

Amelogenin-deficient Mice Display an Amelogenesis Imperfecta Phenotype*

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Dental enamel is the hardest tissue in the body and cannot be replaced or repaired, because the enamel secreting cells are lost at tooth eruption. X-linked amelogenesis imperfecta (MIM 301200), a phenotypically diverse hereditary disorder affecting enamel development, is caused by deletions or point mutations in the human X-chromosomal amelogenin gene. Although the precise functions of the amelogenin proteins in enamel formation are not well defined, these proteins constitute 90% of the enamel organic matrix. We have disrupted the amelogenin locus to generate amelogenin null mice, which display distinctly abnormal teeth as early as 2 weeks of age with chalky-white discoloration. Microradiography revealed broken tips of incisors and molars and scanning electron microscopy analysis indicated disorganized hypoplastic enamel. The amelogenin null phenotype reveals that the amelogenins are apparently not required for initiation of mineral crystal formation but rather for the organization of crystal pattern and regulation of enamel thickness. These null mice will be useful for understanding the functions of amelogenin proteins during enamel formation and for developing therapeutic approaches for treating this developmental defect that affects the enamel.

Dental enamel, the hard tissue that covers the crown of the tooth, is the most highly mineralized tissue in the body. This mineralized layer is neither replaceable nor repairable, because the ameloblast cells that synthesize enamel are lost at tooth eruption. Amelogenin proteins constitute 90% of the extracellular matrix secreted by ameloblasts, and these proteins are cleaved in a regulated process during enamel maturation (1, 2). As the proteins are digested and removed, the mineral crystals grow in well organized “prism” patterns, becoming much larger when compared with crystals of bone, dentin, and cementum (3). Whereas amelogenin proteins have been impli-

cated in the regulation of crystal growth, the exact roles of the amelogenins have not yet been clearly defined (4).

X-linked amelogenesis imperfecta (AI)¹ is an inherited enamel defect characterized by phenotypic variability in which patients present with hypoplastic defects (thin pitted or grooved enamel) and/or hypomineralization where the enamel mineral content is decreased. Several mutations in the human X-chromosomal amelogenin (*AMELX*) gene have been reported that lead to this defect (5–10), and therefore, X-linked AI provides strong evidence that amelogenin is critical for normal enamel formation. Furthermore, the marked phenotypic variability resulting from different *AMELX* mutations suggests that various amelogenin proteins or protein domains have different functions during enamel development. This finding is consistent with the observation of extensive alternative splicing of the amelogenin primary transcript, even though the gene is active only in teeth (11–14).

To address the functions of the amelogenins in a systematic way, we have generated a mouse with a null mutation at the amelogenin locus. The enamel layer in the null mice is hypoplastic but has an elemental composition consistent with hydroxyapatite-like mineral, and therefore, the amelogenins are apparently not required for mineral crystal initiation. However, the characteristic prism pattern is completely absent, indicating the importance of amelogenins in enamel organization. In addition, the hypoplastic enamel layer in the null mice illustrates the importance of amelogenins to generate correct enamel thickness, a second proposed function for amelogenin proteins.

EXPERIMENTAL PROCEDURES

Construction of the Targeting Vector—A 13.5-kb clone containing the entire amelogenin gene was isolated from a 129SvJ mouse genomic library (15). The XhopKSMC1TK vector, which contains the thymidine kinase expression cassette (16), was digested with *Bam*HI, and into this site, the 1.7-kb amelogenin gene short arm including 250 bp of the promoter, amelogenin exon 1, intron 1, and part of exon 2 was inserted. Replacing 300 bp of exon 2 and intron 2 was the 1.6-kb *NeoR* cassette from pPGKneobpA/*Xho*I (17) in the reverse orientation. The long arm consisted of the 4.0-kb amelogenin fragment containing the 3' end of intron 2 through the 5' end of intron 6.

Sequence Analysis—DNA sequence was analyzed using DNASIS, version 2.5 (Hitachi Software Engineering Co., Ltd., Alameda, CA). The SPScan module of the GCG suite of programs (Genetics Computer Group,

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¹ The abbreviations used are: AI, amelogenesis imperfecta; kb, kilobase pairs; bp, base pairs; PCR, polymerase chain reaction; ES, embryonic stem; RT, reverse transcriptase; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; MIM, Mendelian Inheritance in Man.

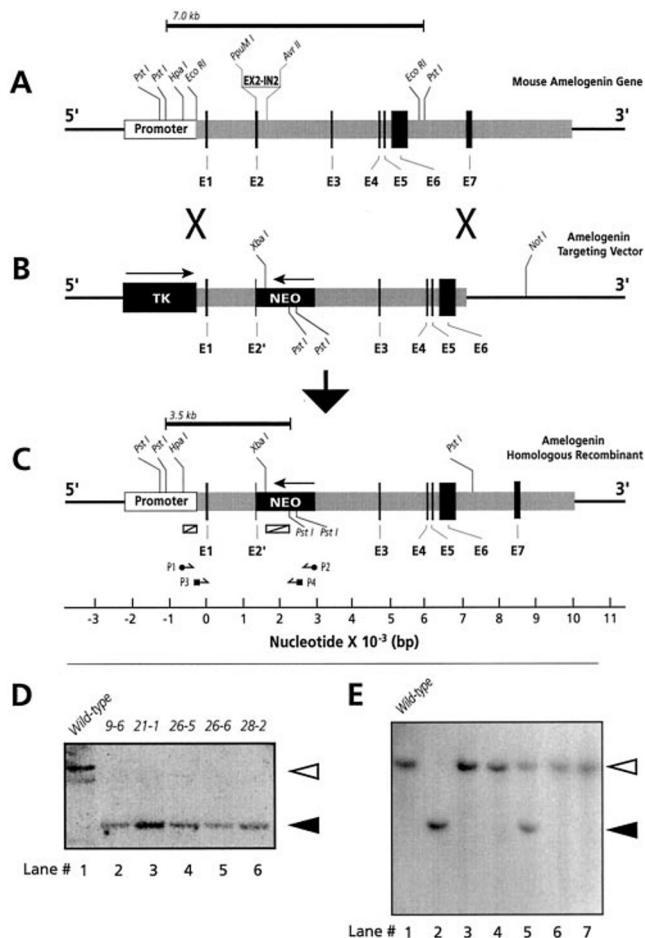


FIG. 1. Generation of amelogenin knockout mice. *A*, a map of the murine amelogenin gene. *B*, the targeting construct included the 6-kb *EcoRI* fragment with a 300-bp region of exon 2 and intron 2 (*EX2-*IN2**) replaced by the *NeoR* cassette in the opposite orientation. *C*, during homologous recombination, the thymidine kinase cassette is removed. The 5'-flanking probe hybridizes to a 7-kb fragment in the wild type gene (*open arrowhead* in *D* and *E*) and a 3.5-kb band in the mutant allele (*closed arrowhead* in *D* and *E*). Primers P1–P4 were used for nested PCR analysis. *D* and *E*, Southern analysis. Southern blots of genomic DNA from individual ES cell clones (*D*) or tail genomic (*E*) DNA taken from littermates. The genomic DNA was digested with *PstI*, and the blots were probed with the 361-bp gene fragment not included in the vector, which revealed several positive ES clones and one null and one heterozygote pup. *E1–E7* are amelogenin gene exons.

Inc., Madison, WI) was used to search for signal peptide homology sequences in the PCR product generated with exons 1 and 6 primers.

Electroporation, Selection, and Generation of Mutant Mice—The targeting vector was linearized by *NotI* digestion, and 120 μ g was electroporated into 2.4×10^7 R-1 embryonic stem (ES) cells as described previously (18). G418 (350 μ g/ml) and gancyclovir (2 μ M) were used for double selection as described previously (18). A total of 185 resistant clones were analyzed for homologous recombination by Southern blot analysis using a 5'-flanking probe (the 361-bp *HpaI-EcoRI* fragment shown in Fig. 1A). Five correctly targeted clones were identified and microinjected into blastocysts, resulting in chimeras from 2 independent clones. Amelogenin \pm mice were generated after a backcross of chimeric male mice with C57Bl/6 females. Amelogenin mutant mice are maintained in C56Bl/6 \times 129/Sv background. All mice were housed in the standard mouse facility (Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) accredited) and were fed an autoclaved diet and water.

Genotype Analysis by PCR and Southern Blot—Genomic DNA isolated from surviving ES clones was digested with *PstI* and blotted to Hybond-N membrane by standard methods (19). The 361-bp *HpaI-EcoRI* fragment just upstream of the targeting construct and the 630-bp *XbaI-PstI* fragment from the neomycin resistance gene were used as probes.

The tail DNA from pups was subjected to nested PCR using primers

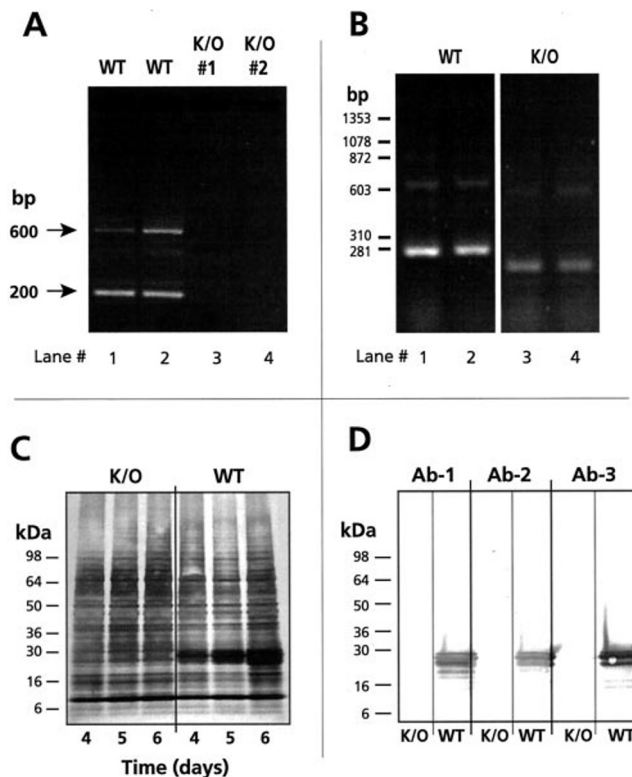


FIG. 2. Analysis of amelogenin RNA and protein. *A*, RNA isolated from tooth germs from 2-day-old mice was subjected to RT-PCR using primers in amelogenin exons 2 and 6. *B*, RT-PCR using primers for exons 1 and 6. *C*, protein analysis by silver stain. Silver-stained polyacrylamide gel of the protein extract from 4–6-day-old $-/-$ (*K/O*) mouse or wild type (*WT*) mouse teeth. *D*, Western blots using $-/-$ tooth or wild type tooth protein extracts. *Ab-1*, polyclonal anti-bovine amelogenin antibodies (Kamiya Biomedical Co.); *Ab-2*, polyclonal anti-rat amelogenin antibodies; *Ab-3*, polyclonal anti-bovine C-terminal region antibodies (41). *K/O*, knockout.

P1–P4 (P1, CAGAGTGGTAATGGAGGACAGAAG; P2, AACTGTTCGC-CAGGCTCAAG; P3, TTTACAAGAATGGGGATTC; and P4, CTTC-CTCGTCTTTACGGTA). PCR cycles consisted of denaturation at 94 $^{\circ}$ C for 1 min, annealing at 55 $^{\circ}$ C for 2 min, and amplification at 72 $^{\circ}$ C for 3 min for 35 cycles. The first amplification was performed using primers P1 and P2. The second amplification was performed using the nested primers P3 and P4 and 10 μ l of a 20-fold dilution of the amplification product from the first reaction.

RNA Isolation and RT-PCR—Teeth were dissected from 1–3-day-old pups, and RNA was isolated using the Trizol reagent exactly as described (Life Technologies, Inc.). First strand cDNA was synthesized at 42 $^{\circ}$ C for 25 min using an oligo(dT)₁₆ primer. PCR amplification was performed using primers for exon 2 (CATGGGGACCTGGATTTT-GTTTG) and exon 6 (TCCCGCTTGGTCTTGTCTGTCTGCT (20, 21)) using the GeneAmp RNA PCR core kit (PE Biosystems, Foster, CA). RT-PCR was also performed with 5' primers for exon 1 (CGGATCAAG-CATCCCTGAGCTTCA) and exon 3 (CTACCACCTCATCCTGGAAGC-CCT). β -Actin primers were used as the positive control for RT-PCR as described previously (13).

Immunohistochemistry—Mandibles dissected from 3-day-old null and wild type mice were fixed overnight in 4% paraformaldehyde, demineralized in 10% EDTA, dehydrated in alcohol, and embedded in glycol methacrylate. 7- μ m sections were incubated with monoclonal bovine anti-amelogenin antibody, previously shown to react with mouse amelogenin (22, 23), polyclonal anti-rat amelogenin, and polyclonal bovine anti-amelogenin antibody (Kamiya Biomedical Company, Seattle, WA) followed by sequential antibody incubations using the avidin-biotin peroxidase method.

Protein Analysis—Developing teeth were dissected from 4–6-day-old pups. The dental pulp soft tissue was removed, and the enamel organ and mineralized tissue were retained for analysis. The samples were suspended in 50 μ l of lysis buffer (10 mM Tris, pH 7.6, 100 mM NaCl, 2

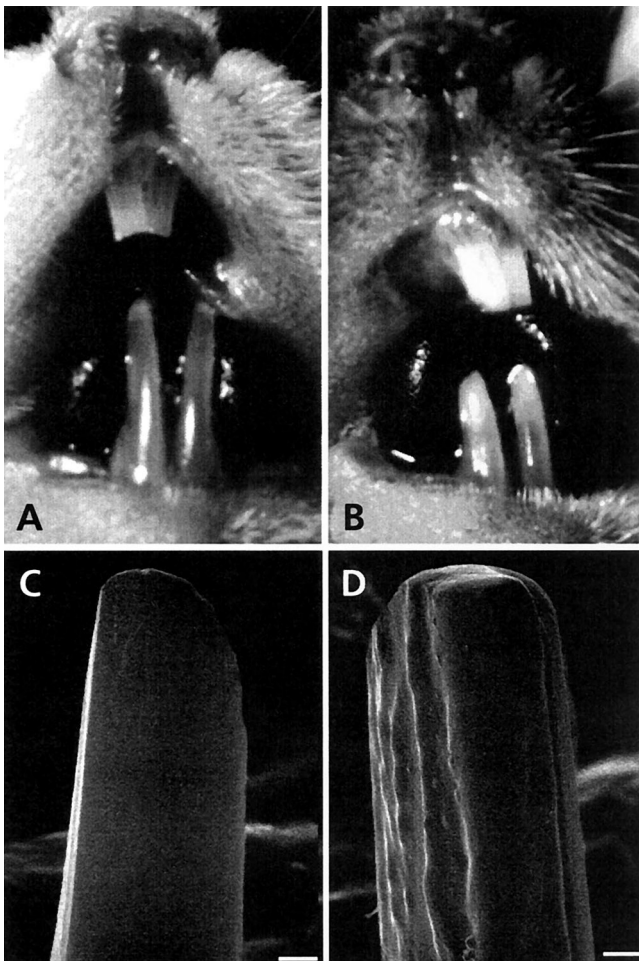


FIG. 3. Incisors of wild type and null mice. Photograph of incisor teeth from wild type (A) and null mice (B). Scanning electron micrograph showing a smooth enamel surface for wild type (C) and marked enamel hypoplasia characterized by a furrowing of the enamel in the incisor from a null mouse (D).

mM EDTA, 0.5% CHAPS, 2 μ M phenylmethylsulfonyl fluoride). The suspension was vortexed and centrifuged, and the extract used for protein analysis by polyacrylamide gel electrophoresis and stained with GelCode Blue Stain Reagent (Pierce, Rockford, IL) or Silver Stain Plus One Kit (Amersham Pharmacia Biotech). For Western analysis, a primary anti-amelogenin antibody was followed with anti-rabbit-Ig-horse-radish peroxidase and 3,3'-diaminobenzidine substrate (Sigma).

Microscopic Analyses of Incisor and Molar Teeth—Mineralized thin sections were cut through mandibles with a slow speed saw and diamond blade for examination by light microscopy. Incisor surfaces were photographed using scanning electron microscopy at 20 kV (Jeol JSM T330A, Jeol, Inc., Peabody, MA), and elemental analysis was performed using energy dispersive spectroscopy (Kevex X-ray, Scotts Valley, CA). Fractured incisors were examined in a Jeol JSM 6300.

RESULTS

Targeted Disruption of the Amelogenin Locus—The mouse amelogenin locus and targeting vector are shown in Fig. 1, A and B. The amelogenin gene includes 7 exons and is located on the X chromosome (21, 24). The DNA sequence of coding exons and upstream regions of this gene are highly conserved, but exons 3–5 and most of 6 can be alternatively spliced in several species (12, 25, 26). Therefore, the exon 2/intron 2 region was chosen as the site to create a 300-bp deletion and for neomycin resistance gene insertion. The location of the deletion between *Pvu*MI and *Avr*II sites (exon 2/intron 2 in Fig. 1A) removes 51 bp of exon 2, including the signal sequence with the exception of one amino acid, and also deletes the first two amino acids of the mature protein. The cassette containing the phosphoglyc-

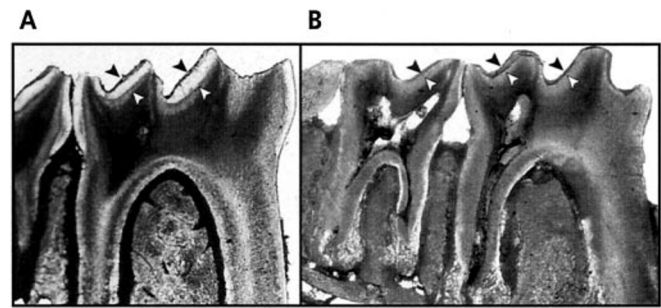


FIG. 4. Light microscopy of mineralized thin sections through mandibular molars from 16-week-old mice. A, wild type dentition shows a normal and relatively even enamel layer over the tooth crown. B, dentition from the null mouse has a markedly reduced enamel thickness. Paired arrowheads indicate thickness of the enamel layer.

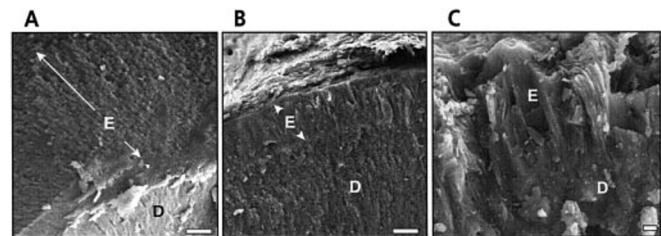


FIG. 5. Scanning electron microscopy of fractured incisors from 16-week-old wild type and null mice. The enamel (E) and junction with dentin (D) are shown. A, wild type mouse. B, the enamel from the null mouse does not have a normal prismatic structure and is markedly reduced in thickness compared with that of the wild type mouse shown at the same magnification as A. C, higher magnification of the enamel layer from the null mouse. Arrowheads indicate enamel thickness. Bars in A and B = 10 μ m; bar in C = 1 μ m.

erate kinase promoter that drives the neomycin resistance gene was inserted in the reverse orientation. The thymidine kinase cassette was inserted at the 5' end of the construct and is removed during homologous recombination (Fig. 1C).

After electroporation of ES cells, five clones surviving G418 and gancyclovir selection were positive by Southern blot analysis of genomic DNA (Fig. 1D). Independent chimeras were obtained from two clones that resulted in germ-line transmission of the null mutation, illustrated by the Southern blot of tail DNA hybridized with the radiolabeled 361-bp fragment of the promoter outside of the targeting construct (Fig. 1E) as well as the *NeoR* probe (data not shown). The genotype was verified by Southern blot of nested PCR products and genomic DNA as described above.

Amelogenin Null Mice Lack Functional Amelogenin Protein—RNA isolated from tooth germs of 1–3-day-old pups was analyzed by RT-PCR. The use of primers located in exons 2 and 6 of the mouse amelogenin cDNA resulted in two major PCR products obtained from wild type mice, which was expected because of the alternative splice pattern of amelogenin primary transcript (25). No product was seen following RT-PCR of RNA from $-/-$ mouse teeth using these primers (Fig. 2A). However, when using primers for exon 3 and exon 6 for RT-PCR, the products of the expected size were obtained, and therefore, we wondered whether alternative splicing could result in the skipping of the partially deleted exon/*NeoR* gene insert region. RT-PCR using exons 1 and 6 primers resulted in a 550-bp PCR product, which would be the size expected if exon 2 was skipped (Fig. 2B). The DNA sequence of this PCR product revealed that exon 1 was spliced to exon 3 in the amelogenin transcripts in the mutant mice.

If a translation product were to be made from this RNA lacking exon 2, it would lack the entire signal sequence including the normal translation start site. To determine whether an N-terminal truncated amelogenin was produced, we used a

monoclonal and three polyclonal anti-amelogenin antibodies in immunohistochemistry experiments, and none of them produced detectable activity in the $-/-$ mice, but high activity was observed in wild type tooth enamel (data not shown). Computer analysis of the aberrantly spliced RNA to search for a signal peptide for secretion did not yield a functional sequence.

To verify these results, protein extracts were made from developing teeth for Coomassie Blue (date not shown), silver stain, and Western blot analysis (Fig. 2, C and D). New proteins were not detected in developing teeth from the mutated animals, and Coomassie Blue and silver staining revealed a lack of the principal 26-kDa band seen in wild type mice. Truncated amelogenins were not detected by Western blot analysis using three anti-amelogenin antibodies (Fig. 2D).

Enamel Phenotype of Amelogenin Null Mice—The amelogenin null mice are fertile, and newborns appear normal. However, as early as 2 weeks of age, incisors from $-/-$ mice display chalky white incisors. In Fig. 3, the incisors from $-/-$ mice are compared with those of wild type mice. Scanning electron microscopic analysis of incisors revealed additionally a rough and knobby surface in the null mice (compare Fig. 3, C with D). By Faxitron analysis, incisor tips and molar cusps of $-/-$ mice have an abraded appearance compared with wild type or heterozygous controls with frequent rounds of breakage and regrowth of incisors (data not shown).

Mineralized thin sections through the mandibular molars from null mice showed an enamel thickness with <10% of normal enamel (Fig. 4). Scanning electron microscopic analysis of fractured incisor teeth revealed a complete lack of a prism pattern, which is the hallmark of organized mineral crystals in normal enamel (Figs. 5, A and B). Flat plate-like structures extended perpendicular to the dental enamel junction to the enamel surface in the mutant mice (Fig. 5C). However, elemental analysis indicated that the composition was similar to that of hydroxyapatite (2.42 Ca/P ratio), indicating the formation of mineral in the absence of the amelogenin protein.

DISCUSSION

Amelogenins are highly conserved proteins that constitute 90% of the enamel organic matrix. To evaluate amelogenin function *in vivo*, we generated a mouse with a null mutation at the amelogenin locus, which resulted in enamel hypoplasia in which the enamel appears as an abnormally thin layer covering the dentin. This enamel layer also lacks the normal highly organized prism structure of the mineral. The null mice do not have detectable amelogenin protein, and because amelogenin is expressed at high levels only by the enamel forming ameloblasts, the phenotype is restricted to dental structures and corresponds with the human enamel defect AI. In humans, AI by definition is not a syndrome but affects only dental enamel (27). The null mouse described here combines the various human phenotypes into one mouse model containing both disorganized and thin enamel but also reveals mineral crystal initiation commenced in the absence of detectable amelogenin protein.

Amelogenins are the major product of ameloblast cells, which reside within the tooth germ until shortly before tooth eruption. Processing of the amelogenins by enamel proteases, which are also products of ameloblasts, begins shortly after secretion (2, 28, 29) and, by the end of the maturation stage, results in enamel with ~95% mineral and <1% protein (1, 30). Experimental approaches have indicated that the amelogenins play an important role in amelogenesis as antisense inhibition of amelogenin mRNA in tooth organ culture led to disrupted mineral formation (31) and amelogenin ribozyme injection into developing murine mandibles produced a phenotype with enamel mineral abnormalities (32).

Amelogenins are thought to self-aggregate as shown in several *in vitro* studies (33–35). Amelogenin self-assembly *in vitro* resulted in the formation of structures referred to as nanospheres that were proposed as the functional components of secretory stage enamel (33). This model suggested that the N-terminal domain of amelogenin is involved with the formation of nanospheres, whereas the C-terminal region contributes to stability and homogeneity in size of nanospheres, preventing mineral crystal fusion to form larger structures prematurely (36). Transgenic mice that express an amelogenin protein with a mutated N or C terminus further demonstrated the importance of these proteins in enamel biomineralization (37). In addition, the N-terminal region of amelogenin shows lectin-like activity *in vitro* (38) and, therefore, may be involved in binding to the glycosylated enamelin proteins found at the enamel junction with dentin. Amelogenin could be functionally important in defining and developing the structural stability required at the enamel dentin junction. Taken together, proteolytic processing, aggregation, and lectin-like properties of amelogenin are likely to be essential for the formation of the mineral phase of enamel in which enamel mineral crystals are much larger than those of other tissues, such as dentin, cementum, and bone (3).

Assigning exact roles to amelogenins has been complicated by the fact that the amelogenin primary transcript is alternatively spliced to form at least three different mRNA species in human and at least nine different mRNA species in mouse (11, 14). Therefore, amelogenin protein heterogeneity arises from not only proteolysis but also from multiple mRNAs because of both alternative splicing and Y-chromosomal amelogenin gene transcription in human males. The nonidentical gene on the human Y chromosome is thought to contribute ~10% amelogenin transcripts and presumably proteins (11, 39, 40).

To delineate precise functions of the amelogenins, we chose the relatively simple system of the knockout mouse. The mouse with a single X-chromosomal amelogenin gene has the advantage that its phenotype should be invariant and, in fact, is consistently expressed in molars and incisors of $-/-$ mice. To understand the role of the individual amelogenin proteins, further studies will include the creation of transgenic mice expressing individual amelogenins, and these transgenic mice can be mated with the amelogenin null mouse to evaluate rescue of the phenotype. Understanding the functions of these prevalent enamel proteins will lead to a greater understanding of how an organic matrix can direct a highly ordered mineral, which although being subject to major masticatory stresses, will last a lifetime. Functional studies will also provide important information for biomimetic approaches and a development of clinical intervention for the treatment of defective enamel.

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