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Spatial- and temporal-restricted pattern for amelogenin gene expression during mouse molar tooth organogenesis

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Summary

Position- and time-restricted amelogenin gene transcription was analysed in developing tooth organs using *in situ* hybridization with asymmetric complementary RNA probes produced from a cDNA specific to the mouse $26\times10^3 M_r$ amelogenin. *In situ* analysis was performed on developmentally staged fetal and neonatal mouse mandibular first (M_1) and maxillary first (M^1) molar tooth organs using serial sections and three-dimensional reconstruction. Amelogenin mRNA was first detected in a cluster of ameloblasts along one cusp of the M_1 molar at the newborn stage of development. In subsequent developmental stages, amelogenin transcripts were detected within foci of ameloblasts lining each of the five cusps comprising the molar crown form. The number of amelogenin transcripts appeared to be position-dependent, being more abundant on one cusp surface while reduced along the opposite surface. Amelogenin gene transcription was found to be bilaterally symmetric between the developing right and left M_1 molars, and complementary between the M^1 and M_1 developing molars; indicating position-restricted gene expression resulting in organ stereoisomerism. The application of *in situ* hybridization to forming tooth organ geometry provides a novel strategy to define epithelial–mesenchymal signal(s) which are believed to be responsible for organ morphogenesis, as well as for temporal- and spatial-restricted tissue-specific expression of enamel extracellular matrix.

Key words: *in situ* hybridization, amelogenin gene, epithelial differentiation, extracellular matrix, epithelial–mesenchymal interaction, biomineralization.

Introduction

Numerous approaches have been used to investigate and understand the processes of determination and differentiation during development (Holtfreter, 1934; Spemann, 1938; Saxen & Troivonen, 1962). One strategy has been to identify proteins and their mRNAs which are cell-type specific and to use these as probes to localize spatially and temporally expression of these unique gene products during development. One useful model is the developing mammalian tooth organ which is dependent upon reciprocal instructive signals for morphogenesis and restricted patterns of gene expression. In this model, neural-crest-derived signal(s) specify the shape for the various classes of teeth (molariform *versus* incisorform) as well as determine ectodermal-derived ameloblasts to express amelogenin gene products (Kollar & Baird, 1969, 1970; Slavkin, 1974; Slavkin & Bringas, 1976; Snead *et al.* 1984; Snead *et al.* 1987). Amelogenin is a proline-rich polypeptide which is implicated in the control of enamel biomineralization. Analysis of the expression of amelogenin gene products by ameloblasts during mouse tooth organogenesis permits studies of time- and position-dependent determination and differentiation which are coupled to the generation of organ stereoisomerism.

The use of *in situ* hybridization with cells, tissues and organs in order to study development- and/or spatial-restricted gene expression has proven useful in several systems (Angerer *et al.* 1983; Angerer *et al.* 1985; Gresik *et al.* 1985; Han *et al.* 1987; Lewis *et al.* 1986; Binder *et al.* 1986; Weeks & Melton, 1987). Presently, few studies have used this approach to examine the transcription of mRNAs for proteins that are associated with forming extracellular matrices which undergo biomineralization. The production and characterization of a cDNA for mouse amelo-

genin (Snead *et al.* 1983; 1985*a*), the identification of *de novo* amelogenin gene expression during development (Snead *et al.* 1984) and the characterization of an intrinsic genetic program for epithelial-derived extracellular matrix biomineralization (Bringas *et al.* 1987; Evans *et al.* 1988), provide the framework to investigate the temporal- and spatial-restricted pattern for amelogenin expression during enamel extracellular matrix production and biomineralization.

In this report, we describe results from *in situ* hybridization studies of mouse tooth organogenesis using asymmetric cRNA probes specific for a mouse 26×10^3 M_r amelogenin protein (Snead *et al.* 1985*a*). We demonstrate that specific hybridization, in the absence of cross-hybridization or hydroxyapatite mineral-phase entrapment, can be obtained by a careful employment of probes and hybridization conditions. Furthermore, we describe the three-dimensional pattern for the temporal- and position-restricted expression of amelogenin mRNA during maxillary first (M^1) and mandibular first (M_1) molar tooth development. We observe regionally regulated expression resulting in coordinated amelogenin gene expression between ameloblasts of the developing M^1 and M_1 molars, as well as for ameloblasts from the left or the right molars. This geometric pattern of gene expression demonstrates intraorgan and interorgan regional regulation of epithelial determination and differentiation. The recognition of precise temporal and spatial regulation for amelogenin gene expression provides opportunities to pursue studies examining the nature of the cellular and molecular mechanisms responsible for ectomesenchyme-derived instructive signal(s) which induce the determination and differentiation of ameloblasts within the developing enamel organ.

Materials and methods

Animal tissues

Mandibles and maxillae were dissected from timed-pregnant Swiss–Webster mice (plug = day 0) and staged according to Theiler (1972) from 16 days *in utero* through 2 days postnatal development.

Preparation of tissue sections

In order to retain target mRNA in a hybridizable state, the recommendations of Angerer *et al.* (1986) were followed. Tissues were fixed in 1% glutaraldehyde in phosphate-buffered saline at 0°C. Standard procedures for paraffin embedding were used to produce sections of 5 μm in thickness which were mounted on poly-L-lysine-coated glass slides. The major buccal cusp of the mandibular molar was selected as the sampling site. Additionally, serial sections of mandibles or maxillae from selected stages of development were similarly prepared.

Preparation of RNA probes

The insert from pMa5-5, a cDNA specific to the mouse 26×10^3 M_r amelogenin (Snead *et al.* 1983; 1985*a*), was subcloned into an RNA transcription vector (Lau *et al.* 1987) (Promega Biotec, Madison, WI), and sense and antisense complementary RNA (cRNA) hybridization probes were prepared by *in vitro* transcription using SP6 polymerase (Promega Biotec, Madison, Wisconsin) in the presence of [^{35}S]thiophosphate-UTP (New England Nuclear, Boston, MA, 1241 Ci mmol^{-1}; probe specific activity 5×10^8 cts min^{-1} μg^{-1} RNA) as described by Melton *et al.* (1984) and Kreig & Melton (1984). For filter hybridization studies ^{32}P-UTP (New England Nuclear, Boston MA, 600 Ci mmol^{-1}; probe specific activity approximately $1–3\times10^8$ cts min^{-1} μg^{-1} RNA) was used in the polymerization reaction. The DNA template was hydrolysed with DNase free of RNase (Promega Biotec, Madison, WI). Free nucleotides were removed by exclusion chromatography on a RNase-free G-50 column (Boehringer Mannheim Biochemicals, Indianapolis, IN). Purified probes were stored at −90°C, as an ethanol slurry, until used.

RNA extraction and Northern blot analysis

Total nucleic acid was isolated from 2-day postnatal mouse molars, a developmental stage wherein amelogenin mRNA is abundant (Snead *et al.* 1983). The nucleic acid preparation was enriched to messenger RNA (mRNA) by repeated LiCl–ethanol precipitation. This RNA preparation was used without further purification. Duplicate RNA samples were fractionated to size by formaldehyde denaturing agarose electrophoresis. The resulting gel was dried and hybridized directly as described by Kidd and colleagues (Kidd *et al.* 1983) for DNA blot analysis to either sense or antisense ^{32}P-radiolabelled probes as previously described (Snead *et al.* 1983; Lau *et al.* 1987). Post-hybridization washes included RNase hydrolysis (Gibbs & Caskey, 1987) (10 μg ml^{-1} RNase-A, Sigma Chemical, St Louis MO) in 5 × SSC for 20 min at room temperature, followed by two washes at a predetermined stringent criteria of salt and temperature. The stringency for the antisense probe was 15°C below the calculated T_M; however, for the sense probe stringency was relaxed to 25°C below the calculated T_m (Angerer *et al.* 1986; Bodkin & Knudson, 1985).

In situ *hybridization histochemistry*

In situ hybridization histochemistry was performed according to Angerer *et al.* (1983, 1985, 1986) with minor modifications necessitated by the presence of hydroxyapatite crystals in mineralizing dental tissue which were predicted to bind nucleic acids. Briefly, to facilitate target–probe reannealing, tissue sections were digested at 37°C with proteinase-K (10 μg ml^{-1} buffer) for 30 min in NTE buffer (100 mM-NaCl; 10 mM-Tris pH 7·5; 1 mM-EDTA). In order to block nonspecific binding sites, tissue sections were prehybridized for 3–4 h at 37°C in 50 μl of hybridization buffer containing 50% formamide; 10 mM-Tris pH 8·0; 1 mM-EDTA; 0·3 M-NaCl; 1 × Denhardt's solution (0·02% each, Ficoll, BSA, polyvinylpyrrolidone); 100 mM-DTT; 10% dextran sulphate; 500 μg ml^{-1} sheared salmon sperm DNA, in covered Petri dishes. A filter paper, wetted with

this buffer, was used to saturate the atmosphere of the vessel during incubation. The tissue sections were then reannealed at 37°C overnight (16–18 h) with the above mixture containing either the ^{35}S-labelled sense or antisense probes at an excess driver concentration of 0·3 μg ml^{-1} buffer. For each tissue section, approximately 30 μl was used and covered with a siliconized coverslip to prevent evaporation.

Determination of the chemical form of target nucleic acid

The chemical form of the target nucleic acid was determined by hybridizing separate tissue sections which had been treated prior to prehybridization with either RNase (made free of DNase activity by incubation at 60°C for 60 min) or DNase (RNase-free) in 1 × NTE. In either case, a second brief digestion with proteinase-K, in conditions detailed above, removed residual enzyme prior to hybridization to radiolabelled probes.

Post-hybridization wash stringency

Following reannealing, the slides were dipped into 4 × SSC (1 × SSC = 150 mM-sodium chloride, 15 mM-sodium citrate, pH 7·0) to remove the coverslips and excess probe. The tissue sections were digested in 20 μg ml^{-1} RNase-A (Sigma Co. St Louis MI) and 200 i.u. ml^{-1} RNase-T1 (Sigma Co. St Louis MI) in 0·5 M-NaCl, 10 mM-Tris pH 7·5, 1 mM-EDTA (Gibbs & Caskey, 1987). The T_m for the hybrid was calculated using the formula for RNA:RNA hybrids as described by Angerer *et al.* (1986). The final wash stringency was 15°C below the theoretical T_m.

Autoradiography

Sections were dehydrated in graded ethanol containing 0·3 M-ammonium acetate, dipped in NTB-2 (Kodak, Rochester NY) nuclear track emulsion diluted 1:1 with 0·3 M-ammonium acetate and exposed for 3–5 days at 4°C. Silver crystals were precipitated by development in D-19 developer (Kodak, Rochester NY). The tissue sections were counterstained with haematoxylin and eosin and the silver grains visualized by bright-field, dark-field or epifluorescent polarized light microscopy.

Three-dimensional reconstruction

Serial tissue sections of selected stages of developing mouse maxillary or mandibular first molars were prepared for *in situ* analysis and reannealed to antisense cRNA as described. *Camera lucida* drawings of the resulting tissue sections were prepared and scored for relative density of silver grains in the epithelial compartment using a three-tier scale. Aided by distinct microanatomic landmarks (Gaunt, 1955), the drawings were serially reassembled along the Z-coordinates proportional to their X and Y relative enlargement.

Results

Northern blot analysis

Mouse amelogenin mRNA was detected by reannealing to antisense cRNA probe and the resulting hybridization signal is shown in Fig. 1. The size of the amelogenin mRNA is approximately 13S, sufficient to encode for the 26×10^3 M_r amelogenin. Even under relaxed conditions, the sense cRNA failed to reanneal thus indicating an absence of complementary sequence in cells of the developing dental organ (data not shown).

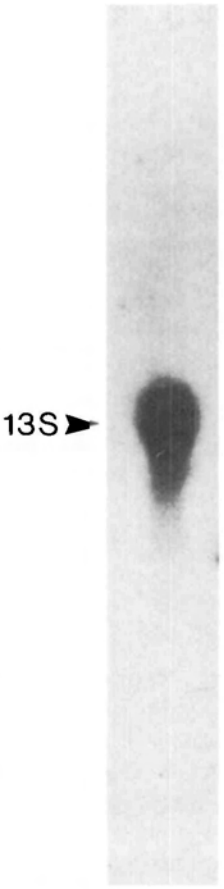

Fig. 1. Northern blot hybridization using asymmetric complementary amelogenin RNA probes. RNA was isolated and resolved in size by denaturing agarose gel electrophoresis and hybridized directly in the gel to anti-sense polarity cRNAs as detailed in 'Material and Methods'.

Fidelity of in situ *hybridization*

The discrimination of antisense *versus* sense amelogenin cRNA probes to reanneal solely to amelogenin mRNA in ameloblasts along the major buccal cusp of one-day postnatal M_1 molar is shown in Fig. 2A–D. The antisense probe yields hybridization signals localized to ameloblasts engaged in enamel formation at this developmental stage and anatomic position within the organ (Fig. 2A). Ameloblasts along the contralateral surface do not express amelogenin mRNA. Ameloblasts from the adjacent enamel-free zone express lower, but detectable, levels of amelogenin mRNA (Fig. 2A, asterisk). The antisense probe does not hybridize to preameloblasts, odontoblasts or other cells of the developing dental organ (Fig. 2A). Moreover, there are scarce grains localized over the enamel or dentin extracellular matrix which contains hydroxyapatite mineral. The sense cRNA probe fails to demonstrate a hybridization signal amongst either

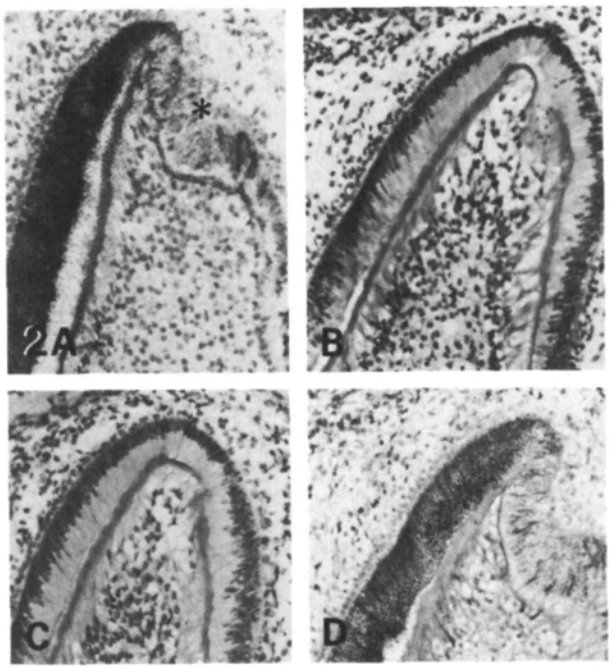

Fig. 2. *In situ* hybridization to serial tissue sections taken from the major buccal cusp of a 1-day postnatal mouse first mandibular molar. Asymmetric cRNA probes were synthesized to mouse amelogenin cDNA template using ^{35}S-nucleotides. Hybridization and washes were as detailed in 'Materials and Methods'. The antisense probe reanneals only to cells expressing amelogenin mRNA (A), the sense probe failed to reanneal to any complementary sequences (B). Tissue section treated with RNAase (C) or DNAase (D) prior to hybridization with antisense probes demonstrate that the tissue target is RNA. (*), enamel-free zone. ×180.

differentiated ameloblasts or other phenotypes within the developing dental organ (Fig. 2B). Pretreatment of tissue sections with RNase abates the hybridization signal (Fig. 2C) whereas the contribution from DNA:RNA hybrids appears to be minimal since the hybridization signal is relatively unaffected by DNase digestion (Fig. 2D), thus demonstrating that the signal is due to RNA:RNA hybrids presumably localized to the cytoplasm. Finally, the fidelity of hybridization for the antisense probe was corroborated by the demonstration of a sigmoid melting profile with an inflection at the theoretical T_M as compared to a flat melting profile for the sense probe (data not shown).

Temporal- and spatial-restricted amelogenin expression

The temporal- and spatial-restricted pattern of amelogenin gene expression during M_1 development is shown in Fig. 3. To facilitate comparison between different developmental stages, the enamel-free zone of the major buccal cusp, or its progenitor region, was sampled. No hybridization signal for the antisense amelogenin probe is detected in 16, 17 and 18 days gestation age organs (Fig. 3A,B, and C, respectively). However, at the newborn stage of development, *de novo* amelogenin gene expression is detected in ameloblasts confined to the cuspal eminence (Fig. 3D,H). No hybridization signal was observed when tissue sections, adjacent to those used for antisense probe analysis, were separately hybridized to the sense probe under identical conditions (data not shown).

In situ hybridization to antisense polarity probes demonstrates spatially restricted expression of amelogenin in developing M_1 molar from the right compared to the left mandibular quadrant (Fig. 4B and C, respectively) or from the M^1 compared to M_1 molars (Fig. 4A and B, respectively). Expression of amelogenin mRNA demonstrates a mirror image for hybridization signal for ameloblasts from the left M_1 molar (Fig. 4B) when compared with ameloblasts from the right M_1 molar (Fig. 4C). Furthermore, the amelogenin hybridization signal within ameloblasts from M^1 (Fig. 4A) compared to M_1 molar (Fig. 4B) demonstrates coordinated and complementary patterns for amelogenin gene expression. In contrast, for all cases examined, the sense orientation of the amelogenin cRNA probe failed to hybridize to any cell of the developing molar organ.

Geometric coordination

Serial tissue sections of selected developmental stages of M^1 and M_1 molars were analysed. The topology of the ameloblasts and a three-tiered scale for the intensity of the hybridization signal was recorded. The M_1 molar at the newborn stage of development (Fig. 5A, upper construction) demonstrates two small foci of differentiated ameloblasts (shown in red), while the adjacent sheet of preameloblasts (shown in white) has yet to become transcriptionally active for amelogenin gene expression. Within 24 h of *in vivo* development (Fig. 5A, lower construction), organ growth (see also Fig. 3A–D) has appreciably changed the topology of the ameloblasts. At this developmental stage, more ameloblasts are transcribing the amelogenin gene (Fig. 5A, lower construction, shown in red), although some cells have yet to express amelogenin mRNA (shown in white).

Analysis of one-day postnatal M^1 and M_1 molars reveals a novel geometric pattern for amelogenin gene expression. For surfaces which will oppose one another during mastication relatively high levels of expression are detected, whereas the opposite surfaces express relatively low levels (Fig. 5B; also compare Fig. 4A to B). Therefore, the varying intensity of hybridization signal for anatomic regions of ameloblasts depicts a mosaic pattern of amelogenin transcription within this continuous sheet of epi-

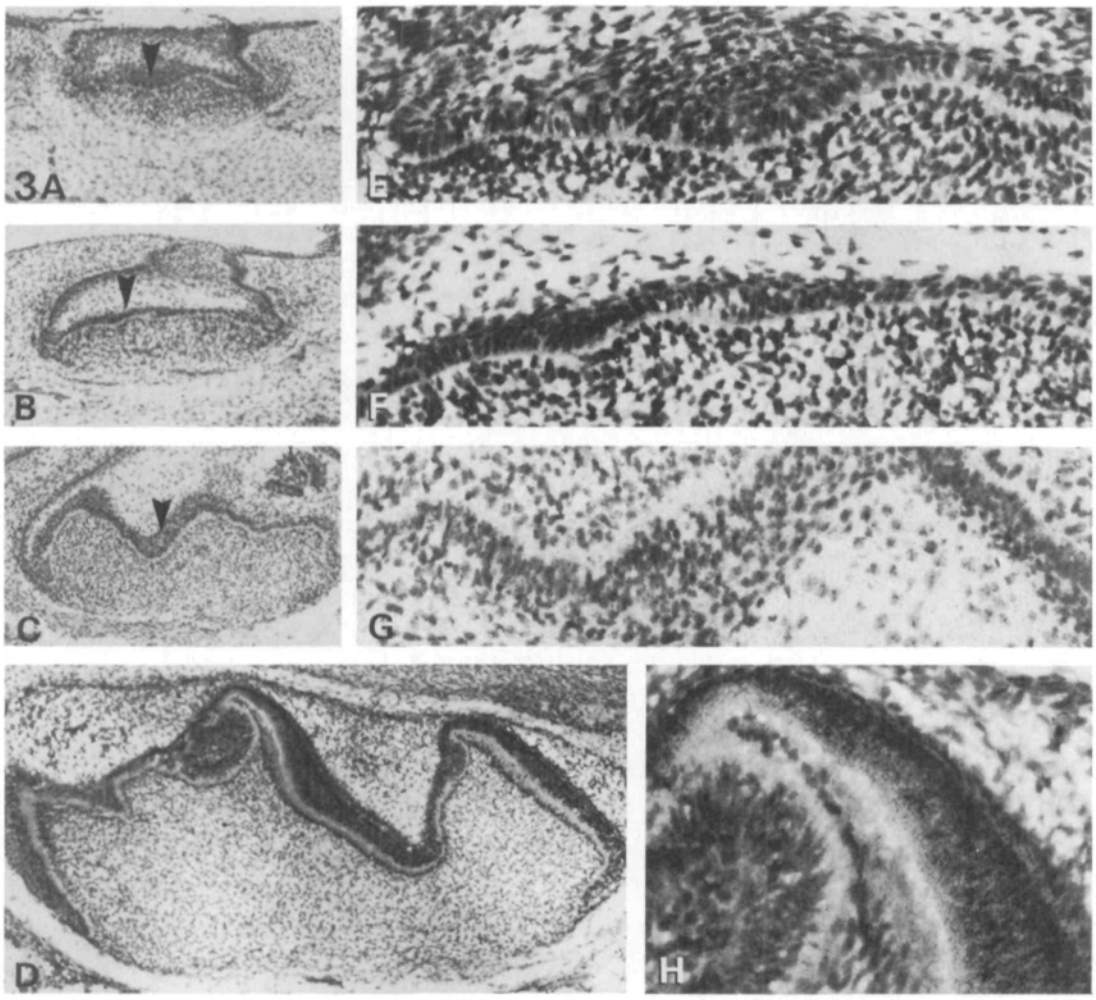

Fig. 3. *In situ* analysis of developmentally staged first mandibular mouse molars. Sections from the major buccal cusp of the developmentally staged first mandibular molar at 16 days gestational age (A,E); 17 days gestational age (B,F); 18 days gestational age (C,G) and the newborn stage of development (D,H) were prepared and hybridized to antisense cRNA probes as described in 'Materials and Methods'. Amelogenin messenger RNA is first detectable at the newborn stage of develoment (D,H). Magnification: left panels ×57; right panels ×180. Arrowhead indicates region selected for paired high-power field.

thelium; ameloblasts expressing high levels of amelogenin mRNA are adjacent to ameloblasts expressing very low levels (Fig. 5A & B).

Discussion

Northern blot hybridization

Using stringent hybridization conditions, the antisense cRNA detected amelogenin mRNA from amongst a crude nucleic acid preparation without transferring the target sequence to nitrocellulose (see Fig. 1). Omitting the transfer step results in a reduction in time and cost without sacrificing fidelity of detection (Kidd *et al.* 1983; Snead *et al.* 1985*b*).

Fidelity of in situ *hybridization*

The selective detection of amelogenin mRNA with opposite polarity cRNA during blot hybridization does not ensure similar differential hybridization signals may be achieved in tissue sections, especially those containing hydroxyapatite crystals. Therefore, the fidelity of *in situ* hybridization to tissue sections was assessed using several experimental strategies. First, hybridization of one-day postnatal molars with antisense probes detected amelogenin mRNA targets only in functional ameloblasts, e.g. those ameloblasts engaged in enamel extracellular matrix biogenesis. Adjacent tissue sections hybridized to the sense probe failed to demonstrate a specific signal (compare Fig. 2A to B). Second, for hybrids formed between the antisense probe with tissue mRNA targets, the melting profile demonstrated a typical sigmoid melting curve with an inflection at the theoretical T_m (Angerer *et al.* 1986). Third, pretreatment of the

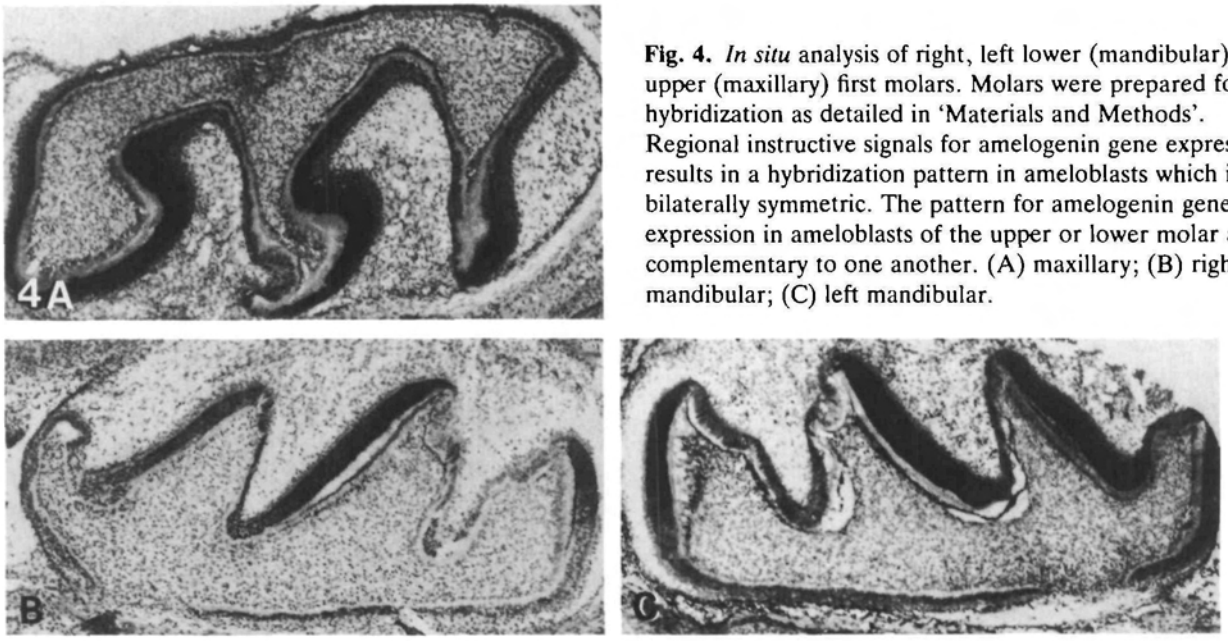

Fig. 4. *In situ* analysis of right, left lower (mandibular) or upper (maxillary) first molars. Molars were prepared for hybridization as detailed in 'Materials and Methods'. Regional instructive signals for amelogenin gene expression results in a hybridization pattern in ameloblasts which is bilaterally symmetric. The pattern for amelogenin gene expression in ameloblasts of the upper or lower molar are complementary to one another. (A) maxillary; (B) right mandibular; (C) left mandibular.

tissue section with RNase abated the hybridization signal, whereas, pretreatment with DNase had minimal effect on the hybridization signal intensity (see Fig. 2C and D). This observation indicates that the hybridization signal is due to RNA:RNA hybrids and not solely to nuclear localized genomic DNA:RNA hybrids. Moreover, the mouse genome has been shown to contain only one copy of the amelogenin gene which is not amplified during differentiation of oral epithelial cells to ameloblasts (Snead & Lau, 1988).

The addition of a post-hybridization wash consisting of RNase-A and T-1 (Gibbs & Caskey, 1987) was found to be a necessary step toward the reduction of spurious hybridization signals which were localized principally to mineralized extracellular matrices, presumably due to their hydroxyapatite crystals. Hydroxyapatite is a common material used for nucleic acid fractionation and under the condition employed for hybridization, single-stranded RNA probes are anticipated to be retained. Increasing the DTT concentration failed to reduce the nonspecific signals (data not shown) although such steps are generally helpful when using thiophosphate radiolabelled probes. These simple modifications should be adaptable to other tissue sections containing bone or other mineralized tissue when *in situ* hybridization is to be used.

Spatial- and temporal-restricted signal(s) regulate amelogenin gene expression

Tooth organogenesis is dependent on reciprocal, sequential, epithelial–mesenchymal interactions (Kollar & Baird, 1969, 1970; Thesleff & Hurmerinta, 1981; Slavkin, 1979; Kollar, 1983). The accumulated data suggest that these interactions provide specific signal(s) which regulate morphogenesis (Kollar & Baird, 1969, 1970) and induce tissue-specific gene expression (Slavkin *et al.* 1981; Snead *et al.* 1984). The exact nature of the signal(s) for any organ model remains unknown. The developing mouse molar is an excellent model for the analysis of these epigenetic signal(s). The result of the present *in situ* hybridization analysis suggests that instructive interactions provide position-dependent and time-dependent signal(s) restricting the transcription of the amelogenin gene. In this study, ameloblasts of the mandibular first molar do not express amelogenin mRNA until the newborn stage of development (see Fig. 3). This confirms a previous observation of *de novo* amelogenin gene expression at the newborn stage of development which was based on hybridization analysis of cytoplasmic RNA, a method which must 'average' expression among all cells of the developing organ (Snead *et al.* 1984). *In situ* analysis permits transcription to be discriminated on a cell-by-cell basis thus demonstrating that amelogenin gene transcription is initiated in a focus of ameloblasts near the tip of the molar cusp which subsequently spreads among adjacent ameloblasts lining the slope of the cusp (see Fig. 5). From 16 days *in utero* through the newborn stage of development, the M_1 organ undergoes extensive growth and morphogenesis which are illustrated at the same magnification in Fig. 3A–D. Within 24 h, at the one-day postnatal age, the morphology of the tooth organ and the distribution of ameloblasts engaged in amelogenin gene expression have been altered by growth from that of the newborn stage (compare the upper with the lower construction shown in Fig. 5A). Ameloblasts are the differentiated

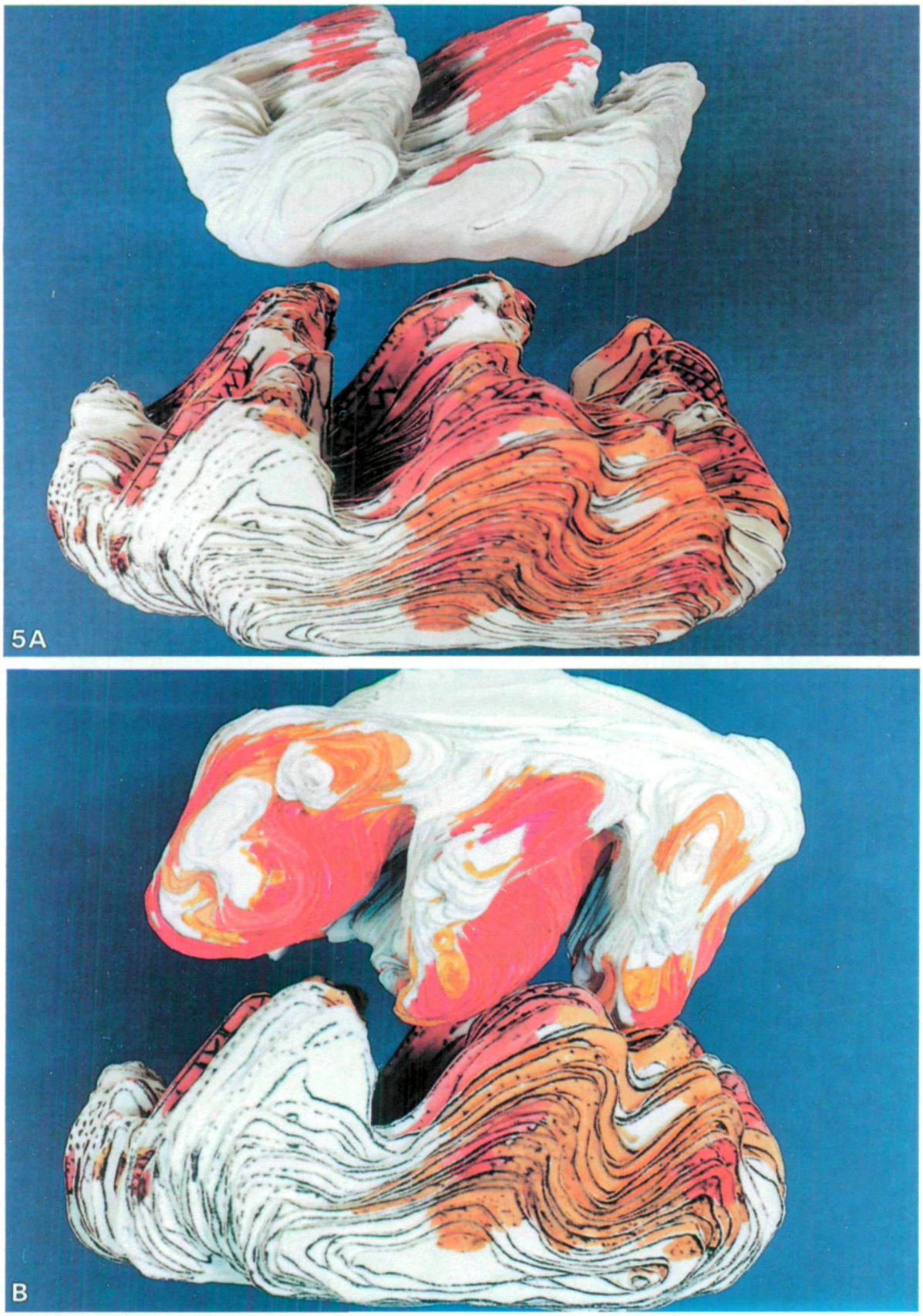

Fig. 5. Three-dimensional reconstruction of mouse molars analysed with *in situ* hybridization. Reconstruction of serial tissue sections of newborn (upper construction) or one-day postnatal mandibular first molar tooth organ (lower construction) following reannealing to antisense cRNA probe (panel A). Organ reconstructions from maxillary (upper reconstruction) and mandibular (lower reconstruction) one-day postnatal molars which are placed in approximate anatomic opposition as they would appear functionally in the postnatal stage of development (panel B). The red areas of the model represent heavy hybridization signal; orange represents moderate hybridization signal; white represents no detectable signal over background.

members of a continuous sheet of epithelial cells linked by gap junctions, yet within this sheet there exists a variation in the relative level of amelogenin mRNA expression (see Fig. 5A and B). Apparently not all ameloblasts are transcriptionally identical; ameloblasts along one surface of the cusp are active whereas those along the opposite surface are inactive. Furthermore, the tip of each cusp, which forms the so-called 'enamel-free zone', expresses amelogenin mRNA at lower levels than ameloblasts along the slope of the cusp (see Fig. 2). Although enhanced local degradation of mRNA remains to be excluded, this explanation seems unlikely since the regional quantitative differences observed for amelogenin gene transcription are ultimately reflected in the final thickness of enamel on rodent molars (Lange & Hammarstrom, 1984).

Geometric-coordinated amelogenin gene expression

Molar organogenesis is initiated early during *in utero* development at unique regions within the growing maxillae and mandible. By the early postnatal stage of development, each molar tooth will complement a counterpart in the opposite jaw during mastication. Moreover, there are local regional differences in the morphogenetic determinants of tooth shape, which result in the various classes of teeth, such as incisors *versus* molars. Apparently, epigenetic signal(s) modulate the genetic program for tooth development resulting in complex developmental patterns for the dentition. Thus, each tooth organ must initiate development, undergo morphogenesis, complete cytodifferentiation for tissue-specific gene expression, biomineralize and finally interdigitate with an opposing member. All these steps are occurring within the craniofacial complex which is itself growing in three dimensions.

The pattern of ameloblasts engaged in amelogenin transcription is coordinated between the left and right M_1 (see Fig. 4B & C), as is the pattern for maxillary and mandibular molars which demonstrate geometric complementation between organs residing in opposite branchial arch derivatives (see Fig. 4A & B and Fig. 5B). Thus, *in situ* analysis demonstrates precise regional restriction of amelogenin gene expression between ameloblasts from organs derived from either side of the midline or from opposite jaws suggesting that epithelial–mesenchymal-derived signal(s) are instructive for patterning coordinated inter-organ gene expression.

That regional-restricted signal(s) specify when and where ameloblasts lining a cusp will become transcriptionally active is reflected in the observation that amelogenin gene expression is initiated individually for each cusp of the molar (see Fig. 5A). This example of heterochrony may reflect the ontogeny of molar teeth which represents an evolutionary tendency for single cusp teeth to fuse to form structures with multiple cusps (Gaunt, 1955). The data presented suggest that each of the cusps of the molar retains an ancestral order for the initiation of ameloblast differentiation. This has been demonstrated in the developing incisor tooth organ which is analogous to a single cusp of the molar. In the incisor, the lingual ameloblasts never produce an enamel matrix (Amar & Ruch, 1987), yet the labial oral epithelia differentiates to ameloblasts and forms an enamel matrix.

Instructive signals

How epithelial–mesenchymal interactions provide specific signal(s) responsible for intraorgan and inter-organ regulation of amelogenin gene expression remains an enigma. We observe that amelogenin gene expression is initiated within a small focus of cells near the tip of each cusp suggesting (i) that the instructive signal(s) for ameloblast differentiation may either be exchanged repeatedly between cells of the epithelium and ectomesenchyme or (ii) the signal may be exchanged only once between but a few cells with the signal being subsequently propagated through gap junctions within the epithelium. In preliminary experiments, we have attempted to identify the manner of signal propagation by surgically removing cells from both germ layers in the region where amelogenin mRNA is first detected (see Fig. 5). We observe that cells approximating the surgical site nevertheless become determined and differentiate to express amelogenin, thus suggesting that the former rather than latter manner for signal propagation is operating.

Epithelial–mesenchymal interactions have previously been proposed to operate through cell–cell contact (Slavkin *et al.* 1974; 1976), cell–matrix contacts (Grobstein, 1975; Hay, 1981), diffusible morphogens (Smith *et al.* 1983; Salag *et al.* 1985), internal clocks, or combination of these. Thus, the interactions are complex and elucidating their molecular mechanism through simple experiments remain illusive. However, the ability to follow the effect of the signal, as measured by gene expression on a cell-by-cell basis, has improved. One appealing hypothesis is that the regionally restricted amelogenin expression is tied to receptor-mediated interaction between cell adhesion molecules (CAMs) (Crossin & Edelman, 1986) or substrate adhesion molecules (SAMs) (reviewed in Bissel *et al.* 1982; Hynes, 1987; Slavkin *et al.* 1988; Horowitz *et al.* 1986; Chiquet-Ehrishman *et al.* 1986; Tamkun *et al.* 1986). Interaction of the receptor with ligands located on either adjacent cells or within the matrix thus provides the positional clues for phenotype determination (Wolpert, 1971). A number of CAMs and SAMs molecules have been

identified during tooth organogenesis (Chiquet-Ehrishman *et al.* 1986; Slavkin et al. 1988; Thesleff *et al.* 1988). It is now possible to sever experimentally such interactions (Gallin *et al.* 1986) and record the change in cell phenotype using *in situ* hybridization. Such an approach may permit the elucidation of molecular mechanism(s) operating during epithelial–mesenchymal interactions which specify tissue-, time-, position-, and geometric-restricted expression of enamel gene products.

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