

ARTICLE

## Expression of Alternatively Spliced RNA Transcripts of Amelogenin Gene Exons 8 and 9 and Its End Products in the Rat Incisor

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**SUMMARY** In addition to seven known exons of the amelogenin gene, recent studies have identified two exons downstream of amelogenin exon 7 in genomic DNA of mouse and rat. Here the spatial and temporal expression of mRNAs and of the translated proteins derived from alternative splicing of the amelogenin gene ending with exon 8 and exon 9 were examined by in situ hybridization (ISH) and immunohistochemistry (IHC). RNA signals for exons 8 and 9 were expressed in the ameloblast layer extending from early presecretory to postsecretory transitional stages of amelogenesis. IHC of amelogenin proteins that include sequences encoded by these exons demonstrated identical localization of these proteins in the ameloblast layer corresponding to RNA signals identified by ISH. There was intense immunostaining of the enamel matrix secreted by these cells. Western blotting analysis of rat enamel proteins revealed three distinct protein bands with sequences encoded by the new exons. These data confirmed the existence of the transcripts of alternatively spliced mRNAs coding for exons 8 and 9 of the amelogenin gene in rat tooth germs and suggest that the translated proteins contribute to the heterogeneity of amelogenins and have some significant roles in enamel formation and mineralization.

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**KEY WORDS**

amelogenin  
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in situ hybridization  
immunohistochemistry

TOOTH ENAMEL is a highly mineralized tissue that is formed by ameloblasts derived from the inner enamel epithelium of the tooth germ. Amelogenins are major protein components of developing enamel matrix. They are hydrophobic proteins with high concentrations of proline (Eastoe 1979; Termine et al. 1980; Fincham et al. 1982; Fincham 1983) and are believed to play important roles in enamel mineralization and in formation and elongation of enamel crystals, while displaying heterogeneous molecular forms. High mo-

lecular weight nascent amelogenin (27 kD) adsorbs strongly onto hydroxyapatite crystals and inhibit their growth except for their elongation on the c-axis. In contrast, degraded smaller molecular weight amelogenins have lower affinity for the apatite crystal surface (Aoba et al. 1987). The heterogeneity of amelogenin proteins in developing enamel matrix is due in part to proteolytic degradation of secreted proteins (Shimizu et al. 1979; Carter et al. 1979; Fincham et al. 1991) and to alternative splicing of the amelogenin transcripts (Shimokawa et al. 1987b; Young et al. 1987; Gibson et al. 1991; Lau et al. 1992; Yamakoshi et al. 1994). The remarkable homology between alternatively spliced sequences for mouse, bovine, and human amelogenin (Salido et al. 1992), and for mouse and rat amelogenin (Li et al. 1995), suggests an essential role of the alternatively spliced amelogenin genes in enamel formation.

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Seven alternative splicing patterns of amelogenin mRNA and seven exons were identified in mouse (Simmer et al. 1994). Moreover, four alternative splicing patterns with novel 3' alternatively spliced sequences were identified in the rat incisor (Li et al. 1995) and two additional exons, exons 8 and 9, were elucidated in later studies (Li et al. 1998). Despite an accumulation of evidence showing the presence of these new exons of the amelogenin gene, the expression pattern of the newly identified alternatively spliced transcripts has not been shown in vivo.

In this article we report the spatial and temporal expression of alternatively spliced transcripts encoded by exons 8 and 9 in the rat incisor by in situ hybridization (ISH). We also demonstrate the localization of the amelogenin protein using an antibody raised against an oligopeptide predicted by exons 8 and 9. Western blotting analysis of enamel proteins using the same antibody was done to determine the molecular weight of amelogenin proteins translated from the mRNA coding for exons 8 and 9.

## Materials and Methods

### Probes for ISH

A 156-bp rat cDNA probe, corresponding to coding regions of the amelogenin gene exons 8 and 9, was generated by following method. Two primers, Exon-89-U derived from nucleotides 585–671 and Exon-89-L derived from nucleotides 655–740 of the full-length rat amelogenin sequence, were synthesized by Genemed Synthesis (South San Francisco, CA). The adaptors for EcoR I restrictive cutting sites were included at the 5' ends of these two nucleotides. Two hundred picomoles of each nucleotide were mixed in a PCR reaction buffer (Gibco; Rockville, MD) containing 1.5 mM MgCl<sub>2</sub> and 0.3 mM dNTP. The reaction was heated at 94C for 5 min and cooled to 42C over 30 min. Five units of Taq polymerase (Gibco) were added to the reaction and the reaction was incubated at 95C for 30 sec, 18 cycles of incubation at 95C for 30 sec, 42C for 1 min, and 72C for 2 min, followed by a final incubation at 72C for 10 min. The DNA was extracted and purified by phenol–chloroform and ethanol precipitation. The purified DNA and PCR II vector (Invitrogen; Carlsbad, CA) were digested by 10 U of EcoR I (Roche Diagnostics; Mannheim, Germany) and the plasmid was dephosphorylated by alkaline phosphatase (Roche) to decrease self-ligation. After purification by phenol–chloroform extraction the DNA and the vector were ligated using T4 ligase (Gibco). The ligated plasmid was transformed into TOP10 *E. coli* (Invitrogen). The plasmid DNA was purified from *E. coli* using a QIAprep Spin Miniprep Kit (Qiagen; Valencia, CA). After linearization of plasmid with the restriction enzymes Spe I and EcoR V (New England Bio Labs; Beverly, MA), digoxigenin-labeled single-stranded antisense and sense probes were generated using T7 and Sp6 RNA polymerases (Roche), respectively, using a DIG RNA Labeling Kit (Roche).

### Antibody for IHC

A polyclonal antibody against a peptide (RHPLNMETTTEK) matching the predicted sequence of the terminal three amino acids in exon 8 and the entire coding region of exon 9 was conjugated to keyhole limpet hemocyanin (KLH) and generated in the rabbit by Genemed Synthesis. The serum and pre-immunized serum were purified by ammonium sulfate precipitation, followed by protein A affinity chromatography. The antibody was further purified by peptide affinity chromatography using the same peptide sequence as used to generate the antibody. The peptide was coupled to a HiTrap NHS-activated affinity column (Pharmacia; Piscataway, NJ) according to the protocol provided by the manufacturer (Li et al. 1998).

### Tissue Preparation

Under anesthesia, six 3-week-old Wistar rats were vascularly perfused with 4% paraformaldehyde solution in 0.1 M phosphate buffer (pH 7.4), mandibles dissected free of soft tissue, and further fixed in the same fixative overnight at 4C. The specimens were then decalcified in 8% EDTA (pH 7.4) at 4C and embedded in paraffin. Serial paraffin sections (4 μm) were prepared for ISH or IHC.

Some of the mandibles were dehydrated through a graded series of *N,N*-dimethylformamide and embedded in glycol methacrylate (GMA) (Oken; Tokyo, Japan), which was polymerized under UV light at 4C.

These animal experiments were approved by the Institutional Ethical Committee for Animal Experiments and properly carried out under the control of the Guideline for Animal Experimentation in Tokyo Medical and Dental University.

### In Situ Hybridization

After treatment with proteinase K (1.0 μg/ml) for 10 min, the paraffin sections were postfixed in 4% paraformaldehyde for 10 min and dipped in 0.1 M triethanolamine containing 0.5% acetic anhydride for 15 min.

Hybridization was performed at 40C using the denatured amelogenin exons 8 and 9 antisense or sense probe in hybridization solution (50% formamide, 0.6 M NaCl, 1 × Denhardt's solution, 10 mM Tris buffer, 1.0 mM EDTA, 10% dextran, 200 μg/ml yeast tRNA) for 20 hr. After post-hybridization treatments including RNase A treatment (5.0 μg/ml, 30 min, 37C) followed by thorough washes, the sections were reacted with anti-digoxigenin antibody conjugated with alkaline phosphatase (1:500) (Roche). The locus of hybridization was demonstrated by treating sections with 5-β-bromo-4-chloro-3-indolylphosphate and nitroblue tetrazolium solution according to the manufacturer's instructions (Roche).

### Immunohistochemistry

**ABC Method on Paraffin Sections.** Paraffin sections were pretreated with 0.3% H<sub>2</sub>O<sub>2</sub>–methanol solution to inhibit endogenous peroxidase, followed by a mixture of 1% bovine serum albumin and 2% normal goat serum solution to prevent nonspecific binding of antisera. A polyclonal antibody raised in rabbit against oligopeptide (RHPLNMETTTEK) matching the predicted amino acid sequence (1:100) and

anti-porcine 25-kD amelogenin polyclonal antibody (Uchida et al. 1991b) (1:5000) were respectively used as the primary antibody, and a biotinylated anti-rabbit IgG antibody (1:500) was used as the secondary antibody. Immunoreactive sites were further treated with a Vectastain ABC kit (Vector; Burlingame, CA) and visualized by treating with 3,3'-diaminobenzidine tetrahydrochloride.

**Colloidal Gold–Silver Enhancement Method on GMA Sections.** Immunolocalization of the amelogenin exons 8 and 9 translated proteins was also pursued by immunogold–silver staining method on GMA-embedded specimens, according to Uchida et al. (1991b). After a blocking treatment, 0.5- $\mu$ m-thick GMA sections were stained with the antibody against the predicted oligopeptide (RHPLNMETTTEK) (1:500) and then with goat anti-rabbit IgG conjugated to 5-nm colloidal gold particles (1:200) (EY Laboratories; San Mateo, CA). Silver intensification was carried out for 14–21 min at room temperature according to Uchida et al. (1991a) until sufficient blackening of immunoreactive sites could be obtained.

### Immunoblotting

Three-week-old Wistar rats ( $n = 20$ ) were sacrificed by an overdose of ether anesthesia and enamel matrix was collected from the apical half of the incisors with a scalpel. Collected enamel matrix was washed with normal saline and dissolved in 2% acetic acid. After removing cell debris by centrifugation (3000 rpm, 15 min at 4C), sample solutions were prepared according to the method of Laemmli (1970). Sample solutions and molecular weight marker (Protein Molecular Weight Standards; Invitrogen) were applied on 10–20% gradient polyacrylamide gels, and the proteins were separated by SDS-PAGE at 30 mA for 1.5 hr.

The proteins separated by SDS-PAGE were transferred to a polyvinylidene difluoride (PVDF) membrane (Clear Blot membrane P; ATTO, Tokyo, Japan) by a semi-dry Western blotting method and processed for immunostaining with a polyclonal antibody against oligopeptide (RHPLNMETTTEK) (1:1000) by the ABC method, as already described for tissue staining. The membranes were also stained with Coomassie Brilliant Blue R-250 to detect all transferred proteins.

## Results

### In Situ Hybridization

The in situ signals of the mRNA coding for amelogenin gene exons 8 and 9 first appeared in the cytoplasm of the cells of the inner enamel epithelium (Figure 1a). No significant in situ signals were depicted in the opposing preodontoblasts. The mRNA levels in preameloblasts became stronger toward the incisal direction, and the most intense signals appeared in the cytoplasm of secretory ameloblasts, except in the Tome's process regions at the distal end (Figure 1b). The signals became weaker in the late secretory stage and disappeared in the postsecretory transitional stage (Figure 2a). Sections hybridized with sense probes displayed no positive signals (Figure 1c).

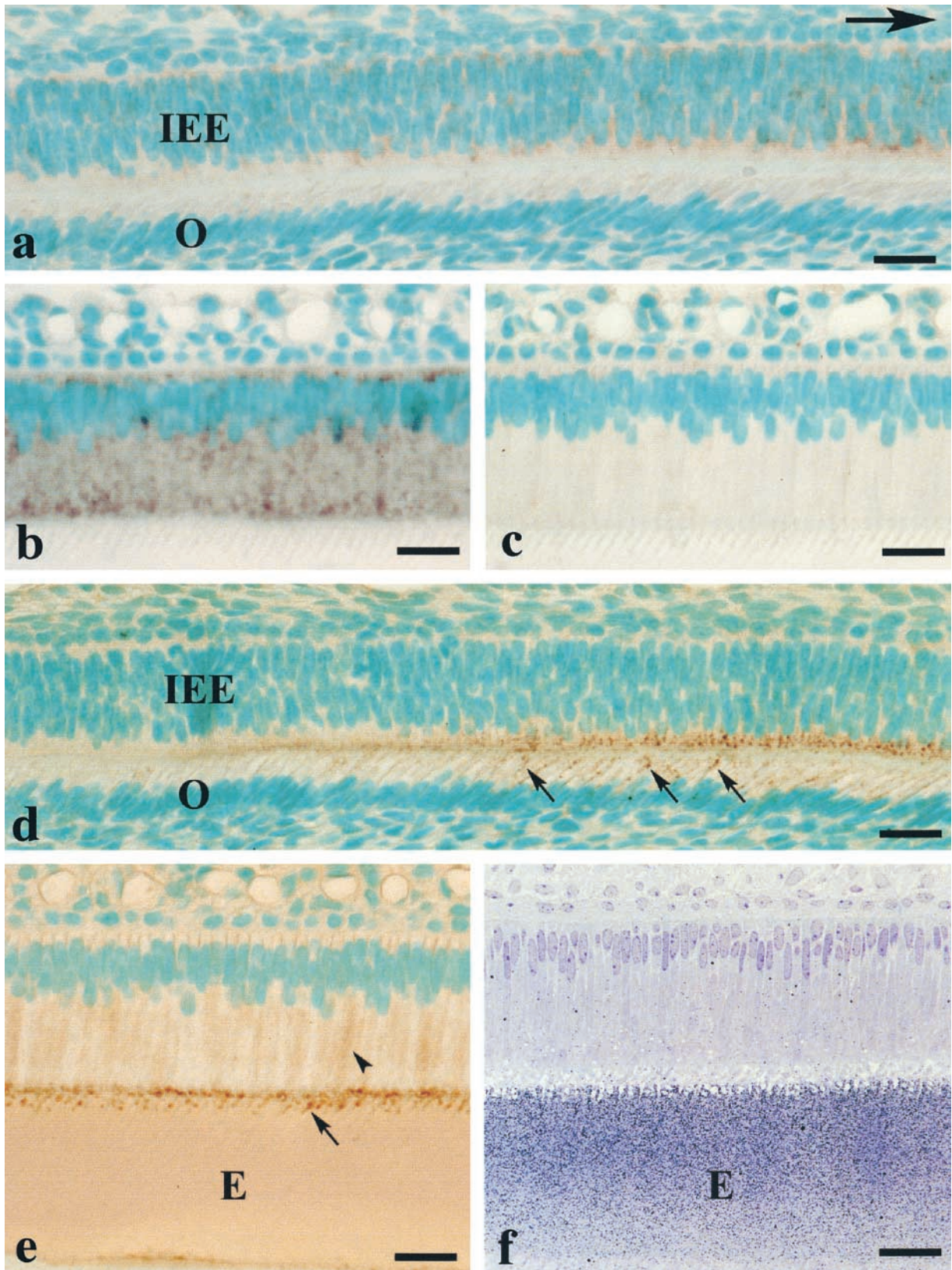
### IHC on Paraffin Sections

In rat incisors, immunoreactions for the amelogenin exons 8 and 9 translated proteins first appeared in the distal cytoplasm and in distal intercellular spaces of the cells of the inner enamel epithelium or in early pre-secretory ameloblasts facing a thin fibrous layer of future mantle dentin (Figure 1d). The cytoplasmic reactions for the amelogenin exons 8 and 9 translated proteins became stronger in the more incisally located preameloblasts, in which supranuclear cytoplasm (Golgi regions) began to show some immunoreactivity. Granular immunoreactions were also visible in the intercellular spaces of these preameloblasts and in the non-mineralized fibrous extracellular matrix lying between the preameloblast and preodontoblast layers (Figure 1d). In the molar tooth germ, intense immunoreactions for the amelogenin exons 8 and 9 translated proteins were localized along the fibrous thin layer lying between the preameloblast and preodontoblast cell layers (Figure 3). At high magnifications, intense immunoreactions were located in the supranuclear and distal cytoplasm of preameloblasts. Extracellular granular reactions were also located in the fibrous matrix of future mantle dentin (Figure 3, inset).

Immediately after the formation of a distinct layer of mineralized dentin, a thin layer of enamel started to form and showed strong reactions for the amelogenin exons 8 and 9 translated protein. The tall secretory ameloblasts maintained moderate immunoreactions in the Golgi regions throughout the secretory stage and further demonstrated more distinct reactions in the Tome's processes inserting in the newly formed enamel matrix (Figure 1e). The newly formed enamel surface showed intense immunoreactions, but the sub-surface and deeper layers of secretory enamel showed reactions only slightly above background density (Figure 1e). No other regions of the enamel organ displayed significant reactions.

Towards the end of the secretory stage, intense reactions at the distal cytoplasm of ameloblasts began to diminish in accord with the retraction of the Tome's processes. Immunoreactions in the ameloblast layer disappeared by the end of the transitional stage (Figure 2b). In some cases, however, granular immunoreactions for exons 8 and 9 translated proteins were noted in the intercellular regions of the transitional ameloblasts (Figure 2b). Thereafter, no immunoreactions became detectable in the enamel organ of rat incisors throughout the maturation stage of amelogenesis (Figure 2c). In contrast, immunoreactivity of the remaining enamel matrix was gradually intensified until the entire matrix finally disappeared by decalcification (Figure 2c).

Localization patterns of immunoreactions for the 25-kD amelogenin were similar to those of the amelogenin exons 8 and 9 translated proteins throughout



the presecretory and secretory stages, but with much stronger reactions in the Golgi regions of the cytoplasm. In the early maturation stage, however, distinct granular immunoreactions for 25-kD amelogenin, presumably corresponding to the Golgi and lysosomal structures, remained in the supranuclear and distal cytoplasm of the ameloblasts, facing the remaining enamel matrix layers after EDTA decalcification (Figure 2d). No immunoreactivity was detectable in the ameloblasts at the more advanced stages of maturation, when the underlying enamel matrix had been totally lost by decalcification.

#### IHC on GMA Sections

Immunoreactions for the amelogenin exons 8 and 9 translated proteins were localized in a manner comparable to those shown in paraffin sections in the extracellular spaces of the inner enamel epithelium and presecretory ameloblasts. However, the immunoreactions in growing enamel layers differed markedly from those on paraffin sections. Silver grains representing the immunoreactive sites were evenly distributed throughout the secretory enamel layers from the dentinoenamel junction to the growing enamel surface (Figure 1f). No significant immunoreactions could be detected in the cytoplasm of secretory ameloblasts, including the Tome's processes. The intensity of immunoreactions on enamel matrix remained consistent until the remaining matrix disappeared by decalcification in the maturation stage.

#### Immunoblotting

The Coomassie Brilliant Blue (CBB)-stained blotted lane (Figure 4) showed several clear bands ranging from approximately 14 kD to 28 kD. Immunoblotting with the anti-25-kD amelogenin antibody showed positive reactions coinciding with these CBB-stained bands. Immunoblotting with the antibody against the oligopeptide containing the amino acids encoded by amelogenin gene exons 8 and 9 revealed three distinct bands of approximately 29, 28, and 26 kD, reactive for the antibody. The highest molecular weight band

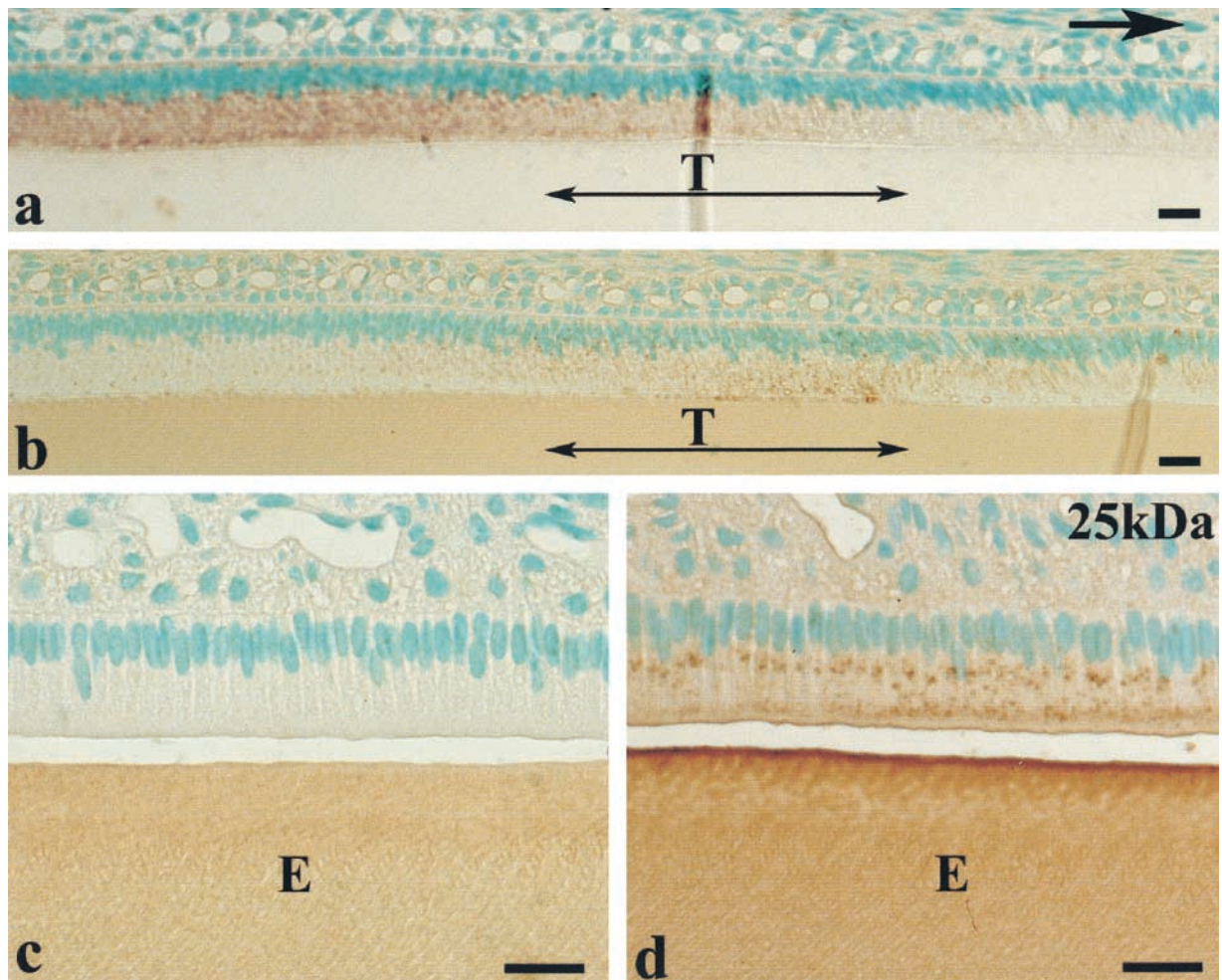
(29 kD) was undetectable either by CBB staining or by the antibody against 25-kD amelogenin.

#### Discussion

In these experiments, a stage-specific expression of mRNAs coding for the exons 8 and 9 of the amelogenin gene, as well as synthesis and secretion of the proteins translated from these messages, has been confirmed in the molar and incisor tooth germs of the rat, starting from early presecretory to postsecretory transitional stages of amelogenesis. The distribution pattern of the amelogenin proteins containing the amino acid sequences encoded by these exons, as shown by IHC, coincided well with that of mRNA expression in the ameloblast layer except at the early stage of development, as shown in Figure 1a. Because in situ signal is lacking throughout the odontoblastic cell layer, immunoreactions in the preodontoblast layer apparently represent the enamel proteins being synthesized by the cells of inner enamel epithelium and/or preameloblasts, which show weak but significant ISH signals for the amelogenin coding for exons 8 and 9. This assumption is supported by previous reports on IHC evidence of penetration of amelogenin towards the dental pulp in early stages of amelogenesis (Inage et al. 1989; Inai et al. 1991).

Using the probe generated from bovine amelogenin cDNA coding for exons 1–7 (Shimokawa et al. 1987a), Inage et al. (1996) showed a precise mapping of amelogenin mRNA expression in rat incisor. In their studies the amelogenin mRNA signals started to appear in the cells of the inner enamel epithelium close to the preameloblasts and lasted until the ameloblasts entered the initial stage of enamel maturation, although the signal expression became weaker during the transitional stage. A similar pattern of amelogenin mRNA expression has been reported to appear in rat molars (Fong et al. 1996), hamster molars (Karg et al. 1997), and mouse molars (Hu et al. 2001). By electron microscopic immunocytochemistry of amelogenins combined with autoradiography, Nanci et al. (1987)

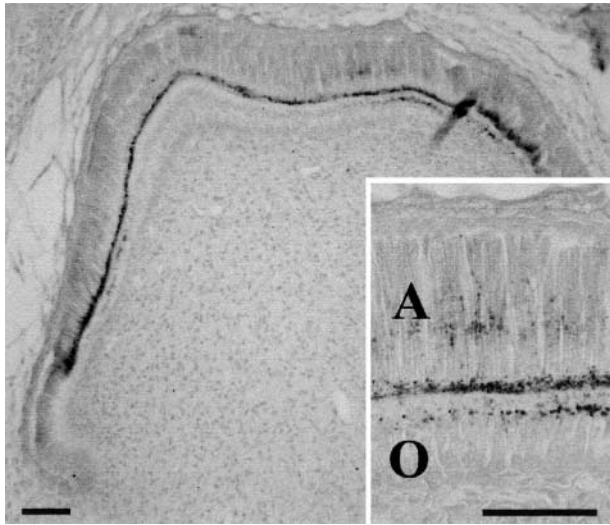
**Figure 1** Expression of mRNA coding for the amelogenin gene exons 8 and 9 by ISH (a–c) and immunolocalization of the translated proteins by the anti-oligopeptide (RHPLNMETTTEK) antibody (d–f) in longitudinal sections of the rat incisor. Arrow indicates incisal direction. (a) Weak but significant RNA signals (dark violet) first appear in the cytoplasm of inner enamel epithelium (IEE) and become gradually intense towards the incisal direction (large arrow). O, preodontoblast. (b) In the secretory stage, ameloblasts show intense RNA signals throughout the cytoplasm except in the region of Tome's processes at the distal end. (c) Absence of signal expression with sense probe. (d) Immunoreactions (brown) for amelogenin exons 8 and 9 translated proteins is located in the distal ends of inner enamel epithelium (IEE). Extracellular immunoreactions (arrows) are also located in the preodontoblast layer (O). (e) Immunoreactions for amelogenin exons 8 and 9 translated proteins in secretory ameloblasts showing moderate staining in the supranuclear and Golgi regions (arrowhead) and intense reactions in the Tome's process regions at the distal cell end (arrow). Enamel matrix (E) is only lightly stained (paraffin section). (f) Immunoreactions for amelogenin exons 8 and 9 translated proteins on GMA section. The whole thickness of secretory enamel layers (E) is evenly stained with the antibody. No significant reaction is seen in the cytoplasm of ameloblasts. Bars = 20  $\mu$ m.



**Figure 2** Changes in signal expression of mRNA coding for the amelogenin gene exons 8 and 9 (a) and immunolocalization of translated proteins (b,c) viewed in longitudinal sections of a rat incisor from late secretory to early maturation stages. Arrow indicates incisal direction. (a) Signal expression in late secretory stage ameloblasts and its disappearance by the end of the transitional stage (T). (b) Immunolocalization of translated proteins in the adjacent section. Note granular immunoreactions in the intercellular regions of ameloblasts at the transitional stage (T). (c) Absence of immunoreactions in the ameloblasts at early maturation and elevated immunoreactivity in the remaining enamel matrix (E). (d) Immunolocalization of amelogenins in the region similar to c, stained with anti-25-kD amelogenin antibody. Intense reactions are localized in the supranuclear Golgi region and distal cytoplasm of ameloblasts. Remaining enamel matrix (E) is also intensely stained. Bars = 20  $\mu$ m.

indicated that postsecretory maturation stage ameloblasts continue to synthesize and secrete minute amounts of amelogenins throughout the maturation stage, up to the point at which enamel matrix is lost by decalcification. In this experiment we could also localize intense immunoreactivity for 25-kD amelogenin in the supranuclear and distal cytoplasm of maturation stage ameloblasts (Figure 2d), indicating continued synthesis and secretion of enamel matrix proteins by the ameloblasts in early stages of enamel maturation. Absence of *in situ* signals of mRNA coding for exons 8 and 9 as well as immunoreactions for its translated proteins in the ameloblasts at early maturation may indicate different roles of the amelogenin proteins be-

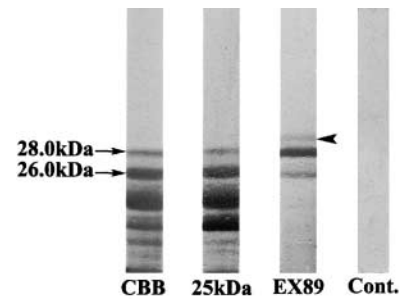
tween those translated from the mRNAs coding for exons 1–7 and for 8 and 9. Nanci et al. (1987) suggested that some proportion of the immunoreactions in maturation stage ameloblasts represent endocytosed enamel proteins. In this context, absence of immunoreactions to the antibody against exons 8 and 9 translated peptide in the maturation stage ameloblasts may also be an indication of a smaller proportion of the protein present in the enamel matrix relative to the other amelogenins detectable by the anti-25-kD amelogenin antibody. In presecretory and secretory stages of amelogenesis, immunolocalization of the amelogenin proteins encoded by mRNA of exons 8 and 9 coincides well with that of 25-kD amelogenin proteins



**Figure 3** Immunolocalization of translated proteins by an anti-oligopeptide (RHPLNMETTTEK) antibody in the third molar tooth germ of a 3-week-old rat. Reactions are located in the supranuclear and distal cytoplasm of preameloblasts (A). Extracellular granular reactions are closely associated with preodontoblasts (O). (Inset) Higher magnification. Bars = 50  $\mu$ m.

(data not shown). We suggest that the synthesis of exons 8 and 9 translated proteins begins in the cells of the inner enamel epithelium or early presecretory ameloblasts and secreted together with the other known enamel proteins, at least until the end of the stage of matrix formation. Granular immunoreactions that appeared in the intercellular spaces of transitional ameloblasts, as shown in Figure 2b, most likely represent the enamel proteins containing exons 8 and 9 translated proteins being extruded from the underlying enamel as well as those erratically secreted by the transitional ameloblasts.

It is noteworthy that, in paraffin sections, intense immunoreactions for exons 8 and 9 amelogenin translated proteins have been limited to Tome's processes of ameloblasts and the layer of the enamel nearest to the surface during the stage of matrix formation (Figure 1e). This finding may indicate extremely rapid degradation of the amelogenin proteins containing the transcripts of these exons after secretion. It is also possible to interpret this phenomenon to indicate that at least part of the translated proteins serve as membrane constituents of ameloblasts rather than as enamel matrix proteins (Li et al. 1998). However, the same antibody stains the entire thickness of secretory enamel layers more or less evenly when applied on GMA sections (Figure 1f). Uchida (2000) has recently claimed that, in paraffin sections, the immature enamel matrix is an exceptionally difficult tissue for the antibodies to penetrate and hence the intensity of immunoreactions



**Figure 4** Immunoblotting analysis of immature enamel matrix of rat incisor. Anti-25-kD amelogenin antibody-positive bands (Lane 25 kD) coincide well with those stained with Coomassie Brilliant Blue-stained bands (Lane CBB) of 14.3–28.0 kD. With anti-oligopeptide (RHPLNMETTTEK) antibody (Lane EX89), three clear bands of 29.0, 28.0, and 26.0 kD are confirmed. The highest band (arrow) is not depicted either by CBB or anti-25-kD antibody. The lane reacted with preimmunized rabbit serum shows no positive bands (Lane Cont.).

over secretory enamel does not reflect the relative amounts of antigens within the enamel matrix, at least in paraffin sections.

In maturation stage enamel, the rather intense immunoreactions over the enamel matrix remaining after decalcification (Figure 2c) are thought to reflect an increased permeability of the maturing enamel matrix to the antigens. On GMA sections, on the other hand, antigen–antibody interaction takes place only at the very surface of the sections, regardless of the types of tissues embedded. Therefore, the staining reactions of the matrix on GMA sections are thought to reflect actual relative concentrations of amelogenins within the tissues more precisely than on paraffin sections. It is noteworthy, however, that reactions for exons 8 and 9 translated proteins associated with the secretory pathway of secretory ameloblasts were demonstrable only on paraffin sections. Absence of comparable immunoreactions in GMA sections is attributable to the scarcity of the exposed antigens on section surface.

The result of immunoblotting analysis has confirmed the existence of the amelogenin proteins translated from the mRNA coding for exons 8 and 9 in the secreted enamel matrix. CBB staining and immunostaining with anti-25-kD amelogenin antibody showed several clear bands ranging from approximately 14 kD to 28 kD, as has been reported by Aoba et al. (1992). In contrast, the antibody against a peptide (RHPLNMETTTEK) matching the sequence of the terminal three amino acids in exon 8 and the entire coding region of exon 9 reacted specifically with three protein bands of approximately 26, 28, and 29 kD. The amount of 29-kD protein in secretory enamel matrix appears to be very small because it is not depicted by CBB. Li et al. (1995) reported the four alternative

splicing patterns coding for exons 8 and 9, and estimated the theoretical molecular weights of their translated products as 23.0, 20.2, 9.4, and 6.6 kD, respectively, based on the amino acid sequence. Since the molecular weight of amelogenins by SDS-PAGE is known to appear larger than the weight calculated from the amino acid sequence (Chen et al. 2000), two of the three proteins of 26 kD, 28 kD, and 29 kD detected by immunoblotting may correspond to the estimated 23.0- and 20.2-kD proteins. The other one would either be the new translated protein from the yet unknown alternatively spliced transcripts or the aggregate of the degraded amelogenins. The absence of lower molecular protein bands corresponding to the estimated proteins of 9.4 kD and 6.6 kD by Western blotting may also be attributable to limitations in the amount of the available proteins used for the analysis.

The roles of these and other alternatively spliced amelogenins in tooth formation are not known. However, the stage-specific localization of the exons 8 and 9 amelogenin transcripts in the secretory ameloblasts suggests a role in enamel formation. Further studies, including the generation of transgenic mice with specific deletions of amelogenin exons 8 and 9, will be needed to define the specific role of these amelogenins in tooth formation.

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