Identification and Characterization of AJ18, a Novel Zinc Finger Transcription Factor Expressed During Skeletal Development

by

Andrew H. Jheon

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Graduate Department of Biochemistry
University of Toronto

© Copyright by Andrew H. Jheon 2001
The author has granted a non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of this thesis in microform, paper or electronic formats.

The author retains ownership of the copyright in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author's permission.

L'auteur a accordé une licence non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de cette thèse sous la forme de microfiche/film, de reproduction sur papier ou sur format électronique.

L’auteur conserve la propriété du droit d'auteur qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.
Identification and Characterization of AJ18, a Novel Zinc Finger Transcription Factor Expressed During Skeletal Development

Andrew H. Jheon, Ph.D., 2001
Graduate Department of Biochemistry, University of Toronto

Abstract

In response to osteo-inductive signals generated by the bone morphogenetic proteins (BMPs), undifferentiated mesenchymal stem cells can differentiate into osteoblasts. However, the molecular mechanisms that coordinate and direct osteogenesis are largely unknown. Differential display was utilized to identify a gene, provisionally named AJ18, whose expression is responsive to BMP-7 and up-regulated during osteoblastic differentiation in fetal rat calvarial cells (FRCCs). AJ18 encodes a novel protein comprising a Krüppel-associated box (KRAB) domain, which is a well-characterized transcription repressor motif, and 11 successive C2H2 zinc finger motifs. Using a target detection assay, a consensus DNA-binding site with the sequence 5'-CCACA-3' was revealed. This sequence is present in the enhancer for Runx2 (OSE2, osteoblast-specific element 2; 5'-ACCACA-3'), a master gene for osteogenesis. By using a transient transfection assay AJ18 expression was found to abrogate the transactivation activity of Runx2 in a dose-dependent manner.

To study the regulation of AJ18 gene expression, a rat genomic library was screened to obtain the 5'-flanking region of AJ18. Mapping of the transcription start site showed that the AJ18 gene contains an unusually long 2.3 kb 5'-untranslated region (5'-UTR) with potential for strong secondary structure. Chimeric constructs encompassing the immediate promoter region (-77 to +177) revealed strong transcriptional activity when ligated to a luciferase reporter gene in transient transfection assays. Addition of ~2 kb of upstream sequence did not increase this activity. Comparison of mouse and rat AJ18 promoters however revealed high sequence identity.
that included several responsive elements for proteins such as Runx2, NFκB, Smads, Sp1, and Ets1.

The expression of AJ18 mRNA and protein at various stages of mouse development revealed high levels in brain, kidney, and mineralized tissues during embryonic development. In developing endochondral bone, AJ18 staining was strong in proliferating and pre-hypertrophic chondrocytes, and osteoblasts, with low or no staining in hypertrophic chondrocytes. Nuclear staining was also observed in differentiating cells that form the mineralized tissues of teeth. Notably, the expression of AJ18 was similar to the expression of BMP-7 consistent with its perceived role as a transcriptional factor that regulates developmental processes downstream of BMP-7.

These studies, therefore, have identified the first KRAB/C2H2 zinc finger gene to be implicated in skeletal development, and have characterized the first promoter sequence for a gene belonging to the large KRAB/C2H2 zinc finger family.
Acknowledgements

I have always felt that an acknowledgements page in PhD dissertations was a little over the top. Sure, people who have done great things deserve to thank others. Great things like winning the Nobel Peace Prize or winning an internationally coveted Juno award. This definitely does not apply to a PhD student. However, I have realized the error of my ways. Although completing a PhD program may not be as noble as generating world peace or being Celine Dion, it sure was difficult but satisfying in its own gut-twisting ways. And it does feel like an accomplishment to me now. Thus, I find myself compelled and shamelessly wanting to acknowledge some great people who have helped me.

My parents have provided everything for me. I learn from them constantly. The majority of my thoughts and ideas are from my parents and I love them enormously for that. I hope that they are proud of me and find comfort during their work that I plan to spoil them silly in the future. My parents even provided me with an amazing sister, Catherine, who teaches me the important things in life like eating olives and how to win arguments (Secret is volume).

Julia, with her important tips on the distribution of hair mousse and reminding me of what I have said, I would be crazy to not want her in my life for a long time.

My supervisor Dr. Jaro Sodek. Is there a better barometer to what a great person he is than his daughter training for the triathlon with him, and his son taking him to a Maple Leafs playoff game with his one spare ticket? How about Jaro’s past and present graduate students getting drunk at a bar discussing, of all things, the virtues of the legendary Jaro? I have gleaned so much from Jaro and science is probably the least important of what I have learned from him.

There are too many people in the CIHR group to mention. So many role models here. Moreover, I have met people that will be friends for a long time. However, I must mention Billy T.A. Teo, without whom I probably would have completed my PhD two years earlier. No...a year and a half. Also, Kam-Ling, Drs. Bernhard Ganss, Sela Cheifetz, and Richard Kim who have taught me all the lab techniques that I know. Lots of love here. Kam-Ling, the foundation of the Sodek lab. Thank you so much for everything but especially for not retiring early.

Lastly, I would like to thank my supervisory committee, Drs. Sela Cheifetz (again), Jacqueline Segall, and Arun Seth for being fair and sufficiently tough on me.

So here it is, the acknowledgements page overrun with sentimentality and slices of cheddar. It does appear though that this is the correct and only way to complete one’s PhD.
# Table of Contents

Abstract .......................................................................................................................... ii
Acknowledgements ......................................................................................................... iv
Table of Contents ........................................................................................................... v
List of Figures .................................................................................................................. vii
List of Abbreviations ....................................................................................................... viii
Preface ............................................................................................................................. xi

## Chapter 1: Introduction

### 1A. Bone: A mineralized connective tissue

1A.1. Bone Cells.............................................................................................................. 2
    1A.1.1. Osteoblasts ..................................................................................................... 5
    1A.1.2. Osteoclasts .................................................................................................. 7
1A.2. Bone matrix proteins ............................................................................................ 9
    1A.2.1. Collagen ...................................................................................................... 10
    1A.2.2. Noncollagenous Proteins .......................................................................... 12
        1A.2.2a. Proteoglycans ....................................................................................... 13
        1A.2.2b. Glycoproteins ....................................................................................... 14
        1A.2.2c. RGD-Containing Glycoproteins ......................................................... 15
        1A.2.2d. γ-Carboxyglutamic Acid (Gla)-Containing Proteins ...................... 18
1A.3. Osteoblastic Differentiation ................................................................................. 19
    1A.3.1. Bone Morphogenetic Proteins .................................................................. 20
    1A.3.2. Smad Signaling ........................................................................................... 22
    1A.3.3. Runx2 ......................................................................................................... 23
    1A.3.4. Other Factors ............................................................................................. 26

### 1B. Gene Regulation

1B.1. Nucleosome ......................................................................................................... 28
1B.2. Transcription Factors .......................................................................................... 34
    1B.2.1. Basal Factors ............................................................................................. 34
    1B.2.2. Gene Regulatory Factors .......................................................................... 37
        1B.2.2a. C3H2 Zinc Fingers Proteins ................................................................. 39
        1B.2.2b. KRAB/C2H2 Zinc Fingers Proteins .................................................... 42

## Hypotheses and Objectives of Thesis

Chapter 2: Identification and Characterization of a Novel Gene
Involved in Bone Differentiation ............................................................................... 46

2.1. Summary ................................................................................................................. 47
2.2. Introduction .......................................................................................................................... 48

2.3. Experimental procedures ..................................................................................................... 50
  2.3a. Cell Culture ......................................................................................................................... 50
  2.3b. RNA Extraction .................................................................................................................... 50
  2.3c. Differential Display .............................................................................................................. 51
  2.3d. Northern Blot Hybridization ............................................................................................... 51
  2.3e. 5'-Rapid Amplification of cDNA Ends (5'-RACE). ............................................................... 52
  2.3f. RT-PCR and Southern Blot ................................................................................................. 52
  2.3g. Bacterial Expression and Target Detection Assay ............................................................... 53
  2.3h. Anti-AJ18 Polyclonal Antibodies ....................................................................................... 54
  2.3i. Immunohistochemical Analysis ........................................................................................... 55
  2.3j. Transient transfections and fluorescence microscopy ........................................................ 56
  2.3k. Transcription Assay ........................................................................................................... 56
  2.3l. Alkaline Phosphatase Activity ............................................................................................ 57
  2.3m. Sequence Analysis ............................................................................................................ 57

2.4 Results .................................................................................................................................... 58
  2.4a. Identification and Sequence Analysis of AJ18 .................................................................... 58
  2.4b. AJ18 is Expressed During Osteoblast Differentiation and Bone Development ................ 62
  2.4c. AJ18 is Localized to the Nucleus through its Zinc Finger Region ....................................... 66
  2.4d. AJ18 Shows Selective Binding to dsDNA ......................................................................... 66
  2.4e. AJ18 is Co-Expressed with Runx2 and Modulates its Transcriptional Activity ............... 70
  2.4f. Over-expression of AJ18 Suppresses Alkaline Phosphatase Expression ............................. 74

2.5. Discussion ............................................................................................................................ 77

Chapter 3: Characterization of the 5'-Flanking Region of the Rat AJ18 Gene ......................... 82

3.1. Summary .................................................................................................................................. 83

3.2. Introduction ............................................................................................................................ 84

3.3. Experimental procedures ...................................................................................................... 87
  3.3a. Rat Genomic Library Screen .............................................................................................. 87
  3.3b. Cell Culture ......................................................................................................................... 87
  3.3c. RNA Extraction ................................................................................................................... 88
  3.3d. PCR Reactions ..................................................................................................................... 88
  3.3e. Primer Extension Analysis ................................................................................................... 88
  3.3f. 3'-Rapid Amplification of cDNA Ends (3'-RACE) and Southern Blot Hybridization. ...... 89
  3.3g. DNA-Luciferase Constructs ............................................................................................... 89
  3.3h. Transient Transfection Assays ........................................................................................... 90
  3.3i. Sequence Analysis .............................................................................................................. 91

3.4 Results .................................................................................................................................... 92
  3.4a. Cloning of Rat AJ18 Promoter ........................................................................................... 92
  3.4b. Mapping of the Transcription Initiation Site ..................................................................... 92
  3.4c. Genomic Organization of AJ18 .......................................................................................... 94
Chapter 4: Temporal and Spatial Expression of AJ18

4.1. Summary .................................................................................................................. 114
4.2. Introduction .............................................................................................................. 116
4.3. Experimental methods ............................................................................................ 118
  4.3a. Mouse Genomic Library Screen ............................................................................ 118
  4.3b. Mouse Tissue Preparation .................................................................................... 118
  4.3c. RNA Extraction .................................................................................................... 118
  4.3d. Northern Blot Hybridization ............................................................................... 119
  4.3e. Anti-AJ18 Polyclonal Antibodies ......................................................................... 119
  4.3f. Immunohistochemical Analysis ........................................................................... 119
4.4. Results ...................................................................................................................... 121
  4.4a. Identification of Mouse AJ18 .............................................................................. 121
  4.4b. Preliminary Northern Blot Hybridization ............................................................. 121
  4.4c. Immunohistochemical Staining ........................................................................... 124
    4.4c-i. Expression of AJ18 Protein in Cartilage and Bone ......................................... 125
    4.4c-ii. Expression of AJ18 Protein in Developing Teeth ........................................... 131
    4.4c-iii. Expression in the Brain and Epithelium ....................................................... 134
    4.4c-iv. Expression in the Kidney, Eye, and Whisker Follicles .................................... 136
4.5. Discussion ................................................................................................................. 138

Chapter 5: Summary and Future Directions .................................................................... 142

5A. Functional Characterization of AJ18 ....................................................................... 146
  5A.1. Interaction with Runx2 ...................................................................................... 148
  5A.2. Determination of Zinc Finger Motifs Involved in DNA Interactions .................. 149
  5A.3. Target Genes and Downstream Effects of AJ18 ................................................. 150
5B. Regulation of AJ18 Expression ............................................................................... 151
  5B.1. Identification of Regulators of AJ18 .................................................................... 152
    5B.1.1. BMP-7 Regulation of AJ18 ........................................................................... 152
  5B.2. Tissue-specific Regulation .................................................................................. 153
  5B.3. Regulation of AJ18 Translation .......................................................................... 153
5C. Transgenic Mouse Models ....................................................................................... 155

References .................................................................................................................... 157
List of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fig. 1.1</td>
<td>Endochondral bone formation.</td>
<td>4</td>
</tr>
<tr>
<td>Fig. 1.2</td>
<td>C2H2 zinc finger motifs.</td>
<td>40</td>
</tr>
<tr>
<td>Fig. 2.1</td>
<td>Identification of a 313 bp gene fragment up-regulated by BMP-7 and Dex.</td>
<td>59</td>
</tr>
<tr>
<td>Fig. 2.2</td>
<td>Identification of a 313 bp gene fragment up-regulated in differentiating bone cells</td>
<td>60</td>
</tr>
<tr>
<td>Fig. 2.3</td>
<td>Primary structure of rat AJ18.</td>
<td>61</td>
</tr>
<tr>
<td>Fig. 2.4</td>
<td>Amino acid sequence alignment of multiple KRAB domains.</td>
<td>63</td>
</tr>
<tr>
<td>Fig. 2.5</td>
<td>Temporal expression of AJ18 and bone-associated genes during FRCC differentiation</td>
<td>64</td>
</tr>
<tr>
<td>Fig. 2.6</td>
<td>AJ18 is differentially expressed during rat development.</td>
<td>65</td>
</tr>
<tr>
<td>Fig. 2.7</td>
<td>Expression of AJ18 protein in the growth plate of rat tibiae.</td>
<td>67</td>
</tr>
<tr>
<td>Fig. 2.8</td>
<td>KRAB domain is not required for nuclear localization.</td>
<td>68</td>
</tr>
<tr>
<td>Fig. 2.9</td>
<td>Bacterially expressed AJ18 binds dsDNA in the presence of zinc.</td>
<td>71</td>
</tr>
<tr>
<td>Fig. 2.10</td>
<td>AJ18 and Cbfa1 are co-expressed during RBMC differentiation and mouse embryonic development</td>
<td>72</td>
</tr>
<tr>
<td>Fig. 2.11</td>
<td>AJ18 represses Runx2/Osf2 transactivation of 6xOSE2 in a dose-dependent manner</td>
<td>73</td>
</tr>
<tr>
<td>Fig. 2.12</td>
<td>Alkaline phosphatase activity is repressed by AJ18 in C3H10T1/2 cells treated with BMP-7.</td>
<td>75</td>
</tr>
<tr>
<td>Fig. 3.1</td>
<td>Transcription initiation site is mapped within a 975 bp region.</td>
<td>93</td>
</tr>
<tr>
<td>Fig. 3.2</td>
<td>Identification of the transcription initiation site by primer extension.</td>
<td>95</td>
</tr>
<tr>
<td>Fig. 3.3</td>
<td>Mapping the 3'-UTR by 3'-RACE.</td>
<td>96</td>
</tr>
<tr>
<td>Fig. 3.4</td>
<td>Genomic organization of AJ18.</td>
<td>97</td>
</tr>
<tr>
<td>Fig. 3.5</td>
<td>Identification of 28 upstream ATG sites and a putative IRES in the 5'-UTR of rat AJ18</td>
<td>99</td>
</tr>
<tr>
<td>Fig. 3.6</td>
<td>Identification of a novel gene upstream of AJ18.</td>
<td>101</td>
</tr>
<tr>
<td>Fig. 3.7</td>
<td>Nucleotide sequence of the 5' flanking region of rat and mouse AJ18.</td>
<td>102</td>
</tr>
</tbody>
</table>
Fig. 3.8. *AJ18* promoter shows transcription activity ........................................ 104

Fig. 3.9. *AJ18* gene shows high transcription activity in bone cells .................. 105

Fig 4.1. Sequence alignment of rat and mouse AJ18 ........................................ 122

Fig. 4.2. Preliminary Northern blot analyses of AJ18 mRNA expression during mouse development ........................................................................................................ 123

Fig. 4.3. Immunostaining of AJ18 in jaw bones and Meckel’s cartilage ............... 126

Fig. 4.4. Immunostaining of AJ18 in developing rib bones ............................. 127

Fig. 4.5. Immunostaining of AJ18 in an embryonic rib bone ......................... 129

Fig. 4.6. Immunostaining of AJ18 in long bone of a 4-week old mouse ............ 130

Fig. 4.7. Immunostaining of AJ18 in developing teeth .................................. 132

Fig. 4.8. Immunostaining of AJ18 in molar and incisor of a 4-week old mouse ... 133

Fig. 4.9. Immunostaining of AJ18 in the brain ............................................. 135

Fig. 4.10. Immunostaining of AJ18 in tissues that express BMP-7 .................... 137

Fig. 5.1. Summary of AJ18 regulation in osteogenesis .................................... 147
**List of Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA</td>
<td>hydroxyapatite</td>
</tr>
<tr>
<td>PCP</td>
<td>procollagen C-proteinase</td>
</tr>
<tr>
<td>BMP</td>
<td>bone morphogenetic protein</td>
</tr>
<tr>
<td>NCP</td>
<td>non-collagenous proteins</td>
</tr>
<tr>
<td>gla</td>
<td>γ-carboxyglutamic acid</td>
</tr>
<tr>
<td>SLRPs</td>
<td>small, leucine rich proteoglycans</td>
</tr>
<tr>
<td>GAG</td>
<td>glycosaminoglycans</td>
</tr>
<tr>
<td>ALP</td>
<td>alkaline phosphatase</td>
</tr>
<tr>
<td>ON</td>
<td>osteonectin</td>
</tr>
<tr>
<td>SPARC</td>
<td>secreted protein acidic and rich in cysteine</td>
</tr>
<tr>
<td>BSP</td>
<td>bone sialoprotein</td>
</tr>
<tr>
<td>OC</td>
<td>osteocalcin</td>
</tr>
<tr>
<td>OPN</td>
<td>osteopontin</td>
</tr>
<tr>
<td>Runx2</td>
<td>runt domain homeobox gene2</td>
</tr>
<tr>
<td>Cbfa</td>
<td>core binding factor α</td>
</tr>
<tr>
<td>Osf</td>
<td>osteogenic specific factor</td>
</tr>
<tr>
<td>OSE2</td>
<td>osteoblast-specific cis-acting element2</td>
</tr>
<tr>
<td>TGF-β</td>
<td>transforming growth factor-β</td>
</tr>
<tr>
<td>OP-1</td>
<td>osteogenic protein-1</td>
</tr>
<tr>
<td>HAT</td>
<td>histone acetylase</td>
</tr>
<tr>
<td>HDAC</td>
<td>histone deacetylase</td>
</tr>
<tr>
<td>RNAP</td>
<td>RNA polymerase</td>
</tr>
<tr>
<td>TBP</td>
<td>TATA-binding protein</td>
</tr>
<tr>
<td>TAFs</td>
<td>TBP-associated factors</td>
</tr>
<tr>
<td>TRF</td>
<td>TBP-related factor</td>
</tr>
<tr>
<td>UTR</td>
<td>untranslated region</td>
</tr>
<tr>
<td>HLH</td>
<td>helix-loop-helix</td>
</tr>
<tr>
<td>KRAB</td>
<td>Krüppel-associated box</td>
</tr>
<tr>
<td>FRCC</td>
<td>fetal rat calvarial cell</td>
</tr>
<tr>
<td>RBMC</td>
<td>rat bone marrow cell</td>
</tr>
<tr>
<td>MEM</td>
<td>minimal essential medium</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl-β-D-thiogalactoside</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>DAPI</td>
<td>4′,6′-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>kb</td>
<td>kilobase(s)</td>
</tr>
<tr>
<td>bp</td>
<td>base pair(s)</td>
</tr>
<tr>
<td>ds</td>
<td>double-stranded</td>
</tr>
<tr>
<td>dpc</td>
<td>days postcoitum</td>
</tr>
</tbody>
</table>
Preface

Bone cells are highly specialized cells that are derived from mesenchymal stem cells. However, the molecular factors and pathways involved in osteogenesis are largely unknown. This thesis will describe the identification and characterization of a novel gene, provisionally named AJ18. AJ18 appears to be a transcription factor that plays a role in osteogenic differentiation.

Chapter One is in two parts. The first part is an introduction to the field of bone research. The composition of bone, including the balance between the actions of bone-forming osteoblasts and bone-resorbing osteoclasts, is first described. Bone is essentially comprised of hydroxyapatite crystals embedded within the extracellular matrix. The matrix is composed of collagenous and noncollagenous proteins. The expression of matrix proteins by osteoblasts is utilized as markers of various stages of bone differentiation. Subsequently, the factors that are essential for the differentiation of bone are described. BMPs are primordial signals for bone formation, whose signals are transduced to the nucleus by Smad proteins. Runx2 is a master gene of osteogenesis. The introduction on bone is closed with a description of other recently identified factors that may be important for osteogenesis. The second part of the introduction describes the regulation of genes. Nucleosomes and the process of transcription, including basal and gene regulatory transcription factors, are briefly discussed. The introduction on gene regulation is closed with a description of KRAB domains and C2H2 zinc finger motifs, protein structures that are present in AJ18.

Chapter Two describes the identification and initial characterization of AJ18. A consensus DNA-binding element was determined and a potential role for AJ18 is shown.
Chapter Three describes the 5’-flanking region of *AJ18*, which was isolated and sequenced as a first step towards characterizing the upstream regulators of AJ18.

Chapter Four shows the temporal and spatial expression of AJ18 during mouse development. The expression of AJ18 protein was determined by immunostaining with polyclonal antibodies raised against peptide sequences specific for rat AJ18.

Chapter Five, perhaps the most important chapter, discusses future experiments that may be performed to further characterize the upstream regulators and downstream effects of AJ18.
Chapter 1: Introduction
1A. Bone: A mineralized connective tissue

Bone is a specialized connective tissue that comprises the largest proportion of connective tissue mass in vertebrates. Together with cartilage, bone makes up the skeletal system, and is required for three major functions: it has a primary mechanical function in body support and is the site of muscle attachment required for movement and locomotion; it protects vital soft tissue organs and bone marrow; and it also has a metabolic function as the major calcium reservoir of the body required for calcium homeostasis in the serum and other body fluids. Over 99% of total body calcium is stored in the skeleton. Unlike most other connective tissue matrices, the bone matrix has the ability to mineralize by the formation of hydroxyapatite (HA; \( Ca_{10}(PO_4)_{6}(OH)_2 \)) crystals. The HA crystals provide the strength and resilience for the mechanical functions of bone tissues while providing reserves, not only of calcium and phosphate ions, but also of magnesium, sodium, carbonate and fluoride ions, that are substituted for calcium and phosphate ions in the HA crystal lattice. Bone tissues are unique in that they are constantly regenerated throughout life as a consequence of remodeling and turnover. To maintain tissue dynamics, large numbers of new cells must be recruited continuously. These include osteoblastic cells, which are derived from mesenchymal cells and are responsible for bone formation, and osteoclasts, which are derived from hematopoietic cells and are responsible for bone resorption (Baron, 1999). There is a fine balance between bone formation and resorption, and a loss of this balance leads to pathological disorders, such as osteopetrosis, in which bone mass is inappropriately increased, and osteoporosis, where bone mass is lost (Mundy, 1999).

The skeleton is composed of flat and long bones. Flat bones such as the skull bones, scapula, mandible, and ilium are produced by intramembranous bone formation, while long bones such as tibia, femur, and humerus are generated by a process of endochondral bone
formation (reviewed by Baron, 1999). In intramembranous bone formation, mesenchymal precursor cells condense and differentiate directly into osteoblastic cells, which produce and mineralize a bone matrix within the condensate. In endochondral bone formation, the condensing mesenchymal cells first form a cartilaginous tissue anlagen in which the cartilage cells hypertrophy and the surrounding matrix is mineralized. The mineralized matrix is then subjected to vascular invasion, which brings osteoclast and osteoblast precursors to resorb the mineralized cartilage and replace it with bone tissue in a process reminiscent of bone remodeling. Vascular invasion in both intramembranous and endochondral bone is also associated with the formation of hematopoietic marrow tissue (Reddi, 1992). A layer of proliferative cartilage cells and expanding cartilage matrix at each end of the long bones is responsible for longitudinal growth of long bone. Between the cartilage and the bone is the growth plate (Fig. 1.1) where the active transition between cartilage and bone occurs. In addition, perichondral bone is also formed around the cartilage anlagen and this becomes periosteal bone following the replacement of cartilage. Two discrete mineralized surfaces in bone are in contact with soft tissue. On the external surface is the periosteum, which contacts fibrous connective tissue, and on the internal surface is the endosteum, which contacts the hematopoietic bone marrow. These surfaces are lined with osteogenic cells, called the periosteal and endosteal bone cells, respectively (Baron, 1999).

Microscopically, the structure of the bone matrix differs in rapidly growing embryonic bone tissues compared to the more slowly deposited adult bone. Woven or embryonic bone is described as irregular bone characterized by large, randomly-arranged collagen fibers and an unevenly calcified ECM in which mineral crystals are distributed within and between the collagen fibrils. As the skeleton matures, lamellar bone or adult bone replaces woven bone.
Fig. 1.1. **Endochondral bone formation.** On the *left* is a schematic representation and on the *right* is a light micrograph (Huntziker, 1994) of the growth plate of long bone. In the proliferative zone are chondrocytes that are lined up in long columns, indicative of their high mitotic activity. These cells undergo hypertrophy and form calcified cartilage. The mineralized cartilage is resorbed by osteoclasts and subsequently replaced by bone formed by osteoblasts.
Lamellar bone is laid down in layers or lamellae, in which the collagen is organized in layers of collagen fibers aligned parallel to each other. This fibre organization allows the highest density of collagen per unit volume of tissue and all the mineral is essentially within the fibrils. Relative to woven bone, lamellar bone is a much stronger bone. Mature bones in higher vertebrates are primarily composed of lamellar bone, which are present in two distinct structures described as cortical and trabecular bone. Both types of bone comprise the same cell types and the same matrix elements, but display structural and functional differences. Approximately 85% of the volume of cortical or compact bone is mineralized, whereas only 20% of the volume of trabecular bone is mineralized. The remaining volume is comprised of bone marrow, blood vessels, and extracellular matrix (ECM). Many bones, such as vertebrae and the long bones, consist of an outer cortex of cortical or compact bone with a central core of trabecular bone. The cortical bone fulfills mainly a mechanical and protective function while trabecular bone has in addition an important metabolic function (Baron, 1999).

1A.1. Bone Cells

While bone remodeling during growth occurs on all surfaces, most of the turnover in adult bone occurs on endosteal surfaces where they interface with bone marrow. To maintain bone mass, it is imperative that the activities of the bone-forming osteoblasts and the bone-resorbing osteoclasts are carefully balanced during remodeling and turnover.

1A.1.1. Osteoblasts

The bone-forming cells are the osteoblasts. Osteoblasts act coordinately to synthesize the organic bone matrix and are responsible for its subsequent mineralization. They are derived from
mesenchymal stem cells (bone marrow stromal stem cells or connective tissue mesenchymal stem cells), which under the appropriate guidance of cytokines and hormones, proliferate and differentiate along a linear sequence progressing from osteoprogenitors to preosteoblasts, to osteoblasts. Osteoblasts may then be retained on the bone surface (bone-lining cells) or become embedded in the matrix (osteocytes), or undergo apoptosis. Osteoblasts are found in clusters along the bone surface (100-400 cells per bone-forming site), and are characterized by a cuboidal shape and a round nucleus, located at the base of the cell opposite to the bone surface. They have a strongly basophilic cytoplasm, and a prominent Golgi complex located between the nucleus and the apex of the cell (Döty and Schofield, 1976). Junctional complexes (gap junctions) are often found between the osteoblasts and osteocytes providing direct communication between cells throughout bone. Osteoblasts first express the organic components of the bone matrix, which form an unmineralized osteoid layer that is subsequently mineralized in a time-dependent manner ensuring a separation between the bone-forming cells and the mineralized matrix. Thus, there is a time lag between bone matrix formation and its subsequent mineralization (24 hours in rat, about 15 days in humans). Located behind the osteoblasts are usually one or two layers of cells comprising activated mesenchymal cells and preosteoblasts (reviewed by Aubin et al., 1993).

Osteoblasts are responsible for the synthesis of most of the proteins in the bone matrix, including collagen (mainly type I with small amounts of type V), and noncollagenous proteins such as bone sialoprotein, osteopontin, and osteocalcin. These and other bone matrix proteins are discussed in detail below. Active osteoblasts contain a large number of mitochondria, a wide Golgi zone, a well-developed endoplasmic reticulum, and possess polarity such that they secrete bone matrix proteins subjacent to the osteoid. The osteoblasts retreat as the matrix is formed and
mineralized. Although the mechanism whereby mineralization occurs is not fully understood, osteoblasts are able to control the mineralization process by expressing proteins that can bind to HA. These proteins regulate the nucleation and growth of mineral crystals. Osteoblasts also express alkaline phosphatase, which can increase the local concentration of inorganic phosphate by hydrolysis of organic phosphates, including pyrophosphate inhibitors of mineral formation (discussed below).

Osteocytes are derived from about 10-20% of osteoblasts and perhaps due to the loss of cell polarity, become entirely surrounded by the mineralized matrix (Menton et al., 1984). Osteocytes maintain cellular continuity with other osteocytes, as well as osteoblasts, by forming gap junctions via extensive cell processes that run through small channels called canaliculi within bone tissue (Doty, 1981). Although the function of osteocytes is not entirely clear, they respond to physical stimulation and may mediate "osteolytic" resorption and bone formation, depending on whether they are subjected to compressive or tensional forces (Lanyon, 1992; Pead et al., 1988).

The role of lining cells is also unclear. Lining cells are derived from osteoblasts that have ceased their activity and flattened out on the surface of bone that is no longer undergoing formation or resorption. Lining cells cover 80% of trabecular bone surfaces and about 95% of the intracortical bone surfaces (Parfitt, 1983), and as such may provide a protective and sensotorial layer (Heersche, 1989).

1A.1-2. Osteoclasts

Osteoclasts are multinucleated cells that have the unique ability to resorb mineralized bone. The activity of osteoclastic cells, relative to the activity of osteoblasts, dictates the
development of osteoporotic diseases, characterized by a decreased bone mass, which increases susceptibility to bone fractures.

Osteoclasts are derived from myeloid precursors that give rise to the monocyte/macrophage cell lineage (Udagawa et al., 1990). Under the guidance of hormones and local cytokines, some of which act indirectly through osteoblastic cells, osteoclast precursors migrate to the bone surface and fuse to form large multinucleated cells, which can contain more than 20 nuclei. Osteoclasts are generally observed within resorptive lacuna or pits, formed as a result of the cells' resorptive activity. The cells are polarized and form a characteristic "ruffled membrane" on their resorptive surface. This ruffled membrane probably represents the transport of acidifying vesicles along microtubules and their polarized insertion into the plasma membrane. A "sealing zone" surrounds the ruffled membrane and generates an isolated extracellular microenvironment between the osteoclast and the bone surface (reviewed by Teitelbaum, 2000).

Bone resorption involves demineralization, which occurs through acidification of the isolated extracellular microenvironment. This process is mediated by a vacuolar adenosine triphosphatase (H⁺-ATPase or V-ATPase) in the ruffled membrane of the cell (Blair et al., 1989). An energy independent Cl⁻/HCO₃⁻ exchanger on the antiresorptive surface of the cell maintains the intra-osteoclastic pH (reviewed by Baron et al., 1993). The resorptive microenvironment has a pH of ~4.5, due to the secretion of H⁺ and Cl⁻ ions. The acidic milieu demineralizes the bone exposing the organic matrix. The matrix is subsequently degraded in a temporal manner by lysosomal proteases, such as cathepsin K (Gowen et al., 1999) and tartrate-resistant acid phosphatase (Hammarstrom et al., 1971), as well as non-lysosomal enzymes, including the cysteine proteinases and matrix metalloproteinases (Everts et al., 1998). The products of bone
degradation are endocytosed by the osteoclast through the ruffled border, which acts as a specialized secondary lysosome, and transported out of the cell at its antiresorptive surface (Nesbitt and Horton, 1997).

In vitro studies of the maturation of myeloid cells into osteoclasts have shown that this process requires bone marrow stromal cells, including osteoblasts and its precursors (Udagawa et al., 1990). The stromal cells express two molecules that are essential for osteoclastogenesis: macrophage colony-stimulating factor (M-CSF) and a cell-surface receptor for activation of nuclear factor kappa B (NFκB) ligand (RANKL; also known as OPGL or TRANCE; reviewed by Teitelbaum, 2000). Although M-CSF is a secreted product, osteoclastogenesis requires contact between osteoclast precursors and stromal cells or osteoblasts (Udagawa et al., 1990) to allow interaction between RANKL and its cognate receptor on the cell surface of osteoclast precursors. Notably, complete osteoclastic differentiation can occur in vitro with pure populations of monocytes/macrophages exposed only to M-CSF and soluble RANKL (Lacey et al., 1998). This observation underscores the pivotal role of these signaling molecules in osteoclastogenesis and in mediating the coupling between the activities of osteoblasts and osteoclasts in regulating bone mass.

1A.2. Bone matrix proteins

The progression of bone formation can be best followed by the analyses of proteins present in the bone extracellular matrix (ECM). Considerable advances have been made in the past two decades in identifying and elucidating potential functions of bone ECM constituents.

Proteins that contribute to the matrix include structural proteins such as collagen, and noncollagenous proteoglycans and matricellular proteins. Most of the proteins present in the
bone matrix are produced by osteoblasts. However, several proteins that are preferentially adsorbed by the bone mineral are derived from the serum and tissue fluids. They include proteins such as α2-HS-glycoprotein and albumin, which are synthesized in the liver, and matrix gla protein, a potent inhibitor of mineralization produced by soft connective tissues and cartilage (Luo et al., 1997).

1A.2.1. Collagen

Collagens are a family of fibrous proteins that have a well-defined structure. These proteins are found in all multicellular animals, and are secreted by connective tissue cells. As a major component of skin and bone, the collagens are the most abundant proteins in vertebrates, constituting 25% of the total protein mass. Collagen molecules are composed of three ~100 kDa α-chains, characterized by a Gly-X-Y repeating triplet (where X and Y are commonly proline and hydroxyproline residues, respectively). The individual α-chains fold in a left-handed polypropyl helix and wind together to form an extended, right-handed superhelical structure that characterizes collagenous proteins. The fixed ring structure of proline and hydroxyproline residues stabilizes the left-handed helix of the α-chains, whereas the glycine residues, present at every third amino acid position, allow the three α-chains to pack close together to form the collagen helix (Prockop et al., 1979). Certain proline and lysine residues become hydroxylated in the lumen of the endoplasmic reticulum to produce hydroxyproline and hydroxylysine, respectively. Hydroxyproline residues form hydrogen bonds that stabilize the triple helix, whereas lysine and hydroxylysine residues are important for intra- and inter-molecular cross-linking of collagen fibrils. The cross-links are generated by lysyl oxidase modifications of some lysines, forming allysine (and hydroxyallysines), which can condense together as intramolecular
aldol condensation products or can form intermolecular cross-links with lysines (and hydroxylysines) through Schiff-base formation. These cross-links undergo further interactions (maturation) that can ultimately lead to the formation of pyridinium ring structures (pyridinolines; Eyre et al., 1988). Since some of the cross-link structures are unique to bone they can be analyzed as collagen degradation products in urine to provide a measure of bone resorption in the body (Eyre, 1997).

Collagen comprises the major (80-90%) organic component in mineralized bone tissues. Although 20 distinct types of collagen molecules have been identified to date, type I collagen is the main collagen in mineralized bone (95%). A small amount of type V collagen (<5%) forms heterotypic fibres with type I collagen. Smaller amounts of type III and XII may also be present. Collagens provide the basic structural integrity of connective tissues (reviewed by Sodek and McKee, 2000).

Type I collagen, which is responsible for the structural integrity of many connective tissues, including bone, is composed of two \( \alpha 1(I) \) chains and one \( \alpha 2(I) \) chain that are named according to their elution from an ion-exchange column. Each polypeptide \( \alpha \)-chain is derived from precursor pro-\( \alpha \) chains. In type I collagen, two identical pro-\( \alpha 1(I) \) chains, and one structurally similar but genetically different pro-\( \alpha 2(I) \) chain, combine in the rough endoplasmic reticulum to form a triple helical procollagen molecule (Hu et al., 1995). As the procollagen molecule is secreted, specific peptidases such as procollagen C-proteinase (PCP) cleave the procollagen to produce collagen molecules. Interestingly, PCP was identified to be the gene product of \textit{bone morphogenetic protein-1 (BMP-1)} (Kessler et al., 1996), which has been implicated in de novo endochondral bone formation (Wozney et al., 1988). It appears that BMP-1/PCP has the ability to cleave procollagen as well as chordin (Blitz et al., 2000), an antagonist
of other BMP family members, which are important in bone formation. This suggests that BMP-1/PCP is important for the link between matrix deposition/maturation and pattern formation. The BMPs are discussed in more detail below.

Upon secretion and activation, the collagen molecules self-assemble in a characteristically staggered arrangement of collagen fibrils. Each collagen molecule is incorporated into a parallel array so that it extends beyond its neighbour by a quarter of its length (Trus and Piez, 1976). The gaps created between successive molecules give rise to the striated appearance of a negatively-stained collagen fibril. It is within these gap regions that mineralization is believed to first occur. In lamellar bone, the collagen fibrils show a plywood-like arrangement, in which successive layers of fibrils are laid down nearly at right angles to each other. Most of the mineral in lamellar bone resides within the collagen fibers. Mutations affecting type I collagen cause osteogenesis imperfecta, characterized by brittle bones that easily fracture. It is evident from such diseases that type I collagen serves as the infrastructure on which mineralization occurs (Christoffersen and Landis, 1991).

1A.2-2. Noncollagenous Proteins

Noncollagenous proteins (NCPs) comprise 10% to 15% of the total bone protein content. NCPs are believed to have important roles in the organization of the collagen matrix and in regulating the formation and growth of the HA crystals. Osteoblastic cells secrete the majority of the NCPs, but due to the highly vascularized nature of bone tissue and the ability of HA crystals to adsorb and concentrate exogenous material, about 25-35% of bone NCPs are exogenously derived. This exogenous fraction is largely composed of serum-derived proteins such as albumin and α2-HS-glycoprotein, which are acidic in character, present at higher concentrations in bone
than serum, and bind to HA. The remainder of the exogenous fraction is composed of growth factors and a large variety of molecules present at trace amounts that exert effects on matrix mineralization and influence local bone cell activity (Boskey, 1996). α2-HS-glycoprotein, which is the human analogue of fetuin, contains a TGF-β/BMP receptor-like domain, and so may regulate bone formation by antagonizing the effects of BMPs (Binkert et al., 1999; Demetriou et al., 1996).

The study of bone proteins has been technically difficult because of the interactive properties of the constituent proteins and the tight association of these proteins with the mineral phase of the bone matrix. The isolation of bone proteins was facilitated by the development of dissociative extraction procedures (Linde et al., 1980; Termine et al., 1981). This allowed a more comprehensive characterization of the proteins to be achieved.

The endogenously synthesized noncollagenous bone proteins can be broken down into four general groups: proteoglycans, glycosylated proteins, glycosylated proteins with potential cell-attachment activities (RGD-motif), and γ-carboxyglutamic acid (gla)-containing proteins.

1A.2-2a. Proteoglycans

The bone matrix contains several proteoglycans, macromolecules that contain acidic polysaccharide side chains attached to a central core protein. Biglycan and decorin are two small proteoglycans that are produced and localized in bone and other tissues (Bianco et al., 1990). Together, the two proteins can comprise ~10% of the non-collagen proteins in bone, but this decreases with age. Both proteoglycans consist of a core protein of approximately 46 kDa that contains leucine-rich repeats that are characteristic of the SLRPs (small, leucine rich proteoglycans). They contain either one (decorin) or two (biglycan) glycosaminoglycans (GAG)
chains, which in bone is in the form of chondroitin sulphate, differentiating them from the same proteins found in soft connective tissues and cartilage where the GAG chains are prominently dermanan sulphate. Decorin appears to be involved in the regulation of collagen formation (Vogel and Trotter, 1987) by binding within the gap region of collagen fibrils, and “decorating” the fibril surface (Scott et al., 1989). Biglycan appears to be a positive regulator of bone formation as deletion of the biglycan gene in mice leads to a significant decrease in the development of trabecular bone and an osteoporosis-like phenotype (Xu et al., 1998).

**IA.2-2b. Glycoproteins**

A major marker of bone formation is the synthesis of high levels of alkaline phosphatase (ALP), although ALP is also expressed in a number of other tissues. In bone, ALP is found mainly on the surface of osteoblasts bound via a phosphoinositide (PI) linkage, and technically is not a bone matrix protein. However, ALP can be released from the cells by PI-specific phospholipase C (Noda et al., 1987). ALP has been hypothesized to be involved in the mineralizing process by hydrolyzing organic phosphates to elevate the local phosphate concentration to a level required for initiation of HA crystal formation. In support of this hypothesis, studies using antisense RNA (Tori et al., 1996) or inhibitors of ALP activity (Bellows et al., 1991; Bellows et al., 1992; Tenenbaum, 1987) have shown that inhibition of ALP activity reduces β-glycerophosphate hydrolysis and calcium accumulation during the initiation phase of mineralization in cultures of calvarial cells. Genetic analyses of the human hereditary disease hypophosphatasia, characterized by subnormal serum levels of ALP and defective bone mineralization resulting in rickets or osteomalacia, showed the presence of missense mutations in the *ALP* gene (Henthorn et al., 1992; Weiss et al., 1988; Zurutuza et al., 1999). Furthermore,
ALP knockout mice show defects in bone mineralization characteristic of hypophosphatasia such as spontaneous fractures, skeletal deformations, and areas of hypo-mineralization (Fedde et al., 1999; Waymire et al., 1995).

Osteonectin (ON) or SPARC (secreted protein acidic and rich in cysteine) is the most abundant NCP produced by osteoblasts, accounting for about 2% of the total protein in developing bones of larger animals. ON was first identified from subperiosteal bovine bone and called osteonectin based on its ability to bind collagen and HA in vitro (Termine et al., 1981). Although originally thought to be specific to bone, ON was subsequently identified in a wide variety of cell types throughout invertebrate and vertebrate organisms, suggestive of a more basic functional role (Yan and Sage, 1999). For example, fibroblastic cells isolated from ON-null mice show an increased rate of proliferation (Bradshaw et al., 1999), consistent with the inhibition of proliferation by ON observed in vitro (Sage et al., 1995). As well, the presence of two Ca$^{2+}$-binding EF-hand sites are suggestive of a regulatory role such as has been shown in Xenopus development (Huynh et al., 1999). In addition to the formation of cataracts in the eyes of ON knockout mice (Gilmour et al., 1998; Norose et al., 1998), the mice also had a decrease in bone formation and osteopenia demonstrating the importance of ON in bone (Delany et al., 2000).

1A.2-2c. RGD-Containing Glycoproteins

Two major sialoproteins, bone sialoprotein (BSP) and osteopontin (OPN), containing polyacidic amino acid hydroxyapatite-binding motifs and an RGD cell-attachment motif have been identified in bone. BSP and OPN together comprise most of the mineral-associated proteins in bone. The nascent proteins are enriched in acidic amino acids and are highly glycosylated and
phosphorylated. The RGD motifs in both proteins are recognized primarily by the α₅β₃ vitronectin receptors.

BSP, which constitutes 8-12% of the total NCPs in bone (Fisher et al., 1987; Fisher et al., 1990; Fisher et al., 1983), was first isolated from bovine cortical bone (reviewed by Herring, 1972) as a 23 kDa glycoprotein with high sialic acid content. However, it was not until subsequent development of dissociative extraction procedures that the intact form of BSP was isolated. Newly deposited BSP was purified from 1-2 mm of the outer section of fetal calf periosteal bone, a site of rapid bone growth (Fisher et al., 1983). However, subsequent studies indicated that the preparation was contaminated with a second sialoprotein, OPN (Franzen and Heinegard, 1985).

BSP is an acidic glycoprotein with a protein core of 33 kDa that migrates at an apparent $M_r$ of 60-80 kDa on SDS-PAGE due to its acidic nature and its high carbohydrate content, which includes sialic acid, glucosamine, and galactosamine (Fisher et al., 1983). Several serines are phosphorylated and a number of tyrosines surrounding the RGD motif are sulphated. BSP has generated considerable interest as a potential nucleator of HA crystals and as a specific marker for osteoblast differentiation (reviewed by Ganss et al., 1999). Studies in vitro and in vivo showed that its expression, which is restricted to osteoblasts, is initiated co-incident with the onset of mineralization of bone tissue (Chen et al., 1992; Yao et al., 1994). In a steady-state system where calcium and phosphate concentrations are maintained below the threshold for spontaneous precipitation, BSP is effective in promoting the nucleation of HA crystals in an agarose gel (Hunter and Goldberg, 1993). Stretches of successive glutamic acid residues present in BSP are responsible for its nucleating activity (Goldberg et al., 1996).
OPN was first separated from BSP in demineralized extracts of bovine bone through the use of cation exchange-column chromatography (Franzen and Heinegard, 1985). The name "osteopontin" was introduced to reflect the potential of this protein to serve as a bridge between cells and HA through its RGD motif and stretches of polyaspartic acid residues, respectively (Oldberg et al., 1986). The 34 kDa nascent protein is acidic, and the secreted protein is extensively modified by post-translational events, including glycosylation and extensive phosphorylation. The protein migrates at an apparent $M_r$ of 45-75 kDa on SDS-PAGE (reviewed by Sodek et al., 2000). Although many similarities exist between BSP and OPN, it is clear that they have distinct functions. Unlike BSP, which has been shown to promote nucleation of HA crystals (Hunter and Goldberg, 1993), OPN is a potent inhibitor of nucleation in steady-state gelatin gels (Boskey et al., 1993), in agarose gels, and in solution systems (Goldberg and Hunter, 1995; Hunter et al., 1996) with calcium and phosphate concentrations above the threshold of spontaneous precipitation.

BSP and OPN differ markedly in their tissue distribution. BSP expression is essentially restricted to mineralized tissues, whereas OPN is also expressed by many other tissues and cells, reflecting more diverse functions (reviewed by Sodek et al., 2000). Osteopontin has been shown to be involved in the regulation of immune reactions, in preventing bacterial infection, in regulating ectopic mineral formation, and in cancer metastasis. These and other effects on basic cell activities, such as migration, proliferation, apoptosis, and phagocytosis, involve signaling through different integrin receptors and CD44 (reviewed by Sodek et al., 2000). Furthermore, different post-transcriptionally modified forms of OPN have been identified, which may have discrete functions in different tissues. An intracellular form of OPN has been identified in osteogenic cultures of fetal rat calvarial cells (Zohar et al., 1997).
Despite strong evidence for BSP and OPN acting as a nucleator and inhibitor of HA crystal formation, respectively, BSP and OPN knockout mice have not been reported to have skeletal abnormalities (Liaw et al., 1998).

1A.2-2d. $\gamma$-Carboxyglutamic Acid (Gla)-Containing Proteins

Osteocalcin (OC), also known as bone $\gamma$-carboxyglutamic acid (gla) protein, is a small, acidic, 5.8 kDa protein that is characteristically modified by vitamin K-dependent carboxylating enzymes converting two to three glutamic acid residues into gla groups. These gla groups bind calcium, and allow OC to bind to HA crystals with high affinity. OC represents ~15% of the noncollagenous proteins and was the first noncollagenous protein to be characterized. Despite extensive studies, the role of OC in bone formation and remodeling is not clear. Treatment with the vitamin K antagonist warfarin reduces osteocalcin levels in bone, which become hypermineralized (Price and Williamson, 1981). OC knockout mice show an osteopetrotic phenotype (Ducy et al., 1996). Gene ablation of matrix gla protein, a similar protein to OC, results in ectopic calcification of arteries and cartilage, eventually leading to death as a result of the arteries cracking (Luo et al., 1997). The above results suggest a role of OC in limiting the mineralization process. However, the regulation of OC gene expression by hormones such as vitamin D3 (Demay et al., 1989) and PTH (Yu and Chandrasekhar, 1997), together with the ability of OC to act as a chemoattractant to osteoclast precursors (Mundy and Poser, 1983), also suggests a role for OC in bone resorption (Glowacki et al., 1991).

Extensive studies on the OC gene show a highly conserved region that is required for the basal expression of OC in osteoblasts, called the "OC Box" (Heinrichs et al., 1993). Two regulatory elements called osteoblast-specific element1 (OSE1) and element2 (OSE2) serve to
strongly increase OC transcription in osteoblasts (Ducy and Karsenty, 1995). Recently, osteoblast-specific factor1 (Osf1; Schinke and Karsenty, 1999) and factor2 (Osf2/Runx2; Ducy et al., 1997) have been identified that bind to OSE1 and OSE2, respectively. These factors are discussed in detail below.

1A.3. Osteoblastic Differentiation

Understanding the origin and differentiation of osteoblastic cells has been hampered both by the lack of a detailed understanding of osteoblast functions and by the lack of a criteria for identifying osteoblastic cells at early developmental stages. ALP, collagen, and noncollagenous proteins, discussed above, are generally expressed in bone cells already relatively mature (Aubin et al., 1993; Yao et al., 1994) such as preosteoblasts, osteoblasts, osteocytes, and bone-lining cells. These cells are the most differentiated and easily recognizable cell types in the osteoblast family. In comparison to other cell lineages such as the hematopoietic cell lineage, which has contributed greatly to the knowledge of the osteoclast lineage, relatively little is known about the osteogenic cell lineage.

Undifferentiated mesenchymal stem cells residing in the stromal tissues surrounding bone marrow and in other connective tissue compartments, such as the periosteal layer of bone, eventually differentiate into osteoblasts. Mesenchymal stem cells are characterized by their ability to self-renew, high proliferative capacity, and multi-potentiality. Such cells have been isolated from stromal cell populations of the bone marrow (Haynesworth et al., 1992) and more recently from embryonic calvarial cells (Zohar et al., 1997). These mesenchymal precursor cells can differentiate into at least five discrete cell lineages: osteoblasts, chondroblasts, fibroblasts, adipoblasts, and myoblasts (Aubin and Kahn, 1996). Instructional signals from cell-cell and cell-
matrix interactions and from diffusible molecules are believed to be important determinants for
directing tissue formation and organogenesis in these multipotent stem cells. The osteogenic
pathway is further complicated by the possible existence of at least two types of osteoprogenitor
cells, a determined osteogenic precursor cell (DOPC) and an inducible osteogenic precursor cell
(IOPC; Friedenstein, 1976; Owen, 1985; Owen and Friedenstein, 1988). DOPCs are stromal cells
present in bone marrow. These cells are clonogenic, have fibroblastic morphology, are highly
proliferative, and are capable of giving rise to bone, cartilage, fibrous connective tissue, muscle,
and fat when implanted in vivo. IOPCs, in addition to their presence in osteogenic tissues, are
also found in non-osteogenic tissues such as muscle and soft connective tissues. Upon the
influence of osteo-inducing agents, the IOPCs can form ectopic bone and cartilage.

A major area of research has been the search for molecules instrumental in influencing
the mesenchymal stem cells towards the osteogenic lineage pathway. Currently, two factors
appear fundamental for the differentiation of mesenchymal cells to osteoblasts: bone
morphogenetic proteins (BMPs) and Runx2. BMPs are the primordial signals for bone formation
and Runx2 is a transcription factor that is essential for bone formation.

1A.3.1. Bone Morphogenetic Proteins

In 1965, Urist demonstrated that demineralized bone contain factors with an ability to
induce ectopic bone formation when implanted in ectopic sites in mammals (Urist, 1965). Subsequently a similar activity was demonstrated in demineralized dentin (Huggins et al., 1970). Urist was unable to isolate BMPs mainly due to a lack of a reproducible, quantitative bioassay for measuring bone growth. Almost 20 years later, Sampath and Reddi (1981) developed an in vivo bone induction assay that involved measuring the activity of alkaline phosphatase and
calcium content at these ectopic implant sites. By utilizing this assay, the first BMPs were isolated and sequenced (Ozkaynak et al., 1990; Wozney et al., 1988). This unique bone inductive activity, which has also been demonstrated in vitro (Sampath et al., 1992; Wozney, 1992), indicates that BMPs provide the primordial signals for osteo-differentiation. This is supported by the BMP-induced expression of Runx2 (Ducy et al., 1997), which has been identified as a master gene for osteogenesis (Rodan and Harada, 1997). Runx2 is discussed in detail below.

BMPs belong to the large TGF-β superfamily of secreted cytokines. Members of this superfamily are dimeric proteins that share a high degree of identity within the COOH-terminal domains of the subunits most noticeably in the conservation of 7 cysteine residues. Based on the crystallization studies of BMP-7/OP-1 (Griffith et al., 1994) and TGF-β2 (Daopin et al., 1992; Schlunegger and Grutter, 1992), six cysteine residues are closely grouped to form a rigid cysteine knot, while the last cysteine residue is involved in a disulfide linkage to form homo or hetero-dimeric proteins.

As members of the TGF-β superfamily, BMPs influence many other developmental processes besides bone induction. BMP-2 and BMP-4 are 92% identical and are mammalian homologues of the Drosophila decapentaplegic (dpp) gene. In fact human BMP-4 and Drosophila dpp are functionally conserved as demonstrated by the induction of endochondral bone formation in mammals by ectopic implantation of dpp, and the rescue of dpp-null mutants with a BMP-4 transgene (Padgett et al., 1993; Sampath et al., 1993). Consistent with the requirement of dpp in the induction of ventral mesoderm in Drosophila (Ferguson and Anderson, 1992), disruption of the BMP-4 gene is embryonic lethal in mice (Winnier et al., 1995). These mice show mesoderm development defects and a null mutation of the BMP-2 gene also results in nonviable mice with defects in amnion/chorion and cardiac development (Zhang and Bradley,
BMPs 5-8 share 75% identity and are mammalian homologues of the \textit{Drosophila 60A} gene. Mutations in the \textit{Bmp-5} gene cause effects in cartilage and bone and are responsible for the reduced size of the external ear in the short ear mouse (Kingsley, 1994). BMP-7/OP-1 knockout mutants die at birth due to severe defects in kidney development but also show defects in eye and skeletal development (Dudley et al., 1995; Luo et al., 1995). From the gene ablation studies of various BMPs, it is clear that the ability of BMPs to induce and influence bone formation and patterning is not their only role.

\textbf{1A.3-2. Smad Signaling}

BMPs transmit their signals through type I and type II serine/threonine kinase transmembrane receptors (ten Dijke et al., 1996). Subsequent to the binding of BMPs, the type II receptor phosphorylates the conserved GS box of the type I receptor and thereby activates this receptor (Wrana et al., 1994). The Smad family of proteins mediates signaling by these receptors (Heldin et al., 1997; Kretzschmar and Massague, 1998). BMP signals are mediated by Smad1, Smad5 or Smad8 (Hoodless et al., 1996; Liu et al., 1996; Macias-Silva et al., 1998; Nishimura et al., 1998) whereas TGF-\( \beta \) and activin signals are mediated by Smad2 or Smad3 (Eppert et al., 1996; Liu et al., 1997; Nakao et al., 1997). The Smads share extensive similarity in the amino-terminal MH1 (MAD homology 1) domain and the carboxy-terminal MH2 domain, which are separated by a linker region. Direct phosphorylation of serine residues in an "SSXS" motif in the MH2 domain by the activated type I receptors (Abdollah et al., 1997) is thought to relieve the inhibitory interaction with the MH1 domain (Hata et al., 1997) allowing the formation of active Smad heteromers comprising receptor-regulated Smads associated with a common partner,
Smad4 (Lagna et al., 1996; Nakao et al., 1997). The Smad complex then translocates into the nucleus (Zhang et al., 1997) to influence the transcription of target genes.

1A.3-3. Runx2

The search for target genes of BMPs that promote the downstream effects on osteogenesis has identified several genes that may be important in mineralized tissue formation. Of particular importance in BMP-induced osteogenic differentiation is the expression of Runx2, which is critical for bone formation. Thus, both intramembranous and endochondral ossification are blocked in Runx2 knockout mice due to the maturational arrest of osteoblastic cells (Komori et al., 1997; Otto et al., 1997). The Runx2-null mice die shortly after birth as a consequence of respiratory problems associated with the absence of ossified ribs, which are needed to maintain the integrity of the thoracic cavity. In Runx2 +/- heterozygous mice, Otto and colleagues noticed abnormalities, the most prominent being hypoplasia of the clavicle and the delayed development of membranous bones, phenotypes that are typical features of the human disease cleidocranial dysplasia (CCD), an autosomal dominant bone disorder (Mundlos et al., 1997; Otto et al., 1997). Deletions, insertions, or mutations that inactivated one allele of the Runx2 gene were shown to be the cause of the CCD syndrome in humans (Lee et al., 1997; Mundlos et al., 1997). Runx2 therefore is considered to be a master gene for osteogenesis (reviewed by Ducy and Karsenty, 1998; Karsenty, 1998; Rodan and Harada, 1997), complementing the identification of PPARγ and the myogenic transcription factors as master genes in adipogenesis (Loftus and Lane, 1997), and myogenesis (Arnold and Winter, 1998), respectively.

Runx proteins, also known as PEBP2/Cbf/AML, are sequence-specific DNA-binding proteins originally identified as the polyomavirus enhancer binding protein (PEBP) and later as
the Moloney murine leukemia virus enhancer core binding protein. Runx binds the consensus DNA element 5'-ACCCPuCPu-3' through two subunits, α and β. The α-subunit shares a conserved 128 amino acid DNA-binding region called the runt domain because of its homology to the Drosophila pair-rule gene run. The runt domain also mediates heterodimerization with the β-subunit, which allows the α-subunit to bind to DNA (Wheeler et al., 2000). The α-subunits are encoded by three distinct genes, \( \text{Runx1} \) (PEBP2αB/Cbfα2/AML1), \( \text{Runx2} \) (PEBP2αA/Cbfα1/AML3), and \( \text{Runx3} \) (PEBP2αC/Cbfα3/AML2). Thus far, only one gene encoding the β-subunit (CBFβ) has been described in mammals (Ogawa et al., 1993; Wang et al., 1993).

A splice variant of Runx2, called Osf2 or osteocalcin specific factor-2, is expressed abundantly in skeletal tissues. Osf2 was identified as a factor that bound to the OSE2 of the osteocalcin gene (Ducy et al., 1997), specifically to the DNA sequence 5'-AACCACA-3', which corresponds well with previously characterized DNA-binding sites for the Runx family. This minimal Runx2 binding site is present in many of the promoters of the bone matrix proteins. The presence of multiple transcripts generated from the Runx2 gene [Runx2/PEBPα2A, (Ogawa et al., 1993), Runx2/Osf2 (Ducy et al., 1997), Runx2/til-1, (Stewart et al., 1996)] has complicated the analysis of the precise role of Runx2 isoforms in osteogenesis. The three splice variants exhibit different effects during osteoblast differentiation (Harada et al., 1999) and their functions remain to be elucidated.

In addition to the interaction with the CBFβ protein, Runx2 can interact with a number of other proteins to alter transcriptional activity of target genes. Runx proteins are currently viewed as context-dependent regulators, which activate or repress transcription depending upon the organization of a particular promoter in a specific cell at a certain time. For example,
Runx2/PEBP2αA interacts with Ets1, a transcription factor involved in bone development (reviewed by Raouf and Seth, 2000), to synergistically induce osteopontin gene expression (Sato et al., 1998). Transactivation of the osteopontin promoter is dependent on the distance between the Runx2/PEBP2αA and Ets1 DNA-binding sites. Each protein binds independently to its cognate DNA-binding element but the distance between the sites appears to determine the direct interaction between Runx2/PEBP2αA and Ets1. Similarly, Runx and Smad proteins have been shown to form complexes to stimulate transcription of target genes (Hanai et al., 1999). Specifically, Runx3 interacts with Smad3 (involved in TGF-β/activin pathways) to stimulate transcription of the germline Ig Cα promoter in a cooperative manner, and the binding of both factors to their specific binding sites is essential for transactivation. Furthermore, the cooperation between Runx2 and Smad5 (involved in BMP pathways) induces osteoblast-specific gene expression in a myogenic cell line, C2C12 (Lee et al., 2000). The transactivation of genes by Runx2 and its interacting proteins appears to be through the recruitment of histone acetylases (HATs) such as CBP/p300 to the promoter (Kitabayashi et al., 1998).

Runx proteins can also repress transcription. Thus, Runx proteins can interact with the Groucho/TLE1 class of corepressors through a conserved VWRPY C-terminal sequence motif that is present in all runt-domain proteins (Aronson et al., 1997). Alternatively, Runx proteins can repress transcription through a Groucho/TLE1-independent mechanism (Lutterbach et al., 2000). Both modes of repression appear to involve histone deacetylase (HDAC) activity (Kitabayashi et al., 1998; Wheeler et al., 2000).

Recent studies have shown that two additional upstream enhancers for Runx2 are present in the mouse osteocalcin promoter that appear to function cooperatively with the proximal OSE in regulating osteocalcin transcription (Javed et al., 1999). Since Runx proteins can interact with
various HATs, HDACs, and the nuclear matrix (Zeng et al., 1998; Zeng et al., 1997), it has been proposed that Runx2 exerts its transcriptional regulation, in part, through rearrangement of the chromatin structure (Lian et al., 2001; Stein et al., 1999).

In addition to the Runx2 enhancer, OSE2 (5'-ACCACA-3'), a second osteoblast-specific cis-acting element, OSE1, was also identified in the osteocalcin gene. Although an osteogenic specific factor1 (Osfl) has been found to bind the OSE1 (5'-TTACATCA-3'), Osfl has yet to be cloned and sequenced (Schinke and Karsenty, 1999). It is possible that the OSE1 may be as important as OSE2 in controlling osteoblast-specific expression.

1A.3-4. Other Factors

Although considerable advances have been made in recent years, the underlying molecular mechanisms whereby Runx2, and BMP/Smads in combination with Runx2, control osteoblast gene expression, are still poorly understood. Only a few putative, immediate target genes been identified to date, and some of these are described below.

TIEG (TGF-β-inducible early gene), a putative transcription factor, was identified in osteoblasts using differential display-PCR (Subramaniam et al., 1995). The expression of TIEG is up-regulated within one hour after cells are treated with TGF-β or BMP-2 (Subramaniam et al., 1995), and the over-expression of TIEG mimics the effects of TGF-β in human osteoblasts (Hefferan et al., 2000). TIEG possesses three zinc finger motifs and several proline-rich Src-homology-3 (SH3) binding motifs, and is believed to play a role in apoptosis, tumor suppression, in addition to its putative role in osteogenesis (Subramaniam et al., 1995; Subramaniam et al., 1998).
BORG (BMP/OP responsive gene) has been identified as a non-coding RNA that is up-regulated within 2 hours of BMP treatment (Takeda et al., 1998). Forced expression of BORG will induce transdifferentiation of C2C12 myogenic cells into osteoblastic cells, whereas blockage of BORG by antisense oligonucleotides inhibits osteogenic differentiation (Takeda et al., 1998).

TAK1, a novel member of the mitogen-activated protein kinase kinase kinase (MAPKKK) family, was identified to be a potential mediator of TGF-β and BMP-4 signal transduction (Yamaguchi et al., 1995). TAK1 along with its activator, TAB1 (Shibuya et al., 1996), is required for the expression of ventral mesoderm marker genes induced by Smad1 or Smad5 (Shibuya et al., 1998). However, the role of TAK1/TAB1 in osteogenesis is currently unknown.

Dlx5 (or distal-less 5) is a homeobox-containing gene that is up-regulated within one hour of BMP treatment in MC3T3-E1 osteoblastic cells (Miyama et al., 1999). Dlx5 is expressed at high levels in bone and brain (Chen et al., 1996), and the over-expression of Dlx5 in MC3T3-E1 cells results in higher alkaline phosphatase (ALP) activity, osteocalcin (OC) production, and mineralization of extracellular matrix, suggesting that Dlx5 is a positive regulator of osteoblastic differentiation (Miyama et al., 1999). Several other homeobox genes such as Hox, Msx, and Mixl have also been implicated in BMP signaling and bone formation although their precise roles have not been clarified (Iimura et al., 1996; Iimura et al., 1994; Mead et al., 1996).

LMP-1 (or LIM mineralization-1) protein is a novel LIM domain containing protein that is induced by BMP-6 in secondary cultures of fetal rat calvarial cells (Boden et al., 1998). The LIM domain is a zinc finger structure involved in protein-protein interactions that is present in several types of proteins including homeodomain- and kinase-containing proteins. The inhibition
of LMP-1 expression by antisense oligonucleotides blocked osteoblastic differentiation in vitro, while conversely, the over-expression of LMP-1 was sufficient to initiate bone nodule formation in vitro and in vivo (Boden et al., 1998). BMP-6 is expressed in hypertrophic chondrocytes (Carey and Liu, 1995), and is part of a regulatory loop involving PTHrP and Ihh that regulates the growth of long bones (Lanske et al., 1996; Vortkamp et al., 1996). It appears that LMP-1 provides an important signal, downstream of BMP-6, coordinating the linkage between cartilage maturation and the initiation of bone formation.

The Id family of genes, Id1, Id2, and Id3, which encode negative regulators of basic HLH proteins, were identified by differential display RT-PCR experiments, as targets of BMP-4 in ES stem cells (Hollnagel et al., 1999). Id family members form heterodimers with the ubiquitously expressed E proteins (E2A, E2-2, and HEB) and inhibit their ability to dimerize with the tissue-restricted bHLH proteins. This prevents the bHLH proteins from activating expression of target genes. Down-regulation of Id genes is necessary for terminal differentiation in developmental processes such as myogenesis (Jen et al., 1992). This indicates that the repression of genes is important for directing differentiation of a multipotent stem cell along a specific pathway.

**1B. Gene Regulation**

With over 30 000 genes in the human genome, transcription must be carefully regulated in accordance with cellular function. For all genes characterized to date, there is a regulatory region of DNA near the transcription start site of the gene that binds the general or basal transcription factors required for transcription. In addition, sites of gene-specific regulation can be found either within this proximal promoter region or at many distances upstream from the transcription start site, as well as within intron sequences downstream of the start site. Thus, the
promoters of eukaryotic genes are divided into the core promoter, which binds the general transcription factors, and distal regulatory elements that bind gene- and/or tissue-specific transcription factors that enhance or repress transcription.

Transcription from DNA templates requires the activity of RNA polymerases (RNAPs). In eukaryotes, RNA products are synthesized by three different RNAPs. RNAP I synthesizes the 5.8S, 18S, and 28S ribosomal RNAs (rRNAs), RNAP II synthesizes messenger RNA (mRNA) and most small nuclear RNAs (snRNA), and RNAP III synthesizes transfer RNA (tRNA) and a variety of other small cellular (i.e., 5S rRNA and U6 RNA) and viral RNAs. Each of these RNAPs is a large multi-subunit protein complex or molecular machine that is specialized to transcribe DNA to RNA. The backbone model of yeast RNAP II was derived from x-ray diffraction data resolved to 3 angstroms (Cramer et al., 2000). Due to the highly conserved nature of archaeal, as well as all three eukaryotic RNAPs, this crystal structure, in which 10 of the 12 subunits comprising yeast RNAP II was crystallized, represents the "core" RNAP. RNAP II is described as consisting of "jaws" to hook on to the downstream DNA, "clamps" that securely fasten the DNA, and pores where nascent RNA and substrates for polymerization can exit and enter, respectively (Cramer et al., 2000). However, the regulation of transcription is controlled by transcription factors. In RNAP II promoters, a unique protein-DNA complex is formed by the interaction of the TATA-binding protein (TBP) with its cognate TATA element. This initial complex serves as a platform for the assembly of the remaining transcription machinery, which includes the general transcription factors and RNAP II. Recently, the concept of sequential assembly has been replaced by the recognition of a RNAP II holoenzyme (Ossipow et al., 1995). Since activators and suppressors of transcription can control the temporo-spatial transcription of
each gene by altering nucleosome structure and interactions of the genes with the nuclear matrix they ultimately determine whether a specific gene is transcribed.

1B.1. Nucleosome

Genomic DNA in eukaryotic cells is present in the nucleus and packaged into chromatin, which allows about three billion base pairs of nucleotides to reside comfortably inside a 0.5 cubic nanometer nucleus of a human cell. Chromatin is composed of repeating nucleosome complexes comprising DNA and eight histone molecules assembled into a core octamer. The nucleosomes are joined by a “linker histone” (Kornberg and Lorch, 1999). As universal components of eukaryotic chromosomes, histones were once believed to be the genetic material. Subsequently, histones were believed to be a diverse family of specific gene regulators. However, only five histone types (H1, H2A, H2B, H3, and H4) have been identified (Philips and Johns, 1965). A nucleosome is composed of two molecules each of H2A, H2B, H3, H4, collectively called the “core histone complex”, with the H1 histone binding DNA in the region between two nucleosomes and referred to as the “linker histone”. Histones are small proteins of 11 to 22 kDa that are rich in basic arginine and lysine residues, and the octomeric nucleosome interacts with about 146 bp of DNA. The histone-DNA interaction is confined to the phosphodiester backbones of the DNA strands. Thus, the lack of contacts with DNA bases allows the lack of sequence specificity between histones and DNA. The packaging of gene promoters in nucleosomes prevents the initiation of transcription and therefore, nucleosomes are viewed as general gene repressors and a focal point of transcription control (Kornberg and Lorch, 1999).
There are a number of biochemical activities that can alter the stability of the nucleosome. All histones contain unstructured amino termini of about 15 to 30 amino acid residues that are commonly referred to as “tails”. These tails are the major sites of histone modifications such as acetylation, phosphorylation, or methylation (reviewed by Strahl and Allis, 2000). Of these modifications, histone acetylation has been the most extensively studied and is currently best understood. The chromatin and specifically the nucleosomes’ repressive effects can be relieved by histone acetyltransferase (HAT) to activate the transactivation of a gene and conversely, histone deacetylases (HDAC) repress gene activity.

Although the relationship between histone acetylation and transcription had long been suspected, the discovery that transcription factors can have HAT activity provided key evidence of their ability to regulate nucleosome binding and, as a consequence, the modulation of chromatin structure (reviewed by Strahl and Allis, 2000). Hyperacetylated regions of the genome are correlated with regions of transcriptional activity; conversely, hypoacetylated regions are generally transcriptionally inert. Furthermore, lysine to glutamine mutations in the N-terminal tail of yeast histones H3 and H4, which mimic lysine acetylation, alleviate the need for transcriptional coactivators of certain genes (Bannister and Miska, 2000). However, this relationship remained unclear until Gcn5, a yeast protein, which is a positive transcriptional regulator of many genes, was shown to possess HAT activity (Brownell et al., 1996) and stimulation of transcription by Gcn5 was shown to require this HAT activity (Kuo et al., 1998). This was the first indication that transcriptional coactivators, such as Gcn5, might function via modification of histones. Chromatin in the vicinity of a Gcn5-dependent promoter showed an increase in the level of H3 histone acetylation upon induction of gene expression (Kuo et al., 1998). Histone acetylation results in decreased affinity between core histone subunits and DNA,
and is correlated with transcriptional activation. The importance of the HAT activity in the transactivation of genes was reinforced with similar results on mammalian transcription factors. The human coactivator, CBP/p300, was identified as a HAT whose activity is closely correlated with its effect on transcription (Bannister and Kouzarides, 1996; Ogrzyzko et al., 1996). Smad proteins, which transduce BMP and TGF-β signaling, recruit CBP/p300 for transactivation (Janknecht et al., 1998; Pouponnot et al., 1998). Runx2 also has the ability to recruit CBP/p300 (Kitabayashi et al., 1998). The binding of HATs to the histone tails appears to be mediated in part by a conserved bromodomain, an approximately 110 amino acid residue region that forms four α-helices and is present in many proteins (Dhalluin et al., 1999; Jeanmougin et al., 1997; Winston and Allis, 1999). Bromodomains will be discussed in more detail below.

The connection between acetylation and transcription is further shown by the repression of transcription by deacetylation. Proteins originally identified as corepressors have now been shown to possess deacetylase activity. The connection between deacetylation and repression of transcription was clearly shown with the isolation of a human histone deacetylase, HDAC1 (reviewed by Struhl, 1998). Many additional HDACs have been identified and all are present in multiprotein complexes with important functional consequences. First, isolated HDACs cannot deacetylate nucleosomes alone; second, the complexes contain other proteins previously implicated in transcription repression; and third, the HDACs interact with DNA-binding proteins that recruit the HDACs to promoters (reviewed by Struhl, 1998). TIF1β, a corepressor of KRAB/C2H2 zinc finger containing proteins (also referred to as KRAB/C2H2 proteins) contains motifs that may be involved in recruiting HDACs, and subsequently repress or silence gene expression (Nielsen et al., 1999). KRAB/C2H2 proteins and the TIF1β co-repressor are discussed in more detail below. Notably, gene silencing is an entirely different mechanism from repression.
by gene-specific DNA-binding proteins, and results from the formation of heterochromatin, a condensed structure that spreads from defined sites such as silencer DNA elements and chromosome ends (telomeres), inactivating the genes it encompasses (Kornberg and Lorch, 1999).

Although the acetylation and deacetylation of the histone tails clearly contributes to the transactivation and repression of genes, respectively, it is unlikely to disrupt the structure of the core particle, as the sites of acetylation in the tails lie outside the core particle and make little contribution to its structure. Chromatin-remodeling complexes are required for nucleosome disruption and displacement to produce naked or nuclease-hypersensitive DNA regions required for the initiation of transcription. The SWI/SNF complex, which comprises 11 protein subunits, has a molecular mass of about 2 MDa, and contains Swi2/Snf2, a DNA-dependent ATPase as its catalytic subunit (reviewed by Bjorklund et al., 1999). The SWI/SNF complex can alter the structure of nucleosomes in an ATP-dependent manner and facilitate the binding of factors such as Gal4 and TATA-binding protein (TBP) to their target sites in the nucleosomal template (Cote et al., 1994; Imbalzano et al., 1994). This complex has been found to physically and functionally associate with the RNAP II carboxy-terminal domain (CTD), which explains how this complex is brought to the promoter region, although other mechanisms could be involved (Wilson et al., 1996). Similarly, NURF or nucleosome remodeling factor is a complex of 500 kDa, originally identified as an activity that facilitated transcription factor binding to chromatin in an ATP-dependent manner (Tsukiyama et al., 1994). However, unlike the SWI/SNF complex, which is stimulated by DNA, NURF is stimulated by a properly assembled nucleosome. Furthermore, NURF contains a distantly related ATPase to Swi2/Snf2, called ISWI (reviewed by Kornberg and Lorch, 1999). There are fundamental differences in the actions of SWI/SNF-related and
ISWI-containing complexes. SWI/SNF-related complexes destabilize the nucleosome thus disrupting DNA-histone contacts. In contrast, ISWI-containing complexes cause no perturbation to the nucleosome, but nonetheless are able to create nucleosome-free regions, apparently enabling movement or sliding of histone octamers on the DNA (reviewed by Kornberg and Lorch, 1999).

The combination of histone covalent modifications, including acetylation, phosphorylation, and methylation, appears to encode a histone 'language' on the histone tail domains that is read by proteins or protein modules to remodel chromatin, and ultimately control transcription (Strahl and Allis, 2000).

1B.2. Transcription Factors

Transcription factors are proteins that are required to initiate and regulate transcription in eukaryotes. Transcription factors usually bind specific sequences of DNA within the promoter of target genes, and are usually divided into two classes: gene regulatory proteins such as activators or suppressors of transcription that regulate specific genes, and general or basal transcription factors, which are ubiquitous and required for the transcription of all genes (Pugh, 1996). For convenience, the promoter of a gene is usually divided into the core promoter where the general transcription factors interact, and distal regulatory elements, which provide the binding sites for gene-specific transcription factors.

1B.2-1. Basal Factors

The core promoter of RNAP II-transcribed genes is defined as the minimal DNA sequence that is both necessary and sufficient to direct transcription by the general
transcriptional machinery. The most common core element is the TATA-box, found about 30 bp upstream from the transcription initiation site (+1), and a less well defined element called an initiator element (Inr), which is a short, weakly conserved DNA sequence that encompasses the transcription start site and is present in TATA-less as well as TATA-containing promoters (Goodrich et al., 1996). RNAP II-mediated transcription appears to be initiated by the binding of TATA-binding protein (TBP) to the TATA element, which serves as a platform for the assembly of the remaining transcription machinery. The view that a preinitiation complex is assembled stepwise from individual basal factors is derived from in vitro assembly of purified factors. However, protein purification tends to strip away all but the most tightly interacting proteins. Consequently, complexes stable in vivo may be disrupted upon purification. Thus, the concept of sequential assembly has been replaced with the concept of a holoenzyme, supported by recognition of a RNAP II holoenzyme, which contains all components required for promoter-specific transcription initiation (Ossipow et al., 1995).

The general transcription factors, along with RNAP II comprise the basal transcription machine. These factors include TFIIA, TFIIB, TFIID, TFIIE, TFIIF, and TFIIH, and approximately 50 additional polypeptides, which are involved in the formation of a transcriptional complex. Many of these general transcription factors can be fractionated using a phosphocellulose column. By eluting with increasing amounts of potassium chloride, nuclear extracts are separated into 4 fractions, termed A, B, C, and D (reviewed by Hernandez, 1993). The “D fraction” contains TFIID, a factor that binds to the TATA-box element present in a large number of RNAP II promoters. TBP was cloned from yeast when it was observed that a 27-kDa protein (TBP) could functionally replace mammalian TFIID in a reconstituted in vitro transcription system. Like yeast TBP, Drosophila and human TBP are small proteins (38 kDa)
that can, in combination with TFIIA and TFIIB, mediate basal RNAP II transcription in place of TFIID, an approximately 750 kDa complex. However, unlike TFIID, TBP cannot respond to transcriptional activators. Biochemical evidence revealed that TFIID is a large complex consisting of TBP and a number of TBP-associated factors (TAFs; Hernandez, 1993).

TFIID is one of the most extensively studied general transcription factors. It is a RNAP II-specific complex consisting of TBP and 10-12 TAFs (Burley and Roeder, 1996; Lee and Young, 1998). The role of TBP was originally assumed to be restricted to genes possessing TATA boxes in their promoters, as the name suggests. However, the requirement of TBP for RNAP III transcription was demonstrated for the transcription of the yeast U6 RNA gene (Margottin et al., 1991), as well as the transcription of ribosomal RNA mediated by RNAP I and transcription of TATA-less genes mediated by RNAP II (Cormack and Struhl, 1992). TBP is involved in the transcription mediated by archael RNAP and all three eukaryotic RNAPs, and the specificity of TBP is acquired through the association of TBP with the various TAFs (reviewed by Hernandez, 1993).

TBP is well conserved from archaeb to humans. This is not surprising considering the level of conservation of the RNAPs, as well as the seemingly generic nature of TBP. The structure of TBP was first resolved in Arabidopsis and shown to be a symmetrical saddle-like structure (Nikolov et al., 1992). The concave under-side, lined with β-sheets, contacts the DNA in the minor groove (Kim et al., 1993), while the convex upper-side, lined with α-helices, is important for protein-protein interactions (Nikolov and Burley, 1994). The interaction of TBP to the TATA box region forms an acute bend, believed to aid in the “melting” of the double stranded DNA prior to initiation of RNAP II transcription (Kim et al., 1993).
Interestingly, TBP-related factor (TRF), a protein whose primary sequence is highly similar to TBP was identified in *Drosophila* (Crowley et al., 1993). TRF was believed to be a TBP descendent that had evolved into a more typical tissue-specific regulatory transcription factor, and not a part of the basal transcription machine. TRF, unlike TBP, is specifically localized to genes transcribed by RNAP III on salivary polytene chromosomes, and specifically expressed in the central nervous system and the brain (Hansen et al., 1997). Furthermore, recent studies show that TBP-related factor (TRF), and not TBP, is the protein required for RNAP III recruitment and transcription in *Drosophila* (Hansen et al., 1997; Takada et al., 2000). TRF can also replace TBP in regulating RNAP II mediated transcription (Holmes and Tjian, 2000). Therefore, it appears that “general transcriptional factors” may confer gene- and tissue-specificity without the presence of additional regulatory transcription factors as previously believed.

**1B.2-2. Gene Regulatory Factors**

The promoters of eukaryotic genes are characterized by the presence of positive and negative regulatory sequences. Although the promoter is considered to extend upstream from the transcription start site, regulatory sequences are not restricted to this region and can also be found within untranslated regions (UTR) such as the 5'-UTR and introns. Positive and negative regulators of transcription are gene and tissue specific factors that bind specifically to DNA to affect transcription. These transcription factors are categorized according to the structure of their DNA-binding domain, and the characteristics of the major classes are summarized below. The C2H2 zinc finger transcription factors comprise the largest family of transcription factors (Tupler et al., 2001), and will be discussed in greater detail.
The homeodomain is a conserved region of about 60 amino acid residues that was first identified as being present in a class of genes critical in *Drosophila* development, called the homeotic selector genes (Wilson and Desplan, 1995). The homeodomain alone is able to form a stable, folded structure that can bind DNA. However, other regions present in these proteins appear to modulate the precise DNA-binding specificity. The homeodomain is distantly related to the helix-turn-helix protein structure, as seen from crystal structure analysis (Kissinger et al., 1990). Originally identified in bacterial cells and later in eukaryotes, the helix-turn-helix motif was the first DNA-binding protein motif to be identified. Its structure is one of the simplest, comprising two α-helices connected by a short stretch of amino acids, which constitutes a “turn”. The C-terminal helix, called the recognition helix, fits into the major groove of DNA.

The leucine-zipper motif is characterized by four or five leucine residues, spaced seven amino acid residues apart (heptad repeats) in an α-helix; thus, leucine is repeated approximately every two turns of an α-helix. This heptad repeat is the fundamental requirement for the formation of coiled-coil structures, as originally demonstrated in α-fibrous proteins such as tropomyosin (Sodek et al., 1972). The DNA-binding domain of leucine zipper proteins consists of 60 to 80 amino acid residues and contains two subdomains: the leucine zipper region, which is actually required for homo- or hetero-dimerization, and a basic region, which contacts the DNA-binding domain (Landschulz et al., 1988).

The basic helix-loop-helix (HLH) motif, similar to the leucine-zipper motif, contains a basic region of about 15 residues that contacts DNA and an N-terminal region that mediates dimerization (Voronova and Baltimore, 1990). The HLH motif, which mediates dimerization, consists of a short α-helix connected by a loop to a second, longer α-helix. As with leucine zipper proteins, basic HLH proteins function as homo- or hetero-dimers (Murre et al., 1989).
four muscle-specific transcription factors considered to be the master genes for muscle differentiation, MyoD, Myf5, MRF4, and myogenin, all possess basic HLH motifs (Arnold and Winter, 1998).

1B.2-2a. C2H2 Zinc Finger Proteins

The zinc finger proteins are characterized by their utilization of zinc ions as structural components. More than 10 different classes of zinc-binding motifs have been identified and partially characterized (reviewed by Berg and Shi, 1996). A structural role for zinc in Xenopus TFIIIA was proposed (Hanas et al., 1983); subsequently, the C2H2 zinc finger motif was revealed (Miller et al., 1985). The C2H2 class of zinc fingers has been characterized extensively.

The C2H2 (also known as TFIIIA/Krüppel) zinc finger motif is the most abundant DNA-binding motif in eukaryotic transcription factors (Berg and Shi, 1996; Tupler et al., 2001). This motif, which is generally present in tandem arrays, has the sequences, Y/F-X-C-X$_2$-C-X$_3$-F-X$_5$-L-X$_2$-H-X$_3$-H where X represents variable amino acid residues. The structure of each zinc finger motif consists of two antiparallel β-strands followed by an α-helix (reviewed by Berg and Shi, 1996). The two conserved cysteine and histidine side chains coordinate the zinc ion, and along with the three other conserved residues within the motif that pack to form a hydrophobic core, stabilize the structure (Fig. 1.2).

A polypeptide containing three C2H2 zinc fingers of Zif268/Egr1 was the first co-crystal structure (Zif268-DNA complex) to be determined; the zinc fingers wrap around the DNA double helix binding within the major groove (Pavletich and Pabo, 1991; Fig. 1.2C). In this structure, each zinc finger appears to bind DNA independently, contacting the DNA mainly at three amino acid positions: -1, +3 and +6, commonly called the contact residues, where +1 is the
Fig. 1.2. C2H2 zinc finger motif. A, diagram of the primary sequence of a C2H2 zinc finger motif. The circled zinc ion (Zn) is tetrahedrally coordinated by two cysteine and two histidine residues. Conserved amino acids are circled. B, the C2H2 zinc finger motif comprises a short antiparallel β-sheet formed by two β-strands (arrows) and hairpin turn (curved line), followed by an α-helix (blue helix) that usually fits into the major groove of the target DNA. Conserved amino acids are underlined. Amino acids at positions −1, +3, +6 are primarily responsible for interactions with DNA. C, co-crystal structure of Zif268/DNA as determined by Pavletch and Pabo (1991). Note the α-helices wrapped around the major groove of the DNA. N denotes the N-terminus.
first residue of the α-helical region (Fig. 1.2B). The β-sheet of each zinc finger is not involved in specific recognition of DNA. The relatively simple mode of interaction in the Zif268-DNA complex suggested a simple recognition code for DNA-C2H2 zinc fingers. However, this recognition code is clearly more complex since the base contact by one amino acid can be affected by neighboring amino acids. For example, the amino acid residue at position +2 often modulates the specificity of the interaction of a C2H2 zinc finger with DNA. Furthermore, selection for C2H2 zinc fingers from randomized phage-display libraries that bind to a specific DNA sequence failed to deduce any comprehensive recognition code rule, although these experiments were limited by the size of the libraries (Choo and Klug, 1994; Choo and Klug, 1994). Moreover, mutational analyses showed that the linkers between the C2H2 zinc fingers make an important contribution to the affinity of the DNA interaction (Choo and Klug, 1993; Clemens et al., 1994). Thus, the importance of context-dependent DNA-C2H2 interactions has yet to be fully appreciated.

The difficulties in understanding the rules of how C2H2 zinc finger proteins interact with duplex DNA is exacerbated by the fact that the physiological roles of most C2H2 zinc finger proteins is not well understood. In addition to interactions with DNA duplexes, C2H2 zinc fingers have been shown to bind to RNA, DNA-RNA hybrids, and even macromolecules other than nucleic acids (reviewed by Berg and Shi, 1996).

The C2H2 zinc finger proteins comprise a large family of genes that is divided into two classes according to the number of zinc finger motifs contained within the protein sequence (Pieler and Bellefroid, 1994). In one class are the zinc finger genes that code for proteins such as Egr1, Sp1, and WT1, which have fewer than 5 zinc finger motifs. The proteins in the group have generally been identified as transcriptional activators or repressors involved in the regulation of
cell proliferation and differentiation. The proteins expressed by the second class of zinc finger genes have more than 5 zinc finger motifs. Although these genes are more abundant, apart from TFIIBA, which binds to both the 5S RNA gene and to 5S RNA (Theunissen et al., 1992) and MZF1, which regulates the CD34 gene (Morris et al., 1995), the physiological function(s) of the proteins expressed by these genes is largely unknown.

1B.2-2b. KRAB/C2H2 Zinc Finger Proteins

The Krüppel-associated box or KRAB domain has been shown to be present in one-third of all C2H2 zinc finger proteins (Bellefroid et al., 1991) making this family one of the most widely distributed transcriptional repression domains yet identified in mammals (Margolin et al., 1994; Witzgall et al., 1994). KRAB domains are not found in yeast and appear to have evolved with multicellular organisms as a transcriptional repressor (Margolin et al., 1994). When fused to a heterologous DNA-binding motif, the ~75 amino acid residues that comprise the KRAB domain represses both basal and activated transcription in transfected cells in a dose dependent manner, and over large distances (Deuschle et al., 1995; Moosmann et al., 1997; Pengue et al., 1994). The KRAB domains can be separated into 3 subfamilies based on nucleic acid sequence alignment (Mark et al., 1999): subfamilies containing a KRAB A box alone, both A and B boxes, or an A box with a divergent B box. Notably, the A domain alone is sufficient for repressor activity whereas the B domain has a lesser contribution (Margolin et al., 1994; Pengue et al., 1994; Witzgall et al., 1994). Insights into the molecular mechanism underlying this silencing activity came from the identification of a nuclear protein, TIF1β, also named KAP-1 (Friedman et al., 1996) or KRIP-1 (Kim et al., 1996). The KRAB domain associates with TIF1β (Friedman et al., 1996; Kim et al., 1996; Le Douarin et al., 1998; Moosmann et al., 1996), which serves as a
universal co-repressor for KRAB-containing transcription factors involved in silencing RNAP II- and III-, but not RNAP I-, dependent transcription (Moosmann et al., 1997).

Based on sequence analysis, TIF1β was also identified as a member of the transcriptional intermediary factor 1 (TIF1) family (Le Douarin et al., 1996). In addition to TIF1β, the family includes TIF1α, a putative nuclear receptor cofactor (Le Douarin et al., 1998; Zhong et al., 1999) and TIF1γ, whose function is unknown (Venturini et al., 1999). These three proteins are defined by the presence of three conserved regions: an amino-terminal RBCC (RING finger, B boxes, coiled coil) motif, which may be involved in intermolecular interactions that influence the targeting of subnuclear structures (Saurin et al., 1996), a PHD finger motif, and a bromodomain. The latter two motifs are often associated and are present in a number of transcriptional cofactors acting at the chromatin level (Aasland et al., 1995; Jeanmougin et al., 1997). The bromodomain has been shown to interact with lysine-acetylated peptides derived from histone H3 and H4, suggesting a chromatin-targeting function for this highly conserved domain (Dhalluin et al., 1999; Winston and Allis, 1999). Supporting the hypothesis that TIF1β may exert its co-repressor function by a chromatin-mediated mechanism, TIF1β is known to associate with and phosphorylate members of the heterochromatin protein 1 (HP1) family, a class of nonhistone proteins with a well-established function in heterochromatin-mediated silencing in Drosophila (Nielsen et al., 1999). Thus, TIF1β may mediate the repression function of the KRAB domain by HP1 interaction and histone deacetylation to induce formation of heterochromatin (Nielsen et al., 1999).

Although KRAB/C2H2 proteins comprise two well-characterized motifs and make up one of the largest protein families, the function of these proteins in vivo is still unclear. KRAB domains are exclusively associated with C2H2 zinc finger proteins and with those proteins...
containing five or more C\textsubscript{2}H\textsubscript{2} zinc finger motifs. These proteins are most likely sequence-specific transcription repressors based on recent results (Zheng et al., 2000). However, the numerous C\textsubscript{2}H\textsubscript{2} zinc finger motifs along with the variable linker region joining the KRAB domain and C\textsubscript{2}H\textsubscript{2} zinc fingers suggest other roles. Furthermore, no promoter has been reported for any member of the KRAB/C\textsubscript{2}H\textsubscript{2} family of proteins.
Hypotheses and Objectives of Thesis

The ability of BMPs to induce osteogenic differentiation has been of fundamental interest in the understanding of bone formation as well as of more general interest in utilizing these powerful cytokines to repair and regenerate bone that has been lost through trauma and disease. Although the sequence of events that is induced by BMPs has been well documented from a biological perspective the molecular basis of these actions of BMPs is still poorly defined. In part, this is due to the variability of responses to BMPs that are dependent on the nature of the responding cell type and also because the continuous presence of BMPs is required for optimal promotion of the osteogenic response. While the identification of immediate gene targets for BMPs in osteogenic systems could provide important insights into the molecular mechanism that govern the osteogenic effects of BMPs, few target genes had been identified when the studies reported in this thesis were initiated. Thus, it was initially hypothesized that the identification and characterization of gene targets for BMPs would reveal novel pathways linking the effects of BMP with osteogenic genes acting downstream of BMP. The rationale for these studies was based on the importance of BMPs in promoting bone formation and the need to relate the effects of BMP with phenotypic changes associated with the differentiation of osteogenic cells. The objectives, therefore, were to utilize differential display to identify genes that were either up or down-regulated as an immediate response to treatment with BMP. Because of its availability and its strong osteogenic activity BMP-7/OP-1 was chosen for these studies.
Chapter 2: Identification and Characterization of a Novel Gene

Involved in Bone Differentiation


Andrew Jheon performed all experiments except for the experiment shown in Fig. 2.7, which was performed by Mr. Jun Chen.

The nucleotide sequence reported in this Chapter has been submitted to the GenBank™/EBI Data Bank with accession number AF321874.
2.1. Summary

Osteogenic differentiation involves a cascade of coordinated gene expression that regulates cell proliferation and matrix protein formation in a defined temporo-spatial manner. A novel zinc finger transcription factor (AJ18) that is responsive to BMP-7 was identified using differential display. AJ18 was shown to be induced during differentiation of bone cells in vitro and in vivo. The 64 kDa protein, encoded by a 7 kb mRNA, contains a Krüppel-associated box (KRAB) domain followed by 11 successive C2H2 zinc finger motifs. AJ18 mRNA is expressed in embryonic tibiae and calvariae in a developmentally regulated manner. There is little expression of AJ18 in these tissues in neonate and adult animals. AJ18 is expressed in kidney and brain of embryonic and neonate animals as well as in the brain of adult animals. During osteogenic differentiation in vitro AJ18 mRNA is expressed as cells approach confluence and declines as bone formation occurs. Using bacterially expressed, His-tagged AJ18 in a target detection assay, a consensus binding sequence of 5'-CCACA-3' was identified. This target site forms part of the consensus element for Runx2, a master gene for osteogenic differentiation. Over-expression of AJ18 in transient transfection assays suppressed Runx2-mediated transactivation of an osteocalcin promoter construct and reduced alkaline phosphatase activity in BMP-induced C3H10T1/2 cells. The studies described in this Chapter, therefore, have identified a novel zinc finger transcription factor that is highly expressed in bone, and has the ability to modulate Runx2 activity and osteogenic differentiation.
2.2. Introduction

The characterization of bone morphogenetic proteins (BMPs), their serine/threonine kinase membrane receptors, and downstream Smad effectors (reviewed by Derynck, 1998; Heldin et al., 1997; Massague, 1998) along with the identification of Runx2/Cbfa1/Osf2 (Runt domain factor2/core binding factor al/osteoblast-specific factor2; reviewed by Karsenty, 1999) as a master gene for osteogenesis (Rodan and Harada, 1997) has established a template for osteogenic differentiation. However, the molecular mechanisms linking the osteogenic effects of BMPs and Runx2 with the expression of a bone matrix by osteoblastic cells are still largely unknown.

BMPs were originally identified by their ability to induce ectopic bone formation (Sampath and Reddi, 1981; Urist, 1965). This unique bone-inductive activity indicates that BMPs provide the primordial signals for osteodifferentiation. This is supported by the BMP-induced expression of Runx2/Cbfa1. As a sub-group of the TGF-β superfamily, BMPs signal through type I and type II serine/threonine receptors on the cell surface (ten Dijke et al., 1996). Upon ligand stimulation, the type I receptor phosphorylates the family of Smad proteins. Smad1, Smad5, and Smad8 mediate BMP signaling (Hoodless et al., 1996; Kawai et al., 2000; Nishimura et al., 1998) whereas Smad2 and Smad3 mediate TGF-β signaling (Macias-Silva et al., 1996; Zhang et al., 1996). These receptor-regulated Smads form a complex with the common partner, Smad4, and translocate to the nucleus, where they interact with other transcription factors, including Xenopus FAST1 and its mammalian homologues and also the c-Jun/c-Fos complex, to regulate the transcription of target genes. A murine isoform of Runx2 was first identified as a DNA-binding protein that recognizes an osteoblast-specific enhancer element (OSE2; 5’-AACCACA-3’) of the osteocalcin gene (Ducy and Karsenty, 1995). Runx2-
dependent gene expression increases in parallel with osteogenic differentiation (Ducy et al., 1997; Ji et al., 1998), and Runx2-deficient mice fail to develop skeletal tissue (Komori et al., 1997; Otto et al., 1997). Moreover, Runx and Smads have been shown to interact functionally and to stimulate transcription of the germline Ig Cα promoter for which binding of both factors to their specific binding sites is essential (Hanai et al., 1999). Although the Runx/Smad complex likely regulates genes involved in the differentiation of bone, target genes for this complex have yet to be identified.

As an approach towards characterization of genes involved in osteoblastic differentiation, differential display was used to identify genes responsive to BMP-7 (osteogenic protein-1; OP-1) and up-regulated during the differentiation of fetal rat calvarial cells (FRCCs). In this Chapter the identification and characterization of AJ18, a C2H2 zinc finger transcription factor is described. AJ18 is transiently up-regulated during osteoblastic differentiation and appears to modulate osteogenic differentiation through effects on Runx2 activity.
2.3. Experimental procedures

2.3a. Cell Culture

Primary fetal rat calvarial cells (FRCCs) and rat bone marrow cells (RBMCs) were prepared and cultured as described previously (Yao et al., 1994). FRCCs, RBMCs, and the rat osteosarcoma cell line, ROS 17/2.8, were grown in α-minimal essential medium (α-MEM; Sigma, St. Louis, MO) containing 10% fetal bovine serum and antibiotics (100 μg/ml penicillin G, 50 μg/ml gentamycin and 0.3 μg/ml fungizone). Dexamethasone (Dex) at a final concentration of 10 nM was added to RBMC cultures. For BMP-induction experiments, pre-confluent FRCCs (80% confluent) were treated with BMP-7 (400 ng/ml) or Dex (10 nM) for 20 min and 3 h. For time-course experiments, FRCCs and RBMCs were plated at 90000 cells/60mm dish; 50 μg/ml of ascorbate was added at confluence; 10 mM of β-glycerophosphate was added at the onset of nodule formation. The mouse fibroblast-like cell line, C3H10T1/2, was obtained at passage 8 from the American Type Culture Collection (Rockville, MD). All experiments on these cells, which were maintained in 10% fetal bovine serum and basal medium essential (BME; Life Technologies, Ontario, Canada), were performed between passage 10 and 15. All cells were grown in a humidified air/CO₂ (19:1) mixture at 37°C.

2.3b. RNA Extraction

Total RNA was extracted from cultured cells and rat tissues using the thiocyanate-phenol-chloroform extraction method as described by Chomczynski and Sacchi (Chomczynski and Sacchi, 1987). Various tissues from developing embryonic and adult rats were prepared as described previously by Chen et al. (1993).
2.3c. Differential Display

Differential display was performed using the RNAimage kit (GenHunter, Nashville, TN) following manufacturer's instructions. Briefly, 200 ng or 1 μg of total RNA was treated with DNase I (MessageClean Kit; GenHunter) and reverse transcribed in 3 separate reactions using poly-dT primers with dA, dC, or dG at the 3'-end. Polymerase chain reaction (PCR) in the presence of $^{35}$S-dATP was performed on each complementary DNA (cDNA)/RNA hybrid subpopulation using the corresponding poly-dT primer in combination with 20 different arbitrary 13-mer primers. The $^{35}$S-labeled, randomly generated gene fragments were separated on a 6% denaturing polyacrylamide gel. The gel was dried and exposed to autoradiographic film. Gene fragments from differentially expressed genes were cut out of the gel, reamplified using the same set of poly-dT and arbitrary primers, subcloned into pBluescript II SK plasmid (Strategene, La Jolla, CA), and sequenced using the T7 sequencing kit (Amersham Pharmacia).

2.3d. Northern Blot Hybridization

Samples of total RNA (15 μg) were separated on a 1.2% agarose-formaldehyde gel and blotted onto a nylon filter (GeneScreen, NEN, Boston, MA) with 10x SSC (1x SSC is 1.5 mM sodium citrate, 150 mM NaCl). A mouse embryonic total RNA blot was purchased from Clontech. Preparation of cDNA fragments coding for bone sialoprotein, osteopontin, osteocalcin, osteonectin/SPARC, collagen-1, alkaline phosphatase, and glyceraldehydes-3-phosphate dehydrogenase are described in Li et al. (1996); mouse Runx2/Osfl (pLA-Oa4) cDNA was provided by Dr. G. Karsenty (Baylor College of Medicine, Houston, TX); and Egr1 and Ets1 cDNAs were provided by Dr. A. Seth (University of Toronto). cDNA fragments were labeled with $^{32}$P-dCTP using the T7 Quickprime kit (Amersham Pharmacia). Hybridization was
performed overnight at 42 °C in a solution containing 5x SSC, 50% formamide, 10x Denhardt's solution, 0.1% sodium dodecyl sulfate (SDS) and 0.25 mg/ml salmon sperm DNA. The blot was washed twice in 2x SSC, 0.1% SDS and twice in 0.1x SSC, 0.1% SDS at 55 °C for 30 min.

2.3e. 5'-Rapid Amplification of cDNA Ends (5'-RACE)

The Marathon cDNA amplification kit (Clontech, Palo Alto, CA) was used according to manufacturer's instructions. Briefly, using 1 µg of poly-A RNA from rat brain, a library of adapter-ligated double-stranded cDNA was constructed. Antisense oligonucleotide (5'-ACTGATTGGCTGACCCAGAGTAT-3’) specific for the 3'-UTR of AJ18 was used with the kit primer, AP-2, to PCR-amplify the upstream sequence. The 5'-RACE product was subcloned into pBluescript II SK plasmid (Strategene) and sequenced using the T7 sequencing kit (Amersham Pharmacia).

2.3f. RT-PCR and Southern Blot

Total RNA (1 µg) from various rat tissues and RBMCs was reverse-transcribed using M-MLV Reverse Transcriptase (Life Technologies). AJ18 specific primers (5'-GGAGAACTAAGAAGGGAAATGGCTG-3’ and 5’-CAGGCTTCTCCCCCCTTCAGACACCT-3’), rat Runx2 primers (5’-AACCACCATGGTGAGATCAT-3’ and 5’-TGAGGCGGACACCTACTCTCATA-3’), and β-actin specific primers (5’-CACCCTGTGCTGCTCACCAGA-3’ and 5’-ACCTGCGCGTCAGGAGCAGCTC-3’) were used to PCR-amplify AJ18, Runx2, and β-actin, respectively. The PCR products were amplified for 22 cycles with Taq polymerase (Life Technologies) and separated on a 1.5% agarose gel. The gel was placed in denaturation buffer (0.5 M NaOH, 1.5 M NaCl) for 30 min and washed in
neutralization buffer (0.5 M Tris, 0.2x SSC) for 30 min. The DNA was transferred onto nylon filter, a probe was synthesized using the full length AJ18 or mouse cDNA as template, and hybridization was performed essentially as described in *Northern blot hybridization* above.

2.3g. *Bacterial Expression and Target Detection Assay*

The cDNA sequence encoding full-length AJ18 (amino acids 1-560) was PCR amplified using primers (5'-CCGCATGCCTGTGGATTTGCTGGC-3' and 5'-CCTCTCTGCTTGTCCTGGATCA-3') and inserted in-frame into *SphI* and *SmaI* sites downstream of the sequence encoding the N-terminal 6xHis tag of pQE32 (Qiagen, Ontario, Canada) to produce His-AJ18 (His-AJ). cDNA encoding truncated AJ18 (His-ZF; amino acid 166-560) was prepared by excising the linker (located between the KRAB domain and the first zinc finger motif) and zinc finger region fragment with *SacI* and *SalI*, and ligating the fragment in frame into pQE32. The *E. coli* strain, M15(pREP4) (Qiagen), was transformed with full-length or truncated AJ18-pQE32 constructs and plated on Luria-Bertani (LB) agarose (Difco Laboratories, Detroit, MI) plates containing 25 μg/ml of kanamycin and 100 μg/ml of ampicillin. Single colonies were grown in culture and cells were harvested five hours after addition of isopropyl-β-D-thiogalactoside (IPTG) to 1 mM to induce expression of His-AJ and His-ZF. Whole cell lysates were separated by SDS-PAGE and transferred to a nitrocellulose membrane (Schleicher and Schuell, Keene, NH). The target detection assay was adapted from the methods described by Thiesen and Bach (1990), and Sukegawa and Blobel (1993). The membrane was treated with 10 mL of 50 mM Tris-Cl, 100 mM KCl, 1% Triton-X-100, 10% glycerol (pH 7.5) in the absence (+50 mM EDTA+10 mM DTT) or presence (+1 mM ZnCl2) of zinc at 4°C overnight. Double-stranded (ds) DNA was prepared from oligonucleotide TDA (5'-
CGCTCTAGAACTAGTGATC-N12-ATCGATACCGTCGACCGA-') by using KS primer (5'-TCGAGGTGCACGATCGATCAG-3') in a 50 μL reaction containing 1 μM TDA oligonucleotide, 1 μM KS primer, 1.5 μM MgCl2, 1 mM dNTPs, 10 μCi α32P-dCTP (Amersham Pharmacia Biotech, Quebec, Canada), 1x PCR buffer, and 2.5 U of Taq polymerase. One amplification cycle was carried out as follows: heated to 94°C for 30 s, annealed at 52°C for 2 min and extended at 72°C for 10 min. DNA was purified though a ProbeQuant column (Amersham Pharmacia Biotech) and 1x10^5 cpm/mL was hybridized to the membrane in renaturation buffer (see above) in the absence (+10 mM EDTA+2 mM DTT) and presence (+0.1 mM ZnCl2) of zinc at 4°C overnight. The membrane was washed for 6 h at 4°C in 100 mM KCl, and the amount of DNA bound was visualized using a PhosphorImager and the ImageQuant software (Molecular Dynamics, Sunnyvale, CA). The positive band was aligned to the blot and excised. The excised membrane was washed in 400 mM KCl and then bound DNA was eluted with 500 μL of 1 M KCl. The DNA present in 5 μL was amplified by PCR in the presence of SK (5'-CGCTCTAGAACTAGTGATC-3') and KS primers with the reaction condition being similar to those described above but amplified for 20 cycles. The amplified product was used for the next round of selection and was repeated for a total of 5 rounds. After 5 rounds, the product was PCR-amplified for 35 cycles, 5'-end phosphorylated using T4 polynucleotide kinase (Life Technologies), ligated into pBluescript and the clones sequenced.

2.3h. Anti-AJ18 Polyclonal Antibodies

Peptides spanning amino acid residues 2-13 (AVDALLARGTEP; Anti-AJ18-1) and 158-169 (EDGIPTDPELEK; Anti-AJ18-2) of the AJ18 sequence were synthesized. The C-terminus of the peptides was conjugated to keyhole limpet hemocyanin (KLH) protein and to bovine
serum albumin (BSA) by Alberta Peptide Institute (Alberta, Canada). The KLH-peptides were used by Cedarlane Labs (Ontario, Canada) to generate antiserum in rabbits from which affinity-purified antibodies were isolated using BSA-immobilized to CNBR-activated Sepharose 4B (Amersham Pharmacia Biotech). The IgG was eluted from the column using glycine-HCl buffer (0.05 M glycine, 0.15 M NaCl, pH 2.3), and was immediately adjusted to pH 7 with 0.1 N NaOH. Anti-HisG monoclonal antibody was purchased from Invitrogen (Carlsbad, CA).

2.3i. **Immunohistochemical Analysis**

Immunoperoxidase staining for AJ18 protein in formalin-fixed paraffin-embedded sections of tibia from 4-week old rats was performed using the Vectastain ABC kit (Vector Laboratories, Burlingame, CA) following the manufacturer’s instructions. Tibia were isolated from a 4-week old rat and fixed in 4% paraformaldehyde-PBS at 4°C. The tissues were demineralized in 12.5% (w/v) EDTA (pH 7.4), with the solution changed every 2nd day for 2-3 weeks. The embryos and tissues were subsequently embedded in paraffin. Serial 12 μm-thick sections were mounted on Superfrost/Plus glass slides (Fisher, Ontario, Canada), dewaxed, and rehydrated through graded alcohols to water. Sections were incubated in blocking solution (5% BSA, 2% normal goat serum) for 1 hr. Affinity-purified anti-AJ18-1, or anti-AJ18-2, antibodies were applied, and tissue sections were incubated for 1 h. The sections were washed and treated with biotinylated anti-rabbit IgG for 30 min, followed by incubation with peroxidase-labeled streptavidin for 30 min, and subsequently incubated with diaminobenzidine tetrahydrochloride (DAB) and H2O2 for 15 min. All incubations were performed at room temperature (21°C). Sections were counterstained with hematoxylin. The stained sections were visualized under a
light microscope (Eclipse 400; Nikon Canada Inc., Mississauga, ON) and photographed using a Coolpix 950 digital camera (Nikon Canada Inc., Mississauga, ON).

2.3j. Transient transfections and fluorescence microscopy

Full-length AJ18 and truncated AJ18 were PCR amplified with KlenTaq (Clontech) using antisense primer 5'-GCGGTACCGGGATGAATTAAGGTCTCAGGCTTCTTCT-3' and sense primers 5'-AAGGTACCGCCACCATGGCTGTGGATTTGCTGGCTGCTCGA-3' and 5'-AAGGTACCGCCACCATGTATCACACTCAGAGAAAGATTATA-3', respectively. KpnI-specific sites were incorporated into the primers (italicized). The fragments were digested with KpnI and inserted into a KpnI site of the pEGFP-N1 plasmid (Clontech). Plasmids containing fragments inserted in the sense orientation and in-frame were selected by sequencing to produce AJ18-GFP and ZF-GFP. ROS 17/2.8 cells were plated at 20 000 cells/well on 8-well chamber slides and grown overnight. Cells were transfected with 2 µg of plasmid using LipofectAMINE 2000 (Life Technologies), grown for 24 h, and fixed in 4% paraformaldehyde-phosphate buffered saline (PBS). The nuclei were stained with 4',6'-diamidino-2-phenylindole (DAPI) for 5 min and visualized under a fluorescent microscope and photographed.

2.3k. Transcription Assay

AJ18 was PCR amplified using antisense primer (5'-GCGGTACCTGGATCGAGGATTAAGGTCTCAGGTC-3') and primers described above and inserted into a KpnI site of the pcDNA plasmid (Invitrogen) to produce AJ18-pcDNA (amino acids 1-560) and ZF-pcDNA (amino acids 191-560). Sense and antisense plasmids of AJ18-pcDNA and ZF-pcDNA were purified using a midi-prep procedure (Qiagen). C3H10T1/2 cells
were plated at 50 000 cells/well in 24-well dishes and grown for 24 h. Total DNA (2 µg), including 0.5 µg of p6OSE2-luc (6xOSE2), 0.3 µg of Runx2/Osf2 (both plasmids generously provided by Dr. G. Karsenty), 0.01-1 µg of AJ18-pcDNA (sense, AJ18-S; antisense, AJ18-AS) or 0.1 µg or 1 µg of ZF-pcDNA (ZF-S), and 0.2 µg of pSV-β-Gal (Amersham Pharmacia Biotech) was introduced into cells by transfection with LipofectAMINE 2000. The cells were grown for 48 h. Luciferase assays were performed as described previously (Kim and Sodek, 1999). The ratio between the activities of luciferase and β-galactosidase were used to normalize for transfection efficiency.

2.3l. Alkaline Phosphatase Activity

Plasmids were transfected into C3H10T1/2 cells as described in the transcription assay above. BMP-7 was added 5 h after transfection and the cells were grown for 48 h. The cells were fixed in 4% paraformaldehyde and alkaline phosphatase activity was observed by staining the cells using Naphthol AS-MX Phosphate and Fast Red TR (Sigma) in 100 µL of 1 M Tris-Cl and 0.1 M MgCl2. Total alkaline phosphatase activity was measured as described by Li et al. (1996).

2.3m. Sequence Analysis

A search of the non-redundant and EST sequence databases (GenBank™/EBI Data Bank) for the 5'-RACE product was performed using the BLAST program (Altschul et al., 1990). The open reading frame and its translated product was determined using the Analyze program (MacMolly Tetra V2.5). Amino acid sequences of KRAB domains were retrieved from GenBank™/EBI and aligned using the ClustalW algorithm (Thompson et al., 1994).
2.4 Results

2.4a. Identification and Sequence Analysis of AJ18

Differential display was performed with total RNA from proliferating (80% confluence) FRCCs treated with BMP-7 or Dex (Fig. 2.1A), and with total RNA from proliferating (80%) and differentiating (confluent) FRCCs (Fig. 2.2A). A 313 bp gene fragment was identified that was temporally up-regulated after 20 min treatment of cells with BMP-7 or Dex (Fig. 2.1A), and up-regulated during osteoblastic differentiation (Fig. 2.2A). The 313 bp gene fragment was subcloned and subsequently used as a probe to perform Northern blot hybridization to confirm the differential display results (Figs. 2.1B & 2.2B). An mRNA of approximately 7 kb was visualized and a comparison to 18S rRNA by densitometric analysis showed expression at 3-fold higher levels after 20 min with BMP-7 or Dex treatment (Fig. 2.1B). Interestingly, Egr1 showed a similar expression profile with 2.5-fold higher levels after 20 min treatment (Fig. 2.1B). The same 7 kb mRNA showed a 5-fold higher level of expression in differentiating FRCCs relative to proliferating FRCCs (Fig. 2.2B). In comparison, Egr1 again showed a similar expression profile whereas expression levels of another transcription factor, Ets1, were not altered significantly. A 2514 bp fragment was generated extending upstream of the 313 bp gene fragment using 5'-RACE. Sequence analysis revealed an open reading frame (ORF) of 1680 bp encoding a protein of 560 amino acid residues extending from the first ATG codon located at nucleotide 175, to a termination TGA codon present at nucleotide 1855 (Fig. 2.3). The amino-terminal 85 amino acids constitute a KRAB domain and is followed by series of 11 C2H2 zinc finger motifs that begin at amino acid 220 and extend almost to the end of the protein sequence (Fig. 2.3). Alignment of the amino-terminal sequence of AJ18 with the KRAB domains of several C2H2 zinc finger proteins revealed at least 2 distinct subfamilies of KRAB/C2H2 proteins as suggested
Fig. 2.1. Identification of a 313 bp gene fragment up-regulated by BMP-7 and Dex. A, differential display was performed using primers polyT-A (5'-AAGCTTTTTTTTTTTTA-3') and arbitrary primer 18 (5'-AAGCTTTCTCGAC-3'). Experiments were performed in duplicate using 0.2 µg (0.2) or 1 µg (1) of total RNA from FRCCs that had not been treated (-) or had been treated with BMP-7 (B) or Dex (D) for 20 min or 3 h. A differentially amplified 313 bp fragment (marked by an arrow) separated on a 6% sequencing gel was identified by radioautography and excised from the gel, re-amplified, cloned and sequenced. B, Northern blot hybridization was performed on the original RNA isolated from the FRCCs using the 313 bp gene fragment to probe for the expression of the corresponding mRNA. A ~7 kb mRNA, provisionally named AJ18, was identified by the 313 bp probe and was expressed at approximately 3-fold higher levels in BMP-7 and Dex treated cells relative to non-treated cells. Early growth response-1 (Egr1) factor showed a similar expression profile to AJ18. A cDNA probe to 18S rRNA was used as a control for RNA loading.
Fig. 2.2. Identification of a 313 bp gene fragment up-regulated in differentiating bone cells. 

**A**, differential display was performed as describe in Fig. 2.1. except 0.2 μg (0.2) or 1 μg (1) of starting total RNA was isolated from proliferating (P) and confluent (C) FRCCs. A differentially amplified 313 bp fragment (marked by an arrow) was identical in sequence to the fragment describe in Fig. 2.1. ~7 kb mRNA, provisionally named AJ18, was expressed at approximately 5-fold higher levels in confluent cells (C) relative to proliferating cells (P). Egr1 showed a similar expression profile to AJ18, whereas the expression of Ets1, was not altered significantly. A cDNA probe to 18S rRNA was used as a control for RNA loading.
Fig. 2.3. **Primary structure of rat AJ18.** Nucleotide and predicted amino acid sequence of rat AJ18. Krüppel-associated box A (KRAB A) domain is *dot underlined*; KRAB B domain is *dash underlined*; 11 conserved C$_2$H$_2$ zinc finger motifs are *underlined*. A polyadenylation signal (AATAAA) in the 3' untranslated region is *underlined*. The 313bp gene fragment isolated from the differential display is in *italics.*
by Mark et al. (1999; Fig. 2.4). Those sequences shown above the AJ18 sequence represent a subfamily of proteins containing KRAB A box alone, whereas sequences below AJ18 represent a subfamily of proteins with both KRAB A and B box domains. Thus, sequence determination suggests that AJ18 is a 64 kDa protein comprising a transcriptional repressor, KRAB domain and 11 DNA-binding, C2H2 zinc finger motifs.

2.4b. AJ18 is Expressed During Osteoblast Differentiation and Bone Development

Northern hybridization analysis of total RNA from FRCCs at various stages of osteodifferentiation showed that AJ18 is first detected as cells approach confluence. The RNA reaches maximal levels as the cells begin to form multilayers and subsequently declines as the mineralization of the bone-like nodules begins (Fig. 2.5). These stages of osteodifferentiation are characterized by the expression of bone matrix proteins (reviewed by Stein and Lian, 1993) with alkaline phosphatase and osteopontin mRNAs being expressed as early differentiation markers; the increase in collagen mRNA reflecting early bone nodule formation; and the expression of bone sialoprotein and osteocalcin mRNAs as early and late indicators of mineralization (Yao et al., 1994), respectively. Notably osteopontin expression is markedly elevated in response to mineralization, whereas the mRNAs for the other matrix proteins, with the exception of osteonectin/SPARC, are down-regulated. In vivo, AJ18 mRNA expression could be observed by Northern hybridization of RNA from embryonic rat calvaria and tibia at the time of bone formation. Little expression was evident in neonate and adult bone tissues (Fig. 2.6). The expression of AJ18 mRNA was not limited to bone but also occurred in kidney and brain during embryonic development. Strong expression continued in the adult rat brain (Fig. 2.6).
Fig. 2.4. Amino acid sequence alignment of multiple KRAB domains. The KRAB domains of 16 zinc finger proteins are aligned with AJ18, using the ClustalW program. All sequences show a conserved KRAB A box domain. Amino acid sequences shown above the AJ18 sequence lack the KRAB B box, while sequences beneath the AJ18 sequence (underlined) show conservation of the KRAB B box domain. The consensus sequence is shown at the bottom, and conserved sequences are on a gray background. Data base accession numbers for the zinc finger proteins are as follows: MZF13, AAF79949; ZNF136, P52737; KRAZ1, AB024224; MZF31, AAF79951; ZFP93, Q61116; rKr2, AAB60512; ZFP97, NP_053895; HZF4, Q14588; ZNF45, NP_003416; HZF6, AAD12728; ZNF85, Q03923; ZNF91, Q05481; KOX1, g549835; MZF22, AAF79950; KOX31, Q06730; and ZFP228, Q9UJU3.
Fig. 2.5. Temporal expression of AJ18 and bone-associated genes during FRCC differentiation. Total RNA (15 μg) extracted at various days during FRCC differentiation was hybridized with AJ18 cDNA probe. The blot was stripped and re-hybridized with cDNA from bone sialoprotein (BSP), osteopontin (OPN), osteocalcin (OC), osteonectin/SPARC (ON), collagen-1 (COL), and alkaline phosphatase (ALP). Glyceraldehydephosphate dehydrogenase (GAPDH) was used as loading controls.
Fig. 2.6. AJ18 is differentially expressed during rat development. Various tissues were isolated from rats at different embryonic (E), neonate (N), and adult stages (A). A, the expression levels of AJ18 mRNA from calvaria (C), tibia (T), liver (L), kidney (K), and brain (B) were analyzed by RT-PCR and Southern blot hybridization. Levels of β-actin were used as loading controls for PCR. B, to compare expression of AJ18 in a range of tissues from an adult rat, total RNA was isolated from various tissues and the expression of AJ18 mRNA was analyzed by Northern blot hybridization. Lanes 1-9 in the top panel contain total RNA from muscle (M), kidney, liver, lung (Lu), brain, ovary (O), spleen (S), intestines (I), and heart (H), respectively. The lower panel shows ethidium bromide-stained 28S and 18S rRNA, which were used as loading controls.
expression of AJ18 protein was shown by immunohistochemical staining of sections of tibia from 4-week old rats with anti-AJ18 polyclonal antibodies (Fig. 2.7). AJ18 protein was detected in the nuclei of hypertrophic chondrocytes and osteoblastic cells in the transition zone of the growth plate where osteogenesis is still evident. Two distinct polyclonal antibodies were raised from peptides specific for AJ18 (shown in Fig. 4.1). Both antisera gave identical results.

2.4c. AJ18 is Localized to the Nucleus through its Zinc Finger Region

To confirm nuclear localization of AJ18 in osteoblastic cells, AJ18 was tagged with GFP to monitor its localization by fluorescence. ROS 17/2.8 cells at 50% confluence were transfected with an empty pEGFP-N1 vector, or the same vector in which either a full-length AJ18 cDNA (AJ-GFP) or a truncated AJ18 cDNA (ZF-GFP) was incorporated. ZF-GFP lacked the coding region for the KRAB domain and 120 of the 150 amino acids comprising the linker region (the region between the KRAB domain and the first zinc finger motif), but retained the sequence for all the zinc fingers. After 24 hours, the cells were fixed and the intracellular location of AJ18 was visualized by fluorescence microscopy (Fig. 2.8). Whereas cells transfected with the empty vector showed a generalized fluorescence in the cytoplasm and nucleus, cells transfected with either AJ-GFP or ZF-GFP showed distinct nuclear localization of fluorescence. Thus, the KRAB domain was not required for nuclear localization whereas the zinc finger region and/or remaining 30 amino acids of the linker region appeared to be sufficient for directing nuclear localization of AJ18.

2.4d. AJ18 Shows Selective Binding to dsDNA

The presence of zinc finger motifs in AJ18 suggests that this protein binds to dsDNA. Therefore, to determine the DNA-binding ability of AJ18, a His-tagged version of AJ18 (His-
Fig. 2.7. Expression of AJ18 protein in the growth plate of rat tibiae. The left panel shows a section of the tibial growth plate that was taken from a 4-week old rat and immunostained with an affinity-purified anti-AJ18 polyclonal antibody. AJ18 is localized to the nucleus in hypertrophic chondrocytes (gray arrowheads) and osteogenic cells (black arrowheads) on the surface of newly-forming bone, as shown by a red/brown stain. The right panel shows immunostaining without primary anti-AJ18 antibody. The sections were counterstained with hematoxylin, which stains nuclei.
Fig. 2.8. **KRAB domain is not required for nuclear localization.** The *left panels* show full-length AJ18-GFP fusion protein, truncated AJ18 (ZF-GFP) or empty pEGFP vector expressed in ROS 17/2.8 cells and visualized under blue fluorescence. The *right panels* show the corresponding nuclear staining as detected with DAPI. Note the nuclear location of the GFP for both the full-length and truncated AJ18-GFP fusion proteins whereas the GFP protein expressed alone is found throughout the cell.
AJ) was prepared by expressing the protein in bacteria. SDS-PAGE analyses revealed a major IPTG-induced protein band of 64 kDa, corresponding to the hypothetical molecular mass of AJ18 (Fig. 2.9A, I vs. NI). In addition, a second band was also evident at ~35 kDa. To express protein comprising the zinc finger domain without the KRAB domain, AJ18 was truncated at the 5'-end creating His-ZF, producing a construct similar to ZF-GFP described above. His-ZF contains 50 amino acids from the linker region. A protein band consistent with the predicted size of the truncated protein (His-ZF; 44 kDa) was induced with IPTG (Fig. 2.9B). Western blot analyses showed that both the 64 kDa and 35 kDa proteins generated by His-AJ18 and the 44 kDa protein generated by His-ZF were recognized by a monoclonal anti-HisG antibody (His) and a polyclonal antibody raised to a peptide corresponding to amino acids 158-169 in the AJ18 protein sequence. Thus, the 35 kDa protein appears to be a spontaneously truncated form of AJ18 that represents the amino-terminal half of the protein and retains the His-tag. Theoretically, this spontaneously truncated form would contain the first three zinc finger motifs of AJ18.

To study DNA-binding with the target detection assay, His-tagged proteins were transferred to nitrocellulose and renatured in the absence and presence of zinc ions prior to hybridization with radiolabeled dsDNA. Randomized 12-mer ds-oligonucleotides with flanking primer sequences were incubated with the nitrocellulose-immobilized His-AJ18 and His-ZF in the absence and presence of zinc (Figs. 2.9A & 2.9B). This same randomized 12-mer library was used to identify a consensus DNA-binding sequence of 5'-CAATG-3' for Krox-26, a putative transcription activator containing 5 C2H2 zinc finger motifs (personal communications, Dr. Bernhard Ganss, University of Toronto). Zinc-dependent binding of ds-oligonucleotides was observed in all three bands identified by immunoblotting. After 5 cycles of hybridization, the DNA bound to His-AJ18 and His-ZF was eluted, amplified, subcloned, and sequenced. The
DNA bound to the truncated, 35 kDa form of His-AJ18 was not analyzed at this time. Out of 48 clones sequenced, sequence alignment revealed the presence of a DNA-binding site of 5' - CCACA-3' in 20 of these clones (Fig. 2.9C). Since a “CCACA” sequence is typically found within the consensus element (OSE2) utilized by Runx2, the ability of an OSE2 ds-oligonucleotide to bind to the immobilized His-AJ18 was investigated and shown to bind to His-AJ18 under stringent binding conditions of the target detection assay (Fig. 2.9D). Notably, the spontaneously truncated form of His-AJ18 also bound OSE2, suggesting that the interaction between AJ18 and OSE2 may be through the first three zinc finger motifs.

2.4e. AJ18 is Co-Expressed with Runx2 and Modulates its Transcriptional Activity

Since the DNA-binding studies suggested that AJ18 might modulate the activity of Runx2 through the OSE2, the temporal expression of AJ18 and Runx2 was examined in primary rat bone marrow cells (Yao et al., 1994), grown in the presence of 10 nM of Dexamethasone to stimulate osteogenic differentiation (Fig. 2.10A). Similar expression profiles for the two proteins were evident with both AJ18 and Runx2 mRNA being expressed at maximal levels as the cells reach confluence and begin to differentiate. While AJ18 and Runx2 expression was down-regulated as mineralized bone nodules were being formed, both mRNAs increased again at 21 days, after bone nodule formation had been completed. The maximal expression of these transcription factors at the onset of bone formation corresponded to their expression profile in vivo (Fig. 2.10B). To determine whether AJ18 might disrupt Runx2 transcriptional activity by possibly competing for the OSE2, transient transfection assays were performed using an osteocalcin-luciferase reporter construct in which six OSE2 sequences had been incorporated (Ducy and Karsenty, 1995). When a full-length AJ18 expression vector (AJ18-S) was co-
Fig. 2.9. Bacterially expressed AJ18 binds dsDNA in the presence of zinc. A, Cell lysates from non-induced (NI) and IPTG-induced (I) M15 bacteria containing expression plasmid for 6xHis-AJ18 were separated on 10% SDS-PAGE and stained with coomassie blue. A second gel loaded with the same samples was transferred to a nitrocellulose membrane and Western blot hybridization performed using an affinity-purified polyclonal AJ18 antibody (AJ) and an anti-His monoclonal antibody (His). Both antibodies revealed a 64 kDa protein corresponding to full-length 6xHis-AJ18 fusion protein (His-AJ; indicated by an arrow), and a 35 kDa fragment. A target detection assay was also performed in the presence (+Zn) and absence of zinc (-Zn) after the proteins were transferred to a nitrocellulose membrane. Radiolabeled ds-oligonucleotide probes selectively hybridized to the 64 and 35 kDa proteins in the presence of zinc. B, to determine whether the KRAB domain was required for DNA binding, the cDNA coding for the amino terminal half of AJ18 was removed using restriction enzymes SacI and SalI, and the truncated AJ18 retaining the 11 zinc finger motifs re-inserted into pQE32. The target detection assay was repeated revealing zinc-dependent binding to the truncated 6xHis-AJ18 fusion protein (His-ZF; indicated by an arrow).
C

1. CACCCACCTCTTTAA  
2. TACAGTCCCAACCG  
3. TATTTGAAACCACG  
4. TGCCATAACCCCG (2)  
5. TACCAACACATGG (2)  
6. CGTTATTGACCAACA  
7. TTGCAACCAACCAG  
8. CCACATGAAACCATA  
9. CCACATACTTTAA  
10. CACACATTGCCATA  
11. CACACATGTATGCA  
12. TGCAGGATGAAACC  
13. CTGTCAGAGCCA  
14. CTCACAATTCGCA  
15. TAGGCAACCTAGG  
16. TAGGCCAGCACATGG (2)  
17. TGCATAACCCCG

Consensus: cCACa

D

<p>| | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>103</td>
<td>77</td>
<td>50</td>
<td>34</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

His-AJ

Fig. 2.9. (cont'd) Bacterially expressed AJ18 binds dsDNA in the presence of zinc. C, a consensus DNA binding site (in **bold**) was identified by aligning the sequences of 20 ds-oligonucleotides that bound to AJ18 in the target detection assay. Sequences 4, 5, and 16 appeared twice. One nucleotide from each flanking primer sequence was included in the sequences. D, a radiolabeled ds-oligonucleotide encompassing the Runx2 regulatory element (OSE2) was incubated with immobilized His-AJ, and shown to bind under the target detection assay conditions (indicated by an **arrow**).
Fig. 2.10. AJ18 and Cbfa1 are co-expressed during RBMC differentiation and mouse embryonic development. A, levels of mRNA for AJ18 and Runx2 were determined by RT-PCR and Southern blot hybridization analyses of total RNA isolated from RBMCs over the course of osteogenic differentiation. Levels of β-actin were used for RNA template controls. B, levels of mRNA expression of AJ18 and Runx2 were determined by Northern blot hybridization on total RNA isolated from mouse embryos at days 7, 11, 15, and 17.
transfected with the Runx2/Osf2 expression vector, the induction of transcription observed with Runx2/Osf2 was markedly suppressed in a dose-dependent manner, whereas the expression vector with anti-sense AJ18 (AJ18-AS) was without effect (Fig. 2.11). Similarly, a dose-dependent reduction of Runx2/Osf2-induced transcription was observed with co-transfection of truncated AJ18 (ZF-S).

2.4f. Over-expression of AJ18 Suppresses Alkaline Phosphatase Expression

Since alkaline phosphatase is an early marker of osteogenesis that is induced downstream of Runx2, following stimulation of C3H10T1/2 cells with BMP (Ducy et al., 1997), the effects of AJ18 on the BMP-7 induced osteogenesis in these cells were examined. When C3H10T1/2 cells were transiently transfected with the AJ18 expression vector, the induction of alkaline phosphatase by BMP-7 (400 ng/ml), observed in non-transfected cells and cells transfected with the empty vector, was suppressed. This was evident by qualitative examination of cultures stained for alkaline phosphatase activity. Quantitative assessment of alkaline phosphatase activity indicated a reduction in alkaline phosphatase activity of almost 40% (Fig. 2.12).
Fig. 2.11. AJ18 represses Runx2/Osf2 transactivation of 6xOSE2 in a dose-dependent manner. p6OSE2-luc (containing 6 copies of OSE2; 6xOSE2) was transfected with Runx2/Osf2 into C3H10T1/2 fibroblast-like cells with increasing amounts (0.01-1 µg) of AJ18-S, 0.1 µg or 1 µg of truncated AJ18 (ZF-S), or without (-) AJ18-S, ZF-S or AJ18-AS. Resultant luciferase activities, normalized to β-galactosidase activities, are expressed relative to the level of luciferase activity observed with cells transfected with 6xOSE2 alone.
Aikaüne phosphate activity is repressed by AJ18 in C3H10T1/2 cells treated with BMP-7. A, C3H10T1/2 cells were transfected with AJ18-pcDNA or empty vector and grown with (+) or without (-) BMP-7. The cells were fixed and stained for alkaline phosphatase (ALP) activity. B, C3H10T1/2 cells were transfected as in A, and the level of alkaline phosphatase activity was measured using a soluble assay as described in Experimental procedures. This experiment was done in triplicate.
2.5. Discussion

Although the presence of more than one hundred members of the KRAB/C2H2 zinc finger protein family have been estimated (Bellefroid et al., 1989; Bellefroid et al., 1991), little is known of their biological function. Moreover, few target DNA sequences or target genes have been identified for KRAB/C2H2 zinc finger proteins (Mark et al., 1999). This study describes the isolation and characterization of a novel zinc finger transcription factor, provisionally named AJ18, that is up-regulated by BMP-7 after 20 min of treatment, developmentally expressed in bone and appears to regulate osteoblastic differentiation. AJ18 contains 11 C2H2 zinc finger motifs and an N-terminal Krüppel-associated box domain (KRAB), which is believed to function as a repressor of transcription. The zinc finger region of the molecule has been shown to be involved in nuclear targeting and for binding of AJ18 to dsDNA containing a ‘5-CCACA-3’ sequence, including the OSE2 for Runx2. These studies further show that AJ18 can suppress the activity of Runx2, a transcription factor that is crucial for bone development (Rodan and Harada, 1997), and alkaline phosphatase activity in BMP-7 stimulated C3H10T1/2 cells. This study appears to be the first to identify a KRAB/C2H2 protein that may be involved in regulating osteoblastic differentiation.

The C2H2 or TFIID/Krüppel type zinc finger proteins comprise a large family of genes that is divided into two classes according to the number of zinc finger motifs contained within the protein sequence (Pieler and Bellefroid, 1994). In one class are zinc finger genes that code for proteins such as Egr1, Sp1, and WT1, with fewer than five zinc finger motifs. The proteins in this group have generally been identified as transcriptional activators or repressors involved in cell proliferation and differentiation. The proteins expressed by the second class of zinc finger genes have more than five zinc finger motifs and include AJ18. Although these genes are more
abundant, apart from TFIIIA, which binds to both the 5S RNA gene and to 5S RNA (Theunissen et al., 1992) and MZF1, which regulates the CD34 gene (Morris et al., 1995), the biological function of the proteins expressed by these genes is largely unknown.

Approximately one-third of all C2H2 zinc finger proteins contain a KRAB domain, which is not present in yeast proteins, and which appears to have evolved with multicellular organisms as a transcriptional repressor (Margolin et al., 1994). KRAB domains can be separated into 3 subfamilies based on nucleic acid sequence alignment (Mark et al., 1999): subfamilies containing a KRAB A box alone, both A and B boxes, or an A box with a divergent B box. Based on amino acid (Fig. 2.4) and nucleic acid (data not shown) sequence alignments, AJI8 appears to be a member of the subfamily of genes possessing a classical KRAB A and divergent B box. Notably, the A domain alone is sufficient for repressor activity whereas the B domain has a lesser contribution (Margolin et al., 1994; Pengue et al., 1994; Witzgall et al., 1994). The KRAB domain associates with TIF1β/KAP-1 (Friedman et al., 1996; Le Douarin et al., 1998; Moosmann et al., 1996), which serves as a universal co-repressor for KRAB-containing transcription factors involved in silencing RNA pol II- and III-, but not pol I-, dependent transcription (Moosmann et al., 1997). TIF1β contains a bromodomain, which has been implicated in the interaction with histone tail domains of H3 and H4 (Dhalluin et al., 1999; Winston and Allis, 1999). TIF1β is also known to associate with and phosphorylate members of the heterochromatin protein 1 (HP1) family, a class of nonhistone proteins with a well-established function in heterochromatin-mediated silencing in Drosophila (Nielsen et al., 1999). Thus, TIF1β appears to mediate the repression/silencing function of the KRAB domain by HP1 interaction and histone deacetylation to induce formation of heterochromatin (Nielsen et al., 1999).
In regard to the multiple zinc finger motifs present in the KRAB/C2H2 proteins, until recently, neither target genes nor DNA target sequences had been identified for these transcription factors. Characterization of ZBRK1, a novel 60 kDa zinc finger protein comprising a KRAB domain and eight zinc fingers, revealed a DNA target sequence of 5'-'GGGxxxCAGxxxTTT-3' found in the growth regulatory gene GADD45. In addition, ZBRK interacts with BRCA1, which is required as a co-repressor in the regulation of genes involved in cell growth and differentiation (Zheng et al., 2000).

Consistent with KRAB/C2H2 proteins acting as transcription factors it was shown with an AJ18-GFP fusion protein and immunohistochemical analysis, that AJ18 is localized to the nucleus (Figs. 2.7 & 2.8) and that AJ18 binds a DNA-binding site of sequence 5'-CCACA-3' (Fig. 2.9C). Moreover, analyses of the expression of a truncated form of AJ18 lacking the N-terminal KRAB domain revealed that the KRAB motif is not required for nuclear targeting or for DNA-binding. Interestingly, the DNA-binding sequence for AJ18 is present within the consensus binding sequence for Runx2 (OSE2), which is present in the promoters of several genes, including osteopontin and osteocalcin that are involved in bone formation (Ducy et al., 1997). These studies show that the OSE2 binds to AJ18 in the presence of Zn\(^{2+}\) under high stringency conditions of the target detection assay (Fig. 2.8D). Interestingly, the spontaneously truncated form of His-AJ18 (35 kDa form), which in theory should contain the first three zinc finger motifs, also bound OSE2 suggesting that these three zinc finger motifs may be important for binding to the OSE2. Notably, 28 of the 48 clones selected by the target detection assay did not contain the sequence, 5'-CCACA-3'. This suggests that AJ18 through its 11 zinc finger motifs (and possibly its linker region) may recognize other or longer DNA-binding sequences. A future
study on the interaction of DNA through the numerous zinc finger motifs of AJ18 is described in Chapter 5.

Notably, attempts to establish conditions for electrophoretic mobility shift assays were not successful, suggesting that stabilization of the protein structure, afforded by the target detection assay, may be required for DNA-binding. In this regard, ZNF74, a KRAB/C2H2 protein whose primary sequence is similar to AJ18, has been shown to interact strongly with the nuclear matrix (Grondin et al., 1996). As a consequence, a more detailed study of the possible competition between AJ18 and Runx2 for the OSE2 could not be performed as planned. However, the interaction of AJ18 with wild-type and mutagenized OSE2, which can be studied with procedures utilized in the target detection assay, are important experiments that would demonstrate the specificity of the AJ18-OSE2 interaction.

In vivo, full-length AJ18 could strongly suppress transcriptional activity induced by Runx2 in a 6xOSE2-luciferase reporter gene in a dose-dependent manner (Fig. 2.11). The suppression could be mediated by the KRAB domain recruiting co-repressors such as TIF1β or involve competition of the AJ18 and Runx2/Osf2 for OSE2 binding sequence. However, since truncated AJ18, which lacks the KRAB domain, also suppressed Runx2-induced transcription of the 6xOSE2-luciferase reporter gene, the modulation of Runx2/Osf2 appears to involve competition between AJ18 and Runx2 for the OSE2 (Fig. 2.11). Because of the low transfection efficiency of the ROS17/2.8 cells used in transient transfection assays, these studies have not been able to determine whether AJ18 suppresses the endogenous expression of either osteocalcin or osteopontin. This question might be answered from the analysis of AJ18 effects following stable transfection of AJ18 cDNA into an osteogenic cell line as described in Chapter 5.
That AJ18 may modulate Runx2-mediated osteogenic differentiation is indicated by the suppression of alkaline phosphatase expression in BMP-7 stimulated CH310T1/2 cells transfected with an AJ18 expression vector (Fig. 2.12). Although not an immediate target of Runx2, alkaline phosphatase is as an early marker of osteogenic differentiation and is required for mineralization (Lee et al., 1999). Analysis of the temporal expression of AJ18 and Runx2 during bone formation in vivo and in vitro, is also consistent with an interactive role of these transcription factors (Fig. 2.10), although the relative level of expression of the two proteins within the same cell is currently unknown. Both AJ18 and Runx2 are up-regulated early and are maximally expressed as osteoblastic differentiation occurs; the mRNA expression of both proteins being down-regulated as bone tissue formation is underway. However, the expression of AJ18 in other embryonic tissues, including kidney and brain, indicates a more general role for AJ18 in organogenesis.

In summary, the studies in this Chapter have described the characterization of a novel zinc finger transcription factor, AJ18, which is expressed early in bone formation and has the potential to modulate the osteo-inductive activities of Runx2.
Chapter 3: Characterization of the 5’-Flanking Region of the Rat

AJ18 Gene

Andrew Jheon performed all experiments presented in this Chapter, except the rat genomic library screen and the experiment presented in Fig. 3.7. These experiments were performed under my supervision by Drs. Naoto Suzuki and Takehisa Nishiyama (Nihon University, Tokyo, Japan), respectively. Drs. Suzuki and Nishiyama were visiting scientists in the laboratory.
3.1. Summary

Krüppel-associated box (KRAB) domains are present in one-third of all C2H2 zinc finger containing proteins, making the KRAB/C2H2 proteins one of the largest known families of putative transcription repressors. AJ18 has been identified as a novel KRAB/C2H2 gene that is involved in the differentiation of osteogenic cells. To study the regulation of expression of the AJ18 gene, the 5'-flanking region of the AJ18 gene was obtained by screening a rat genomic library. This region was sequenced, and the transcription start site mapped by primer extension. The AJ18 gene consists of at least 4 exons, the first exon coding for an unusually long 2.3 kb 5'-UTR region. A putative internal ribosome entry site (IRES), immediately upstream of the translation initiation site, is indicated from the complementarity of a 12 nucleotide sequence with a region in the rat 18S rRNA. Partial sequence analysis indicated that a novel gene, provisionally named AJ17, which encodes another putative zinc finger transcription factor is present 1.5 kb upstream of the transcription start site of AJ18. Chimeric constructs encompassing the region surrounding the transcription start site (-77 to +171), as well as constructs with additional 1.9 kb upstream from this region revealed strong transcriptional activity when ligated to a luciferase reporter gene and tested in transient transfection assays. This activity was lost on deletion of the 5'-flanking region to -77. In addition, luciferase activity was progressively lost with the inclusion of downstream sequences extending into the 5'-UTR. High conservation between rodent AJ18 promoters is apparent from comparisons of the rat sequence with a sequence retrieved from the murine genomic databases. Several known response elements for proteins such as Runx2, NFκB, Smads, Sp1, and Ets1 are retained within the conserved sequences. Notably, this is the first gene promoter from the large KRAB/C2H2 zinc finger family of proteins to be identified and characterized.
3.2. Introduction

Differentiation of multipotent stem cells into discrete cell lineages occurs during organogenesis and embryological development (Hall and Watt, 1989; Potten and Loeffler, 1990). Thus, mesenchymal tissues such as bone, cartilage, muscle, and fat are believed to be derived from a common precursor cell that proliferates and commits to specific lineages. The lineages are guided by environmental influences and molecular signals which initiate a cascade of gene activities (Aronow et al., 1989). Elucidating molecular mechanisms that determine tissue specification, therefore, is fundamental to understanding the potential of precursor cells to form specific tissues. While the molecular events involved in the differentiation of hematopoietic cells have been extensively studied, it is only recently that comparable progress has been made in understanding the differentiation of mesenchymal cells. Studies of adipogenesis (Loftus and Lane, 1997), myogenesis (Arnold and Winter, 1998) and osteogenesis (Ducy and Karsenty, 1998; Karsenty, 1998; Rodan and Harada, 1997) have identified "master genes" that are crucial for the normal development of these tissues. In osteogenesis, the expression of Runx2 is critical for bone formation (Ducy et al., 1997; Komori et al., 1997; Otto et al., 1997; Rodan and Harada, 1997). Thus, ablation of the Runx2 gene in mice completely abolishes bone formation while mutations in the human gene have been linked to the heritable disease cleidocranial dysplasia (CCD), which is characterized by patent fontanelles, supernumary teeth, hypoplasia/aplasia of clavicles, short stature, and other changes in skeletal patterning and growth (Mundlos et al., 1997; Otto et al., 1997). Moreover, the expression of Runx2 is up-regulated when osteogenesis is induced in undifferentiated cells by treatment with bone morphogenetic proteins (BMPs; Ducy et al., 1997; Lee et al., 2000; Lee et al., 1999), which have been recognized as important osteoinductive molecules. Although the characterization of BMPs and their signaling pathways
(reviewed by Kawabata et al., 1998; Wrana, 2000), together with the recent identification of Runx2 as a master gene in osteogenesis (Rodan and Harada, 1997) has greatly increased the knowledge on osteoblast differentiation, the molecular mechanisms that link the bone-inductive activities of BMPs and Runx2, and other genes involved in promoting osteogenesis, are largely unknown.

Developmentally regulated genes and transcription factors that regulate the expression of differentiation-associated genes appear to be the most useful for defining the early stages of osteo-differentiation (Erlebacher et al., 1995). Many of the developmental genes, including homeobox genes such as hoxa2, hoxd13 and hoxa13, dlx5, msx1 and msx2, as well as BMPs are involved in skeletal patterning (Cohn and Tickle, 1996; Hogan, 1996). However, the same genes are also expressed during the development of other organs and tissues. Similarly, different classes of transcription factors such as the zinc finger, helix-loop-helix, and leucine zipper proteins have broad targets of regulation. In the previous Chapter, a novel gene provisionally named AJ18, was identified as an immediate-early responsive gene to bone morphogenetic protein-7 (BMP-7; Jheon et al., 2000). This gene was identified by a differential display approach to analyse RNAs from fetal rat calvarial cells (FRCCs). AJ18 comprises a Krüppel-associated box (KRAB) domain followed by 11 successive C2H2 zinc finger motifs. AJ18 is the first member of the large family of KRAB/C2H2 zinc finger proteins reported to be expressed in bone cells. Preliminary studies indicate that AJ18 may affect osteogenic differentiation through an ability to modulate Runx2 activity (Chapter 2; Jheon et al., 2001).

As a first step towards characterization of upstream regulators of AJ18, a genomic library was screened. The rat AJ18 gene including the 5'-flanking region was cloned. The genomic organization of the AJ18 gene was defined, the transcription start site mapped, and the region
encompassing the start site was shown to possess strong transcriptional activity in osteoblastic cells.
3.3. Experimental procedures

3.3a. Rat Genomic Library Screen

A rat genomic library prepared from rat testis DNA and assembled into λ phage DASH II vector (Strategene, La Jolla, CA) was screened by plaque hybridization to AJ18 (Sambrook et al., 1989). The 461 bp fragment used as the probe was prepared by digesting rat AJ18 cDNA with PstI. The gel-purified cDNA fragment was labeled with 32P-dCTP using the T7 Quickprime kit (Amersham Pharmacia). Positive clones from tertiary screens were isolated, purified, and characterized by restriction endonuclease digests. XhoI-released inserts were separated on an agarose gel, gel-purified, and subcloned into pBluescript II SK (Strategene). Southern blot hybridization was performed as described in Chapter 2, using a cDNA probe specific to the 5'- or 3'-UTR of AJ18. A 240 bp, 5'-UTR specific cDNA fragment was obtained from XhoI digests of AJ18 cDNA, and the 3'-UTR specific cDNA is described in Chapter 2.

3.3b. Cell Culture

Primary fetal rat calvarial cells (FRCCs) and rat bone marrow cells (RBMCs) were prepared and cultured as described previously (Yao et al., 1994). FRCCs, RBMCs, and the rat osteosarcoma cell line, ROS 17/2.8, were grown in 10% fetal bovine serum, α-minimal essential medium (α-MEM) (Sigma, St. Louis, MO), and antibiotics (100 μg/ml penicillin G, 50 μg/ml gentamycin and 0.3 μg/ml fungizone). The mouse fibroblast-like cell line, C3H10T1/2, was obtained at passage 8 from the American Type Culture Collection (Rockville, MD), and maintained in 10% fetal bovine serum and basal medium essential (BME; Life Technologies, Ontario, Canada). Experiments using C3H10T1/2 cells were performed using cultures between passage 10 and 15. All cells were grown in a humidified air/CO2 (19:1) mixture at 37°C.
3.3c. RNA Extraction

Total RNA was isolated from FRCCs and RBMCs using the thiocyanate-phenol-chloroform extraction method as described by Chomczynski and Sacchi (1987).

3.3d. PCR Reactions

Total RNA (1 μg) from FRCCs was reverse-transcribed using an antisense oligonucleotide (5’-CTCAAATCCTCTCTCGACTACACAT-3’) specific for the 5’-UTR of AJ18 and Moloney Reverse Transcriptase (Life Technologies, ON, Canada). Various combinations of AJ18 specific primers (antisense, A) 5’-AGAGTTCTGAAAGCTGAGTCGGA-3’; B) 5’-TACCAATAGTCTTTGTGCAGCTACA-3’; sense, 1) 5’-CTGATTTCTCCATGTTCCATGT-3’; 2) 5’-GCTGCACAAGACTATTGGTATC-3’; 3) 5’-TCTTTCCCCCTCTAGCTTTTCTCT-3’; 4) 5’-CTGTTGGAGATATCTTGCACTGTC-3’; 5) 5’-GTATGGGGCTGTTTCAGACTGTC-3’; 6) 5’-GTTTCTAGAAACTCTCCTGTCTTGT-3’) were used to amplify PCR products for 35 cycles with Taq polymerase (Life Technologies). The amplified products were separated on a 1.5% agarose gel, and photographed.

3.3e. Primer Extension Analysis

Primer extension was based on methodology described in Ausubel et al. (1992). Briefly, the complement of oligonucleotide 5 (above), was end-labeled using γ-32P-ATP (Amersham Pharmacia Biotech, Quebec, Canada), and T4 kinase (Life Technologies) and hybridized to 20μg of DNase I-treated (Amersham Pharmacia Biotech), total RNA prepared from FRCCs and RBMCs in 150 mM KCl, 10 mM Tris-HCl and 1 mM EDTA. The RNA and oligonucleotide were precipitated, re-suspended in 1x Reverse Transcriptase buffer, and incubated with 200U M-
MLV Reverse Transcriptase (Gibco BRL) at 42°C for 1 h. The same oligonucleotide that was used for the primer extension experiment, and plasmid X/X (pX/X), which contains the XhoI-digested AJ18 gene insert were used to obtain a sequencing ladder. The primer extension product was separated concurrently with the sequencing ladder on a 5% denaturing polyacrylamide gel.

3.3f. 3'-Rapid Amplification of cDNA Ends (3'-RACE) and Southern Blot Hybridization

The Marathon cDNA amplification kit (Clontech, Palo Alto, CA) was used according to manufacturer's instructions. Briefly, using 1 µg of poly-A RNA from rat brain, a library of adapter-ligated double-strand cDNA was constructed. A sense oligonucleotide specific for the 3'-UTR of AJ18 (5'-GACAGATCCACTACAGAGGAAAA-3' at nucleotide position 2232 of the previously reported sequence; Fig. 2.3), was used with the kit primer, AP-2, to PCR-amplify the downstream sequence. The 3'-RACE product was separated by agarose gel electrophoresis and Southern blot hybridization was performed as described in Chapter 2, using a cDNA probe generated from the 3'-UTR of AJ18.

3.3g. DNA-Luciferase Constructs

Chimeric constructs encompassing various lengths of the rat AJ18 promoter extending ~2 kb upstream and ~1 kb downstream of the transcription start site, were cloned into the pGL3-Basic (pluc) vector (Promega, Madison, WI). A combination of PCR and restriction enzyme digests was used to generate the desired fragments using pX/X as template. Construct p(-1822/-284) was prepared by HindIII digests of pX/X and ligation of the fragment into pGL3; constructs p(-77/+171), p(-77/+451), p(-77/+505), and p(-77/+657) were prepared by PCR using sense primer, -77 (5'-GGGGTACCAGCAGCGATCTGTATTCTTAG-3'), in combination with
antisense primers, +171 (5'-GGGGTACCACTAGGAGAGAAAGGCT-3'), +451 (5'-GGGGTACCATAGGCTTTTTTCTTCCACC-3'), +505 (5'-GGGGTACCATCAACCAACATGAGTCCCTG-3'), +657 (5'-GGGGTACCCTCATAGGTTAGATGCCC-3'), respectively. The PCR-amplified fragments were digested with KpnI and inserted into pGL3; constructs p(-1886/+171) and p(-1484/+171) were prepared by PCR using antisense primer, +171, in combination with sense primers, -1886 (5'-GGGGTACCTCGGTAAATCTGCCTTGAGACA-3') and -1484 (5'-GGGGTACCTGCGGTAAATCTGCCTTGAGACA-3'), respectively; construct p(-77/+955) was prepared by PCR using primer -77 and primer +955 (5'-ATGCTAGCTCACTTTCAAAGCGCTCAA-3'), digested with KpnI and NheI, and inserted into pGL3.

3.3h. Transient Transfection Assays

ROS 17/2.8 and C3H10T1/2 cells were plated at 50 000 cells/well in 24-well dishes and grown for 24 h. A total of 2 μg of DNA, including various AJ18 gene fusion-luciferase reporter constructs described above, 0.2 μg of pSV-β-Gal (Amersham Pharmacia Biotech), and varying amounts of pBluescript II SK plasmid (Strategene), was transfected using LipofectAMINE 2000 and the cells were grown for 48 h. Luciferase assays were performed as described previously (Kim and Sodek, 1999). The ratio between the activities of luciferase and β-galactosidase were used to normalize for transfection efficiency.
3.3i. Sequence Analysis

The mouse AJ18 genomic sequence was retrieved from the genomic databases (GenBank™/EBI Data Bank) using the BLAST program (Altschul et al., 1990). Rat and mouse AJ18 genomic sequences were aligned using the ClustalW algorithm (Thompson et al., 1994).
3.4 Results

3.4a. Cloning of Rat AJ18 Promoter

To isolate AJ18 genomic clones, a rat genomic library was screened by plaque hybridization with rat AJ18 cDNA probe, which targeted the sequence between the KRAB domain and C2H2 zinc finger motifs. Four independent lambda clones were obtained from an initial screen of \( \sim 1 \times 10^6 \) recombinant phage plaques. Three clones were 10 kb in size and determined to be identical based on restriction enzyme digests (\( \lambda 6.8 \); data not shown). The fourth clone (\( \lambda 4.2 \)) was 4 kb and hybridized to the 3'-UTR AJ18 cDNA probe (data not shown). A \( \sim 5 \) kb fragment excised with \( XhoI \) from \( \lambda 6.8 \) was recognized with the 5'-UTR AJ18 probe, and subcloned into Bluescript plasmid (pX/X). Sequence comparison between pX/X and previously obtained AJ18 cDNA (Chapter 2; Jheon et al., 2001) showed that the subcloned genomic fragment extends 5 kb upstream from the translation start site, and contains a 1004 bp intron (intron i) in the 5'-UTR (Fig. 3.1A).

3.4b. Mapping of the Transcription Initiation Site

In previous studies the size of the AJ18 mRNA was determined from Northern hybridizations to be approximately 7 kb, whereas the cDNA used to characterize the coding region of AJ18 was only 2.5 kb (Chapter 2; Jheon et al., 2001). Consequently, large 5'-UTR and/or 3'-UTR sequences were expected. However, previous studies using 5'-RACE did not extend the 5'-UTR beyond 175 bps from the translation start site (Chapter 2; Jheon et al., 2001). Examination of the genomic sequence revealed a region of high GC content located approximately 200 bp upstream of the translation start site, which may have caused premature termination of the 5'-RACE amplifications. Therefore to determine the length of the 5'-UTR and
Fig. 3.1. Transcription initiation site is mapped within a 975 bp region. A, X/X was digested from plage clone λ6.8, inserted into Bluescript plasmid (pX/X), and sequenced. The restriction enzymes indicated on the map are X, Xhol; B, BamHI; S, SalI; P, PstI; Bg, BglII. A 1004 bp intron (intron i) was identified within the 5'-UTR by sequence comparison with AJ18 cDNA. Partial coding sequence of AJ18 is shown as a gray box; 5'-UTR is shown as a white box; genomic sequence is shown as a solid line. Various sense (A-6) and antisense primers (A, B) were synthesized spanning the region upstream of the translation start site (ATG) in an attempt to identify the region containing the transcription initiation site by PCR. B, PCR was performed using various primer combinations on total RNA isolated from FRCCs with (+) or without (-) reverse transcription. The expected PCR products from the various primer combinations are: A1, 901 bp; A2, 997 bp; B3, 486 bp; B4, 581 bp; B5, 790 bp; B6, 1765 bp.
locate the region of the transcription start site, RT-PCR was performed on total RNA isolated from FRCCs using various combinations of primers corresponding to regions upstream from the previously determined 5'-UTR (Fig. 3.1). Amplified cDNA fragments were obtained using primer combinations A1, A2, B3, B4, and B5, but not with the B6 primer combination, suggesting that the 5'-UTR does indeed extend much further upstream with the transcription start site being present between primer sites 5 and 6. Since the size of the amplicons generated by A1, A2, B3, B4 and B5 corresponded with the distance between the primer sites in the genomic sequence it was deduced that introns were not present in this region of the 5'-UTR. To identify the transcription start site, the antisense oligonucleotide of the sense oligonucleotide designated as 5) was synthesized and primer extension experiments performed on total RNA isolated form FRCCs and RBMCs. A major radiolabelled primer extension product was obtained (Fig. 3.2). By comparing this product to a sequencing ladder prepared using the same antisense primer used in the primer extension experiment and plasmid X/X as template, the transcription start site was mapped to a cytosine residue and designated as position +1. Thus, the 5'-UTR of AJ18 was determined to extend 2.3 kb and to be encoded by two exons separated by intron i (Fig. 3.4A).

3.4c. Genomic Organization of AJ18

To complete the genomic structure of AJ18, the length of the 3'-UTR was determined using 3'-RACE. A sense primer located 370 bp downstream from the translation stop codon was used to amplify two major cDNA transcripts of ~300 bp and ~3 kb, both of which were recognized by an AJ18 3'-UTR specific cDNA probe (Fig. 3.3). Although this would suggest that two mRNAs with a short and a long 3'-UTR might be generated for AJ18, only one ~7 kb mRNA has been observed so far and this corresponds to the transcript with a long 3'-UTR. Because the intensity
Fig. 3.2. Identification of the transcription initiation site by primer extension. An antisense oligonucleotide targeted to a sequence in the 5'-UTR of AJ18 (underlined) was end-labeled using γ-32P-ATP and primer extension was performed on total RNA isolated from FRCCs and RBMCs. The primer extension reaction products (PE) and sequencing reaction products using the same primer were separated concurrently by electrophoresis. The major cDNA fragment from the primer extension reaction is indicated with an arrow, and the transcription start site was designated +1.
Fig. 3.3. **Mapping the 3'-UTR by 3'-RACE.** 3'-RACE was performed using mRNA isolated from FRCCs. cDNA fragments were separated on an agarose gel (*left panel*), transferred onto nylon membrane, and Southern blot hybridization was performed using a probe specific to the 3'-UTR of AJ18 (*right panel*). The two cDNA fragments (~3 kb and ~300 bp) identified with the AJ18 probe are indicated with *arrows*. 
Fig. 3.4. Genomic organization of AJ18. A, the AJ18 gene consists of at least 4 exons (I-IV) that are separated by 3 introns (i-iii) indicated as gray boxes and solid lines, respectively. The restriction enzymes indicated on the map of phage clone, λ6.8, isolated from the rat genomic library screen are: X, XhoI; Xb, XbaI; E, EcoRI; H, HindIII, B, BamHI. B, AJ18 protein is encoded by an open reading frame comprising exons II, III, and IV. Solid lines indicate the 5'- and 3'-UTR sequences.
of the 7 kb mRNA on Northern blots is relatively weak, it is conceivable that the presence of a minor transcript would not be detected.

From the analyses of the UTRs, the AJ18 gene can be shown to comprise 4 exons separated by 3 introns. The sizes of introns ii and iii were deduced from comparisons with AJ18 mouse genomic sequence (Chapter 4). The 5'-UTR is 2.3 kb, the open reading frame (ORF) is 1.7 kb, and the 3'-UTR is 3.4 kb (Fig 3.4A). AJ18 protein comprising the KRAB domain, a linker, and 11 C_2H_2 zinc finger motifs is encoded by exons 2 and 3 (KRAB domain), and exon 4 (linker + 11 C_2H_2 zinc fingers; Fig. 3.4B).

The 5'-UTR of AJ18 revealed several GC-rich regions and 28 ATG (or AUG) sites upstream of the previously reported ATG site of the ORF (Fig. 2.3). All 28 ATG sites are subsequently followed by an in-frame translation stop codons (TGA, TAA or TAG), which would generate short peptide fragments (Fig. 3.5A). Notably, the translation start site previously identified for AJ18 contains the sequence, GAAATGG, which is a “strong” ATG start codon based on the presence of nucleotides R^3 and G^4 (Kozak, 1999). A sequence representing a putative internal ribosome entry site (IRES), with a complementary sequence to rat 18S rRNA, was identified near the previously reported AJ18 translation initiation site (Fig. 3.5B). This region may direct translation at this ATG site. Sequences complementary to 18S rRNA were not present around the other 28 upstream ATG sites.

3.4d. Identification of a Second Novel C_2H_2 Zinc Finger Gene

Analysis of the sequence upstream of AJ18, obtained from the ~5 kb fragment excised with XhoI from λ6.8 genomic clone, revealed a partial ORF, encoding a second novel C_2H_2 zinc finger protein. This putative zinc finger protein-encoding gene, provisionally named AJ17, is
Fig. 3.5. Identification of 28 upstream ATG sites and a putative IRES in the 5'-UTR of rat AJ18. A, ATG sites (↓) and stop codons, TAA, TAG, and TGA (↑) upstream of the authentic translation start site (+1) in the 5'-UTR of rat AJ18 are indicated for the three reading frames (1, 2, 3). B, the region encompassing the translation initiation site of AJ18 mRNA shows complementarity to a sequence within rat 18S rRNA (nt 1329-1355). AUG (ATG) in bold represents the translation initiation site.
present approximately 1.5 kb upstream of the AJ18 transcription start site (Fig. 3.4A). To
determine whether the expression of AJ17 and AJ18 are linked in osteogenesis, a cDNA probe
encompassing the partial ORF of AJ17 was synthesized and the temporal expression of AJ17
mRNA during osteogenic differentiation of FRCCs (Chapter 2; Jheon et al., 2001) was analyzed
by Northern blot hybridization (Fig. 3.6). A single ~2.5 kb AJ17 mRNA was detected but, in
contrast to AJ18, its expression remained fairly constant over the 28-day time period.

3.4e. Mouse and Rat AJ18 Genomic Sequences

The nucleotide sequence corresponding to rat AJ18 promoter sequence was used in a
BLAST search of High Throughput Genomic (HTG) databases, which contain 'unfinished' or
'single-pass' DNA sequences generated by high-throughput sequencing centers, and the
homologous mouse AJ18 gene (Genbank Accession No. AC023427) was retrieved. The 5'-
flanking regions of the rat and mouse genes were aligned using the ClustalW program, and found
to be highly conserved, sharing several consensus binding elements for transcription factors such
as Runx2, NFκB, Smads, Ets1, and Sp1 (Fig. 3.7).

3.4f. Promoter Region Possesses Transcriptional Activity

Examination of the nucleotide sequence 5’ to the transcription start site of the rat AJ18
failed to identify a consensus sequence corresponding to a TATA box. Moreover, an initiator
element (Inr) could not be located. The sequence surrounding the transcription start site was
analyzed to verify that this region, which lacks the elements that are characteristic of a
transcriptional start site, has promoter activity, and to locate the region of the proximal promoter.
Various rat AJ18 gene fragments were ligated into the promoter-less pGL3 luciferase reporter
Fig. 3.6. Identification of a novel gene upstream of \textit{AJ18}. A 191 bp DNA fragment from the open reading frame of AJ17 was excised from pX/X using restriction enzymes \textit{XhoI} and \textit{EcoRI}, and used to synthesis radiolabeled probes. Northern blot analysis of total RNA isolated on various days of FRCC differentiation was performed. AJ17 mRNA is \textit{\textasciitilde}2.5 kb while AJ18 mRNA is \textit{\textasciitilde}7 kb. Glyceraldehydephosphate dehydrogenase (GAPDH) was used as loading controls.
Fig. 3.7. Nucleotide sequence of the 5' flanking region of rat and mouse AJ18. The rat AJ18 gene isolated from the rat genomic library screen was sequenced. The sequence of mouse AJ18 gene was retrieved using the BLAST program (Genbank Accession No. AC023427), and aligned using the ClustalW algorithm. Putative Rnu2, Smad, Ets1, and NFkB binding sites are indicated in boxes. A putative Sp1 binding site is indicated with a dashed box. The transcription start site (+1) of the rat AJ18 gene is indicated with an arrow.
vector and the luciferase activities measured in ROS 17/2.8 cells (Fig. 3.8). Transcription activities of constructs p(-1886/+171), p(-1484/+171), and p(-77/+171) were 5- to 8-fold higher than the activity of a pGL3 luciferase reporter plasmid (pluc). This is comparable to the luciferase activity of the thymidine kinase promoter construct (pTK-luc). As expected, the fragment that lacked the transcription start site, construct p(-1822/-284), did not support the expression of the reporter gene. Inclusion of additional 5'-UTR sequence downstream of nt +171 resulted in a progressive decrease in relative luciferase levels. Constructs p(-77/+451) and p(-77/+505) produced about 2-fold luciferase activity, whereas constructs p(-1886/+955), p(-77/+657), and p(-77/+955) showed luciferase activities below the levels of pluc. The progressive loss of luciferase activity is suggestive of a translational effect due to interference from ATG sites upstream of the authentic translation start site rather than a transcriptional effect due to the presence of a transcriptional repressor element.

3.4g. Transcription Activity of Rat AJ18 is Higher in Osteoblastic Cells

To determine whether the promoter activity was influenced by the differentiation state of the cells, the relative luciferase activity levels of rat AJ18-luciferase constructs, p(-1494/+171) and p(-77/+505) were analyzed in undifferentiated C3H10T1/2 cells and in osteoblastic ROS 17/2.8 cells. For both constructs, transcription activity was found to be 100-fold higher in the ROS 17/2.8 cells (Fig. 3.9).
Fig. 3.8. *AJ18* promoter shows transcription activity. Variable lengths of the *AJ18* gene were inserted in a luciferase reporter vector, transfected into ROS 17/2.8 cells, and the transcription activities measured. Resultant luciferase activities, normalized to β-galactosidase activities, are expressed relative to the level of luciferase activity observed with cells transfected with pluc alone.
Fig. 3.9. AJ18 gene shows high transcription activity in bone cells. The transcription activity of luciferase constructs p(-1494/+171) and p(-77/+505) was measured in ROS 17/2.8 and C3H10T1/2 cells. Resultant luciferase activities are expressed relative to the level of luciferase activities observed in ROS 17/2.8 cells.
3.5. Discussion

AJ18 was initially identified using differential display in a screen to identify genes that were regulated by BMP-7 treatment of osteogenic FRCCs (Chapter 2; Jheon et al., 2000). Subsequent studies in vitro and in vivo showed that AJ18 is expressed as a ~7kb mRNA that appears, together with Runx2, early in osteogenic differentiation and is down-regulated as bone formation commences (Chapter 2; Jheon et al., 2001). A target detection assay identified a nucleotide sequence 5'-CCACA-3' that is also present within enhancer element of OSE2, through which AJ18 could potentially modulate Runx2 transcriptional activity. Moreover, expression of AJ18 in undifferentiated C3H10T1/2 cells suppressed BMP-7 induced alkaline phosphatase activity, indicating that AJ18 has an important role in osteogenic differentiation. Although the complete ORF was determined from these studies, the limits of the 5’- and 3’-UTR regions were not determined.

To investigate regulatory pathways that control the expression of the AJ18 gene, a rat genomic library was screened and a phage clone extending 10 kb upstream from the translation start site was subcloned and sequenced. The genomic structure of AJ18 was mapped using 3'-RACE, and a combination of RT-PCR (Fig. 3.1) and primer extension experiments (Fig. 3.2) to determine the size of the 3’- and 5’-UTRs, respectively, and to locate the transcription start site. By comparing the cDNA sequence corresponding to the ~7 kb mRNA with the genomic sequence, the AJ18 gene was shown to comprise four exons and three introns. An unusually long (2.3 kb) 5’-UTR is coded by the first two exons, which are separated by a 1 kb intron. A 1.5 kb intergenic region was identified extending from the transcription start site of AJ18 to an upstream gene, AJ17, which also appears to code for a novel zinc finger protein. Although the minimal promoter of AJ18 appears to lack TATA-box and Inr elements, high transcriptional
activity was evident in chimeric reporter constructs containing the region encompassing nucleotides -77 to +171 (Fig. 3.8). Furthermore, luciferase activity was progressively lost when constructs included sequences extending downstream into the 5'-UTR. This may be due to the interference on translation by the numerous ATG sites upstream of the authentic translation start site.

3.5a. Characterization of the 5'-UTR and Transcription Start Site

The unusually long 5'-UTR of AJ18 containing GC-rich sequences interfered with previous attempts to determine the size of the UTR and the site of transcription initiation. The presence of stable secondary structure formed by GC-rich regions appeared to preclude successful 5'-RACE, as only short ~175 bp extensions of the ORF were generated (Chapter 2; Jheon et al., 2001). By using a series of PCR amplifications, which can be performed at higher temperatures to unfold more stable secondary structures, in combination with primer extension analysis, the extent of the 5'-UTR region and the transcription start site were determined in the studies described in this Chapter. However, if the long 5'-UTR in AJ18 is characteristic of the KRAB/C2H2 family of genes, this could explain the prior absence of a reported promoter sequence for this large family of proteins.

The 5'-UTR identified in this study contains 28 ATG (or AUG) sequences upstream of the previously reported ATG site of the ORF in AJ18, which challenges the theory of the ribosome-scanning mechanism for the initiation of translation. Briefly, the scanning theory suggests that a preinitiation complex comprising a small (40S) ribosomal subunit, tRNA, and other factors is recruited by the cap structure at the 5'-end of mRNA, and migrates linearly until it encounters the first ATG codon, which is recognized by basepairing with the anticodon in Met-
tRNA (reviewed by Kozak, 1999). Recently, there has been evidence suggesting that the ribosome-scanning model for translation initiation may be an inadequate model to explain the initiation of translation of all genes. Construction of "5'-end enriched" cDNA libraries showed that only 63% of 954 species of 5'-UTR contained a single ATG start codon (Suzuki et al., 2000). The remaining 37% of the 5'-UTR species contained additional upstream ATG codon sites and did not contain an in-frame terminator codon downstream of the upstream ATG. Moreover, a putative breast cancer suppressor gene (Genbank Accession No. AU076409) was found to contain 26 upstream ATG codons while the promoter of the Runxl gene contains 15 upstream ATGs (Pozner et al., 2000). Therefore, translation from a large number of 5'-UTR species (37%) conflicts with the scanning translation initiation model, in which translation is initiated from the first ATG codon (Kozak, 1999). While leaky scanning or pre-initiation by the 40S ribosome are two mechanisms by which translation initiation at a second or third downstream ATG codon could occur (Kozak, 1999), access to internal translation start sites has been postulated to occur via ribosome shunting, which in A18 may be facilitated by the formation of stable secondary structure in the GC-rich regions. In ribosome shunting, the 40S ribosome bypasses large segments of the mRNA to reach the initiation codon (Yueh and Schneider, 2000). Alternatively, a cap-independent mechanism may exist for translation initiation through direct internal binding of an internal ribosome entry site (IRES) (Gray and Wickens, 1998; Jackson and Kaminski, 1995). Although the IRES sequences characterized to date are dissimilar, it has been postulated that the 18S rRNA within the 40S ribosomal subunit can recruit mRNA through sequences that are complementary to the IRES sequences (Chappell et al., 2000; Owens et al., 2001; Zhou et al., 2001), which allow the IRES and 18S rRNA to interact directly (Hu et al., 1999; Tranque et al., 1998). In fact, an IRES may be as short as
stretches of 9 nucleotides that can initiate translation via cap-independent mechanisms (Chappell et al., 2000). Thus, while the scanning model is currently the best characterized mechanism for translation initiation (Kozak, 2000), there is strong evidence of alternative mechanisms, which could explain the location of the translational initiation site in AJ18. Notably, the translation start site identified for AJ18 is a “strong” ATG start codon based on the presence of nucleotides R\(^{-3}\) and G\(^{-4}\) (Kozak, 1999). Furthermore, all upstream ATG sequences present in the AJ18 gene are followed by subsequent in-frame translation stop codons, which would generate short peptides. Therefore, there are at least two postulated models of translation initiation for the expression of AJ18 protein: 1) a cap-independent mechanism that occurs through the interaction of the 40S ribosome complex to an IRES encompassing the rat AJ18 initiation codon, or 2) a cap-dependent mechanism where strong secondary structure within the 5′-UTR of AJ18 mRNA results in ribosome shunting allowing translation initiation at a downstream initiation codon. Further experiments are required to make any conclusions, but AJ18 and perhaps the family of KRAB/C\(_2\)H\(_2\) genes may be important tools for elucidating various known or novel mechanisms, or a combination of these mechanisms of translation initiation.

Approximately 1.5 kb upstream of the transcription start site for AJ18, a second novel C\(_2\)H\(_2\) zinc finger gene, provisionally named AJ17, was identified and partially sequenced. AJ17 contains at least 7 C\(_2\)H\(_2\) zinc finger motifs and is most likely another member of the KRAB/C\(_2\)H\(_2\) zinc finger gene based on its close proximity to AJ18. Northern hybridization experiments showed that AJ17 and AJ18 are encoded by two separate mRNA species of ~2.5 kb and ~7 kb, respectively, and show different expression profiles during FRCC maturation (Fig. 3.6).
3.5b. Characterization of the 3' -UTR

The 3'-UTR of AJ18 was determined to be 3.4 kb based on 3'-RACE experiments (Fig. 3.3). The 3.4 kb 3'-UTR, along with the 1.7 ORF and 2.3 kb 5'-UTR would result in a 7.4 kb mRNA, which corresponds with the observed ~7 kb AJ18 mRNA. A possible, alternative termination site resulting in a shorter 700 bp 3'-UTR was also observed (Fig. 3.3). Although the sequence AAUAAA, is near the end of the 700 bp 3'-UTR, it is present ~250 nucleotides upstream of the putative poly-A tail (Fig. 2.3). The AAUAAA hexamer is required for cleavage and polyadenylation of the 3' end, and is present in the region from 11 to 30 nucleotides upstream of the site of poly(A) addition (Conway and Wickens, 1987). Furthermore, there has been no evidence of a second AJ18 transcript of 4.7 kb (0.7 kb 3'-UTR + 1.7 kb ORF + 2.3 kb 5'-UTR). Although it is conceivable that the presence of a minor transcript would not be detected, as the intensity of the major ~7 kb mRNA on Northern blots is relatively weak, it is more likely that the 700 bp 3'-UTR does not represent an authentic termination product. Interestingly, the poly-TA primer utilized in the differential display experiments that originally identified AJ18 appears to have anchored at the end of this 700 bp 3'-UTR (Chapter 2; Jheon et al., 2001), and explains why the complete 3'-UTR was not obtained previously.

3.5c. Characterization of the AJ18 Promoter

Rat and mouse AJ18 gene expression appears to be mediated by a TATA-less and Inr-less promoter, which is well conserved between the two species (Fig. 3.7). Thus, transcription may be mediated through an Sp1 site near the transcription start site. Promoter constructs containing sequences extending upstream from nt +171 demonstrated high promoter activity, comparable to the constitutively active thymidine kinase promoter (TK-luc; Fig. 3.8). However, this
transcriptional activity was progressively lost in constructs in which additional sequence extending downstream of nt +171 and in the 5'-UTR was included (Fig. 3.8). Although this may be due to a repressor element, the gradual decline in luciferase activities suggests that the decrease is more likely due to the length (the presence of numerous ATG sites) and/or the presence of extensive secondary structure in the 5'-UTR of AJ18, which may affect translation. As the levels of luciferase activities measured in transient transfection assays are dependent on transcription and translation, the effects of the 5'-UTR of AJ18 on these events remain to be determined.

The region upstream of the transcription start site of the rat AJ18 gene includes various consensus-binding sites for regulatory proteins such as Runx2, Ets1, Smads, Sp1, and NFκB that are also conserved in the mouse gene. The regulation of AJ18 through the Runx2 and Smads sites might be anticipated in view of the relationship between the expression of AJ18 and Runx2 and the osteogenic activity of BMPs. However, transcription regulation of promoter constructs containing these responsive elements was not observed in cells treated with BMP-7 or transfected with Runx2 expression vector (data not shown). Similarly, transfection of an expression vector for Ets1, a transcription factor involved in bone development (reviewed in Raouf and Seth, 2000), did not significantly alter transcription of AJ18 (data not shown). Current studies are directed at the effects of NFκB, which has been implicated in bone resorption (Iotsova et al., 1997) and at the interactive effects of BMPs, Runx2 and Ets1. In this regard, there is a Smad binding site separated by 9 bp from an Ets1 binding site (Fig. 3.7). It has been previously shown that Ets1 interacts with Runx2 for the synergistic transactivation of the osteopontin gene (Sato et al., 1998), while Smads have been shown to interact with the Runx proteins (Hanai et al., 1999). Therefore, it is possible that Smads and Ets1 may interact directly
or indirectly, with Runx2 as a mediator to induce the transcription of bone related genes such as AJ18.

Although AJ18 mRNA is expressed in various developing rat tissues, AJ18 was identified in a primary bone cell preparation (FRCCs) and is expressed highly in bone and brain tissues. The transcription activity of AJ18 appears to be significantly greater in osteoblastic cells compared to undifferentiated fibroblastic cells, supported by the 100-fold higher luciferase activity for constructs p(-1494/+171) and p(-77/+505) in ROS 17/2.8 cells relative to that seen in C3H10T1/2 cells. This suggests that the region encompassing nucleotides -77 to +171, which includes Runx2 sites (OSE2) at nucleotides -30 and -73 (Fig. 3.6) may, in part, be important for the bone-specific expression of AJ18.

In summary, these studies have characterized the 5'-flanking region of AJ18, which is the first promoter described for the large family of KRAB/C2H2 zinc finger genes.
Chapter 4: Temporal and Spatial Expression of AJ18

Andrew Jheon performed experiments shown in Figs. 1 and 2. Dr. Bernhard Ganss and William Teo dissected the mouse tissues and Jun Chen performed the immunostaining. Andrew Jheon was responsible for interpretation of data presented in Figs. 4.3 to 4.10.
4.1. Summary

Osteoblastic differentiation requires the coordinated regulation of specific genes that direct mesenchymal stem cells towards the bone cell lineage. Rat AJ18 is a putative transcriptional repressor that was initially identified as an immediate-early responsive gene to bone morphogenetic protein-7 (BMP-7). AJ18 appears to be involved in osteoblastic differentiation through its ability to bind to the osteoblast-specific element2 (OSE2), and to modulate transactivation by Runx2. To determine the temporal and spatial expression profile of AJ18 during mouse development, Northern blot hybridization analyses were performed in concert with immunohistochemical experiments using affinity-purified anti-AJ18 antibodies. Whereas AJ18 mRNA expression was low in most tissues during embryonic development, high levels were observed in brain, kidney, and bone tissues of 17 days postcoitum (17 dpc) embryonic mice. Thereafter, expression of AJ18 declined, except in brain where levels remained high. Immunohistochemical analysis for AJ18 in 16 dpc embryos showed strong staining of nuclei in brain, kidney and skeletal tissues, consistent with the expression of mRNA in these tissues. In addition, strong staining was also evident in various epithelia. In developing endochondral bone, AJ18 staining was strong in proliferating and pre-hypertrophic chondrocytes, and osteoblasts with low or no staining in hypertrophic chondrocytes. In long bone of 4-week old mice, some pre-hypertrophic and hypertrophic chondrocytes stained for AJ18, with strong staining in osteoblasts. In developing embryonic teeth, staining for AJ18 was observed in nuclei of cells that are destined to form enamel, dentin, and alveolar bone. In teeth of 4-week old mice, staining was observed in ameloblasts, odontoblasts, cementoblasts, alveolar bone cells, and periodontal ligament cells. Notably, the expression of AJ18 mRNA in mouse embryos including expression in developing glomeruli of the kidney, the eye, developing bone and teeth, and an
absence of expression in the liver is similar to that observed previously for the expression of BMP-7 mRNA (Helder et al., 1995). Thus, the expression of AJ18 is consistent with its perceived role as a transcriptional factor that regulates developmental processes downstream of BMP-7.
4.2. Introduction

Organogenesis involves temporally-regulated morphogenesis of cells that differentiate to form organ-specific structures in the appropriate location (reviewed by Peters and Balling, 1999). At the earliest stages of organ development, coordinated sequential and reciprocal interactions occur between adjacent epithelial and mesenchymal tissues, and it is evident that growth factor-mediated signaling is important and necessary for the regulation of the epithelial-mesenchymal interactions. The majority of these growth factors are encoded by a small number of conserved multigene families, which include the bone morphogenetic proteins (BMPs). Although BMPs provide the primordial signals for bone formation, the importance of BMPs in epithelial-mesenchymal interactions has also been demonstrated. For example, BMP-4 expression in dental epithelium induces its own expression along with transcription factors Msx1, Msx2, and Egr1 in mesenchyme (Vainio et al., 1993). Moreover, BMP-7 is expressed in both the ureteric epithelium and the undifferentiated mesenchyme of the kidney, and is required for the continued proliferation and differentiation of mesenchymal and/or epithelial cells (Dudley et al., 1995; Luo et al., 1995). BMP-7 is also required for the development of mesenchyme and epithelium during eye development.

AJ18, a member of the growing family of KRAB/C2H2 zinc finger genes, was originally identified in fetal rat calvarial cells (FRCCs) as a target gene for BMP-7 (Chapter 2; Jheon et al., 2000). Subsequent studies have shown that the protein is differentially regulated during osteogenic differentiation, that it has DNA-binding activity, and that it can modulate Runx2-mediated transactivation through the OSE2 (Chapter 2; Jheon et al., 2001). To determine how the expression of AJ18 is regulated during osteogenic differentiation the rat AJ18 gene was isolated and the 5'-flanking region characterized (Chapter 3). The intergenic region upstream of the
transcriptional start site is relatively short and contains consensus elements for transcription factors, such as Runx2, Ets1, Smads, Sp1, and NFκB. These factors are known to be involved in the differentiation of osteoblastic cells.

To determine the physiological significance of AJ18 in bone development, preliminary analyses of AJ18 mRNA expression were performed in rat tissues by Northern blot hybridization (Chapter 2; Jheon et al., 2001). Whereas expression of AJ18 was relatively strong in embryonic bone, expression was also observed in other tissues, including brain and kidney. In this chapter, a combination of Northern hybridization and immunohistochemistry was used to analyze the temporal and spatial expression of AJ18 in mouse embryos to provide a more comprehensive and detailed analyses of tissues and cells that express AJ18 during embryonic development. The isolation and sequencing of mouse AJ18 confirmed that polyclonal antibodies specific for rat AJ18 would recognize the mouse protein. Mouse AJ18 was later found to be the same gene as Kid3, which was isolated from a mouse genomic library, and as shown for AJ18, is expressed highly in brain and is differentially expressed during kidney development (Watson, 2000). The studies presented in this chapter have revealed an expression pattern of AJ18 that is similar to that reported for BMP-7, which affects tissue and organ development during embryogenesis. These observations support the possibility that AJ18 is a downstream target of BMP-7.
4.3. Experimental methods

4.3a. Mouse Genomic Library Screen

To characterize the mouse AJ18 gene, a mouse genomic library was prepared from 129SvJ mouse spleen DNA assembled into λ phage FIX II vector (Strategene, La Jolla, CA) and screened by plaque hybridization (Sambrook et al., 1989). The library was screened with a 32P-dCTP-labeled cDNA probe, which was digested from rat AJ18 cDNA by PstI, generating a 461 bp fragment. After tertiary screening, positive clones were isolated, purified, and characterized by restriction endonuclease digests. EcoRI digested fragments were separated on an agarose gel, subcloned into pBluescript II SK (Strategene), and sequenced.

4.3b. Mouse Tissue Preparation

Tissues were isolated from mice at various stages of development. Tissues from brain, heart, lung, skeletal muscle, cartilage, liver, thymus, kidney, calvaria, and long bones were dissected from mice at embryonic stages 15 and 17 dpc, and neonate stages day 2, 7, 15, and 23. Mouse embryos at 16 dpc, and a mandible and tibiae from a 4-week old mouse were isolated, and fixed in 4% paraformaldehyde-PBS at 4°C. The mandible and tibiae were demineralized in 12.5% (w/v) EDTA (pH 7.4), with the solution changed every 2nd day for 2-3 weeks. The embryos and tissues were embedded in paraffin. Serial 12 µm-thick sections were mounted on Superfrost/Plus glass slides (Fisher, Ontario, Canada), and stored at 4°C until use.

4.3c. RNA Extraction

Total RNA was isolated from mouse tissues using the thiocyanate-phenol-chloroform extraction method as described by Chomczynski and Sacchi (Chomczynski and Sacchi, 1987).
4.3d. *Northern Blot Hybridization*

Northern blot hybridization was performed as described in Chapter 2. A cDNA fragment of 700 bp within the 3'-UTR of AJ18 was PCR-amplified from the isolated mouse λ clone using primers 5'-GGCACACCTTTTCATCTACCGCTCATCC-3 and 5'-ACGCCTGTATCCATCCCCACTGTTAAG-3', and used as the template for probe synthesis.

4.3e. *Anti-AJ18 Polyclonal Antibodies*

Polyclonal antibodies specific for AJ18 were raised and affinity-purified as described in Chapter 2.

4.3f. *Immunohistochemical Analysis*

Immunoperoxidase staining for AJ18 protein in tissue sections from 11, 12, 13, 14, 15 and 16 dpc mouse embryos, and 4-week mouse teeth and tibia was performed using the Vectastain ABC kit (Vector Laboratories, Burlingame, CA) following the manufacturer's instructions. Tissue sections, prepared as described above, were rehydrated through incubation in graded alcohols to water, and incubated in blocking solution (5% BSA, 2% normal goat serum) for 1 hr. Affinity-purified anti-AJ18-1, or anti-AJ18-2, antibodies were applied, and tissue sections were incubated for 1 h. The sections were washed and treated with biotinylated anti-rabbit IgG for 30 min, followed by incubation with peroxidase-labeled streptavidin for 30 min, and subsequently incubated with diaminobenzidine tetrahydrochloride (DAB) and H₂O₂ for 15 min. All incubations were performed at room temperature (21°C). Some of the sections were counterstained with hematoxylin. All sections were visualized under a light microscope (Eclipse...
400; Nikon Canada Inc., Mississauga, ON) and photographed using a Coolpix 950 digital camera (Nikon Canada Inc., Mississauga, ON).
4.4. Results

4.4a. Identification of Mouse AJ18

The sequence of the mouse AJ18 ORF was deduced from a \( \sim 10 \) kb \( \lambda \) clone that was isolated from mouse genomic screens. Due to a high conservation of the nucleotide sequence between mouse and rat AJ18, the mouse AJ18 gene was readily identified and the sequence of the ORF determined from a comparison of the mouse genomic sequence to the rat cDNA. This was simplified, in part, as the 11 C\(_2\)H\(_2\) zinc finger motifs are over 97% identical and are encoded by a single exon. In total, the translated protein sequences of rat and mouse AJ18 share 91% identity and 94% similarity (Fig. 4.1). Whereas the N-terminus, KRAB domain, and zinc finger regions of mouse and rat AJ18 are almost perfectly conserved, showing between 97% to 100% identity, the linker sequence between the KRAB domain and zinc finger motifs and the C-terminus show relatively low conservation with 72% and 79% identity, respectively.

4.4b. Preliminary Northern Blot Hybridization

In previous studies expression of AJ18 mRNA in whole mouse embryos was first detected in 11 dpc embryos, reached a peak at 15 dpc, and decreased in 17 dpc embryos (Fig. 2.10B). To determine the developmental expression of AJ18 mRNA in different mouse tissues, total RNA extracted from tissues dissected at various stages of embryonic and neonate development and Northern blot analyses performed (Fig. 4.2). In 15 dpc embryos, expression of AJ18 mRNA was evident in all tissues and organs analyzed, except the liver (Fig. 4.2). In 17 dpc embryos, relatively high expression of AJ18 mRNA was indicated in brain, kidney, and bone tissues. Thereafter, in day 2 and day 7 neonates (Fig. 4.2), in 15- and 23-day old mice (data not
Fig 4.1. Sequence alignment of rat and mouse AJ18. A, alignment of the predicted amino acid sequence of rat and mouse AJ18. The KRAB A box is indicated with a *dash-dotted line*, the modified KRAB B box with a **dashed line**, and the C,H zinc finger motifs are indicated with *lines*. Synthesized peptides 1 and 2 for raising anti-AJ18-1 and anti-AJ18-2 polyclonal antibodies, respectively are indicated with *gray lines*. B, schematic representation of rat and mouse AJ18. AJ18 was divided into 5 regions: N-terminus (N), KRAB A and B (K A+B), linker (L), zinc fingers (ZFs), and the C-terminus (C). Degrees of identity and similarities are listed for each region. Rat and mouse AJ18 protein sequences showed 91% identity and 94% similarity.
Fig. 4.2. Preliminary Northern blot analyses of AJ18 mRNA expression during mouse development. Various tissues were isolated from mouse embryos and neonates, and total RNA extracted for Northern blot analyses. Blots were hybridized with radiolabeled probes synthesized from AJ18 cDNA. A cDNA probe for Glyceraldehydephosphate dehydrogenase (GAPDH) was used as a control for RNA loading. Mouse embryos 15 dpc, E15; 17 dpc, E17; mouse neonates day 2, D2; neonates day 7, D7.
shown), and in adult rats (Fig. 2.6B), AJ18 mRNA was expressed highly in the brain, with expression barely detectable in bone and other tissues. Notably, AJ18 mRNA was not detected in liver at any of the developmental stages analyzed (Fig. 4.2 & Fig. 2.6).

4.4c. Immunohistochemical Staining

In concert with Northern blot analyses, immunohistochemistry was performed on tissue sections of whole mouse embryos at various developmental stages, as well as in neonate tissues. As the mouse and rat AJ18 sequences are highly conserved, the anti-rat AJ18 polyclonal antibodies raised to synthetic peptides 1 (residues 2-13) and 2 (residues 158-169) should recognize both rat and mouse AJ18 protein. Affinity-purified anti-AJ18-1 and anti-AJ18-2 antibodies recognized mouse AJ18 protein (data not shown), although anti-AJ18-1 showed higher immunoreactivity, consistent with the 100% conservation of sequence between residues 2-13 (Fig. 4.1). Therefore, all the tissue sections presented hereafter were immunostained using affinity-purified anti-AJ18-1 antibody. Strongest staining was obtained for all tissues at day 16 dpc at which time skeletal tissues could be analyzed at different developmental stages in the ribs. Consequently results are shown primarily for tissues at this stage (Figs. 4.3-4.5, 4.7, 4.8, 4.10). Although staining for AJ18 was observed in the same tissues beginning at 13 to 14 dpc mouse embryos, staining intensity was lower relative to 16 dpc mouse embryos, and the differential expression within and between tissues was less clear. To analyze expression of AJ18 in tibial bone and teeth, sections of tibia and teeth from a 4-week old mouse were immunostained, respectively (Figs. 4.6 & 4.9).
Expression of AJ18 Protein in Cartilage and Bone

Immunostaining of AJ18 was high in skeletal tissues of 16 dpc mouse embryos, as shown for bone of the maxilla and mandible, Meckel's cartilage, and rib bones (Figs. 4.3-4.5 & 4.7). During embryonic development, mesenchymal cells in the facial region condense to form nodules of differentiated osteoblasts that form the maxillary and mandibular bones of the upper and lower jaws, respectively. Expression of AJ18 was evident in the nuclei of mesenchymal cells adjacent to the bone surfaces with stronger staining of the osteoblasts lining mineralized bone surfaces (Figs. 4.3A & C). AJ18 was also detected within some newly-formed osteocytes, but no staining was apparent in the endothelial cells surrounding blood vessels or in loose connective tissue (Fig. 4.3C). AJ18 was detected within the nuclei of the small chondrocytes of Meckel's cartilage, which form the template for mandibular bone (Fig. 4.3E). In identically prepared control sections, lacking the incubation with the primary antibody, no red-brown staining from the peroxidase activity was observed (Figs. 4.3 B, D & F).

As the individual rib bones are formed at different times, a progression of endochondral bone development can be seen at a single time point in a sagittal section through a series of ribs. Thus, staining for AJ18 in the 10th, 11th, and 12th rib bone shows protein in nuclei of cartilage and bone cells (Fig. 4.4A). In the 12th rib, the section shows nuclear staining of AJ18 in pre-hypertrophic chondrocytes (Fig. 4.4B). In the 11th rib, which is further developed, the cartilage cells have undergone hypertrophy and cell nuclei show sporadic staining for AJ18 (Fig. 4.4C). At this stage the cartilage has started to mineralize and osteogenic periosteal cells have condensed and are expressing AJ18 on the surface of the mineralizing bone. In the more advanced 10th rib, endochondral bone has formed centrally, replacing the mineralized cartilage, and the formation of periosteal bone is well-established (Fig. 4.4D). Staining for AJ18 is
Fig. 4.3. Immunostaining of AJ18 in jaw bones and Meckel’s cartilage. Light micrographs of maxillary and mandibular bones of a 16 dpc mouse embryo immunostained for AJ18. A, C, regions of bone development in the maxilla and mandible, respectively, show AJ18 staining in osteoblasts (black arrowheads) on the surface of mineralized bone (Mb) and osteocytes (white arrowheads) embedded within bone. Capillaries are identified with asterisks. E, immunostaining of AJ18 in Meckel’s cartilage (MC) and the surrounding mineralized bone (Mb) of the mandible showing staining in the nuclei of chondrocytes (white arrowheads) and osteoblasts (black arrowheads), respectively. B, D, F, show sections immunostained omitting primary antibody as negative controls. All sections were counterstained with hematoxylin. Bars: 40 μm.
Fig. 4.4. Immunostaining of AJ18 in developing rib bones. Light micrographs of developing rib bones of a 16 dpc mouse embryo immunostained for AJ18. A, developing 10th, 11th and 12th rib bones (D, C, B, respectively) shows staining for AJ18. Strong staining for AJ18 is observed in the epithelium (Ep) and is also evident in muscle (Mu). B, higher magnification of the 12th rib shows staining for AJ18 in prehypertrophic chondrocytes (PC; black arrowheads). C, higher magnification of the 11th rib shows staining for AJ18 in some hypertrophic chondrocytes (HC; gray arrowheads) and in the developing osteoblasts (black arrowheads) on surface of the periosteum (P). D, higher magnification of the 10th rib shows staining for AJ18 in osteoblasts (black arrowheads) present in the bone marrow (BM) and the surface of mineralizing bone in the periosteum (P), and in osteocytes (gray arrowheads) embedded in periosteal bone. E, higher magnification showing staining for AJ18 in the epithelium (Ep). White bar: 30 μm, black bars: 10 μm.
observed in cells present in bone marrow, in the periosteal cells on the surface of the rib bone, and in osteocytes present within periosteal bone. In these sections comparable staining is present in muscle tissue surrounding the ribs (Fig. 4.4A) and is particularly strong in the outer epithelial layer of the embryo (Fig. 4.4E). In both muscle and especially in the epithelium, diffuse staining was seen in the cells, with nuclear staining in the epithelium being strongest in the basal cell layer.

A sagittal section through the growth plate of the 3rd rib bone from a 16 dpc mouse embryo showed the developmental progression of cartilage cells from proliferating chondrocytes through the formation of columnar proliferating chondrocytes to hypertrophic chondrocytes in the mineralized cartilage, which is replaced by epiphyseal bone (Fig. 4.5A). The strongest staining was seen in the proliferating chondrocytes with lower expression in hypertrophic chondrocytes (Figs. 4.5A & B), consistent with the staining in the 11th and 12th ribs. However, staining was high in the osteogenic cells in the primary spongiosa, immediately below the calcified cartilage and in the subperiosteal bone collar formed by periosteal cells (Fig. 4.5C). Staining in muscle was also observed.

In rodents, formation of the long bones in the limbs occurs continuously and the epiphysis does not close. To study AJ18 in endochondral bones, sections of tibiae from a 4-week old mouse were immunostained (Fig. 4.6A). AJ18 staining was observed in some pre-hypertrophic and hypertrophic chondrocytes (Fig. 4.6B) with strong staining visible in osteoblasts (Fig. 4.5C). Staining for AJ18 was also present in the cartilage cells at the periarticular surface (Fig. 4.5D). No staining for AJ18 was observed in osteocytes embedded within cortical bone (Fig. 4.5E). These results were identical to those obtained in tibiae of a 4-week old rat (Fig. 2.7).
Fig. 4.5. Immunostaining of AJ18 in an embryonic rib bone. A, sagittal sections of the 3rd rib shows staining for AJ18 in the growth plate (GP) of a 16 dpc mouse embryo. Mesenchymal cells (M) lie adjacent to the rib. B, higher magnification shows staining for AJ18 within prehypertrophic chondrocytes (PC; black arrowheads) and low or no expression within hypertrophic chondrocytes (HC; gray arrowheads). C, higher magnification of the expression of AJ18 in osteoblasts (black arrowheads) within the bone marrow (BM) below the zone of calcifying cartilage (ZC), and in the periosteum (P) adjacent to the subperiosteal bone collar (BC). Mesenchymal cells (M) also shows some staining. White bar: 40 μm, black bars: 20 μm.
Fig. 4.6. Immunostaining of AJ18 in long bone of 4-week old mouse. A, sagittal sections of tibial bone from a 4-week old mouse shows staining for AJ18 within epiphysis (Ep) comprising articular cartilage (AC) and secondary center of ossification (2nd), and the growth plate (GP). Cortical bone (Cb) shows an absence of staining for AJ18. B-E, shows higher magnification of regions within the tibiae. B, staining for AJ18 is seen in some of the prehypertrophic chondrocytes (gray arrowheads) and hypertrophic chondrocytes (black arrowheads) within the growth plate. C, staining for AJ18 in osteoblastic cells (black arrowheads) is strong within the bone marrow (BM) below the zone of calcifying cartilage (ZC). D, staining for AJ18 is observed within cells of articular cartilage (black arrowheads). E, shows lack of AJ18 staining in osteocytes (black arrowheads) embedded in cortical bone. Gray bars: 10 μm, black bar: 200 μm.
4.4c-ii. Expression of AJ18 Protein in Developing Teeth

Three principal stages (bud, cap, and bell), are recognized in the formation of teeth. The bud stage begins with the incursion of epithelial cells into the mesenchyme of the jaw. The cap stage shows a condensation of cells and the formation of the dental papilla and dental follicle. The outer epithelial ingrowth resembles a cap sitting on the dental papilla and is called the dental organ. The dental organ, dental papilla, and dental follicle constitute the tooth germ, and gives rise to enamel, dentin-pulp, and supporting structures of the tooth, respectively (reviewed by ten Cate, 1994). Continued growth of the tooth germ produces the bell stage (Fig 4.7), named for the resemblance of the dental organ to a bell as the crescent-shaped area of the epithelial cap deepens. At the early bell stage, the epithelial cells start to differentiate forming the internal and external dental epithelia. Immunostaining for AJ18 was weak prior to the bell stage at which time AJ18 was expressed primarily in the nuclei of the internal dental epithelium, especially around the cervical loop region of the dental organ (Fig. 4.7). As well AJ18 was expressed in the external dental epithelium, which is contiguous with the oral epithelium and in some of the mesenchymal cells in the dental papilla.

Expression of AJ18 was also studied in the incisors and fully developed molars of a 4-week old mouse (Fig. 4.8A). In sections through the molar roots, staining for AJ18 was seen in the dental papilla with strong staining in the odontoblasts lining the dentin. Strong staining was also apparent in the cells of the periodontal ligament and in osteogenic cells lining the surface of the alveolar bone. However, nuclear staining of osteocytes was weak (Fig. 4.8B). In the area of the cervical loop staining of the dental follicle cells, which differentiate into cementoblasts, staining for AJ18 was also seen (Fig. 4.8B). In the continuously erupting incisor tooth, a progression of continuous cell differentiation for the formation of dentin and enamel is
Fig. 4.7. Immunostaining of AJ18 in developing teeth. Light micrograph of the early bell stage of molar tooth development from the maxilla of a 16 dpc mouse embryo. The nuclei in the oral epithelium (OE), which gives rise to the dental organ (DO) are lightly stained. Some staining is also evident in nuclei of condensing mesenchymal cells in the dental papilla (DP), which forms a crescent shaped area as the cap stage is replaced by the bell stage. Strongest staining is observed in the cervical loop region of the dental organ (black arrowheads), and is also present at lower intensities in epithelial cells forming the internal (IDE) and external (EDE) dental epithelium. Section was counterstained with hematoxylin. Bar: 20 μm.
Fig. 4.8. Immunostaining of AJ18 in molar and incisor of 4-week old mouse. Light micrographs of the mandible from a 4-week old mouse. A, shows staining for AJ18 within molars (Mo) and in the incisor (In). Odontoblasts, which forms the dentin (De), and ameloblasts, which forms the enamel (En) show strong staining for AJ18. B-E, regions show higher magnification. B, staining for AJ18 in the nuclei of odontoblasts (O) in the first molar tooth root. Nuclear staining is also seen in cementoblasts in cementum (C) and in periodontal ligament (PL) fibroblasts. Osteoblasts (black arrowheads) on the surface of the alveolar bone (Alv) also stain, whereas osteocytes (gray arrowheads) are weakly stained. C, staining for AJ18 is strong in cells of the dental follicle (DF) and odontoblasts (O) adjacent to newly-formed dentin (De) with less staining in dental papilla (DP). D, E, shows staining for AJ18 in ameloblasts (Am) at different stages of maturation. Ameloblasts actively forming enamel (En), shown in D, show lateral organization with the immunoreactive nuclei polarized at the interface with the stellate reticulum (Sr), which also shows staining. At a late stage, shown in E, the immunostained ameloblasts undergo apoptosis and appear less organized. Black bars: 10 μm, gray bars: 40 μm
present only on the underside of the mouse mandibular incisor. AJ18 staining was observed in the cells of the dental papilla with strong staining in the newly-differentiating odontoblasts (Fig. 4.8C), which lose the staining after the formation of dentin has started. Anterior to this region the nuclei of ameloblasts, with their elongated cell bodies, are strongly stained (Figs. 4.7D & E). Moreover, the staining intensity appeared to be retained as the ameloblasts progress from the secretory stage where they appear highly ordered (Fig. 4.7D), to the post-maturation stage where they undergo apoptosis and the columnar arrangement becomes disorganized (Fig. 4.7E).

4.4c-iii. Expression in the Brain and Epithelium

Expression of AJ18 mRNA was highest in brain in the embryo and especially post-natally when expression in other tissues declined markedly (Fig. 4.2). Immunostaining of sections of brain in 16 dpc embryos showed variable staining in brain cells with the strongest staining in the condensing cells forming the future cerebral cortex, midbrain, and tentorium cerebelli (Figs. 4.9A & C). Generally, staining in the brain was not as strong as seen in the osteogenic cells forming the calvarial bone and much lower than the staining seen in the epithelium covering the cranium (Fig. 4.9B). Since AJ18 mRNA expression appeared stronger in brain compared to bone it is possible that protein translation in brain cells is less efficient than in bone cells, or AJ18 may be highly expressed in specific regions of the brain, which were not included in the sections analyzed. Relatively strong staining was observed in the outer epithelium surrounding the embryo (Fig. 4.9B). Although nuclear staining was present in the basal cells of the epithelium, staining in the outer layers was diffuse, possibly due to cell death.
Fig. 4.9. Immunostaining of AJ18 in the brain. A, staining for AJ18 in the brain of a 16 dpc mouse embryo is variable, with strong staining apparent in condensing cells separating distinct regions of the brain, namely the future cerebral cortex (CC), midbrain (MB), and tentorium cerebelli (TC). B, higher magnification of region showing strong staining for AJ18 in the outer epithelium (Ep) and in osteoblastic cells (black arrowheads) forming the intramembranous calvarial bone (Ca). Nuclear staining of cells in tentorium cerebelli is observed below the calvarium (gray arrowheads). C, strong staining for AJ18 is observed in condensing cells (black arrowheads) shown at higher magnification. Black bars: 40 μm, white bar: 200 μm.
Northern blot analyses showed AJ18 mRNA expression in the kidney of mouse embryos at 15 and 17 dpc. Consistent with these results relatively high immunostaining for AJ18 was observed in cells within developing glomeruli of the kidney (Fig. 4.10A). The specific expression of AJ18 mRNA within the developing glomeruli was also detected by in situ hybridization in 19 dpc rat embryos (personal communications, Dr. Mark DeCastekeler, Vanderbilt University). In the eye, specific staining was found in cells within the sclera, choroids, and vitreous body, with less staining in the retina (Fig. 4.8C). AJ18 staining was also observed in epithelial cells forming the whisker follicles (Fig. 4.8E). The strong staining of nuclei in the whisker follicles is consistent with the general staining of epithelial cells, including the epidermal cells (Figs 4.4 & 4.9B), and epithelial cells lining the intestine and lung alveoli (results not shown).
Fig. 4.10. Immunostaining of AJ18 in tissues that express BMP-7. Light micrographs of 16 dpc mouse embryo sections. A, staining for AJ18 is observed in developing glomeruli of kidney (black arrowheads). C, staining for AJ18 is observed in the various layers of the eye including the sclera (Sc), choroid (Ch), and the vitreous body (VB), with less staining in the retina (Re). E, staining for AJ18 is seen in cells of a whisker follicle (black arrowheads). B, D, E, show sections immunostained with secondary antibody alone as negative controls. B, section was counterstained with hematoxylin. Black bars; 40 μm, white bars: 10 μm.
4.5. Discussion

Previous studies have characterized AJ18 as a zinc finger transcription factor that is up-regulated in osteogenic cells induced to differentiate with BMP-7. Consistent with the expression of AJ18 mRNA during osteogenic differentiation by FRCCs in vitro (Fig. 2.5), AJ18 mRNA was expressed early during the formation of endochondral and intramembranous bones (tibia and calvaria, respectively) in mouse embryos. Expression of AJ18 mRNA was also detected in other tissues and organs with maximal expression during embryonic development. While significant expression of AJ18 occurred post-natally in brain, which together with mineralized tissues has the highest expression of AJ18, expression was lost in other tissues. As expected for a transcription factor, immunohistochemical staining for AJ18 protein was largely restricted to cell nuclei, with the relative intensity of staining in the tissues approximating the level of mRNA determined by Northern blot hybridization.

Comparison of the protein sequences for AJ18 in rat and mouse revealed high conservation of amino acids (97% to 100%) in the region of the C2H2 zinc fingers and the KRAB domain, signifying the functional importance of these regions in mediating DNA-binding and transcriptional activity. In the linker region between the KRAB domain and the zinc fingers, and at the carboxy-terminal region, sequence conservation was much lower (72% to 79%), indicating that the precise structure of these regions is of lesser functional importance. In previous studies (Chapter 2; Jheon et al., 2001) the C2H2 zinc finger motifs of AJ18 has been shown to bind to DNA with a preference for sequences containing 5'-CCACA-3', which is present within the OSE2 through which Runx2 mediates transcriptional activity (Ducy et al., 1997). AJ18 was found to suppress Runx2-mediated transactivation through the OSE2. While gene targets for AJ18 have not been identified, forced expression of AJ18 in C3H10T1/2 fibroblast-like cells
induced towards osteogenesis by BMP-7, suppressed the expression of alkaline phosphatase, an early marker of osteogenic differentiation.

Although BMPs are characterized by their bone-inductive activity (Rodan and Harada, 1997) as members of the TGF-β superfamily of cytokines they have a broader role in developmental processes involving different tissues and organs (Hogan, 1996). Thus, it is of interest that BMP-7, which was originally used to identify AJ18 (Chapter 2; Jheon et al., 2000), has a similar expression pattern to AJ18 in mouse embryos. BMP-7 mRNA is expressed in the glomeruli of kidney, in the eye, and in whisker follicles, and its expression is absent in the liver of 17.5 dpc mouse embryos (Helder et al., 1995). Studies presented in this Chapter show that the expression of AJ18 in 16 dpc mouse embryos corresponds with the results reported by Helder et al. (1995). In addition, BMP-7 is expressed in the brain, in mineralized tissues such as in hypertrophic chondrocytes, osteoblasts, and periosteum, and in the inner dental epithelium with expression in a few cells in the dental pulp (Helder et al., 1995). These are all tissues in which AJ18 showed positive staining. In 12- to 18-day mice, BMP-7 is expressed in all bony tissues, and in the odontoblasts and ameloblasts of teeth (Thomadakis et al., 1999). Although AJ18 mRNA expression was markedly reduced in all post-natal tissues, except brain, and was difficult to detect by Northern hybridization, AJ18 protein was detectable by immunostaining in 4-week old mice. AJ18 was present in the nuclei of chondrocytes and osteoblasts in tibiae, and in the odontoblasts and ameloblasts in molar and incisor teeth. Taken together, the temporal and spatial expression of AJ18 at various developmental stages closely mimics the expression of BMP-7 in mouse tissues, consistent with the original identification of AJ18 as an immediate-early responsive gene to BMP-7 (Fig. 2.1). Similar to BMP-7, therefore, AJ18 may modulate the differentiation of bone and cartilage cells, but its expression in various tissues suggests
functional roles in the development of other tissues, which may be revealed by generating mice with a targeted disruption of the AJ18 gene. While significant developmental effects have been observed in BMP-7 knockout mice in only three organs (eyes, kidneys, and bone; Dudley et al., 1995; Luo et al., 1995), other BMPs may compensate for the absence of BMP-7 in the formation of other tissues.

From the immunohistochemical analysis of endochondral bone in 16 dpc mouse embryos, it is evident that AJ18 is initially expressed by proliferating chondrocytes and pre-hypertrophic chondrocytes, with sporadic expression in hypertrophic chondrocytes. The formation of cartilage in endochondral bones is controlled by a negative regulatory feedback loop involving PTH-related protein (PTHrP) and Indian hedgehog (Ihh) (Lanske et al., 1996; Vortkamp et al., 1996). Ihh is expressed in the pre-hypertrophic chondrocytes present in the growth plate, where it regulates the rate of hypertrophic chondrocyte differentiation (Vortkamp et al., 1996). Ihh induces the expression of PTHrP in the peri-articular perichondrium, which signals to its receptors expressed in pre-hypertrophic chondrocytes and inhibits the expression of Ihh thereby blocking hypertrophic chondrocyte differentiation (Lanske et al., 1996; Vortkamp et al., 1996). Thus, ablation of the PTH/PTHrP receptor results in mice with accelerated differentiation of chondrocytes within the growth plate of bone (Lanske et al., 1996). The inhibition of Ihh by PTHrP appears to be indirect, and through the direct down-regulation of BMP-6 (Grimsrud et al., 1999). The spatial expression pattern of AJ18 within the growth plate of bone and the apparent functional redundancy of BMPs suggests that AJ18 may be a possible downstream target for BMP-6 in this cartilage negative feedback loop.

BMP-7 has also been shown to inhibit the terminal differentiation of chondrocytes in the peri-articular region, independent of the Ihh/PTHrP/BMP-6 negative feedback loop via unknown
inhibitory factors (Haaijman et al., 1999). Peri-articular cartilage remains unmineralized, and it is the loss of peri-articular cartilage due to new bone formation that leads to joint diseases such as osteoarthritis (Goldring, 1999). Thus, AJ18, which is also expressed in cartilage cells present at the peri-articular region, may play a role in the inhibition of the terminal differentiation of these chondrocytes under the control of BMP-7. That AJ18 expression is lost in hypertrophic chondrocytes, in which Runx2 expression is induced (Kim et al., 1999), further suggests that AJ18 and Runx2 have antagonistic activities, as evident in the AJ18 suppression of Runx2 transcriptional activity (Chapter 2; Jheon et al., 2001). Thus, whereas the over-expression of Runx2 in pre-hypertrophic chondrocytes induces differentiation to hypertrophic chondrocytes (Takeda et al., 2001), the over-expression of AJ18 in pre-hypertrophic chondrocytes may inhibit hypertrophic chondrocyte differentiation. Therefore, it would be interesting to determine the phenotypic effects of AJ18 over-expression in a chondrogenic cell line such as CFK2 (Bernier and Goltzman, 1993), along with its effects on Runx2 and Ihh expression.

These studies have shown that AJ18 has a broad expression profile in embryonic tissues, consistent with the concept that AJ18 acts downstream of BMP-7 in tissue morphogenesis.
Chapter 5: Summary and Future Directions
Elucidating the mechanisms that control the coordinated cascade of cellular events and the regulation of genes involved in osteogenesis is fundamental to understanding the formation of mineralized tissues and the aberrations associated with inherited and acquired bone diseases. Although relatively few molecular signals that control these events are known (Ducy et al., 2000; Erlebacher et al., 1995; Karsenty, 2000), the characterization of BMPs and their downstream effectors (Smads), together with the recent identification of Runx2 as a “master gene” of osteogenesis (Rodan and Harada, 1997) have provided a basic paradigm for studying osteogenic differentiation. Using BMP-7 to induce bone cell differentiation, and differential display to identify the genes regulated during osteoblast differentiation, a novel gene provisionally named AJ18 that is up-regulated during osteoblast differentiation and responsive to BMP-7 was identified from cultures of FRCCs. AJ18 encodes a 64 kDa protein comprising a KRAB domain at the N-terminus, and a linker region followed by 11 successive C2H2 zinc finger motifs. The presence of the transcription repression KRAB domain and C2H2 DNA-binding zinc finger motifs was indicative of a transcription repression function for AJ18. Using a target detection assay, a consensus-binding site (5'-CCACA-3') for zinc finger DNA-binding domain of AJ18 has been identified. The binding site forms part of the osteoblast-specific element2 (OSE2; 5'-ACCACA-3') that is recognized by Runx2. Since the temporal expression of AJ18 and Runx2 in osteogenic differentiation in vitro was similar, the effects of AJ18 on Runx2-mediated transcription were analyzed in transient transfection assays. Using a luciferase reporter plasmid containing six-tandem repeats of OSE2 fused upstream of a minimal osteocalcin promoter, over-expression of AJ18 was found to abrogate the transactivation activity of Runx2 in a dose-dependent manner. Since the suppressor activity of AJ18 did not require the KRAB domain, it is possible that AJ18 competes with Runx2 for binding to the OSE2 element. Consistent with the
ability of AJ18 to repress Runx2-mediated transactivation through the OSE2 is the decrease in activity of alkaline phosphatase resultant from the over-expression of AJ18 during BMP-induced osteogenesis in undifferentiated fibroblastic cells.

To understand how the expression of AJ18 is regulated, a rat genomic library screen was performed to isolate the 5'-flanking region and to elucidate the genomic organization of AJ18. A short intergenic region of 1.5 kb was identified between AJ18 and another putative zinc finger transcription factor gene provisionally named AJ17, and partially sequenced. The 5'-flanking region of AJ18 was shown to be active in transcription assays, with strong activity obtained with a minimal promoter encompassing nucleotides −77 to +171. The rat AJ18 gene consists of at least 4 exons, the first exon coding for an unusually long 2.3 kb 5'-UTR region. The presence of multiple ATG codons upstream of the authentic initiator ATG codon suggests the translation of AJ18 occurs via a mechanism other than the ribosome-scanning mechanism. Interestingly, a putative IRES was identified near the authentic ATG codon that showed complementarity to rat 18S rRNA sequence. The initiation of translation via an IRES is supported by the GC-rich regions present in the 5'-UTR, and the gradual decline in luciferase activity with the addition of sequence extending downstream of the minimal promoter into the 5'-UTR. The minimal promoter of AJ18 appears to lack a TATA box and an initiator (Inr) element, and transcription initiation may be mediated through the putative Sp1 site present near the transcription start site. Consensus binding-sites for transcription factors such as Runx2, NFκB, Smads, and Ets1 were identified within and upstream of the minimal promoter. Although preliminary analyses have failed to show regulation through individual Runx2 and Smad sites, the transcription activity was shown to be dramatically increased in osteoblastic cells compared to undifferentiated C3H10T1/2 cells, indicating that transcriptional regulation of AJ18 in osteogenic cells may
involve the coordinated activities of the transcription factors acting through one or more enhancer elements. Thus, interactions between Smads and Runx proteins have been shown in the regulation of the germline Ig Cα promoter (Hanai et al., 1999), while Ets1 interacts with Runx2 in the regulation of the osteopontin gene (Sato et al., 1998). Recent studies have also shown that Runx2-mediated up-regulation of the osteocalcin gene involves cooperative interactions with three OSE2 sites in a mechanism involving interaction of the Runx2 with the nuclear matrix (Javed et al., 1999). These studies also emphasize the importance of studying transcriptional regulation in the context of the nuclear matrix, which may also stabilize interactions between transcription factors and DNA. For example, the KRAB/C2H2 protein ZNF74, which has a primary sequence that is similar to AJ18, has been shown to interact strongly with the nuclear matrix (Grondin et al., 1996). In this regard, a requisite for interactions of AJ18 with other transcription factors or the nuclear matrix for binding to DNA could explain the lack of success with electrophoretic gel mobility shift experiments.

Notably, the characterization of the promoter region of AJ18 appears to be the first reported for a member of the large KRAB/C2H2 family of genes. Thus, it is conceivable that the unusually long 5′-UTR in combination with regions of high GC content in the AJ18 gene, which proved problematic in determining the AJ18 promoter, may be a characteristic of the KRAB/C2H2 family of genes.

The temporal and spatial expression of AJ18 in mineralized and other tissues is strikingly similar to the expression of BMP-7, further supporting AJ18 as a target gene of BMP-7. The expression of AJ18 in various tissues suggests a role for AJ18 in epithelial-mesenchymal interactions during organogenesis. BMP-7 has been implicated as an important signaling protein between the epithelium and mesenchyme during early eye and kidney development (Dudley et
al., 1995; Luo et al., 1995). Furthermore, BMP-4 expressed by dental epithelium induces the expression of at least three transcription factors (Msx1, Msx2, and Egr1) in mesenchyme (Vainio et al., 1993). Egr1, whose expression profile is similar to AJ18 (Figs. 2.1 & 2.2), was identified as a target gene for BMP-7 (Fig. 2.1). This may suggest that Egr1 and AJ18 cooperates downstream of BMP-7 to regulate epithelial-mesenchymal interactions. Furthermore, Runx2, which is induced by BMPs (Ducy et al., 1997) and has an expression profile similar in developing tooth structures (Jiang et al., 1999) to AJ18, has also been implicated as an important factor that regulates the expression of molecules derived from mesenchyme that act reciprocally on dental epithelium to control its growth and differentiation (D'Souza et al., 1999).

The regulation and possible functions of AJ18 are summarized (Fig. 5.1). Possible future experiments to elucidate the function(s) of AJ18, which may then be extended to the KRAB/C2H2 family of proteins are discussed below.

5A. Functional Characterization of AJ18

The function of AJ18 and the KRAB/C2H2 family of proteins in vivo, is still unclear. Although the data presented in this thesis (Chapter 2; Jheon et al., 2001) and by others (Zheng et al., 2000) demonstrate that the KRAB/C2H2 proteins are sequence-specific transcription repressors, the numerous C2H2 zinc finger motifs along with the highly variable linker and C-terminal regions suggest other possible roles. For example, the KRAB/C2H2 protein Kid-1 that contains 13 zinc finger motifs, binds to heteroduplex DNA (Elser et al., 1997) and appears to dissociate the nucleolus (Huang et al., 1999). ZNF74, a KRAB domain protein containing 12 C2H2 zinc finger motifs, has an affinity for RNA homopolymers and interacts strongly with the nuclear matrix (Grondin et al., 1996), and can interact with the hyperphosphorylated form of
Fig. 5.1. Summary of AJ18 regulation in osteogenesis. A-D, schematic linking the induction of AJ18 expression by BMP-7 with the downstream effects of AJ18 on regulation of osteogenic genes through specific enhancers/repressors for AJ18 (SE) or through an OSE2. 

A, binding of BMP-7 invokes a signal cascade comprising transmembrane receptors and the translocation of receptor-dependent Smads to the nucleus. Other exogenous signals, including other BMP members such as BMP-6, may also be involved. B, Smad proteins, as well as other transcription factors whose consensus sequences are present in the promoter of AJ18, may regulate the transcription of AJ18 hnRNA, which has an unusually long 5'-UTR (exon I). C, AJ18 hnRNA is exported from the nucleus where introns would be spliced, and the mRNA could form extensive secondary structure in the GC-rich regions of exon I. AJ18, comprising a KRAB domain and 11 C$_2$H$_2$ zinc finger motifs is translated from the mRNA utilizing a putative IRES. AJ18 localizes to the nucleus independent of its KRAB domain where it regulates the transcription of target genes. D, by recognizing the consensus sequence 5'-CCACA-3', which is a partial OSE2, AJ18 can modulate the transactivation of Runx2 and other OSE2-regulated genes. Alternatively AJ18 alone, or through interactions with other transcription factors or its putative co-repressor TIF5, may enhance or repress the transcription of target genes that are associated with osteogenic differentiation through a specific element encompassing the 5'-CCACA-3'.
RNA polymerase II (Grondin et al., 1997). Furthermore, in addition to the interaction through the KRAB domain to TIF1β, various proteins interact with KRAB/C₂H₂ proteins. Thus ZBRK1, a KRAB domain protein containing 8 C₂H₂ zinc finger motifs, interacts with BRCA1 to down-regulate the GADD45 gene (Zheng et al., 2000). The direct binding of BRCA1 appears to involve some of the C₂H₂ zinc fingers and the C-terminus of ZBRK1. Also, RbaK, a KRAB/C₂H₂ protein that represses E2F-dependent genes interacts directly with the retinoblastoma gene product (RB) through its linker region between the KRAB domain and zinc finger motifs (Skapek et al., 2000).

Therefore, it would be of interest to identify proteins that may interact with the linker or C-terminus region of AJ18, particularly in context with osteogenic differentiation. This would be best achieved through yeast 2-hybrid screens. Briefly, this would involve the fusion of the linker (amino acids 82-219) or C-terminus (amino acids 521-560) region to the GAL4 DNA-binding domain to generate the "bait", which would be used to screen a target library generated from osteogenic cells expressing cellular proteins fused to the GAL4 activation domain. Potential difficulties due to the interactions with endogenous yeast proteins are not anticipated as KRAB/C₂H₂ proteins are exclusive to multicellular organisms and not present in yeast. However, it should be noted that although the linker and/or C-terminus regions are the likely protein-protein interaction regions, the C₂H₂ zinc finger motifs might also be involved in protein interactions.

5A.1. Interaction with Runx2

Runx2 is a strong candidate to be identified as a protein interacting with linker region of AJ18. This hypothesis arises in part from the observation that there is high sequence similarity
within the linker region of AJ18 to an Ets-related protein called Elf-1 (61% similarity between amino-acids 132-165). A 118-amino-acid sequence that encompasses the 34-amino acid region conserved in Elf-1 and AJ18 has been implicated in the interaction between Elf-1 and Runx1/AML1 (Mao et al., 1999). Thus, rather than there being a mutually exclusive binding of AJ18 and Runx2 to the OSE2, the mechanism of suppression of Runx2 transcriptional activation could alternatively involve direct interaction of AJ18 and Runx2, with the consensus elements in polymeric OSE2 aiding the recruitment of various transcription factors. Consistent with Runx2 acting as a context-dependent transcription factor, AJ18 could suppress transcription by competing with a binding partner of Runx2 that induces transcription. Such potential interactions between AJ18 and Runx2 can be studied using immunoadsorption experiments, which are routinely used to provide evidence of specific interaction between proteins. By incubating AJ18 and radiolabelled Runx2, translated in vitro under physiological conditions, the putative complex could be immunoadsorbed to resin-bound, affinity-purified, anti-AJ18 antibodies. Should other proteins be required for the formation of the AJ18-Runx2 complex, the binding could be performed in the presence of nuclear extracts derived from bone cell lines.

5A.2. Determination of Zinc Finger Motifs Involved in DNA Interactions

Although the target detection assay was successfully used to identify a DNA sequence recognized by the zinc finger domain of AJ18, as few as one or two zinc fingers could recognize this target sequence. With 11 C2H2 zinc finger motifs in AJ18, it is probable that DNA recognition sequence is much longer than the 5’-CCACA-3’ identified or that multiple 5’-CCACA-3’ sequences are involved. Notably, 5’-CCACA-3’ sequences were present in the polymeric OSE2 used to assess transcriptional suppression of Runx2 by AJ18. Although a 15
nucleotide target was determined for the KRAB/C2H2 protein, ZBRK1, which has 8 zinc fingers (Zheng et al., 2000), identification of long sequences by target detection assays is normally limited by the exponential increase in the number of variant target DNA sequences needed with the addition of each nucleotide. Consequently, further characterization of the C2H2 zinc finger motifs involved in DNA recognition may best be achieved through the use of deletion mutants. By deleting single, or combinations of zinc fingers using various oligonucleotide primers, the contribution of specific C2H2 zinc fingers to the binding of the 5'-CCACA-3' can be determined and the identification of other binding sequences determined using the target detection assay. Notably, a spontaneously truncated (35 kDa) form of His-tagged AJ18, which theoretically should contain the first three zinc finger motifs, was shown to bind to OSE2 (Fig. 2D). This suggests that the interaction of AJ18 to OSE2 is specifically through these three zinc fingers.

5A.3. Target Genes and Downstream Effects of AJ18

Although AJ18 can recognize the OSE2 (5'-ACCACA-3'), an immediate target gene regulated by AJ18 has yet to be identified. Since the OSE2 sequence is present within the promoters of many bone-related as well as many other genes, identifying potential gene targets for AJ18 will require methodology allowing a general screen for AJ18-regulated genes. Furthermore, the potential for multiple genes to be regulated by AJ18 warrants a more detailed analysis of the downstream effects of AJ18 on osteogenesis. This can also help dissect out regulatory cascades involved in osteogenic differentiation. Although suppression of Runx2-mediated transcription has been shown in a mechanistic model and has been linked to a downstream suppression of alkaline phosphatase, the ultimate effects of AJ18 on osteogenesis are not known. To extend these studies, therefore, the effects of AJ18 stably transfected into an
osteogenic cell line, will be determined. These studies will utilize a clonal rat bone marrow cell line, established in the laboratory from a mixed cell line provided by Dr S. Pitaru (University of Tel Aviv, Israel). These cells undergo spontaneous differentiation and within 14 days form bone-like nodules in vitro. Therefore, using sense and antisense expression vectors to increase and decrease expression, respectively, the effects of changing AJ18 expression levels on the temporal expression of bone-related mRNAs including Runx2, BSP, OC, ALP, COLI, and OPN can be determined. Furthermore, the number, size, and rate of formation of bone-like nodules, representing the number of precursor cells capable of differentiating into bone-forming osteoblasts, can be readily measured.

To identify immediate target genes, AJ18 will be cloned into a Tet-ON gene expression vector (Clontech) in which the expression of AJ18 is regulated by tetracycline. Following isolation of both non-osseous (i.e. C3H10T1/2 or C2C12) and osseous cell lines (i.e. RBMC) stably expressing Tet-AJ18, the effects of AJ18 on gene expression can be analyzed at various time points determined by the addition of tetracycline. Using RNA isolated from cells after 30 min or 3 hours of tetracycline treatment, experiments would initially determine the effects of AJ18 expression on known genes involved in osteogenic differentiation (mentioned above). Then, a broader screen would be attempted using differential display and/or cDNA microarray analyses to identify genes up- or down-regulated in response to AJ18 expression.

5B. Regulation of AJ18 Expression

The promoter sequence of AJ18 is the first promoter for the large KRAB/C2H2 family of genes to be reported. Difficulties in cloning arose due to the unexpected and unusually long 5'
UTR, which may be a characteristic of KRAB/C2H2 proteins, and the reason behind the lack of a reported promoter for this gene family.

5B.1. Identification of Regulators of AJ18

Various consensus DNA-binding elements for transcription factors such as Runx2, NFκB, Ets1, Smads, and Sp1 were identified within the 5'-flanking region of AJ18. Luciferase activities may be measured by co-transfection of these transcription factors alone or in combination, with the AJ18 promoter-luciferase reporter constructs.

5B.1-1. BMP-7 Regulation of AJ18

AJ18 was identified as an immediate-early target gene for BMP-7 in FRCCs. The BMP-7 induction of AJ18 was also shown in C2C12 myogenic cells and in mesangial cells (data not shown and personal communication from Dr. Mark DeCasteker, Vanderbilt University, respectively). Although the primordial function of BMP-7 and other BMPs appears to be to induce bone formation, BMP-7, as well as AJ18, are expressed in many tissues and are clearly multifunctional. Although preliminary experiments failed to show effects on AJ18 transcriptional activity with BMP-7 treatment, these experiments were performed using luciferase constructs containing incomplete 5'-UTRs of AJ18. Furthermore, the Smad sites identified in the promoter are Smad 2 and 3 sites, which have been implicated in TGF-β signaling as opposed to BMP-signaling. Sequence analysis of the complete 5'-UTR revealed the presence of a Smad1 consensus binding element (5'-GCCGnCGC-3'; Kusanagi et al., 2000) at nucleotide +2094 of AJ18. Therefore, transcriptional regulation of AJ18 by BMP-7 may occur through elements present within the 5'-UTR of the AJ18 gene.
BMP-7, as well as other factors, may regulate AJ18 through post-transcriptional effects including RNA and protein stability, and protein modifications. Analysis of the sequenced 3'-UTR revealed the presence of a key AU-rich sequence motif (5'-UAUUUAU-3') that has been implicated in mRNA degradation (Zubiaga et al., 1995). Furthermore, analyses of the amino acid sequence of AJ18 showed the presence of a possible PEST protein instability sequence at amino acids 153-169, suggesting that the protein may undergo rapid turnover (Rechsteiner and Rogers, 1996). The presence of 17 putative casein kinase II and 6 putative protein kinase C phosphorylation sites suggests that AJ18 activity may be modulated by phosphorylation.

5B.2. Tissue-specific Regulation

The presence of two OSE2 sites at nucleotide positions -30 and -73 may be important for the 100-fold higher transcription activity in bone cells relative to fibroblast-like cells. To determine the importance of these sites for tissue specificity, mutations can be introduced at the OSE2 sites in plasmids p(-1494/+171) and p(-77/+505) and the transcription activities compared with wild-type plasmids in osseous and nonosseous cells.

5B.3. Regulation of AJ18 Translation

The 5'-UTR of AJ18 is unusually long at 2.3 kb with GC-rich regions, and a putative IRES near the authentic ATG start codon. The minimal promoter, p(77/+171), ligated upstream of the luciferase gene results in high transcription activity. However, the progressive decline of luciferase activities when additional sequence is included downstream into the 5'-UTR [p(-77/+451), p(-77/+505), p(-77/+657) and p(-77/+955)], suggests that this decline may be due to effects on translation rather than transcription. Thus, with an incomplete 5'-UTR present (minus
IRES) the chimeric AJ18-luciferase mRNA may be translated via the ribosome scanning mechanism. However, the inclusion of the full 2.3 kb 5'-UTR would restore luciferase activity as translation initiation occurs through the IRES rather than through the ribosome scanning mechanism. The following experiments could test this hypothesis. Firstly, construct p(-77/+2300) upstream of the luciferase gene would be prepared and its luciferase activity measured. Based on the hypothesis, luciferase activity should be restored with the presence of the putative IRES. Secondly, to determine that the length of the 5'-UTR affects translation and not transcription, total RNA prepared from cells transiently transfected with the various luciferase reporter constructs would be analyzed on Northern blots using a probe specific to the luciferase mRNA. If transcription is not affected, there should not be a significant difference in the mRNA expression levels between the chimeric luciferase constructs. Thirdly, to determine whether translation is affected, the mRNAs of the above constructs can be synthesized and in vitro translation experiments performed to determine the synthesis of luciferase by incorporating radiolabel or performing a luciferase assay.

To determine the importance of the putative IRES, mutations can be introduced at this site in the various plasmids and the experiments described above repeated. Additionally, the putative IRES can be inserted into a bicistronic construct and the ability of IRES to initiate translation analyzed. This could be achieved by constructing a bicistronic gene comprising the chloramphenicol acetyltransferase (CAT) and luciferase (LUC) genes separated by the putative AJ18 IRES. Thus, CAT would be translated via the ribosome-scanning mechanism while LUC would be translated via the IRES, if it is indeed functional; the activities of CAT and LUC can be readily determined.
5C. Transgenic Mouse Models

To determine the functionality of AJ18 in the context of general and specific tissue development in vivo, transgenic approaches can be used. General effects can be determined by gene knockouts, through the use of targeted deletion/replacement, while tissues specific effects can be achieved by creating tissue-specific promoter-driven constructs that will express wild-type or mutant AJ18. The first steps for preparing knockout mice have been completed. A mouse AJ18 genomic clone was isolated from a genomic library screen (Chapter 4), and constructs prepared. Embryonic stem (ES) cells are currently being screened for cells in which homologous recombination of AJ18 has been achieved. As AJ18 is a potential target gene for BMP-7, and BMP-7 and AJ18 have similar expression profiles (Chapter 4), it is anticipated that AJ18 knockout mice will show a similar phenotype as the BMP-7 knockout mice. This, in conjunction with promoter analyses described above, would provide strong evidence that AJ18 is a target gene of BMP-7. In addition to the knockout experiments, tissue-specific effects of AJ18 can be analyzed using the mouse Runx2 promoter (Accession no. AF155361) to direct the expression to osteogenic systems. Over- and under-expression of AJ18 can be achieved using sense and antisense cDNAs driven by the Runx2 promoter. Alternatively, expressing a dominant-negative mutant of AJ18, in which the KRAB domain and or the DNA-binding region is removed, could be used to assess the functionality of AJ18. Depending upon whether AJ18 promotes or suppresses bone formation, osteopetrotic or osteoporotic phenotypes, respectively, would be expected in these studies. Thus, the combination of AJ18 ablation and misexpression studies should greatly assist in deciphering the function of AJ18 in vivo.

In summary, these studies have characterized a novel zinc finger transcription factor that is expressed during tissue development in a pattern that is consistent with its potential role in
mediating effects of BMP-7 in epithelial-mesenchymal interactions in general, and specifically during osteogenic differentiation. Future studies should further establish the relationship between BMP-7 and downstream target genes involved in osteogenic differentiation, providing further insights into molecular mechanisms that control osteogenesis.
References


159


Conway, L., and Wickens, M. (1987) Analysis of mRNA 3' end formation by modification interference: the only modifications which prevent processing lie in AAUAAA and the poly(A) site. EBMO J. 6, 4177-84.


Grimsrud, C. D., Romano, P. R., D'Souza, M., Puzas, J. E., Reynolds, P. R., Rosier, R. N., and O'Keefe, R. J. (1999) BMP-6 is an autocrine stimulator of chondrocyte differentiation. J. Bone Miner. Res. 14, 475-82.


Zbang, H., and Bradley, A. (1996) Mice deficient for BMP2 are nonviable and have defects in amnion/chorion and cardiac development. Development 122, 2977-86.


