Antisense inhibition of AMEL translation demonstrates supramolecular controls for enamel HAP crystal growth during embryonic mouse molar development

Thomas Diekwisch, Sasson David, Pablo Bringas Jr., Valentino Santos and Harold C. Slavkin*

Center for Craniofacial Molecular Biology, School of Dentistry, University of Southern California, 2250 Alcazar Street CSA/103, Los Angeles, CA 90033, USA

*Author for correspondence

Summary

During tooth development, enamel organ epithelial cells express a tissue-specific gene product (amelogenin) which presumably functions to control calcium hydroxyapatite crystal growth patterns during enamel biomineralization. The present studies were designed to test the hypothesis that amelogenin as a supramolecular aggregate regulates crystal growth during enamel biomineralization. Antisense oligodeoxynucleotide strategy was used in a simple organ culture system to inhibit amelogenin translation. Under these experimental conditions, antisense treatment prior to and during amelogenin expression resulted in inhibition of amelogenin translation products within immunoprecipitated [35S]methionine metabolically labeled proteins. To determine the efficiency of antisense treatment in this model system, digoxigenin-labeled oligodeoxynucleotides were observed to diffuse throughout the tooth explants including the target ameloblast cells within 24 hours. Ultrastructural analyses of amelogenin supramolecular assembly as electron-dense stippled materials in antisense treated cultures demonstrated dysmorphology of the extracellular enamel matrix with a significant reduction in crystal length and width. We conclude that secreted extracellular proteins form a supramolecular aggregate, which controls both the orientation and dimensions of enamel crystal formation during tooth development.

Key words: amelogenin, antisense, biomineralization, tooth development, enamel

Introduction

Biomineralization is a cell- and/or extracellular matrix (ECM)-mediated process in which inorganic ions are assembled into a biological structure (Young, 1975; Young and Brown, 1982). Cell- and ECM-mediated biomineralization assumes that biologically associated inorganic crystals grow within a preconstructed organic framework within cells or in ECM secreted by cells (Lowenstam, 1981; Lowenstam and Weiner, 1989). Lowenstam (1981) concluded that different organisms (procaryotes and eucaryotes) use common strategies for mineral deposition and raised the possibilities for highly conserved processes throughout evolution that mediate biomineralization. Curiously, inorganic mineral deposition in organic matrices mostly occurs intracellularly throughout evolution (see Lowenstam and Weiner, 1989). Anionic macromolecules (e.g. phospholipids, phosphoproteins, glycoproteins) as constituents of organic intra- and/or ECM appear to function as templates for inorganic deposition, thereby serving as nucleation sites for crystal growth in supersaturated microenvironments (Fleisch, 1982; Lowenstam and Weiner, 1989; Williams, 1989). Whereas there is extensive descriptive information regarding invertebrate and vertebrate biomineralization during development, very little is known about how specific molecules regulate tissue-specific biomineralization.

Phylogenetically, enamel biomineralization is highly conserved during vertebrate evolution: adults frogs (Rana pippens), reptiles and mammals produce amelogenins (AMEL), whereas sharks, bony fish and amphibian larvae (Rana pippens) do not appear to produce AMEL (Deutsch, 1989; Deutsch et al., 1991; Slavkin et al., 1984; Herold et al., 1989). Recent studies of mouse enamel formation have provided molecular characterization of an AMEL cDNA, the genomic localization of AMEL to the X-chromosome in mice, and the deduced amino acid sequence of the AMEL protein (Snead et al., 1983, 1985; Lau et al., 1988). Ultrastructural studies described the initial enamel ECM to consist of a stippled or finely granular, electron-dense material localized between the distal extensions of the secretory ameloblasts and the initial mineralization region (Watson, 1960; Fearnhead, 1960; Reith, 1967; Slavkin et al., 1976; Nanci et al., 1984, 1985). Comparable electron-dense mate-
MATERIALS AND METHODS

Microdissection and organ culture

Timed-pregnant, Swiss-Webster strain mice were used in this study (Simonsen Labs, Gilroy, California). E15 embryos were dissected from uterine decidua and developmentally staged by external features (Theiler, 1972). Cap stage M1 molars were isolated and cultured as explants for periods up to 21 days in our modification of the Trowell organ culture system using serumless, chemically defined medium. The details of our modifications have been described in several publications (Bringas et al., 1987; Evans et al., 1988). The dissected molar explants were oriented on a Millicell filter so that left and right quadrant tooth organs could be identified.

Synthetic AMEL sense and antisense oligonucleotides

Based upon the nucleic acid sequence data for mouse AMEL (Snead et al., 1985), we synthesized a penultimate oligomer (15mer) sense and antisense oligonucleotides (ODN) targeted to 5′ codons of the mouse AMEL precursor mRNA beginning with the highly conserved initiating AUG codon (Fincham et al., 1981, 1992) using a PCR Mate EP 391 DNA Synthesizer (Applied Biosystems, Foster City, CA) and subsequently purified the ODN products using a cartridge technique (Beaucage and Caruthers, 1981; Matteucci and Caruthers, 1981; Zon et al., 1985). Subsequent experiments used HPLC-purified ODN synthesized by an outside vendor (Biosynthesis, Lewisville). The nucleic acid sequence used for the construction of the ODN were as follows: (i) antisense sequence was: 5′ AGG TGG TAG GGG CAT 3′, and (ii) sense sequence: 5′ ATG CCC CTA CCA CCT 3′. Both the sense and antisense probes were dissolved in double distilled water and quantitated by optical density at OD260. The antisense and sense ODN were used at a concentration of 30 µM and added every other day to the culture medium.

Diffusion analysis using digoxigenin antisense AMEL ODN labeling

To evaluate the efficiency of synthetic ODN diffusion E15, molars were cultured for 5 days and then treated with labeled ODN for 24 hours. AMEL transcripts were first expressed in this model system, as observed with in situ hybridization, at 5 days in vitro (data not shown). The 15mer antisense AMEL ODN were tailed with digoxigenin-11-dUTP and terminal transferase using a Boehringer Mannheim kit (DNA Labeling and Detection Kit, Non-radioactive; Boehringer Mannheim, Indianapolis, IN). The ODN were purified by alcohol precipitation (100% ethanol, overnight) and centrifugation in a Sephadex®-column for 2 minutes at 1000×g. The labeled ODN were added into the culture medium at a concentration of 30 µM and incubated for 24 hours. Control groups were treated with unlabeled ODN at the same concentration. After incubation, the cultured molar explants were immediately frozen, stored at ~80°C and subsequently cut into frozen sections. Sections were not fixed. Sections were directly used for immunodetection with anti-digoxigenin antibody (Boehringer Mannheim, Indianapolis, IN) at a concentration of 1:5000. The immunoreaction was carried out according to the instructions of the labeling kit data sheet provided by the vendor.

35S-metabolic labeling for AMEL immunoprecipitation

E15 molar explants cultured for 12 days were metabolically labeled with [35S]methionine (specific activity 3,000 Ci/mM, 80 µCi/ml, New England Nuclear, Boston) using a methionine-deficient medium (RPMI-1640, GIBCO) for 4 hours, chased for 1 hour with non-radioactive methionine (10 nM), and then washed with PBS (phosphate-buffered saline, pH 7.4). Explants were then homogenized and proteins were extracted with acetic acid and immunoprecipitated with AMEL antibodies as previously described (Slavkin et al., 1982, 1988). Antisense and sense ODN were used at concentrations of 30 µM and medium was changed every other day. Each culture dish contained 5 molar explants. Ten dishes of cultured explants were pooled for each treatment.
Light and transmission electron microscopy

Cultured molar explants were processed for anhydrous fixation (Landis, 1983; Evans et al., 1988), or Karnovsky fixation (Luft, 1961; Yamada et al., 1980). Anhydrous fixation was used to conserve the initial HAP crystal structure position for subsequent crystal measurements, whereas organic matrix structures was optimally examined using Karnovsky-fixed specimens. After embedding in Epon 812 and thin sectioning, observations were made on a JEOL 1200EX transmission electron microscope at 80 kV.

Ultrastructural sampling criteria

After polymerization, the Epon blocks were trimmed and regularly re-embedded to ensure that the mesio-buccal cusps of the M1 were routinely used for semithin and thin sectioning. The mesiobuccal cusp was oriented vertically to the block axis. Only the middle vertical plane was used for thin sectioning to ensure comparable sampling. Twelve blocks per experimental group were analyzed; 5 grids per block, 4 sections per grid, and 5 electron photomicrographs of randomly selected areas near the forming dentine-enamel junction were used and processed. For HAP crystal measurements, we analyzed crystals in areas where the enamel width was 1 µm thickness. Photomicrographs were taken at 100,000× magnification and enlarged on high-contrast paper.

Ultrastructural supramolecular pattern analyses

To analyze ultrastructural differences in the organization of enamel stippled materials, presumably representing supramolecular aggregates of AMEL between antisense ODN-treated and control groups, we digitized electron photomicrograph images at 500,000× magnification and calculated moment invariances. Moments are descriptive numbers that reflect the qualitative deviations of points in a finite area. According to a uniqueness theorem (Papoulis, 1965), a continuous function of finite areas contains moments of all orders. After calculating moment descriptors per image, these were centralized and then normalized to adjust for differences between the gray-tone levels of different pictures. In order to discriminate for texture, skewness, variance and other pattern characteristics, a set of moment invariants were calculated that are independent of translation, rotation or scale change as previously described by Gonzalez (1987). The results were analyzed using ANOVA with a statistical software package (Epistat®).

RESULTS

Enamel biomineralization during molar tooth development in serumless, chemically defined medium

AMEL transcription and translation occurs at birth in M1 tooth organs (Snead et al., 1984, 1988). When E15 cap stage M1 were cultured in serumless, chemically defined medium, AMEL transcription and translation was observed at 5 days in vitro (data not shown). In this in vitro model system, initial enamel biomineralization was first observed at 12 days (Evans et al., 1988; Slavkin et al., 1992; Figs 1A-F, 7A). Von Kossa staining for calcium phosphate salt precipitation demonstrated initial dentine and enamel biomineralization along the messio-buccal cusp of the explants cultured for 12 days (Fig. 1C). Ultrastructural observations found enamel HAP crystals (en) forming along the initial dentine-enamel junction (dej) (Fig. 1D,E). Previous immunocytochemical studies reported that AMEL antigens were localized within ameloblast-derived extracellular electron-dense granular material in vivo and in vitro (see Nanci et al., 1984, 1985; Slavkin et al., 1988) (Figs 1E, 7A).

Exogenous oligodeoxynucleotides diffuse throughout molar explants

Digoxigenin-labeled ODN were uniformly distributed throughout all cells within molar explants after 5 days in culture including the ameloblast target cells as well as other epithelial and mesenchymal cells. In this in vitro model, AMEL (Fig. 2A, amel) transcription and translation were expressed at 5 days (data not shown). Controls with unlabelled antisense ODN probes were negative (Fig. 2B). These results suggest that ODN diffused throughout the tooth organ and was localized within ameloblast target cells within 24 hours in culture.

Antisense AMEL inhibition of translation

Studies were designed to determine AMEL translation arrest in E15 M1 explants cultured for 12 days in serumless medium. Cultured explants were metabolically labeled with [35S]methionine for 4 hours, chased for 1 hour in nonlabeled methionine-containing medium and extensively washed with PBS. Homogenized explants were extracted with acetic acid and then processed for immunoprecipitation using anti-AMEL antibodies (Slavkin et al., 1982, 1988). Analyses were performed on either solubilized immunoprecipitated proteins resolved with one-dimensional gel electrophoresis and subsequent fluorography (Fig. 3) or immunoprecipitated residues (Table 1).

Comparisons were performed to evaluate the antisense inhibition of AMEL translation by immunoprecipitation (Table 1). AMEL translation was inhibited approximately 46% in the antisense ODN-treated compared to the control groups (Table 1, Fig. 3, lanes 1 and 4), whereas in the sense ODN-treated groups AMEL translation was inhibited 31% compared to the control groups (Table 1, Fig. 3, lanes 1,3 and 6).

AMEL antisense inhibition reduces HAP crystal size and orientation

Average HAP crystal length in molar explants cultured for
12 days were significantly reduced in the antisense-treated compared to the control groups. The average HAP crystal length in the control groups was 254.5 nm (61.9 nm s.d.) (Fig. 4A,C and E), compared to 92.5 nm (28.4 nm s.d.) in the antisense ODN-treated groups (Fig. 4B,D and F). Whereas these results could have varied due to slightly different planes of sectioning, the criteria and number of samples enhanced the analyses for variations.

Crystal diameter was examined as a variable independent from the plane of sectioning. The average HAP crystal diameter was 12 nm (2.1 nm s.d.) in the control group, 12.1 nm (4.5 nm s.d.) in the sense ODN-treated group and 5 nm (1.9 nm s.d.) in the antisense ODN-treated group (Figs 4E,F, 5A-C, 6). Measurements for HAP crystal diameter represented a mean crystal width and thickness. Analyses of these results suggested a highly significant (P<0.0001) inhibition of HAP crystal size in the antisense ODN-treated groups (Fig. 6). A difference in crystal orientation was observed (Fig. 4A-F); crystals in the control group were oriented parallel to each other, whereas crystals in the anti-
Inhibition of amelogenin translation

Fig. 2. Digoxigenin-labeled AMEL ODN was used to determine diffusion patterns throughout the entire tooth organ explant within a 24 hour period of observation. (A) Survey of digoxigenin-labeled probe showed diffusion throughout the E15 M1 explant; labeled-ODN was localized in ameloblast target cells (amel), as well as within other ectodermally derived epithelial and adjacent ectomesenchymal cells (i.e. preodontoblasts, odontoblasts and dental pulp mesenchyme cells). (B) Negatively stained control. Magnifications 50x.

Antisense ODN produced disorganization of AMEL supramolecular aggregates within electron-dense patterns of stippled material

Comparisons of stippled material showed reduced compartments and less well-defined electron-dense AMEL aggregates in the antisense ODN-treated groups compared to either sense or control groups (Fig. 7A-G). The analyses of the differences were derived from computer-assisted digitization of image patterns by feature extraction and subsequent moment invariance analyses. The differences between the three groups were statistically significant: P<0.0001 (Fig. 8). Relatively low values were correlated with high levels of organization in the sense and control groups, whereas the antisense ODN-treated groups showed high values correlated with low levels of AMEL supramolecular organization patterns (Fig. 8). Antisense ODN-treatment reduced the supramolecular aggregation within the electron-dense stippled materials within the enamel ECM compartment (Fig. 7F,G).

DISCUSSION

The present study utilized an in vitro model, which is permissive for enamel biomineralization and which has been shown to result in similar physical-chemical properties of mineralized enamel as in vivo controls (Bringas et al., 1987; Evans et al., 1988). An antisense strategy was applied to inhibit selectively AMEL translation during E15 M1 explant development in serumless, chemically defined medium. The effects of a selective inhibition of AMEL translation products were studied to deduce the function of this gene product (AMEL) in vitro. Our study provides the first evidence from antisense AMEL ODN-treatment demonstrating 46% AMEL translation arrest and a significant decrease in enamel HAP crystal length, width and orientation. Our results complement the hypothesis that AMEL regulates HAP crystal growth (see recent review by Fincham et al., 1992).

Our data demonstrated a down-regulation of enamel HAP crystal size after antisense ODN inhibition of AMEL (Figs 4-6) and supports the hypothesis that organic ECM constituents influence the manner in which biominerals crystallize (crystal habit and growth) during development (Aoba et al., 1989; Deutsch, 1989; Doi et al., 1984; Eastoe, 1979; Fincham et al., 1992; Limeback, 1991; Slavkin et al., 1992).

Fig. 3. Antisense ODN inhibition of AMEL translation. E15 M1 were cultured for 12 days and labeled with [35S]methionine for 4 hours using a methionine-deficient medium, chased for 1 hour with non-radioactive methionine and then washed with PBS. Explants were then homogenized and proteins were extracted with acetic acid and then immunoprecipitated with anti-AMEL antibodies. Immunoprecipitation of radiolabeled AMEL translation products from either sense- or antisense-treated cultures was analyzed by one-dimensional gel electrophoresis. Fluorographs were exposed for 5 days. The major AMEL migrated at a position of approximately 23,000. Lane 1, control and precipitated with AMEL antibody; lane 2, sense-treated and preimmune antibody; lane 3, sense-treated and precipitated with primary antibody; lane 4, antisense-treated and precipitated with AMEL antibody; lane 5, antisense-treated and precipitated with preimmune antibody; and lane 6, antisense-treated and precipitated with AMEL antibody. Molecular weight markers p27, p18 and p15.
Fig. 4. Antisense ODN-treatment resulted in decreased HAP crystal length and orientation in E15 M1 explants cultured for 12 days. (A) Average enamel crystal lengths in ultrathin sections of the control group were 254.5±1.9 nm s.d. (A,C and E). Crystal lengths in the antisense ODN-treated group were 92.5±28.4 nm s.d. (B,D and F). (C,D) The length of a selected crystal in the control group (C) and in the antisense ODN-treated group (D) is indicated (|—|^). Magnifications were 50,000× (A,B); 100,000× (C,D); and 300,000× (E,F). Bar, 200 nm (A,B), 100 nm (C,D), and 50 nm (E,F).

Table 1. Antisense AMEL ODN-treatment down-regulates AMEL translation

<table>
<thead>
<tr>
<th>Experimental groups</th>
<th>AA Extract^a^ (total protein, µg)</th>
<th>AA Extract^b^ (total cpm)</th>
<th>Specific activity^c^</th>
<th>AMEL PPT^d^ (µg protein)</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>15.04</td>
<td>117.5</td>
<td>1.563</td>
<td>4.305</td>
<td>0.0</td>
</tr>
<tr>
<td>Sense</td>
<td>13.94</td>
<td>177.6</td>
<td>2.548</td>
<td>2.964</td>
<td>31.15</td>
</tr>
<tr>
<td>Antisense</td>
<td>14.63</td>
<td>196.8</td>
<td>2.690</td>
<td>2.307</td>
<td>46.41</td>
</tr>
</tbody>
</table>

^a^Total acetic acid (AA) protein extract per molar
^b^Total cpm per molar
^c^Specific activity cpm/mg protein/molar/4 hours
^d^Pre-immune antibodies were used as a control
^%^ Inhibition of AMEL translation compared to the untreated control group
Although general principles of procaryotic and eucaryotic biomineralization remain to be discovered (Lowenstam, 1981; Lowenstam and Weiner, 1989; Mann et al., 1989), recent investigations have provided new ideas for possible mechanisms. One new concept to explain phenomena of cell- and ECM-mediated biomineralization was developed in studies on spicule matrix proteins of sea urchin embryos (Decker et al., 1987; Wilt et al., 1992). The striking observation was that the spicule was not assembled within an intracellular vacuole, but formed within the ECM during development. It was concluded (Wilt et al., 1992) that the initial stages of spicule formation take place within the cytoplasm, and that the supramolecular assembly of the spicule structure was formed in the ECM compartment controlled in its habit and growth by unique ECM proteins (Wilt et al., 1992).

In the mouse mandibular first molar (M1) model, ECM biomineralization starts prior to birth; prior to the transcription and translation of *AMEL* (see Snead et al., 1984, 1988; Slavkin et al., 1988, 1992). Ultrastructural and immunocytochemical analyses of enamel tissue-specific biomineralization demonstrated that preameloblasts released electron-dense granular or stippled materials from intracellular secretory vesicles into the forming ECM (Figs 1E, 7A) and these AMEL-enriched aggregates associated with the HAP crystals (Figs 1E,F, 7A) forming along the dentine-enamel junction (see Nanci et al., 1984, 1985; Slavkin et al., 1976, 1988). Since *AMEL* transcription and translation were detected at birth in the mouse M1 tooth organ (Snead et al., 1984, 1988; Slavkin et al., 1988), after initial HAP crystal formations, we argue that AMEL is not involved in the de novo nucleation of HAP as suggested by Glimcher (1979), but rather functions to control the growth parameters of developing enamel as previously suggested (Aoba et al., 1989; Eastoe, 1965, 1979; Fincham et al., 1989, 1991, 1992; Snead et al., 1988). Recently, Deutsch and his colleagues (1991) isolated, cloned and sequenced bovine enamelin cDNA assumed to function as the de novo nucleation anionic glycoprotein for enamel HAP crystal formation. Enamelin expression appears to precede *AMEL* transcription in enamel development (Termine et al., 1980; Deutsch, 1989; Deutsch et al., 1991; Slavkin et al., 1988).

The concept of an organic ECM being involved in enamel HAP crystal formation was introduced by Eastoe (1965). Whereas Eastoe (1965, 1979) postulated that the biological function of the AMEL-rich ECM is a space-filling thixotropic gel, Warshawsky (1989) later suggested that AMEL functioned to protect developing HAP crystallites and served as a packing and/or filling material. The inhibitory effect of AMEL on HAP crystal growth in supersaturated solutions has more recently been interpreted to suggest that AMEL functions to control specifically the growth rates and patterns of enamel crystallites (Doi et al., 1984, Aoba et al., 1989). Based upon these reports and the evidence provided by Addadi and Weiner (1991), we also assume that AMEL proteins form supramolecular aggregates in the ECM and these aggregates provide two specific functions: (i) to create compartments that control for a pre-defined enamel HAP crystal shape, and (ii) to provide a
Fig. 7
physical intercystalline microenvironment which restricts lateral growth in either the a- or b-axes but enhances unidirectional growth along the c-axis.

The developing enamel organic matrix as visualized in transmission electron micrographs as electron-dense, granular or stippled materials appeared to be highly organized with regular, annular or hexagonal shapes in cross-section (Fig. 7A,B-E). Two lines of evidence support our conclusion that these electron-dense granular structures contain AMEL: (i) ultrastructural immunolocalization of AMEL antigens were demonstrated within these supramolecular aggregates (see Slavkin et al., 1988; Nanci et al., 1984, 1985); and (ii) ultrastructural autoradiographic evidence revealed that \[^{13}H\]tryptophan was metabolically incorporated into forming electron-dense granular material during de novo enamel development (Slavkin et al., 1976). We suggest that after de novo nucleation of crystal growth presumably mediated by enamelin (Deutsch et al., 1991; Slavkin et al., 1988), AMEL supramolecular aggregates provide ECM controls to regulate patterns of enamel HAP crystal growth. This model predicts that AMEL controls crystal growth by limiting growth along the c-axis as mediated by the physical and chemical properties of the supramolecular aggregate. This model also offers explanations for the effects of antisense AMEL inhibition on HAP crystal growth as reported in the present study. If the dimensions and/or composition of the enamel organic matrix were reduced by antisense-mediated inhibition of AMEL translation products, prior to and during the formation of the dentine-enamel junction, enamel HAP crystals will either not grow (in the case of a 100% inhibition of AMEL translation), or will be reduced in length, width and orientation (in the case of a partial inhibition of AMEL translation as was observed in the present studies) (see Figs 4-7). Our interpretations support earlier investigations from Nylen (1979), Gustavsen and Silness (1969), and Silness and Gustavsen (1970), who reported that the changing dimensions of the organic compartments closely correlated with the dimensions of enamel HAP crystals; changing in parallel during the process of enamel maturation.

AMEL represents an extracellular matrix protein, which forms a supramolecular aggregating network and is, therefore, a model to examine the physical and chemical specificities for the development of tissue-specific enamel bio-

**Fig. 7.** Comparison of the supramolecular assembly patterns formed from electron-dense granular or stippled materials between antisense ODN-treated, sense ODN-treated and control groups. (A) Enamel electron-dense stippled materials (st) were located between the dentine-enamel junction (dej) and the secretory end of the ameloblast cells (amel). Electron-dense secretory vesicles (v) are associated with the stippled material in the forming enamel matrix. The organic matrix in the control group (B,C) and the sense-treated group (D,E) and shows a regular, network-like pattern, while stippled materials in the antisense-ODN-treated group (F,G) have smaller compartments and are more diffuse. (B,D and F) show initial sites of secreted stippled materials in association with collagen fibers (c).

Representative electron micrographs of Karnovsky fixed material, magnification 50,000× (A) and 100,000× (B-G). Bar, 200 nm (A) and 100 nm (B-G).

**Fig. 8.** Comparison of the relative units of invariance in stippled enamel materials between the control group and the sense ODN-treated and antisense ODN-treated groups. We observed a 2-fold increase in disorganization in the antisense ODN-treated group. The moment descriptor-pattern analyses were performed on 100,000× magnification electron photomicrographic images of enamel electron-dense stippled materials. The images of the antisense ODN-treated groups have significantly higher numbers for reduced organization as compared to either the sense or the control groups (P<0.0001).

mineralization (see recent review by Fincham et al., 1992). AMEL proteins aggregate or disaggregate dependent upon either pH or temperature in vivo and in vitro (Limeback, 1991; Limeback and Simic, 1990; Nikiforuk and Simmons, 1965; Lyaruu et al., 1982). Following secretion from pre-ameloblast and ameloblast cells, AMEL forms aggregates (i.e. electron-dense granular or stippled materials) (Slavkin et al., 1976; Nanci et al., 1985; Slavkin et al., 1988), and this supramolecular aggregation may be due to a slight change in extracellular micromenvironment pH. Significant changes in pH during enamel crystal formation occur since increased numbers of protons are generated (e.g. derived from histidine residues in AMEL) during HAP crystal formation. The appearance of the electron-dense granular or stippled material observed with electron microscopy has been found to be temperature-dependent (Lyaruu et al., 1984), and this structure has been correlated to the temperature- and pH-dependency of aggregation-disaggregation phenomena observed with AMEL proteins (Nikiforuk and Simmons, 1965; Lyaruu et al., 1982).

A number of technical caveats must be considered when evaluating the results from antisense inhibition investigations using complex organ culture models. Whereas antisense strategies are potentially significant approaches to analyze developmental problems of structure/function relationships through transitory "knock-out" or down-regulation of translation for specific transcripts of interest (see Melton, 1988; Stein and Cohen, 1988; Moffat, 1991), the precise mechanisms of action, putative side-effects, and/or specificities are as yet not fully understood (Boiziau et al., 1991). Previous studies demonstrated that the most efficient translation arrest was produced when the antisense ODN was designed to hybridize with the 5′ codons of the precursor targeted mRNA, and in particular with sequences
adjacent to the AUG initiation codon (Minshull and Hunt, 1986; Shakin-Esheleman and Liebhaber, 1988). One critical issue is to design oligonucleotide analogs that are short enough to enter target cells in sufficient concentration, yet which can also provide unique specificity for hybridization to the complementary endogenous mRNA in the cytoplasm. An ODN of 15 base pairs in length is an ideal candidate and tertiary structures (Zamecnik 1991).

Another major factor to consider is the effectiveness of antisense ODN to block specific translation. Inhibition of translation by antisense ODN may involve RNAase-H, an RNAase which hydrolyses the DNA part of RNA/DNA hybrids (Haeuptle et al., 1986). In addition, the RNA secondary structure may play an important role in antisense studies (Verspieber et al., 1990). Clearly a number of problematic issues are to be found in most if not all antisense inhibition studies: (i) the ODN may not enter the cell, (ii) the ODN may be promptly degraded, and/or (iii) the sequence region selected for inhibition is free of secondary and tertiary structures (Zamecnik 1991).

To address some of these caveats during the formulation of our investigations, we initially designed a study to determine the relative patterns for AMEL ODN diffusion in our simple culture model. The results obtained indicated that the synthetic ODN diffused throughout the molar explants within 24 hours and were localized within all cells including the target ameloblast cells (see Fig. 2A and B). Therefore, in the present studies, the 15mer synthetic oligos readily diffused throughout the explants, entered all cells and were not completely degraded by 24 hours in the culture model.

Precedent for using antisense ODN strategies to down-regulate specific translation products during development have been reported. Florini and colleagues (1991) demonstrated that the 5′ 15mer antisense ODN directed against IGF-II inhibited endogenous IGF-II production and myogenic differentiation. Phosphothionate ODNs complementary to the 3′ region of nerve growth factor receptor (NGFR) mRNA inhibited NGFR expression and tubular morphogenesis in kidney organ culture explants (Sariola et al., 1991). During embryonic cardiac development a phosphoramide-modified ODN complementary to TGF-β1 mRNA inhibited normal epithelial-mesenchymal transformations by 80% (Runyan, 1991). Recently, Krommiller and his colleagues (1992) demonstrated that E9 embryonic mouse mandibular explants when cultured in the presence of a 5′ 15mer antisense EGF ODN completely inhibited tooth formation.

In summary, the present study provides additional evidence to support the hypothesis that AMEL functions in early enamel HAP crystal formation to control mineral growth and habit. AMEL appears to control the size and directions of crystal growth during enamel development. It is not clear, however, if the control of HAP crystal growth is a unique function of specific domains within the primary structure of AMEL or if crystal growth is controlled by multiple AMEL proteins resulting from alternative spliced transcripts (Gibson et al. 1991), or whether non-AMEL proteins involved in other examples of tissue-specific biomineralization in different species also possess these functions. The increased generation of deduced primary structures from cDNA sequences, representing ECM molecules associated with biomineralization from procaryotic as well as eucaryotic models, should facilitate searches for highly conserved regions related to either calcium carbonate- or calcium hydroxyapatite-mediated biomineralization.

The authors would like to express their sincere appreciation to Dr Alan G. Fincham for his continued intellectual contributions to questions of enamel biomineralization in our laboratory. We also wish to acknowledge the excellent technical support of Mr Steven Gennett who developed the image analysis programs that allowed us to correlate patterns of electron-dense granular or stippled materials with mathematical functions. We would like to thank Mr Constant Crohin for processing numerous photographs for morphometrical evaluation. These studies were supported in part by funds from the National Institute For Dental Research (NIDR), NIH, USPHS, grants DE-02848 and DE-06425 (H. C. S.). T. D. was supported by Postdoctoral Fellowship DFG grant Di 427/1-1.

REFERENCES


Irritation of amelogenin translation 481


