SCREENING OF POTENTIAL UPSTREAM REGULATORS AND IDENTIFICATION OF DNA BINDING SITES FOR THE TOOTH TRANSCRIPTION FACTOR KROX-26

by

WILLIAM J. TEO
2001

A thesis submitted in conformity with the requirements for the Degree of Master of Science, School of Graduate Studies and Faculty of Dentistry, University of Toronto.

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Abstract

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Krox-26 is a novel 25.8 kDa protein comprising five C2H2 zinc fingers. Previous temporo-spatial data from in-situ hybridization and Northern blot analyses suggest that the expression of Krox-26 mRNA coincides with that of Msx-1, BMP-2, BMP-4, FGF-4, and FGF-8 during early tooth development. To screen for Krox-26 regulatory molecules, semi-quantitative RT-PCR, Northern blot, and in-situ hybridization analyses were performed on Dental Papilla Mesenchymal cells, mouse E10.5 mandibles, and mouse E15 molar tooth germ cultures, treated with BMP-2, BMP-4, FGF-4, or FGF-8. Results from these analyses failed to show BMPs or FGFs regulation on Krox-26 mRNA expression in these systems. As a putative zinc finger transcription factor, Krox-26 is anticipated to bind specific DNA sequences through its zinc finger motifs. To identify a target DNA sequence for Krox-26, a recombinant 6xHis-tagged Krox-26 protein was expressed and purified for Target Detection Assay. The resulting Krox-26 target DNA sequence of 5'-cAATg-3' was identified.
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<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>μl</td>
<td>microliter</td>
</tr>
<tr>
<td>APS</td>
<td>ammonium persulfate</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>DEPC</td>
<td>diethylpyrocarbonate</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>dsDNA</td>
<td>double stranded DNA</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>E</td>
<td>mouse embryonic day</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EMSA</td>
<td>electrophoretic mobility shift assay</td>
</tr>
<tr>
<td>FCS</td>
<td>fetal calf serum</td>
</tr>
<tr>
<td>FPLC</td>
<td>fast protein liquid chromatography</td>
</tr>
<tr>
<td>h</td>
<td>hour(s)</td>
</tr>
<tr>
<td>HG-DME</td>
<td>Dulbecco’s Minimum Eagle (high glucose)</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl-β-D-thiogalactoside</td>
</tr>
<tr>
<td>kb</td>
<td>kilobase pairs</td>
</tr>
<tr>
<td>kDa</td>
<td>kiloDalton</td>
</tr>
<tr>
<td>mA</td>
<td>milliampere</td>
</tr>
<tr>
<td>min</td>
<td>minute(s)</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>Ni-NTA</td>
<td>nickel-nitrilotriacetic acid</td>
</tr>
<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered solution</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>rpm</td>
<td>revolution per minute</td>
</tr>
<tr>
<td>s</td>
<td>second(s)</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecysulphate</td>
</tr>
<tr>
<td>SSC</td>
<td>standard saline-citrate</td>
</tr>
<tr>
<td>TBE</td>
<td>tris, boric acid, and EDTA</td>
</tr>
<tr>
<td>TDA</td>
<td>target detection assay</td>
</tr>
<tr>
<td>TE</td>
<td>Tris-Cl, EDTA</td>
</tr>
<tr>
<td>TEMED</td>
<td>N, N, N', N'-tetramethylethlenediamine</td>
</tr>
<tr>
<td>Tris</td>
<td>tris(hydroxymethyl)aminomethane</td>
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Preface

Tooth development has been studied extensively over the past four decades. Early experiments on tooth development relied entirely on techniques such as tissue grafting, tissue recombination, and cell and organ culture. Much of the earlier work provided a foundation for an understanding that tooth development begins with epithelial-mesenchymal tissue interactions, followed by morphogenesis, differentiation, and growth. More recently, the development of molecular techniques has led to the discovery of various growth factors, signaling molecules, and transcription factors that regulate tooth development. From their initial discoveries, subsequent studies have focused on the characterization of these molecules.

The expression of a novel zinc finger transcription factor, Krox-26, has recently been reported in developing rodent teeth. As a putative transcription factor, Krox-26 is expected to regulate downstream gene transcription. Conversely, various upstream signaling molecules will regulate Krox-26 expression. Thus, this thesis attempts to place Krox-26 within the cascade of molecular interactions during tooth development.

Chapter 1 of this thesis will briefly introduce the general principle of organ development, followed by the origin of odontogenic cells, the development of tooth at cellular and molecular levels, and the details on the discovery of Krox-26. Chapter 2 will focus on screening of possible signaling molecules affecting Krox-26 mRNA expression. Chapter 3 describes studies designed to identify the target DNA sequence recognized by the Krox-26 protein.
Chapter 1: Introduction
1.1 Principles of Development

The process of development encompasses three objectives: 1) generation of cellular organization, 2) production of cellular diversification, and 3) maintenance of growth to ensure reproduction. Cellular organization into tissues and organs is accomplished by morphogenesis while cellular diversification is achieved by differentiation (Gilbert, 1994). The dawn of animal development is marked by the fusion of gametes from the sperm and the egg, forming a zygote. Subsequent events and stages leading to the formation of organs are known as embryogenesis (Carlson, 1988). Four cellular events form the basis of embryogenesis – blastulation, gastrulation, organogenesis, and gametogenesis. Following fertilization, the zygote is rapidly cleaved into smaller cells, called blastomeres. Collectively, at the end of cleavage blastomeres give rise to a hollow sphere called the blastula, a process known as blastulation. Subsequently, during gastrulation cell division slows down and through a process called primary induction, the blastomeres undergo rearrangements resulting in the establishment of three germ layers called the ectoderm, mesoderm, and endoderm. The ectoderm refers to the outer layer of cells which give rise to epidermis and nervous system. The mesoderm consists of the middle layer of cells that are responsible for the formation of blood cells, heart, kidneys, gonads, and connective tissues such as bone, muscles, and tendons. The innermost cell layer, the endoderm, produces the lining of the digestive tube and its associated organs such as pancreas and liver (Gilbert, 1994). Following primary induction, organogenesis takes place via secondary induction, whereby the cells of the distinct germ layers interact and form specialized organs such as limbs, eyes, and teeth (Gilbert, 1994).
1.2 Tooth Development

1.2.1 The Origin of Odontogenic Cells

Vertebrate teeth are organs that develop from secondary induction between the oral epithelium and its adjacent dental mesenchyme (Mina and Kollar, 1987; Lumsden, 1988; Thesleff and Sharpe, 1997). Several studies have concluded that mesenchymal cells beneath the epithelium of the first branchial arch of mammalian embryos are of neural crest origin (Osumi-Yamashita et al., 1994). The oral epithelium, however, originates from the stomodeal or pharyngeal epithelium of the developing embryo (Thesleff and Sharpe, 1997; Kettunen et al., 2000). Once the migration of neural crest cells to the first brachial arch is complete, a group of neural crest cells interacts with the overlying oral epithelium promoting a band of epithelial cells to invaginate the mesodermal layer, forming the dental ledge or the dental lamina. Subsequently, a series of additional interactions between the dental mesenchyme and the dental epithelium lead to the formation of teeth (Ruch, 1985). The architecture of every mature tooth consists of a core, calcified material called dentine and an outer, harder material called enamel. Dentine is produced by mesenchymal-derived odontoblasts while enamel is formed by epithelial-derived ameloblasts (Carlson, 1988).

1.2.2 Cellular Events in Tooth Morphogenesis

Tooth development can be divided into different stages based on morphological changes (Fig. 1-1). The first distinct morphological feature indicative of tooth formation in mice is the formation of a dental lamina at embryonic day 10-11 (E10-11), caused by the thickening of the dental epithelium. As the dental lamina grows, the epithelium invaginating the underlying mesenchyme forms an epithelial bud, which is known as the
Figure 1-1. Tooth developmental stages.
Temporo-spatial expression of BMP-2, BMP-4, FGF-4, FGF-8, Msx-1, and Krox-26 are shown localized within different cell types of developing tooth.

oe - oral epithelium; de - dental epithelium; bm - basement membrane; dm - dental mesenchyme; sr - stellate reticulum; ek - enamel knot; eoe - outer enamel epithelium; lee - inner enamel epithelium; dp - dental papilla; ds - dental sac; si - stratum intermedium; ob - odontoblasts; ab - ameloblast; en - enamel; dn - dentin; pdn - predentin; cm - cementum; cb - cementoblasts; pl - periodontal ligament
bud stage (E11-13). Subsequently, the mesenchymal cells condense around the bud and form the dental papilla, which gives rise to both the dental pulp and dentin-secreting odontoblasts (Peters and Balling, 1999). It is believed that two cell adhesion molecules, the heparan sulfate proteoglycan syndecan-1 and the matrix protein tenascin, are involved in mesenchymal cell condensation (Thesleff et al., 1995). At the cap stage (E13-15), the enamel knot appears within the epithelial compartment. The bell stage (E15 to E17) is characterized by rapid cell proliferation, which is then followed by the differentiation stage (E17 to postnatal day 1, P1), and tooth crown formation (P1 onwards; Kettunen and Thesleff, 1998). Various molecular factors including BMP-2, BMP-4, FGF-4, FGF-8, Msx-1, and Krox-26, which are expressed in a temporo-spatial manner during tooth formation, are discussed below.

The reciprocal interaction between the oral epithelium and its adjacent mesenchyme demonstrates that inductive and competence capacities of each tissue are temporally and spatially restricted. Mina and Kollar (1987) used tissue recombination experiments to show that non-dental, neural crest-derived mesenchyme from mice could be induced to express the tooth phenotype by oral epithelium isolated from the mandibular arch between E9.0 and E11.5. Thus, the so-called odontogenic potential resides in the epithelium layer at this stage. However, after E11.5, the mesenchyme acquires odontogenic potential and the epithelium loses its inductive capability. Thus, mesenchyme at this stage, combined with a non-dental epithelium, can induce tooth formation (Vahtokari et al., 1996).

Information for the development of individual teeth, molars or incisors, resides in the mesenchyme. Through similar tissue recombination experiments as mentioned above,
the mesenchyme from a molar tooth bud in combination with epithelium from an incisor resulted in the development of a molar (Kollar and Baird, 1969). Conversely, an incisor develops when incisor mesenchyme is combined with molar epithelium. These two experiments demonstrate the importance of the mesenchyme in determining whether a tooth becomes a molar or an incisor.

Around E14.5, the odontogenic potential is switched to a specific group of signaling epithelial cells that form a structure known as the enamel knot (Vaahtokari et al., 1996). Initially identified in histological sections of developing teeth at the cap stage, the enamel knot was described as a distinct morphological structure of unknown function. This structure is now recognized as the organizing center of tooth crown morphogenesis where cell apoptosis and proliferation occur simultaneously to control the growth and formation of molar tooth cusps (Jernvall et al., 1994; Vaahtokari et al., 1996).

The regulation of inductive and competence properties of cells during every stage of tooth development are attributed to various signaling molecules present within the dental epithelium and mesenchyme (Dassule and McMahon, 1998). Some of these molecules are reviewed below.

1.2.3 Molecular Events in Tooth Morphogenesis

In the effort to gain insight into the molecular mechanisms of tooth development, many studies have focused on the analysis of expression patterns of genes encoding extracellular signaling molecules, cell surface molecules, and transcription factors as potential regulators of tooth morphogenesis (Thesleff et al., 1995; Vainio et al., 1993; Hogan et al., 1994). In-situ hybridization analyses of these potential regulators have shown a temporo-spatial expression of mRNA by the epithelial or mesenchymal tissue.
(Thesleff and Sharpe, 1997). The localization of specific signals within these tissue layers indicates a potential function for expressed genes that participate in the regulatory and growth stages in tooth development. Some of the well-studied signaling molecules and transcription factors that are essential to the construction of teeth are discussed below.

**a. Signaling Molecules**

**i) Bone Morphogenetic Proteins (BMPs)**

The bone morphogenetic proteins (BMPs) form one of the largest families of signaling molecules involved in embryonic development and tissue regeneration. Originally, BMPs were discovered by their ability to induce ectopic bone formation when implanted into muscle in vivo (Urist, 1965). Cloning and sequencing of the first BMPs revealed these factors to be a subclass of the transforming growth factor β (TGF-β) superfamily (Hogan, 1996). In addition to inducing ectopic bone formation, BMPs also have other roles throughout embryogenesis. For example, BMPs mediate the inductive interactions between the epithelial and the mesenchymal cells and regulate cell proliferation and apoptosis. More specifically, in organs such as limbs and teeth, BMPs are expressed in small groups of cells known as organizing centers (Hogan, 1996). BMPs have been fairly conserved throughout evolution and related proteins to mammalian BMPs are present in *Xenopus* and *Drosophila* (Kingsley, 1994).

Several members of the BMP family, particularly BMP-2, BMP-4, and BMP-7, are expressed in the developing molar tooth germ. The expression of BMP-2 and BMP-4 at various developmental stages during tooth formation in mice is summarized in Figure 1-1. BMP-4 and BMP-7 are produced by both dental epithelium and dental mesenchyme while BMP-2 is found only in the dental epithelium (Maas and Bei, 1997). Mice deficient
for these BMPs have major developmental defects due to inductive failures during embryogenesis (Zhang and Bradley, 1996; Winnier et al., 1995). Both BMP-2 and BMP-4 deficient mice die prior to tooth formation while the development of teeth in BMP-7 knockout mice appeared normal. These analyses show that certain BMPs play a pivotal role in the mediation of ectodermal-mesodermal signaling.

BMP-2 expression appears in the molar epithelium of mice at the late dental lamina stage (E11; Åberg et al., 1997). At the early bud stage around E12, BMP-2 is found throughout the bud. At E13, or late bud stage, the expression of BMP-2 is localized to the tip of the tooth bud. Later at E14, BMP-2 expression is restricted to the enamel knot. During the late cap stage (E15 to E15.5), BMP-2 spreads to the neighboring inner dental epithelium as the enamel knot starts to disappear. By early bell stage around E16, the expression of BMP-2 shifts to the central cells of the dental papilla mesenchyme. Subsequently, BMP-2 expression appears in the odontoblasts in post-natal day 1 (P1) mice, when predentine secretion begins. In the subsequent days, BMP-2 is only found in functional ameloblasts as it disappears from the most advanced odontoblasts in the cuspal region. At post-natal day 4 (P4), transcripts of BMP-2 are only detected in the apical portion of the crown where differentiation continues (Åberg et al., 1997). In addition, BMP-2 is also expressed between E11 and E13 in the epithelium of diastema primordia, regions devoid of teeth, which form transiently in mice (Maas and Bei, 1997).

Similarly, BMP-4 expression first appears in the oral epithelium during the formation of dental lamina around E11 (Maas and Bei, 1997; Åberg et al., 1997). At E12, BMP-4 transcripts are found in both the dental epithelium and mesenchyme. During the bud stage from E12 to E13, BMP-4 expression localizes to the condensed dental
mesenchyme only (Vainio et al., 1993). This shift of BMP-4 expression coincides with the shift in odontogenic potential from dental epithelial to dental mesenchyme that was described by Mina and Kollar (1987) in tissue recombination experiments. In the dental mesenchyme, BMP-4 is found to induce and maintain its own expression. Thesleff and colleagues (1995) showed that agarose beads, soaked in recombinant BMP-4 and implanted into isolated E11.5 dental mesenchyme, induced BMP-4 expression and this auto-induction spread throughout the mesenchyme through a series of cell-cell interactions (Vainio et al., 1993). By the cap stage (E14-E15), the expression of BMP-4 remains intense in the dental papilla mesenchyme and it reappears in the epithelium of the enamel knot (Åberg et al., 1997). With the disappearance of the enamel knot during the late cap and bell stage (E15-E19), BMP-4 expression appears stronger in the cuspal region of the dental papilla. Between P1 and P4, BMP-4 transcripts can be detected in both the ameloblast and odontoblast layers. As development advances, BMP-4 expression decreases in the odontoblasts but persists in the ameloblast cell layer (Åberg et al., 1997). In the diastema regions between E11 and E13, the expression of BMP-4 is confined to the dental mesenchyme, as opposed to the dental epithelium for BMP-2 (Maas and Bei, 1997).

Of special interest is the expression of both BMP-2 and BMP-4 in the enamel knot, since this is considered the organizing center for tooth differentiation and growth. The enamel knot controls tooth shape by balancing cell proliferation with apoptosis. In this regard, BMP-4 also plays a role in regulating apoptosis in the rhombomeres and interdigital mesenchyme (Graham et al., 1994; Zou and Niswander, 1996).
**ii) Fibroblasts Growth Factors (FGFs)**

Fibroblasts growth factors (FGFs) comprise a group of heparin binding growth factors that are known to regulate multiple cellular events during development and promote the growth and differentiation of mesodermal, neuroectodermal, and ectodermal cells in vitro (Wilkie et al., 1995). Roles for FGFs as inductive signals were identified in mesoderm formation in *Xenopus* embryos (Slack, 1994), early morphogenesis in mammals (Feldman et al., 1995), and organ development in *Drosophila* (Sutherland et al., 1996). Currently, expression of FGF-3, FGF-4, FGF-7, FGF-8, FGF-9, and FGF-10 has been reported in the developing tooth (Wilkinson et al., 1989; Niswander and Martin, 1992; Neubuser et al., 1997; Kettunen and Thesleff, 1998; Kettunen et al., 2000). Of particular interest, both FGF-4 and FGF-8 have been shown to be a part of complex signaling networks during organ development such as limbs and midbrain morphogenesis (Cohn et al., 1995; Tickle, 1995; Crossley et al., 1996). During tooth development, FGF-4 has been found to stimulate cell proliferation in the dental epithelium and mesenchyme (Jernvall et al., 1994). The expression of FGF-4 and FGF-8 at various developmental stages during tooth formation is shown in Figure 1-1.

Prior to the morphological appearance of molar teeth in mice at E10, both FGF-8 and FGF-9, but not FGF-4, are expressed in the oral epithelium. At the initiation stage, E11, transcripts of both FGF-8 and FGF-9 are found localized in the thickened presumptive dental epithelium, with FGF-8 mRNAs present in higher abundance (Kettunen and Thesleff, 1998). During the bud stage, E12-13, the expression of FGF-8 is present mainly in the distal part of the tooth bud, while at the late bud stage, FGF-8 expression is downregulated. This coincides with the shift of odontogenic potential from
dental epithelium to dental mesenchyme (Mina and Kollar, 1987; Lumsden, 1988). In comparison, the expression of both FGF-9 and FGF-4 are upregulated in the primary enamel knot at E13 and remain restricted to this area until the cap stage at E14 (Kettunen and Thesleff, 1998), at which time the expression of FGF-8 is undetectable. At the early bell stage, E15, expression of FGF-9 transcripts spreads from within the inner enamel epithelium into the surrounding cells while FGF-4 expression persists within the primary enamel knot. During the late bell stage, at E16-18, expression of FGF-4 extends to the secondary enamel knots (Kettunen and Thesleff, 1998).

Since in-situ hybridization data show that the expression of FGF-4 is localized within the enamel knots, it has been suggested that FGF-4 regulates tooth cusp formation during molar development (Jernvall et al., 1994; Vaahkari et al., 1996; Kettunen and Thesleff, 1998). Also it has been suggested that FGF-4 regulates tooth cusp formation by inhibiting untimely apoptosis in dental epithelium (Vaahkari et al., 1996; Jernvall et al., 1994; Jernvall et al., 1998). In the incisor tooth germ, similar expression patterns for FGF-4, FGF-8, and FGF-9 mRNAs are observed. Since there are no secondary enamel knots in the developing incisor, the expression of FGF-4 disappears after the cap stage, E14 (Kettunen and Thesleff, 1998).

**iii) Wnt**

Wnt proteins are another example of signaling molecules that play a significant role in developmental processes during embryonic induction, generation of cell polarity, and specification of cell fate. Mutations in Wnt show remarkable phenotypic defects in the mouse, *Caenorhabditis elegans*, and *Drosophila* (Cadigan and Nusse, 1997). Wnt is homologous to Wnt-1, originally known as int-1 in mice and wingless (wg) in *Drosophila*.
musse and Varmus, 1982; Cabrera et al. 1987). Wg is the best-understood Wnt family member, and defects in wg signaling result in a wingless phenotype in *Drosophila* (Wieschaus and Riggleman, 1987; Perrimon et al. 1989). In mice, mutations in Wnt-7a resulted in ventralized limbs (Parr and McMahon, 1995) and mutations in Wnt-4 resulted in the absence of kidneys (Starks et al., 1994). Most of these defects suggest that Wnt functions in the mesenchymal-epithelial transitions during organ formation.

During tooth development in mice, several Wnt family members have been identified: Wnt-3, Wnt-4, Wnt-5a, Wnt-6, Wnt-7b, Wnt-10a, and Wnt-10b (Sarkar and Sharpe, 1999). Of these, Wnt10a and Wnt10b are present in the molar dental epithelium while Wnt-3 and Wnt-7b localize within the oral epithelium, during the initiation stage at E11.5 (Dassule and McMahon, 1998; Sarkar and Sharpe, 1999). Wnt-6 expression at this time can be detected in both the oral and dental epithelium while Wnt-5a transcripts are widespread in the mandibular mesenchyme (Sarkar and Sharpe, 1999). At the bud stage (E13.5), Wnt-6, Wnt-7b and Wnt-4 are expressed throughout the molar tooth bud epithelium while both Wnt-10a and Wnt-10b transcripts localize in the tip of budding epithelium. Expression of Wnt-5a begins to localize around the tooth bud mesenchyme while Wnt-3 mRNA is not detectable at this stage. At E14.5, cap stage, Wnt-3 expression resides exclusively in the primary molar enamel knots while the expression of Wnt-6, Wnt-10a and Wnt-10b are present stronger in these structures than the enamel epithelium (Dassule and McMahon, 1998; Sarkar and Sharpe, 1999). Both Wnt-7b and Wnt-4 are absent from the enamel knots but show the same level of expression throughout the entire enamel epithelium. In the mesenchyme, expression of Wnt-5a at the cap stage is localized at the tip of dental papilla and around the dental follicle (Sarkar and Sharpe, 1999).
early bell stage (E15.5), both Wnt-3 and Wnt10-b are absent from external enamel epithelium but are found in the internal enamel epithelium. Wnt-6, however, is found in both the internal and external enamel epithelium. At this time, Wnt-5a transcripts are also abundant around the dental follicle and papilla, but remain absent from the tooth germ epithelium. Both Wnt-4 and Wnt-7b are expressed at low levels throughout the epithelium of the tooth germ (Sarkar and Sharpe, 1999).

iv) Hedgehog

Members of the Hedgehog family of signaling molecules are found to mediate the patterning processes during invertebrate and vertebrate development. Various types of rigorously studied hedgehog proteins include the Drosophila hedgehog (hh), mouse Sonic Hedgehog (Shh), zebrafish Shh, and zebrafish Tiggy-winkle Hedgehog (twhh). The Drosophila hh gene is responsible for the establishment of body segmentation polarity and imaginal disc patterning, where future wings, legs, and eyes develop. In both the mouse and the zebrafish, Shh is expressed in different organizing centers that regulate embryonic polarity (Hammerschmidt et al., 1997). In the mouse, Shh influences the development of the somites, regulates anterior-posterior polarity, and controls the ventralization of the central nervous system (Johnson and Tabin, 1995; Riddle et al., 1993).

In the developing tooth of a mouse, Shh is expressed in the epithelium thickening of the early dental lamina stage, E11.0 (Bitgood and McMahon, 1995). However, its expression is not uniform throughout the epithelium, but rather appears stronger in the mesial part of the tooth. This expression pattern within the epithelium persists well into the bud and cap stages. By late cap stage, Shh transcripts can be seen in the epithelium
across the circular axis that runs parallel to the mandibular arch, with strongest expression on the mesial aspects of the developing cusps (Bitgood and McMahon, 1995). At the bell stage, Shh mRNAs localize within the differentiating ameloblasts.

Shh expression during tooth development closely resembles that of the BMP-2 in the epithelium, but shows proximal apposition to the BMP-4 expression in the mesenchyme. This correlation of Shh expression with that of the BMPs suggests that Shh participates in the epithelial-mesenchymal interactions, in addition to its possible role in regulating the polarity of tooth cusps (Bitgood and McMahon, 1995).

b. Transcription Factors

During tooth development, various structural and signaling molecules have been found to influence epithelial and mesenchymal interactions, morphogenesis, and differentiation. These molecules, which can affect the expression of downstream genes, are themselves regulated by transcription factors that can activate or repress gene expression. Transcription factors are proteins that bind to specific sites in the gene promoter. The complex formed between a transcription factor and target DNA acts as a switch that allows (or prevents) the transcription of the gene into RNA that is catalyzed by an enzyme called RNA polymerase II. Sometimes RNA itself is the final product but in most cases, it serves as a template for synthesis of a specific protein. In turn, the protein may be of structural importance or may function as a growth factor, a signaling molecule, or a transcription factor. In general, transcription factors are responsible for gene expression at the right time and place. Transcription factors are grouped into different classes based on their structural motifs: homeodomain, helix-loop-helix, leucine zipper, and C₂H₂ zinc fingers, to name a few.
CzH2 zinc fingers are the most prevalent DNA-binding motifs found in eukaryotic transcription factors (Wolfe et al., 1999). These motifs are commonly found in animals, plants, and fungi, but the number of finger repeats varies among different genes (Bohm et al., 1997; Takatsuji, 1998). The CzH2 (or Krüppel) motif was first observed in the transcription factor IIIA (TFIIIA) of Xenopus laevis, containing tandem arrays of nine CzH2 motifs. Thus far, CzH2 zinc finger containing proteins possess as few as two to as many as 37 zinc fingers (Rhodes and Klug, 1993). In the CzH2 zinc finger proteins, each of the zinc fingers fold in the presence of zinc (Frankel et al., 1987) to form a compact double-stranded antiparallel β-sheet and the α-helix domain (Pavletich et al., 1991). Most of the zinc finger structural stability is provided by a conserved hydrophobic core that flanks the zinc-binding site and by the zinc coordination between the two cysteines at one end of the β-sheet and two histidines at the C-terminal of the α-helix (Fig. 3-1). In the absence of zinc, the fingers are unfolded (Frankel et al., 1987) and substituting a residue besides cysteine or histidine at one of the ligand positions results in a loss of function (Cook et al., 1994; Thukral et al., 1991). X-ray crystallography analyses on the transcription factor Zif268 (Egr1,Krox-24), which contains three CzH2 zinc fingers, revealed that the zinc finger region curls around the major groove of DNA helix (Pavletich and Pabo, 1991). 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 first residue of the α-helix. This interaction is facilitated through formation of hydrogen bonds and Van der Waals forces (Klug, 1999).

Although CzH2 zinc finger containing proteins are the most abundant family of transcription factors to date, no family member has been identified or demonstrated to
play a role in tooth formation. However, several transcription factors containing other motifs besides C2H2 zinc fingers, have been identified by their response to signaling molecules during tooth development, or by the tooth-specific phenotypes caused by their targeted inactivation. Some of these transcription factors are discussed below.

**i) Lymphoid enhancer-binding factor 1 (Lef1)**

In contrast to the BMP and FGF signaling, nothing is known about any transcription factors that mediate the epithelial-mesenchymal interactions at the late bud or early cap stage of tooth development (Peters and Balling, 1999). One candidate transcription factor that may play a role is lymphoid enhancer-binding factor 1 (Lef1).

Lef1 is a member of the high mobility group (HMG) family of proteins (Kratochwil et al., 1996). It is a cell type-specific transcription factor found in lymphocytes of the adult mouse, and in the neural crest, tooth germs, mesencephalon, whisker follicles, and other sites during embryogenesis (Travis et al., 1991; Waterman et al., 1991; Zhou et al., 1995). Mice, deficient in Lef1, were found with tooth development arrest prior to the formation of a mesenchymal dental papilla at E13, the bud stage. In general, Lef1 was found to be necessary for ectodermal-mesenchymal tissue interactions (van Genderen et al., 1994).

The expression of Lef1 begins as early as E10 in the mouse. Between E10 and E11, Lef1 is strongly expressed in the thickened oral epithelium (Kratochwil et al., 1996). At E12, the bud stage, the expression of Lef1 transcripts shifts to the condensing mesenchyme surrounding the invaginated epithelial tooth bud. The shift at this time coincides with the shift in odontogenic potential and BMP-4 expression from the dental epithelium to dental mesenchyme (Mina and Kollar, 1987; Vainio et al., 1993). At E13,
both the mesenchyme and epithelium express Lef1 mRNA around the tooth bud. During the subsequent cap and bell stages (E14-E16), Lef1 expression persists in both tissues, including the dental papilla mesenchyme, preameloblasts, and predontoblasts (Kratochwil et al., 1996).

A previous report suggests that BMP-4 regulates the expression of Lef1 (Kratochwil et al., 1996). Implanted agarose beads soaked with recombinant BMP-4 induced Lef1 expression in E11 dental mesenchyme culture, although Lef1 transcripts were not detectable in E11 dental mesenchyme treated with bovine serum albumin (BSA) coated beads. This data suggests that Lef1 acts downstream of BMP-4 (Kratochwil et al., 1996).

**ii) Msx**

The Msx genes, members of the homeobox gene family, appear to play a critical role in the specification of neural crest and tooth development (Sharpe, 1995). Homeobox genes contain sequences that are related to *Drosophila* muscle segment homeobox (msh) (Sharpe, 1995). Both Msx-1 and Msx-2 are expressed in either neural crest cells or orofacial structures (Sharpe, 1995).

In tooth development, Msx-1 is expressed strongly and exclusively in the dental mesenchyme throughout the bud, cap, and bell stages of odontogenesis (MacKenzie et al., 1991). Similarly, Msx-2 expression is found beneath the dental lamina at E11.5 and later in the dental papilla mesenchyme and in the enamel knot up to late cap and bell stages. As ameloblast and odontoblast differentiation proceeds, expression of Msx-2 disappears (MacKenzie et al., 1992). Mice deficient in Msx-1 exhibit developmental
arrest at the bud stage of molar teeth and with a complete absence of incisors (Satokata and Maas, 1994).

Signaling by BMPs affects gene transcription and culminates in alterations of gene expression. BMP-4 can stimulate the expression of Msx-1 and Msx-2 in the dental mesenchyme (Vainio et al., 1993). It has been shown that the expression of BMP-4 is reduced in Msx-1 mutant tooth mesenchyme but is preserved in Msx-1 mutant epithelium (Bei et al., 1996; Chen et al., 1996). This suggests that Msx-1 is essential for the expression of BMP-4 in the dental mesenchyme and that Msx-1 functions upstream of BMP-4 in the dental mesenchyme. However, in the epithelium, BMP-4 acts upstream of Msx-1 because BMP-4 does not require Msx-1 for its expression (Bei and Maas, 1998). Although BMP-4 has the ability to induce its own expression in the dental mesenchyme (Vainio et al., 1993), it fails to do so in Msx-1 mutant dental mesenchyme. This indicates that a functional positive feedback loop exists between Msx-1 and mesenchymal BMP-4 expression.

One member of the FGFs has been associated with Msx signaling pathway. FGF-8 in the epithelium induces Msx-1 expression in the dental mesenchyme, mimicking the inductive potential of the oral ectoderm. Bead implantation experiments have demonstrated that FGF-8 is able to induce the expression of FGF-3 in wildtype dental mesenchyme in an Msx-1 dependent manner (Chen et al., 1996). FGF-8 has also been proposed to act antagonistically with BMP-4 in specification of tooth initiation sites (Neubüser et al., 1997; Kettunen and Thesleff, 1998). Another member of the FGFs, FGF-4, has been found to stimulate cell proliferation (Jernvall et al., 1994) and to induce syndecan-1 expression in the dental mesenchyme (Chen et al., 1996).
iii) Distalless (Dlx)

Like the Msx genes, Dlx-related genes belong to the family of homeobox transcription factors. Dlx genes have been implicated in neural crest specification and tooth development (Sharpe, 1995). Dlx was originally identified in Drosophila where it is essential for limb development. Currently, there are six Dlx family members (Dlx1, Dlx2, Dlx3, Dlx5, Dlx6, and Dlx7) known in mammals and they have been found to play significant roles during tooth development (Zhao et al., 2000). In Dlx1/Dlx2 double-knockout mice, only maxillary molars were absent while all other teeth were unaffected (Qiu et al., 1997). However, neither tooth morphology or location was affected in mice deficient in Dlx5 (Depew et al., 1999).

In mice, all six Dlx genes are expressed in the mesenchyme of the first branchial arch at E9.5, with Dlx3 additionally expressed in the mandibular epithelium. A similar pattern of expression was observed at E10.5 except that Dlx1 and Dlx2 were expressed in both the maxillary process and the mandibular arch while others were expressed only in the mandibular arch (Zhao et al., 2000). By E11.5, only Dlx2 and Dlx3 were expressed in the early epithelial thickenings of both molars and incisors. At bud stage, the expression of both Dlx2 and Dlx3 persisted within the similar location with additional expression of Dlx2 found in the dental mesenchyme (Zhao et al., 2000). At cap (E14.5) and early bell (E15.5) stages, Dlx1 and Dlx6 were expressed in the dental follicle while Dlx3, Dlx5, and Dlx7 were localized in the dental papilla. None of the Dlx genes was found in the enamel knot of the developing tooth. However, their expression in the dental mesenchyme raises the possibility that these genes may induce or maintain the enamel knots (Zhao et al., 2000).
iv) Pax

The mammalian Pax gene family consists of nine unlinked family members, Pax1 to Pax9. Each member contains a DNA-binding domain known as the paired domain with gene sequence homology to the Drosophila paired gene (Maas and Bei, 1997). Of the nine members, only the closely related Pax1 and Pax9 genes have been implicated in tooth formation. Mutant mice lacking Pax1 show craniofacial and other skeletal defects (Maas and Bei, 1997). Mice deficient of Pax-9 lack all teeth and exhibit a number of other developmental defects (Peters et al., 1998; Peters and Balling, 1999).

Data from in-situ hybridizations showed that prior to any morphological manifestations, the expression of Pax9 is located at the prospective site of tooth formation within the mesenchymal tissue (Neubuser et al., 1997). In mice, the expression levels of Pax9 are detectable in the dental mesenchyme from E10.0 to E16.5, and maximal between E12.5 to E14.5 (Maas and Bei, 1997; Peters and Balling, 1999). Pax9 knockout mice exhibit an arrest in molar tooth development at the bud stage, phenotypically similar to that observed in Msx1 mutants (Maas and Bei, 1997).

v) Barx1

BarH-like homeobox-1 (Barx1) is a member of the subfamily defined by Drosophila BarH-1 and BarH-2 genes, which are determinants of photoreceptor and sensory organ differentiation in fly (Kojima et al., 1991; Higashijima et al., 1992). In contrast to other transcription factors involved in tooth development, Barx1 expression is restricted to anlagen of molars and is absent in developing incisors (Tissier-Seta et al., 1995). In developing molars, expression of Barx1 is detected weakly within the dental mesenchyme from lamina to cap stage. At the bell stage, the expression of Barx1
becomes restricted and reaches a peak in the dental papilla of the molars (Tissier-Seta et al., 1995). The restricted expression of Barx1 in molars suggests that this positional information is responsible for the morphological differences between the molars and incisors.

1.3 Discovery and Initial Characterization of Krox-26

In an attempt to identify novel genes that are involved in tooth formation, Matsuki et al. (1995) prepared a cDNA library from the apical portions of 3-4 week old non-calcified pulp of rat incisors. The apical portions of these incisors were chosen because they contain tissue at a variety of developmental stages. Partial sequences of cDNA clones from the cDNA library were determined. Out of the 400 cDNA clones randomly selected from the library, a clone designated as Y150, containing a partial sequence of a novel zinc finger protein with two cysteine and two histidine residues (C\(_{2}\)H\(_{2}\)) was identified. Subsequently, the homologous full-length cDNA of Y150 from the mouse was cloned and sequenced (Ganss et al., in preparation). The cDNA is 2.4 kb long, encoding a protein of 224 amino acid residues with a predicted molecular mass of 25.8 kDa. The conceptual translation of the cDNA sequence revealed the presence of five consecutive zinc finger motifs (Fig. 1-2), homologous to the \emph{Drosophila} Krüppel segmentation gene, as the only apparent functional domain. Since the zinc finger domains in mouse Y150 contain linker sequences typical for the Krox family of transcription factors, this gene has been re-named Krox-26.

Northern analysis of mRNA from whole mouse embryos of developmental stages E7 to E17, showed the presence of one Krox-26 transcript (2.4 kb) with maximal expression between E11 and E15 (Ganss et al., in preparation). The timing of Krox-26
expression coincides with the major molecular events occurring during tooth morphogenesis and general organ formation. Northern analysis of RNA isolated from specific tissues during mouse embryonic development revealed that Krox-26 mRNA is expressed in several tissues including calvaria, brain, kidneys and salivary glands, but more strongly in the incisor (Lee et al., 1997). In-situ hybridization studies showed that Krox-26 is initially expressed at E12.5 in the dental mesenchyme, underlying the thickening epithelial layer. At E13.5, Krox-26 expression shifts into the inner enamel epithelium and the enamel knot area of developing molars. This pattern of expression persists at E14.5. In 3-week old rats, the expression of the Krox-26 transcript is observed in the stratum intermedium and in the ameloblasts on the enamel side of the apical portion of incisors (Lee et al. 1997). The addition of antisense oligonucleotides to Krox-26 mRNA in mouse molar tooth germ cultures caused a reduction of molar tooth size and general malformations of the tooth structure with an apparent disorganization to the ameloblast layer (Lee et al., 1997). Collectively, these results suggest that Krox-26 is involved in early events of tooth formation as well as proliferation or differentiation of ameloblasts.
Hypothesis and Objectives

Upstream Studies

Temporo-spatial expression data from in-situ hybridization and Northern blot hybridization experiments suggest that Krox-26 expression localizes within the tooth organ and its developmental pattern coincides with that of another transcription factor, Msx-1. As Msx-1 is upregulated by BMP-4 and FGF-8, Krox-26 may also be affected by these factors. In addition, both BMP-2 and FGF-4 are expressed in close proximity to that of the Krox-26 in the dental organ. Therefore, the objective of the upstream studies is to screen potential growth factors such as BMP-2, BMP-4, FGF-4, and FGF-8 in regulating the expression of Krox-26.

Downstream Studies

Krox-26 is a zinc finger transcription factor found in developing tooth. It is anticipated to bind to specific sequences within the promoter of target genes and to regulate the expression of these genes. The objective of downstream studies is to identify potential DNA binding sequences for Krox-26 using the Target Detection Assay. This assay requires a functional Krox-26 protein along with a pool of random sequences of dsDNA.
Chapter 2: Screening of Potential Upstream Regulators of Krox-26.

All experiments in this chapter were performed by William Teo except for the RT-PCR on DPM cells grown at high serum conditions, which was performed by Tiffany Poon (section 2.2.1a) and the whole mount in-situ hybridization on bead implanted mouse E10.5 mandibles, which was performed by Hong Hong Chen and Dr. Bernhard Ganss (sections 2.2.5b).
2.1 Introduction

Tooth development involves a cascade of coordinated events that begins with interaction between the dental epithelium and dental mesenchyme. However, only recent studies have begun to investigate the molecular basis of tooth initiation, morphogenesis, and differentiation. Currently, several growth factors, signaling molecules, and transcription factors have been identified during the process of tooth development. Of these, Bone Morphogenetic Protein 2 (BMP-2), BMP-4, Fibroblast Growth Factor 4 (FGF-4), and FGF-8 are well-characterized and studies of their temporo-spatial expression during the growth and differentiation of a tooth organ suggest synergistic functions (Thesleff et al., 1995). Expression of both BMP-2 and BMP-4, which are members of the transforming growth factor-β (TGF-β) superfamily of secreted cytokines, is associated with epithelial-mesenchymal interactions (Vainio et al., 1993). In vitro studies have shown that BMP-2 and BMP-4 in the epithelium regulate the expression of genes such as the homeobox-containing transcription factors Msx-1 and Msx-2 in dental mesenchyme (Vainio et al., 1993; Thesleff et al., 1995). Notably, when BMP-coated beads were placed on dental mesenchyme, their effects were similar to that of dental epithelium, indicating that these molecules mediate the major interactions with the underlying mesenchyme.

The family of Fibroblast Growth Factors (FGFs), and specifically FGF-4 and FGF-8, are another group of molecules associated with epithelial-mesenchymal interactions. Both FGF-4 and FGF-8 are epithelial signals mediating tissue interactions during tooth development and their functions appear, in part, to be redundant (Kettunen and Thesleff, 1998). In vitro experiments showed that FGF-4 stimulates cell proliferation
in the dental epithelium and mesenchyme. In addition, it has also been suggested that FGF-4 regulates the formation of the tooth cusp during molar tooth development (Jernvall et al., 1994; Niswander and Martin, 1992).

Krox-26, a novel zinc finger transcription factor, shows a temporo-spatial expression pattern similar to Msx-1, until the bud stage of tooth development (Fig. 1-1). In addition, the expression of BMP-2, BMP-4, FGF-4, and FGF-8 occurs in close proximity to Krox-26 during the early stages of tooth development (Fig. 1-1). Some studies have suggested that BMP-2, BMP-4, FGF-4 and FGF-8 regulate Msx-1 expression (Vainio, 1993; Neubüser et al., 1997; Kettunen and Thesleff, 1998).

In an initial screen to identify upstream regulators of Krox-26, the effects of BMPs and FGFs on the expression of Krox-26 mRNA were studied. Various cell and organ culture systems, such as Dental Papilla Mesenchymal (DPM) cells, mouse E10.5 mandibles and E15 molar tooth germs were treated with BMPs and FGFs. Semiquantitative RT-PCR, Northern blot hybridization, and in-situ hybridization were utilized to determine the effects of these cytokines on the expression of Krox-26 mRNA.
2.2 Materials and Methods

2.2.1 Cell culture

a) DPM cell culture at high serum concentration and total RNA isolation

Dental papilla mesenchyme (DPM) cells originated from the tooth germ of an E18 heterozygous immortal mouse, a transgenic mouse harboring the SV40 large T antigen (Jat et al., 1991). DPM cells were obtained from Dr. M. Zeichner-David at the University of Southern California. DPM cells were seeded at a density of \(1.0 \times 10^6\) cells on a 100 mm cell culture dish (Falcon 3003; Becton Dickinson and Company, Franklin Lakes, NJ) in high glucose Dulbecco’s modified Eagle (HG-DME) medium. This medium was supplemented with 5 U/ml mouse gamma interferon (IFN-\(\gamma\); Roche Diagnostics, Laval, PQ) to enhance large T antigen expression under proliferating conditions, and 10% fetal calf serum (FCS). The cultures were incubated overnight at 33°C in a humidified atmosphere of 95% air and 5% \(\text{CO}_2\). Following incubation, the cells were trypsinized and re-seeded into a 6-well plate (Falcon 3046; Becton Dickinson and Company) at a density of \(5.0 \times 10^5\) cells. Cells were treated with or without 100 ng/ml of BMP-2 or BMP-4 (Genetics Institute, Cambridge, MA), or 10 ng/ml of either FGF-4 or FGF-8 (BioShop Canada, Burlington, ON) for 30 minutes (min), 3 hours (h), or 8 h. Total RNA was obtained from cells using Trizol reagent (Life Technologies, Burlington, ON) according to the manufacturer’s protocol. Once isolated, total RNA was stored at -80°C for later use.

b) DPM cell culture at low serum concentration and total RNA isolation

For experiments using low serum conditions, DPM cells were grown as described above except that 1% FCS was used. Cells were treated with or without 200 ng/ml of
either BMP-2 or BMP-4 or 20 ng/ml of either FGF-4 or FGF-8 for 30 min, 1 h, or 3 h. Total RNA was obtained using the StrataPrep® Total RNA MicroPrep Kit (Stratagene, La Jolla, CA) according to manufacturer's protocol.

2.2.2 Organ Culture

a) E15 mouse molar tooth germ isolation, culture, and total RNA extraction

E15 mouse embryos were obtained from timed-pregnant female CD-1 mice (Charles River Laboratories, St. Constant, PQ). The microdissection procedure for obtaining mandibles was carried out in BGJb medium (Life Technologies) as described by Slavkin et al. (2000). Molar tooth germs were isolated from the distal ends of E15 mouse embryos mandibles, and placed on 6 mm diameter discs of Millipore filter (type AA, 0.8μm pore size; Millipore, Bedford, MA) supported by stainless steel grids (30 mesh, openings 0.015 in; Ferrier Wire, Toronto, ON). The molar tooth germs were placed in Falcon 3037 organ culture dishes (Becton Dickinson) containing 200 μl of BGJb medium and 100 μg/ml ascorbic acid and 100 U/ml penicillin and 100 μg/ml streptomycin (Life Technologies). The cultures were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO₂ for 24 h prior to treatment with or without 200 ng/ml of BMP-2 or BMP-4, or 20 ng/ml of FGF-4 or FGF-8. Total RNA from cultures treated for 1-6 h was harvested using the StrataPrep® Total RNA MicroPrep Kit.

b) E10.5 mouse mandibular isolation, culture, treatment, and total RNA extraction

Mouse mandibles were isolated and harvested from E10.5 mouse embryos as described above, except that the cultures were incubated for 5 days prior to treatment with or without 400 ng/ml of BMP-2 or BMP-4, or 40 ng/ml of FGF-4 or FGF-8. Total
RNA from cultures treated for 1, 3, and 6 h was harvested using the StrataPrep® Total RNA Microprep Kit.

2.2.3 RT-PCR for cell and organ cultures

First strand cDNA synthesis was performed from DNase I treated total RNA isolated from cell and organ cultures using random hexamer primers and Superscript II RNase H - Reverse Transcriptase (Life Technologies). cDNA fragments of Krox-26, Msx-1, β-actin, and 18S rRNA were PCR-amplified using specific primers and cycle numbers within the linear amplification range. Annealing temperatures, number of cycles, and expected product sizes are summarized in Table 2-1. Quantification of amplified cDNA products was performed by densitometry using ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

<table>
<thead>
<tr>
<th>Reverse primer sequence 5' to 3'</th>
<th>18S rRNA</th>
<th>β-Actin</th>
<th>Msx-1</th>
<th>Krox-26</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward primer sequence 5' to 3'</td>
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<td>ACCTGGGCCGTACGAGCATGCT</td>
<td>AGAACGACGTACCCTGTC-TATTGCCGCAG</td>
<td>ACTTCAGGAGTTTCCAT-GACCAGTGCCT</td>
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<tr>
<td>PCR annealing temperature</td>
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<td>58°C/20sec</td>
<td>64°C/20sec</td>
<td>68°C/20sec</td>
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<tr>
<td>PCR cycles</td>
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<td>25</td>
<td>35</td>
<td>30</td>
</tr>
<tr>
<td>Amplicon size</td>
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<td>400bp</td>
<td>664bp</td>
<td>550bp</td>
</tr>
</tbody>
</table>

Table 2-1. A summary of PCR conditions for semiquantitative RT-PCR.

2.2.4 Northern blot analysis on BMP-2 treated E11.5 mouse mandibles

Ten mouse mandibles were isolated from E11.5 mouse embryos as described above, except that the cultures were incubated for 3 days prior to treatment with or without 400 ng/ml of BMP-2 for 3 hours. Total RNA from the mandibles was isolated using the phenol-chloroform extraction method (Chomczynski and Sacchi, 1987). Total
RNA (10 to 15 μg) was isolated and separated on a 1% formaldehyde/agarose gel and blotted onto Hybond membrane (Amersham Pharmacia Biotech). 32P-dCTP labeled probes for Krox-26, GAPDH, and Msx-1 were synthesized using the T7 Quickprime kit, according to manufacturer's protocol (Amersham Pharmacia Biotech). Hybridization was performed overnight at 68 °C in 10X SSC, 0.1% SDS. The blot was washed in 2X SSC and 0.1% SDS, followed by 0.2X SSC and 0.1% SDS at 55 °C for 30 min. The blot was exposed to a PhosphorScreen overnight and the screen was scanned using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

2.2.5 Bead implantation experiment

a) Preparation and implantation of beads

Preparation of Affi-Gel Blue Gel beads (100-200 mesh; BioRad, Hercules, CA) coated with 200 ng/μl of BMP-2 or BMP-4, or 20 ng/μl of FGF-4 and FGF-8 was performed as described by Slavkin et al. (2000). The beads coated with growth factors were placed within the mesenchyme of the prospective molar region on the left half of the mandible cultures (described above) while beads untreated with BMPs or FGFs were placed on the right half. Each treatment was performed on two separate mandible cultures, where one mandible culture was probed for Msx-1 mRNA and another for Krox-26 mRNA. After 24 h, the explants were fixed in 4% paraformaldehyde/PBS at 4°C, overnight, and gradually transferred to methanol and stored at -20°C until later use.

b) Whole mount in-situ hybridization

A full-length 2.1 kb antisense RNA probe for Krox-26 was generated from a KpnI and SacI Krox-26 fragment cloned into pBS-ISH (Ganss et al., in preparation). Briefly,
the pBS-ISH vector containing Krox-26 was linearized with KpnI and the digoxigenin-labeled RNA probe was synthesized using T3 RNA Polymerase (Promega, Madison, WI) and the DIG RNA labeling kit (Roche Diagnostics; according to manufacturer’s protocol). A 670 bp Msx-1 cDNA fragment was generated by RT-PCR from DPM cells using forward (5'-AGAAGCAGTACCTGTCTATTTG CCGAG-3') and reverse (5'-CAGCAGAATTAGGGCTGAAGGAGAC-3') primers, and cloned into pPCR-Script™ Amp SK(+) vector (Stratagene). The vector containing the Msx-1 fragment was linearized with BamHI and the digoxigenin-labeled RNA probe synthesized with T3 RNA polymerase as described above. Explants stored in -20°C were rehydrated with successive incubations in 75%, 50%, and 25% methanol/PBT (PBS + 0.1% Tween-20). The explants were washed in PBT and treated with 20 ng/ml proteinase K for 20 min at 37°C, and hybridized overnight at 68°C in hybridization buffer (50% formamide, 0.75 M NaCl, 100 μg/ml tRNA, 0.05% heparin, 0.1% BSA, 20% SDS, and 10 mM PIPES, 1 mM EDTA, pH 6.8) using 20 ng/ml of Krox-26 or 30 ng/ml of Msx-1 digoxigenin-labeled RNA probes. Explants were washed and incubated with 1:5000 alkaline-phosphatase-coupled antidigoxigenin antibody (Roche Diagnostics) overnight at 4°C. Quenching of endogenous alkaline phosphatase was performed using 2 mM levamisole hydrochloride. Colour development on whole mount explants was developed using BCIP/NBT Combo kit (Gibco BRL) for purple alkaline phosphatase substrate.

2.2.6 Alkaline Phosphatase Assay of C2C12 cells treated with BMP-2 and BMP-4

The mouse myoblast-like cell line, C2C12, was obtained from the American Type Culture Collection (Rockville, MD). The cells were seeded at 6.4x10³ cells/well in a 96-well plate and grown in DMEM containing 5% FCS. The cells were treated with or
without 100, 200, or 400 ng/ml of BMP-2 or BMP-4 at confluence. Media containing BMP-2 or BMP-4 were replenished after 48 h. C2C12 cells were cultured at 37°C in a humidified atmosphere of 5% CO₂ in air. C2C12 cells treated with BMP-2 or BMP-4 were examined histochemically after 72 h for alkaline phosphatase activity. Briefly, the cells were fixed in 4% paraformaldehyde/PBS for 15 min at room temperature. After washing with PBS, the cells were incubated with a mixture of 0.1 mg/ml naphthol AS-MX phosphate (Sigma), 0.5% N, N-dimethylformamide, 2 mM MgCl₂, and 0.6 mg/ml of fast blue BB salt (Sigma) in 0.1 M Tris-Cl, pH 8.5. Color development was allowed to proceed at room temperature with periodic observations under the microscope to monitor staining intensity, and the reaction stopped by rinsing the cultures with dH₂O.

2.2.7 Cell Proliferation Assay on DPM cells treated with FGF-4 and FGF-8

DPM cells were grown in HG-DME medium containing 5 U/ml mouse IFN-γ, 1% FCS at 33°C in a humidified atmosphere containing 5% CO₂. The cells were transferred into 6-well plates at a density of 5.0x10⁵ cells/well. Following overnight incubation, the cells were treated with or without 20 ng/ml of FGF-4 or FGF-8. Cells incubated in medium containing 2% FCS were used as positive controls. Each treatment was performed in triplicate. All groups of cells were maintained in proliferative state at 33°C in a humidified atmosphere containing 5% CO₂. Cells were trypsinized and counted after 24, 48, and 72 h using a Coulter counter (Beckman Coulter, Mississauga, ON).
2.3 Results

2.3.1 Cell and organ culture systems

Regulation of Krox-26 mRNA expression by BMP-2, BMP-4, FGF-4, and FGF-8, which were selected based on their similar temporo-spatial expression pattern to Krox-26 during early tooth development, was determined using three tooth-specific culture systems: dental papilla mesenchyme (DPM) cells, mouse E15 molar tooth germs, and mouse E10.5 mandibles.

DPM cells isolated from heterozygous immortomouse E18 tooth germ and immortalized using a temperature-sensitive SV40 large T-antigen-containing retroviral vector were obtained from Dr. M. Zeichner-David (University of Southern California). DPM cells were chosen for two reasons: 1) DPM cells are odontogenic cells derived from the tooth organ, and thus, are likely to be responsive to BMPs and FGFs, which may subsequently regulate the mRNA expression of Krox-26; 2) an initial RT-PCR and Northern blot analysis verified that Krox-26 mRNA was expressed in DPM cells (Ganss et al., in preparation). DPM cells cultured at 33°C in the presence of IFN-γ show a fibroblast-like morphology (Fig. 2-1).

Tooth germs and mandibles were dissected from E15 and E10.5 mouse embryos (Fig. 2-2), respectively, and cultivated on a Millipore filter supported by a stainless steel grid (Thesleff, 1976). These organ cultures are systems that allow screening of possible regulatory effects of BMP-2, -4, or FGF-4, -8 on Krox-26 mRNA expression during epithelial-mesenchymal interactions.
Figure 2-1. Morphology of DPM cells.
DPM cells in culture medium assume fibroblast-like cell morphology. Rounded cells with bright, white outer rings are indicative of their detachment from the surface of the culture plate.

Figure 2-2. Mouse E10.5 mandible and E15 molar tooth germ cultures.
Mouse E10.5 mandibles along with their tongues (left panel) and mouse E15 molars (right panel) were placed on filters supported by wire mesh. Mouse E15 molar tooth germ shown in the right panel originated from the region indicated in the mouse E10.5 mandible. Both mandible and molar cultures were grown in the same media containing ascorbic acid and antibiotics.

2.3.2 BMP-2 and BMP-4 induced alkaline phosphatase activity in C2C12 cells

Alkaline phosphatase assay was performed to confirm the functionality of BMP-2 and BMP-4 used in subsequent experiments. C2C12 myoblast-like cells were chosen as a cell culture system due to their ability to differentiate towards the osteoblast lineage with BMP-2 or BMP-4 treatment (Katagiri et al., 1994). Alkaline phosphatase activity was
measured as an early marker of bone formation. Various concentrations of BMP-2 or BMP-4 (100, 200, and 400 ng/ml) were used. Alkaline phosphatase activity of differentiated C2C12 cells could be detected at BMP concentrations as low as 100 ng/ml after 3 days of culture (Fig. 2-3).

![Control vs BMP Treated](image)

**Figure 2-3. Alkaline phosphatase activity in C2C12 cells treated with BMP.**
Alkaline phosphatase activity of BMP-2 (100 ng/ml) treated C2C12 cells can be visually detected by the purple staining of fast blue BB salt (right panel). Similar results were obtained for 200 ng/ml and 400 ng/ml of BMP-2 or BMP-4 treated C2C12 cells (data not shown). C2C12 cells that did not receive any BMP treatment show an absence of alkaline phosphatase activity (left panel).

### 2.3.3 Characterization of Krox-26 mRNA expression

To investigate the possible effects of BMP-2, -4, or FGF-4, -8 on Krox-26 mRNA expression, several techniques were used: 1) RT-PCR on treated DPM cells, molar and mandible organ cultures; 2) in-situ hybridization on mandibles implanted with coated beads; and 3) Northern blot analysis of total RNA isolated from BMP-2 treated mandibles. In all cases, Msx-1 mRNA expression served as a positive control. Initial RT-PCR screening using Msx-1 specific primers confirmed the expression of Msx-1 within the cell and organ culture systems (data not shown).
a) RT-PCR on BMP-2, BMP-4, FGF-4, or FGF-8 treated cultures

Total RNA from DPM cells cultured at a high serum concentration (10% FCS) and treated with BMPs or FGFs were isolated, and the expression of Krox-26, Msx-1, and β-actin analyzed over an 8-hour treatment period (Fig. 2-4). Semi-quantitative analysis of the expression of Krox-26 and Msx-1 was performed in comparison to β-actin expression. BMP-2, -4, or FGF-4, -8 did not appear to regulate the expression levels of Krox-26 or Msx-1 mRNAs in DPM cells grown in medium containing 10% FCS.

![Table](image)

**Figure 2-4. RT-PCR analyses of DPM cells grown under high serum conditions treated with BMP-2, BMP-4, FGF4, or FGF8.**

cDNA products specific for Krox-26, Msx-1, and mouse β-actin were amplified by RT-PCR from DPM cells cultured in medium containing 10% FCS. DPM cells were treated with 100 ng/ml BMP-2 or BMP-4, or 10 ng/ml FGF-4 or FGF-8 for 30 min, 3 h, and 8 h, and total RNA was isolated. PCR was performed using Krox-26 primers on total RNA without reverse transcriptase (-RT) to control for genomic DNA contamination.

To minimize possible serum effects due to the presence of 10% FCS, RT-PCR was performed on total RNA isolated from BMP-2, -4, and FGF-4, -8-treated DPM cells grown in medium containing 1% FCS (Fig. 2-5). RT-PCR was also performed on total RNA isolated from organ cultures (E15 mouse molar tooth germ and E10.5 mouse mandibles), which were grown in the absence of FCS (Fig. 2-6 for molars and Fig. 2-7 for mandibles). Total RNA isolated from cell and organ cultures were treated with DNase I prior to cDNA synthesis. However, a DNA fragment was PCR-amplified with specific primers for Krox-26 from total RNA without the cDNA synthesis (-RT). Thus, the
removal of genomic DNA was incomplete (data not shown for Msx-1 and 18S rRNA).
To correct for genomic DNA contamination, the levels amplified from -RT groups (genomic Krox-26 DNA) were subtracted from amplified cDNA (Krox-26 cDNA), and this value was subsequently normalized to 18S rRNA. The levels of amplified products were quantitated by densitometry with the ImageQuant software.

BMP-2, -4, and FGF-4, -8 did not appear to have any significant effects on the expression of Krox-26 and Msx-1 mRNAs in DPM cells grown in medium containing 1% FCS (Fig. 2-5). Expression of Krox-26 mRNA reached a peak after 1 hour in all

![Relative Krox-26 mRNA expression levels](image)

![Relative Msx-1 mRNA expression levels](image)

**Figure 2-5.** RT-PCR analyses of DPM cells grown in low serum (1% FCS) conditions treated with BMP-2, BMP-4, FGF-4, or FGF-8.

C DNA products specific for Krox-26, Msx-1, and 18S rRNA were amplified by RT-PCR from DPM cells cultured in medium containing 1% FCS. DPM cells were treated with 200 ng/ml BMP-2 or BMP-4, or 20 ng/ml FGF-4 or FGF-8 for 30 min, 1 h, and 3 h, and total RNA isolated. PCR was performed using Krox-26 primers on total RNA without reverse transcriptase (-RT) to check for genomic DNA contamination. Results from densitometry analysis on amplified targets are shown in the graphs. mRNA expression for Krox-26 and Msx-1 was determined relative to 18S rRNA.
treated and non-treated cells, suggesting that 1% FCS alone influenced Krox-26 expression. A possible serum effect was also evident in the gradual increase in the expression of Msx-1 mRNA independent of BMP or FGF treatment.

RT-PCR was performed on total RNA isolated from mouse E15 molar tooth germs treated with BMP-2, BMP-4, FGF-4 or FGF-8 for 1 to 6 hours (Fig. 2-6). The mRNA levels of Krox-26 and Msx-1 were normalized to 18S rRNA and the results plotted on a line graph (Fig. 2-6). In comparison to the control, the expression of Krox-26 mRNA increased approximately 1.5-fold after 2 hours of treatment with BMP-4, FGF-4, or FGF-8, but declined after 3 hours. Expression of Krox-26 mRNA was about 2-fold higher in comparison to the control after 4 hours of treatment with BMP-2 or BMP-4, and subsequently declined to control levels after 6 hours. The mRNA expression of Msx-1 showed the highest level in control cells after 2 hours, which gradually declined thereafter. Msx-1 mRNA expression was about 1.5-fold higher after 1 hour treatment with FGF-4 relative to control cells, and expression levels also declined thereafter. Expression of Msx-1 mRNA in cultures treated with FGF-8 was about 2-fold higher relative to controls after 5 hours. In comparison to the control, the expression levels of Msx-1 mRNA increased after 5 hours of treatment with BMP-4 (~1.5-fold) or FGF-4 (~1.5-fold). Thus, in the molar tooth germ culture, treatment with BMPs and FGFs appeared to regulate Krox-26 and Msx-1 mRNA expression.

RT-PCR was also performed on total RNA isolated from mouse E10.5 mandibles treated with BMP-2, BMP-4, FGF-4 or FGF-8 for 1 to 6 hours (Fig. 2-7). The mRNA levels of Krox-26 and Msx-1 were normalized to 18S rRNA and the results plotted on a line graph (Fig. 2-7). Msx-1 mRNA expression levels were lower in cultures treated for 6
Figure 2-6. RT-PCR analyses to mouse E15 molar tooth germ treated with BMP-2, BMP-4, FGF-4, or FGF-8.
cDNA products specific for Krox-26, Msx-1, and 18S rRNA were amplified by RT-PCR from mouse E15 molar tooth germs treated with 200 ng/ml BMP-2 or BMP-4, or 20 ng/ml FGF-4 or FGF-8 for up to 6 hours with total RNA isolated at every hour. PCR was performed using Krox-26 primers on total RNA without reverse transcriptase (-RT) to check for genomic DNA contamination. Results from densitometry analysis on amplified targets are shown in the graphs. mRNA expression for Krox-26 and Msx-1 was determined relative to 18S rRNA.

Expression levels of Krox-26 mRNA were ~4-fold higher in cultures treated for 3 hours with BMP-2, BMP-4, FGF-4, or FGF-8 relative to control cultures (Fig. 2-7).
with BMP-2, relative to control cultures. After 6 hours, expression of Krox-26 mRNA was lower in all treated groups. Thus, in the mandible culture, treatment with BMPs and FGFs showed possible regulation of Krox-26 and Msx-1 mRNA expression.

<table>
<thead>
<tr>
<th>mRNA</th>
<th>1 hour</th>
<th>3 hours</th>
<th>6 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>Krox-26</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Msx-1</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>18S rRNA</td>
<td></td>
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<tr>
<td>-RT</td>
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![Relative Krox-26 mRNA expression levels](image)

![Relative Msx-1 mRNA expression levels](image)

Figure 2-7. RT-PCR analyses of mouse E10.5 mandibles treated with BMP-2, BMP-4, FGF-4, or FGF8. cDNA products specific for Krox-26, Msx-1, and 18S rRNA were amplified by RT-PCR from mouse E10.5 mandibles treated with 400 ng/ml BMP-2 or BMP-4, or 40 ng/ml FGF-4 or FGF-8 for up to 6 hours with total RNA isolated at 1, 3 and 6 hours. PCR was performed using Krox-26 primers on total RNA without reverse transcription to check for genomic DNA contamination. Results from densitometry analysis on amplified targets are shown in the graphs. mRNA expression for Krox-26 and Msx-1 was determined relative to 18S rRNA.

**b) Northern blot analysis on BMP-2 treated mouse E11.5 mandibles**

Northern blot analysis was performed on total RNA isolated from five mouse E11.5 mandibles with or without BMP-2 treatment for 3 hours (Fig. 2-8). Qualitative observation suggested that BMP-2 did not have any significant effects on the expression
of Krox-26 or Msx-1 mRNA. However, upon quantitative determination by densitometry, Krox-26 and Msx-1 mRNA expression appeared to be slightly downregulated (1.3-fold and 1.1-fold, respectively) by BMP-2 treatment compared to the non-treated tissues.

**Figure 2-8. Northern blot analysis on mouse E11.5 mandible treated with BMP-2.**
Total RNA was electrophoresed on a 1% formaldehyde/agarose gel, blotted onto nylon membrane and hybridized with $^{32}$P-labeled probes for Msx-1, Krox-26, and GAPDH. Quantitative mRNA expression of Krox-26 and Msx-1 relative to the expression of GAPDH was determined by densitometry.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Treated</th>
</tr>
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<tbody>
<tr>
<td>Krox-26</td>
<td>0.904</td>
<td>0.708</td>
</tr>
<tr>
<td>Ratio to GAPDH</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Msx-1</td>
<td>0.975</td>
<td>0.903</td>
</tr>
<tr>
<td>Ratio to GAPDH</td>
<td></td>
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<tr>
<td>GAPDH</td>
<td></td>
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</tr>
</tbody>
</table>

c) **In-situ hybridization of Krox-26 and Msx-1 of mouse E10.5 mandibles treated with BMP-2, BMP-4, FGF-4 or FGF-8.**

In an attempt to analyze the responsiveness of Krox-26 mRNA expression in individual cells within the organ cultures rather than isolating RNA from the entire organ, in-situ hybridization was performed. Beads coated with BMPs and FGFs were implanted in the mesenchyme layer of mouse E10.5 mandible cultures. Preliminary in-situ hybridization was performed with antisense DIG-labeled RNA probes specific for Krox-26 and Msx-1. Signals specific for Krox-26 and Msx-1 mRNA could not be detected in cultures treated with BMP or FGF-coated beads (Fig. 2-9).
Figure 2-9. In-situ hybridization experiment on mouse E10.5 mandibles treated with BMP-2, BMP-4, FGF-4, and FGF-8.
In each picture, a bead treated with BMP-2, BMP-4, FGF-4, or FGF-8 was implanted into the mesenchyme layer of left mandible, denoted with (+). Control bead without BMPs or FGFs treatment was implanted into the mesenchyme layer of the right mandible, denoted with (-). DIG-labeled RNA probes against Msx-1 and Krox-26 mRNAs were synthesized and hybridized with the mandibles.
2.3.4 Effects of FGFs on DPM cells

The viability of DPM cells seemed compromised by the low serum (1% FCS) conditions observed in previous experiment. To determine the survival of the DPM cells grown in low serum conditions, a cell proliferation assay was performed. DPM cells were grown in medium containing 1% FCS and treated with FGF-4 and FGF-8, which are positive regulators of cell proliferation. As a control, DPM cells were also grown in medium containing 2% FCS. After 24, 48, and 72 hours, the number of DPM cells was determined with a Coulter counter (Fig. 2-10). After 48 hours, the number of cells in all four groups decreased relative to the number of cells after 24 hours suggesting cell death. However, after 72 h the number of DPM cells was similar or increased relative to the number of cells present after 48 hours. After 72 h, cell proliferation was increased by treatment with 2% FCS in the positive control, but no obvious stimulating effects of FGF-4 or FGF-8 on cell proliferation could be observed (Fig. 2-10).

![Cell Proliferation Assay](image)

**Figure 2-10.** Cell proliferation assay on DPM cells treated with FGF-4 or FGF-8.

For this assay, DPM cells were treated with 20 ng/ml of FGF-4 or FGF-8 for 24, 48, and 72 hours. Control cells were cultured in medium containing 1% FCS while cells grown in medium containing 2% FCS served as a positive control. At the end of 24, 48 or 72 hours, DPM cells from each treatment group were trypsinized and counted on a Coulter counter.
2.4 Discussion

Krox-26, a novel zinc finger containing protein, was originally identified from the rat tooth organ (Matsuki et al., 1995). The full-length sequence of mouse Krox-26 was subsequently obtained. In-situ hybridization studies showed the expression of Krox-26 in the dental mesenchyme of mouse E12.5, and in the inner enamel epithelium and the enamel knot area of developing molars in mouse E13.5 (Lee et al., 1997). Krox-26 mRNA is predominantly expressed in the stratum intermedium and in the ameloblasts of incisors of 3-week old rats (Lee et al., 1997). The temporo-spatial expression of Krox-26 suggests a possible regulation by odontogenic factors such as BMP-2, BMP-4, FGF-4, and FGF-8. These secreted signaling molecules are important in early dental epithelial-mesenchymal interactions and are involved in tooth morphogenesis as described previously (Fig. 1-1).

In an initial screen to identify upstream regulators of Krox-26, the effects of BMPs and FGFs on the expression of Krox-26 mRNA were studied. Total RNA was isolated from several tooth-specific cell and organ culture systems treated with BMPs and FGFs. BMP-2, BMP-4, FGF-4, and FGF-8 were selected as likely candidates to regulate Krox-26 based on their expression patterns in developing tooth organs. Msx-1, a homeobox-containing transcription factor has been reported to be upregulated by BMP-2, BMP-4, FGF-4 and FGF-8 (Vainio, 1993; Neubüser et al., 1997; Kettunen and Thesleff, 1998; Jernvall et al., 1994; Niswander and Martin, 1992). Therefore, Msx-1 was used as a positive control in all experiments performed in this chapter. RT-PCR, Northern blot hybridization, and in-situ hybridization were performed to determine the levels of Krox-26 with and without BMP or FGF treatment.
DPM cells, derived from the murine dental organ, were grown in 10% serum and treated with BMPs and FGFs. From RT-PCR studies, BMP-2, BMP-4, FGF-4, and FGF-8 did not appear to regulate the expression of Krox-26 and Msx-1 mRNA (Fig. 2-4). As Msx-1 has been shown previously to be a target gene of BMP-4 and FGF-8, and the failure of Msx-1 to respond to BMP-4 and FGF-8 may involve two possible explanations. Firstly, components present in the serum may exert undesired effects such as antagonism of BMPs or FGFs. The second and more obvious reason may be that DPM cells are not responsive to BMPs or FGFs. In an attempt to address the first possibility, DPM cells were grown and treated with BMPs and FGFs in low serum (1% FCS) conditions, and RT-PCR performed (Fig. 2-5). Although by qualitative observation, Krox-26 mRNA appeared to be upregulated in all treated groups compared to the control, this apparent up-regulation was not observed when normalized to the levels of 18S rRNA. In fact, Krox-26 expression reached a peak after 1 hour in all treated and non-treated cells, suggesting serum effects even at 1% serum. Furthermore, Msx-1 mRNA was not induced by BMP-4 or FGF-8.

In an attempt to understand the non-responsiveness of Msx-1 mRNA expression to BMP-4 or FGF-8, the viability of the DPM cells in low serum conditions was analyzed. It was observed previously that higher numbers of detached cells were present when DPM cells were incubated in low serum conditions compared to high serum conditions (data not shown). Therefore, to determine the viability of the DPM cells grown in medium containing 1% FCS, a cell proliferation assay was performed on FGF-4 or FGF-8 treated DPM cells (Fig. 2-10). Results indicated that the DPM cells required at least 72 hours to adapt to 1% serum conditions. Furthermore, FGF-4 and FGF-8 did not
positively regulate cell proliferation in DPM cells as shown previously in other systems (Jernvall et al., 1994; Kettunen et al., 1998; Wilkie et al., 1995). Taken together, DPM cells do not appear to be a suitable cell system for the study of BMP or FGF regulation in low serum conditions.

To further investigate the effects of BMP-2, BMP-4, FGF-4, and FGF-8 on the expression of Krox-26, organ culture systems were used. Both the mouse E10.5 mandibles and E15 molar tooth germ culture systems provide an environment that is closer to the in vivo situation and allows to study the effects of these growth factors on Krox-26 expression. Organ cultures were treated with BMPs and FGFs and total RNA isolated. A limitation of these organ cultures is the small amount of tissues resulting in low amounts of total RNA isolated. As described for the analyses of total RNA isolated from DPM cells, RT-PCR was performed. Northern blot hybridization was also performed on total RNA isolated from five mandibles with or without BMP treatment.

Results from molar and mandible cultures treated with BMPs and FGFs indicated a possible regulation of Krox-26 and Msx-1 mRNA expression. In the molar tooth germ culture, Krox-26 mRNA expression was upregulated by BMPs and FGFs at various time points (Fig. 2-6). Msx-1 mRNA was transiently upregulated with BMP-4, FGF-4, and FGF-8. In the mandible culture, the expression levels of Krox-26 mRNA were upregulated after 3 hours when treated with BMP-2 and FGF-4 (Fig. 2-7). However, Msx-1 mRNA expression was downregulated after 3-hour treatment with BMPs and FGFs, which is inconsistent with previously reported results (Vainio, 1993; Neubüser et al., 1997; Kettunen and Thesleff, 1998; Jernvall et al., 1994; Niswander and Martin, 1992). Although BMPs and FGFs appeared to regulate the expression of Krox-26 mRNA
in both organ systems at certain time points, an obvious regulatory trend was not observed. Msx-1 was also upregulated in the molar cultures at certain time points, as expected. However, in mandible cultures, Msx-1 appeared to be downregulated by BMPs and FGFs. Taken together, the regulatory effects of BMPs and FGFs on Krox-26 mRNA expression were variable and relatively minor in these systems.

Since our initial RT-PCR analyses had indicated a more pronounced effect of BMP-2 on Krox-26 mRNA expression after 3 hours (Fig. 2-7), a Northern blot analysis was performed on total RNA isolated from five mouse E11.5 mandibles treated with or without BMP-2 to confirm this observation. From the five mandibles in each treatment group, less than 10µg of total RNA could be isolated. Densitometry analysis of the hybridization signals showed that Krox-26 and Msx-1 mRNA expression appeared to be slightly downregulated with BMP-2 treatment. This result is contradictory to the findings that BMP-2 upregulates Msx-1 expression (Vainio et al., 1993; Thesleff et al., 1995).

Due to the conflicting data from RT-PCR and Northern blot analyses of organ cultures, in-situ hybridization was performed. This experiment provided an alternative approach and was favored, as it would identify specific cells within the organ cultures responsive to BMPs and FGFs. Beads coated with or without BMPs or FGFs were implanted into the mandibles. Control and treated beads were implanted on opposite sides of the same mandible to minimize variability within different organ preparations. No significant effects of Krox-26 or Msx-1 mRNA expression by BMPs or FGFs were observed (Fig. 2-9). These mandible organ cultures were used previously to show the upregulation of Msx-1 in response to BMP-2 and BMP-4 (Vainio et al., 1993; Thesleff et al., 1995), FGF-4 (Jernvall et al., 1994), and FGF-8 (Neubüser et al., 1997; Kettunen and
Thesleff, 1998). The in-situ hybridization protocol requires further optimization as no signals were detected for Krox-26 or Msx-1 in treated or non-treated mandibles. A similar study by Dassule and McMahon (1998) used beads treated with BMP-2, Shh, or Wnt10b to study their effects on gene expression in mandible organ cultures. Out of 144 mandibles implanted with beads, 33 mandibles or ~25% failed to show proper development. Since our experiments were performed on only one mandible per treatment, the failure to detect Msx-1 or Krox-26 signals may be remedied by increasing the number of cultured mandibles.

In summary, semi-quantitative RT-PCR, Northern blot hybridization analysis, and in-situ hybridization experiments using DPM cells and organ cultures failed to consistently show regulation of Krox-26 by BMPs or FGFs. These experiments suggest that Krox-26 may not be regulated by BMPs and FGFs in tooth-specific cells. However, the regulation of Msx-1 mRNA expression was also not consistently observed, suggesting that further modifications to the experimental conditions may be necessary. The DPM cells require further characterization when grown in low serum conditions and their responsiveness to BMPs and FGFs remains to be confirmed. The organ cultures (mandible E10.5 and molar E15.5) are well characterized, but are technically challenging to isolate and maintain, and thus less replicate organs were available per treatment group. In the scope of this thesis, it was therefore not possible to establish an effect of BMPs or FGFs on Krox-26 mRNA expression in several tooth cell and organ culture systems.
Chapter 3: Identification of DNA Binding Sites for Krox-26

William Teo performed all experiments in this chapter except for the construction of a Krox-26 expression vector, which was performed by Dr. Bernhard Ganss (section 3.2.1a)
3.1 Introduction

A novel C$_2$H$_2$ zinc finger transcription factor, Krox-26, has recently been discovered from apical portions of 3-4 week old non-calcified pulp of rat incisors (Matsuki et al., 1995). Sequence analysis revealed that Krox-26 contains five consecutive C$_2$H$_2$ zinc finger motifs, linked by a highly conserved linker sequence of TGEKPF/Y (Fig. 3-1). In Krox-26, no other known protein motifs are found besides the zinc finger motifs. As a putative transcription factor, Krox-26 is anticipated to bind to specific DNA sequences in target genes through the zinc finger motifs in which three amino acids from each zinc finger contact three adjacent bases in DNA (Klug, 1999). Prior to Krox-26 discovery, no C$_2$H$_2$ zinc finger family member of proteins had been identified in tooth.

Figure 3-1. Amino acid sequence of Krox-26
Amino acid sequence of Krox-26 showing five consecutive C$_2$H$_2$ motifs, with conserved linker sequence of TGEKPY.

C$_2$H$_2$ zinc finger containing proteins can be divided into two subclasses. One subclass contains proteins comprising five or less zinc fingers whereas the second subclass contains proteins comprising more than five zinc finger motifs (Pieler and Bellefroid, 1994). The proteins with more than five zinc finger motifs are usually associated with conserved repressor domains such BTB/POZ (Albagli et al., 1995),
WRPW (Dawson et al., 1995), SNAG (Grimes et al., 1996), SCAN (Williams et al., 1995), and Krüppel-associated box (KRAB) (Peng et al., 2000). Among the repressor domains, the KRAB domain is the most well characterized protein motif and present in one-third of all C2H2 zinc finger-containing proteins (Losson, 1997). Since Krox-26 contains five C2H2 zinc fingers without any known repressor domains, it appears likely that Krox-26 functions as a transcriptional activator.

As a putative zinc finger transcription factor, the target DNA sequence for Krox-26 has yet to be identified. Therefore, to identify DNA binding sequences, recombinant Krox-26 was expressed in bacteria and, following purification, was used in a target detection assay (Thiesen and Bach, 1990; Sukegawa and Blobel, 1993). A consensus-binding site was determined which may be useful in identifying target genes of Krox-26.
3.2 Materials and Methods

3.2.1 Expression, Purification and Characterization of Krox-26

a) Construction of a Krox-26 expression vector

Krox-26 full-length cDNA was amplified via PCR from randomly-primed cDNA synthesized from total RNA of P19 mouse embryonic carcinoma cells using primers Y150-3’4 (5’-TTACCGAAAGC7TTGCCTACGCACCAT-3’) and Y150-5’3 (5’-GGAGAATTCTCATTAATACAGGAAGAACTGA-3’). The amplified cDNA was digested with HindIII and EcoRI and cloned into pBluescript® SK (Stratagene). The restriction enzymes EcoRI and XhoI were used to excise the full length Krox-26 cDNA, which was subsequently ligated in-frame, downstream of the 6x-Histidine tag in the expression vector pRSET A (Invitrogen, Carlsbad, CA) by standard molecular biology methods (Ausubel et al. 1993). The correct sequence of the resulting plasmid was confirmed by sequencing using the T7 DNA Polymerase Sequencing kit (Amersham Pharmacia Biotech) according to manufacturer’s protocol.

b) Expression of 6xHis-tagged Krox-26 protein

i) Transformation of BL21(DE3)pLysS competent cells

Transformation of BL21(DE3)pLysS cells with Krox-26 in pRSET A vector was performed according to Invitrogen’s transformation protocol. Briefly, a vial of the One Shot® BL21(DE3)pLysS Competent Cells (Invitrogen) was transferred from -80°C and thawed on ice. Krox-26 in pRSET A (10 ng) was added to the cells and incubated on ice for 30 min. The cells were heat shocked at 42°C for 30 seconds (s) and immediately placed on ice. 250 µL of pre-warmed SOC medium (37°C) supplied in the transformation
kit (Invitrogen), was added to the cells. The vial was incubated at 37°C in a shaking incubator (250 rpm) for 1 h.

ii) Growth and induction

The entire transformation reaction (300 μL) was added to 3 L of pre-warmed (37°C) Luria-Bertani (LB) medium containing 100 μg/ml ampicillin. The culture was incubated at 37°C in a shaking incubator at 250 rpm until the culture reached an optical density (λ=600 nm) of approximately 0.5. A sample (100 ml) of the 3 L culture was pelleted by centrifugation at 6000 rpm for 20 min at 4°C and stored at -80°C. These cells were used to assess the level of Krox-26 expression before induction. IPTG (0.5 mM final concentration) was added to the remaining culture and the incubation was continued at 37°C and with agitation at 250 rpm for 3 h. The bacterial cells were harvested by centrifugation at 6000 rpm for 20 min at 4°C. The cell pellet from the induced culture was stored at -80°C.

iii) Cell lysis and preliminary protein analysis

Frozen cell pellets, before and after induction, were thawed and lysed using buffer B (8 M urea, 0.1 M NaH₂PO₄, 0.01 M Tris·HCl, pH 8.0) at 5 ml/g wet weight of the cell pellet. Cell clumps were dispersed by repeated pipetting and sonicated to a homogenous viscosity. Cell debris was removed by centrifugation at 10,000 rpm for 20 min at 4°C. 12 μl of bacterial lysates were mixed with 3 μl of 5x SDS-PAGE sample buffer (15% β-mercaptoethanol, 15% SDS, 1.5% bromophenol blue, 50% glycerol), boiled for 2 min, centrifuged at 14 000 rpm for 1 min, and separated by 10% SDS-PAGE. The gel was electrophoresed at 130 V for 45 min and stained with Coomassie Brilliant Blue.
c) Purification of 6xHis-tagged Krox-26 protein under denaturing conditions

i) Gravity flow affinity column chromatography

The bacterial cell lysate was mixed with 50% nickel-bound nitrilotriacetic acid (Ni-NTA) agarose (Qiagen, Mississauga, ON) at 4:1 (lysate: Ni-NTA) ratio and incubated on a rocking platform at 4°C for 1 h. The lysate-resin mixture was then loaded into an empty 10 ml Poly-Prep column (Bio-Rad Laboratories, Hercules, CA). Once the flow through was collected, the Ni-NTA column was washed with 6 x 2 ml buffer C (8 M urea, 0.1 M NaH₂PO₄, 0.01 M Tris·HCl, pH 6.3). Bound materials were eluted with 2 x 2 ml buffer D (8 M urea, 0.1 M NaH₂PO₄, 0.01 M Tris·HCl, pH 5.9) followed by 2 x 2 ml buffer E (8 M urea, 0.1 M NaH₂PO₄, 0.01 M Tris·HCl, pH 4.5) and 1 x 2 ml buffer F (6 M guanidinium-HCl, 0.2 M acetic acid, pH 2). All fractions were collected, concentrated using the methanol-chloroform extraction/precipitation method described below, and aliquots analyzed by 10% SDS-PAGE.

ii) FPLC purification

A 10 ml FPLC column (C10/10; Amersham Pharmacia Biotech) was packed with 50% Ni-NTA Superflow (Qiagen) according to manufacturer's protocol. The FPLC column was equilibrated with 25 ml buffer B (described above) at a flow rate of 1 ml/min. The lysate (3 ml) was diluted with 2 ml of buffer B, filtered through a 0.45 μm filter (Millipore), and loaded onto the column. The column was washed with 20 ml of buffer B, and the protein was eluted with a linear pH gradient (pH 8 to pH 2) using mixtures of buffer B and buffer F at a flow rate maintained at 0.5 ml/min. Collected fractions (0.5 ml) were concentrated using the methanol-chloroform method as described below and analyzed by 10% SDS-PAGE.
iii) Gel Electro-elution

Cell lysate from IPTG-induced bacteria was separated by SDS-PAGE with pre-stained standard low molecular weight protein markers (Bio-Rad) included in an adjacent lane. The pre-stained marker facilitated locating the position of 6xHis-tagged Krox-26 protein on the gel so that the region of interest could be excised without staining the gel. The gel piece was cut into small pieces and placed into a glass tube fitted with a membrane cap from the Electro-eluter (Model 422, Bio-Rad). The glass tube containing the gel pieces was placed into the buffer chamber containing elution buffer (25 mM Tris, 192 mM glycine, 0.1% SDS). Elution was performed at 10 mA/glass tube for 5 h with vigorous stirring of the chamber buffer according to manufacturer's protocol. The eluted 6xHis-tagged Krox-26 protein was collected in 600 µl of fresh elution buffer and analyzed by 10% SDS-PAGE.

iv) Concentrating protein using methanol-chloroform method

Recombinant Krox-26 protein was concentrated using the methanol-chloroform method as described by Wessel and Flügge (1984). In a 1.5 ml microfuge tube, 750 µl of methanol:chloroform (v/v, 4:1) solution and 450 µl of water were added to 150 µl of protein solution, and briefly vortexed. The mixture was centrifuged at 14,000 rpm for 3 min at room temperature. The upper aqueous phase was carefully removed and discarded, taking care to leave the protein at the interphase. Methanol (450 µl) was added to the tube, briefly vortexed, and centrifuged at 14,000 rpm for 3 min at room temperature. The supernatant was removed leaving a protein pellet, which was solubilized in 15 µl of water.
d) Characterization of recombinant Krox-26 protein

i) Production and affinity purification of polyclonal antiKrox-26 antibody

A peptide specific for Krox-26 (amino acid residues 18-30, KAYESKRKTARQR) was synthesized and conjugated to keyhole limpet hemocyanin (KLH) protein and bovine serum albumin (BSA) by Alberta Peptide Institute (Edmonton, AB). The KLH-peptide was used by Cedarlane Laboratories (Hornby, ON) to generate antiserum in New Zealand white, female rabbits from which affinity-purified antibody was isolated using gravity flow column chromatography packed with cyanogen-bromide (CNBr) Sepharose beads (Amersham Pharmacia Biotech) bound to BSA-peptide. The bound IgG was eluted with elution buffer (50 mM glycine, 150 mM NaCl, 0.05% Tween-20, pH 2.3), collected in 1 ml fractions, and was immediately neutralized with 1M Tris.

ii) Enzyme-Linked Immuno-Adsorbent Assay (ELISA)

ELISA was performed according to Rennard et al. (1980). Briefly, Krox-26 peptide conjugated to BSA (2 µg/ml) in Voller’s buffer (15 mM Na₂CO₃, 3 mM NaN₃, 35 mM NaNHCO₃, pH 9.2) was used to coat the wells of a 96-well plate by overnight incubation at 4°C (200 µl per well). Affinity-purified anti-Krox-26 from each collected fraction was serially diluted in PBS-T (1:10, 1:100, 1:200, and 1:500), incubated for 1 h at room temperature in the Krox26-BSA-coated wells. The wells were washed 3x with PBS-T. Secondary goat-antirabbit antiserum coupled to horseradish peroxidase (HRP) diluted in PBS-T (1:2000) was added to the to the wells and incubated for 1 h at room temperature. The wells were washed 3x with PBS-T. Color development was performed using a solution comprising 1 mg of phenyldiamine dissolved in 100 µl methanol, 9.9 ml water, and 10 µl of 30% hydrogen peroxide, and the reaction stopped with 8 M sulfuric
acid when the desired intensity was achieved. The optical density ($\lambda=492$ nm) was measured in a Titertek® Multiskan Microplate Reader II (Labsystems, Finland).

iii) Western blot analysis

Recombinant 6xHis-tagged Krox-26 protein was separated by SDS-PAGE and transferred onto nitrocellulose membrane (Schleicher and Schuell, Keene, NH) by electrophoresis using a Semi-dry blotter (LTF Labortechnik, Wasserburg, Germany). The blot was rinsed 2x in TBS-T (20 mM Tris-Cl, pH 7.5; 150 mM NaCl; 0.05% Tween-20) and incubated with affinity-purified anti-Krox-26 (diluted 1:100 in TBS-T) or monoclonal anti-HisG antibody (diluted 1:5000 in TBS-T; Invitrogen) for 1 h at room temperature. The blot was washed 5x with TBS-T, and incubated with secondary goat anti-rabbit antibody coupled to HRP (diluted 1:2000 in TBS-T) or secondary goat anti-mouse (diluted 1:2000 in TBS-T) for Krox-26 or 6xHis, respectively, for 45 min at room temperature. The blot was washed 4x with TBS-T and 2x in TBS buffer (10 mM Tris-Cl, pH 7.5; 150 mM NaCl). Color development was performed using HRP staining solution [18 mg 4-chloro-1-naphthol dissolved in 6 ml methanol, 24 ml Tris-saline (0.9% NaCl, 0.1 M Tris-Cl, pH 8.0), and 60 µl 30% hydrogen peroxide], and the color reaction stopped with several washes with water.

3.2.2 Target Detection Assay

The target detection assay (TDA) was modified from experiments performed by Thiesen and Bach (1990) and Sukegawa and Blobel (1993).
a) Preparation of randomized dsDNA target

Three oligonucleotides (TDA-9, TDA-15, TDA-21) were designed comprising random 9, 15, or 21 nucleotides, respectively, flanked by sequences specific for primers, TDA-1-5' (5'-TGGGCGCATGCGCTAATG-3'), and TDA-1-3' (5'-CTACCGCTGGCTAGTTA-3'). TDA-9, TDA-15, and TDA-21 were converted into dsDNAs in a 50 μl reaction containing 2 μM oligonucleotide, 1 μM TDA-1-3', 1.5 μM MgCl₂, 1 mM dNTPs, 1x PCR buffer, and 1 U of Taq polymerase (MBI Fermentas, Burlington, ON) by denaturing at 94°C for 30 s, annealing at 45°C for 2 min, and extension at 67°C for 10 min. The dsDNA was purified using ProbeQuant G-50 micro-column (Amersham Pharmacia Biotech).

To produce radiolabeled dsDNA targets from TDA-9, TDA-15, and TDA-21, the reaction described above was performed except for the replacement of 1 mM dNTP with the addition of 750 μM d(ATG)TPs and 40 μCi α³²P-dCTP (Amersham Pharmacia Biotech).

b) Renaturation of recombinant 6xHis-tagged Krox-26 and binding to dsDNA targets

Recombinant 6xHis-tagged Krox-26 protein (200 ng/μl) purified by gel electrophoresis was separated by SDS-PAGE and transferred onto nitrocellulose membrane (Schleicher and Schuell, Keene, NH). The nitrocellulose blot was subsequently cut into strips (1 lane/strip). The strips were incubated overnight at 4°C in a renaturation buffer (50 mM Tris-Cl, pH 7.5, 100 mM KCl, 1% Triton-X-100, 10% glycerol) with (1 mM ZnCl₂) or without (50 mM EDTA, 10 mM DTT) Zn²⁺. After renaturation, the strips were transferred into reaction buffer (50 mM Tris-Cl, 100 mM KCl, 0.1% Triton-X-100, 10% glycerol) with (0.1 mM ZnCl₂) or without (10 mM EDTA, 2 mM DTT) Zn²⁺, and
incubated for 2 h at 4°C. Radiolabeled dsDNA from TDA-9, TDA-15, TDA-21 targets were added to the reaction buffer and incubated on a rocker overnight at 4°C. The blots were washed 4x in reaction buffer, and exposed to Kodak XAR-5 X-ray film (Eastman-Kodak, Rochester, NY) overnight at -80°C with intensifying screen.

c) Elution of bound DNA

To determine the optimal elution conditions, various salt and phenanthroline concentrations in reaction buffer (Table 3-1) were utilized to elute radiolabeled dsDNA bound to recombinant 6xHis-tagged Krox-26 immobilized on nitrocellulose membrane strips. The activity of the eluted radiolabeled dsDNA was measured using a scintillation counter (LS5000TD, Beckman, Mississauga, ON).

<table>
<thead>
<tr>
<th>Composition of elution buffer</th>
<th>[KCl]</th>
<th>[o-phenanthroline]</th>
</tr>
</thead>
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<tr>
<td>9</td>
<td>2 M</td>
<td>100 mM</td>
</tr>
</tbody>
</table>

Table 3-1. Composition of DNA elution buffer optimized for TDA.
Summary of various elution conditions of random oligonucleotides bound to immobilized recombinant Krox-26 protein.

d) DNA elution and amplification

The optimal elution condition was determined using a DNA elution assay as described above. The first TDA cycle then involved four steps: 1) subsequent to renaturation and dsDNA interaction to membrane-immobilized recombinant 6xHis-
tagged Krox-26, the strips were washed 3x with reaction buffer containing 100 mM KCl; 2) strongly bound dsDNAs were subsequently eluted from the washed strip with 1 ml reaction buffer containing 1 M KCl and 1mM phenanthroline; 3) eluted dsDNAs were amplified by PCR in a 50 μl reaction containing 5 μl eluted dsDNA, 1 μM TDA-1-5', 1 μM TDA-1-3', 1x PCR buffer (100 mM Tris, 15 mM MgCl₂), 1 mM dNTPs, and 1 U Taq polymerase for 20 cycles with denaturation at 94°C for 20 s, annealing at 54°C for 30 s, and extension at 72°C for 10 s; 4) 10 μl of the amplified product was analyzed on a 6% polyacrylamide gel stained with ethidium bromide.

**e) Second and subsequent TDA cycles**

In the second and subsequent TDA cycles, 4 μl of the amplified product was hybridized to a fresh nitrocellulose strip containing immobilized 6xHis-tagged Krox-26. Bound dsDNA was eluted and amplified as described above, except that the wash conditions for the second and subsequent TDA cycles contained 1 mM phenanthroline with varying concentration of KCl (2nd cycle, 100 mM KCl; 3rd cycle, 200 mM KCl; 4th cycle, 400 mM KCl; 5th cycle, 600 mM KCl). Additionally, 35 cycles of PCR amplification was performed for TDA cycles 4 and 5.

**f) Cloning and sequencing of DNA amplicons from TDA**

The amplified products from the 5th TDA cycle (10 μl) were blunt-end ligated into PCR-Script™ Amp cloning vector following the manufacturer’s protocol (Catalog #211188; Revision #069010, Stratagene). Clones were sequenced using the T7 DNA Polymerase Sequencing kit (Amersham Pharmacia Biotech).
g) DNA sequence analysis

ClustalW Sequence Alignment (Thompson et al., 1994), a subprogram utilized by BioEdit software (Hall, 1999), was used to construct multiple alignments of DNA sequences.

h) Electrophoretic Mobility Shift Assay (EMSA)

EMSA was performed as described in Promega's Gel Shift Assay Systems (Technical Bulletin no. 110; Promega). Briefly, oligonucleotides specific for Sp1 (5'-GG TACCCGGGGATCGGGGCGGGGATCCTCTAG-3' and its reverse complement), TDA-15 (5'-CTAGAGGATCGCCCGCCCGATCCCCGGGTACC-3' and its reverse complement), and a five tandem repeat of the sequence, CAATG (5'-GCGGATCCCAATGGCCAATGATCAATGCGCAATGCGCAATGTACAATGGGATCCATA-3' and its reverse complement) were annealed and end-labeled using 20 pmole dsDNA, 5 μCi [γ-32P]ATP, 10 U T4 polynucleotide kinase (MBI Fermentas) in 1x kinase buffer (MBI Fermentas). End labeled-dsDNA was purified using ProbeQuant G-50 micro-columns (Amersham Pharmacia Biotech) and counted by scintillation counter. 32P-labeled dsDNA (150 000 cpm) was mixed with 200 ng of recombinant 6xHis-tagged Krox-26 purified by electro-elution, or recombinant Sp1 protein (Promega) in binding buffer containing 25 mM HEPES (pH 7.5), 50 mM KCl, 4 mM MgCl₂, 10% glycerol, 25 mM ZnSO₄ for 20 min at room temperature. The samples were separated on a 4% polyacrylamide gel with 0.5x TBE, dried, and exposed to Kodak XAR-5 X-ray film (Eastman-Kodak, Rochester, NY) overnight at -80°C with intensifying screen.
3.3 Results

3.3.1 Expression, characterization and purification of Krox-26

a) Expression and characterization of 6xHis-tagged Krox-26 protein

Initial attempts to express Krox-26 protein using several different vectors and bacterial expression systems were unsuccessful (Table 3-2). However, when Krox-26 cDNA encompassing the open reading frame of the Krox-26 protein was cloned in frame into a pRSET expression vector (Invitrogen), downstream of a 6xHis-tag sequence, expression was achieved in E. Coli BL21(DE3)pLysS competent cells (Invitrogen).

<table>
<thead>
<tr>
<th>Expression system</th>
<th>Vector</th>
<th>E. coli strain</th>
</tr>
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<tbody>
<tr>
<td>A</td>
<td>pRSET A (Invitrogen)</td>
<td>(i) Epicurian Coli® BL21 (Stratagene)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(ii) Epicurian Coli® BL21(DE3) (Stratagene)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(iii) BL21(DE3)pLysS (Invitrogen)</td>
</tr>
<tr>
<td>B</td>
<td>pRSET C (Invitrogen)</td>
<td>(i) Epicurian Coli® BL21(DE3) (Stratagene)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(ii) Epicurian Coli® BL21Gold (Stratagene)</td>
</tr>
<tr>
<td>C</td>
<td>QIAexpress pQE (Qiagen Inc.)</td>
<td>(i) M15[pREP4] (Qiagen Inc.)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(ii) SG13009[pREP4] (Qiagen Inc.)</td>
</tr>
</tbody>
</table>

Table 3-2. Various bacterial expression systems tested for the expression of recombinant Krox-26 protein.

All expression systems listed failed to express recombinant Krox-26 protein except for BL21(DE3)pLysS transformed with pRSET A.

To characterize the expressed protein the BL21(DE3)pLysS cells were incubated for 3 hours after IPTG induction and cell proteins extracted. Analysis of the cellular proteins by SDS-PAGE and Coomassie blue staining revealed a protein band with a molecular mass of approximately 34 kDa that was increased following IPTG induction (Fig 3-2A). The size of the protein corresponded to the molecular mass calculated for the amino acid sequence encoded by the Krox-26 cDNA including the His-tag sequence. To
confirm that the protein was Krox-26, Western blot analyses were performed. Following
electrophoretic separation by SDS-PAGE, the protein extracts were transferred to a
nitrocellulose membrane and stained with Ponceau S to demonstrate successful transfer
to the membrane (Fig 3-2B). The transferred proteins were then probed, separately, with
polyclonal rabbit anti-Krox-26 antibodies and a monoclonal anti-HisG antibody. With
both antibodies a single major band was observed corresponding to the 34 kDa protein
with a minor band migrating at approximately 28 kDa (Fig 3-2C&D). These results
identified the 34 kDa protein as His-tagged Krox-26, the 28 kDa possibly representing a
truncated form of His-tagged Krox-26. To determine whether the expressed 34 kDa
protein could bind DNA, separate blots were probed with a $^{32}$P-labeled randomized pool
of double stranded TDA-15 DNA in the presence and absence of zinc ions. Following
radioautography a strong band was observed at 34 kDa, with a minor band at 28 kDa, in
blots incubated in the presence, but not in the absence, of zinc (Fig 3-2F).

![Figure 3-2. Characterization of bacterially expressed 6xHis-tagged Krox-26 protein.](image)

Each blot shows whole lysate before (left) and after (right) the induction of Krox-26
expression by IPTG. The coomassie stained gel (A) shows an induced band of approximately
34 kDa. Ponceau S staining (B) on Western blot shows successful transfer of proteins onto
nitrocellulose blot. Western blot analysis using antiKrox-26 (C) and antiHisG (D) antibodies
show color reaction to the 34 kD bands. Zinc binding analysis shows the presence of 34 kD
band in the presence of zinc (F) and not in the absence of zinc (E).
**b) Purification of 6xHis-tagged Krox-26 protein via Ni-NTA**

Recombinant 6xHis-tagged Krox-26 protein was purified by affinity chromatography for use in a target detection assay (TDA) using Ni-NTA Agarose or Ni-NTA Superflow (Fig. 3-3). In both systems, elution of 6xHis-tagged Krox-26 from the Ni-NTA matrices was performed by gradually decreasing the pH of the elution buffer. SDS-PAGE analyses of protein bound to the Ni-NTA Agarose showed that the addition of buffer E (pH 4.5; fraction 9) eluted a protein of 34 kDa, the predicted molecular mass of recombinant Krox-26 (Fig. 3-3A). A second protein of 28 kDa was also present in this fraction. Most of the other proteins were eluted in earlier fractions (fractions 1 to 6) with buffer C at pH 6.3. Purification of recombinant Krox-26 by FPLC through a column packed with Ni-NTA Superflow using a continuous linear pH gradient between pH 8 and pH 2 showed the elution of 34 kDa and 28 kDa proteins, as the pH of the eluting buffer approached pH 2.0 (Fig. 3-3B). Most of the other proteins were eluted in earlier fractions (data not shown for fractions 1 to 9, fractions 10 to 19). To identify the recombinant Krox-26 within the fractions collected, Western blot analyses with polyclonal antiKrox-26 (Fig. 3-3C) and monoclonal antiHisG (Fig. 3-3D) antibodies were performed. AntiKrox-26 and antiHisG recognized both the 34 kDa and 28 kDa proteins, containing the proteins as full-length and a possible C-terminally truncated form of Krox-26, respectively.
Figure 3-3. Purification and Western blot analyses of recombinant Krox-26 protein. (A) A Coomassie-stained gel of fractions collected from purification of 6xHis-tagged Krox-26 protein by Ni-NTA Agarose affinity chromatography. The recombinant Krox-26 protein was eluted between fractions 7 and 10 (pH 5.9 to pH 4.5). (B) A Coomassie-stained gel of fractions collected from purification of 6xHis-tagged Krox-26 protein by FPLC with Ni-NTA Superflow showing eluted recombinant Krox-26 protein mainly between fractions 20 and 23. Western blot analyses using (C) antiKrox-26 and (D) antiHisG antibodies confirmed the presence of 6xHis-tagged Krox-26 protein. Note that Western blots in (C) and (D) correspond to fractions 10-22. Arrows indicate the full-length recombinant Krox-26 protein of 34 kDa and a potentially truncated form of 28 kDa.
**c) Purification of the 34 kDa 6xHis-tagged Krox-26 protein via electro-elution**

The 34 kDa recombinant 6xHis-tagged Krox-26 protein was further purified from the eluted fractions by electro-elution. Western blot analyses with antiKrox-26 (Fig. 3-4B) and antiHisG (Fig. 3-4C) antibodies were performed to reconfirm the identity of the purified 34 kDa recombinant Krox-26 protein (Coomassie stained gel, Fig. 3-4A). DNA binding analysis was performed and the 34 kDa recombinant Krox-26 was shown to bind DNA in a zinc-dependent manner (Figs. 3-4D & E).

![Figure 3-4. Characterization of the 34 kDa 6xHis-tagged Krox-26 protein purified by electro-elution.](image)

(A) Coomassie-stained gel of the electro-eluted 34 kDa recombinant Krox-26 protein. Western blot analyses using (B) antiKrox-26 and (C) antiHisG antibodies against purified 6xHis-tagged Krox-26 protein. The 34 kDa recombinant Krox-26 protein bound radiolabeled dsDNA in the (E) presence but not in the (D) absence of zinc.

**3.3.2 Target Detection Assay**

In an attempt to identify DNA binding sites for Krox-26, a target detection assay was utilized. Briefly, dsDNAs composed of randomized nucleotides (TDA-9, TDA-15, and TDA-21) were bound to membrane-immobilized recombinant Krox-26 in the presence of zinc. The membrane was washed with various concentrations of KCl and phenanthroline that were determined as described below. The bound DNA was eluted,
amplified by PCR, and re-bound to the membrane-immobilized recombinant Krox-26. This procedure was repeated for 5 cycles to identify a DNA binding element for Krox-26.

a) Determination of optimal elution conditions

To determine the optimal conditions for the washing and elution of bound randomized DNAs, radiolabeled dsDNA (TDA-9, TDA-15, and TDA-21) were bound to membrane-immobilized recombinant Krox-26 protein and eluted using various KCl and phenanthroline concentrations (Fig. 3-5). The radioactivity of the eluted DNA was measured on a scintillation counter and the remaining DNA bound to recombinant Krox-26 protein was visualized by autoradiography. The majority of the bound DNA was removed with 100 mM KCl + 1 mM phenanthroline (Fig. 3-5). To select for DNA that interacts strongly with recombinant Krox-26 protein, increasing wash stringencies were utilized with each successive cycle: 1st cycle, 100 mM (without phenanthroline); 2nd cycle, 100 mM KCl; 3rd cycle, 200 mM KCl; 4th cycle, 400 mM KCl; 5th cycle, 600 mM KCl. The remaining bound DNA at each cycle was eluted with 1 M KCl + 1 mM phenanthroline.
Figure 3-5. Elution profile of $^{32}$P-labeled DNA bound to recombinant Krox-26 protein.
The amount of radiolabeled DNA eluted at specific concentrations of KCl in the presence of phenanthroline is shown. The radiographs show the remaining radiolabeled DNA bound to recombinant Krox-26 protein on the nitrocellulose blot. A, B, and C refer to radiolabeled dsDNA: TDA-15, TDA-9, and TDA-21, respectively.

b) DNA amplification from five TDA cycles

DNA binding to 6xHis-tagged Krox-26 protein, elution, and PCR amplification was performed for five cycles. At each cycle, the eluted and PCR-amplified DNA was separated by PAGE (Fig. 3-6). A 57 bp DNA fragment was amplified at each cycle that corresponds to the theoretical size of TDA-21. Five cycles of selection were also performed with dsDNA prepared from TDA-9 and TDA-15 (data not shown).
Figure 3-6. PCR amplification of eluted dsDNA from each of the five TDA cycles. Eluted dsDNA from TDA-21 (57bp) was analyzed on 6% polyacrylamide gel at each TDA cycle. The PCR-# corresponds to the amplified dsDNA from TDA cycle # (e.g. PCR-1 originates from TDA cycle 1).

c) DNA sequence alignment

After the 5th cycle of selection, the eluted and amplified DNA was cloned and forty clones selected for sequencing. The DNA sequences (from TDA-9, TDA-15, and TDA-21) along with their reverse complementary strands were aligned and a consensus DNA binding site of sequence 5'-cAATg-3' was identified in ten of the sequences (Fig. 3-7).

![DNA sequence alignment](image)

Figure 3-7. Consensus DNA binding site of Krox-26. Sequence alignment performed on 40 DNA sequences from TDA using ClustalW shows 10 DNA sequences with a consensus sequence of cAATg.
d) Electrophoretic Mobility Shift Assay (EMSA) on recombinant Krox-26 and SP1 proteins.

To further demonstrate Krox-26 binding to DNA, Electrophoretic mobility shift assays were performed using the recombinant 6xHis-tagged Krox-26 protein purified by electro-elution and a synthetic oligonucleotide comprised of five tandem repeats of the identified consensus sequence, 5'-cAATg-3' (Fig. 3-8). These experiments were performed at 4°C (Fig. 3-8A) and at room temperature (Fig. 3-8B). However, interaction between Krox-26 and the synthesized oligonucleotide (5xCAATG) was not observed as a gel shift. In contrast, an interaction between Sp1 transcription factor and its target DNA, which was used as a positive control, was observed. Although only a small amount of the radiolabeled cDNA was shifted, some Sp1 also bound to dsDNA derived from TDA-15 (Fig. 3-8A) in EMSAs whereas Krox-26 protein did not show interaction with TDA-15. dsDNA derived from TDA-15 comprises randomized nucleotides and therefore was used as a positive control for DNA binding of Krox-26 and Sp1.
Figure 3-8. EMSA of Krox-26 binding to target DNA sequence identified by target detection assay. Electrophoretic Mobility Shift Assay was performed on various protein and DNA complexes at (A) 4°C and (B) room temperature. Arrows indicate retarded migration of complexes in electrical field.

<table>
<thead>
<tr>
<th>Lane #</th>
<th>Protein</th>
<th>DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Krox-26</td>
<td>(CAATG)$_5$</td>
</tr>
<tr>
<td>2</td>
<td>Krox-26</td>
<td>TDA-15</td>
</tr>
<tr>
<td>3</td>
<td>Sp1</td>
<td>Sp1 target site</td>
</tr>
<tr>
<td>4</td>
<td>Sp1</td>
<td>TDA-15</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>(CAATG)$_5$</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>Sp1 target site</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td>TDA-15</td>
</tr>
</tbody>
</table>
3.4 Discussion

Krox-26, as a putative zinc-finger transcription factor, is expected to bind to specific DNA sequences. An expression system was developed to produce recombinant Krox-26 protein, which could be used for studies of DNA binding. Although initial attempts to express recombinant Krox-26 in different prokaryotic expression systems were unsuccessful, expression of a 34 kDa protein, corresponding to the size anticipated for Krox-26, was achieved using a pRSET A vector transfected into BL21 (DE3)LysS cells. The expressed protein was shown to be His-tagged Krox-26 using affinity-purified rabbit antibodies raised against a Krox-26 peptide and a monoclonal antibody to the His tag sequence. Moreover, the 34 kDa protein was shown to selectively bind DNA in the presence of zinc ions and, using a target detection assay, a consensus DNA binding sequence “5'-cAATg-3” identified. However, binding to this consensus sequence could not be demonstrated in Electrophoretic Mobility Shift (EMSA) assays.

Earlier attempts to express Krox-26 with other expression vectors and strains of bacteria failed to yield any recombinant protein past two rounds of induction. This phenomenon is generally observed in "leaky" expression systems if the expressed protein is toxic for the host cells. Thus, a tight regulation of Krox-26 expression before IPTG induction appears necessary for its successful expression. In the pRSET A vector, transcription of Krox-26 is mediated by T7 RNA polymerase, which is not found in the host cells. The *E. coli* BL21(DE3)pLysS strain offers two regulatory mechanisms that reduce leaky expression of recombinant His-tagged Krox-26 mediated by T7 RNA polymerase. These cells contain the DE3 bacteriophage lambda lysogen that allows the regulation of T7 RNA polymerase expression by an IPTG-inducible *lacUV5* promoter.
(Studier and Moffat, 1985). Furthermore, the BL21(DE3)pLysS strain expresses a low level of T7 lysozyme, which will degrade basal levels of T7 RNA polymerase (Studier et al., 1990). These features allow the expression of toxic proteins such as Krox-26. Once expression was induced, the bacteria were collected, lysed, and the lysate analyzed by SDS-PAGE. A 34 kDa protein, corresponding to the calculated molecular mass of His-Krox-26, was induced after the addition of IPTG. This 34 kDa protein was recognized by antibodies specific for Krox-26 and the 6xHis tag, and bound DNA in a zinc-dependent manner (Fig. 3-2).

Recombinant His-tagged Krox-26 protein was purified through Ni-NTA affinity chromatography (Fig. 3-3). However, the appearance of a second protein band of 28 kDa in addition to the 34 kDa was observed in the eluted fractions. Both 34 kDa and 28 kDa proteins bands were recognized by anti-Krox-26 and anti-His antibodies suggesting that the expression and/or purification process resulted in a C-terminally truncated recombinant His-tagged Krox-26 protein product. Thus, the full-length 34 kDa recombinant His-tagged Krox-26 protein was further purified by electro-elution and the electro-eluted protein used for the subsequent target detection assay.

Random sequence oligonucleotides of various lengths (TDA-9, TDA-15, TDA-21) were used for the target detection assay. Krox-26 contains five consecutive C2H2 (Krüppel-type) zinc fingers. Each finger in this class of proteins has been shown to bind to three bases in the major groove of dsDNA via three amino acids (Wolfe et al., 1999). Theoretically, Krox-26 should therefore recognize a 15 bp DNA target sequence. However, steric restraints often cause the zinc fingers to get "out of register" with the DNA helix after more than three zinc fingers are bound to DNA. Thus, if may be possible
that Krox-26 only utilizes three of its five zinc fingers to effectively bind DNA. However, it is also possible that an individual finger in Krox-26 will recognize sequences longer than 3 bp and that these individual recognition sequences are separated by one or more nucleotides. Krox-26 may therefore require more than 15 randomized bp to effectively scan for and bind to its recognition sequence.

An elution profile was determined (Fig. 3-5) and the conditions utilized for the five cycles of the target detection assay. The stringency of DNA elution was increased with each cycle by pre-elution washes with increased salt concentrations and phenanthroline. Phenanthroline is a chelator of zinc ions, which are required for the proper conformation of the zinc finger motifs. Thus, only the DNA that interacts strongly with Krox-26 was isolated. The increasingly stringent selection of bound DNA was supported by the need for increasing numbers of PCR amplification cycles in the 4th and 5th cycles. A sequence alignment of 40 sequences identified by the target detection assay showed that 25% of these sequences contained a short consensus sequence of 5'-cAATg-3'. Unfortunately, BLAST searches could not be utilized, as there is a minimum requirement of 8 nucleotides. However, the sequence 5'-cAATg-3' is similar to the consensus binding site of a CCAAT/enhancer binding protein (C/EBP), also known as "CCAAT box". Thus, there is a possibility that Krox-26 may interact with C/EBPs or compete for a similar binding site. Briefly, C/EBPs comprise a family of transcription factors that have been found to play critical roles in normal cellular differentiation and function in a variety of tissues (Lekstrom-Himes and Xanthopoulos, 1998). To date, C/EBPs have only been found to interact with its own family members and other transcription factors of the NF-κB and Fos/Jun families (Vinson et al., 1993). Interaction
of C/EBPs with Krüppel-type transcription factors or competition for DNA binding sites between these two proteins has not been documented.

To test if the sequence identified by the target detection assay, 5'-cAATg-3', could interact with Krox-26, EMSAs were performed with a synthesized oligonucleotide comprised of 5 tandem repeats of the CAATG sequence. A well-studied zinc finger transcription factor, Sp1, with its known target sequence was used as a positive control. Results showed an interaction between Sp1 and its target sequence, whereas interaction was not observed with Krox-26 and its target oligonucleotides (Fig. 3-7). TDA-15, an oligonucleotide containing 15 randomized nucleotides also showed interaction with Sp1 protein, whereas interaction was not observed with Krox-26. A theoretical target sequence of 5'-AGA AG/AA AAA AAA AAA-3' for Krox-26 protein was predicted based on the amino acid sequence of the 5 zinc fingers (Klug, personal communications). However, this oligonucleotide was unable to bind to the recombinant Krox-26 protein in EMSAs either (data not shown). The absence of DNA interaction with recombinant Krox-26 in EMSAs suggests that the binding affinity of Krox-26 to its target sequence may be too low to be detected via EMSA. Since Krox-26 binds DNA in zinc dependent manner when immobilized on nitrocellulose, a "matrix" may be necessary for it to bind to DNA. This matrix may be present in vivo in form of nucleosomes and the supramolecular nuclear matrix architecture, which is missing in EMSAs, precluding proper DNA binding in solution.

Since purified recombinant Krox-26 protein was extracted under denaturing conditions, refolding of its structure to native conformation is necessary for proper interaction of its zinc fingers to DNA. Recombinant Krox-26 protein in EMSAs may be
inactive due to improper folding of its zinc fingers structure in solution. Since the binding of immobilized Krox-26 protein to labeled dsDNA was observed in the presence of zinc, this suggests that the zinc finger structure was properly folded (Fig. 3-2). Thus, proper refolding of denatured recombinant Krox-26 protein may require some means of immobilization. To obtain successful results from EMSAs, nuclear extracts from mammalian cells over-expressing Krox-26 protein may be a better source of active protein compared to the purified protein from bacterially expressed sources due to the presence of cofactors from nuclear extracts that are necessary for proper functions of the protein.

Instability of the DNA-protein complex could also be due to binding buffer conditions, resulting in the formation of protein aggregates. To investigate this possibility, it was attempted to determine the molecular size of Krox-26 in solution by gel filtration using Sephadex G-100 (AP Biotech; data not shown). The Sephadex G-100 is able to separate proteins of molecular masses ranging from 5 kDa to 150 kDa. However, Krox-26 protein could not be recovered from the column, indicating that the protein may have been degraded during the chromatography process.

The unusually high theoretical isoelectric point (pI) of recombinant Krox-26 protein may be another reason for the failure to detect DNA-protein interaction by EMSAs. A high pI may cause the Krox-26 protein to carry a net charge that will compromise proper migration of the complex in the buffer system used for EMSAs. In this context, it should be noted that DNA interaction with transcription factors such as Msx-1 and Msx-2, which possess comparable pI values to recombinant Krox-26, and whose binding sites are known, have not been observed using EMSA. Several selected
transcription factors and their pl values are listed in Table 3-3. Note the unusually high pl of Krox-26, Msx-1, and Msx-2.

<table>
<thead>
<tr>
<th>Transcription Factors</th>
<th>Isoelectric Point</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ace-1</td>
<td>6.43</td>
</tr>
<tr>
<td>BCL-5</td>
<td>7.79</td>
</tr>
<tr>
<td>EGR-1</td>
<td>8.25</td>
</tr>
<tr>
<td>EGR-3</td>
<td>8.35</td>
</tr>
<tr>
<td>erythroid Krüppel-like factor EKLF</td>
<td>6.71</td>
</tr>
<tr>
<td>Gli-1</td>
<td>7.62</td>
</tr>
<tr>
<td>Gli-3</td>
<td>7.42</td>
</tr>
<tr>
<td>Recombinant 6xHisKrox-26 protein in pRSET A</td>
<td>9.96</td>
</tr>
<tr>
<td>Human EZF</td>
<td>8.53</td>
</tr>
<tr>
<td>Lef-1</td>
<td>6.99</td>
</tr>
<tr>
<td>Msx-1</td>
<td>10.51</td>
</tr>
<tr>
<td>Msx-2</td>
<td>9.95</td>
</tr>
<tr>
<td>Sp-1</td>
<td>7.96</td>
</tr>
<tr>
<td>WT-1</td>
<td>6.01</td>
</tr>
<tr>
<td>ZNF-207 (mouse)</td>
<td>9.36</td>
</tr>
<tr>
<td>ZNF-274</td>
<td>8.83</td>
</tr>
</tbody>
</table>

Table 3-3. Isoelectric points comparison for various transcription factors.

In summary, the recombinant 6xHis-tagged Krox-26 protein was successfully expressed and purified. A consensus target sequence of 5'-cAATg-3' was identified by the target detection assay. Although a BLAST search could not be performed on such a short sequence, the 5'-cAATg-3' is strikingly similar to the sequence targeted by the C/EBP family of proteins (5'-CCAAT-3'). Since Krox-26 is a transcription factor containing five C₂H₂ zinc fingers without any known repressor domains, it appears likely that Krox-26 functions as a transcriptional activator.
The studies described in this thesis have shown that recombinantly expressed Krox-26 protein shows selective binding to DNA in the presence of zinc ions. These properties are consistent with Krox-26 acting as a transcription factor that may be involved in the regulation of gene activity required for tooth formation (Chapter 3). However, attempts to show that Krox-26 itself may be regulated in a temporo-spatial manner by odontogenic growth factors, as part of the cascade of gene activations that regulate odontogenesis, were largely unsuccessful. Since the regulation of Msx-1 was not clearly demonstrated, it would appear that a combination of the systems used for these studies and the low amounts of material available for analyses have compromised these studies.

To more clearly demonstrate that Krox-26 is regulated during tooth morphogenesis, improved methodology and different strategies will need to be developed. While tooth germ cultures and cultures of embryonic mouse mandibles have been used successfully in other studies, the DPM cell line appears to have limited value for the analysis of Krox-26 regulation. DPM cells did not survive the transfer to differentiation condition media (data not shown) and no definitive response could be achieved with Msx-1, whose regulation has been well-documented in odontogenesis (Vainio et al., 1993; Chen et al., 1996). Despite their utility, the organs in culture systems do not develop properly in many cases and provide only small amounts of RNA for analysis. Increasing the number of explants could compensate the complications of organs to develop properly in vitro. The small amount of RNA that can be isolated from these cultures necessitates the use of RT-PCR, which is highly sensitive but has limited accuracy without the introduction of "mimics" to ensure uniform amplification (Cheifetz,
personal communications). The semi-quantitative RT-PCR approach used in the thesis generated variable results. It was therefore not possible to confirm a regulation of Krox-26 mRNA expression by BMP-2, BMP-4, FGF-4, or FGF-8.

The strategy applied in this thesis was a "candidate" approach and was used because the expression patterns of Krox-26 and BMPs or FGFs suggested a regulation by these molecules. A more systematic approach that can be used to identify regulators of the Krox-26 gene is to isolate and characterize the Krox-26 gene promoter. This can be achieved by screening a genomic mouse library using a mouse probe generated from the 5'-end of the cDNA. The promoter can be further characterized by primer extension analysis to determine the transcriptional start site and DNA sequencing to identify conserved binding sites for known transcriptional regulators. To confirm that the 5'-flanking region of Krox-26 is transcriptionally active, transient transfection assays can be performed in cells that express Krox-26. Briefly, the minimal or core promoter of Krox-26 can be determined by ligating various lengths of 5'-flanking region upstream of the luciferase reporter gene in pGL3-Basic plasmid (Promega), and the activity of luciferase measured; the luciferase activity in cell lysates corresponding to promoter activity. To map the promoter region, various truncated fragments of the 5'-flanking region can be generated by unidirectional Exonuclease III digestions (Ausubel et al., 1993). To identify transcription factors that regulate Krox-26 transcription, the sequence of the 5'-flanking region can be analyzed for the existence of consensus binding elements. Once putative regulatory proteins are identified, their effects on the transcriptional activity of Krox-26 can be measured using transient transfection assays in cells stimulated with the effector
molecule. In this way, the regulation of Krox-26, if any, by regulatory factors and the necessary elements in Krox-26 promoter can be determined.

The recombinant 6xHis-tagged Krox-26 protein was successfully expressed and purified. As a zinc finger transcription factor, Krox-26 is expected to bind to dsDNA. To identify target DNA sequence for Krox-26, target detection assay was performed and the resulting sequence of 5'-cAATg-3' was identified. The target sequence 5'-cAATg-3' is strikingly similar to the sequence targeted by the C/EBP family of proteins (5'-CCAAT-3'). To date, C/EBPs have only been found to interact with its own family members and other transcription factors of the NF-κB and Fos/Jun families (Vinson et al., 1993). Interaction of C/EBPs with Krüppel-type transcription factors or competition for DNA binding sites between these two proteins has not been documented.

To further characterize DNA binding by Krox-26, an analysis of mutations in the zinc fingers of the Krox-26 protein can be performed using various oligonucleotides primers. Since Krox-26 contains five consecutive C2H2 zinc finger motifs, mutation to the either histidine or cysteine residues involved in coordinating zinc in will result in a distortion of the general conformation of the zinc finger (Redemann et al., 1988). Target detection assay and electrophoretic mobility shift assay can then be performed using the mutated Krox-26 protein. Thus, the analysis of mutations in the zinc fingers of Krox-26 protein can provide rules that are more specific for its DNA binding properties.

While a potential consensus sequence for Krox-26 binding has been identified by TDA, the length of the sequence is insufficient to screen for potential target genes using data base screening. A more systematic strategy to identify target genes would be to overexpress Krox-26 in eukaryotic cells and determine the changes in overall gene
expression that are caused by Krox-26. This is best achieved using an inducible system such as a tetracycline-regulated gene expression system (Gossen and Bujard, 1995). This system permits Krox-26 gene expression to be regulated by the addition of doxycycline, a derivative of tetracycline. After the induction of Krox-26 expression, total RNA can be isolated and various affected genes, up- or down-regulated by Krox-26, expression can be screened for known genes by Northern hybridization and unknown genes using differential screening methods such as differential display or cDNA microarray analyses.
References


