having the 1.1 kb rat WT OC promoter. Nuclei were incubated with increasing amounts of DNase I from 0 to 5 units per 20 OD260 units of nuclei for 10 minutes at room temperature. DNase I concentrations (U/ml) are designated above the lanes of the Southern blot. The purified DNA was digested with Xba I to detect the transgene either with a Xba I-Nco I (C) or a Xba I- Ban I probe (D) and digested with BamH I to detect the endogenous gene with a Bam HI-Hind III probe (E). M represents markers from λ DNA digested with Hind III and EcoR I. The two DNase I hypersensitive sites (DHS I and DHS II) are indicated by filled arrowheads.
contrast, DNase I hypersensitive sites are not detected in nuclei from either untreated or vitamin D treated cells carrying the mABC OC promoter-CAT transgene (Fig. 3.8A). The specificity of the DNase I hypersensitive sites was confirmed by reprobing the blot with a 0.4 kb fragment (XbaI-BanI) that detected the vector backbone (Fig. 3.7D).

To ensure that the complete loss of DNase I hypersensitivity in mABC is not due to a nonspecific alteration of the promoter, we examined the DNase I profile of mutant BC, which retains significant basal activity and vitamin D responsiveness (Fig. 3.3 and 3.6). Interestingly we observed DNase I hypersensitivity in the distal domain (DHS II), which encompasses the VDRE (Fig. 3.9). Thus there is a correlation between vitamin-D enhanced transcription and the level of DNase I hypersensitivity. The competency of the mBC cell line to respond to vitamin D suggests that the ability of VDR receptor complex to interact with its specific binding sequence in the OC promoter remains intact. Significantly, in mBC, the proximal DHS I that resides over the mutated site C is very weak. Therefore modifications in DNase I hypersensitivity are linked to mutations in Runx sites. Together these results demonstrate that the chromatin structure of the OC promoter requires the integrity of the Runx sites.

The specificity of the contribution of Runx elements to chromatin structure is further demonstrated by the DNase I hypersensitivity of the native gene in the mABC cell line (Fig. 3.8B). The endogenous OC gene in the mABC mutant cell line retains DNase I hypersensitivity, which increases upon treatment with vitamin D. Similar responsiveness of the endogenous OC gene to DNase I was observed in the cell lines carrying the WT or the mBC transgenes (Fig. 3.7E, 3.9B). A schematic illustration summarizing these modifications in DNase I hypersensitivity of the WT and mABC
Figure 3.8 DNase I hypersensitive profile of mABC stable cell line: Nuclei isolated from control and vitamin D treated cells carrying the mABC transgene were digested with DNase I as described in figure 7. Notice the absence of DNase I hypersensitive sites in the transgene (A). Panel B shows the DNase I profile of the endogenous gene as control.
Figure 3.9 DNase I hypersensitivity profile of mBC stable cell line: Nuclei isolated from control and vitamin D treated cells carrying the mBC transgene were digested with DNase I as described in figure 3.7. DNase I hypersensitive sites in the transgene (A). Panel B shows the DNase I profile of the endogenous gene as control.
OC promoters is presented in figure 3.10. These studies provide compelling evidence that Runx factors are determinants of chromatin organization that supports transcriptional activity of the OC promoter.

**DISCUSSION**

The osteocalcin gene promoter provides a blueprint for defining factors that regulate osteoblast-specific expression of the gene. These factors contribute to the complexity of molecular mechanisms associated with development of the osteoblast phenotype. We and others [Banerjee et al., 1997; Banerjee et al., 1996b; Hoffmann et al., 1994; Towler et al., 1994a; Towler and Rodan, 1995; Towler et al., 1994b], have previously shown by deletion analysis of the rat osteocalcin promoter from -1.7 kb to -108 bp that a significant loss of transcriptional activity occurs when the proximal Runx site C is not present. This observation suggests either that there is functional redundancy of the Runx sites or that the distal recognition sites do not contribute to promoter activity. To understand the contribution of Runx factors to regulation of osteocalcin gene transcription, it was necessary to address function of the multiple Runx sites in the native osteocalcin promoter.

Our studies have established the importance of the distal Runx sites A and B to activity of the rat osteocalcin promoter in a genomic context. We used a series of cell lines with stable integrants of the -1.1 kb OC promoter or with site-specific mutations of each Runx sequence alone, as well as combinatorial mutations of two or all three Runx sites. While transient transfection assays have greatly expanded our knowledge of transcriptional mechanisms regulating gene expression, such transfected promoter-
Figure 3.10 Illustration of modifications in chromatin organization of the rat osteocalcin promoter: Top line diagrams a random array of nucleosomes across -1.1 kb of the rat osteocalcin promoter in cells which do not express OC, established by micrococcal nuclease digestion (Montecino 1996). Second line diagrams the actively transcribed OC promoter showing the span of two DNase I hypersensitive (DHS) sites together with regulatory elements in the proximal and distal DHS sites. The extent of DNase I hypersensitivity is compared for basal OC expression (WT-basal) and vitamin D treated (WT-vitamin D enhanced) osteoblasts (ROS 17/2.8) cells. No DHS - nondetectable DNase I HS in non-osseous cells or of the mABC OC promoter in osteoblasts.
reporter constructs may not completely reflect regulation of endogenous genes. In stable integrants of the OC promoter in ROS 17/2.8 cells, mutation of either site A or B results in a significant loss of promoter activity. Mutation of the proximal site C (OSE-2 [Ducy and Karsenty, 1995]) alone has the least effect in modifying bone-specific basal activity of the OC promoter, suggesting strong compensatory activity by the distal Runx sites. However, when we carried out Runx expression studies to confirm the importance of each Runx site, the results demonstrated that all three sites are necessary for maximal basal promoter activity. The more pronounced effects of the Runx site mutations observed when the OC promoter is stably integrated into the genome of ROS 17/2.8 cells suggest that the chromatin context strongly influences activity of the promoter.

Our results indicate that regulatory elements other than Runx sites contribute to osteocalcin gene transcription. We find that in transient assays, 40% of wild type OC promoter activity remains when Runx sites A, B and C are mutated (mABC, Fig. 3.3B), while 20% activity is retained by the mABC mutant in stable cell lines (Fig. 3.3C). This residual promoter activity of mABC is consistent with results from promoter-deletion analysis of the rat OC gene [Hoffmann et al., 2000; Hoffmann et al., 1996] and reflects the contribution of the highly conserved OC Box I (-99/-76) regulatory element, which is also necessary for tissue-specific basal expression.

The major products of the various Runx/AML genes have several shared domains that contribute to transcriptional regulation of tissue-specific genes; the Runx factors may function as architectural proteins that serve to assemble macromolecular complexes involved in gene regulation. These structurally and functionally
homologous segments include the conserved DNA binding runt homology domain, transcriptional activation and suppression domains [Hiebert et al., 1996; Lenny et al., 1995; Meyers et al., 1996], as well as subcellular targeting signals [Ito, 1997; Zeng et al., 1997]. The promoter organizing functions of Runx factors may involve Runx-interacting proteins, including Cbfβ [Banerjee et al., 1996a; Banerjee et al., 1997; Kanno et al., 1998; Speck and Stacy, 1995], ALY [Bruhn et al., 1997], and Groucho/TLE [Aronson et al., 1997; Guo et al., 1998; Levanon et al., 1998; Thirunavukkarasu et al., 1998]. Interestingly, Groucho/TLE proteins have been shown to contact the N-terminus of histone H3 [Fisher and Caudy, 1998; Palaparti et al., 1997]. The Runx class of transcription factors has also been shown to associate with the nuclear matrix [Ito, 1997; Zeng et al., 1997; Zeng et al., 1998], the structural scaffold of the nucleus, through a 31 amino acid nuclear matrix targeting sequence (NMTS) in the C terminus of full length Runx isoforms [Zeng et al., 1997] and unpublished data]. The NMTS directs Runx factors to transcriptionally active subnuclear sites [Zeng et al., 1998], similar to the NMTS-dependent targeting of the rat glucocorticoid receptor [Tang et al., 1998]. Together, the multiple protein-protein interaction domains of Runx factors may operate by a promoter architectural mechanism to functionally support physiologically regulated expression of the tissuespecific osteocalcin gene.

We have shown that mutation of the Runx sites results in a striking loss of responsiveness of the rat OC promoter to vitamin D and other physiological mediators of osteoblast differentiation, including glucocorticoids and TGFβ [Javed et al., 1998]. These signaling molecules regulate OC transcription through, respectively, VDR/RXR,
GR, and AP-1 factors that act at non-Runx elements. Furthermore, mutation of the three Runx sites results in complete loss of DNase I hypersensitivity and the dynamic vitamin D-dependent modifications in chromatin structure, which are essential for normal activity of the OC promoter. Indeed, a similar absence of DNase I hypersensitivity is observed in the silent endogenous OC gene within non-osseous cells (Fig. 3.10). Therefore, the competency of this promoter to undergo chromatin remodeling for maximal transcriptional responsiveness involves key contributions of Runx factors. Our results are consistent with the concept that Runx factors contribute to a promoter conformation that mediates accessibility or recruitment of factors to DNA regulatory elements.

Steroid hormone-dependent transcriptional activation is known to involve modification in chromatin organization. In the rat OC gene, we have established that binding of the vitamin D-liganded VDR/RXR complex to the VDRE in the distal promoter induces architectural changes in chromatin that facilitate requisite interactions with the proximal basal promoter complex [Guo et al., 1997; Zhou et al., 1994]. Glucocorticoid regulation of the MMTV promoter [Beato, 1996; Fragoso et al., 1998; Smith et al., 1997; Truss et al., 1996; Zaret and Yamamoto, 1984] involves the GR-mediated conversion of a repressive chromatin state to an open configuration allowing NF1 and Oct1 access to their binding sites. Subsequently, activation of transcription occurs through interactions of GR with the TFIID basal complex [Archer et al., 1992; Smith and Hager, 1997]. The results presented here suggest that interaction of the OC promoter with the nuclear matrix-associated Runx2 factor is an essential step for steroid hormone-dependent activity of the OC promoter.
The significance of the three Runx sites in the rat osteocalcin promoter, two of which flank the VDRE, in contributing to maximal expression and physiologic responsiveness of the gene is highlighted by the opposing effects of vitamin D on the mouse OC promoter [Clemens et al., 1997; Lian et al., 1997; Zhang et al., 1997a]. Vitamin D does not mediate enhanced activity of the mouse OC promoter [Zhou et al., 1994] and, in fact, the mouse OC VDRE exhibits weak downregulation by the hormone [Lian et al., 1997]. Consistent with this finding, the mouse VDRE sequence is not flanked by two functional Runx sites as occurs in the rat OC promoter. In the mouse OC promoter, the distal Runx site resides upstream of the VDRE in a similar position as in the rat promoter (nt -608 to -602), but site B (-441 to -435 in the rat), which is critically involved in vitamin D regulation of the rat promoter, is not present in the mouse promoter [Frendo et al., 1998]. Mutational analysis of the mouse Runx sites has established that the distal site contributes far less to transcription than the proximal Runx/OSE2 site [Frendo et al., 1998]. Clearly, our studies demonstrate that the distal Runx A and B sites are important for basal activity of the rat OC promoter and essential for vitamin D responsiveness. The subtle differences between mouse and rat in organization of Runx motifs may be necessary to regulate OC expression and responsiveness to physiologic mediators of bone formation and turnover at different levels dependent on the species. Thus, caution must be exercised when generalizing conclusions with respect to regulation of osteocalcin promoters from different species.

Many tissue-specific genes contain multiple Runx sites which are strategically positioned relative to other cis-acting elements [Gao et al., 1998; Ji et al., 1998; Selvamurugan et al., 1998]. This heterogeneity in promoter organization of Runx-
dependent genes, together with a series of context-dependent activation domains in the C-terminus of Runx factors, suggests an inherent difficulty in predicting the transcriptional effect of a given Runx site. This molecular complexity provides the necessary versatility to accommodate the different biological functions of the broad spectrum of Runx-regulated genes. Our studies provide the first evidence that Runx2 factors in osteoblasts regulate bone tissue-specific transcription not only through their DNA binding activity, but also as nuclear matrix associated factors that mediate chromatin organization and facilitate transcriptional activity by association with other transactivating factors.
CHAPTER 4

Basal Activity and Chromatin Remodeling of the Proximal Osteocalcin Promoter Are Dependent on VDR Binding at the Distal Promoter Element.
INTRODUCTION

Bone is one of the major physiologic sites for the biological action of 1,25 dihydroxyvitamin D₃, the active form of vitamin D. This steroid hormone regulates principal events associated with bone formation and resorption. The *in vivo* effects of vitamin D are largely mediated by a specific vitamin D receptor (VDR), which belongs to the steroid and thyroid hormone receptor superfamily [Truss et al., 1995; Mangelsdorf and Evans, 1995; Chambon, 1996]. VDR is a ligand-dependent transcription factor that is capable of recognizing and binding to cis-acting vitamin D responsive elements (VDREs) in the promoter region of target genes to induce or repress their transcription [Tsai and O'Malley, 1994; Darwish and DeLuca, 1993].

Osteocalcin (OC) is a bone-specific protein whose transcription is directly regulated by vitamin D [Lian et al., 1989b; Demay et al., 1989]. Transcriptional analysis has established a positive cis-acting element, located between nucleotides -461 and -445 of the rat osteocalcin promoter, that confers vitamin D responsiveness [Owen et al., 1990c; Terpening et al., 1991; Breen et al., 1994]. Enhancement of OC expression in response to vitamin D occurs only after basal tissue-specific transcription is initiated by bone-specific factors.

Initiation of basal expression, as well as enhancement by vitamin D, of rat osteocalcin gene transcription is accompanied by changes in the structural properties of chromatin [Montecino et al., 1994b; Montecino et al., 1996b]. In proliferating osteoblasts and cell lines that do not express OC, the promoter is uniformly packed in nucleosomes, which suppress transcription. However, in differentiated osteoblasts,
where OC is transcriptionally active, rearrangement of chromatin in the promoter region results in the appearance of two DNase I hypersensitive sites (DHS). These DHS are localized in two regions of the promoter that contain important transcriptional elements: the distal site (-600 to -400bp) spans the vitamin D responsive element (VDRE), two Runx binding sites and a YY1 binding motif, while the proximal site (-170 to -70bp) includes a Runx element, a C/EBP binding site and a Dlx5/Msx2 homeodomain motif. The presence and intensity of these DHS correlate with levels of OC transcription. For instance, in the ROS 24/1 cell line, which does not express OC no DNase I hypersensitivity is detected. However, in confluent cultures of rat osteoblastic ROS 17/2.8 cells, where OC is transcriptionally active, the presence of two DHS is detected. Treatment of cultures with vitamin D results in transcriptional induction of OC and the DHS are significantly intensified [Montecino et al., 1994b]. Furthermore, recent studies have shown that the transcriptionally active rat OC gene promoter is associated with acetylated histones H3 and H4 and the acetylation of these histones is increased in response to vitamin D [Shen et al., 2002].

It is generally accepted that binding of transcription factors to their cognate elements on a promoter allows the recruitment of coactivators, chromatin remodeling complexes and histone modifying enzymes and results in the reorganization of the chromatin necessary for transcriptional activation of a gene [Dynlacht et al., 1991; DiRenzo et al., 2000; Myers and Kornberg, 2000; Narlikar et al., 2002; Kornberg and Lorch, 1999; Cheung et al., 2000; Berger, 2002]. In this study we have addressed the role of vitamin D receptor interaction with the rat osteocalcin VDRE in mediating the chromatin modifications required for OC expression. This VDRE is a multipartite
element with recognition sequences for the transcription factors YY1 and AP-1 partially overlapping the steroid half elements [Owen et al., 1990b; Guo et al., 1997]. Previous studies have shown that forced expression of YY1 decreases vitamin D dependent enhancer activity of the OC gene in a dose dependent manner [Guo et al., 1997] and mutation of the AP-1 binding site severely inhibited vitamin D responsiveness [Ozono et al., 1990]. Liganded VDR/RXR can interact with and recruits to the target gene promoter coactivators such as p300, SRC-1, a member of p160 family that possesses HAT activity, and DRIP, a protein complex that helps the transcription machinery to contend with the nucleosome structure in an, until now, undefined way [Rachez et al., 1999; Freedman, 1999]. The particular coregulatory proteins recruited by VDR may contribute to the specificity of OC transcriptional regulation. Our results demonstrate that mutation of the VDRE results in increased basal activity of the OC gene, although formation of basal DNase I hypersensitivity is not altered. In contrast, transcriptional upregulation and increased accessibility of DNase I in response to vitamin D require an intact VDRE motif. We also find that chromatin remodeling of the proximal OC promoter is altered in the VDRE mutant (mSHE). Thus binding VDR/RXR is determinant to assure a tight control of OC expression and to fine-tune the chromatin changes required for OC expression.
RESULTS

Mutation Of The Vitamin D Responsive Element (VDRE) Results In Increased
Basal Transcriptional Activity Of The OC Promoter

In order to assess the contribution of the VDRE in regulating native OC
promoter activity and its potential role in the chromatin remodeling that accompanies
OC gene expression, we established a series of monoclonal stable cell lines of rat
osteoblastic ROS 17/2.8 cells. These cells harbor a 1.1Kb OC promoter with point
mutations in each of the two Steroid Half Elements of the VDRE (mSHE)(Fig. 4.1), or
in the three Runx binding sites (mABC). Although both YY1 and AP-1 binding sites
partially overlap the VDRE, the introduced mutations abolish VDR binding but they do
not affect binding of AP-1 or YY1 to their respective binding motifs [Aslam et al.,
1999].

The effects of these mutations on OC promoter basal activity were first
analyzed by transient transfection in ROS 24/1 cells, which do not express the VDR
[Baran et al., 1991]. No significant difference could be observed in these cells between
the wild type and mutant OC promoters (Fig. 4.2A). However, significant differences
in promoter activity were noted in ROS 17/2.8 cells, which represent a mature
osteoblast phenotype and express the VDR as well as the endogenous OC gene. The
basal activity of the SHE mutant was higher (2.5x) than wild type, and mABC was
lower (4x) as noted previously (Fig. 4.2B, see also chapter 3). Similar differences in
basal activity were observed in monoclonal stable cell lines carrying either the wild
type or the mutated promoters (Fig. 4.2C). These results suggest that basal levels of
OC transcription are influenced by VDR binding.
Figure 4.1 Rat Osteocalcin promoter: The position of the VDRE relative to Runx, C/EBP and the two primary transcriptional elements required for basal transcription, the OC-box and the TATA box, are indicated. Lower panel shows the wild type and mutated VDRE sequences used to generate the pSHE plasmid. The introduced mutations do not affect the binding to the overlapping YY-1 and AP-1 motifs in the VDRE (Aslam 1999 Endocrinology 140: 63-70).
Figure 4.2 Basal activity of wild-type and mutated OC promoters: Basal activity of plasmids carrying wild-type or mutated OC promoters was assayed by transient transfection in ROS 24/1 (A) or ROS 17/2.8 cells (B) (n=12). Cells were transfected with 1 ug of promoter-reporter construct using SuperFect transfection reagent (Qiagen, Inc., CA). pGEM is an empty vector used as control. Cells were treated with 10^{-8}M 1,25(OH)_{2}D_{3} and harvested 24 h post-transfection to determine CAT activity. Panel (C) average basal activity of five independent monoclonal cell lines carrying the indicated OC-plasmids.
We next tested the ability of vitamin D to induce promoter activity of the stable cell lines (Fig. 4.3). Monoclonal cell lines carrying the mSHE-CAT transgene completely lack responsiveness to vitamin D, confirming the loss of VDR-DNA interaction. A similar loss of responsiveness was also observed with cell lines carrying the mABC transgene (where all Runx binding sites are mutated) as previously reported [Javed et al., 1999]. In contrast, cell lines harboring the wild-type transgene showed a 4-5-fold increase in promoter activity upon treatment with vitamin D (Fig. 4.3A). For comparison, the effectiveness of vitamin D treatment on the activity of the endogenous OC gene in these cell lines was assessed by measuring secreted osteocalcin levels (Fig. 4.3B). All cell lines presented a 3-6-fold induction of OC in response to vitamin D. Thus these data suggest that all cell lines maintain their osteoblastic phenotype and that the loss of vitamin D responsiveness by mSHE and mABC is due to mutation in the promoter.

The observation that mutation of Runx binding sites abolishes vitamin D responsiveness prompted us to test the effect of VDRE mutation on Runx2 dependent activation of the OC promoter (Fig. 4.4). For these experiments we used HeLa cells because they do not express Runx2 (see Fig. 5.6A) and lack any Runx DNA binding activity [Armesilla et al., 1996]. As shown in figure 4.4, VDRE mutation did not abolish Runx2 activation of the OC promoter. In contrast, Runx2 activation of mSHE was significantly higher (25 fold) than wild type (10 fold). Also, the expected loss of Runx2 induction was seen for the mABC promoter. Thus, these findings demonstrate that mutation of the VDRE motif increases basal and Runx stimulated expression of the OC promoter.
Figure 4.3 Vitamin D responsiveness of stable cell lines: (A) Loss of vitamin D responsiveness of the rat OC promoter with mutation either in VDRE (mSHE) or in the three Runx binding sites (mABC). Five independent cell lines for each transgene were treated 3 days after plating with 10^{-8}M 1,25(OH)_{2}D_{3} and assayed 24 h later for CAT activity. The CAT values were normalized to total protein in the cell lysate. Each bar represents the mean value of at least three independent determinations for each cell line. (B) The levels of secreted OC in the culture media were measured by RIA. The values are plotted as fold induction (Vitamin D/Control).
Figure 4.4 Runx2-mediated activation of mutated OC promoters in HeLa cells: The Runx2 expression construct (0.4 μg) and the indicated OC-CAT plasmids (1μg) were co-transfected into HeLa cells and assayed 24 h after transfection. CAT activity was calculated as percent conversion and normalized for luciferase values used as internal control (n=9). The values are plotted as fold induction (Overexpressed Runx2/Control).
Basal DNase I Hypersensitivity Of The OC Promoter Is Unaffected By Mutation Of The VDRE

Previous studies from our laboratory have shown the presence of DNase I-hypersensitive sites in the endogenous OC gene and in transgenes of stable cell lines with an integrated OC promoter [Montecino et al., 1996a]. As noted earlier, this hypersensitivity was absent in stable cell lines carrying the mABC plasmid that harbors mutations in all three Runx sites (Fig. 4.5; see also chapter 3). Because the distal hypersensitive site (DHS II) encompasses the VDRE and vitamin D induction of OC transcriptional activity results in an increased DNase I accessibility of the OC promoter, we examined the effect of the VDRE mutation on nuclease hypersensitivity during basal and vitamin D-induced transcription. Figure 4.5 top panel shows the results of DNase I digestion of nuclei isolated from the ROS 17/2.8 cell line carrying the mSHE plasmid. The two DNase I hypersensitive sites are present both in the control cells and in cells treated with vitamin D. However, a decrease in nuclease accessibility, especially at the distal DHS, was observed after vitamin D treatment (Fig. 4.5 top right panel). The responsiveness of these cell lines to vitamin D treatment was confirmed by analyzing the DNase I digestion profile of the endogenous OC gene. As expected, the nuclease accessibility of the endogenous gene was increased by vitamin D treatment (Fig. 4.5 middle panel). The lower panel in figure 4.5 shows the DNase I profile of a monoclonal stable cell line carrying the mABC plasmid, as it has been previously described (Fig. 3.8) no DNase I hypersensitive sites were present in the mutant promoter. Taken together these results demonstrate that appearance of the two DHS
Figure 4.5 DNase I-hypersensitivity profile of the endogenous OC gene and OC-CAT transgenes: Nuclei isolated from untreated (control) or vitamin D treated (10^{-8} vitamin 1,25(OH)_{2}D_{3} for 24 h) ROS 17/2.8 monoclonal stable cell lines were incubated with increasing amounts of DNase I for 10 min at room temperature. The DNase I concentrations (U/ml) are designated above each lane of the Southern blot. The DNA was then purified and digested with XbaI to detect the transgene or BamHI to detect the endogenous gene. All samples (10μg) were resolved on a 1.2% agarose gel, transferred to a Hybond N+ membrane and hybridized with the corresponding probe. The two DNase I hypersensitive sites (DHS I and DHS II) are indicated by the solid arrows.
associated with initial chromatin remodeling during OC gene activation is independent of VDR interaction.

**Mutation Of VDRE And Runx Elements Alters The Chromatin Structure Of The OC Promoter**

We next examined accessibility of the mutated promoters to restriction endonucleases, as an indication of modifications in nucleosome positioning during basal and vitamin D induced states of OC gene expression. We selected restriction sites in the proximal and distal promoter regions, which undergo extensive remodeling during both OC gene activation and vitamin D mediated enhancement [Montecino et al., 1996b]. The digestions were performed on nuclei isolated from ROS 17/2.8 stable cell lines carrying mSHE (VDRE) and mABC (Runx) transgenes. As shown in figure 4.6A, the endogenous OC gene is highly accessible to all the restriction enzymes tested, and vitamin D treatment results in an enhanced cleavage by PstI, PvuII and HincII. The percent digestion by each enzyme and changes mediated by vitamin D treatment are summarized in Table 4.1. Although the overall pattern of restriction accessibility seen for the mSHE transgene is similar to that of the endogenous OC gene (Fig. 4.6A, 4.6B, Table 4.1), the percent of digestion by PstI, PvuII, and BglII is substantially lower than for the wild-type promoter. Interestingly, the changes are restricted to this region only, as the level of HincII digestion is essentially the same in the wild type and the VDRE mutant promoter (Table 4.1). However, in contrast to the wild type OC promoter, the mutated transgene shows no change in restriction cleavage upon vitamin D treatment, consistent with the loss of vitamin D responsiveness (Fig. 4.3A).
Figure 4.6 Restriction enzyme digestion of endogenous and mutated OC promoters: (A) Restriction digestion profile of endogenous OC promoter from mSHE cell line. ROS 17/2.8 monoclonal stable cell lines were treated with vitamin D for 24 h. Nuclei were isolated and digested with different restriction enzymes for 30 min at 37°C. Purified DNA (10 µg) was digested BamHI to analyze the endogenous OC gene, fractionated electrophoretically in a 1.2% agarose gel, transferred to Hybond N+ membrane and hybridized with a BamHI-HindIII probe.
Figure 4.6 Restriction enzyme digestion of endogenous and mutated OC promoters:
(B) Restriction digestion profile of the mSHE transgene. ROS 17/2.8 monoclonal stable cell lines were treated with vitamin D for 24 h. Nuclei were isolated and digested with different restriction enzymes for 30 min at 37°C. Purified DNA (10 μg) was digested XbaI to analyze the transgene, fractionated electrophoretically in a 1.2% agarose gel, transferred to Hybond N+ membrane and hybridized with a Xba I-Nco I.
Figure 4.6 Restriction enzyme digestion of endogenous and mutated OC promoters: (C) Restriction digestion profile of mABC transgene. Nuclei were isolated and digested with different restriction enzymes for 30 min at 37°C. Purified DNA (10 μg) was digested XbaI to analyze the transgene, fractionated electrophoretically in a 1.2% agarose gel, transferred to Hybond N+ membrane and hybridized with a Xba I-Nco I.
TABLE 4.1
PERCENT OF RESTRICTION ENZYME DIGESTION

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Hinc II</th>
<th>Bgl II</th>
<th>Pvu II a</th>
<th>Pvu II b</th>
<th>Pst I</th>
<th>Pvu II c</th>
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</thead>
<tbody>
<tr>
<td>WT (Endogenous)</td>
<td>C</td>
<td>28±1.4</td>
<td>55±3</td>
<td>nd</td>
<td>66±3.7</td>
<td>67±2</td>
</tr>
<tr>
<td>D</td>
<td>50±0.9</td>
<td>56±2.5</td>
<td>nd</td>
<td>79±4</td>
<td>76±1</td>
<td>na</td>
</tr>
<tr>
<td>mABC</td>
<td>C</td>
<td>9±0.3</td>
<td>31±0.6</td>
<td>8±0.9</td>
<td>18±1.2</td>
<td>22±1</td>
</tr>
<tr>
<td>D</td>
<td>11±1</td>
<td>31±1.5</td>
<td>7±0.5</td>
<td>18±0.6</td>
<td>21±2</td>
<td>12±1</td>
</tr>
<tr>
<td>mSHE</td>
<td>C</td>
<td>33±2.3</td>
<td>40±2.3</td>
<td>12±0.8</td>
<td>30±1</td>
<td>44±1</td>
</tr>
<tr>
<td>D</td>
<td>28±0.7</td>
<td>37±0.8</td>
<td>11±0.5</td>
<td>33±1.4</td>
<td>45±2</td>
<td>18±0.8</td>
</tr>
</tbody>
</table>

nd: no determined
na: no applicable

Restriction enzyme accessibility of wild type and endogenous promoters was assay in three different monoclonal cell lines for each promoter. Digestion product were analyzed by Southern blots (Fig 4,6), quantitated by Phospholmager analysis and expressed as Percent Digestion [100*product/(product + substrate)].
The mABC Runx mutant transgene showed nearly complete loss of cleavage by HincII (9%) and a significantly decreased accessibility to PstI (22% vs 67%) (Fig. 4.6C, Table 4.1). There were no changes in restriction enzyme accessibility upon treatment with vitamin D. These observations are consistent with the low basal promoter activity as well as the lack of vitamin D responsiveness of the OC promoter in the mABC cell lines (Fig. 4.2C, 4.3A). Notably the cleavage pattern observed for the mABC transgene partially resembles the digestion pattern obtained for the transcriptionally inactive OC promoter [Montecino et al., 1996b].

Taken together with the transcription results, these data support the concept that chromatin remodeling at the distal (HincII site) and proximal (PstI site) promoter regions are required for maximal OC expression and that Runx sites are essential for chromatin remodeling of the OC promoter. Moreover, the decreased accessibility of the mSHE OC promoter downstream of the VDRE motif but normal levels of digestion upstream of it suggests that at least two independent chromatin remodeling processes occur at the OC promoter. One of them is dependent of Runx2 binding and results in formation of the two DHS observed in the active OC promoter, as well as a general increase in accessibility of the OC promoter region. The second event is related to VDR binding, and has as a consequence an increased opening of the chromatin towards the transcription initiation site.

**Histone H3 Acetylation At The OC Promoter Is Increased By Mutation Of The VDRE But Unaffected By Runx Mutation**

Remodeling of chromatin structure at the OC promoter in response to vitamin D is accompanied by increased acetylation of both histone H3 and H4 [Shen et al., 2002].
In order to investigate histone modifications during recruitment of Runx2 and VDR to the OC promoter, we performed chromatin immunoprecipitation (ChIP) using antibodies that detect acetylated histones H3 and H4. To distinguish the endogenous OC promoter from the transgene, we designed primers with 3’ nucleotides carrying either the wild type or the mutated sequences (Table 4.2). These primers were first tested for their ability to selectively amplify the mutant versus the wild type gene. As shown (Fig. 4.7, right panel), under the PCR conditions used each set of primers amplified selectively the respective promoter.

We then proceeded to evaluate the acetylation status of the histones associated with each of these promoters. We find acetylation of both H3 and H4 at the endogenous OC locus as previously reported [Shen et al., 2002]. Acetylation levels for histones H3 and H4 at the transgene carrying mutated Runx binding sites (mABC) and acetylation levels for histone H4 at the mSHE transgene are comparable to the levels at the endogenous OC locus (Fig. 4.7). However, acetylated H3 levels at the SHE mutant promoter are higher than the wild type. It has been shown that, in vitro, histone acetylases (HATs) exhibit a differential specificity for the four histones [Roth et al., 2001]; therefore our results suggest that different HAT activities are recruited at the wild type and the mSHE promoter under conditions of basal expression.

Vitamin D treatment results in increased acetylation of both H3 and H4 at the endogenous OC locus [Shen et al., 2002]. However, no significant changes in acetylation were observed at either of the transgene promoters in response to vitamin D (Fig. 4.7). These data are consistent with the loss of vitamin D response for transcription, DNase I and restriction enzyme accessibility exhibited by the mSHE and
Figure 4.7 Association of acetylated Histone H3, H4 and RNA polymerase II with endogenous and mutated OC promoters: Chromatin immunoprecipitation assays were performed with formaldehyde-crosslinked chromatin isolated from control or vitamin D treated monoclonal ROS17/2.8 stable cell lines. The antibodies used were against either acetylated H3 and acetylated H4 or RNA polymerase II. (A) Representative autoradiographs of PCR products obtained from the chromatin immunoprecipitated with the indicated antibodies. The right panel (plasmids) shows the specificity of primers in selective amplification of wild-type versus mutant plasmid (- no PCR). (B) Data in A were quantitated by PhosphoImager analysis and normalized vs. input levels (n=5).


**TABLE 4.2**

**PRIMERS USED FOR PCR REACTIONS**

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence 5’-3’</th>
<th>T Ann. (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AwtF</td>
<td>(-619) GCTCTCCCCCATCAAAACC (-620)</td>
<td>60</td>
</tr>
<tr>
<td>AmF</td>
<td>(-619) GCTCTCCCCCATCAAAAAG (-602)</td>
<td>55</td>
</tr>
<tr>
<td>BwtR</td>
<td>(-421) AAACCCCAAGAAGGAGCGG (-439)</td>
<td>60</td>
</tr>
<tr>
<td>BmR</td>
<td>(-421) AAACCCCAAGAAGGAGCAA (-439)</td>
<td>55</td>
</tr>
<tr>
<td>VDRE.2F</td>
<td>(-475) AGCTGCCCTGCACTGGG (-459)</td>
<td>60</td>
</tr>
<tr>
<td>SHE.2F</td>
<td>(-476) GAGCTGCCCTGCACTGTA (-459)</td>
<td>60</td>
</tr>
<tr>
<td>OC.7R</td>
<td>(-319) CATAGCCTAGAGGAGGTACA (-338)</td>
<td>60</td>
</tr>
<tr>
<td>CwtF</td>
<td>(-141) GTCAACCAACCACAGCATCCTTTG (-119)</td>
<td>60</td>
</tr>
<tr>
<td>LMOC.3</td>
<td>(+20) AGCAGAGAGGAGGTCCTCATG (-1)</td>
<td>60</td>
</tr>
<tr>
<td>LMCAT.3</td>
<td>(+45) TAGCTCCTGAAATCTCGCCAAG (+23)</td>
<td>60</td>
</tr>
<tr>
<td>rMYOG F1</td>
<td>(28) CGACCTGATGGAGCTGTATG (47)</td>
<td>60</td>
</tr>
<tr>
<td>rMYOG R</td>
<td>(201) GGACAATGCTCAGGGGTCC (220)</td>
<td>60</td>
</tr>
</tbody>
</table>
mABC promoters. Taken together our results for DNase I and restriction enzyme accessibility confirm that no changes in chromatin organization of the OC promoter are produced in response to vitamin D treatment when either the VDRE (mSHE) or all the Runx binding sites (mABC) are mutated.

We also compared histone modifications at the myogenin locus, a muscle specific gene, as a prototype for a gene that is inactive in osteoblastic ROS 17/2.8 cells. Acetylation of H3 or H4 was not observed at this locus indicating the silent state of the myogenin gene in osteoblasts (Fig. 4.7).

To confirm that wild type and mutated OC promoters are transcriptionally active, we performed ChIP assays using antibodies specific to hyperphosphorylated RNA polymerase II (RNA pol II), which recognizes the active (elongating) form of the polymerase. Our results show that RNA pol II is present on both the wild type and the mutant promoters; however, the levels are significantly higher on the SHE mutant promoter. Thus the high basal level of transcriptional activity observed for the SHE promoter (Fig. 4.2B, 4.2C) is well correlated with the levels of pol II detected at the promoter. Interestingly, a significant increase in pol II recruitment upon treatment with vitamin D was observed only for the wild-type promoter. Taken together, the levels of H3-H4 acetylation and recruitment of pol II are reflective of the activities of the wild type and mutant promoters either in basal or vitamin D induced conditions.

**DISCUSSION**

The vitamin D element in the bone tissue-specific osteocalcin gene has served as a prototype for understanding molecular mechanisms regulating physiologic
responsiveness of vitamin D-dependent genes in bone cells. Vitamin D promotes cellular differentiation and the significant upregulation of OC transcription, a marker of the mature osteoblast phenotype, reflects this physiological effect on osteoblasts maturation. Vitamin D enhanced activity of the OC gene is tightly regulated to ensure physiological levels of OC in bone cells.

In this study, we found that mutation of the VDRE (mSHE) results in an increased basal activity of the rat OC promoter accompanied by complete loss of vitamin D responsiveness. As mentioned earlier, the rat OC VDRE contains an internal AP-1 and a partially overlapping YY1 element. Previous studies from our group have shown that in proliferating osteoblasts, high expression levels of c-fos and c-jun contribute to suppression of the OC promoter; while in differentiated osteoblasts, fra2 and junB accounted for high levels of OC expression. Moreover, mutation of the AP-1 binding element severely inhibits vitamin D response suggesting a possible interaction between AP-1 and VDR/RXR to facilitate enhancer activity [McCabe et al., 1996; Aslam et al., 1999]. In vitro analysis of the SHE mutant demonstrated that AP-1 binding is not affected [Aslam et al., 1999]. It is possible, however, that in vivo the absence of VDR binding results in an enhanced binding of AP-1 family members to the OC promoter. On the other hand, the YY1 binding site partially overlap VDRE and forced expression of YY1 decreased vitamin D enhanced transcription, suggesting that YY1 competes with VDR for OC binding [Guo et al., 1997]. Recently it has been shown that over-expression of YY1 in the absence of vitamin D induction results in increased OC expression (Montecino personal communication). Therefore mutation of the VDRE may result in YY1 binding and increased promoter activity. An alternative
explanation for these results is based on the observation that many nuclear receptors activate gene expression in response to ligand binding and repress gene expression in its absence [Xu et al., 1999; Torchia et al., 1998; Nagy et al., 1997; Heinzel et al., 1997]. Therefore it is possible that mutation of the VDRE abolishes binding of unliganded VDR to the OC promoter resulting in an increased basal activity as well as a concomitant loss of vitamin D induction.

Ligand binding to nuclear receptors induces a series of conformational changes that allow the formation of dimers and recruitment of co-activator complexes [Glass and Rosenfeld, 2000; Xu et al., 1999; Darimont et al., 1998]. Until now, two distinct classes of nuclear receptor co-activator complexes have been identified. One class contains co-activators that are proposed to act by altering chromatin structure and includes complexes with histone acetyl transferase activity, such as CBP/p300 and p160 (SRC-1/TIF2/AIB1) family proteins [Kamei et al., 1996; Ogryzko et al., 1996; Onate et al., 1995; Voegel et al., 1998; Anzick et al., 1997; Heinzel et al., 1997] and the ATP-dependent chromatin remodeling complex SWI/SNF [Östlund Farrants et al., 1997; Ichinose et al., 1997; Fryer and Archer, 1998]. The second class corresponds to a co-activator complex, DRIP/TRAP/ARC [Fondell et al., 1996; Gu et al., 1999; Ito et al., 1999; Rachez et al., 1999], without known chromatin remodeling activity; it is worth noting that many components of this complex are also present in a mammalian complex that corresponds to the yeast transcriptional mediator complex [Gu et al., 1999].

Analysis of the chromatin structure in stable cell lines carrying the mSHE OC promoter demonstrates that both of the DNase I hypersensitive sites identified in the
endogenous OC promoter are present under basal conditions. However, treatment with vitamin D results in a decreased hypersensitivity, especially at the distal DHS that correlates with the decrease in transcriptional activity observed upon vitamin D treatment of the mSHE cell line. Interestingly a competition model has been proposed to explain the antagonism observed between AP-1 and VDR in genes that contain binding sites for only one of these factors [Kamei et al., 1996]. According to this model, limiting amounts of CBP/p300 or general transcription factors are partitioned by the alternative activation of VDR by ligand or of AP-1 by phosphorylation. It is possible then that activation of VDR results in a loss of co-activators from the OC promoter when the VDRE is mutated.

As has been previously published [Javed et al., 1999] an OC promoter carrying mutations in all Runx sites (mABC) does not respond to vitamin D treatment and does not exhibit DNase I hypersensitivity. In the present work, we show that the accessibility of the mABC promoter to restriction enzymes is dramatically reduced, resembling the digestion pattern obtained when the gene is inactive [Montecino et al., 1996b]. In contrast, stable cell lines carrying the mSHE promoter show a significantly decreased accessibility only in restriction sites located downstream of the VDRE. However, except for acetylated H3 that is increased at the mSHE promoter, histone acetylation levels are similar in mutated and wild type promoters. These results suggest that a different mechanism of chromatin remodeling is responsible for the changes in DNA accessibility.

Taken together our results indicate that the chromatin remodeling of OC promoter required for its full activity and hormonal responsiveness is a complex
process that involves multiple steps and probably the recruitment of different co-activators either simultaneously or in a sequential fashion. Future studies should aim to determine the sequential order of events required for the re-arrangement of chromatin at the OC locus once this gene start to being expressed during osteoblast differentiation.
CHAPTER 5

CCAAT/enhancer-binding Proteins (C/EBP) Beta and Delta Activate Osteocalcin Gene Transcription and Synergize With Runx2 at the C/EBP Element to Regulate Bone-specific Expression.

Gutierrez S, Javed A, Tennant DK, van Rees M, Montecino M, Stein GS, Stein JL, Lian JB.

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Northern blots and electrophoretic mobility shift assays included in this chapter were done by Amjad Javed, Ph.D.
INTRODUCTION

The CCAAT/ enhancer-binding proteins (C/EBPs) comprise a family of transcription factors that are critical for normal cellular differentiation and metabolic functions in a variety of tissues. There are currently six members of the C/EBP family designated as C/EPBα, β, δ, γ, ε and ζ [Lekstrom-Himes and Xanthopoulos, 1998] most of which are expressed in liver, spleen and adipocytic tissues. However, more selective expression in other tissues has been observed among the family members [Alam et al., 1992; Antonson and Xanthopoulos, 1995; Birkenmeier et al., 1989; Morosetti et al., 1997; Wang et al., 1995; Yamanaka et al., 1997]. Isoforms of the C/EBP proteins are known and all function by homo- or heterodimerization with one another and interaction with other transcriptional activators or co-activators such as NF-kB, Stat3, c-Myb, PU.1, SP-1, ATF-2, PPARγ and Runx1 [Lopez-Rodriguez et al., 1997; Oelgeschlager et al., 1996; Shuman et al., 1997; Spiegelman et al., 2000; Stein and Lian, 1993; Zhang et al., 1996a; Zhang et al., 1996b].

Very little is known about the role of C/EBP factors in osteogenesis. Targeted disruptions of C/EBP genes have been performed, but in none of the studies were gross abnormalities of the skeleton observed [Wang et al., 1995; Tanaka et al., 1995; Sterneck et al., 1997; Tanaka et al., 1997]. However, recent studies have identified C/EBP regulation of genes expressed in osteoblasts. The insulin-like growth factor 1 (IGF-1) is a key regulator of osteoblast growth and differentiation [Gabbitas and Canalis, 1998]. C/EBPδ enhances either basal or prostaglandin E2-activated transcription of the insulin-like growth factor I promoter in osteoblasts [Umayahara et
Expression of COX-2 and the α1 subunit of Type I collagen are also regulated in osteoblasts by C/EBP factors [Attard et al., 2000; Ogasawara et al., 2001]. The interaction of C/EBPα with a Runx1 factor [Petrovick et al., 1998] is also particularly relevant for postulating a role for C/EBP factors in osteoblast differentiation.

The Runt related transcription factors (Runx/AML/Cbfα/PEBP2α) represent essential gene regulatory proteins that control lineage commitment for hematopoiesis [Okuda et al., 1996; Speck et al., 1999; Wang et al., 1996] and osteogenesis [Komori et al., 1997; Mundlos et al., 1997]. Runx2 (AML3/Cbfα1/PEBP2αA) is the most abundant Runt related protein in osteogenic and chondrogenic cell lineages [Banerjee et al., 1997; Ducy et al., 1997; Javed et al., 2001]. Genetic ablation of the Runx2 gene causes developmental defects in osteogenesis [Komori et al., 1997] and hereditary mutations in the Runx2 gene are linked to specific ossification defects as observed in cleidocranial dysplasia [Otto et al., 1997]. Runx2 is essential for osteoblast differentiation [Banerjee et al., 1997; Ducy et al., 1997; Komori et al., 1997] and regulates expression of numerous bone-related genes [Banerjee et al., 1997; Jimenez et al., 1999; Javed et al., 2001; Ji et al., 1998; Sato et al., 1998]. The importance of Runx2 in expression of the bone-specific osteocalcin (OC) gene is well documented [Javed et al., 1999; Frendo et al., 1998]. Thus Runx2 performs specialized functions during bone-tissue development and differentiation in vivo. However, it is noteworthy that osteoblast-specific transcription of osteocalcin occurs even in the absence of Runx sites in the rat OC promoter [Hoffmann et al., 1996], suggesting a tissue-specific role for other regulatory factors in osteoblasts.
Activation of tissue specific genes is controlled by combinatorial mechanisms that rely on local features of the promoters, including organization of control elements in the target genes and/or the interplay between DNA binding proteins and various transcriptional co-regulators [Graves, 1998]. Both C/EBPβ and Runx factors have been shown to cooperate with chromatin remodeling factors (p300, SWI/SNF) and other enhancer-binding proteins [Kitabayashi et al., 1998; Kowenz-Leutz and Leutz, 1999]. For example, Ets-1, c-Myb, Sp1 and C/EBP, together with Runx factors, stimulate the transcription of hematopoietic and osteogenic genes [Petrovick et al., 1998; Sun et al., 1995; Zaiman and Lenz, 1996; Zhang et al., 1996a; Zhang et al., 1996b; Gu et al., 2000] while PPARγ and Stat3 interactions with C/EBPα are driving forces for adipocyte differentiation [Rosen et al., 2000]. Given these observations, i.e., the presence of both C/EBPβ and δ and Runx2 in osteoblasts [McCarthy et al., 2000; Banerjee et al., 1997; Ducy et al., 1997] and C/EBPα-Runx1 protein-protein interactions in regulation of a hematopoietic specific gene [Zhang et al., 1996a], we addressed the possible role of C/EBP factors in osteoblasts and in the regulation of a bone specific gene, osteocalcin.

Here we report that C/EBP β and δ, but not α are developmentally expressed during osteoblast differentiation and are upregulated in response to 1,25(OH)2D3, a hormone that promotes osteoblast differentiation. We have identified a C/EBP responsive regulatory element in the proximal promoter of the bone specific osteocalcin gene. Deletion or mutation of this motif abrogates transcriptional enhancement by C/EBP factors. Furthermore, we provide the first demonstration that Runx2 and C/EBPβ physically interact and that C/EBP and Runx proteins act synergistically to
activate the OC promoter. Importantly, this functional synergism is mediated through the C/EBP element. These findings establish for the first time a role of C/EBP in the regulation of an osteoblast specific gene and define a novel mechanism for C/EBP in the regulation of cell type specific gene transcription.

RESULTS

C/EBP Family Members Are Expressed During Osteoblast Differentiation And Are Regulated By Vitamin D3

We initially assessed the expression of C/EBP factors in various bone tissues and during development of the osteoblast phenotype. Figure 5.1A shows that C/EBP β and δ mRNAs are present in calvarial tissue (lane 2) at levels similar to those in a representative soft tissue, muscle (lane 1). C/EBP α is not detected in bone tissue, consistent with its pivotal role for adipogenesis. Osteoblast markers (OC, Runx) that are not present in soft tissues are shown for comparison. The abundance of C/EBP β and δ in bone prompted examination of expression of the C/EBP family members from growth to differentiation stages of osteoblasts in vitro, representing proliferation (days 3-5), matrix maturation (days 7-12), and the mineralization stage (days 19-22), reflected by peak levels of histone H4, alkaline phosphatase (ALP) and osteocalcin (OC), respectively (Fig. 5.1B).

Both C/EBP β and δ mRNAs are detected from the growth to maturation stages and are expressed in a bi-phasic pattern. In contrast, C/EBP α expression is not detected at any stage of osteoblast differentiation, consistent with its absence in bone
Figure 5.1 Bi-phasic expression and vitamin D regulation of C/EBP transcription factors during rat osteoblast differentiation and presence in bone tissues: (A) Total RNA was isolated from various skeletal and soft rat tissues as described in Methods. RNA (10 μg) was resolved in a 1% formaldehyde gel and transferred to nylon membrane. The blots were hybridized with NcoI fragment for C/EBPα, EcoRI-XhoI fragment for C/EBPβ, BamHI-EcoRI fragment for C/EBPδ, EcoRI fragment for alkaline phosphatase, HindIII-XbaI fragment for histone H4, BamHI-XhoI fragment for Runx2, EcoRI-HindIII fragment for GAPDH, and EcoRI-BamHI fragment for the detection of rat OC. C/EBPβ and δ mRNAs are expressed in both soft and skeletal tissues. Lane 1, muscle and lane 2, day 21 fetal calvaria. (B) Primary rat osteoblasts were cultured for the indicated time (days) and total RNA (10 μg) was resolved in a 1% agarose gel and probed with the above mentioned cDNA fragments. The relative positions of 28S, 18S and 5S ribosomal RNA are indicated for reference.
Figure 5.1 Bi-phasic expression and vitamin D regulation of C/EBP transcription factors during rat osteoblast differentiation and presence in bone tissues: (A) Total RNA was isolated from various skeletal and soft rat tissues as described in Methods. RNA (10 μg) was resolved in a 1% formaldehyde gel and transferred to nylon membrane. The blots were hybridized with NcoI fragment for C/EBPα, EcoRI-XhoI fragment for C/EBPβ, BamHI-EcoRI fragment for C/EBPδ, EcoRI fragment for alkaline phosphatase, HindIII-Xbal fragment for histone H4, BamHI-XhoI fragment for Runx2, EcoRI-HindIII fragment for GAPDH, and EcoRI-BamHI fragment for the detection of rat OC. C/EBPβ and δ mRNAs are expressed in both soft and skeletal tissues. Lane 1, muscle and lane 2, day 21 fetal calvaria. (B) Primary rat osteoblasts were cultured for the indicated time (days) and total RNA (10 μg) was resolved in a 1% agarose gel and probed with the above mentioned cDNA fragments. The relative positions of 28S, 18S and 5S ribosomal RNA are indicated for reference.
Figure 5.1 Bi-phasic expression and vitamin D regulation of C/EBP transcription factors during rat osteoblast differentiation and presence in bone tissues: (C) Enhancement of C/EBP factors by vitamin D during osteoblast differentiation. Primary rat calvarial osteoblasts were cultured in vitro and collected at three stages of development: proliferation (day 7), matrix maturation (day 12) and mineralization (day 19). Cells were treated with $10^{-8}$ M vitamin D$_3$ for 24 hours prior to harvesting. Total RNA was isolated as described in Methods. The Northern blotting and probing were performed essentially as described in chapter 2. GAPDH is shown as control for loading of RNA.
tissue. Two sizes of C/EBP β mRNA are present with the larger species appearing more constitutive, while the smaller transcript is expressed during the growth period (days 3-5). The latter transcript is decreased markedly during the matrix maturation stage (days 7-12, when alkaline phosphatase positive cells are forming nodules), followed by a 4-5 fold increase in expression concomitant with mineral deposition and peak levels of osteocalcin and Runx2 expression (Fig. 5.1B). C/EBP δ mRNA is expressed in a similar fashion as C/EBP β, but the larger transcript is detected at very low levels. A significant (5 fold) temporal increase in C/EBP δ mRNA expression is observed during osteoblast differentiation from confluency (day 7) to the mature osteoblasts (day 22). Several Runx2 isoforms, which result from utilization of alternative promoters and differential splicing, are expressed (Fig. 5.1B). The increase in expression of the major Runx2 transcript during later stages of differentiation is consistent with increased Runx2 DNA binding activity in mature osteoblasts [Banerjee et al., 1997]. Thus, the increases in C/EBP β and C/EBP δ in the late stages of osteoblast differentiation appear to parallel peak expression levels for the osteoblast-related osteocalcin gene and the Runx2 transcription factor.

We further assessed the relationship of C/EBP factors to osteoblast differentiation. Vitamin D₃, a hormone that promotes osteoblast differentiation, is a known enhancer of many osteoblast-related genes [Lian et al., 1999a]. Expression of C/EBP β is stimulated by vitamin D₃ at each stage of osteoblast maturation, 6 fold during growth (day 7) and nodule development (day 12) and 3 fold during mineralization (day 19). Vitamin D₃ dependent enhancement of C/EBP δ is similar to that of C/EBP β except the fold stimulation is lower (3 fold at all stages). Treatment of
cells with vitamin D<sub>3</sub> has no effect on the expression of C/EBP α at any stage of differentiation. The differentiation-promoting properties of Vitamin D<sub>3</sub> are reflected by the decrease in histone H4 and the increase in OC expression (Fig. 5.1C). Thus, expression of both C/EBP β and C/EBP δ are strongly enhanced upon treatment with vitamin D<sub>3</sub> in relation to osteoblast differentiation. Taken together these data demonstrate that C/EBP transcription factors are expressed at significant levels in bone tissue, increase during osteoblast differentiation \textit{in vitro} and their expression is upregulated by 1,25(OH)<sub>2</sub>D<sub>3</sub>.

**C/EBP Proteins Activate The Osteocalcin Gene Through A C/EBP Responsive Element In The Proximal Promoter**

The enhanced expression of C/EBP β and δ during mineralization relative to the onset of OC transcription and in response to vitamin D<sub>3</sub> suggests that these C/EBP transcription factors may contribute to osteoblast specific expression of the OC gene. Previous studies using promoter deletion constructs of the rat OC gene have shown that the initial 200 bp of the promoter can confer tissue specific expression [Hoffmann et al., 1996]. This region contains a Runx responsive motif and a homeodomain box that also binds an osteoblast specific complex [Hoffmann et al., 2000]. Sequence analysis of this region reveals the presence of a C/EBP motif (Fig. 5.2). To determine if the C/EBP protein(s) can interact with this element, gel mobility shift analyses were performed using oligonucleotides (see Methods) containing either wild type or mutated C/EBP binding sequences (Fig. 5.2). Using nuclear extracts from day 20 primary rat osteoblasts in which OC is actively expressed, we observed two major protein-DNA complexes (Fig. 5.3). Specificity of these protein-DNA interactions was confirmed by
C/EBP consensus: 5' TTG CG CAA 3'

Probes used for Binding and Competition Assay:

**OC WT**: GGT TTGACCTATA**TTGCGCA**CATGACCCCAA

**OC mt**: GGT TTGACCTA ga ctagt CATGACCCCAA

Figure 5.2 A consensus C/EBP responsive motif is present in the proximal regulatory region of the rat OC gene: Regulatory sequences and their cognate binding factors in the -1.1 kb OC promoter are shown. A putative C/EBP motif is contiguous to the OC Box, a 24 bp element containing a homeodomain binding site. Position and nucleotide sequence for the binding of the C/EBP transcription factors are shown below. Sequence of the osteocalcin C/EBP motif differs from consensus by a single base. Recognition motifs for Runx factors [sites A (-605 to -599), B (-441 to -435) and C (-136 to -130)] and the vitamin D response element (VDRE) (-461 to -441) are shown and the positioned nucleosome between sites B and C is indicated. Oligonucleotides corresponding to wild type or mutated C/EBP sites used for electrophoretic mobility shift assays are shown in the lower panel.
Figure 5.3 Sequence specific protein-DNA interactions at the rat OC C/EBP element: (A) Oligonucleotides corresponding to wild type or mutated C/EBP sites from the OC gene were incubated with increasing concentrations (0-10 μg) of nuclear protein from day 20 primary rat osteoblast cultures. (B) The specificity of the complexes is further demonstrated by competition assays. Oligonucleotides carrying the wild type C/EBP site from the OC gene were incubated with 6 μg of nuclear proteins. The concentration ranges of wild type or mutant cold competitors are indicated at the top of each lane.
oligonucleotide competition assays. Addition of unlabeled wild type oligonucleotide, but not mutant oligo, inhibited the binding of the protein complexes to the labeled probe (Fig. 5.3B). Taken together these results indicate that C/EBP proteins interact in a sequence specific manner with their cognate element in the proximal promoter of the bone-specific rat OC gene.

The presence of C/EBP in the protein complex binding to the OC promoter element suggests transcriptional regulation of the OC gene by C/EBP factors. This possibility was experimentally addressed by assessing the effect of forced expression of C/EBP β and δ on activity of full length (-1.1kb) and proximal (-208 bp) OC promoter-CAT reporter gene constructs in the osteoblastic ROS 17/2.8 cell line (Fig. 5.4). The results indicate that C/EBP β and δ significantly enhance OC promoter activity, 4-5 fold on the full-length promoter (Fig. 5.4A, left panel) and 8 fold on the proximal promoter segments (Fig. 5.4A, right panel). Western blot analysis shows that C/EBP β and C/EBP δ proteins of the expected sizes are expressed (Fig. 5.4B). Thus both C/EBP β and δ are potent activators of osteocalcin gene transcription.

In order to establish that the C/EBP element in the proximal region directly mediates the enhancement of promoter activity by C/EBPs, site directed mutagenesis was performed. The C/EBP element in -208 OC-CAT was mutated (see Fig. 5.2) using the same oligonucleotides used in the gel shift assays. The results show that mutation of the C/EBP element blocks the C/EBP mediated stimulation (6-8 fold) observed with the wild type promoter (Fig. 5.5). Therefore responsiveness of the OC promoter to C/EBP transcription factors is dependent on the integrity of the C/EBP element in the basal promoter region.
Figure 5.4 C/EBP class of transcription factors regulates osteocalcin gene expression: (A) ROS 17/2.8 cells were transiently co-transfected with 0.75 μg of C/EBPβ or δ expression plasmids and 2.5 μg of -1.1 kb OC-CAT (left panel) or -208 OC-CAT reporter constructs (right panel). Cells were harvested 24 hours post transfection and CAT activities were determined. The data were normalized to values for RSV-luciferase activity as an internal control. Pooled data from 3 independent experiments are presented as % CAT conversions. Each bar represents LS±SEM (n=18). (B) ROS17/2.8 cells plated in 100 mm dishes were transiently transfected in parallel with 10 μg of either empty vector or C/EBPβ and δ expression constructs. Thirty (30) μg protein was resolved on 10% SDS-PAGE. Lamin B antigen is shown as a loading control.
Figure 5.5 C/EBP mediated activation of the rat OC promoter requires the integrity of the C/EBP responsive motif: Constructs carrying wild type or mutated C/EBP elements in the proximal promoter of the rat OC gene (-208 OC-CAT) or empty vector control (pGEM-CAT) were transiently co-transfected with C/EBPβ expression plasmid into ROS 17/2.8 cells. Reporter activities were determined 24 hours post transfection and normalized to luciferase values used for transfection control (n=6).
Functional Synergism Between Runx And C/EBP Proteins Is Mediated Through The C/EBP Element

Runx2 is a well-characterized regulator of OC gene transcription and a Runx responsive motif is located in close proximity to the C/EBP element in the proximal OC promoter. Therefore, functional interaction between these proteins in transcriptional regulation of the OC gene was tested. For these studies we selected HeLa cells, which lack Runx2 (Fig. 5.6A) and have no Runx DNA binding activity [Armesilla et al., 1996]. Consistent with these observations, we find low basal activity of the -208 OC promoter in HeLa cells compared to osteoblastic ROS 17/2.8 cells (Fig. 5.6B), which express both C/EBP β and Runx2 (Fig. 5.6A). Interestingly, HeLa cells have significant levels of C/EBP β (Fig. 5.6A). Based upon preliminary examination of dose-dependent effects of Runx2 and C/EBP β individually on activity of the -208 OC promoter, we used a suboptimal concentration of each expression plasmid (0.4 μg) for these studies. Expression of either C/EBP β or Runx2 in HeLa cells stimulates OC promoter activity 2-4 fold. However, co-expression of Runx2 and C/EBP β results in a massive activation (30-40 fold) of the OC promoter (Fig. 5.7) demonstrating a functional synergism between these two proteins. This functional synergy between two positive regulators of OC transcription is consistent with increased expression of C/EBP β and δ, Runx2 and OC during late stages of osteoblast differentiation.

To investigate the specific contribution of the Runx and C/EBP elements and their cognate factors to synergistic activation of the OC promoter, a series of promoter constructs bearing mutations in either Runx or C/EBP binding sites were generated (Fig. 5.8). Each of these constructs was tested for responsiveness to C/EBP and/or...
Figure 5.6 Expression of the rat OC promoter in osseous and non-osseous cells: (A) Total cellular protein (30 μg) from HeLa or ROS 17/2.8 cells were resolved on 10% SDS-PAGE. Levels of endogenous C/EBPβ and Runx2 proteins were determined by probing the blots with anti-C/EBPβ and anti-Runx2 antibodies. Lamin B is shown as loading control. (B) HeLa or ROS 17/2.8 cells were transfected with 2 µg of -208 OC-CAT constructs. CAT activities were determined 24 hours post transfection and presented as % CAT conversion.
Figure 5.7 CCAAT Enhancer Binding Protein β (C/EBPβ) and Runx2 synergistically activate the osteocalcin promoter: Functional interaction between C/EBPβ and Runx2 was determined by transient co-expression of C/EBPβ and Runx2 constructs (0.4 μg each) with 1 μg of indicated CAT reporter along with RSV-luciferase construct (100 ng) into HeLa cells. CAT activities were determined 24 hours post transfection and normalized to luciferase values. Data are presented as % CAT conversions (n=18, from 3 independent experiments).
Figure 5.8 Illustration of mutations in the proximal OC promoter: Top line diagram shows the relative positions and sequences of Runx and C/EBP responsive elements with mutated sequences shown in lower case. Constructs carrying mutation in either Runx or C/EBP elements alone or combined are indicated.
Runx2. Data pooled from four independent experiments show a consistent 30-40 fold synergistic enhancement of the wild type OC promoter by C/EBPβ and Runx2 (Fig. 5.9). Mutation of the Runx site did not affect the synergistic response (23-26 fold enhancement). In contrast, we observed a loss of this functional synergism upon mutation of the C/EBP motif. A similar loss of synergistic activity was observed when both C/EBP and Runx sites were mutated (Fig. 5.9). These findings suggest that the C/EBP regulatory element is required for the synergistic enhancer activity involving Runx2.

For further insight into the mechanisms involved in Runx2-C/EBP functional synergism, we tested a series of carboxy terminal deletion mutants of Runx2 (Fig. 5.10A). Both mutant Runx2 proteins (1-361 and 1-230) are expressed (Fig. 5.10B), enter the nucleus (data not shown) and retain DNA binding activity. Figure 5.10C shows that both mutants also retain functional activity on the OC promoter but at a lower level than wild type Runx2. When the mutant Runx2 (1-361) was co-expressed with either C/EBP β or δ, synergistic activation (40-80 fold) of the OC promoter was observed. However, this synergism did not occur with the mutant Runx2 (1-230). These Runx mutational studies suggest that a Runx2-C/EBP interaction is required to support synergistic activation of the OC promoter.

To determine whether functional synergism between Runx2 and C/EBP requires a physical interaction, we performed co-immunoprecipitation studies. WT Runx2 and Runx2 (1-361), which lacks the carboxy terminus, each form a complex with C/EBPβ, while Runx2 (1-230) fails to interact (Fig. 5.11). The level of C/EBPβ co-
Figure 5.9 Functional synergism between Runx2 and C/EBP factors is mediated by a C/EBP-responsive motif in the rat OC promoter: HeLa cells were transiently co-transfected with 1 µg of either wild type or mutated OC promoter constructs (carrying mutation in either C/EBP or Runx sites individually or together) and 0.4 µg of C/EBPβ or Runx2 expression constructs as indicated. Reporter activities were determined 24 hours post transfection and normalized to luciferase values used for transfection control.
Figure 5.10 Functional synergy between C/EBP and Runx2 requires C/EBP interacting motif: (A) Deletion mutants of Runx2 are shown diagrammatically. The runt homology DNA binding domain (RHD), nuclear localization (NLS) and putative C/EBP interacting region are indicated. Panel (B) shows western blot analysis of Xpress-tagged Runx2 protein expressed in HeLa cells. Tubulin is shown as a loading control.
Figure 5.10 Functional synergy between C/EBP and Runx2 requires C/EBP interacting motif: (C) Functional synergism is demonstrated in HeLa cells which were transiently co-transfected with 0.4 μg each of CMV empty vector, C/EBPβ, C/EBPδ, Runx2 full length and deletion mutant expression plasmid and 1 μg of the -208 OC-CAT constructs, as well as 100 ng of a RSV-luciferase plasmid. Cells were harvested 24 hours after transfection and assayed as described in methods. Data are presented as fold induction (expression constructs/empty vector control, n=12 from 4 independent experiments).
Figure 5.11 Co-immunoprecipitation of Runx2 and C/EBPβ: Direct interaction between Runx2 and C/EBPβ is shown by co-immunoprecipitation. HeLa cells were co-transfected with C/EBPβ and the Xpress tagged deletion constructs of Runx2 (as indicated) lysed 24 hrs later and immunoprecipitated with αXpress antibody or mouse IgG as described in methods section. Immunoprecipitated complexes were resolved on 12% SDS-PAGE followed by western blotting using either αXpressHRP or αC/EBPβ.
immunoprecipitated are very low compared to the total level of protein detected by western blot in the cell lysate, therefore it is possible that the conditions for immunoprecipitation are not appropriate to conserve the protein-protein interaction or that the actual amount of Runx2-C/EBP that interact in vivo is a small fraction of the total. Due to lack of antibodies that immunoprecipitate the endogenous proteins we could not determine if this interaction actually occurs in osteoblastic cells.

The absence of C/EBP β in the immunoprecipitated Runx2 (1-230) complex indicates that amino acids 230-361 of Runx2 are required for this interaction, consistent with the functional activity data (Fig. 5.10C). It was previously shown that the runt homology domain (RHD) of Runx1 supports interaction with C/EBP α [Petrovick et al., 1998; Zhang et al., 1996a]. However, our Runx2 deletion analysis reveals that the RHD of Runx2 is not sufficient for C/EBP β interaction (Fig. 5.11). Our findings are consistent with the recent co-crystal structure of the RHD with C/EBP β (bZIP), which demonstrates a lack of interaction of these two domains [Tahirov et al., 2001]. Taken together, our results suggest that the synergism observed on the OC promoter requires an interaction between Runx2 and C/EBPβ that involves a region of Runx2 outside the DNA binding domain.

To establish that the Runx2-C/EBP protein-protein interaction can occur when C/EBP proteins are bound to its regulatory element, we performed gel mobility shift assays with the C/EBP motif of the OC promoter (Fig. 5.12). We compared nuclear extracts from HeLa cells lacking Runx2 and mature osteoblasts, which contains both Runx and C/EBP factors (see Fig. 5.6A). We find similar C/EBP complexes formed with both nuclear extracts; however, addition of antibody against Runx2 resulted in
Figure 5.12 Runx2-C/EBP interaction at the C/EBP element: A C/EBP oligonucleotide (see Fig. 2 legend) was incubated with 10 μg of nuclear extracts from HeLa cells (lanes 1 and 2) or day 20 rat osteoblasts (lanes 3 and 4). Nuclear extracts were preincubated with 1 μl of Runx2 antibody for 20 minutes at 37°C (lanes 2, 4 and 5). Brackets show C/EBP complexes; arrowhead indicates supershifted band.
supershift only with the bone cell extracts. These results provide evidence for a Runx2-C/EBP interaction that is independent of a Runx DNA binding site.

**DISCUSSION**

Our studies demonstrate that the C/EBP transcription factors support osteoblast specific gene expression and may play an important regulatory role during osteoblast differentiation. C/EBPβ and δ, but not C/EBP α, are expressed in skeletal tissues and are developmentally regulated during osteoblast maturation. Vitamin D₃, a positive regulator of osteoblast differentiation and of the bone-specific osteocalcin gene, also increases expression of C/EBP factors. We find that osteocalcin is a downstream C/EBP target gene, strongly upregulated in response to forced expression of C/EBP family members. The level of enhancement is equivalent to that observed for the bone-related Runx2 transcription factor. More importantly, we also demonstrate that Runx2 and C/EBPβ and δ functionally cooperate for positive regulation of the OC gene and that the synergism is mediated through a physical interaction between Runx and C/EBP at the C/EBP element. We propose that C/EBP activity may be physiologically relevant to the spatio-temporal regulation of Runx2 dependent genes in mature osteoblasts.

Transcription of the OC gene is stringently regulated during osteoblast differentiation [Stein and Lian, 1993] but the mechanisms involved have not been completely elucidated. OC gene induction is coupled to a post-proliferative increase in Runx2 DNA binding activity [Banerjee et al., 1997; Ducy et al., 1997]. However, Runx proteins are present in skeletal progenitor cells and immature proliferating
osteoblasts, in which OC gene expression is not activated [Banerjee et al., 1997]. Thus other factors are contributing to strong suppression of OC transcription in such cells, as well as to maximal levels of OC expression in mature osteoblasts. Our studies indicate that the activities of Runx proteins and transcriptional activation of Runx responsive genes during skeletal development may be regulated in part by controlling cellular levels of Runx or C/EBP proteins and/or functional cooperation between Runx and C/EBP transcription factors. In support of this mechanism are the relative expression levels of each factor during osteoblast differentiation. Although C/EBPβ and δ are present in proliferating osteoblasts (consistent with their role in cell growth), the low Runx levels may be insufficient to produce a synergistic effect. In contrast, when the cellular levels of both factors are elevated during the mineralization stage, synergy may account for the massive and tissue specific induction of OC gene expression. Notably, C/EBPδ expression is positively regulated by Runx2 [McCarthy et al., 2000], which may provide the biological assurance of high C/EBP levels during osteoblast differentiation when bone specific gene expression is required.

For definitive assessment of the contribution of Runx2 and C/EBP proteins and their synergistic effect on the OC promoter, we carried out studies not only in bone cells, but also in HeLa cells which have a zero background for Runx factors [Armesilla et al., 1996]. Synergistic activation of the OC promoter occurred only when both factors were expressed in HeLa cells. This situation is analogous to mature osteoblasts where both proteins are maximally present. Based upon previous observations of the critical role of the Runx site in maintaining active chromatin conformation of the OC promoter [Javed et al., 1999], we anticipated that synergism would involve the Runx
element located 30 bp upstream of the C/EBP site. Our transient transfection studies of the OC promoter with mutated elements indicate a role for the C/EBP element in mediating synergistic activation with Runx2. However, these studies do not exclude the contribution of the Runx2 binding site to regulation of OC transcription in osteoblasts.

The mechanisms by which C/EBP and Runx factors together result in a synergistic activation of tissue-specific genes may involve modifications in chromatin structure. Several studies have shown that the Runx and C/EBP classes of transcription factors can each form regulatory complexes with proteins that influence chromatin remodeling. Runx factors facilitate the formation and maintenance of transcriptionally active chromatin [Javed et al., 1999; Lutterbach et al., 1998; Lian et al., 2001] and are known to interact with co-regulatory proteins possessing histone acetyltransferase [Kitabayashi et al., 1998] or histone deacetylase activity [Lutterbach et al., 1998] [Chen et al., 1999]. Recent studies from our laboratories indicate that Runx factors regulate tissue-specific and vitamin D mediated gene expression of osteocalcin by modifying chromatin organization [Javed et al., 1999]. More importantly, C/EBPβ has recently been shown to recruit the SWI/SNF complex to modify chromatin and regulate transcription of myeloid genes [Kowenz-Leutz and Leutz, 1999]. Numerous studies have established that chromatin remodeling and modifications in nucleosomal organization are necessary for bone specific activation of the osteocalcin gene [Javed et al., 1999; Montecino et al., 1996b; Montecino et al., 1999a; Montecino et al., 1999b]. Hence, C/EBP-dependent Runx synergism during development of the osteoblast
phenotype may result from temporal modifications in the chromatin-related interactions among C/EBP, Runx, SWI/SNF and/or other components of the nuclear architecture.

In summary, the data presented here address the mechanisms critical for the robust activation of the osteocalcin gene during osteoblast differentiation. Our studies indicate that these mechanisms require a C/EBP response element and synergism of C/EBP and Runx factors that may be facilitated by their regulated levels of expression in mature osteoblasts. The concept that lineage specific gene expression depends on the combination of factors, rather than being controlled by a single master regulator, is reinforced by these studies.
CHAPTER 6

General Discussion
GENERAL DISCUSSION

The regulation of gene expression in eukaryotes is highly complex and often occurs through the coordinated action of multiple transcription factors. Combinatorial regulation of transcription has several advantages, including the control of gene expression in response to a variety of signals and the usage of a limited number of transcription factors to create many combinations of regulatory complexes whose activities are modulated by diverse sets of conditions. This model implies that ultimately the arrangement of cis-regulatory sequences in the promoter of a gene will determine the type of signals to which it will respond [Pilpel et al., 2001; Yuh et al., 1998].

The osteocalcin (OC) gene serves as a paradigm to study tissue specific as well as hormone induced gene expression. Both transcriptional activity and mRNA levels of the OC gene are undetectable in early osteoprogenitors and detected at extremely low levels in postproliferative osteoblasts, but with the onset of osteoblast differentiation osteocalcin expression is induced, reaching its peak in the mineralizing osteoblast [Bellows et al., 1986; Owen et al., 1990a]. The studies presented in this thesis have provided novel insight into regulatory mechanisms that contribute to OC expression. In particular, we demonstrate that Runx2 is essential for both maximal transcriptional activity and for the chromatin remodeling that occurs when the OC promoter is activated. Our results also showed that binding of vitamin D receptor (VDR) is required to achieve a tight regulation of the OC expression and to complete the chromatin remodeling of the proximal promoter region during the process of OC
transcriptional activation. Furthermore, by sequence analysis of the proximal OC promoter sequence we found a consensus element for the C/EBP family of transcription factors in close proximity to the Runx binding motif. Northern blot analysis showed that during differentiation of the primary osteoblast two members of this family: C/EBP β and C/EBP δ are being expressed. Finally, we demonstrate that Runx2 and C/EBPβ or δ act synergistically to enhance OC transcription and this synergism is mediated through the C/EBP element in the OC promoter.

Runx2 is considered a master gene for bone development. While Runx2 binding to the OC promoter is most probably the signal of cell type specificity for the expression of OC, binding of C/EBP will maximize the levels OC in mature osteoblasts. This combined action for maximal activation of OC is indeed supported by our demonstration of functional synergism exhibited by C/EBP and Runx 2 transcription factors on the OC promoter. Interestingly, expression of both C/EBPβ and osteocalcin are upregulated by vitamin D, a positive modulator of bone formation. This could well be seen as a concerted effort where an additional signal, the presence of a steroid hormone, not only enhances osteocalcin expression by directly binding to its promoter, but it also increases the expression of the positive regulators involved in OC transcription. Given these observations higher basal activity of the VDRE mutant OC promoter (mSHE) is somewhat surprising. However, it is important to note that the mSHE promoter has only lost the signal for hormonal responsiveness but signaling from other queues is intact. Therefore, the VDRE mutation transform the arrangement of the cis-regulatory signals at the OC promoter, from one design to respond to the
presence of steroid hormones to an arrangement that only specify the expression of this promoter in bone cells.

Because eukaryotic DNA is packaged with histones to form nucleosomes, the accessibility of transcription factors to their binding sites is limited. Therefore, transcription factors must have the capacity to bind their respective DNA elements even when they are organized into nucleosomes, or alternatively the DNA must be made accessible by remodeling of the chromatin. Indeed, examples for both types of transcription factors have been identified [Östlund Farrants et al., 1997; Wechsler et al., 1994]. The biological significance of this functional division between factors is best seen on the induction of the MMTV gene [Di Croce et al., 1999; Fletcher et al., 2000]. The promoter of this gene contains five binding sites for glucocorticoid receptor (GR) and two for the nuclear factor 1 (NF1); GR can bind its cognate element in a nucleosome, while NF1 cannot. Consequently, it has been shown that when the promoter is packed into nucleosomes, GR is able to bind at least two of these five sites. Once GR is bound to the promoter, it recruits chromatin-modifying activities that increase DNA accessibility and facilitate the binding of NF1 [Cordingley et al., 1987; Archer et al., 1991; Pina et al., 1990].

Previously, it has been shown that in proliferating osteoblasts the OC promoter is uniformly packaged in nucleosomes and no nuclease hypersensitivity is detected. In contrast, when osteocalcin gene expression is up-regulated postproliferatively and vitamin D enhancement of transcription occurs, two DNase I hypersensitive sites are detected, designated as the distal site (-600 to -400) and the proximal site (-170 to -70) [Montecino et al., 1996b; Montecino et al., 1994b]. This demonstrates that the
transition of the OC gene from transcriptionally inactive to active is accompanied by changes in chromatin structure of the gene promoter. The studies in this thesis demonstrate that a key player in OC chromatin remodeling is Runx2; in fact, mutation of Runx binding sites results in a complete loss of DNase I hypersensitivity and a reduced accessibility to restriction enzymes (Fig. 3.8, 4.5, 4.6C). However, no significant alteration in basal histone acetylation is observed (Fig. 4.7). These results suggest that chromatin remodeling at the OC locus is a step-wise process, where some of the steps involved (e.g., DNase I hypersensitivity) require binding of Runx2 while others (histone acetylation) do not. These results also suggest that the generation of hypersensitive sites most probably results from the action of ATP-dependent chromatin remodeling factors or histone modifying complexes other than histone acetyltransferases and that histone acetylation of the OC promoter, although is not sufficient to support full transcriptional activation of the OC gene, it most probably required for the binding of transcription factors and/or chromatin remodeling complexes that will complete the OC activation process.

VDRE mutation (mSHE) did not affect basal DNase I hypersensitivity; however, restriction enzyme accessibility of the proximal promoter region was significantly reduced. This observation is consistent with the ability of VDR to interact with and recruit components of the basal transcription machinery as well as coregulators that act on chromatin [MacDonald et al., 1995b; Rachez and Freedman, 2000]. A striking characteristic of the mSHE promoter is increased H3 acetylation compared to the wild type promoter. It has been reported that, at least in vitro, histone acetyltransferases exhibit substrate specificity. For example, GCN5 and some acetylases
associated with the basal transcription machinery preferentially acetylate histone H3 [Roth et al., 2001]. Therefore, the increased H3 acetylation levels at the mSHE promoter most probably reflect the recruitment of a different histone acetylase complex or, alternatively, it may be the result of the acetylase activity associated with the RNA polymerase complexes that are present at very high levels at this promoter (Fig 4.7).

Vitamin D enhanced activation of the wild type OC promoter is mediated by histone acetylation (Fig. 4.7, [Shen et al., 2002]). Our results demonstrated that this event is accompanied by increased restriction enzyme accessibility for some of the enzymes tested (Fig. 4.6A). However, consistent with the lack of vitamin D responsiveness of the mutant promoters (mSHE and mABC), no significant changes either in restriction enzyme accessibility or histone acetylation were observed. These results suggest that binding of VDR results in the recruitment of additional histone acetyltransferase complex to the OC promoter.

The sequence of events associated with gene activation has been studied in several genes; for example, in the cell cycle and developmentally regulated yeast gene HO the first step in its activation is the binding of the transcription factor Swi5. This binding is required for the association of the SWI/SNF complex, which in turn is required for the recruitment of SAGA a histone acetylase complex. Finally the transcription factor SBF binds to the promoter, but this happens only after both chromatin-modifying activities have been recruited to the HO promoter. This linear pathway of ordered recruitment (Swi5, SWI/SNF, SAGA, SBF) occurs only in mother cells. In daughter cells, the Ash1 repressor associates with the HO promoter shortly after Swi5 binding and blocks the recruitment of SWI/SNF and all the subsequent
events [Cosma et al., 1999]. In contrast, for the interferon β (IFN β) promoter the histone acetylase GCN 5 is first recruited to the nucleosome-free enhancer region of the gene. This leads to acetylation of a strategically positioned nucleosome, which masks the TATA box. The next step is the binding of CBP-pol II holoenzyme complex, followed by SWI/SNF recruitment resulting in chromatin remodeling that shifts the nucleosome 36 bp downstream, facilitating TBP binding [Lomvardas and Thanos, 2001]. This program of sequential recruitment culminates in the binding of TFIID to the promoter and the activation of transcription [Agalioti et al., 2000]. Therefore, it appears that there is no obligate order for function of ATP-dependent remodelers and covalent modifiers that is general for all promoters. The precise order seems to depend upon the nature of the promoter, the complement of transcription factors present, and the chromatin structure in which the promoter resides. Moreover, it seems that there is no set order of action for chromatin-modifying complexes and complexes in the general transcription machinery [Soutoglou and Talianidis, 2002; Agalioti et al., 2000].

We can imagine that for the OC gene one of the first steps would be histone acetylation. Chromatin accessibility data suggest that in the proximal OC promoter both the Runx and C/EBP binding sites are located in the nucleosomal linker region, it is possible therefore that Runx and/or C/EBP binding will be one of the earliest events in OC activation (Fig. 6.1). This binding of C/EBP could result in the recruitment of a histone acetyltransferase activity such as p300, which is known to interact with C/EBP β [Mink et al., 1997], and the subsequent acetylation of the promoter. Runx2 can then be recruited to its cognate binding sites and bring along others coactivators (SWI/SNF, CBP). This in turn will nucleate the binding of VDR/RXR and/or other transcription
Figure 6.1 Postulated model for Osteocalcin Gene Activation: Runx2 and C/EBP nucleate the recruitment of transcriptional co-activators and additional transcription factors that render the OC gene transcriptionally active and responsive to steroid hormones. TF: Transcription Factor, HAT: Histone Acetyltransferase.
factors and finally results in the binding of RNA polymerase. According to this model, C/EBP is a family of transcription factors that are critical for normal cellular differentiation and metabolic functions in a variety of tissues on the Runx mutant promoter (mABC) only binding of C/EBP and acetylation of the promoter occurs before the recruitment of RNA polymerase; therefore, a low transcriptional activity would be observed (Fig 6.1). In contrast on the VDRE mutant promoter (mSHE) sequential recruitment of C/EBP, p300, Runx and SWI/SNF would render the promoter fully competent for transcription and recruitment of RNA polymerase will result in a high transcriptional activity (Fig.6.1).

In summary, our results indicate that transcriptional activation of OC gene is a tightly regulated process that involves the recruitment of multiple transcription factors and chromatin remodeling complexes. Future studies should aim to identify which factors are recruited in vivo and in what sequence these factors bind to the OC promoter.
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