Cloning and Characterization of the Murine Ameloblastin Promoter*

Sangeeta Dhamija‡, Ying Liu§, Yoshihiko Yamada¶, Malcolm L. Sneed¶, and Paul H. Krebsbach‡‡

The molecular mechanisms directing the highly restricted expression pattern of murine ameloblastin were characterized by cloning and functional analysis of the ameloblastin promoter. The transcription start site, mapped by primer extension, was located 19 base pairs (bp) 5' of the published cDNA. The promoter was analyzed in a mouse ameloblast-like cell line (LS8) and was compared with promoter activity in primary gingival fibroblasts and pulp fibroblasts. Sequential 5'-deletion mutants encompassing DNA sequences from −1616 to −781 bp exhibited high promoter activity in LS8 cells, whereas the promoter activity decreased to 50% of the full-length construct in the −781- and −477-bp regions. The −217-bp promoter region regained promoter activity that approached the activity of the full-length promoter construct, suggesting that both positive and negative cis-acting regions may be involved in ameloblastin transcriptional regulation. Activity of the ameloblastin promoter in gingival and pulp fibroblasts was minimal and ranged from 8 to 30% of the activity in ameloblast-like cells. Several DNA-protein complexes were formed between functionally important promoter fragments and nuclear extracts from LS8 cells. The inactivity of promoter constructs in pulp and gingival fibroblasts as well as the absence of similar DNA-protein complexes from these cells suggest that regulatory regions of the murine ameloblastin promoter may function in a cell-specific manner.

The developing mammalian dentition provides a valuable model system for investigating tissue-specific gene regulation, morphogenesis, and biomineralization. Tooth development is dependent on the coordinated expression of many genes, some of which are unique to the developing tooth (1, 2). Ameloblastin is one of this group of tooth-specific genes that displays a unique and specific developmental expression pattern. Ameloblastin is principally expressed in the enamel-producing ameloblasts and is present in the developing enamel matrix (3, 4). The initial cloning, immunolocalization, and chromosomal mapping studies revealed that the rat ameloblastin gene encodes an open reading frame of 422 amino acids corresponding to a putative protein of 45 kDa (5). In humans, ameloblastin maps to chromosome 4q21 in a locus that is linked to an autosomal dominant form of amelogenesis imperfecta, a disease that adversely affects enamel formation and function (6). Thus, ameloblastin is considered a candidate gene for this inherited defect in humans.

High resolution electron microscopy provides evidence that ameloblastin accumulates near the crystal growth sites in developing enamel (4). The nascent ameloblastin protein is hypothesized to play a role in enamel crystal formation at these sites by a poorly understood mechanism that involves the rapid processing of ameloblastin to lower molecular weight fragments deeper within the tissue (4, 7). Ameloblastin is also transiently expressed in pre-odontoblasts prior to the initiation of amelogensis (8, 9). However, once amelogensis is initiated, ameloblastin expression is terminated in the dentin-producing odontoblasts and continues to be strongly expressed in ameloblasts. A converse phenomenon is observed with the odontoblast-enriched gene dentin sialophosphoprotein. Dentin sialophosphoprotein, once considered to be expressed exclusively by odontoblasts, has recently been determined to be also transiently expressed by ameloblasts prior to odontoblast differentiation (10). Taken together, these observations suggest that tooth-specific matrix genes including ameloblastin and dentin sialophosphoprotein may function as signaling molecules between ameloblasts and odontoblasts in initiating enamel and dentin development in addition to their primary role in biomineralization.

Isolation and functional characterization of transcriptional regulatory elements are prerequisites for understanding cell type-specific gene expression. The focus of this paper is upon the ability of the ameloblastin promoter to respond to developmental signals that regulate the expression of ameloblastin, a protein believed to be essential to the formation and function of the enamel extracellular matrix. Here we describe the cloning, sequencing, and functional analysis of the murine ameloblastin promoter in several odontogenic cell lines. Transfection analyses suggest that ameloblast-like cells express the trans-acting factors necessary to direct transcription of ameloblastin. In contrast, gingival fibroblasts and pulp fibroblasts lack the necessary transcriptional machinery needed for ameloblastin expression, suggesting that regulatory regions of the murine ameloblastin promoter may function in a cell-specific manner.

**EXPERIMENTAL PROCEDURES**

Isolation of the Murine Ameloblastin 5'-Flanking Region—The murine 129-strain genomic library cloned in the Lambda FIX II vector (Stratagene, La Jolla, CA) was screened with a 1.9-kb1 ameloblastin

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*This work was supported by National Institutes of Health Grant R29 DE12502 from the NIDCR and the University of Michigan, Office of the Vice President for Research (to P. H. K.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡Recipient of National Institutes of Health Independent Scientist Award R02 DE00426 sponsored by NIDCR. To whom correspondence should be addressed: Dept. of Oral Medicine, Pathology, and Oncology, School of Dentistry, Rm. 4207, University of Michigan, Ann Arbor, MI 48109-1078. Tel.: 734-764-1543; Fax: 734-764-2469; E-mail: paulk@umich.edu.

‡‡Printed in U.S.A.
cDNA probe to obtain genomic clones of murine ameloblastin. The probe was labeled with \( {\alpha}^{32}P \) dATP (3000 Ci/ml) to a specific activity of \( >1 \times 10^7 \) dpm/µg using a random primer DNA labeling kit (Stratagene). Plaque lifting, prehybridization, hybridization, washings of the filters, and autoradiography were performed either according to the manufacturer's protocol of Chemical Research (11). Putative positive clones were purified by secondary and tertiary rounds of screenings, and genomic DNA inserts were subcloned into pCR\( ^{TM} \) II plasmid vectors (Invitrogen, Carlsbad, CA) for restriction mapping and sequencing. To identify 5′-flanking regions containing clones, the positive clones were PCR-amplified using a vector-specific (T7) probe and a gene-specific (L18) primer labeled with T4 polynucleotide kinase (Invitrogen, Carlsbad, CA) and designed p2544. Sequencing of both strands of plasmid p2544 using vector-specific M13 reverse and M13 forward primers revealed that it contained about 100 bp of the 5′-untranslated region of exon 1 as well as the translation start codon (see Fig. 1). A 1.6-kb fragment 10 bp upstream of the translation start site was generated by SstI digestion. This fragment was subcloned into the Smal site of the promoterless firefly luciferase reporter gene vector pGL3-Basic (Promega) in both the sense and antisense orientations to obtain plasmids pSD069 and pSD070, respectively. Sequencing was performed on both strands of the reporter plasmids using Luc+ vector primers RV3 and GL2, followed by nested primers on both strands. Sequencing data were analyzed and assembled using MacVector\( ^{TM} \) 6.0 and AssemblyLIGN software (Oxford Molecular Limited).

**Construction of Deletion Mutant Ameloblastin Constructs—**Progressive 5′-deleted deletions were made using exonuclease III and S1 nuclease (Erase-a-Base, Promega). Briefly, plasmid pSD070 was digested with SacI and NheI, generating 3′ and 5′ extensions, respectively. DNA was extracted with phenol/chloroform and was digested with exonuclease III (USB). Putative positive clones were purified by secondary and tertiary rounds of screenings, and genomic DNA inserts were subcloned into pCR\( ^{TM} \) II plasmid vectors (Invitrogen, Carlsbad, CA) for restriction mapping and sequencing. To identify 5′-flanking regions containing clones, the positive clones were PCR-amplified using a vector-specific (T7) probe and a gene-specific (L18) primer labeled with T4 polynucleotide kinase (Invitrogen, Carlsbad, CA) and designed p2544. Sequencing of both strands of plasmid p2544 using vector-specific M13 reverse and M13 forward primers revealed that it contained about 100 bp of the 5′-untranslated region of exon 1 as well as the translation start codon (see Fig. 1). A 1.6-kb fragment 10 bp upstream of the translation start site was generated by SstI digestion. This fragment was subcloned into the Smal site of the promoterless firefly luciferase reporter gene vector pGL3-Basic (Promega) in both the sense and antisense orientations to obtain plasmids pSD069 and pSD070, respectively. Sequencing was performed on both strands of the reporter plasmids using Luc+ vector primers RV3 and GL2, followed by nested primers on both strands. Sequencing data were analyzed and assembled using MacVector\( ^{TM} \) 6.0 and AssemblyLIGN software (Oxford Molecular Limited).

**Results—**

**Cloning and Sequencing of Ameloblastin 5′-Flanking Sequence—**A murine 129-strain genomic library was screened with a full-length rat ameloblastin cDNA. Several overlapping clones were identified that collectively spanned the ameloblastin coding region and included a 5′-flanking sequence. In this study, a λ genomic DNA clone (SD99) that contains 2.5 kb of immediate 5′-flanking ameloblastin sequence was analyzed. Clone SD99 was verified by PCR analysis using two nested minus DNA strand primers and a T7 primer complementary to sequence in the λ vector arm (Fig. 1). A 2.5-kb fragment was subcloned into the pCR\( ^{TM} \) II vector, and the orientation was verified by sequencing both DNA strands (Fig. 2).

**The murine ameloblastin promoter contains several putative cis-acting regulatory elements including AP-1, TCF-1, CACC binding sites, and sites for two zinc finger proteins, CF2-II and Krox-20 (14, 15). The Bovine Retinoblastoma virus (BRV) provides two potential osteoblast specific element 2 sites known to be functional in other mineralized tissues such as bone and mesenchymal condensation regions involved in early chondrogenesis (16).** One AP-1 site and two osteoblast specific element 2 sites were identified in a reverse and complementary orientation. A canonical TATA box was not identified, although a TAAATATAA motif is located upstream of the transcription start site.
The full-length promoter construct (−1616/+57) was consistently highly expressed in LS8 cells. Progressive 5′-deletion mutations of the full-length promoter revealed a bimodal pattern of functional activity in transfected LS8 cells (Fig. 5). A progressive reduction in promoter activity was detected as the 5′-deletion mutations approached −477 bp, relative to the transcription start site. Further deletions from −477 to −217 bp lead to an increase in promoter activity approaching that of the full-length promoter. Deletions to −50 bp lead to near complete ablation of promoter activity in LS8 cells. In striking contrast, no significant or differential expression between ameloblastin constructs was observed in ameloblastin transfections into either human gingival fibroblasts or human pulp fibroblasts (Fig. 5). Transfections of the ameloblastin promoter constructs into gingival fibroblasts and pulp fibroblasts showed minimal promoter activity that was between 8 and 30% of the activity in LS8 cells. Furthermore, no notable differences were observed between deletion mutant constructs in gingival or pulp fibroblasts that do not express ameloblastin.

Identification of Specific DNA-Protein Complexes from Ameloblast-like Cells—The transient transfection data indicated that both positive and negative regulatory elements exist within the murine ameloblastin promoter. Additionally, the differential expression of ameloblastin promoter constructs between cells derived from different oral tissues suggested that the gingival and pulp fibroblasts lacked the necessary trans-acting factors to activate the ameloblastin promoter constructs. To explore whether DNA-protein complexes differed between ameloblastin-expressing and non-expressing cells, electrophoretic mobility shift assays were performed using oligonucleotide probes generated from functionally important regions of the ameloblastin promoter and nuclear extracts derived from LS8 and pulp fibroblasts. As shown in Fig. 5, the single largest decrease in promoter activity was observed when promoter sequences between −217 and −100 were deleted. EMSA experiments using double-stranded oligonucleotide probes spanning a portion of this region illustrate clear differences in putative transcription factor binding in this region (Fig. 6, A–C). Distinct DNA-protein complexes were identified with extracts from LS8 cells and pulp fibroblasts, suggesting that differences in trans-acting factors between the two cell types dictate the cell-specific promoter activity observed in ameloblast-like cells. Further evidence for the specificity of putative transcription factor binding is shown in Fig. 7 where DNA-protein complexes are diminished by the presence of low level excess unlabeled probe.

**DISCUSSION**

To initiate studies directed at identifying signaling pathways involved in the complex development of the mammalian dentition, we have isolated, sequenced, and characterized the functional activity of the murine ameloblastin promoter in cells derived from three different oral tissues. A single transcription start site was mapped, and promoter constructs containing up to 1600 bp of 5′-flanking sequence were active in ameloblast-like LS8 cells but were inactive in non-ameloblastin-expressing gingival and pulp cells. Promoter activity was altered in LS8 cells by progressive 5′-deletion mutations and was characterized by a bimodal pattern of activity. We found that deletions from −1616 to −477 bp produced a gradual decrease in promoter activity. However, further deletion to −217 bp restored full promoter activity. These data suggest that transcriptional repressive element(s) exist between −477 and −217 bp of the ameloblastin promoter. However, significant promoter activity was detected between −217 and −100, suggesting that this region contains information necessary for cell type-specific transcription in cultured cells.

Despite the recent identification of specific transcription factor and growth factor function in early tooth morphogenesis, the targets of many of these factors and their ability to influence the expression of specific extracellular matrix proteins of the tooth are unknown (17–20). Both the enamel and dentin are formed by the elaboration of a tissue-specific extracellular matrix that directs the orientation of the inorganic hydroxyapatite crystallites that, in turn, affect their biomechanical prop-
appropriate. The first exon sequence is in italics dashed underline transcription initiation site (g
cDNA, was end-labeled with [TCC C-3]
nucleotide primer L-18 (5
by primer extension analysis. For the primer extension reaction, oligo-
murine ameloblastin gene. The transcription start site was mapped
isms by which cell-signaling factors influence transcriptional
growth, and termination.
ities (21). It is critical, therefore, to control when and where
enamel-specific proteins are expressed and secreted into the
matrix and where they regulate interactions during the process
of biomineralization (22, 23). The biomechanical properties of
enamel are a summation of the regulated expression of struc-
tural genes required for enamel formation. Thus, identifying
the ameloblastin promoter, it is possible that new factors or
unique combinations of known factors direct the highly re-
stricted expression of ameloblastin to the developing tooth.
Ameloblastin Promoter Characterization

FIG. 2. Immediate 5’-flanking nucleotide sequence of the murine ameloblastin gene. The numbering of nucleotides starts at the
transcription initiation site (+1), which is indicated by a bent arrow. Each core motif is underlined, with an arrow to indicate the orientation where
appropriate. The first exon sequence is in italics. Oligonucleotides corresponding to the promoter sequence used for EMSA are designated with a
dashed underline. The remainder of the 5’-flanking sequence is entered in GenBank™ with accession number AF126544.

FIG. 3. Determination of the transcription start site of the murine ameloblastin gene. The transcription start site was mapped by primer extension analysis. For the primer extension reaction, oligo-
nucleotide primer L-18 (5’-CTT AGA TGC TGA CAT TCA CTG TGC TGC TCC C-3’), complementary to nucleotides of the published ameloblastin
cDNA, was end-labeled with [γ-32P]ATP and hybridized with 10 μg of
total RNA from mouse incisor or murine bone marrow stromal cells.
Lanes 1–4, the nucleotide of the sequencing reaction using primer L-18 and a genomic clone p2544; lane 5, primer extension reaction with no
RNA added; lane 6, primer extension with murine incisor RNA; lane 7, primer extension using murine bone marrow stromal cell RNA. The
arrow designates the primer extension product, and the asterisk indicates the nucleotide position of the transcription start site.

FIG. 4. Expression of ameloblastin mRNA in cell lines. cDNA was generated from total RNA derived from LS8 and gingival fibro-
blasts and was amplified by PCR. Primers spanned intron/exon bound-
aries (see “Experimental Procedures”) and were used to amplify a product of 691 bp (arrow). Ameloblastin is expressed in LS8 cells (lane 3) but not in gingival fibroblasts (lane 4). Lane 1, 500-bp molecular weight ladder; lane 2, PCR product using plasmid Y224 (ameloblastin cDNA) as the template; lane 3, PCR product using LS8-derived cDNA as the template; lane 4, gingival fibroblast cDNA as the template; lane 5, no cDNA template control; lane 6, 250-bp molecular weight ladder.

FIG. 5. Differential ameloblastin promoter activity in vitro. A, partial restriction map of the 5’-flanking region of the murine amelo-
blastin gene. The vertical dashed line designates the 3’ end of the ameloblastin sequence at nucleotide position +57, relative to the transcrip-
tion start site. B, schematic representation of the ameloblastin reporter constructs used in transient transfection analysis of promoter
activity in three different cell lines derived from oral tissues. The ameloblastin luciferase constructs were co-transfected with a control
plasmid (pRLTK) and assayed 48 h posttransfection. Percent luciferase
activity elicited by each deletion mutant is expressed as a percentage of the
activity obtained by the control plasmid. The open bars designate
activity in pulp fibroblasts, the gray bars designate activity in gingival fibroblasts, and the black bars designate activity in LS8 cells. Error bars represent the standard error for three samples in at least four independent experiments.

Our isolation and characterization of a functional ameloblas-
tin promoter will facilitate new investigations into the mecha-
nisms by which cell-signaling factors influence transcriptional
events of this tooth-specific gene. The presence of common DNA
sequences within promoter and enhancer elements of different
genes makes interpretation of developmental and tissue-spe-
cific gene expression quite complex. Because only a limited
number of cis- and trans-acting factors are defined, diversity in
gene expression is likely because of differences in promoter
context. Variations in promoter context may be the result of
temporal or tissue-specific expression of transcription factors.
Although several common regulatory motifs were identified in
the ameloblastin promoter, it is possible that new factors or
unique combinations of known factors direct the highly re-
stricted expression of ameloblastin to the developing tooth.
The observation that the ameloblastin promoter constructs displayed a bimodal pattern of activity indicates that both
positive and negative transcriptional events participate in the
regulation of the murine ameloblastin promoter. These ele-
ments likely function exclusively within the context of this
specialized cell type. As an initial test of this hypothesis, we
compared the DNA binding ability of nuclear extracts from LS8
and pulp fibroblasts. In these EMSA experiments, different DNA-protein complexes formed between functionally important promoter regions and putative transcription factors from both the LS8 and pulp fibroblasts. The distinct complexes formed with LS8 extracts may be because of the presence of more abundant positive transactivating proteins in the LS8 cells. Alternatively, negative transactivating factors or more general factors in the absence of positive factors may be contributing to the complexes formed in pulp fibroblasts.

The murine and bovine amelogenin promoters have also been characterized (24, 25) and together with the ameloblastin promoter characterized in this study provide valuable molecular tools required to define the genetic hierarchies involved in organogenesis of the tooth as well as contribute toward the basic understanding of cell-specific transcriptional mechanisms. However, because our current knowledge of possible cooperative or synergistic interactions between transcription factors in the developing tooth is incomplete, it is conceivable that additional regulatory elements may be identified in the regulation of ameloblastin. Likewise, because the function of transgenes in transfected cells does not always mimic the expression in the complex milieu of a developing organ (26, 27), ameloblastin transgene expression must also be tested in the context of a developing animal.

Acknowledgments—We thank Dr. R. Bruce Rutherford and Renny Franceschi for helpful discussions and critical reading of the manuscript and Dr. Rutherford for providing the gingival and pulp fibroblasts.

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