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Review

Amelogenin gene splice products: potential signaling molecules

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Abstract. The amelogenins, the major proteins of the developing tooth enamel matrix, are highly conserved throughout most species studied. The gene structure is similar, with a set of seven exons and intervening introns, and remarkable conservation of particular exon sizes over divergent species. Studies of exon skipping and consequent alternative gene splicing suggest that, in vertebrates, exon definition is crucial. In this mechanism, exon size is important. If too small, an exon can be readily skipped, if too large, internal cryptic splice sites may be utilized. Other factors, such as intron length and specific nucleotide sequences at the splice boundaries also modulate splicing efficiency, but amelogenin gene splicing conforms well to the generalized exon length model. Exons 1, 2 and 7 are not subject to splicing that affects the secreted protein product, but exons 3, 4 and 5 are at the lower boundary of exon size, rendering them, 4 and 5 especially, subject to skipping. On the other hand, exon 6 is very long and has cryptic splicing sites that can be used. In the mouse, nine distinct splice product proteins have been detected. The question now is the functions of these products. The larger forms, those that contain the intact proline-rich, hydrophobic exon 6 domains, are important for enamel mineralization. Recent work suggests that the small proteins resulting from deletion of a major part of amelogenin gene exon 6 via utilization of a cryptic site may have signal transduction functions during tooth development. Furthermore, new work also suggests that odontoblasts transiently express the small amelogenins during the period that epithelial-mesenchymal signaling between preodontoblasts and preameloblasts determines the course of tooth development. The same peptides have been demonstrated to act on non-odontogenic cells and effect their phenotypic expression patterns in vitro, and to induce bone formation in implants in vivo.

Key words. Alternative mRNA splicing; amelogenesis; tooth development; epithelial-mesenchymal signaling; bone induction.

Introduction

Proteins are minor constituents of mature vertebrate tooth enamel, accounting perhaps for about 1% of the enamel. However, in the early stages of amelogenesis, enamel consists of 20–30% protein. Amelogenins are the most abundant protein constituents [1] of the developing enamel, and during the first phases of mineral deposition may account for 60–90% of the enamel matrix [2]. The amelogenin aggregates that form the extracellular matrix of the ameloblasts have been hypothesized to create the space and environment conducive to the deposition of the mineral phase. Amelogenin may thus play a major role in the structural organization of the mineral within the developing enamel and might also regulate the nucleation and growth pattern of the enamel hydroxyapatite crystals [3]. Amelogenin biochemistry is quite complex because the secreted molecules interact to form a matrix which is then degraded and removed by a variety of matrix proteinases during the process of replacement

by the mineral phase as the enamel matures. At any given time between the initiation of amelogenin secretion from the ameloblasts, of epithelial origin, and the final death of the ameloblasts [4, 5] and maturation of the enamel upon tooth eruption, the matrix contains a mixture of amelogenin degradation products, produced by a range of specific enzymatic cleavages. Thus, protein isolates from the enamel, especially developing enamel, always contain complex mixtures of amelogenin and amelogenin degradation products as well as other proteins. The amelogenin and its principal degradation products are strongly interactive and self-assemble into aggregates that complicate their isolation and purification for study.

A second complication in studying and understanding amelogenin function is that amelogenin genes, in some species, and in bovine and human in particular, are located on both X and Y chromosomes. The two genes have specific sequence differences so that two distinct isoforms are present in the enamel of males of those species. A third complication is that the gene structure is such that during transcription and nuclear processing, a number of sequences favoring alternative splicing are recognized. As described below, some of these are classical exon-intron boundary splice sites, while others are internal to an exon. These are all very active areas of current investigation and there have been several recent excellent, comprehensive reviews on each of these topics [3, 6–8].

The major emphasis of the mainstream studies on amelogenin has been understanding its function in regulating mineralization [3, 7]. That inquiry is not the subject of this review. As indicated above, transcription of the amelogenin gene and processing of the pre-mRNA yield a number of specific mRNAs that, in turn, produce a variety of specific protein products. Thus, it is appropriate to discuss amelogenin at the mRNA and translated protein levels in the plural. That is, although there may be only a single gene in a given species, that gene will yield a variety of specific gene product amelogenins. In the past decade, new lines of investigation have been developing that lead to the suggestion that the various amelogenins might also function differentially as signaling molecules, with roles in tooth development and cementogenesis. This work is much less well developed and at this time there are, relative to the hundreds of papers on the structural implications of the amelogenins, only a few that have dealt with the topic. These studies have, essentially, gone in two directions. Most of the work has been devoted to the potential role of amelogenins in the process of cementum formation and its attachment to the tooth root surface. The still newer work has begun to explore the specific roles of the small alternatively spliced amelogenin products as signaling molecules at early stages of tooth development. This review will attempt to correlate work on the specific alternatively spliced amelogenin proteins and their potential function during tooth development with the larger body of work on cementogenesis.

The amelogenin gene and its pre-mRNA processing to potential alternative splice product mRNAs

The coding region of the amelogenin gene is highly conserved among very diverse species. The coding regions for human, bovine, porcine, murine, rat, hamster and opossum have all been determined. The human and bovine genes have single copies on both the X and Y chromosomes, but the porcine, murine and rat genes are located only on the X chromosome. The chromosomal location of the opossum amelogenin gene is not known [9]. Both human X/Y and bovine X/Y transcribed mRNAs are isoforms with similar but not identical nucleotide sequences, and similar intron-exon arrangements. Partial nucleotide and corresponding amino acid sequences are available for a number of other species, but the work on the function of the amelogenin peptides has been carried out using the porcine, bovine, rat and murine systems. Therefore, the following discussion focuses on these and the human RNA processing.

The genes of these species all have seven exons within the coding region [10, 11], although early work proposed only six exons for the bovine X amelogenin [12]. Exon 1 is highly conserved and contains most of the 5′-non-coding region. Translation initiation and the signal peptide sequences are invariably encoded in exon 2. The translation stop codon is at the beginning of exon 7. Figure 1 shows the construction of the X and Y chromosome forms of human amelogenin, based on the data of Salido et al. [13]. In the older literature, AMELX and AMELY were used to denote the X and Y amelogenin forms, re-

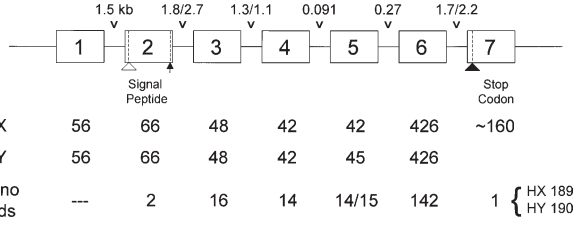

Figure 1. The intron-exon structure of the human amelogenin genes based on the data of Salido et al. [13]. The numbered boxes represent the exons, the line, the introns. Translation of the signal sequence initiates in exon 2 (open triangle) and the signal sequence cleavage point is indicated by an arrow. The translation stop codon is at the beginning of exon 7 (closed triangle). Approximate intron nucleotide sequence lengths are shown above the intron domains. Note that the diagram is not drawn to scale. The exon lengths, HX and HY,are in number of nucleotide bases, the intron lengths are in kilobases. There are a number of nucleotide and consequent amino acid differences at several points when comparing the X and Y chromosomal gene products (see fig. 2) but the exon sizes are tightly controlled.

spectively. Currently they are designated AMGX-Human and AMGY-Human. Both genes have small exons 1–5, and larger exons 6 and 7. The translated proteins begin with the signal peptide, but the tissue forms of the secreted protein begin at the signal peptide cleavage point. Since the initial amino acid sequence of the processed secreted amelogenins in many species, MPLPPHPG, is so highly conserved, numbering the residues customarily begins by designating the N-terminal methionine as residue 1. The various splice product proteins are then identified by the total number of amino acid residues in the secreted product. Thus the maximum, full-length secreted product of AMGX-Human has 189 amino acids (designated HX189). The largest secreted product of AMGY-Human is HY190. The intron nucleotide sequences are much larger and more disparate, and vary considerably between species.

Before discussing specific aspects of amelogenin pre-mRNA alternative splicing, it is worthwhile to consider the factors that have been developed specifically for the general problem of converting the transcribed nuclear pre-mRNA into the functional forms of mRNA.Two systems have been described whereby exons are cut out and spliced together: exon definition [14], and intron definition [15, 16] in which the introns are the target for spliceosome assembly. As emphasized in a recent review by Berget [17], the size of the exon or intron is a key factor in spliceosome formation. In exon definition, adjacent exons are recognized by nuclear arginine-serine (SR) proteins and U1 and U2 small nuclear ribonucleoproteins (snRNPs). After the recognition complex has been formed, additional U2, 4 and 6 snRNPs bind and lead to the recognition of adjacent complexed exons. The looped-out intron is then removed and the adjacent exons are joined. For this complex process to work efficiently, the exons must be small, while the introns are much larger. In that way, by this theory, one avoids joining distant sequences in the intron or an upstream or downstream exon that might also bear a ‘recognition’ nucleotide sequence. The initial and terminal exons each have only a single 5′ or 3′ terminal splice recognition sequence, respectively. In these cases, the 5′ guanosine cap and the 3′ polyadenylation sequences are also proposed to interact with the snRNPs just as the internal exons do to form spliceosome complexes that can be joined to a neighboring exon at one end only. This model puts two constraints on the fidelity of the system. There is an optimum exon size. The exons cannot be too large. If they are, the possibilities of efficient joining to their nearest neighbor exons is reduced. If the exons are too small, spliceosome complex formation is less efficient and an entire exon may be skipped. The second constraint is the specific sequence at the splice point and the possibility of internal exon SR binding sequences of different binding power or strength [18]. If an exon is too long, a cryptic internal recognition sequence with proper polarity may be revealed and thus lead to a deletion of part of an exon. The nucleotide sequence within an intron, particularly in the intron 3′ branch point, may also affect the strength of the exon splicing [19]. In the normal course of exon skipping, all of the coding sequences deleted are at the beginning or end of a codon and do not change the reading frame of the remaining sequence. Intron definition seems to be most common in non-vertebrate systems in which the introns are small while the exons are large. In this case, the spliceosomes form on the intron. The SR proteins do not seem to be present in the lower eukaryotes. They utilize a different set of heterogeneous ribonuclear proteins (hnRNPs) to form the spliceosome on the intron sequence and establish the differential recognition that leads to its removal. The exon and intron definition systems are, in effect, mirror images. The exons are ‘looped out’ but joined as the introns are excised in the intron definition mechanism. The vertebrates use the exon definition system.

The size distribution of vertebrate exons has been considered [17]. Of some 1600 vertebrate exons tabulated, only 3.5% of the exons were larger than 300 nucleotides, while less than 1% were longer than 400 nucleotides. On the other hand, very few exons are in the range below 50 nucleotides, and these are frequently found to be skipped [20, 21]. The majority of vertebrate exons are in the 100–200-nucleotide range.In light of these very general considerations, the amelogenin exon-intron pattern in figure 1 makes perfectly clear that a variety of amelogenin splice product mRNAs should be expected.The nature of the resultant protein forms fit nicely with predictions that could be made from the exon sequence sizes. That is, exons 1, 2 and 7 should be faithfully and fully retained, with no internal cryptic splice sites revealed. Small exons 3, 4 and 5 could be variously skipped and deleted, while the very long (>400 nucleotides) exon 6 could contain a variety of cryptic splice sites and yield additional splice isoforms.

Table 1 lists the splice products determined to be present for the human AMGX/Y and the nucleotide sequences at the exon-intron splice junctions. The most prominent human AMGX mRNA form is comprised from exons 1, 2, 3, 5, 6, 7, deleting exon 4 (80%), leading to protein HX175. HX159 is next most abundant (16%), with the deletion of both exons 3 and 4. Full-length HX189 comprises only 3.5% of the total amelogenin protein [13]. The splicing pattern in AMGY is different, with no detectable full-length HY190, >95% HY176, with the deletion of exon 4, and <2% HY145, in which exons 3, 4 and 5 are deleted. Exon 4 is almost always deleted from the enamel amelogenin mRNA in most species, but the deletion of exon 3 is more variable and appears to be developmental stage dependent in human teeth [10, 22]. Because obtaining human teeth at defined developmental

Table 1. Nucleotide sequences at the boundaries of the primary introns of the human amelogenin genes. AMGX and AMGY [from ref. 13].

Exon 1	Intron 1	Exon 2
----TGC ACT GGA ATA CAT TCA A**AG**----	**GT**ATGTGG--1.5 KB--CATTTC	**AG**----**AA**C CAT CAA GAA---
T T T	*T*	
Exon 2	Intron 2	Exon 3
----GAG CAG CTT TTG CCA TGC C**CT**----	**GT**GAGTAA--1.8/2.7--TGTAAA	**AG**----**CT**A CCA CCT CAT ---
	A	
Exon 3	Intron 3	Exon 4
---- TAT ATC AAC TTC AGC TAT G**AG** ----	**GT**AATTTT--1.3/1.1--TTCTTGT	**AG**----**AA**C TCA CAT TCT---
	C	
Exon 4	Intron 4	Exon 5
---- ATG TTG ACA GGA CTG CAT T**TA**----	**GT**GAGTCT---0.091--- CTCTTA	**AG** --- **GT**G CTT ACC CCT---
		C
Exon 5	Intron 5	Exon 6
---- CAG AGC ------ATA AGG CCA C**CG**----	**GT**ATGTAG---0.27----CTCACC	**AG**--- **TA**C CCT TCC TAT ---
ATG A ***A***		*T*
Exon 6	Intron 6	Exon 7
-AAG ACC AAG CGG GAG GAA G**TG**----	**GT**GAGTAT--0.7/2.2---TTTTTC	**AG**--- **GA**T TAA AAG ATC-
	C	

The coding sequendes of the exons surrounding the introns are shown, written from the 5′–3′ direction for the AMGX gene. Where the AMGY differs in sequence, this is shown with the nucleotide base directly below the nucleotide of AMGX in italics. The nucleotides directly at the exon-intron boundaries are in bold in larger type.

stages is difficult, much more work has been done with bovine, murine and porcine teeth. The translated amino acid sequence data for human AMGX/Y are shown in figure 2. Figure 3 shows the exon nucleotide sequences for human, bovine, porcine and murine amelogenin pre-mRNAs. These data have been compiled in the form developed by Simmer and Snead [6], brought up to date with data from ENTREZ, NCBI (accession numbers provided in the figure legend). The locations of the exon splice boundaries are denoted by the solid vertical lines. These are numbered explicitly. As noted earlier, the >400-nucleotide exon 6 is a good candidate for cryptic splicing. Three such internal splice sites have been confirmed in the mouse and those boundaries are also noted and labeled. Of interest is that in each species examined, the putative internal exon 6 cryptic splice site sequences are highly conserved.

As pointed out by Dominski and Kole [18], the sequence near the 5′ end of an exon is important to the strength of the binding, even at the 3′ exon boundary. The relevant 5′ sequence may involve as many as 20 nucleotides. Dominski and Kole [23] have proposed that the purine content of the 5′ region of an exon is important to the exon boundary strength and of interest is that the 5′ region of exon 6A is richer in purines than comparable cryptic exon 6B, C and D regions. On the other hand, the 3′ boundary of exon 6 (6D) is the most highly conserved sequence between these species and has 25 or 26 purines out of the last 36. All of the intron sequences at the exon-intron boundaries are the classical gt-ag type, including the cryptic exon 6 splice site 'analog intron' boundaries.

The most prominent differences between the species whose nucleotide sequences are shown in figure 3 are in the skipping of exon 4 and the highly repetitive sequences of exon 6B, but exons 3 and 5 are also subject to skipping. Figure 4, modified from that of Hu et al. [24], shows the alternative splicing patterns for mouse amelogenin. Exons 1, 2 and 3, and 6D and 7 are present in all mouse mRNAs found thus far, but nine variant isoforms have been detected, with different combinations of exon 3, 4, and 5 and internal 6A, 6B, and 6C deletions. Oida et al. [25] have just completed a comparable very thorough study of alternative gene expression in the pig. RNA messages with exon distributions 2-3-5-6ABCD-7 (P173), 2-5-6ABCD-7 (P157), 2-3-5-6D-7 (P56) and 2-3-6D-7 (P41) were present. The pig amelogenin gene also expresses exon 4, and amelogenins containing that sequence as translated protein (P190) have also been isolated and

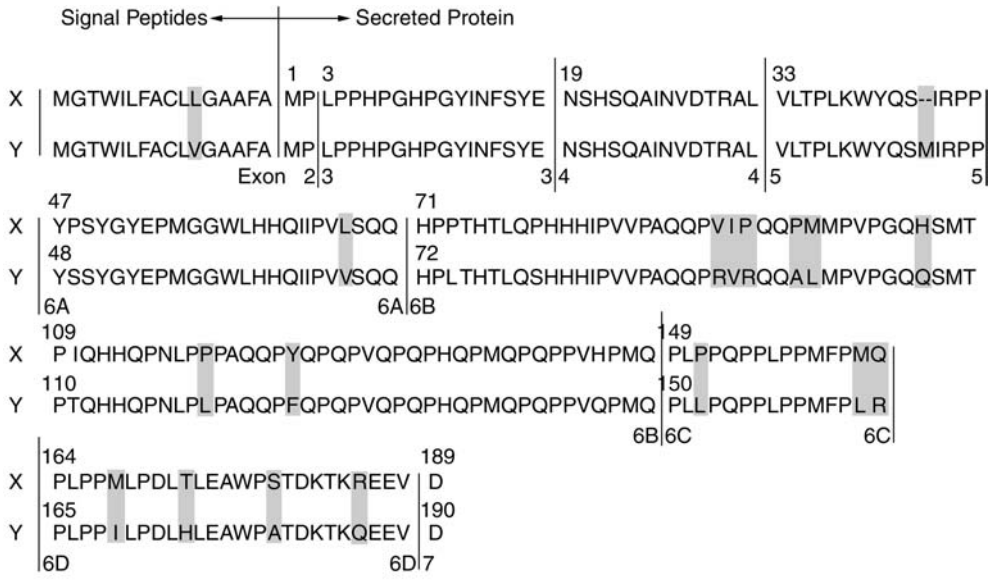

Figure 2. The aligned amino acid sequences of the full-length human X and Y chromosomal amelogenin gene products [13], starting with the signal sequence. The first secreted protein Met is denoted as residue 1, above the sequence. The signal sequence cleavage site is marked, as are the exon boundaries. The points at which individual amino acids differ are highlighted by the light-gray backgrounds. The exon, and cryptic exon 6 splice boundaries are also shown, below each sequence pair.

characterized [26]. In this case, the only one found thus far, the expressed exon 4 peptide has 17 rather than 14 amino acid residues and is not homologous with the exon 4 sequences found in the bovine, human, mouse and rat. In fact, two 'exon 4-like sequences' were found in the pig. The exon termed 4a generates the unique 17-residue sequence, KSGRWGARLTAFVSSVQ. The second putative 'exon 4b' nucleotide sequence would have generated a peptide with the sequence DLYLEAIRIDRTAF, but that peptide, more homologous to the exon 4 peptide sequences of the other species (fig. 2), has not been detected in porcine amelogenins. Exon 4 is usually skipped, but the corresponding protein sequence has been found in amelogenins of the mouse [24] and human [13]. It has not been reported in bovine amelogenins. Recently Yuan et al. [27] developed a model system in which a green fluorescent protein (GFP)-tagged expression vector containing bovine amelogenin exons 3–5 and their introns 3 and 4 were transfected into Chinese hamster ovary (CHO) cells. Other GFP constructs including parts of the bovine amelogenin intron 6 of varying length were prepared to extend the very small 63-nt intron 4. The skipping of exon 4 was then determined by PCR analysis. The length of the intron was indeed important. Increasing the intron size by only 6 nt increased inclusion of exon 4 by 55%, while an inclusion bringing the total intron 4 to 313 nt increased exon 4 inclusion sixfold. Use of this model is an important step forward in determining how sequence size and composition in both introns and exons regulate splicing efficiency, and, possibly, how the control of splicing affects the properties of the resultant enamel.

With the outstanding exception of the internal exon 6 domain denoted as exon 6B, the nucleotide and consequent translated protein sequences of the amelogenin are highly conserved. In exon 6B, codons for triads of repeat sequences PXQ, where X is L, Q or M, may be inserted differentially in different species. The exon-skipping splice patterns are in accord with general predictions based on the exon definition model and mechanism for splicing. In essence, all the expected single and multiple exon deletions have been detected, although there are striking differences in retention or excision of exons 3, 4 and 5, and the internal exon 6 alternative splicing from one species to the next, indicating that some local factors of the splicing mechanism may be variable. Nevertheless, ameloblast-specific splicing factors are not required for

Figure 3. The aligned nucleotide sequences of the human (X, Y), bovine (X, Y), mouse and porcine amelogenin cDNAs. These data have been compiled from latest data entries in the NCBI nucleotide gene bank. They are from the work of Salido et al. [13] (human, accession Nos. NM001142, NM001143), Gibson et al. [11, 12, 88], (bovine, accession Nos. M63499, M63500) and Hu et al. [24, 26] (mouse, accession No. D31768; pig, accession No. AF328419). In all species, the translation start site, signal peptide and signal peptide cleavage points are in exon 2. The very conserved sizes of exons 2, 3, 4, 5, 6A and 6D are evident. The principal deviations are in exons 6B and 6C. The numbers of nucleotides at the end of each line indicate the total number of nucleotides in each exon, or cryptic exon.

```
      EXON1
aaaggatca agcatccct gagtttcaa acagaaact tgcactgaa ---tacattcaaag          HX
-----agaggaccaagcctccctgtgtagcacaaagaaagtttctctgaa---tatatttaaag          HY
-----------------------------------gaaactcactgagaacg-tgtgttcaaag          BX
                                                                          BY
-----atcggatcaagcatccctgagcttcagacagaaactcactgagca---tacactcaaag          M

      EXON 2 ▼      Coding region - Signal Peptide              ▼
aaccatcaagaa-atggggacc tggatttta tttgcctgc ctcctggga gcagctttt gcc-atgcct  HX 6
aaccatcaagaa-atggggacc tggattttg tttgcctgc cttgtggga gcagctttt gcc-atgcct  HY 6
ggctatcaagaa-atggggacc tggattttg tttgcctgc ctcctggga gcagccttc tct-atgcct  BX 6
         aa-atggggacc tggattttc ttttgcctg cccctggga gcagcctat agt-atgcct  BY 6
aaccatcaagaa atggggacc ttgattttg tttgcctgc ctcctggga gcagctttt gct-atgccc  M  6
ggccatcaagaa atggggacc tggattttg tttgcttgc ctcctggga gcagccttc tct-atgcct  P  6

      EXON 3
ctaccacct catcctggg caccctggt tatatcaac ttcagctat gag       HX 48
ctaccacct catcctggg caccctggt tatatcaac ttcagctat gag       HY 48
ctaccacct catcctggg caccctggt tatatcaac ttcagctat gag       BX 48
ctaccacct catcctggg caccctggt tatatcaac ttcagctat gag       BY 48
ctaccacct catcctgga agccctggt tatatcaac ttaagctat gag       M  48
ctaccacct catcctggg caccctggt tatatcaac ttcagctat gag       P  48


      EXON 4
aactcacat tctcaggct atcaatgtt gacaggact gcatta              HX 42
aactcacat tctcaggct atcaatgtt gacaggatt gcttta              HY 42
aactcctat tttcagggt atcagtatt gacgagact gcataa              BX 42
aactcctat tttcagggt atcagtatt gacgagact gcataa              BY 42
aagtcacat tctcaggct atcaatact gacaggact gcatta              M  42
aagagcggg cgctgggga gcgagactt actgctttt gtaagc tccgtccag    P  51

      EXON 5
gtgcttacc cctttgaag tggtaccag agc---ata aggccaccg           HX 42
gtgctcacc cctttgaag tggtaccag agcatgata agaccacca           HY 45
gtgctcacc cctctgaag tggtaccag agcatgata agacacccg           BX 45
gtacttaca cctctgaag tggtatcag aacatgtta agatatccg           BY 45
gtgcttacc cctttgaag tggtaccag agcatgata aggcagccg           M  45
gtgcttacc cctctgaag tggtaccag aacatgata agacatccg           P  45

      EXON 6A
tacccttcc tatggttac gagcccatg ggtggatgg ctgcaccac caaatcatc cccgtgct                        HX 72
tacccttcc tatggttac gagcccatg ggtggatgg ctgcaccac caaatcatc cccgtggt                        HY 72
tacccttcc tatggttac gaacccatg ggtggatgg ctgcaccac caaatcatt cccgtggt      agcag             BX 72
taccccttcc tatggctat gaacccgtt ggtggatgg ctgcaccac caaataatt cctgtggt                       Y  72
tatccttcc tatggttac gaacccatg ggtggatgg ctgcaccac caaatcatc cctgtgct                        M  72
tacacttcc tatgggtac gaacccatg ggtggatgg ctgcaccac caaatcata cccgtggtg tcg---cag             P  69

      EXON 6B
caccccccg actcacacc ctgcagcct catcaccac atcccagtg gtgccagct cagcagcc
caccccctg actcacacc ctgcagtct catcaccac atcccagtg gtgccagct cagcagcc
actccccag aatcacgcc ctgcagcct catcaccac atccccatg gtgccagct cagcagccc gtggtcccc cagcaacca
agtccccag atcatgccc tgcagcctc atcaccaca accccatgg tgccagctc agcagcct
catcccccg agtcacacc cttcagcct catcaccac cttcccgtg gtgccagct caacagcc             agcaacca     M  81
cagactccc cagagtcat gcccttcag cctcatcac cacatcccc atggtgcca gctcaaca

atgatgccc gttcctggc caacactcc atgactcca atccaacac caccagcca aacctccc
ctgatgcct gttcctggc cagcaatcc atgactcca acccaacac catcagcca aacctccct ctgcctgcc cagcagccc     HY 81
atgatgcca gttcctggc caacactcc atgactcca acccaacac caccagcca aacctccc
tgatgccag tt-cctggc caacactca atgactcca atccaacac caccagcca aacctccct ctgcccgcc c             BY 81
atgatgcca gttcctggc caccactcc atgactcca acccaacac catcagcca aacatccc
ccaatgatg ccacttcct ggccaacac tccatgact ccaacccaa caccaccag ccaaacct            ----------    P  72

taccagccc cagcctgtt cagccacag --------- --------- --------- --------- --- cctcaccag cccatgcag HX 45
ttccagccc cagcctgtt cagccacag --------- --------- --------- --------- --- cctcaccag cccatgcag HY 45
ttccagccc cagtccatc cagccgcag --------- --------- --------- --------- --- cctcaccag cccctgcag BX 45
ttccagccc cagcccatc cagccacag --------- --------- --------- --------- --- cctcaccaa --------- BY 36
ttccagcag cccttccag ccccaggcc attccaccc cagtctcat cagcccatg cagccccа
gcccagcag cccttccag ccccagccc gtccagccc cagcctcac cagcccctg cagccccag --- --------- --------- P  63

      EXON 6C
ccccagcca cctgtgcac cccatgcag cccctgccg ccacagcca cctctgcct ccgatgtt            ---------    HX  72
cccctgctg ccacagcca cctctgcct cccctgctg ccacagcca cctctgcct ccaatgtt            ---------    HY  72
cctcaccag cccctgcag cccatgcag cccatgcag cccttgcag ccccgcag ccccgcа
cccatccag cccttgccg ccacagcca cctctgcct ccgatattc cccatgcag                                  BX 135
cccctgcag ccccagcca cctgtgcac cccatccag cgcttgcca ccacagcca cctctgcc          c cccatgcaa    BY  81
cccctggca ccacagcca cctctgcct ccactgttc tccatgcag                                           M   45
tcacccatg caccccatc cagcccttg ctgccacag ccacctctg cctccgatg ttc                             P   57

      EXON 6D                                                                               EXON 7
cccctgcct cccatgctt cctgatctg actctggaa gcttggcca tcaacagac aagaccaag cgggaggaa gtg  HX 75   gat taaaag    HX 3
cccctgccc cccatactt cctgatctg actctggaa gcttggcca gcaacagac aagaccaа                         gat taaaag    HY 3
cctctgccc cccatgctt cctgacctg cctctggaa gcttggcca qcaacagac aagaccaа                         gat taaaag    BX 3
cctctgccc cctgtgctt cctgacctc cctctggaa gcttggcca caacagaca agaccaag                        gat taaaag    BY 3
cccctgtcc cccattctt cctgagctg cctctggaa gcttggcca gcgacagac aagaccaа                         gat taaaag    M  3
tccatgcag tctctgctt cctgacctg cctctggaa gcttggcca gcaacagac aagaccaag cgggaggaa gtg  P  75   gat taaaag    P  3
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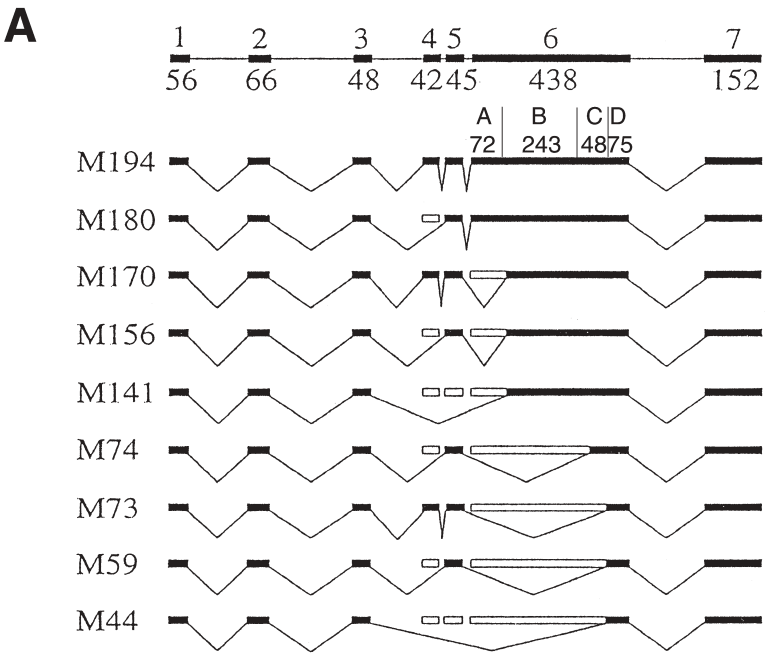


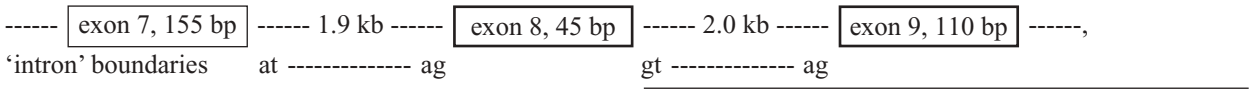
B

Splice Junction	REGULAR Exon / Intron / Exon 5'---3'		CRYPTIC Exon / Intron / Exon 5'---3'	
1-2	ag/gt---ag/aa	All	--	
2-3	ct /gt---ag/ct	All	--	
3-4	ag/gt---ag/aa	M 194, 170, 73	--	
3-5	ag/gt---ag/gt	M 156, 74, 59	--	
3-6B	--		ag/gt---ag/ca	M 141
3-6D	--		ag/gt---ag/cc	M 44
4-5	tg /gt---ag/gt	M 194, 170, 73	--	
5-6A	cg/gt---ag/ta	M 194, 180	--	
5-6B	--		cg/gt---ag/ca	M 170, 156
5-6C	--		cg/gt---ag/ca	M 174
5-6D	--		cg/gt---ag/cc	M 73, 59
6D-7	tg /gt---ag/ta	All	--	

Figure 4. (*A*) The splicing pattern of the mouse amelogenin gene [adapted with permission from ref. 24]. Each of the nine gene products has been detected as protein. (*B*) The pre-mRNA nucleotide splice junctions for each of the peptide products shown in *A*. All the splice junctions have gt-ag intron boundaries, including the 'cryptic' sites deleting 6A, 6AB and 6ABC.

correct splicing of exons 3–5 in amelogenin pre-mRNA [11]. As the techniques for detecting low levels of the putative alternatively spliced mRNAs and the corresponding peptides have increased in sensitivity, more alternatively spliced forms of amelogenin message/protein have been determined to be present in ameloblasts and secreted enamel matrix. Thus, one might expect to encounter low levels of any one of the possible splice products in an ameloblast-enamel matrix preparation. Furthermore, the ratios of these products are likely to be different at different tooth developmental stages, as has been shown already for the exon 3 translated product [11, 22].

The discussion above follows the usual interpretation of the amelogenin splicing data relevant to the nucleotide sequences embracing exons 1 through 7 in the amelogenin pre-mRNA. Two papers from the DenBesten laboratory [28, 29] indicate that two additional potential exons exist in the usually untranslated 3′ end of the amelogenin pre-mRNA transcript. In the rat, mouse and human, a long nucleotide sequence follows exon 7. As depicted here:



this domain contains additional exons, 8 and 9, that can be detected as expressed protein. These additional sequences, as protein, are only found when exon 7, containing a stop codon, is spliced out. The putative 'intron' sequence between exons 7 and 8 does not follow the 'gt-ag' rule, and, in fact, rat amelogenin never splices between exons 7 and 8. Li [private communication] suggests that exons 8 and 9 should be named exons 7′ and 8, as they form two alternatively spliced variants (···6-7·· or ····6-8-9). Thus far, exons 8 and 9 always appear together, as might be expected since the intron 8–9 has the 'classical' nucleotides at the intron boundaries. In the 6-8-9 variant, the entire sequence, intron 6-exon7-intron 7, is removed. Both exon 7 and exon 9 have non-coding regions and poly(A) tails. Since the inclusion of exon 7 is the most common amelogenin isoform and its inclusion terminates translation, the skipping of exon 7 must be rare. How common the skipping of exon 7 is and the role it may play are questions under debate and further study.

Is the expression of the amelogenin gene restricted to ameloblasts?

The consensus conclusion from many years of study has been that the amelogenins are expressed only by ameloblasts. Immunohistochemical studies [30–34] demonstrated that amelogenin peptides can be detected in dentin in a zone near the dentino-enamel junction, in the clear layer just beneath the preameloblasts and in the mantle dentin. Low molecular-size amelogenins and amelogenin degradation peptides were postulated to diffuse through the basement membrane separating preameloblasts from preodontoblasts and become trapped in the forming dentin matrix. This position was supported by many in situ hybridization and PCR studies that readily detected amelogenin mRNA in ameloblasts, but failed to detect any amelogenin signals in the adjacent odontoblasts [34–42].

My laboratory came upon this question accidentally, a byproduct of a study designed to follow up on the observations of Urist [43–45] concerning the bone morphogenetic protein, BMP. He showed that demineralized dentin matrix had a stronger and more stable ability than demineralized bone matrix to induce bone formation at ectopic sites in in vivo implants. Implants of demineralized dentin matrix into muscle induced cartilage formation, which, following the endochondral model ultimately produced a vascularized marrow containing lamellar bone modeled on the matrix. In attempting to show that not every mineralized tissue had this property, he also implanted HCl-demineralized mature enamel into similar ectopic sites [45, 46]. Surprisingly, although the activity was low, when HCl-demineralized enamel matrix was implanted into muscle, it attracted host mesenchymal cells that grew on the surfaces of the enamel prism sheaths and caused them to 'differentiate into a bizarre mixture of cellular and acellular hard tissue resembling the bone of certain fishes' [45]. The partially calcified enamel-dentin complex in unerupted teeth at the bell stage had a higher BMP-like activity than the comparable dentin-enamel complex from mature erupted teeth, and both had a higher activity than bone [46]. One focus of my laboratory has been the study of the non-collagenous proteins of the dentin matrix, with emphasis on the mineralization-related phosphoproteins. It seemed obvious that high levels of BMP-like factors must be present in the extracts obtained during dentin demineralization, and we decided to isolate them. The fact that we were searching for the 'BMP' and a bone-inductive material was crucial in the design of the experiments. Thus, the assays used to follow the fractionation of the dentin extracts were for chondrogenesis and the appearance of cartilage phenotypic macromolecules [47–51]. Not until we came to the very end of the isolation work did we discover that the bone-inductive factors were related to the amelogenins [52]. On SDS gels, the active peptides from bovine and rat dentins had apparent molecular weights of the order of 6000 to 10,000.

The finding that the 'active' peptides were low molecular-weight amelogenins was startling, although not inconsistent with the observation [45] that demineralized enamel matrix had some activity as an implant material. Furthermore, the fact that they represented a very small fraction of the total soluble proteins extracted was consistent with being enamel matrix remnants. However, the exhaustive protocols followed for the preparation of the bovine dentin matrix [52] prior to final extraction seemed to preclude that possibility. Sequencing showed that the bovine peptides contained the amino-terminal sequence MPLPPHP-(exons 2–3), and an internal sequence released by trypsin digestion, WYQSMI-(BX, exon 5). The intact peptide size, in the 6- to 10-kDa range, was consistent with the 'LRAP' amelogenin isoform, the translation of mRNA comprised from exons 2, 3, 5, 6D, 7. Thus, we hypothesized that the peptides might be the products of translation of specific spliced mRNAs, and not proteolytic fragments of ameloblast-produced amelogenins.

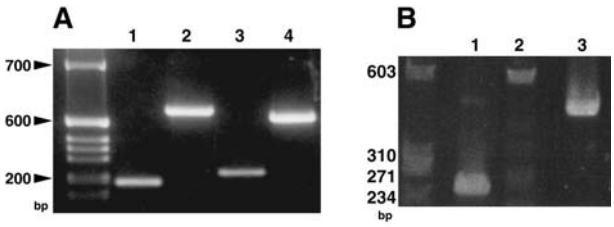


Figure 5. (*A*) RT-PCR of rat incisor odontoblast-pulp cDNA using the exon 2 sequence as forward primer and exon 6D,7 as the reverse primer [with permission, fig. 2B from ref. 58]. Lane 1, exons 2, 3, 5, 6D, 7 [A-4], LRAP]; lane 2, ex. 2, 3, 4, 5, 6, 7; lane 3, ex. 2, 3,4, 5, 6D, 7; lane 4, ex. 2, 3, 5, 6, 7. (*B*) RT- PCR of porcine odontoblast-pulp cDNA using exon 2 and 7 primers showing the presence of amelogenin mRNA in the porcine odontoblasts [with permission, fig. 4B from ref. 25]. Lane 1, forward primer exon 5, reverse primer exon 6; lane 2, forward primer exon 2; reverse primer exon 7; lane 3, GAPDH primers.

Several studies of supposedly dentin-specific extracellular matrix proteins, notably dentin matrix protein 1, DMP1, had shown that alveolar bone and enamel contained both mRNA and translated proteins [53–56]. That is, the dentin proteins were transiently expressed in the odontogenic dental epithelium as well as in the alveolar bone. On this basis, we considered that the amelogenins with BMP-like activity that we found in the dentin might, in reciprocal fashion, be transiently expressed in the odontoblasts during the early stages of tooth morphogenesis. A rat incisor odontoblast-pulp cDNA library that had served as a source for the cloning of the rat dentin matrix proteins [57] was available, so it was probed for the presence of the amelogenins [58]. As shown in figure 5A, PCR products corresponding to the proteins encoded by exons 2, 3, 4, 5, 6ABCD, 7 (R195), 2, 3, 5, 6ABCD, 7 (R181), 2, 3, 4, 5, 6D, 7 (R73) and 2, 3, 5, 6D, 7 (R59) were detected. A similar result, confirming our data and hypothesis, was obtained by Oida et al. [25] for the RT-PCR of porcine odontoblasts (fig. 5B). Two primer pairs were used, one set generated a 236-base pair amplification product comprised from exons 5 and 6 only, while the second set spanned the sequences from exon 2 to exon 7 and generated multiple amplification products from alternatively spliced mRNA transcripts.The porcine exons (2, 3, 5, 6ABCD, 7), (2, 5, 6ABCD, 7), (2, 3, 5, 6D, 7) and (2, 3, 6D, 7) were all expressed in the odontoblasts. In the case of the rat incisor RT-PCR, the initial very weak bands in the initial odontoblast-pulp RT-PCR were isolated and reamplified before they were strongly represented as in figure 5A [58]. In the porcine study, the appearance of the odontoblast 'amelogenins' was not evident at 20 cycles of amplification, but did appear after 30 cycles. The concentration of message in the porcine ameloblasts was estimated as close to 1000 times that of the expression in the porcine odontoblasts. Similarly, Dey et al. [59], in a study of the rat incisor odontoblast-specific cDNA clones, identified the presence of amelogenin cDNA at low levels in a rat incisor odontoblast cDNA library. These data are consistent with the attempts at in situ hybridization. For example, although Wurtz et al. [39] had observed a very low level of amelogenin probe staining in the odontoblasts of postnatal day 2 rat molars (fig. 6B), they attributed that to background staining as compared to the much more intense labeling in the ameloblasts. They concluded that there was no amelogenin production in the odontoblasts in their study. However, the localization of the 'background' stain along the distal side of the mandibular molars and mesial side of the maxillary rat molars was not uniform along all parts of the odontoblast layer and, in fact, the 'background

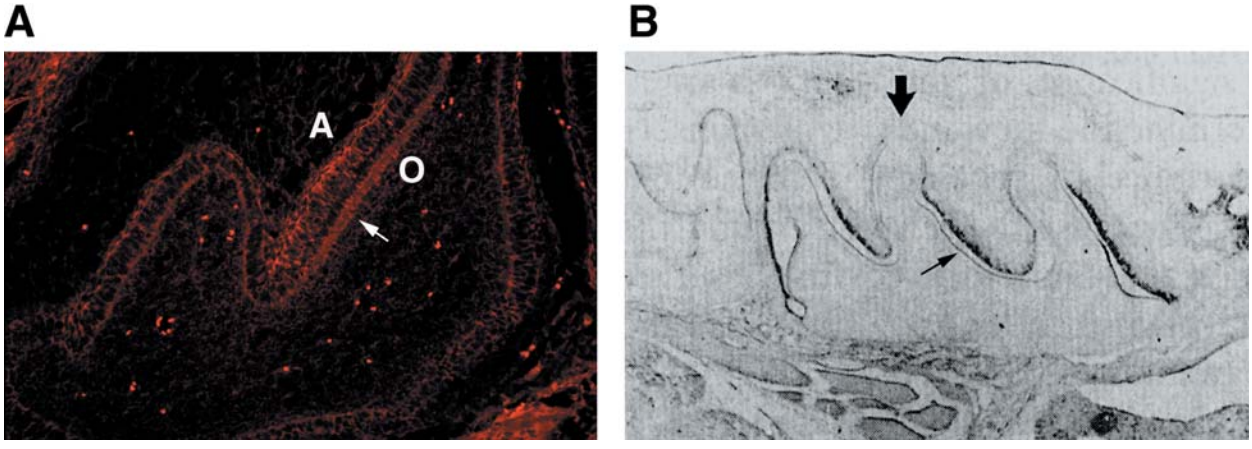


Figure 6. (*A*) In situ hybridization of the amelogenins in the first mandibular molar of a postnatal day 2 mouse, probe specific to exon 5-6D boundary [A+4/A-4, LRAP]. A, ameloblast layer; O, odontoblast layer. White arrow points to region of maximal odontoblast labeling [Kulkarni et al., unpublished data]. (*B*) Labeling of the LRAP component in postnatal day 5 rat molar, with a radiolabeled LRAP oligo. Broad arrow, inner enamel epithelium cells with little or no amelogenin mRNA; narrow arrow, LRAP mRNA labeling in odontoblast layer. Note that labeling is least where more dentin is present [with permission from ref. 39, fig. 3].

stain' location was developmental stage dependent. Preliminary reexamination of this problem [Kulkarni et al., unpublished data] using immunofluorescence labeling and a riboprobe specific for the exon 5-6D boundary nucleotide sequence showed the same kind of location-dependent distribution of amelogenin in the odontoblasts of 1- and 2-day postnatal first mandibular mouse molars (fig. 6A). One problem with these data still under investigation is that the cells of the stratum intermedium also show considerable fluorescence. The control sense-strand probe showed only a faint background fluorescence in all cells. Nevertheless, although the early work had established the dogma that the amelogenins were expressed as protein only in the odontogenic epithelium and arrived in the mantle dentin only by diffusion, one might now safely conclude that, indeed, both near full-length and exon 6ABC-deleted amelogenin mRNAs are transiently expressed at a low level in odontoblasts in a developmentally regulated manner.

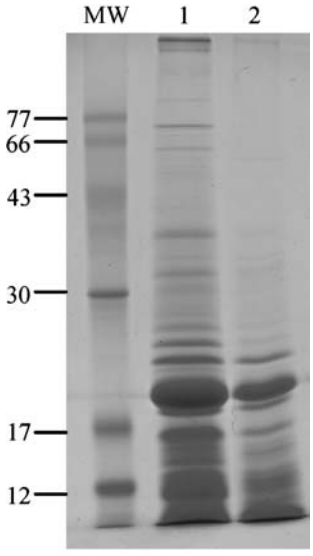


Figure 7. A comparison of the SDS gel electrophoretic patterns of EMDOGAIN (lane 1) and porcine developing enamel matrix (lane 2). Stained with Coomassie blue. Ten micrograms of each preparation was electrophoresed. The EMD was enriched in the amelogenin components [reproduced with permission from ref. 68].

Potential functions of the amelogenins

Cementogenesis

In addition to the direct role that amelogenins are postulated to have in the organization and mineralization of the enamel matrix [3], they, or other protein components of the enamel, have been proposed to have signaling effects, particularly with regard to development of the acellular cementum. In the complex process of cementum formation [60], the proteins were thought to be released from Hertwig's epithelial root sheath, the apical extension of the cervical loop inner enamel epithelium after completion of the crown [61, 62]. Amelogenins were among them [63]. Hammarström [64] showed by immunohistochemistry that human teeth express amelogenin during root formation.An acellular cementum-like layer was formed when the mesenchymal cells of the dental follicle were exposed to an enamel surface in the presence of enamel matrix proteins. When the enamel matrix proteins were placed in experimental cavities in the roots of monkey incisors, the acellular cementum was firmly attached to the underlying root dentin surface. If the follicular cells were exposed to the dentin in the absence of the enamel matrix proteins, then a cellular, poorly attached hard tissue was formed [65]. Studies such as these led to the development of a porcine enamel matrix derivative, called EMDOGAIN (EMD), that was essentially a mixture of amelogenins [64, 66, 67]. Maycock et al. [68] compared EMD with porcine developing enamel matrix. SDS gel electrophoresis (fig. 7) showed that EMD was very heterogenous, but that the amelogenin components, degradation products and perhaps specific splice products, were concentrated in the EMD. Western blotting with various enamel protein antibodies showed that essentially all of the Coomassie-stained bands in EMD of 40 kDa or less (fig. 7, lane 1) were amelogenin related, whereas none were reactive with either anti-enamelin or anti-amelin antibodies. On the other hand, zymography showed the EMD to have metalloendoprotease and serine protease activities.

The complexity of both component composition and proteolytic activity of EMD underscores the difficulty of relating the biological activity of EMD to any single component. Moreover, in vitro studies of the effects of EMD on cells and tissues have revealed additional important points. One of the most important is that EMD has differential effects on mesenchymal and epithelial cells. When EMD was applied to mesenchymal human periodontal ligament cells, cell attachment rate, growth and metabolism were all significantly increased. Parallel cultures of epithelial cells with EMD showed that cell proliferation and growth were inhibited [69]. EMD treatment of oral epithelial (SCC25) cells led to inhibition of cell division, with a concomitant arrest of the cell cycle at the G1 phase, although the number of apoptotic cells did not increase. These data suggested that EMD acted as a cytostatic agent, rather than a cytotoxic agent, on oral epithelial cells [70]. Furthermore, EMD does not act identically on all mesenchymal cells: its action may depend on the stage of cell maturation and on cell type [71]. Quite clearly, studies of the action of EMD and/or unfractionated extracts of enamel matrix are appropriate in assessing the clinical effectiveness of the EMD preparation in tissue regeneration. However, the potential highly variable activities of the multitude of enamel matrix proteins

suggests that such studies may not be very productive in leading to mechanisms of action.

Osteogenic activity of EMD

The method of delivery of enamel matrix to target cells may be as important as cell type and developmental stage. Wang [72] reported that amelogenin in a plaster of Paris carrier had the same bone inductive activity as similar soluble dentin protein implants into a mouse thigh muscle pouch. Boyan et al. [73], on the other hand, found EMD alone or in an osteoinductive-inactive demineralized freeze-dried bone allograft to be inactive in comparable implants. If the demineralized freeze-dried bone was osteoinductive, low levels of EMD had no effect on activity, but at high amounts of EMD per implant (4 mg), the EMD enhanced net bone induction, new bone area and cortical bone formation. The conclusion drawn was that EMD was not osteoinductive, but it was osteopromotive above some threshold concentration as long as osteoinductive factors were present. Such factors could arise from the surrounding substrate cells in vivo or in culture. As Lyngstadaas et al. [69] have suggested for PDL cells, EMD interactions with mesenchymal cells could initiate signaling pathways leading to secretion of transforming growth factor-β1, interleukin-6 or platelet-derived growth factor or similar autocrine growth factors which then permit tissue regeneration, depending on the particular cells involved. Epithelial cell growth, in the tooth system, is inhibited by the same factors.

Potential functions of the amelogenin peptides created by gene splicing

The complications noted above suggest that alternative methods of studying particular amelogenin effects in vitro and in vivo must be selected. Studies are in progress in a number of laboratories making use of amelogenin knockout mouse models, and these studies support the position that amelogenins are indeed signaling molecules important for proper tooth development and periodontal attachment [74–76]. Amelogenin over-expression is also being used. For example, the over-expression of bovine LRAP (exons 2, 3, 5, 6D, 7) in wild-type and amelogenin-null mice yielded enamel that displayed a cobbled or globular appearing surface in contrast to the smooth enamel surface in untreated wild-type mice [76]. These studies are in their initial stages and will not be discussed further.

Our laboratory has taken a reductionist approach, whereby the effects of particular components are assessed individually in different systems, as an appropriate path for determining the basic interactions of amelogenin components with cells and in developing teeth. This section focuses on the use of defined peptide fractions or specific recombinant amelogenins. One problem with such studies is that the recombinant peptides used have not been subject to any possible post-translational modifications. Another is that during their production, the peptides are subjected to denaturing solvents at some point during purification and may therefore be in some non-native folded or non-native aggregated state. The other side of the coin is the assay system. What are the appropriate tests for activity? As mentioned earlier, the discovery of the mesenchymal cell interaction with the amelogenins specifically, in contrast to some activity of the intact dental epithelium, arose from studies of the chondrogenic-inducing potential of the peptides on low-passage muscle fibroblasts (EMFs) grown out from neonatal rat muscle explants. The basic assays selected were therefore incorporation of ^{35}S-sulfate into proteoglycan, the production of type II collagen and alkaline phosphatase activity.

Chondrogenic and osteogenic activities

Rat incisor amelogenins corresponding to the secreted form of the product of exons 2, 3, 5, 6D, 7 (LRAP, [A-4]), and exons 2, 3, 4, 5, 6D, 7 ([A+4]) were prepared as the glutathione S-transferase (GST) fusion proteins. The forms tested were either the thrombin-cleaved and reverse-phase chromatographically purified amelogenins or the GST-[A+/-4] constructs. Figure 8 shows the sulfate incorporation data for the purified peptides [58]. The peptides had activities comparable to recombinant human BMP-2, with sulfate incorporation about three times that of the negative control. [A+4] and [A-4] incorporated statistically significant different levels of sulfate at comparable weight concentrations at their maximum incorporation concentrations. There was a definite maximum concentration of the peptides above which activity diminished. In separate experiments, enamel matrix derivative (EMD without the commercial carrier used in EMDOGAIN) was used. A response was not obtained until concentrations of the order of 500 µg/ml EMD were applied to the cells. This result made clear that the higher-weight amelogenins and their major degradation products did not have the bioactivity of the small alternatively spliced mRNAtranslated proteins.

Alkaline phosphatase activity was tested in fibroblast-like C2C12 cells, derived from a fetal mouse myoblast cell line. In this study (fig. 9), B. Han, M. E. Nimni and A. Veis [unpublished data] used [A+4] and [A-4] as the intact GST-fusion proteins; therefore, the weight concentrations used were four to five times higher than in the [A+/-4] sulfate incorporation studies shown in figure 8. The GST-fusion proteins were active. However, in measurements made 48 h after the fusion proteins had been added to the cells, the peptides had different levels of activity. In both cases, the peptides showed a distinct maxi-

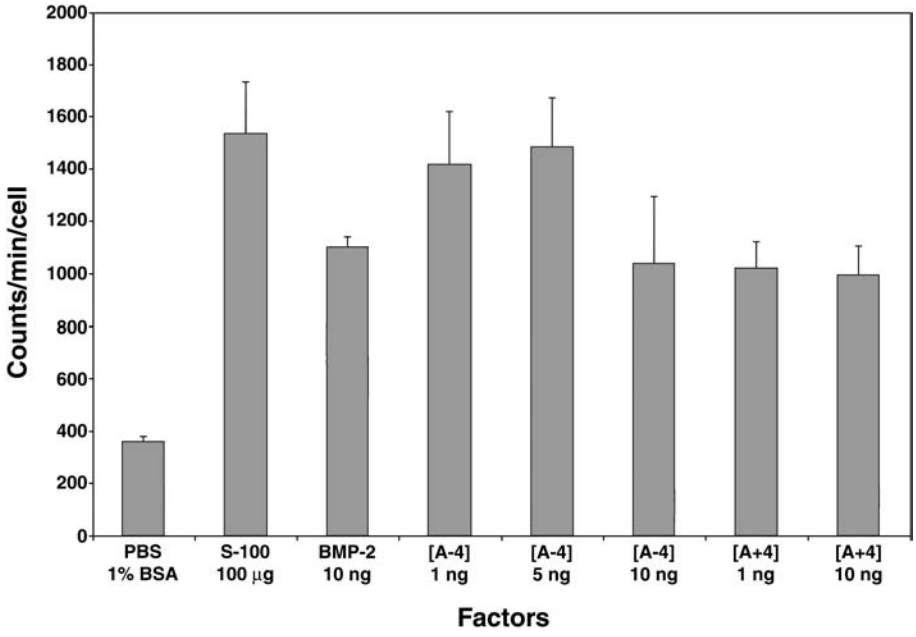


Figure 8. In vitro incorporation of $[^{35}S]SO_4$ into proteoglycan by confluent embryonic rat muscle fibroblasts upon the addition of recombinant amelogenins to the cultures for a 20-h period in 0.5% fetal bovine serum [reproduced with permission from ref. 58]. S-100 is the first crude active fraction isolated from a total dentin extract upon S-100 gel filtration chromatography. BMP-2 is the standard recombinant human BMP-2 positive control, 1% BSA is the negative control. Note that in this assay, [A+4] and [A-4] have statistically significantly different activities on a weight concentration basis.

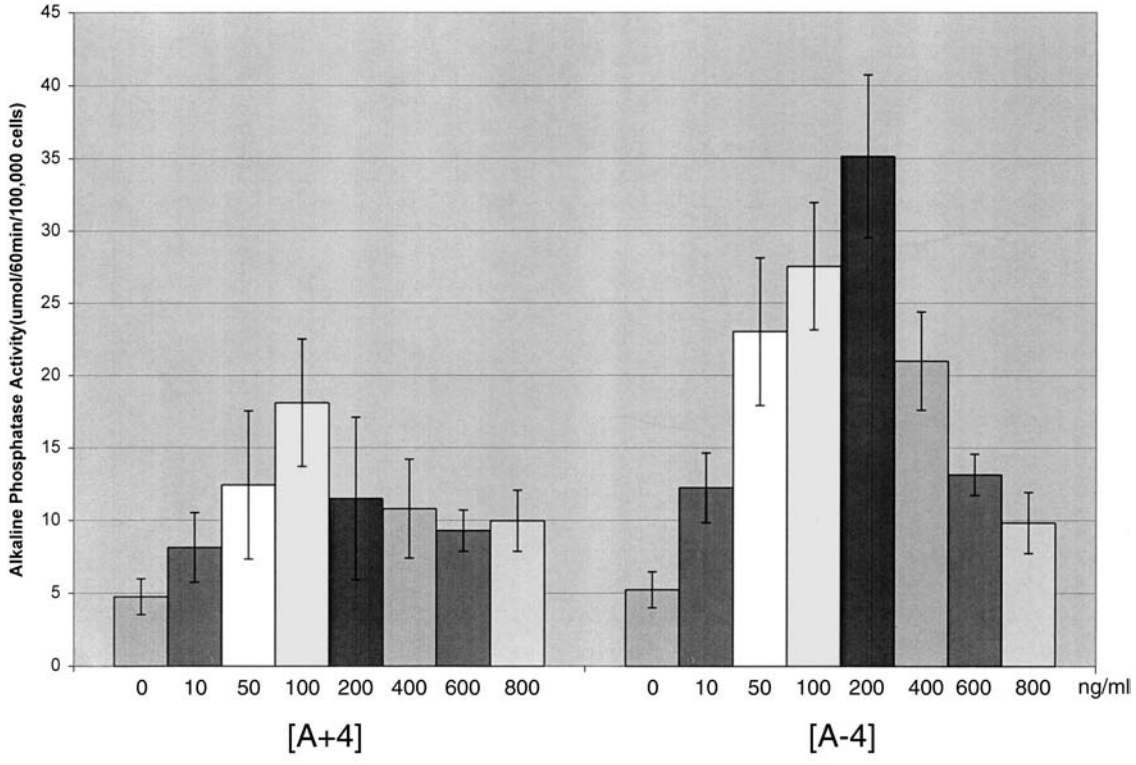


Figure 9. The concentration-dependent effects of GST-[A+4] and GST-[A-4] fusion proteins on the induction of alkaline phosphatase activity in C2C12 cells after 48 h culture. The concentrations of the GST-[A+/-4] were in weight concentrations so that the molar concentration of the [A-4] fusion protein is about 5% higher than the [A+4] fusion protein at each point indicated, not sufficient to account for the difference in activity [Han et al., unpublished data].

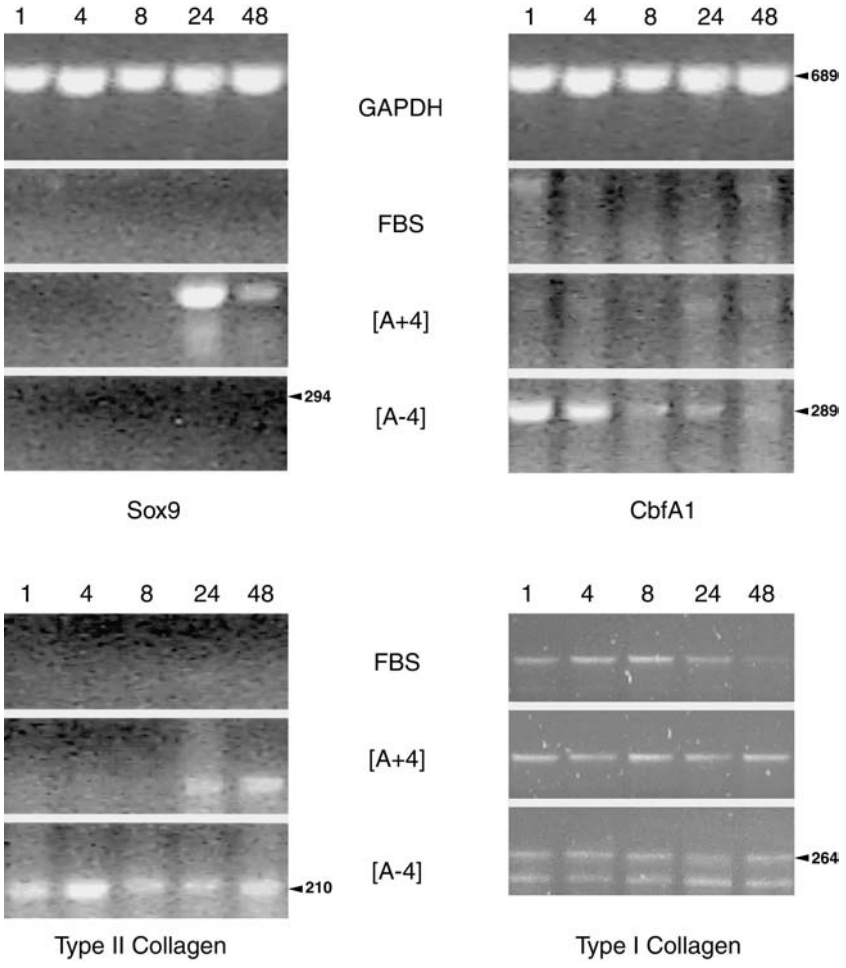


Figure 10. Time course of the production oftranscription factors Cbfa1 and Sox9 and type I and II collagen messages after addition of 10 ng/ml [A+4] and [A-4] to conditioned rat EMF cells. The numbers along the top of each gel lane refer to the time after addition of the factors to the conditioned cells, in hours [adapted with permission from ref. 58].

mum in activity, and higher peptide concentrations were inhibitory, reducing the alkaline phosphatase activity to below negative-control levels.

In the rat EMF culture system [58], [A+4] and [A-4] also induced the appearance of type II collagen message. Several days in culture were required to obtain concentrations of message sufficiently high to detect by Northern analysis, or protein by direct Western blotting with anti-type II collagen antibodies, but PCR analysis provided insight into their mechanisms of action. Figure 10 shows the time course of Sox9 and Cbfa1 mRNA expression after administration of 10 ng/ml [A+4] or [A-4] to the conditioned cells in 0.5% fetal bovine serum (FBS). This is compared to the expression of type II mRNA. The EMFs in culture showed no evidence of type II collagen expression in the conditioned medium alone over the 1–48 h period. [A+4] induced expression of type II collagen in parallel with the expression of Sox9, a transcription factor required for type II collagen expression and entry into the chondrogenic pathway [Han et al., unpublished data]. In contrast, but consistent with all the data indicating different behavior for [A-4] and [A+4], the transcription factor Cbfa1, which appeared to be constituitively expressed at very low levels in the cells in conditioned medium, was upregulated in expression within 1 h of addition of [A–4]. Expression persisted, although the level declined over the 48-h period. Type II collagen message intensity peaked at 4 h and then progressively declined over the time period. Cbfa1 is involved in the transition of chondrogenic cells to the hypertrophic state at the growth plate, and is a transcriptional activator of osteoblastic differentiation [77–80].

In vivo implants into muscle, using a polylactide-polyglycolide carrier confirmed the cell culture data, in that [A+4] and [A-4] were active in inducing cellular ingrowth into the implants, followed by extracellular matrix production, vascularization and mineralization after about 4 weeks [58]. Again, differences were seen between

the activities of [A+4] and [A-4]. [A-4] induced a more copious and diffuse mineral deposition than [A+4], which produced more focal and more highly vascularized mineralized areas. Implants into muscle of GST-[A+4] and GST-[A-4] in collagen sponge carriers [Han et al., unpublished data] similarly showed mineral deposition within the implants, [A-4] >[A+4] > control, after 3 weeks. In onplants, where the collagen sponges were placed subcutaneously directly over the parietal bone of rat calvaria,GST-[A-4] induced new bone formation in the collagen sponge and adjacent to the existing bone. GST-[A+4] in the collagen matrix led to the formation of discrete bone islands, but was accompanied by areas of resorption of the adjacent calvarial bone. These preliminary studies need to be extended to longer time periods and more complete analysis, but show that in the appropriate delivery systems, in vivo, the recombinant amelogenin peptides do have osteogenic-inductive activity.

Role in tooth development: epithelial-mesenchymal signaling

From the studies that have shown the presence of amelogenins in the clear-cell free zone between developing ameloblasts and odontoblasts and in the mantle dentin [30–32] came the proposal that the amelogenins might have a role in the early signaling leading to the maturation of the preodontoblasts. In light of the evidence indicating that the amelogenins might be transiently synthesized within the odontoblasts, equally possible is that the signaling is a reciprocal event and the amelogenins produced by the odontoblasts might influence ameloblast development as well. The time period in which this influence might operate is probably quite brief. While sequential epithelial-mesenchymal interactions take place from the very first stages of epithelial thickening and continue throughout tooth development [81], use of the amelogenin peptides as signaling molecules must be a late, and probably quite restricted event. In the developing tooth germ, the primary events of cell division in both preodontoblasts and preameloblasts take place in the cervical loop region where the mesenchymal dental papilla (DP) and the inner enamel epithelium (IEE) cells face each other across an intact basement membrane (fig. 11). As the cells in each layer mature, the basement membrane degrades, and at this point, amelogenin-related peptide signaling between the cell layers is likely to begin. The odontoblasts mature first and begin to secrete the predentin matrix and finally the mineralized dentin. Ameloblast maturation, characterized by the secretion of the enamel matrix in the region of the Tomes' processes, does not begin until mineralized dentin separates the predentin from the preameloblasts. Thus, the window of time for amelogenin-related epithelial-mesenchymal communication between the IEE and DP cells is probably from the time of initiation of basement membrane breakdown to the formation of mineralized dentin. After the cell division process is completed, maturation of the cells is an inexorable process [82]. On this basis, one might suggest that the preameloblasts signal the underlying preodontoblasts to begin terminal differentiation, while the odontoblasts, in turn, secrete a signal to inhibit ameloblast entry into terminal differentiation until the dentin layer begins to form, ensuring that the final mineralized enamel will

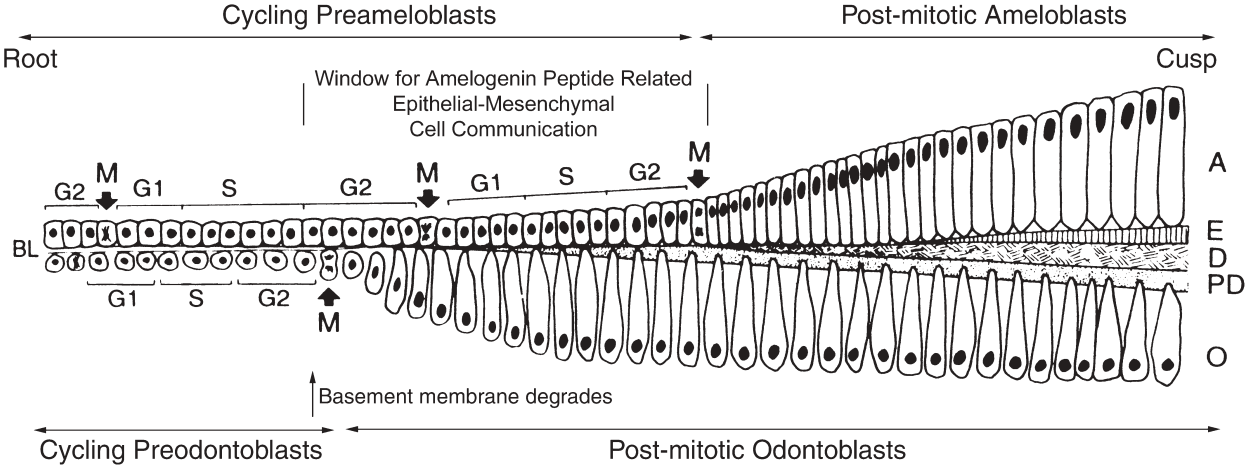


Figure 11. The patterned distribution of differentiating odontoblasts and ameloblasts after completion of their mitotic cycles [modified, with permission from ref. 82, fig. 5]. The putative zone of possible amelogenin-related epithelial-mesenchymal signaling and other direct communication is between the last preodontoblast mitotic cycle and that in the pre-ameloblasts. Other epithelial-mesenchymal signaling events take place beginning from the very first thickening of the dental epithelium at the site of tooth bud formation. The amelogenin peptide signaling and proposed regulation of ameloblast maturation is a late event. This interaction becomes physically blocked when the secreted predentin begins to mineralize and become dentin. A, ameloblasts; BL, basal lamina; E, enamel; D, dentin; PD, predentin; O, odontoblasts.

sit upon a mineralized dentin matrix. That signal could be the secretion of either [A+4] or [A-4] from the preodontoblasts.

To test that hypothesis, the effects of exogenous [A+4] or [A-4] on lower first molars of CD-1 mice at various embryonic stages or post-natal stages were examined in a culture system consisting of a semisolid, serum-free medium supplemented with retinoic acid, ascorbic acid and transferrin [83, 84]. The enamel organ does not synthesize proteins as efficiently as the dental papilla tissue in a serum-free medium [85]. Thus, the effects of the added peptides were essentially restricted to the mesenchyme. Under those circumstances, [A+4] led to the induction of early expression of DMP2, a dentin matrix protein. [A-4], on the other hand, stimulated the expression of cementum attachment protein, in the dental papilla cells and the dental follicle cells surrounding day 1 post-natal tooth germs. Under culture conditions with 2% FBS, enamel organ development proceeded normally in the absence or presence of [A+4], but exogenous [A-4] added to the 2% FBS medium appeared to inhibit preameloblast maturation [86]. These data suggest that, in agreement with the proposals that EMD may have a function in regenerating cementum and periodontal attachment to exposed tooth root surfaces while inhibiting epithelial cell attachment, the more abundantly synthesized [A-4], transiently produced in the maturing odontoblasts, may be inhibitory to maturation of the adjacent ameloblasts. Thus, [A-4] may have opposite effects on epithelial and mesenchymal cells.

In a related study, Six et al. [87] soaked agarose beads with [A+4] or [A-4] and implanted them in cavities drilled through to the surface of the pulp in rat molars. The cavities were placed at the cervical third of the tooth. Cells migrated from the apical region of the pulp to the central part of the root canal. With [A+4], mineralization occurred in the crown part of the pulp and a dentinal bridge closed the cavity within 15 days. Coronal reparative dentin was seen in [A-4] implants, but the major effect was that the mesial root canal became totally filled with reparative dentin by day 30.

Summary

Although the data are still fragmentary, a strong case can be made that the amelogenins have a variety of functions. The primary role may be the provision of the milieu necessary for the development of the mineralized enamel, and that depends on the expression of the full-length amelogenin molecules. The seven-exon amelogenin gene, however, has several short exons with lengths that make them susceptible to exon skipping, while the long exon 6 has several sequences that provide cryptic splice possibilities. In the mouse, all possible splice forms of the mRNA have been found, and most forms also exist in every other species studied. The smaller proteins produced by the minor spliced forms may have specific functions. These functions may relate to the epithelial-mesenchymal signaling that is a prominent part of tooth morphogenesis. In particular, we postulate that the major role may be in a transient preodontoblast signal that inhibits ameloblast maturation and secretion of the amelogenin matrix for mineralization until after a layer of mineralized dentin is formed. That signal may be provided by [A-4], the LRAP peptide form. The expression of [A+4] may promote odontoblast development, as seen in its enhancement of the dentin-specific matrix protein DMP2. Both [A+4] and [A-4] are active, in cultures, at ng/ml levels, appropriate for signaling molecules. Higher concentrations inhibit the signaling. Not clear at this time is if particular amelogenin gene-encoded splice products are involved in the demonstrated positive effect of amelogenin on acellular cementum formation and the periodontal attachment process.

The preliminary studies on the use of [A+4] and [A-4] on tooth repair via dentin bridge formation or pulp capping, and the now well-known differentiation effects on embryonic fibroblasts, and presumably on stem cells, leading to the milieu-dependent pathway leading to cartilage or bone induction, present a possibility for tissue-engineered tooth, cartilage and bone tissue regeneration, or repair based on initiating factors other than the BMPs. The opportunities for use of specific amelogenins in tissue regeneration and repair seem quite open and are worthy of additional study, but this must be done in a framework of further basic investigations of how the amelogenins interact with cells and transduce their signals into different directions.

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