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Origin, Splicing, and Expression of Rodent Amelogenin Exon 8

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Abstract

Amelogenin RNA transcripts undergo extensive alternative splicing, and MMP-20 processes the isoforms following their secretion. Since amelogenins have been ascribed cell-signaling activities, we asked if a lack of proteolytic processing by MMP-20 affects amelogenin signaling and consequently alters amelogenin splice site selection. RT-PCR analyses of amelogenin mRNA between control and *Mmp20*^{-/-} mice revealed no differences in the splicing pattern. We characterized 3 previously unidentified amelogenin alternatively spliced transcripts and demonstrated that exon-8-encoded amelogenin isoforms are processed by MMP-20. Transcripts with exon 8 were expressed approximately five-fold less than those with exon 7. Analyses of the mouse and rat amelogenin gene structures confirmed that exon 8 arose in a duplication of exons 4 through 5, with translocation of the copy downstream of exon 7. No downstream genomic sequences homologous to exons 4-5 were present in the bovine or human amelogenin genes, suggesting that this translocation occurred only in rodents.

Keywords

enamelysin; enamel; amelogenin

Introduction

Secretory-stage ameloblasts are responsible for many important processes necessary for enamel formation, including: alternative mRNA splicing, translation and secretion of enamel proteins (*i.e.*, amelogenin, ameloblastin, enamelin, MMP-20), selective reabsorption and degradation of cleavage products, establishment and regulation of the influx of calcium and phosphate ions, and maintenance of extracellular pH and ionic strength (Simmer and Fincham, 1995; Fincham *et al.*, 1999). Disruptions of any one of these processes may cause enamel defects. For example, the *Mmp20* null mouse and human kindreds with critical *MMP20* mutations have brittle enamel that tends to delaminate from the dentin surface (Caterina *et al.*, 2002; Kim *et al.*, 2005; Ozdemir *et al.*, 2005). It is believed that ameloblasts respond to changes in the extracellular matrix by adjusting their activities to maintain conditions appropriate for biomineralization (Xu *et al.*, 2006).

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The most abundant enamel protein is amelogenin, which is essential for proper enamel formation (Hart *et al.*, 2002; Wright *et al.*, 2003; Kim *et al.*, 2004). However, the roles of specific alternatively spliced amelogenins (Gibson *et al.*, 1991; Lau *et al.*, 1992; Salido *et al.*, 1992) during enamel development remain unclear. In rodents, amelogenin undergoes extensive alternative splicing, and at least 13 different isoforms have been previously characterized in rats (R) and mice (M). The major amelogenin mRNA encodes an amelogenin protein of 180 amino acids (M180) (Snead *et al.*, 1983, 1985). Other alternatively spliced mouse amelogenin transcripts include: M156, M141, M74, M59 (Lau *et al.*, 1992), M194, M44 (Simmer *et al.*, 1994), M170, and M73 (Hu *et al.*, 1997). These splice products are all derived from amelogenin mRNA transcripts ending in exon 7. Additional alternatively spliced amelogenin mRNAs ending in exons 8 and 9 were identified in the rat, including R203, R179, R82, and R57 (Li *et al.*, 1995, 1998). Similar transcripts have recently been identified in mice (Papagerakis *et al.*, 2005). However, the roles of exons 8 and 9 remain an enigma, because extensive characterizations of pig enamel extracts (Yamakoshi *et al.*, 1994, 2004) have not identified amelogenin C-termini homologous to the rodent exon 8-9 encoded segment. To assess the conservation of exons 8 and 9 among mammalian species, we analyzed mouse, rat, bovine, and human amelogenin genomic sequences downstream of exon 7.

We also asked if a relationship exists between amelogenin alternative splicing and proteolysis. If amelogenin cleavage products serve as signaling molecules, as has been proposed to explain the therapeutic effects of Emdogain (Hammarström *et al.*, 1997), and/or if ameloblasts adjust their activities based on the presence of the protein component within the matrix (Xu *et al.*, 2006), then proteolysis within the matrix might affect ameloblast protein expression. To determine if the regulation of alternative splicing by ameloblasts is altered by a failure of MMP-20 to cleave enamel matrix proteins, we compared spliced amelogenin mRNA transcripts and SDS-PAGE enamel protein profiles in mouse molars from control and *Mmp20* null mice. We also raised anti-peptide antibodies to the exon-8-encoded sequence and analyzed differences in cleavage patterns of exon-8-containing amelogenins in normal and enamelysin null mouse molars.

Materials & Methods

The Animal Care Committee of The Forsyth Institute approved the protocol for the handling, care, and usage of animals.

SDS-PAGE

First molars were dissected from *Mmp20*^{+/-} and *Mmp20*^{-/-} mice of specified ages, and all non-mineralized tissues were removed. Care was taken to maintain the samples free of blood contamination. The mineralized developing tooth was placed into gel-loading buffer containing dithiothreitol, bromophenol blue, glycerol, sodium dodecyl sulfate (SDS), and Tris HCl. Electrophoresis was performed with 15% SDS gels, followed by silver-staining (Amersham Biosciences, Piscataway, NJ, USA).

RT-PCR & qPCR

Primer sets (Table 1) were designed by analysis of annealing sites by DNASTar software (Madison, WI, USA). PCR amplifications were performed (at 95°C for 4 min; 30 cycles at 95°C for 45 sec, at 50-65°C for 45 sec, at 72°C for 45 sec; at 72°C for 6 min), and products were separated on a 1% agarose gel. Amplified cDNAs were purified (Qiagen, Valencia, CA, USA) and sequenced. Primers for qPCR analysis of amelogenin expression were: 5'*Amelx* exon-7 (5'-TGGCCAGCGACAGACAA-3'); 3'*Amelx* exon-7 (5'-TATTCCTAAAAACAACCAAAGTATCC-3'); 5'*Amelx* exons 8 & 9 (5'-CTGTCCCCCATTCTTCCT G-3'); 3'*Amelx* exons 8 & 9 (5'-

TGCCCTGGTACCACTTCATAG-3'); 5'EF1 α 1 (5'-AATCCGGCAAGTCCACCACCA-3'); and 3'EF1 α 1 (5'-CATCTCAGCAGCCTCCTTCTCAAA-3'). The PCR temperature profile was: 3 min at 95°C initial melt; then 20 sec at 95°C, 30 sec at 60°C for 45 cycles, then 30 sec at 95°C for 1 cycle, and 1 min at 55°C, followed by stepwise temperature increases from 55°C to 95°C, to generate the melt curve. With each primer set, we generated standard curves using untreated control cDNA preparations and a 10-fold dilution series ranging from 1000 ng/mL to 100 pg/mL. PCR efficiencies and relative expression levels of amelogenin as a function of eEF1 α 1 expression were calculated as previously described (Pfaffl, 2001).

Western Blot Analysis

Exon-8-encoded peptide (CAFSPMKWYQGMTRHP) was synthesized and conjugated to the carrier protein KLH. Antibodies were generated in rabbits by 4 immunizations. Serum was purified by protein G affinity chromatography (Pierce Biotechnology, Rockford, IL, USA). After electrophoresis, proteins were transferred to nitrocellulose membranes. The membranes were incubated with exon-8-specific serum overnight at room temperature. Amelogenins encoded by exon 8 were identified by incubation with secondary antibodies labeled with horseradish peroxidase, followed by processing for chemiluminescence (Amersham, Piscataway, NJ, USA).

Results

Identification of Novel Mouse Amelogenin Alternatively Spliced mRNA Transcripts

We identified and sequenced 3 novel mouse amelogenin RT-PCR products by use of primer 5'Exon 4w/6bc paired with 3'Exon 9. If we assume, as is the case for every other known mouse amelogenin transcript, that these new transcripts also contain exons 1-3, then the new amelogenin transcripts encode proteins containing 217 (M217), 193 (M193), and 96 (M96) amino acids. This brings to 16 the number of amelogenin alternatively spliced mRNA transcripts in rodents. A summary of these transcripts—including their exon structure, encoded amino acids, protein molecular mass, and protein isoelectric point—is provided in Table 1. All calculations omit the signal peptide and include the Ser¹⁶ phosphate.

A Comparison of Amelogenin Alternative Splicing between Controls and *Mmp20*^{-/-} Mice

We designed PCR primer sets (Table 1, bottom) to amplify specific amelogenin splice forms for the purpose of identifying differences in splicing between control and *Mmp20* null mice. We used primer sets to amplify cDNA from first molars of three-day-old mice (Table 2). RT-PCR analysis allowed us to identify 6 of the 13 previously identified alternatively spliced transcripts (Table 2). No differences in splicing were observed between controls and *Mmp20* null mice.

Next, to characterize the relative expression levels of amelogenin transcripts that contain exon 7 vs. those transcripts that contain exons 8 and 9, we performed qPCR on total RNA from first molars extracted from three-, five-, seven-, nine-, and 11-day-old mice. These teeth were in the secretory stage (days 3 & 5), secretory-early maturation stage (day 7), and maturation stages (days 9 & 11) of development. As expected, the overall expression of amelogenin transcripts decreased as development progressed to the maturation stage. The exon-7-containing transcripts were expressed at approximately five-fold-higher levels than the exons-8- and -9-containing transcripts (Fig. 1A).

***In silico* Analysis of Exon 8 and Surrounding Genomic Sequence**

We performed a mouse genome BLASTN search with 3907 bp from the mouse amelogenin gene (GenBank accession NT_039718.4), immediately downstream of exon 7 and extending to the precise end of exon 9. Two Blast hits were observed. The first was identical to the query sequence, and the second showed 91% (191/209) identity, with no gaps, to another region of mouse *Amelx* that included exons 4 and 5 (Fig. 1B). Sequence analysis demonstrated that exon 4 aligned with an identical sequence downstream of exon 7. We designated this second sequence exon 4b. The deduced amino acid sequences for mouse exon 4 and exon 4b were identical: KSHSQAINTDRTAL. Following 96 nucleotides of homologous intron sequence, exon 5 exactly aligned with exon 8. Over half (8/15) of the 15 amino acids encoded by exons 5 and 8 were identical (Fig. 1C). Interestingly, the 7 amino acid changes in mouse exon 8 vs. exon 5 arose from just 8 nucleotide differences (compare Figs. 1B and 1C). A similar result was obtained when the homologous region of rat *Amelx* was analyzed (GenBank accession NW_048039.1). These results essentially confirm what has recently been reported elsewhere (Li *et al.*, 2006). To expand upon these results, we used BLASTN searches to identify amelogenin exon 8 in other non-rodent species. No second region homologous to the exon-4 exon-5 region was identified in the bovine or human amelogenin genes. No exon 8 sequences were identified in the numerous amelogenin cDNA sequences in the non-redundant NCBI database. These findings suggest that exon 8 was generated from exon 5 in a rodent ancestor by the duplication and downstream insertion of a 200- to 300-bp *Amelx* segment containing exons 4 and 5. We speculate that this duplication activated a downstream sequence that became exon 9.

Developmental Assessment of Amelogenins in Control and *Mmp20*^{-/-} First Molars

To assess how the protein component of the enamel matrix changed as enamel development progressed, we performed SDS-PAGE analysis on proteins extracted from first molars from three-, five-, nine-, and 11-day-old control or *Mmp20*^{-/-} mice (Fig. 2A). Three-day-old control mice (*Mmp20*^{+/+}) had 2 prominent bands beneath the 19.2-kDa marker that were missing from the corresponding *Mmp20*^{-/-} lane (Fig. 2A, three-day). These bands persisted in the controls through the maturation stage at day 9 and were degraded by day 11. Enamel proteins from the *Mmp20*^{-/-} mice had a strong band beneath the 24.7-kDa marker that was not present in the controls. This band began to weaken by day 9, but was still present in enamel matrix proteins extracted from the 11-day-old *Mmp20*^{-/-} mice (Fig. 2A, 11-day). Presumably, the secreted, alternatively spliced proteins were not significantly degraded in *Mmp20*^{-/-} enamel until secretion of Kallikrein-4 during the later stages of enamel development.

Western Blot of Proteins Extracted from *Mmp20*^{+/+} and *Mmp20*^{-/-} Day 3 Molars

To identify amelogenin protein expressed from transcripts containing exons 8 and 9, we used an exon-8-specific antiserum (Fig. 2B) to visualize extracted mouse molar proteins. Western blot analysis of amelogenin from *Mmp20*^{+/+} and *Mmp20*^{-/-} molars revealed the presence of at least 3 different amelogenins between 20 and 30 kDa (Fig. 2C). The *Mmp20*^{+/+} lane had an additional band of approximately 18 kDa that likely resulted from hydrolysis by MMP-20. The apparent mobilities and relative quantities of the 3 upper bands in the *Mmp20*^{-/-} lane are consistent with the presence of uncleaved M217, M203, and M179. These bands might be equivalent to the 3 bands previously observed in exon 9 Western blots of rat enamel matrix (Baba *et al.*, 2002). Overall, the amelogenin bands from the *Mmp20*^{+/+} lane appeared to shift downward by 1-2 kDa, relative to the bands present in the *Mmp20*^{-/-} lane. This is consistent with cleavage of these amelogenin isoforms by MMP-20 in a way that released only a short peptide, but did not destroy the epitope for the exon 8 antibody. Since enamelysin typically processes amelogenin from its C-terminus, we suspect that the exon-9-encoded sequence was excised.

Discussion

Sixteen different alternatively spliced amelogenin mRNA transcripts have now been characterized in rodents. All amelogenin mRNAs contain exons 1 through 3, and at least a part of exon 6. Exon 4, when present, is always upstream of exon 6 sequences, so exon 4b, which is downstream of exon 7, is always deleted during splicing. Seven of the amelogenin transcripts contain a novel C-terminus encoded by exons 8 and 9. Exons 8 and 9 are always paired; no transcripts have only exon 8 or only exon 9. Furthermore, exon 7, which encodes only 1 amino acid prior to a stop codon, is never found in the same transcript as exons 8 and 9. Previously, Li *et al.* (1998) predicted that all amelogenin mRNA transcripts ending with exon 7 might alternatively end with exons 8 and 9. Our results lend support to this claim.

The amelogenin alternatively spliced transcripts are expressed by secretory-stage ameloblasts, translated into their corresponding amelogenin isoforms, and secreted together into the enamel matrix. However, the various amelogenin isoforms are not secreted in equal quantities. In fact, most of the amelogenin variants are expressed in minute amounts and have never been detected in enamel protein extracts. Since all of the splice junctions in the amelogenin gene are phase 0 (occur between codons), skipped or included exons always maintain the appropriate reading frame for the downstream exons. While the primary sequences of the predominant amelogenins are highly conserved, the patterns of alternative splicing that generate the less abundant amelogenin isoforms vary widely among mammalian species (Simmer and Snead, 1995). In humans, there are also 2 non-allelic amelogenin genes on the sex chromosomes (Lau *et al.*, 1989), a pattern that is found in many mammalian species. Proteolytic processing increases the heterogeneity further (Bartlett and Simmer, 1999; Simmer and Hu, 2002).

The mouse exon-8-deduced amino acid sequence was compared with the exon-5-deduced amino acid sequences from various species (Sire *et al.*, 2005). Although the gene duplication that generated exon 8 was relatively recent (since the divergence of rodents and human, but before the splitting of mouse and rat), the exon-8-deduced protein sequence has even more amino acid substitutions than does the marsupial (opossum) exon-5-encoded sequence, which diverged much earlier and therefore had more time to mutate. Perhaps the few exon 8 mutations that significantly changed the amino acid sequence were positively selected to reduce interference with the function of the exon-5-encoded region.

Here, we provide evidence showing that amelogenin exon 8 arose from a relatively recent duplication of exon 5, and that exon 8 is present only in rodent amelogenin genes. We identified 3 previously undetected amelogenin isoforms containing the exon-8- and -9-encoded C-terminus, and we determined that no qualitative differences in amelogenin alternative splicing are evident in the *Mmp20*^{-/-} null mouse relative to controls. The relative simplicity of the enamel matrix in the *Mmp20*^{-/-} null mouse was demonstrated, which suggests that analysis of the amelogenin proteins in the null mouse may provide insights into the relative abundance of the various amelogenin isoforms. Finally, we demonstrated that enamelysin processes exon-8-containing amelogenins.

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Figure 1.

Relative abundance of amelogenin transcripts containing exon 7 vs. those containing exons 8 & 9 and alignments of duplicated genome region with separate alignment of exon 5 with exon 8. (A) Quantitative real-time PCR analysis of amelogenin expression in first molars extracted from mice of the indicated age. Note that amelogenin transcripts with exon 7 were significantly more abundant than transcripts with exons 8 and 9. The error bars represent the standard error of the mean from 6 separate samples. EF1 α 1 expression served as the internal reference gene control, so the data were plotted relative to its expression. (B) Alignment of mouse exon 4, intron 4, and exon 5 (X4&5) with mouse exon 4b, intron 4b, and exon 8 (X4b8). The first underlined sequences denote exons 4 and 4b; the second underlined sequences denote exons 5 and 8. Nucleotide differences are italicized and highlighted in bold. Note the very high sequence identity starting from before exon 4 and extending past the aligned exons 5 and 8 sequence. Of 209 nucleotides, 191 are identical (91%), and all align without gaps. (C) Alignment of mouse exon 8 amino acid sequence (mouse 8) with exon 5 sequence from the indicated species. Amino acid differences are italicized and highlighted in bold. Note that when mouse exon 8 is compared with exon 5, just 8 nucleotide differences result in the encoding of 7 different amino acids between exons 5 and 8.

Figure 2.

Extracted amelogenins from control vs. *Mmp20* null mice. **(A)** Enamel proteins from the first molars of three-, five-, nine-, and 11-day-old mice were subjected to electrophoresis on SDS-PAGE gels for the assessment of differences in protein size and abundance between control (+/-, +/+) and *Mmp20* null (-/-) mice. Note that several lower MW bands are missing in the -/- lanes when compared with bands present in the controls. Also, note that the upper bands in the control lanes became progressively weaker as development progressed, while the upper bands in the -/- lanes also faded but remained relatively strong. **(B)** Western blot demonstrating that the generated exon 8 antiserum was specific for exon 8. Amelogenins from the three-day-old control mouse first molar (lane +/-), recombinant mouse amelogenin (rM179) at a concentration of 1 μ g (lane, M1) or 5 μ g (lane, M5), and recombinant pig amelogenin (rP172) at a concentration of 1 μ g (lane, P1) or 5 μ g (lane, P5) were loaded onto the gel. Western blots were performed with antiserum specific for rP172 (top panel) or for mouse exon 8 (bottom panel). Note that neither rM179 nor rP172 contained exon 8, and neither reacted with the exon 8 antiserum. Also note that although lane +/- was under-loaded in the exon 8 panel, the M and P lanes were loaded identically, and the same concentration of exon 8 antiserum was used in (B) and (C). **(C)** Western blot of amelogenins encoded by exon 8. Amelogenins were extracted from three-day-old control (+/-) or *MMP-20* null (-/-) mice for Western blotting with the exon 8 antiserum. Note the apparent shifting of each -/- band to a higher molecular mass, indicating a lack of proteolytic processing. Also note a putative proteolytic cleavage product at approximately 18 kDa in the +/- lane.

Table 1

Amelogenin Alternative Splice Forms and the Primer Sets Used to Identify Any Differences in Splicing between Control and *MMP20* Null Mice

	AA ^b	Exons	Mr (Da) ^c	pI
1 ^d	217	1-6, 8-9	24,648.0	6.89
2	203	1-3, 5-6, 8-9	23,122.4	6.68
3	194	1-7	21,906.8	6.52
4 ^d	193	1-5, 6b-6d, 8-9	21,863.9	7.10
5	180	1-3, 5-7	20,381.2	6.35
6	179	1-3, 5, 6b-6d, 8-9	20,338.2	6.82
7	170	1-5, 6b-6d-7	19,122.7	6.61
8	156	1-3, 5, 6b-6d-7	17,597.1	6.41
9	141	1-3, 6b-6d-7	15,752.8	6.12
10 ^d	96	1-5, 6d, 8-9	11,082.6	6.67
11	82	1-3, 5, 6d, 8-9	9,556.9	6.02
12	74	1-3, 5, 6c-6d, 7	8,430.6	4.84
13	73	1-5, 6d-7	8,341.4	5.53
14	59	1-3, 5, 6d-7	6,815.7	4.84
15	57	1-3, 5, 8-9	6,746.7	7.20
16	44	1-3, 6d-7	4,971.5	4.36
5'-PRIMERS ^e		5'-Exon 2: ATG GGG ACC TGG ATT TTG TTT G 5'-Exon 1,3: ATT TTG TTT GCC TGC CTC CTG 5'-Exon 4w/6a: CAG GCT ATC AAT ACT GAC AGG ACT 5'-Exon 4w/6bc: AGT CAC ATT CTC AGG CTA TCA A 5'-Exon 4 w/7: TCA CAT TCT CAG GCT ATC A 5'-Exon 5: ACC CCT TTG AAG TGG TA 5'-Exon 6b: CAG GCC ATT CCA CCC CAG TCT		
3'-PRIMERS		3'-Exon 9: CTG GGA AGG TAG GAG AGT GGT TG 3'-Exon 8: TGC CCT GGT ACC ACT TCA TAG 3'-Exon 7 w/1,3,6b: TTC CTA AAA ACA ACC AAA GTA TCC 3'-Exon 7 w/5: TGT AGG CAC AAA TCA TTG 3'-Exon 7 w/4: GTA TCC ACT TCG GTT CTC T 3'-Exon 6bc: TGC ATG GAG AAC AGT GGA G 3'-Exon 6a w/1,3: ACA GGG ATG ATT TGG TGG TG 3'-Exon 6a w/4: TGT TGA GAC AGC ACA GGG ATG AT		

^a Schematic displaying an overall view of several *Amelx* primer annealing sites.

^b AA, amino acids; Mr, molecular weight in Daltons.

^c Mr and pI includes the Ser¹⁶ phosphate and was calculated at: <http://scansite.mit.edu/cgi-bin/calcp1>.

^d Newly identified amelogenin transcript.

^e Primer names with a "w/" designate the primer with which it was paired. So, primer 5'Exon 4w/6a was paired with primer 3'Exon 6a w/4.

Table 2
Amelogenin RT-PCR Data of mRNA Extracted from First Molar Enamel Organs of Three-day-old Mice

Primers ^a	PCR Product Size	Possible Splice Products (AA) ^b	Deduced Splice Product ^c from cDNA Sequence (AA)
5'AML 1,3 3'AML 6a	~200 bp	194, 180	180 (no exon 4)
5'AML 4 3'AML 7	~600 bp	194, 170, 73	194 (has exons 4 & 6a)
5'AML 4 3'AML 7	~200 bp	194, 170, 73	73 (PCR band size)
5'AML 4 3'AML6bc	~450 bp	194, 170	194 (has exons 4 & 6a)
5'AML 4 3'AML 6a	~150 bp	194	194
5'AML 1,3 3'AML 7	~600 bp	194, 180, 170, 156, 141, 59, 44	180 (no exon 4, has exons 6a, 7)
5'AML 1,3 3'AML 7	~300 bp	194, 180, 170, 156, 141, 74, 59, 44	59 (has no exon 4, has exon 6d)
5'AML 7 3'AML 7	~200 bp	194, 180, 170, 156, 79, 59	59 (has exons 5, 6d, & 7)
5'AML 2 3'AML 8	~600 bp	203, 179, 82, 57	203 (has all of exon 6)
5'AML 2 3'AML 8	~275 bp	203, 179, 82, 57	82 (has just exon 6d)
5'AML 2 3'AML 9	~650 bp	203, 179, 82, 57	203 (has all of exon 6)
5'AML 2 3'AML 9	~300 bp	203, 179, 82, 57	82 (has just exon 6d)

^aNumbers after the "AML" designation refer to the exon to which each primer binds. Note that certain uninformative primer set results were not included in the Table.

^bAA, amino acids.

^cParentheses contain an explanation of why the indicated splice product was selected (see Table 1).