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Special Review

Control of ameloblast differentiation

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Introduction

A central problem in developmental biology is to understand the initiation and complexities of morphogenesis, of which tooth development (i.e. position, shape, size) is a classic example. Morphogenesis involves reciprocal epithelial-mesenchymal interactions that result in differentiation and the spatial organization of cells to form organs (Spemann and Mangold, 1924; Grobstein, 1953; Gurdon, 1991). During the last decade, a number of investigations have identified an increasing number of specific transcription factors, growth factors, growth factor receptors and cell surface adhesion molecules that provide molecular determinants which regulate allocation, determination and differentiation of specific cell phenotypes within a number of different organ systems.

Amelogenesis is a regulated and sequential developmental cascade that originates in the oral ectodermally-derived odontogenic placode, beginning at day 9 in embryonic mice (E9), and extending through the subsequent stages of tooth morphogenesis to generate an enamel organ epithelium and a number of distinct stages within the epithelial cell lineage that become the ameloblast phenotype. The determination and differentiation of the ameloblast phenotype begins in an early progenitor, representing a minimal proportion of the oral ectodermally-derived odontogenic placode cells. In the continuously erupting rodent incisor teeth, ameloblast differentiation takes place as a complex interplay between self-renewal of stem cells, their progeny and a sequence of epithelial differentiation resulting in the ameloblast phenotype. This complex series of events is at least in part regulated by growth factors, the developmentally ordered appearance of their cognate receptors, transcription factors and a number of cell surface and substrate adhesion molecules during ameloblast differentiation.

To understand the mechanism of early ameloblast differentiation, it is necessary to examine the genes and their gene products associated with the control of the ameloblast phenotype. Moreover, it is not only essential to define the regulation for the sequential expression of ameloblast-specific genes and their products (e.g. tuftelins/enamelins, amelogenins, enamel proteases); it is also important to define those genes which in turn control the ameloblast-specific genes. Therefore, several key questions need to be considered: (i) what are the intrinsic molecular determinants which specify when and where tooth development will be scheduled, (ii) which molecular determinants regulate the timing, positions and forms of sequential tooth development, (iii) what are the molecular controls for ameloblast cell differentiation, and (iv) how do ameloblast cells control enamel matrix formation?

Intrinsic molecular determinants which specify the position and timing of tooth development

The vertebrate hindbrain develops from a metameric organization; as a series of segments or rhombomeres (see review by Krumlauf, 1994). *In situ* hybridization experiments have demonstrated that various homeobox and zinc-finger genes appear to be expressed in patterns that respect rhombomere (r 1-8) borders and boundaries (see Wilkinson *et al.*, 1988, 1989; Bastian and Gruss, 1990; Chisaka and Cappecchi, 1991; Hunt *et al.*, 1991; Nieto *et al.*, 1991; Dolle *et al.*, 1992; Hunt and Krumlauf, 1992; Kuratani and Eichele, 1993; Krumlauf, 1994). It is further established that r2 neuroectoderm transforms into cranial neural crest (CNC) cells which emigrate from the neural tube and subsequently give rise to a number of neuronal as well as non-neuronal connective tissue phenotypes including tooth ectomesenchyme in the first branchial arch (Fig. 1) (see Nichols, 1981, 1986; LeDouarin, 1982; Noden, 1983, 1991; Lumsden, 1988; Serbedzija *et al.*, 1992; LeDouarin *et al.*, 1993; Osumi-Yamashita *et al.*, 1994; Gorlin and Slavkin, in press; Slavkin *et al.*, in press). Evidence supports the contention that a branchial arch developmental code is transferred from the anterior-posterior r2 via CNC to the forming first branchial arch (see Hunt *et al.*, 1991; Nieto *et al.*, 1991; Serbedzija *et al.*, 1992; Osumi-Yamashita *et al.*, 1994). What is not evident is a molecular understanding of the specification of tooth ectomesenchyme and subsequent epithelial-mesenchymal interactions regulating the shape, size and subsequent features of tooth development.

Odontogenic placode and cranial neural crest-derived tooth ectomesenchyme

Experiments in both chicken (LeDouarin, 1982; Noden, 1983; see review by Noden, 1991) and rodent (Tan and Morriss-Kay, 1986; Lumsden, 1988; reviewed in Morriss-Kay and Tuckett, 1989) demonstrate that CNC cells undergo an epithelial-mesenchymal transformation at the lateral margins of the neural folds and migrate ventrally and medially in the developing embryo to form essentially all of the mesenchyme of the craniofacial-oral-dental structures. CNC derived from r2 give rise to the ectomesenchyme of the maxilla, palate and mandible (Morriss-Kay and Tan, 1987; LeDouarin *et al.*, 1993). In the mouse, the emigration of CNC from the neural tube neuroectoderm and subsequent migration of CNC into the first branchial arch as either neuronal or non-neuronal connective tissue cell lineage is initiated at the three to four somite stage (E8) embryo (Nichols, 1981, 1986; Lumsden, 1988; Serbedzija *et al.*, 1992; Osumi-Yamashita *et al.*, 1994). CNC cells are found within the forming first arch within 9 h and by 48 h the cell migration into the arch is completed (see reviews by Noden, 1991; Serbedzija *et al.*, 1992; LeDouarin *et al.*, 1993; Selleck *et al.*, 1993; Osumi-Yamashita *et al.*, 1994).

Subsequent instructive interactions involve the odontogenic placode epithelium in the patterning of tooth and Meckel's cartilage morphogenesis (see Kollar, 1983; Mina and Kollar, 1987; Lumsden, 1988; Hall, 1991; Kollar and Mina, 1991; Ferguson *et al.*, 1992; Thesleff *et al.*, 1992; Jowett *et al.*, 1993). The odontogenic placode provides instructions which control the initial patterning for odontogenesis and skeletogenesis, and also regulate mesenchymal cell proliferation rates (see Mina and Kollar, 1987; Lumsden, 1988; Hall, 1991; Kollar and Mina, 1991); the ectomesenchyme cell lineages in the first branchial arch control patterns of responsive-

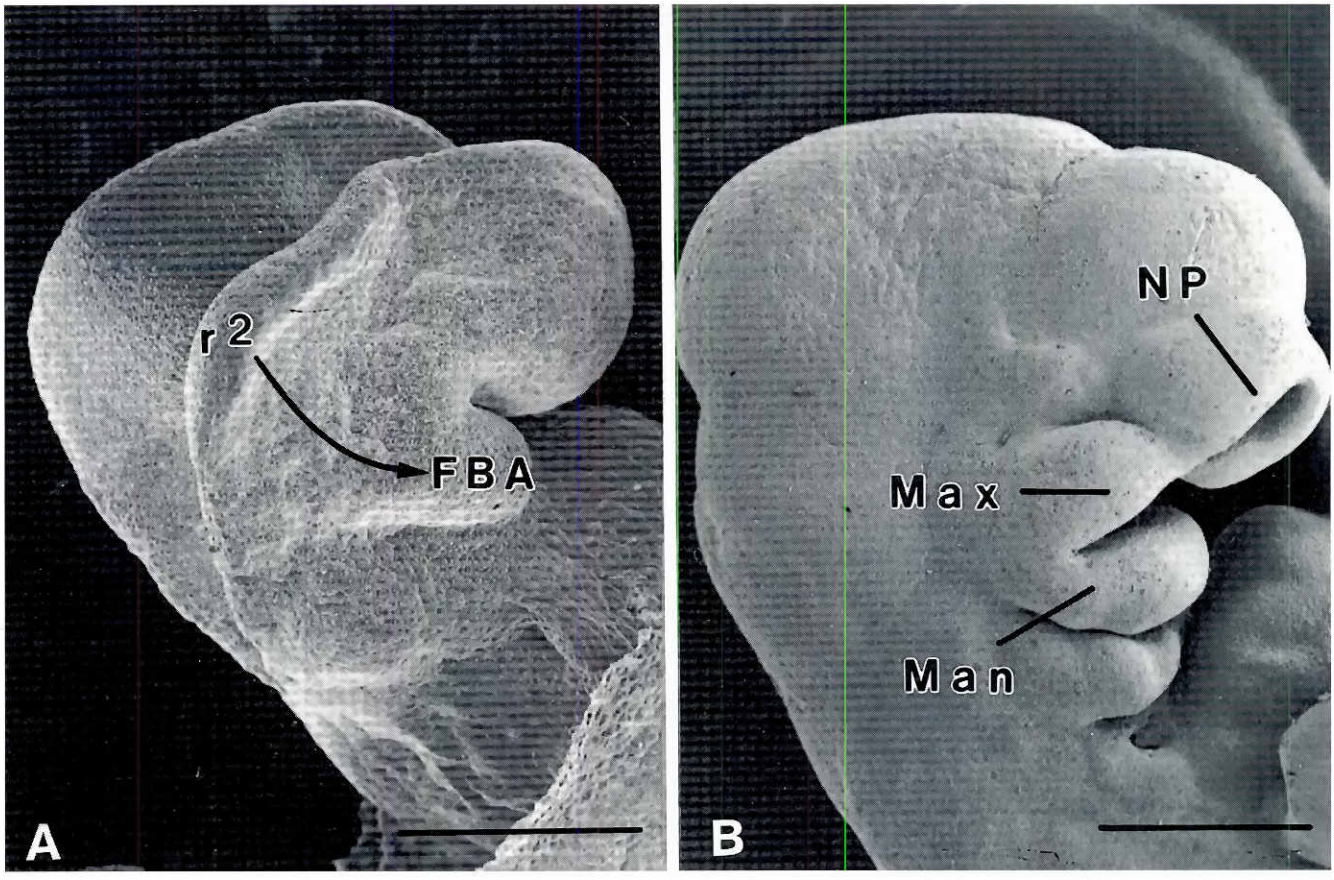

Fig. 1. Determination and differentiation of the ameloblast phenotype during mouse tooth formation. (A) *The rhombomere 2 (r2) within the neuroectoderm of the forming hindbrain provide the origin for the cranial neural crest-derived cell lineages (arrow) for the first branchial arch (FBA). Cranial neural crest cell migrations are initiated from r2 at E7.5-E8 in mouse embryos (arrow) and are completed within 9-12 h. Bar, 200 um.* **(B)** *The first branchial arch gives rise to the dorsal maxillary processes (Max) and the ventral mandibular processes (Man). Rhombomere 2 gives rise to the cranial neural crest-derived ectomesenchyme cells found within the maxillary, mandibular and the nasal processes (NP) (e.g. chondrogenic, osteogenic, odontogenic and connective tissue cell lineages). Bar, 500 μm.*

ness to odontogenic placode-derived factors (see discussions by Graham and Lumsden, 1993; Noden, 1991; Selleck *et al.*, 1993; Vainio *et al.*, 1993). The molecular details for this developmentally coordinated dialogue between the odontogenic placode and the adjacent CNC-derived ectomesenchyme are beginning to be understood (Fig. 2).

Identification of transcription factors involved in odontogenic placode signaling to initiate tooth development

Four different transcription factors have recently been identified and are expressed within the odontogenic placode. These morphoregulatory molecules have been implicated in the control of early epithelial signalling to adjacent ectomesenchyme during the initial specification of tooth development. The suggested significance for these transcription factors is based upon the timing and position of their developmental expression during early tooth formation. Curiously, each of these transcription factors are expressed in a wide variety of different cells and organs during development, suggesting that these proteins may have pleiotropic functions, further confounding the analyses of precisely how they function in the control of tooth development.

First, transgenic mice carrying a homozygous germline mutation in the LEF-1 (lymphoid enhancer-binding factor-1) gene lack teeth at birth (Van Genderen *et al.*, in press); analysis of this null mutation during embryogenesis, indicates that tooth morphogenesis is arrested at the bud stage of development. Second, a closely related gene to LEF-1, termed TCF-1, has been identified which encodes a protein with a DNA-binding domain that is identical with that of LEF-1 (Oosterwegel *et al.*, 1991; van de Wetering *et al.*, 1991). TCF-1 has a developmental expression pattern that overlaps with that of LEF-1 (Oosterwegel *et al.*, 1993). Both LEF-1 and TCF-1 are expressed in odontogenic placode and adjacent CNC-derived ectomesenchymal cells (Van Genderen *et al.*, in press). Third, the homeodomain protein Msx-1 (previously termed Hox-7) is initially expressed in the oral epithelium, presumptive odontogenic placode, prior to tooth initiation, and subsequently is down-regulated in the oral epithelium but induced in the immediately adjacent ectomesenchymal cells (MacKenzie *et al.*, 1991, 1992; Jowett *et al.*, 1993). Transgenic mice carrying a homozygous germline mutation of the homeodomain protein Msx-1 demonstrate the complete absence of incisor tooth anlagen yet bud stage molar tooth development (Satokata and Maas, 1994). Finally, a related homeodomain protein Msx-2 (previously termed Hox-8) is also expressed during tooth development in a sequence complementary to that shown for Msx-1 (MacKenzie *et al.*, 1991, 1992; Jowett *et al.*, 1993). Null mutations of Msx-2 result in the absence of teeth

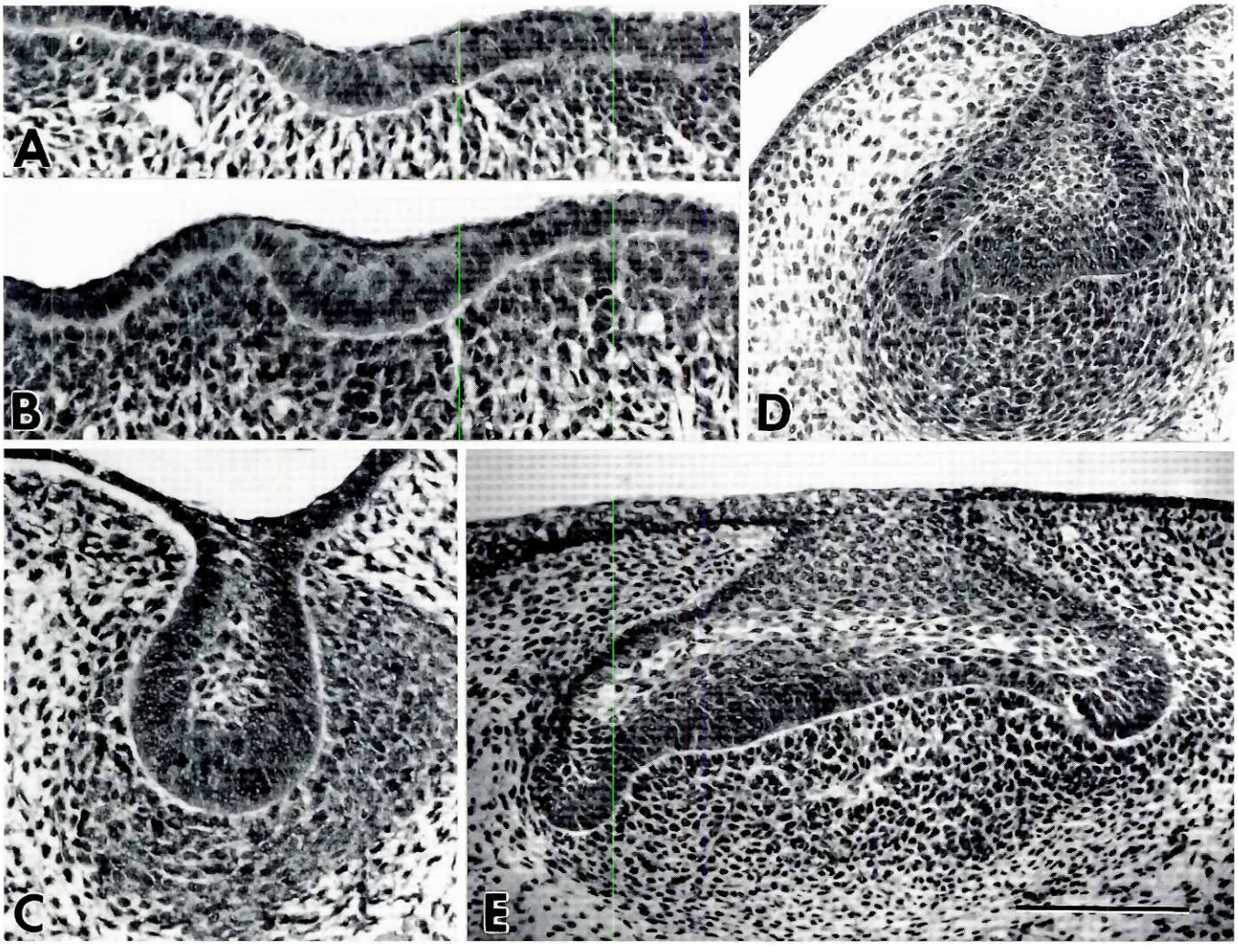

Fig. 2. The ameloblast cell lineage begins within the oral placode epithelium and continues through the subsequent stages of early mouse tooth morphogenesis. (A) *Odontogenic placode (E9/10).* **(B)** *Dental lamina (E10/11).* **(C)** *Bud stage (E12).* **(D)** *Early cap stage (E14).* **(E)** *Cap stage (E15). Bar, 100 μm.*

(R. Maas, personal communication). LEF-1, TCF-1, Msx-1 and Msx-2 are all initially expressed within the oral ectodermally-derived odontogenic placode epithelial cells prior to their expression in the adjacent dental ectomesenchyme cells. None of these transcription factors are unique to odontogenic cells, in that they are expressed in numerous cell types during embryonic, fetal, neonatal and postnatal development (Fig. 3).

Over- or under-expression of growth factors regulates transcriptional factors during early tooth development

Mutations, under-expression, over-expression or "knock-out" (null mutations), in either the odontogenic placode epithelial cells or cranial neural crest-derived ectomesenchymal cells result in tooth dysmorphogenesis (e.g. adontia, hypodontia, oligodontia) and/or amelogenesis imperfecta and dentinogenesis imperfecta. As previously mentioned, adontia results from the null mutations ("knock-out" experiments) of Msx-1, Msx-2 and LEF-1 (Satokata and Maas, 1994; Van Genderen *et al.*, in press). However, it is not known which upstream genes regulate Msx-1, Msx-2 and LEF-1, nor what are the downstream target genes for these transcriptional factors. Which genes within the odontogenic placode signal tooth mesenchyme expression of morphoregulatory molecules? Subsequently, which regulatory genes within the dental mesenchyme signal the induction of the enamel organ, inner enamel epithelium and ameloblast phenotype?

Growth factor-mediated signaling has been implicated in the control of inductive epithelial-mesenchymal interactions. Growth factors may serve as epigenetic controls upstream to Msx-1, Msx-2 or LEF-1 transcription factors. The initial patterning for tooth development resides in the odontogenic placode (Lumsden, 1988). Local thickenings of the oral epithelium in the embryonic day 11 (E11) mouse are the first histological features of tooth development. By E13, the initial dental lamina becomes the bud stage of tooth development and by late E14 to early E15 the cap stage is readily apparent. The odontogenic oral epithelium controls tooth development through E12, thereafter, the adjacent dental ectomesenchyme controls the size and shape of tooth morphogenesis as well as providing the inductive signals for ameloblast-specific gene expression (Kollar, 1983; Mina and Kollar, 1987; Kollar and Mina, 1991; Thesleff *et al.*, 1992;

Couwenhoven and Snead, 1994; Nakamura *et al.*, 1994; Slavkin *et al.*, in press).

The expression of Msx-1 and Msx-2 has been shown to be regulated by epithelial-mesenchymal interactions prior to E13 (Jowett *et al.*, 1993). More recently, BMP-4 has been discovered to induce Msx-1 and Msx-2 expression within odontogenic mesenchyme in the absence of odontogenic epithelium; BMP-4 (a member of the TGF beta family) can substitute for the odontogenic placode in tooth patterning and appears to be a critical signal in the epithelial-mesenchymal inductive pathway (Vainio *et al.*, 1993). Moreover, BMP-4 appears to autoregulate its own gene expression in dental mesenchyme (Vainio *et al.*, 1993); however, BMP-4 does not induce cell proliferation or cell substrate adhesion molecule expression in the dental mesenchyme even though they are known to be controlled by epithelial-mesenchymal interactions in the bud and cap stages of development (Vainio *et al.*, 1993).

Another candidate regulatory growth factor associated with odontogenic placode epithelial signalling is epidermal growth factor (EGF). EGF is expressed in the E9-E11 mouse mandibular epithelium, prior to dental lamina formation (Kronmiller *et al.*, 1991a; Shum *et al.*, 1993). Antisense inhibition or under-expression of EGF during E9 mandibular explant development in organ culture resulted in the inhibition of tooth formation (adontia) (Kronmiller *et al.*, 1991b). EGF is also expressed during subsequent stages of tooth development (Slavkin *et al.*, 1990, 1992; Hu *et al.*, 1992; Shum *et al.*, 1993). Antisense inhibition or under-expression of E10 (42-44 somite pairs) mouse mandibular explant development in serumless medium *in vitro* resulted in hypodontia (Hu *et al.*, 1992; Shum *et al.*, 1993); recovery of abrogated development was achieved using exogenous EGF. Down-regulation of the EGF receptor resulted in hypodontia with delayed morphogenesis and retarded ameloblast differentiation (Hu *et al.*, 1992; Shum *et al.*, 1993). The downstream gene targets for EGF at these sequential stages of tooth development are not as yet known (Slavkin, 1993).

Other candidate morphoregulatory molecules which may control transcription factors associated with the determination and differentiation of the ameloblast phenotype include retinoic acid (RA), platelet-derived growth factor alpha (PDGF-alpha), acidic fibroblast growth factor-1 (Fgf-1-8) and members of the TGF-beta family. Retinoic acid receptor genes are expressed in CNC during mouse craniofacial morphogenesis (Osumi-Yamashita *et al.*, 1990); the expression of RARs and RXRs also correlate with the expression of Msx-1 and Msx-2 in CNC cells during migration (see Takahashi and LeDouarin, 1990; MacKenzie *et al.*, 1991, 1992; Takahashi *et al.*, 1991; Ferguson *et al.*, 1992; Bell *et al.*, 1993). In addition, a mouse mutation, Patch (Ph), appears to be caused by a deletion within the coding region of the alpha-subunit of the PDGF receptor (Stephenson *et al.*, 1991); the Ph mutation perturbs development of the pigment and craniofacial ectomesenchymal cells (e.g. adontia, hypodontia and cartilage ectomesenchyme deficiencies), but this mutation has no affect on the location and size of cranial, spinal and enteric ganglia (Morrison-Graham *et al.*, 1992). The human PDGF-alpha gene maps to chromosome 7p22 (Stenman *et al.*, 1992). These results support the hypothesis that specific ectomesenchymal cell lineages require PDGF-alpha signalling mediated through its cognate receptor, whereas CNC-derived neuronal cell lineages do not appear to require PDGF-alpha signalling for their differentiation. In addition, PDGF-alpha and Fhfs possess pleiotropic functions, serving as mitogens for early tooth epithelial and mesenchymal cell proliferation, and as differentiation factors which induce precocious amelogenin expression during tooth development in serumless, chemically-defined medium (Hu *et al.*, 1993; Chai *et al.*, 1994b; Ashdown *et al.*, 1995).

Transcription factors (e.g. Msx-1, Msx-2, LEF-1, Dlx-2, Evx-1, int-2) and growth factors (e.g. BMP-2, BMP-4, PDGF-alpha, Fgfs, EGF, TGF-alpha, TGF-beta2 and their cognate receptors) are expressed during subsequent stages of tooth epithelial and ectomesenchymal cell differentiation (Slavkin *et al.*, 1990, 1992; Porteus *et al.*, 1991; Robinson *et al.*, 1991; Hu *et al.*, 1992, 1993; MacKenzie *et al.*, 1992; Bulfone *et al.*, 1993; Shum *et al.*, 1993; Chai *et al.*, 1994a,b). For example, Msx-1 and Msx-2 are expressed during the specification of tooth formation prior to the dental lamina (MacKenzie *et al.*, 1992). Dlx-2 is localized in the tooth bud and early cap stages of tooth development and expression continues throughout the cap, bell and crown stages of tooth development (Porteus *et al.*, 1991; Robinson *et al.*, 1991). Dlx-2 is localized within the enamel organ epithelia (Porteus *et al.*, 1991), whereas Dlx-1 is localized to the odontoblasts (Dolle *et al.*, 1992). EGF, TGF-alpha, TGF-beta isotypes, Fgfs, IGF-I and -II and PDGF-alpha are expressed throughout the sequence of stages which include ameloblast differentiation (Slavkin *et al.*, 1990). Therefore, combinatorial interactions between morphoregulatory signalling proteins (e.g. BMP-4, EGF, TGF-beta2) and their suggested downstream transcription factors (e.g. Msx-1, Msx-2, Dlx-1, Dlx-2) may control sequential steps in the process of ameloblast differentiation.

Clinical human genetic evidence provides some support for such an assertion. For example, the Greig cephalopolysyndactyly syndrome is an autosomal dominant disorder affecting craniofacial, dental and limb development; the genetic locus is located at chromosome 7p13 (genes that map to this region include EGF receptor and Hox 1.4) (Vortkamp *et al.*, 1991). Rieger's syndrome is another autosomal dominant craniofacial disorder with abnormal mandibular, tooth (number and size, adontia and hypodontia) development; this syndrome maps to the location of the EGF gene on chromosome 4 (Murray *et al.*, 1992; Slavkin, 1993). Mutations in morphoregulatory

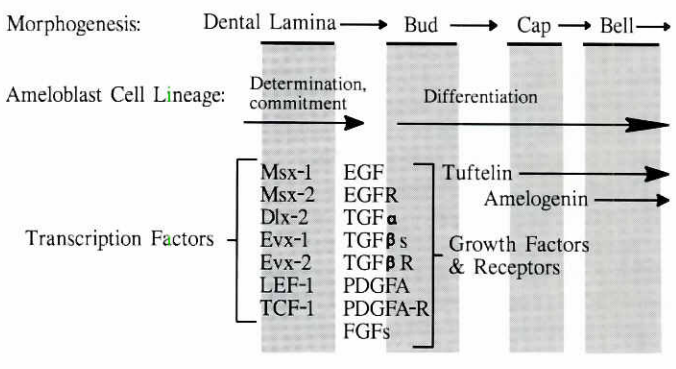

Fig. 3. Genetic controls for sequential embryonic mouse ameloblast differentiation. *During the determination and commitment of the ameloblast cell lineage during initial tooth specification, a number of transcription factors (e.g. MSX-1, Msx-2), growth factors and their cognate receptors (e.g. TGF-betas, TGF-alpha, EGF, PDGF-alpha, FGFs) mediate cell proliferation and cell differentiation prior to and during sequential expression of specific ameloblast-specific gene products (e.g. tuftelin and amelogenins).*

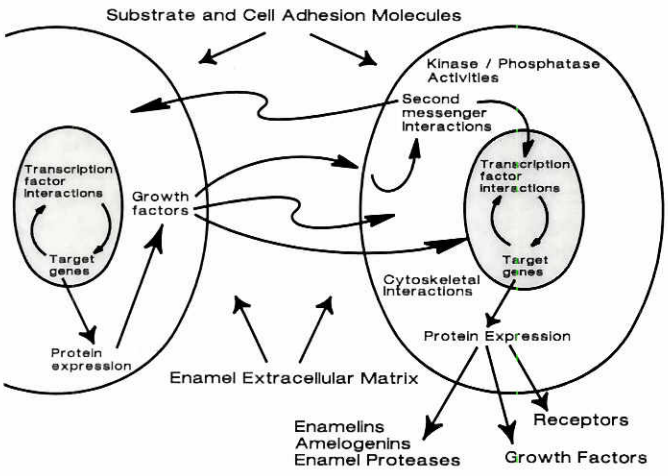

Fig. 4. Signal transduction controls of ameloblast cell differentiation. *An emerging body of evidence suggests a combinatorial control for tooth development, from the initial specification by the ectodermally-derived odontogenic placode to the completion of tooth eruption in postnatal development. Curiously, neither the growth factors, their cognate receptors nor the transcription factors are tooth-specific, but rather the tooth specificity is the resultant from the precise combination(s) of the sequences of gene expression.*

gene expression during the commitment to the fates and cell lineage of CNC derived from r2 neuroectoderm may represent the basis for a number of first branchial arch syndromes and associated tooth dysmorphogenesis (Fig. 4).

Combinatorial interactions between growth factors and transcription factors

One interpretation of the available evidence is to suggest that early expression of growth factor ligand induces downstream transcriptional factors which in turn control a number of other yet related genes including cell and substrate adhesion molecules (see review by Tijan and Maniatis, 1994). The combinations between gene interactions determine the pattern of development. Pursuing such a process predicts that the same genes are expressed in many different developing systems (e.g. LEF-1, TCR-1, Msx-1, Msx-2, PDGF-alpha, Fgfs), yet the combinations before and after their expression provide the specificity to the particular developing system such as the control of ameloblast differentiation during tooth development.

Presently, a major opportunity resides in testing the hypothesis that endogenous autocrine and/or paracrine factors serve as epigenetic signals which either induce or are induced by specific combinations of transcriptional factors and thereby regulate the timing and positional information for tooth development and ameloblast differentiation. Of course, the combinatorial selections may be time and position-specific during the morphological sequence of odontogenic placode signalling, dental lamina stage and the subsequent stages of bud, cap, bell, crown and root formations. If demonstrated, these mechanisms would provide a molecular understanding for a morphogenetic system within a defined developmental field (see discussions by Opitz and Gilbert, 1993) such as the developing human or mouse dentitions.

Growth factor and transcriptional factor transcripts are sequentially expressed in an in vitro model system using serumless, chemically-defined medium permissive for mouse molar tooth morphogenesis: studies of the ameloblast cell lineage in vitro

A simple *in vitro* model has been developed which is permissive for early embryonic mouse mandibular tooth morphogenesis (see papers from our laboratory including Bringas *et al.*, 1987; Evans *et al.*, 1988; Slavkin *et al.*, 1989, 1990; Hu *et al.*, 1992; Shum *et al.*, 1993; Chai *et al.*, 1994a). This model provides opportunities to study intrinsic transcriptional factors and growth factors, as well as the signal transduction resulting from either autocrine and/or paracrine regulation of amelogenesis without the confounding variables of serum, plasma and/or exogenous growth factors as a requirement for development. Further, the E10 (42-44 somite pairs) mandibular or E14/15 cap stage explant models provide experimental approaches to investigate the discrete developmental field associated with tooth formation, i.e. a morphogenetic unit of the embryo in which events are temporally and spatially synchronized, coordinated, and epimorphically hierarchical engaging the constitutive expression of a limited set of gene products which are also used in other permutations in different parts of the embryo (see discussion and commentary by Opitz and Gilbert, 1993).

A survey of selected growth factor transcript expression during tooth development has been published (see Snead *et al.*, 1989; Slavkin *et al.*, 1990, 1992; Hu *et al.*, 1992); IGF-I, IGF-II, bFGF, TGF-beta isoforms, TGF-alpha, EGF, PDGF-alpha and their cognate receptors were identified as well as transcription factors Msx-1, Msx-2, Eve, Dlx-1 and Dlx-2 (see Porteus *et al.*, 1991; Robinson *et al.*, 1991; Dolle *et al.*, 1992; MacKenzie *et al.*, 1992; Bulfone *et al.*, 1993). PCR product sequence analysis was used to confirm the specificity of the amplified transcripts (see methodology in Chai *et al.*, 1994a). We were also able to quantitate changes in EGF transcripts during mandibular and tooth development (see Hu *et al.*, 1992; Shum *et al.*, 1993).

Two studies have recently been completed which illustrate the use of antisense inhibition strategies to investigate the effects of under-expression of specific translation products during tooth development and ameloblast differentiation (see Hu *et al.*, 1992; Shum *et al.*, 1993; Slavkin, 1993; Chai *et al.*, 1994a). In the first study, EGF abrogation induced fusilli-form dysmorphogenesis of Meckel's cartilage, hypodontia and delayed tooth development during embryonic mouse mandibular morphogenesis in a serumless, chemically-defined medium. This *in vitro* model provided a simple culture system to consider inherited human birth defects which are associated with mutations of genomic EGF such as in Rieger's syndrome (Slavkin, 1993); Rieger's syndrome maps to the location of the EGF gene on chromosome 4q25 (Murray *et al.*, 1992).

In the second study, we investigated the functions of the three TGF-beta subtypes found in mammalian tissues. To test the hypothesis that TGF-beta subtypes regulate either Meckel's cartilage or tooth morphogenesis, we designed experiments to compare loss of function effects of each subtype in the simple *in vitro* model. Abrogation of TGF-beta1 using antisense inhibition resulted in an increase in chondrocyte number, a decrease in extracellular matrix and dysmorphology of the rostral region of Meckel's cartilage; tooth development was not effected. Abrogation of TGF-beta2 using antisense inhibition induced a three-fold increase in tooth size, and stimulated tooth development to reach the cap stage as compared to the bud stage in sense-treated or

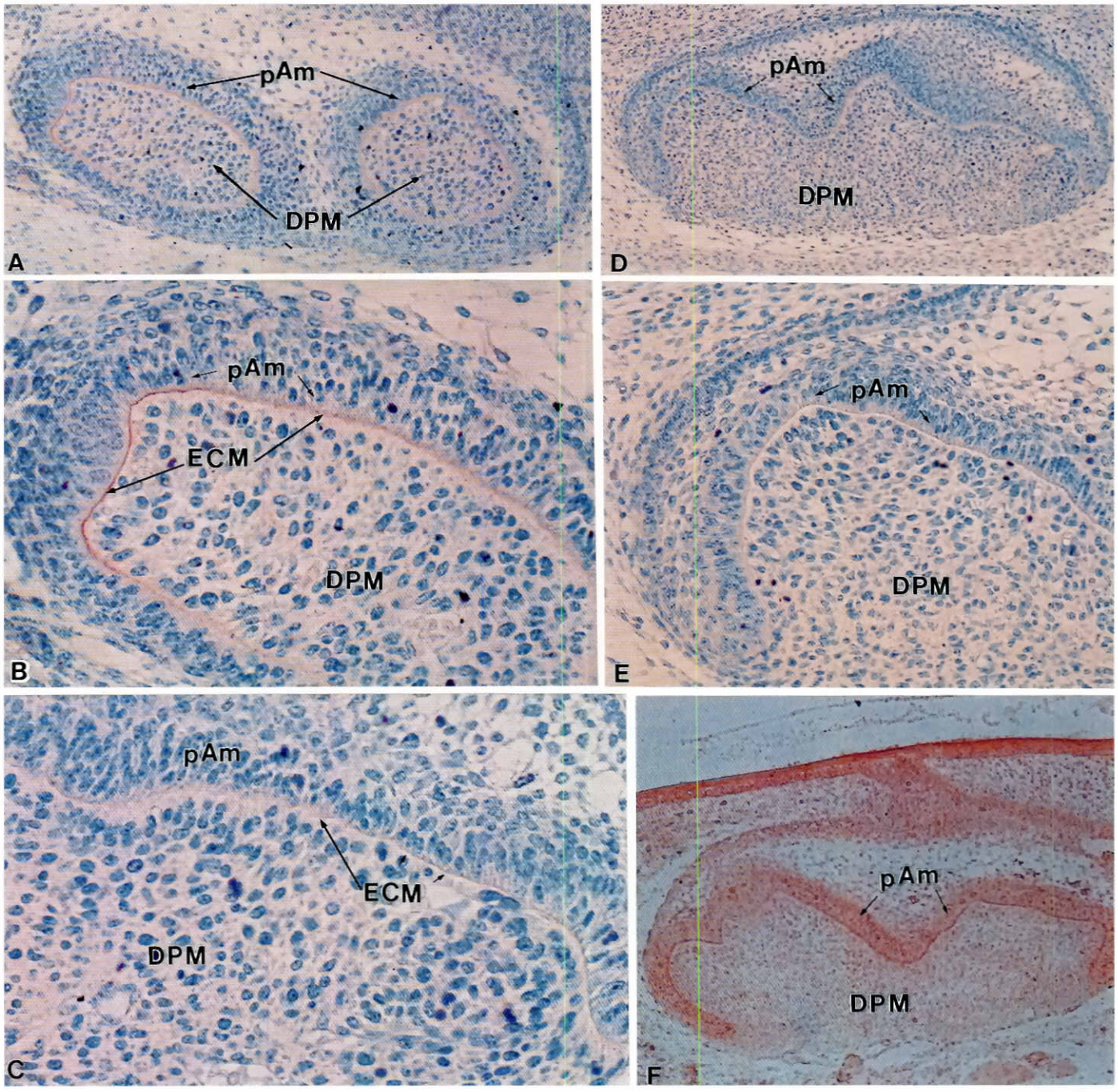

Fig. 5. Expression of tuftelin in E17 mouse molars. *The expression of tuftelin was determined in serial sections of E17 mouse mandibular molar using an antipeptide antibody against tuftelin* **(A,B)**. *No amelogenin staining was detected in same stage teeth using an amelogenin antibody* **(D,E)**. *Mitotic activity in these cells was demonstrated using an antibody against cyclin A courtesy of Dr. Frederick Hall, Childrens Hospital of Los Angeles* **(F)**. *Control using only secondary antibody* **(C)**. *pAM, pre-ameloblast cells; DPM, dental papillae mesenchyme; ECM, extracellular matrix.*

non-treated controls. Down-regulation of this TGF beta isotype induced precocious ameloblast differentiation. Abrogation of TGF-beta3 produced a decrease in the size of Meckel's cartilage, but did not appear to influence tooth development. All abrogations were recovered by the addition of the specific TGF subtype ligand; the other ligands had no effect. We interpret these studies to suggest that specific TGF-beta subtypes regulate specific tissues and sites during embryonic mouse mandibular morphogenesis (Chai *et al.*, 1994a).

Ameloblast cell differentiation

The inner enamel epithelia undergoes a precise sequential developmental program which includes morphological and cyto-

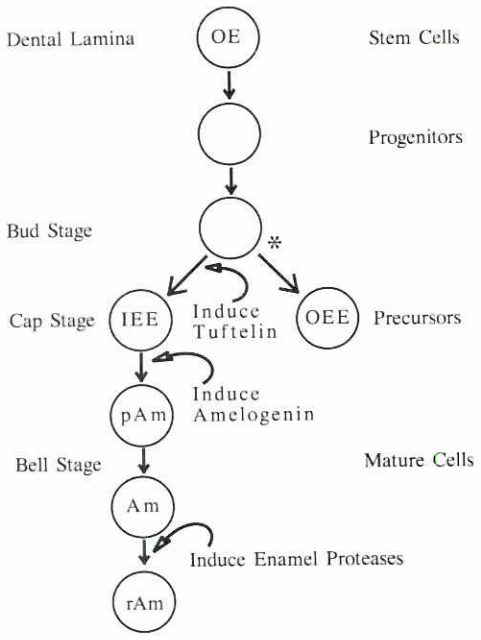

Fig. 6. Ameloblast cell lineage emerges during early tooth morphogenesis. *Stem cells within the odontogenic placode oral epithelium (OE) produce progenitor cells which divide and produce daughter cells which express tuftelin transcripts during the E13 bud stage and amelogenin transcripts during the E15 cap stage of tooth development. Asterisk (*) indicates the formation of inner (IEE) from outer enamel epithelium (OEE) during the late bud/early cap stages of development.*

logical changes resulting in the terminal differentiation of these cells into ameloblasts and the expression of tissue-specific gene products that form the enamel extracellular matrix. The ameloblast phenotype has been characterized as consisting of two major classes of proteins: hydrophobic proteins known as amelogenins (Eastoe 1963, 1965, 1979) and non-amelogenin proteins such as anionic enamel proteins (enamelins, tuft proteins, tuftelin), enamel proteases (Suga, 1970; Robinson *et al.*, 1975; Termine *et al.*, 1980; Carter *et al.*, 1989), proteoglycans and/or sulfated glycoproteins (Yoshiki and Umeda, 1972; Goldberg and Septier, 1987; Kogaya and Furuhashi, 1988; Smith *et al.*, 1993). Amelogenins are the most abundant enamel proteins, comprising approximately 90 % of the proteins secreted by the ameloblast cells (Termine *et al.*, 1980; Fincham and Belcourt, 1985). Sequence comparisons of amelogenins obtained from different species shows striking homology (Fincham *et al.*, 1992). The postulated functions of amelogenins range from calcium-chelating nucleating sites (Glimcher, 1979), inhibitors of crystal growth (Doi *et al.*, 1984; Aoba *et al.*, 1987) and/or regulators for crystal size, growth and orientation (Fearnhead, 1979; Aoba *et al.*, 1987, 1989; Robinson *et al.*, 1989; Fincham *et al.*, 1992). Recent studies by Diekwisch *et al.* (1993) using antisense inhibition provided support for the hypothesis that amelogenins regulate the orientation and dimensions of enamel crystals. In contrast to amelogenins, detailed knowledge for the anionic enamel proteins (enamelins, tuft proteins, tuftelins) has remained elusive. The major factor contributing to the lack of information concerning these proteins has been their under-representation due to the presence of serum proteins (Termine *et al.*, 1980; Fincham *et al.*, 1982; Okamura, 1983; Menanteau *et al.*, 1987; Zeichner-David *et al.*,1987; Limeback and Simic 1989; Strawich and Glimcher 1990; Strawich *et al.*, 1993). The only sequence available for these anionic enamel proteins is that for a bovine tuftelin obtained from a cDNA clone (Deutsch *et al.*, 1987, 1991).

Enamel proteases appear to be required for processing secreted amelogenins in the extracellular matrix and subsequently for amelogenin degradation and removal from the mineralizing matrix during the maturation stages of amelogenesis. If anionic enamel proteins are also processed and degraded remains to be determined. Several investigators have described these enzymes as serine proteases (Moe and Birkedal-Hansen, 1979; Shimizu *et al.*, 1979; Carter *et al.*, 1984, 1989; Crenshaw and Bawden, 1984; Sasaki *et al.*, 1991; Tanabe *et al.*, 1992) and metalloproteases (Overall and Limeback, 1988; DenBesten and Heffernan, 1989; Moradian-Oldak *et al.*, 1994). To date there are no reports for the isolation, purification and characterization of these enzymes. Importance for these enzymes in enamel mineralization is supported by studies of Wright and Butler (1989) and Wright *et al.* (1992b,c) in patients with autosomal inherited hypomaturation Amelogenesis Imperfecta (AI), in which it was suggested that the defect might be a failure to remove amelogenins from the maturing enamel resulting in the inhibition of normal crystallite growth. In addition, studies from DenBesten and Thariani (1992) suggested that fluorosis is the result of a failure to remove amelogenins from the forming enamel.

Cytodifferentiation is not a pre-requisite for ameloblast phenotype expression

Previous studies suggested that transcription of the major amelogenin, as determined by cytoplasmic dot blot hybridization and *in situ* hybridization during mouse mandibular molar development, was restricted to inner enamel epithelial cells that achieved terminal differentiation: polarized, elongated and withdrawn from the cell cycle (Snead *et al.*, 1984, 1987, 1988; Ahmad and Ruch, 1987; Amar *et al.*, 1989). However, recent studies indicate that this is not necessarily the case. With the advent of more sensitive mRNA phenotyping using reverse transcriptase-polymerase chain reaction (RT-PCR) techniques, initial expression of amelogenin transcripts in the mouse molar was determined at E15 (cap stage of tooth development) within progenitor inner enamel epithelial cells (Couwenhoven and Snead, 1994; Nakamura *et al.*, 1994; Zeichner-David *et al.*, 1994). Amelogenin detection by immunological methods was evident in mouse neonatal molars at the bell stage (newborn) (Snead *et al.*, 1987; Slavkin *et al.*, 1988; Nakamura *et al.*, 1994; Zeichner-David *et al.*, 1994). Furthermore, Slavkin *et al.* (1988a) demonstrated the expression of a 46 kDa anionic protein cross-reactive with polyclonal antibodies against enamel proteins in E18 mouse embryos (bell stage). This protein was synthesized and secreted into the extracellular matrix (ECM) by inner enamel epithelial cells associated with an intact basal lamina, defined by Kallenbachís differentiation stages III and IV (1971, 1979). At E19, a second anionic protein (72 kDa) was detected in differentiation zones III-V. This stage included the initiation of mineralization as determined by the appearance of calcium hydroxyapatite crystals, electron-diffraction patterns and von Kossa staining for calcium-salt deposition. Newborn molar stages were associated with the continued expression of amelogenins by ameloblast (Kallenbach differentiation zone VI, secretory ameloblast with Tome's processes).

More recently, Zeichner-David *et al.* (1993, 1994) reported the initial expression of one of the anionic enamel proteins, tuftelin, in

E13 mouse molars (bud stage). The presence of this tuftelin as determined by immunohistochemistry was not apparent until the cap stage or Kallenbach's differentiation zone I-II (E17) (Fig. 5). At this stage, the basal lamina is still present, the inner enamel epithelial cells are not polarized and the cells are still dividing (Zeichner-David *et al.*, 1994). The discrepancy between the detection of transcripts and the detection of translation product might represent a difference in method sensitivity; PCR is extremely sensitive whereas immunodetection requires an increased number of molecules for detection. Another interpretation of these results is that the differences might represent a physiological stage of ameloblast protodifferentiation where very low levels of transcripts are expressed as suggested by Couwenhowen and Snead (1994).

It is evident that inner enamel epithelial cells which are morphologically indistinguishable from other immature epithelial cells and are mitotically active (Panasse, 1962; Kallenbach, 1971; Ahmad and Ruch, 1987; Couwenhoven and Snead, 1994; Zeichner-David *et al.*, 1994) are transcribing tissue-specific tuftelin and amelogenin proteins. This is particularly important in the context of the significance of several *in vitro* experiments in which investigators have cultured either the whole tooth organ as an explant, isolated enamel organ epithelia, enamel organ epithelia recombinations, or have isolated primary cell cultures and maintained them *in vitro*. In recombination studies, enamel organ epithelia at the cap or bell stages have been recombined with mesenchyme obtained from different organs, scoring the results as ameloblast differentiation by the presence of elongated and polarized ameloblast cells (Koch, 1967; Thesleff *et al.*, 1977; Thesleff and Hurmerinta, 1981; Karcher-Djuricic *et al.*, 1985). The newer data as discussed indicates that early cap stage enamel organ epithelia used in recombination experiments was already determined and expressed the biochemical ameloblast phenotype.

Couwenhoven and Snead (1994) recently showed that if the enamel organ epithelia is isolated and placed in culture, it requires the presence of a reconstituted basement membrane gel (Matrigel) to express the amelogenin phenotype. When these investigators placed isolated epithelia derived from dental lamina (E12), bud stage (E13) or early cap stage (E14) in culture, only the cap stage-derived enamel organ epithelium expressed amelogenin in prolonged tissue culture; earlier stages of odontogenic epithelia did not express amelogenins. These experiments suggest that the instructive signal which controls amelogenin transcription occurs prior to or during early cap stage. Furthermore, the inducer for tuftelin transcription is possibly different than that required for amelogenin, since tuftelin and amelogenin are sequentially expressed and tuftelin is expressed at the bud stage (E13) (Zeichner-David *et al.*, 1993, 1994). These results support the hypothesis that multiple, sequential regulatory signals provided by the dental papillae mesenchyme control the biochemical differentiation of inner enamel epithelia into ameloblasts (Fig. 6) (Thesleff and Hurmerinta, 1981; Ruch, 1985, 1988; Lumsden, 1987, 1988; Thesleff *et al.*, 1991).

Dental mesenchyme is necessary for morphogenesis

An interesting observation from Couwenhoven and Snead's (1994) experiments was the fact that even in the permissive culture conditions where amelogenin was expressed, no polarization of the ameloblast cells was evident. Lack of ameloblast elongation and polarization has also been reported in isolated enamel organ epithelial cells placed in primary cell cultures.

Numerous investigators attempted to establish enamel organ epithelia in culture. These culture systems used epithelial cells derived from outgrowths of tooth explants (Limeback, 1985, 1986), outgrowths of isolated epithelial explants (Prime and Reade, 1980; Yamasaki and Pinero, 1989), or isolated cells from epithelia tissues plated in monolayers (MacDougall *et al.*, 1991; Kukita *et al.*, 1992). In addition, immortalized cell lines have been established from developing mouse enamel organ epithelia which maintain some of the biochemical characteristics of ameloblasts (Chen *et al.*, 1992).

In initial primary cell cultures derived from inner enamel epithelia (IEE), three cells types were observed: (i) cells resembling ameloblasts; (ii) small elongated cells, assumed to originate from the stellate reticulum; and (iii) polygonal cells, assumed to originate from the stratum intermedium. The cells resembling mature ameloblast were columnar cells which initially adhered to the culture dishes. These cells, however, degenerated *in vitro* to form aggregates of cellular debris (Prime and Reade, 1980), suggesting terminally differentiated ameloblast cells incapable of further cell

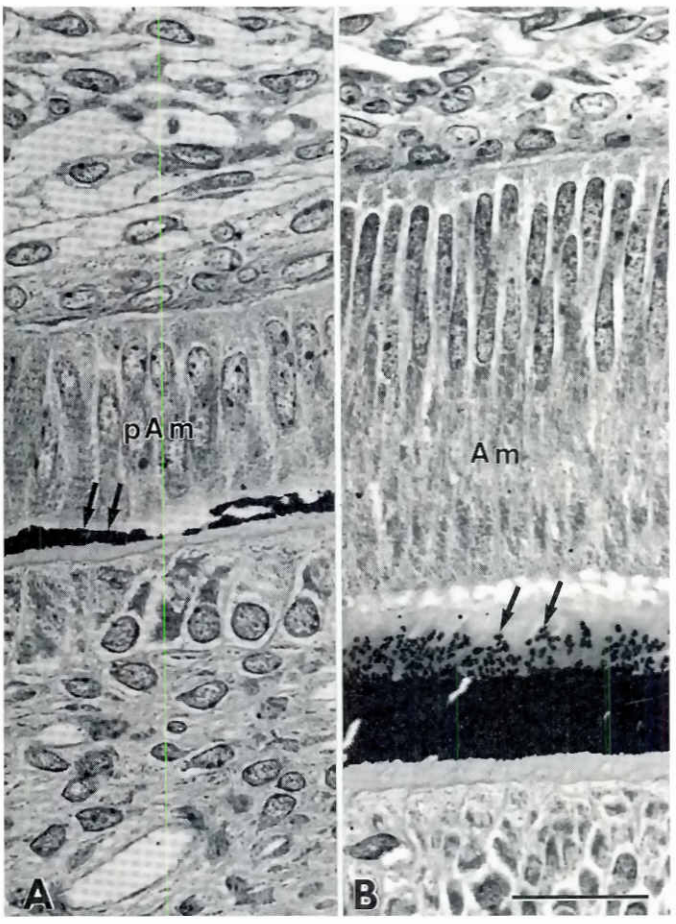

Fig. 7. Initial ameloblast cell functions include the nucleation and growth of enamel hydroxyapatite crystal formations. *Von Kossa histochemistry provides a useful technique to visualize when and where calcium phosphate salts are forming during early tooth development.* **(A)** *Arrows indicate the surface of the mineralizing dentine adjacent to the preameloblasts (pAm).* **(B)** *Overt secretory ameloblast cell differentiation illustrating the initial formation of enamel calcium phosphate deposits (arrows) within the enamel extracellular matrix. Bar, 25 μm.*

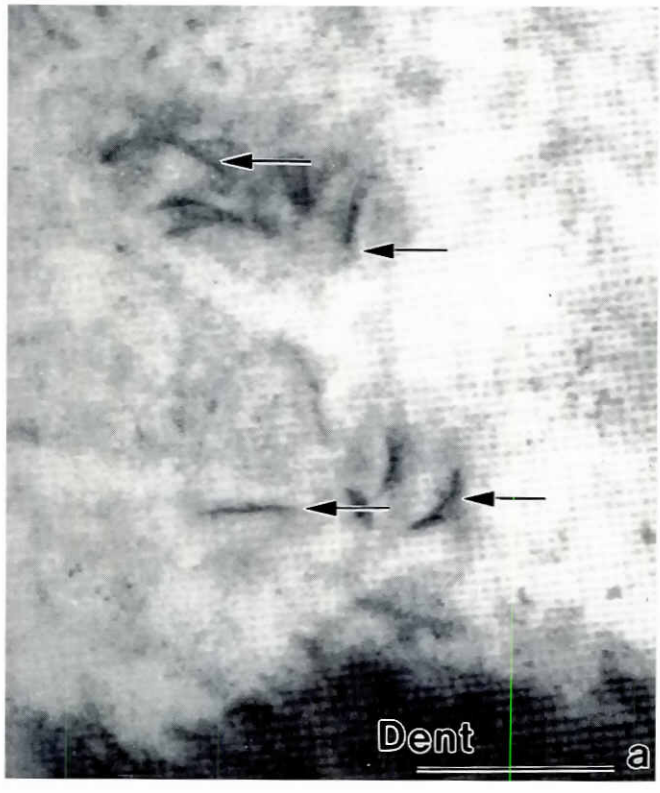

Fig. 8. Initial enamel crystals are not continuous with mineralized dentine crystals. *Short, randomly-oriented initial enamel crystals (arrows) are embedded in an electron-dense material, independent of the mineralized dentine (Dent.), during mouse molar tooth development. Magnification, x250,000; bar, 100 nm.*

division. Limeback *et al.* (1985, 1986) established epithelia cells outgrowths from porcine whole tooth bud explants; the epithelial-like cells were small, polygonal-shaped and synthesized amelogenins.

Kukita *et al.* (1992) isolated 1-2 week rat incisor ameloblasts. Two types of morphologically distinct cells were identified within this primary cell culture system; the major type showed changed phenotype with the addition of high calcium concentrations. The major cell type a large, flat fibroblast-like phenotype expressed amelogenin as determined by immunohistochemical staining using a monoclonal antibody (Kukita *et al.*, 1992). More recently, an ameloblast cell culture system conducive for ameloblast cytodifferentiation, synthesis and secretion of enamel proteins, and production of an enamel extracellular matrix which undergoes the process of biomineralization has been reported by MacDougall *et al.* (1991, 1993, 1994). Enamel organ epithelial (EOE) cells were grown to confluent monolayers which subsequently formed multilayered nodules. Ameloblasts cells are identified on the basis of amelogenin and tuftelin expression using both transcriptional and translational detection assays. These EOE cell cultures produce a mineralized extracellular matrix as determined by von Kossa staining, ultrastructural studies, electron diffraction analysis and X-ray spectrum microanalysis. Ultrastructural analysis identified large "enamel-like" crystals of varying sizes with electron on ring patterns comparable to those obtained for calcium hydroxyapatite crystal standards. In other studies, Chen *et al.* (1992) immortalized mouse EOE cells to establish a stable dental epithelial cell line. EOE cells were transfected by electroporation with the large T antigen from polyoma virus. The resulting cell lines were passaged for up to 4 weeks and expressed both keratin cytoskeletal elements and amelogenin (Chen *et al.*, 1992).

None of the cells in any of the cultures systems described acquired the polarized and elongated characteristics of ameloblast cells. From these studies it is reasonable to suggest that the presence of an underlying tooth mesenchyme and dentine extracellular matrix is necessary for ameloblast cytodifferentiation, but is not essential for the maintenance of the ameloblast biochemical phenotype.

Several questions arise from the previously described studies: what is the significance of ameloblast polarization if cytological changes are not necessary for biochemical expression and enamel biomineralization? Why are the tissue-specific gene products expressed in early ameloblast differentiation when cells are still proliferating? If the function of enamel proteins is related to biomineralization, why are tuftelin and amelogenin transcripts expressed in advance of the initiation of biomineralization? and Could the enamel gene products have functions other than enamel biomineralization?

Previous studies reported that "enamel-like material" known as stippled material was observed in predentin, dentine and along the cell surfaces of odontoblasts (Suga, 1960; Watson, 1960; Reith, 1967; Yamamoto *et al.*, 1980). More recently, immunohistochemical and immunocytochemical techniques detected amelogenin in early presecretory ameloblast cells in the early stages of differentiation (Nanci *et al.*, 1984, 1985, 1987; Slavkin *et al.*, 1988a,b,c; Inai *et al.*, 1991; Nakamura, 1994). The epitopes in the ameloblast antigens were also localized in predentin as well as in the intercellular spaces between odontoblasts; the antigens were further identified in odontoblast lysozymes (Inai *et al.*, 1991; Nakamura *et al.*, 1994), suggesting a translocation of enamel proteins from presecretory ameloblasts to adjacent odontoblasts. If such a translocation is a pre-requisite for further odontoblast differentiation, or if it represents a passive diffusion of enamel proteins without biological relevance remains unknown. However, experiments by Ruch *et al.* (1989) demonstrated that a monoclonal antibody produced against isolated mouse dental papilla mesenchyme cells (MC22-45D) strongly stained the adjacent enamel layer. This antibody was also capable of inhibiting odontoblast terminal differentiation *in vitro*, suggesting a functional role of enamel proteins in odontoblast differentiation. If the protein recognized by this antibody is tuftelin, amelogenin or an as yet unidentified enamel protein remains to be determined.

Positional requirements for ameloblast differentiation

One of the most intriguing aspects of enamel formation is the absence of enamel along the lingual surfaces in rodent incisors (Gaunt, 1956; Cohn, 1957; Warshawsky, 1968) and frogs teeth *(Rana pipiens)* (Gilette, 1955). Although dentine and pre-dentine cover all of the surfaces of the developing tooth, the lingual or "enamel-free" areas are covered by inner enamel epithelial cells which do not differentiate into ameloblast cells. Several investigators have approached this positional puzzle from several different points of view.

It has been established that the dental mesenchyme and/or its extracellular-matrix (ECM) products, pre-dentine and dentine, but not isolated matrix molecules, are necessary for ameloblast terminal differentiation as measured by polarization, elongation and

withdrawal from the cell cycle (Karcher-Djuricic *et al.*, 1985; Lesot *et al.*, 1985). Furthermore, heterotypic tissue recombination experiments demonstrated that dentine ECM in the enamel-free areas is capable of promoting ameloblast terminal differentiation in competent preameloblasts suggesting that the epithelia in these areas is not competent to react to the epigenetic signals of the mesenchyme (Amar and Ruch, 1987; Amar *et al.*, 1987). In contrast, Snead *et al.* (1988) using *in situ* hybridization techniques demonstrated the presence of amelogenin mRNA transcripts in the epithelial cells covering the "enamel-free" areas, ameloblasts and stratum intermedium cells. However, transcriptional activity in these cells was considerably lower than in the ameloblasts covering the labial surfaces. These results are also supported by autoradiography (Glazman *et al.*, 1986), ultrastructural studies (Diab and Zaki, 1991) and immunocytochemical studies (Nakamura *et al.*, 1987; Sakakura *et al.*, 1989). The presence of enamel proteins in this area was demonstrated by immunocytochemistry using a polyclonal antibody against mouse amelogenin (Nakamura *et al.*, 1991). Differences in the relationship between ameloblast cells and stratum intermedium in the enamel forming areas as compared to the enamel free areas were also identified. Ameloblasts in the enamel forming areas contain multiple cell processes connected by desmosomes and gap junctions to the stratum intermedium, suggesting the presence of cell-cell interactions between these two cells types which were absent in the epithelial cells of the enamel-free area. It was suggested that these cell-cell interactions regulate the synthesis and/or secretion pathway of enamel proteins (Nakamura *et al.*, 1991). At present, the factors that regulate positional information for ameloblast differentiation (either by cell-cell interactions with stratum intermedium or other mechanisms) in the enamel-free areas remain unknown.

Ameloblast control matrix formation

Biomineralization is a cell mediated process in which several different macromolecules control the space, position, orientation, size, shape and length of the mineral crystals (Lowenstam and Weiner, 1989). The role of anionic proteins in biomineralization has been established in several different systems ranging from mollusk shells to mammalian mineralized tissues such as bone, dentine and enamel. Enamel biomineralization is mediated by the ameloblasts and the organic extracellular matrix secreted by these cells (Fig. 7). The exact function of these enamel proteins is not well established. Developmental studies suggest that enamel proteins are sequentially expressed with anionic proteins being synthesized prior to the amelogenins. These enamel proteins are deposited along the dentino-enamel junction (DEJ) and appear to function as nucleators for calcium hydroxyapatite formation (Robinson *et al.*, 1977; Deutsch *et al.*, 1984; Slavkin *et al.*, 1988a,c; Deutsch, 1989; Zeichner-David *et al.*, 1994). The more hydrophobic amelogenins are synthesized approximately 48 h after the anionic tuftelins and appear to function as calcium chelating nucleating sites (Glimcher, 1979), inhibitors of crystal growth (Doi *et al.*, 1984; Aoba *et al.*, 1987) and/or regulators of crystal size, growth and orientation (Fearnhead, 1979; Deutsch *et al.*, 1984; Robinsonnd Kirkham, 1984; Robinson *et al.*, 1989; Aoba *et al.*, 1987, 1989; Slavkin *et al.*, 1988a,c; Fincham *et al.*, 1992). Recent studies by Diekwisch *et al.* (1993) using antisense inhibition provide support for the hypothesis that amelogenins control the orientation and size of enamel crystals. The function of amelogenins in enamel biomineralization is further supported by analysis of two cases of X-linked Amelogenesis Imperfecta (AI) (Lagerstrom *et al.*, 1991; Aldred *et al.*, 1992a,b). These studies characterized the clinical AI phenotype as hypoplastic and hypomineralized, and the genotypic defect as a deletion or introduction of a premature stop codon resulting in the absence of amelogenin transcription.

Crystal nucleation is not initiated at the dentino enamel junction

During cytodifferentiation ameloblasts secrete amelogenins and enamelins which regulate tissue-specific biomineralization. Is enamel biomineralization an event entirely restricted to the ameloblast and its secretory products, or is it dependent on the presence of other factors such as mineralized dentine matrix? Transfilter-studies had previously demonstrated the presence of odontoblasts and dentine in juxtaposition to ameloblasts and suggested that the dentine ECM was a requirement for ameloblast cytodifferentiation. Is the presence of dentine matrix mineral a requirement for subsequent enamel crystals formation?

Early explanations of enamel formation suggested that mineralized dentine was a prerequisite for enamel crystal nucleation, in that developmental studies showed that enamel biomineralization followed dentine mineralization (Lehner and Plenk, 1936). This explanation was supported by studies of the delay of enamel formation in rachitic teeth (Pflueger, 1932). Various ultrastructural investigations suggested that either dentine collagen (Boyde, 1964), pre-existing dentine crystals (Bernard, 1972; Reith 1967; Eisenmann and Glick, 1972; Fearnhead, 1979; Arsenault and Robinson, 1989), or enamel matrix proteins (Travis and Glimcher, 1964; Hoehling, 1966) were responsible for initial enamel crystal formation.

Recently, studies in our laboratory localized discrete individual enamel crystals spatially separated from the dentine mineralized matrix along the forming dentine-enamel junction (Fig. 8) (Diekwisch *et al.*, 1995). These results provide direct ultrastructural evidence to support the hypothesis that *de novo* enamel HAP crystal nucleation and growth are independent from the mineralization processes characterized for dentine. In later stages of enamel formation, enamel crystals transformed into long and parallelly oriented HAP crystals and were not visualized as separate from dentine HAP crystals, supporting earlier findings by Arsenault and Robinson (1989) that HAP crystals are formed closely associated with the dentine-enamel junction.

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b:MPLPPHPGHP GYINFOYEVL TPLKWYQSMI RHPYPSYGYE PMGGWLHHQI
m:MPLPPHPGSP GYINLSYEVL TPLKWYQSMI RQPYPSYGYE PMGGWLHHQI
r:MPLPPHPGSP GYINLSYEVL TPLKWYQSMI RQPYPSYGYE PMGGWLHHQI
p:MPLPPHPGHP GYINFOYEVL TPLKWYQNMI RHPYTSYGYE PMGGWLHHQI
h:MPLPPHPGHP GYINFOYEVL TPLKWYQS-I RPPYPSYGYE PMGGWLHHQI

b:IPVVSQQTPQ NHALQPHHHI PMVPAQQPVV PQQPMMPVPG QHSMTPTQHH
m:IPVLSQQHPP SHTLQPHHHL PVVPAQQPVA PQQPMMPVPG HHSMTPTQHH
r:IPVLSQQHPP SHTLQPHHHL PVVPAQQPVA PQQPMMPVPG HHSMTPTQHH
p:IPVVSQQTPQ SHALQPHHHI PMVPAQQPGI PQQPMMPLPG QHSMTPTQHH
h:IPVLSQQHPP THTLQPHHHI PVVPAQQPVI PQQPMMPVPG QHSMTPIQHH

b:QPNLPLPAQQ PFQPQSIQPQ -----PHQPL QPHQPLQPMQ PMQPLQPLQP
m:QPNIPPSAQQ PFQ-QPFQPQ AIPPQSHQPM Q--------- ----------
r:QPNIPPSAQQ PFQ-QPFQPQ AIPPQSHQPM Q--------- ----------
p:QPNLPLPAQQ PFQPQPVQPQ -----PHQPL Q--------- ----------
h:QPNLPPPAQQ PYQPQPVQPQ -----PHQPM Q--------- ----------

b:LQPQPPVHPI QPLPPQPPLP PIFPMQPLPP MLPDLPLEAW PATDKTKREE VD
m:--PQSPLHPM QPLAPQPPLP PLFSMQPLSP ILPELPLEAW PATDKTKREE VD
r:--PQSPLHPM QPLAPQPPLP PLFSMQPLSP ILPELPLEAW PATDKTKREE VD
p:--PQSPMHPI QPLLPQPPLP PMFSMQSL-- -LPDLPLEAW PATDKTKREE VD
h:--PQPPVHPM QPLPPQPPLP PMFPMQPLPP MLPDLTLEAW PSTDKTKREE VD
```

Fig. 9. Homology of amelogenin primary structure for the principal amelogenins. *Abbreviations: b, bovine; m, mouse; r; rat; p, pig; h, human.*

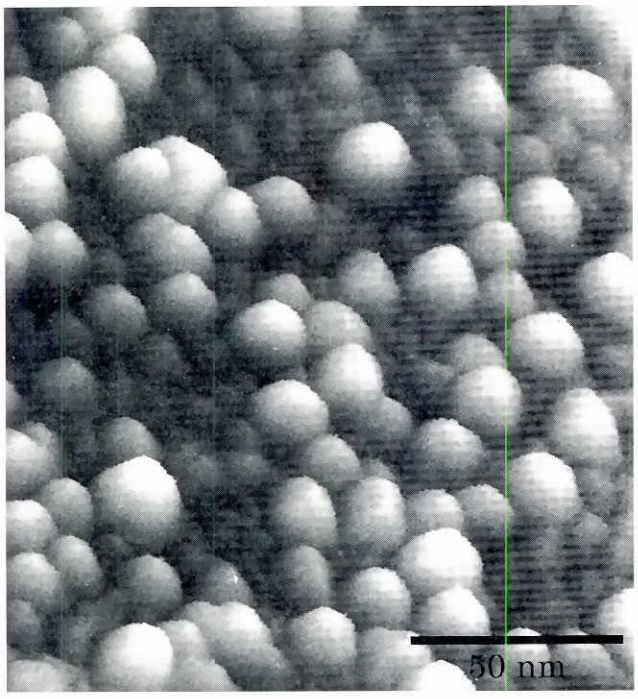

Fig. 10. Atomic force microscopic image of purified recombinant mouse amelogenins (M179) adsorbed onto a mica surface and showing nanosphere formation. *The individual amelogenin nanospheres of circa 20 nanometers in diameter are calculated to be formed by the self-assembly of >100 amelogenin monomers. Bar, 50 nm.*

Additional support for our conclusions comes from the enamel organ epithelial primary cell cultures reported by MacDougall *et al.* (1993, 1994), showing that cells grown in plastic in the absence of dentine or any component derived from dentine produced enamel crystals similar to those observed *in vivo* by electron microscopic and electron diffraction analyses.

Quaternary structure determines amelogenin function

Since the initial identification of "amelogenin" (Eastoe, 1965) proteins as the principal component of the mammalian secretory-stage dental enamel matrix (Eastoe, 1960; Piez, 1961; Glimcher *et al.*, 1961), the issue as to their function in the complex processes of amelogenesis has been widely debated (Eastoe, 1979; Deutsch, 1989; Fincham *et al.*, 1992).

By analogy with other mineralized tissues such as bone or dentine, the view that extracellular matrix proteins of developing enamel would fit somewhere into the family of "structural proteins" (collagens, keratins, elastin etc.) appeared intuitive to early investigators in this field (Block *et al.*, 1949). Astbury (1961) in a discussion of then current ideas on Biological Calcification observed that the X-ray diffraction diagram of a specimen of developing human dental enamel protein (Pautard, 1961) gave a cross-ß diffraction pattern and went on to note: "... it is not a well-defined cross-ß diagram but it is acceptable enough in the original.", thereby sparking off a debate on amelogenin structure which has persisted for over 30 years (Pautard, 1961; Glimcher *et al.*, 1961; Bonar *et al.*, 1965a,b; Termine *et al.*, 1979; Traub *et al.*, 1985; Jodaikin *et al.*, 1986; Zheng *et al.*, 1987; Aoba *et al.*, 1990; Goto *et al.*, 1993).

Currently, complete data on the primary structures of amelogenin proteins from five mammalian species (cow, pig, human, mouse and rat) are now available (see Fig. 9 for details). This evidence serves to emphasize the high degree of amino acid sequence homology between the mammalian species, reaching a level of identity in excess of 80% of total residues. This conserved primary structure is distinguished by several features: (i) high proportion of proline, histidine, glutamine and leucine; (ii) general hydrophobic character, with a distinctively hydrophilic carboxy-terminal motif; and (iii) occurrence of multiple tandem (or triple) repeat sequences such as His-His, Pro-Pro, Gln-Gln, Ile-Ile and Val-Val; the significance of these repeat motifs in the amelogenin structure at the secondary and tertiary levels is presently obscure.

In the case of the bovine (X-197) amelogenin, a remarkable repetitive sequence [(Gln-Pro-X)9] occurs within the "core" of the molecule. Molecular modeling studies suggested that the secondary structure of this motif is that of a "ß-spiral" which, it has been postulated, may function as a calcium transport channel (Renugopalakrishnan *et al.*, 1989). However, this repetitive structure has not yet been identified in the other four mammalian species, casting some doubt on its central role in amelogenesis.

A current search for homologies to amelogenin primary structures within the protein sequence data-bases reveals no related structures, which serves to emphasize the unique primary structure of this group of proteins. Immunohistochemical studies employing both monoclonal and polyclonal antibodies showed that amelogenins are tissue-specific products of ameloblast cells within the inner enamel epithelium (Nanci *et al.*, 1985,1989; Herold *et al.*, 1987). In the mouse, a single copy of the amelogenin gene is localized to the short arm of the X-chromosome (Lau *et al.*, 1989; Chapman *et al.*, 1991), while in other species (bovine, human and some monkeys) two copies of the gene have been identified; one on each of the sex chromosomes (Snead *et al.*, 1985b; Nakahori *et al.*, 1991; Salido *et al.*, 1992). Most recently, studies of amelogenin expression at the mRNA level revealed extensive alternative splicing from the primary transcript with seven alternatively-spliced mRNAs now being identified in the mouse (Lau *et al.*, 1992; Simmer *et al.*, 1994a). Further, it appears likely that similar situations exist in other species (Shimokawa *et al.*, 1987; Young *et al.*, 1987; Gibson *et al.*, 1991a,b).

Recognizing that sequence determines structure and structure determines function, several investigators have sought to define amelogenin structure at the secondary and tertiary levels. These studies have employed NMR, CD, molecular modeling and FTIR techniques (Renugopalakrishnan *et al.*, 1986, 1989; Zheng *et al.*, 1987; Aoba *et al.*, 1990; Goto *et al.*, 1993). Currently, preliminary investigations have provided the following generalizations: (i) amelogenin contains both ß-turn and ß-sheet motifs; (ii) a high proportion of proline (one residue in three) precludes any significant α-helical motif; (iii) six tyrosines of the amino-terminal sequence may be externalized; (iv) in bovine amelogenin, Gln-Pro-X repeat sequence appears to form a spiral structure (ß-spiral); and (v) most of the amelogenin polypeptide chain appears to be in rapid isotropic (i.e. "random") motion (Termine and Torchia, 1980). Recently, NMR studies of recombinant mouse amelogenin (Simmer *et al.*, 1994), isotopically labeled with ^{15}N, confirmed the apparently "unstructured" nature of the amelogenin molecule (D. Torchia, unpublished observations). Ironically, there is a paradox in which the principal amelogenin protein of an extracellular matrix which becomes transformed during amelogenesis into an exqui-

sitely organized bioceramic structure, appears to be largely unstructured at the secondary and tertiary levels.

Amelogenin protein in solution generates temperature-sensitive reversible coacervates (Nikiforuk and Simmons, 1965). The formation of high molecular weight aggregate structures (Bonar *et al.*, 1965a,b), which exhibit properties of redistribution in size-exclusion chromatographic systems (Mechanic, 1971), suggest that hydrophobically mediated inter- and intra-protein-protein interactions are significant to amelogenin tertiary and quaternary structures. This perspective has recently received support from aggregation studies of the physical properties of purified recombinant amelogenins employing dynamic light scattering (DLS), transmission electron microscopy (TEM), high resolution size-exclusion chromatography and atomic force microscopy (AFM) (Fincham *et al.*, 1994; Moradian-Oldak *et al.*, 1994a). Over a wide range of pH and ionic strength, a 179-amino acid recombinant mouse amelogenin ("M179") formed quasi-spherical structures of 18-20 nm diameter ("nanospheres"). Nanospheres were observed under hydrated (AFM and DLS) and dehydrated (TEM) conditions and exhibited size distributions of less than 20% dispersity (see Fig. 10). In addition, a comparison of the TEM image obtained from preparations of recombinant M179 amelogenin with *in vivo* mouse developing enamel (Fig. 11) served to emphasize the similarity of these structures.

Size exclusion chromatographic data for the M179 amelogenin confirmed earlier observations of Mechanic (1971), working with partially purified bovine amelogenins, that aggregation was amelogenin concentration-dependent and reversible. The newer data further suggests that in solution recombinant amelogenin undergoes a process of self-assembly to generate nanosphere structures calculated to incorporate >100 amelogenin monomers (Fincham *et al.*, 1994; Moradian-Oldak *et al.*, 1994a).

These observations of amelogenin quaternary structures, formed *in vitro*, have recently gained greater significance through high resolution TEM studies of *in vivo* preparations of developing mouse enamel, oriented to display the longitudinal view of the early-stage developing mineral crystallites (Fincham *et al.*, unpublished observations). Such preparations have consistently shown rows of structures, of dimensions comparable to the *in vitro* images of amelogenin nanospheres, and oriented in alignment with the developing mineral phase. These observations suggest that specific amelogenin aggregate structures may be directly implicated in the ultrastructural organization of the developing enamel (see previous discussion by Smales, 1975). We now speculate that amelogenin nanospheres collectively function to control enamel crystallite spacing, which is found to be about 20 nm (Diekwisch *et al.*, 1995). When packed into a three-dimensional array, nanospheres may provide oriented anionic channels within the extracellular matrix which serve to facilitate ion transport and crystal growth (Aoba and Moreno, 1991).

Antisense inhibition experiments of amelogenins provide additional support for these conclusions. Experiments were designed to test the hypothesis that amelogenin regulates the size and orientation of enamel hydroxyapatite crystal formations when tooth explants are cultured in serumless medium. Since the major mouse amelogenin gene is induced during early cap stages (Zeichner-David *et al.*, 1993; Couwenhoven and Snead, 1994; Nakamura *et al.*, 1994), E15 molar explants were cultured for extended periods of time in the presence or absence of antisense or sense amelogenin oligonucleotides. Under these experimental conditions, amelogenin translation was arrested by approximately 30% and resulted in a significant decrease in HAP crystal size and crystal orientation (Diekwisch *et al.*, 1993). These results were

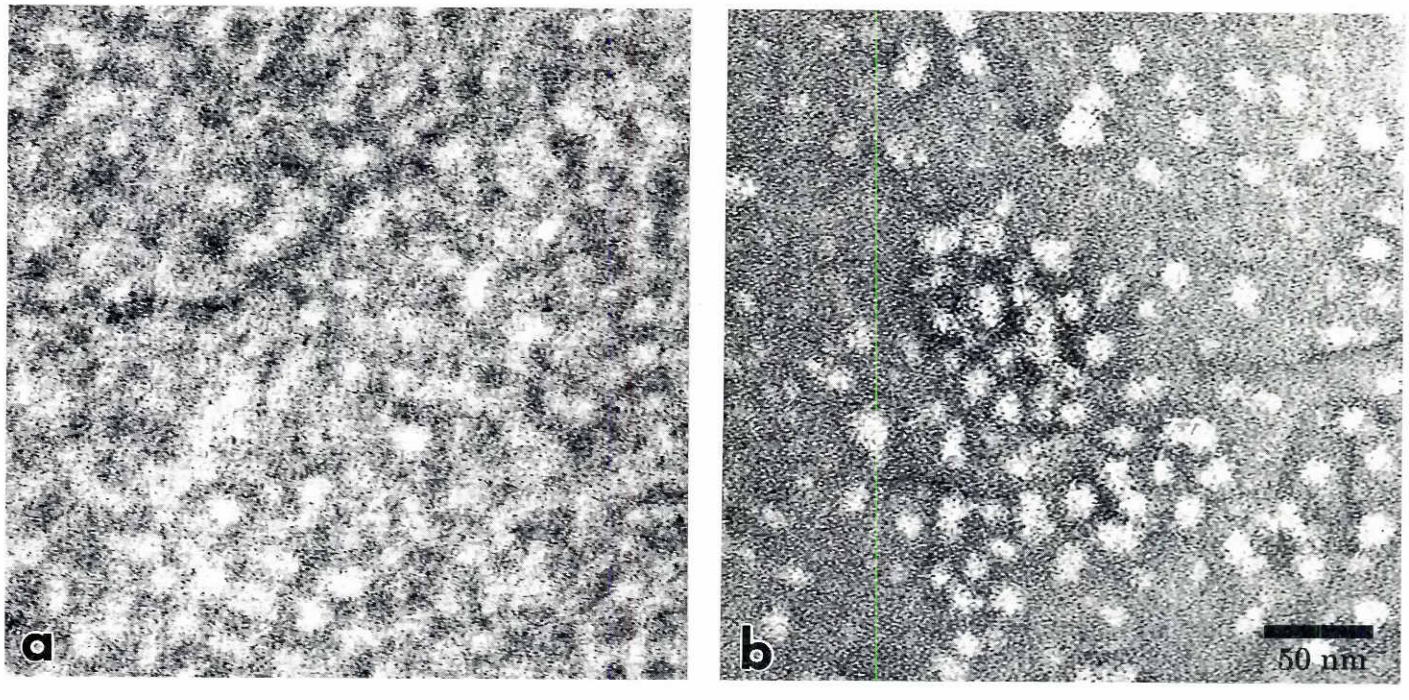

Fig. 11. Nanosphere structures form *in vivo* and *in vitro*. **(a)** *Nanospheres appear as electron-lucent within forming enamel matrix of developing mouse molar teeth as contrasted with uranyl acetate and examined with transmission electron microscopy (TEM).* **(b)** *Nanospheres form in vitro from the self-assembly of recombinant mouse amelogenin (M179). The isolated M179 proteins are sprayed onto a microscope grid and contrasted with uranyl acetate and then examined with TEM. Note the physical similarities between the in vivo and in vitro nanosphere structures.*

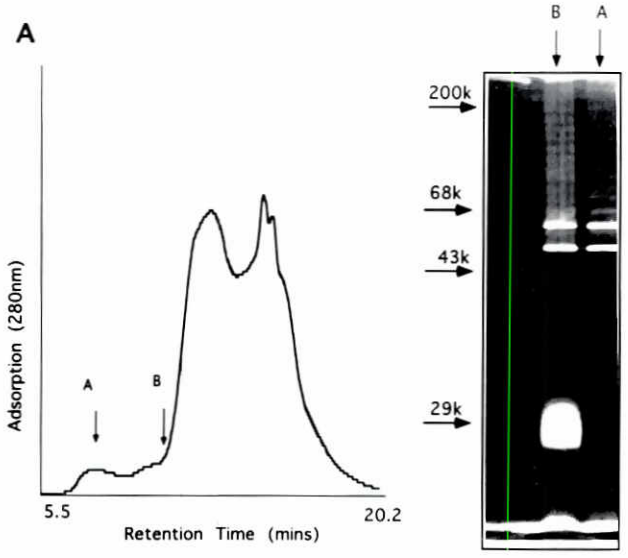

Fig. 12. Isolation of two classes of enamel proteases. *Size-exclusion HPLC chromatography of the acetic acid extract of developing bovine enamel. Two fractions were found to have proteolytic activity ("A" and "B"), eluting before the major amelogenin peaks shown by arrows. Proteins were detected by monitoring absorbance at 280 nm. Insert is a zymogram showing gelatinolytic activities in the eluted fractions "A" and "B".*

interpreted to suggest that the supramolecular organization of the enamel extracellular matrix functions to nucleate HAP crystals and amelogenins primarily control the size and patterns of crystal growth.

Amelogenins are removed from the mineralizing matrix

During the maturation stage of amelogenesis, ameloblast cells change their morphology and function from secretory to resorptive and transporting cells (Kallenbach, 1974). In the late secretory stage, cells reduce their height, and the Tomes' processes become shorter until they disappear. One characteristic of these reduced epithelial cells is a "modulation" activity of an invaginated ruffle-ended apical surface resulting in an alternating pattern of ruffle-ended ameloblasts (RA) and smooth-ended ameloblasts (SA) (Josephsen and Fejerskov, 1977; McKee *et al.*, 1989). The modulations occur in waves, traveling from the least mature to the most mature regions (Smith *et al.*, 1987; Takano *et al.*, 1988). Although the significance of these changes is not known, it has been speculated that they are related to alterations in the permeability of the membrane and calcium transport. The ruffle-ended ameloblasts are tightly coupled, contain many lysosomes, indicate considerable endocytotic activity, calcium binding proteins and membrane-associated calcium ATPases (Josephsen and Fejerskov, 1977; Salama *et al.*, 1987; Berdal *et al.*, 1991). The smooth-ended ameloblast are not coupled, show almost no endocytotic activity and no membrane calcium ATPase. One explanation for modulation is that it is necessary to neutralize the low pH associated with growth of enamel crystals (Smith *et al.*, 1987) which underlies the ruffle-ended ameloblast (Takano *et al.*, 1982). Studies by Sasaki *et al.* (1991) support this hypothesis; they measured the pH of enamel and discovered that the areas covered with SA were more neutral than those areas under RA which were more acidic. Whereas it had previously been assumed that at the maturation stage ameloblast no longer synthesized and secreted enamel proteins, it was demonstrated that this is not necessarily the case (Nanci *et al.*, 1989; Smith and Nanci, 1989). The mechanism(s) that controls the change of ameloblast from elongated, polarized cells to shortened, cuboidal cells and initiates the modulation cycles during enamel maturation is unknown.

As the maturation stage progresses and the inorganic mineral content increases, the organic material needs to be removed from the mineralizing enamel. It was originally believed that this was achieved through a resorptive activity of the ameloblast cells (Reith and Cotty, 1967), presumably via endocytosis and transfer of matrix proteins to the lysosomes (Smith, 1979). This was further supported by the localization of amelogenin antigens within ameloblast lysosomes (Nanci *et al.*, 1985; Inage *et al.*, 1989). More recently, it was suggested that this process is initiated by specific enamel proteases (Suga, 1970; Carter *et al.*, 1989; DenBesten and Heffernan, 1989). The localization of amelogenin antigens in the lysosomes can have two interpretations: (I) due to degradation of amelogenin previously found in the ECM or (ii) an intracellular process to down-regulate newly synthesized amelogenin in secretory ameloblast (Nanci *et al.*, 1989; Smith and Nanci, 1989; Nanci and Smith, 1992). It is assumed that smaller peptides are removed by random diffusion through smooth-ended ameloblast cells (Aoba and Moreno, 1991; McKee *et al.*, 1986), or by endocytosis in the ruffle-ended ameloblast cells (Garant *et al.*, 1984; Goldberg and Sasaki, 1985; Smith *et al.*, 1987; Aoba and Moreno, 1991; Salama *et al.*, 1991). These ameloblast also function to remove water from the ECM, allowing the hydroxyapatite crystals to grow (Robinson *et al.*, 1981a,b; Fincham *et al.*, 1982).

During the dynamic process of amelogenesis, the loss of the enamel organic matrix proteins results from a progressive degradation of the secreted amelogenins by proteinases through mechanisms which are thought to be highly controlled yet are poorly understood (Suga *et al.*, 1970; Robinson *et al.*, 1977; Termine *et al.*, 1980; Fincham *et al.*, 1982; Fincham and Belcourt, 1985). Enamel proteinases have been described by several investigators and were mainly attributed to the serine proteinase class (Moe and Brikedal-Hansen, 1979; Shimizu *et al.*, 1979; Carter *et al.*, 1984, 1989; Crenshaw and Bawden, 1984; Sasaki *et al.*, 1991; Tanabe *et al.*, 1992). Some reports described metalloproteinases in developing enamel, but these enzymes have relatively weak activity against amelogenin (Overall and Limeback, 1988; DenBesten and Heffernan, 1989). In general, characterization of enamel proteinases has been slowed down by technical difficulties of isolation and purification of both the enzyme and the substrate from *in vivo* tissues in sufficient quantities to analyze the solubility and aggregation properties of the amelogenins.

Recently Moradian-Oldak *et al.* (1994b) used recombinant murine amelogenin M179 (Lau *et al.*, 1992; Simmer *et al.*, 1994b) as a substrate to examine the specificity of an enamel proteinase fraction isolated from developing bovine enamel. A proteinase fraction of 48-70 kDa was isolated from developing bovine enamel by size exclusion and reversed-phase HPLC techniques (Fig. 12). Proteolytic activity in the HPLC fractions A and B were visualized by enzymography using gelatin as a substrate (Heussen and Dowdle, 1980). Incubation of M179 with the proteinase fraction "A" generated a major proteolytic product having the N-terminal of the M179 parent protein but with a mass of 18,894.2±3.0 Da as compared to 20,160 Da for the M179 parent molecule. Given an

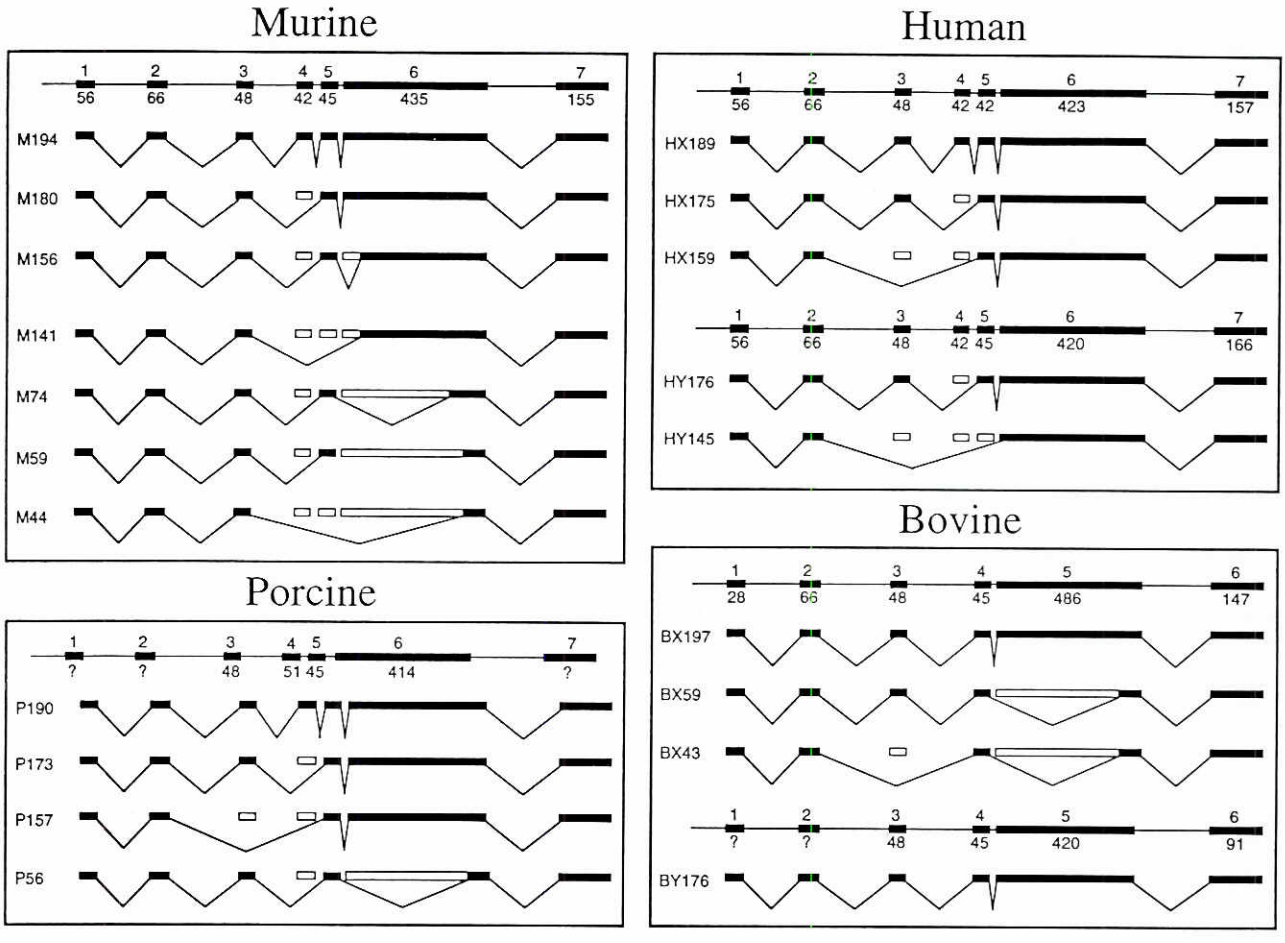

Fig. 13. Alternative splicing characterizes murine, porcine, bovine and human mammalian amelogenin genes. *The intron/exon structure for each of the four mammalian species' amelogenin gene is schematically illustrated at the top of each alternative splicing diagram. The lines correspond to introns and the bars to exons. The exons are numbered above while the number of nucleotides per exon is indicated below each bar. A hollow bar is used to indicate when an exon, or part of an exon has been removed during splicing. Key to abbreviations: Individual amelogenin proteins are denoted by a 1 or 2 letter code indicating the organism and the X and/or Y chromosome expressing the isoform (if ambiguous). The number following the letter indicates the number of amino acid residues present in the secreted protein after removal of the signal peptide. Therefore the HX189 is the 189 residue amelogenin isoform expressed from the human X-chromosomal copy of the gene.*

intact amino-terminal sequence, this mass figure suggests that this product terminates at Pro^{168} (Mr 18,888.9 Da), suggesting that the cleavage takes place at the C-terminal region of the molecule.

The enzyme within fraction "A" is Ca^{2+} dependent; the Ca^{2+} can not be substituted by Zn^{2+}, Mn^{2+} or Co^{2+}; Antipain, aprotinin, leupeptin or other serine proteinase inhibitors did not affect the proteolytic activity of the enzyme. We concluded that this "high molecular weight" proteinase cleaves amelogenin at the carboxy-terminal region and is a specific calcium-dependent metalloproteinase (Moradian-Oldak *et al.*, 1944b).

In other studies, Tanabe *et al.* (1992) reported a 76 kDa proteinase from the outer layer of porcine developing enamel which cleaved the carboxy-terminal peptide of a porcine amelogenin. These investigators suggested that the porcine amelogenin (173 residues) was initially cleaved to a 148 residue through the loss of 25 residues from the carboxy-terminus, suggesting that the processing is mediated by an exopeptidase (carboxy-peptidase). This data opens the possibility of sequential processing with the product described by Tanabe *et al.* (1992) being produced at a later stage of processing, with the initial carboxy-terminal proteolytic product being a 162-residue amelogenin generated by cleavage to the -Pro168-Ala169- locus as described.

Smith *et al.* (1989a,b) reported that the higher molecular weight enamel proteinases are mainly present in the secretory stage of amelogenesis and that this stage contains proteinases which degrade amelogenin less efficiently than those present in the maturation stage. It is therefore reasonable to conclude that amelogenin carboxy-terminal processing occurs during the secretory stage whereas subsequent degradation of amelogenin occurs at the maturation stage.

Sasaki *et al.* (1991) reported amelogenin degradation by an enamel proteinase of 30 kDa having an acidic pH optimum. This enzyme split a 28 kDa bovine amelogenin into two fragments, one of which proved to be identical to the amino-terminal 45-residue TRAP molecule previously described (Fincham *et al.*, 1981, 1983). Current studies in our laboratory also showed that a proteinase

fraction (25-30 kDa) isolated from developing pig enamel cleaves the M179 recombinant amelogenin at the N-terminal region (manuscript in preparation). On the basis of these reports (Sasaki *et al.*, 1991; Tanabe *et al.*, 1992), and the recent data using recombinant M179 amelogenin (Moradian-Oldak *et al.*, 1994b), we suggest that the high molecular weight enamel proteinases (60-70 kDa) are metalloproteinases which cleave amelogenin at the carboxy-terminal region, whereas the lower molecular weight proteinases (25-30 kDa) are serine proteases which either cleave the amino-terminal TRAP region, or completely degrade amelogenin into small fragments.

The fact that the amino- and carboxy-terminal regions of amelogenins are highly conserved between the five mammalian species studied so far (pig, human, mouse, bovine and rat) (Snead *et al.*, 1985; Shimokawa *et al.*, 1987; Yamakoshi *et al.*, 1989; Salido *et al.*, 1992; Bonass *et al.*, 1994) suggests that similar mechanisms for proteolysis exist in the different species, and that "amelogeninases" exist in the enamel extracellular matrix cleaving amelogenin through specific and highly controlled mechanisms. The function of proteolysis with respect to enamel biomineralization remains an intriguing problem yet to be solved.

Alternative splicing of amelogenins

In the early 1980s, protein sequencing methods revealed the primary structures of a number of amelogenin polypeptides (Fukae *et al.*, 1980, 1983; Fincham *et al.*, 1981, 1983; Takagi *et al.*, 1984). Comparing the amino acid sequences of a number of proteolytic cleavage products revealed a surprising pattern. Bovine amelogenin polypeptides were identical at their amino and carboxyl termini but differed by the presence or absence of a polypeptide segment in the middle of the protein. Sasaki *et al.* (1984) was the first to invoke the mechanism of differential RNA processing to explain this phenomenon. Further evidence supporting the alternative RNA splicing hypothesis came from *in vitro* translation of RNA extracted from bovine (Shimokawa *et al.*, 1987; Young *et al.*, 1987) and murine, hamster, and rat enamel organ epithelia (Zeichner-David *et al.*, 1985). These experiments demonstrated that RNA isolated from developing teeth yielded multiple translation products of various sizes. In 1991, definitive evidence derived from the cloning and sequencing of a bovine mRNA encoding a 59 residue amelogenin known as the Leucine-Rich Amelogenin Polypeptide or LRAP was reported (Gibson *et al.*, 1991a).

Alternative splicing of the amelogenin primary RNA transcript is more extensive than was originally imagined. A summary of the amelogenin isoforms generated by alternative RNA splicing is provided in Fig. 13. The gene structures are extrapolated from the human and bovine genomic structures which are deduced by aligning the exons found on cDNAs with the genomic sequence. The long segments of DNA between the short segments of coding region are introns and are framed by consensus sequences that signal a potential splice site.

An interesting feature of the alternative splicing of amelogenins is that the spliced transcript product appears to change with progressive ameloblasts differentiation (DenBesten and Li, 1992; Couwenhowen and Snead, 1994). The proportions of the various amelogenin isoforms would appear to be regulated by specific splicing factors. Another possibility is that multiple promoter and/or polyadenylation/cleavage sites may be utilized. Messenger RNA transcripts expressed from different promoters vary at their 5' ends, whereas multiple polyadenylation/cleavage sites change the transcripts at the 3' end. All transcripts using a given promoter or cleavage site may be spliced along a given pathway. This transcript could be specifically up- or down-regulated by transcription factors, growth factors, or the polyadenylation/cleavage site machinery, which would cause more or less transcripts to be spliced along a given pathway.

Since the mechanism by which amelogenins contribute to enamel biomineralization is unknown, the functional significance of alternative splicing is as yet speculative. In general, alternative splicing allows for the synthesis of a family of proteins from a single gene. The proteins differ from one another by the inclusion or deletion of discrete polypeptide motifs. In systems where functional or binding assays permit the analysis of alternative splicing , it has been shown that each of the isoforms can illustrate different cellular localization (Ushiyama *et al.*, 1993; Montmayeur and Borrelli, 1994), protein-protein interactions (Namba *et al.*, 1993; Spengler *et al.*, 1993; Yan *et al.*, 1993), protein-DNA interactions (Gogos *et al.*, 1992; Hsu *et al.*, 1993), cofactor (Sorimachi *et al.*, 1993) and ligand binding (Gilbert *et al.*, 1993), post-translational modifications (Otterson *et al.*, 1993; Tingley *et al.*, 1993), regulation (Durand *et al.*, 1993) with no detectable change in function (De la Pena *et al.*, 1992). It is assumed that sequential alternative splicing of amelogenins during ameloblast cell differentiation resulting in different isoforms is a significant feature of enamel biomineralization.

Enamel organ dysfunction

Developmental defects of enamel were reported more than 200 hundred years ago associated with several vitamin deficiencies such as rickets and scurvy, as well as with some infectious diseases such as measles and syphilis. At present, there are more than one hundred pathological conditions associated with abnormal enamel development. The etiology of various enamel abnormalities are extremely variable and they include environmental influences, malnutrition, trauma, infection, exposure to certain elements such as fluoride and hereditary disorders (for reviews see: Melnick *et al.*, 1982; Clarkson, 1989; Suckling, 1989).

Fluorosis

Fluorosis is the result of an acute or chronic exposure to fluoride while teeth are developing. Fluorosed teeth are characterized by opacities of the enamel and production of a more porous enamel. It has been postulated that fluorosis accelerates ameloblast modulation and also affects the protease activity in charge for amelogenin degradation during the maturation stages of enamel formation, resulting in the retention of amelogenins in the extracellular matrix (DenBesten and Thariani, 1992).

Amelogenesis imperfecta

The recent technical developments in human molecular genetics resulted in the determination of the genes responsible for many genetics diseases such as cystic fibrosis (Collins, 1992); Alzheimer's disease (Kosik, 1992); malignant hyperthermia (MacLennan and Phillips, 1992); epidermolysis bullosa (Epstein, 1992) and Gaucher disease (Beutler, 1992). Several diseases which affect the human dentition have been reported and their pattern of inheritance determined (for review see Melnick *et al.*, 1982; Jorgenson, 1983). These genetic diseases have been classified by the tissue which they affect: enamel, dentine, cementum, and gingiva. Among the first distinctions to be made is to determine if the defect involves

alterations in other tissues and/or metabolic processes (syndromic), or if the defect is exclusively expressed in specific dental tissues (non-syndromic). Knowledge of the molecular basis for these non-syndromic diseases is just beginning to emerge.

Amelogenesis Imperfecta (AI) represents a genetic and clinically heterogeneous group of enamel defects which occurs in the general population with an incidence of 1:14,000 in the USA (Witkop and Sauk, 1976), while in Sweden the ratio is considerably higher [(1-700), Backman and Holm, 1986; Backman *et al.*, 1993]. The most widely accepted classification of AI is based upon the developmental process which is assumed to be the level of the defect: 1) hypoplastic, reflects defects in the differentiation or viability of ameloblasts with decreased matrix production resulting in a very thin or sometimes absent enamel; 2) hypocalcification, reflects defects in the enamel matrix mineralization resulting in a soft enamel; and 3) hypomaturation, reflects alterations in the enamel rods and rod sheath structures resulting in a deformed enamel particularly susceptible to fracture and chipping from the underlying dentine (Winter and Brook, 1975; Witkop and Sauk, 1976; Melnick *et al.*, 1982).

Lagerstrom *et al.* (1990) performed a linkage study in two families presenting X-linked AI. Although one family presented hypoplastic X-linked dominant, and the other with hypocalcified X-recessive (Backman and Holmgren, 1988), both defects were localized to chromosome Xp22, the same region (Xp22.1-Xp22.3) where the human amelogenin structural gene has been localized (Lau *et al.*, 1989), strongly implicating amelogenin in the etiology of AI. Furthermore, Aldred *et al.* (1992b) analyzed three families with X-linked AI using polymorphic DNA markers flanking the position of AMELX. Using two-point linkage analysis, linkage was established between AI and several of these markers in two of this families supporting the involvement of AMELX in AI. However, the third family did not show linkage to the Xp22 region, but rather to the long arm of chromosome X (Xq22-q28 region) thus indicating locus heterogeneity in X-linked AI. Evidence of the defect being in the amelogenin gene has been provided by Lagerstrom *et al.* (1991) showing that a 5-kb (two exons) interstitial deletion within the amelogenin gene was identified in affected males and heterozygous females in an X-linked recessive hypomineralized AI family (Lagerstrom *et al.*, 1990). Another case in which the disease is associated with a nonsense mutation in exon 5 of the amelogenin gene has been reported by Aldred *et al.* (1992a). This mutation involves a single base deletion (CCCC→CCC) in the exon in affected and carrier individuals. The result of this deletion is to alter the reading frame and introduce an early TGA stop codon (an opal mutation). A small deletion of four amino acids in the signal peptide region of the amelogenin was recently reported in a family with X-linked AI; this deletion might result in an incomplete secretion of amelogenin into the extracellular matrix resulting in enamel hypoplasia (Lagerstrom-Fermer *et al.*, in press).

The autosomal dominant hypocalcification form of AI is the most predominant form of AI [(1:20,000) Witkop and Sauk (1976)]. To date, the only linkage study available has linked a hypoplastic autosomal dominant type of AI to human chromose 4q (Backman *et al.*, in press). No gene associated with the autosomal inherited forms of AI has been published. Wright *et al.* (1989, 1991b,c, 1992, 1993) reported the analysis of enamel proteins and ultrastructure of teeth obtained from a patient with autosomal recessive pigmented hypomaturation AI. Their results suggest that this type of defect is associated with the retention of organic material, possibly related to the inhibition of normal crystallite growth. These results suggest that the defect might be at the level of the enamel protease required to degrade amelogenins from the mineralizing enamel matrix. Ultrastructural analysis of teeth obtained from a patient with an autosomal dominant hypocalcified AI showed that the enamel contained 30% less mineral than normal controls and that this type of AI was different from the hypomaturation or hypoplastic forms of AI (Wright *et al.*, 1993).

Epidermolysis bullosa

The syndromic disease epidermolysis bullosa (EB) is characterized by mechanical fragility, enamel defects and blistering of the skin (Fine, 1986). EB has been divided into three subtypes based on the level of tissue separation following mechanical trauma of the skin (Fine *et al.*, 1991): (i) EB simplex, blistering occurs within the epidermis; (ii) EB junctional, blistering occurs within the basement membrane; and (iii) EB dystrophic, blistering occurs beneath the basement membrane (Fine, 1986). The molecular defects associated with EB have involved keratins (simplex), type VII collagen (dystrophic) and laminin-5 (junctional). EB affected individuals may also present abnormal dental development and dysplastic enamel (Witkop and Sauk, 1976). Enamel hypoplasia is common in patients with junctional EB (Wright *et al.*, 1992a). Teeth from these patients show extensive disruption of ameloblast cell function (Arwill *et al.*, 1965; Gardner and Hudson, 1975); ameloblast degenerate to a reduced enamel epithelium shortly after differentiating and produce a thin layer of enamel extracellular matrix. These results suggest that the expression of laminin-5 during ameloblast differentiation to form a basal lamina is crucial to maintain the ameloblast phenotype (T. Wright, personal communication). The inter-relationship between the severity of the cutaneous skin fragility, the ameloblast cell dysfunction and the molecular defect in the laminin-5 genes is presently being studied.

Tricho-dento-osseous syndrome

Another genetic disease presenting enamel hypoplasia is Tricho-dento-osseous syndrome (TDO). This is an autosomal dominant hereditary disorder characterized by curly hair, enamel hypoplasia, taurodontia and thickened cortical bone (Quattromani *et al.*, 1983; Shapiro *et al.*, 1983). The molecular basis of TDO is not known, however histological observations of TDO teeth show an enamel 90% thinner than normal enamel, whereas the dentine appears normal (Melnick *et al.*, 1977; Wright *et al.*, 1994). Ultrastructural studies suggest that the ameloblast cells are functionally altered and perhaps have a shortened life cycle (Melnick *et al.*, 1977).

Summary

This review highlights a number of advances towards understanding the sequential developmental cascade of events beginning in the oral ectodermally-derived odontogenic placode and culminating in the formation of the mineralized enamel extracellular matrix. Recent discoveries of growth factors, growth factor receptors and transcription factors associated with instructive epithelial-mesenchymal interactions and subsequent controls for ameloblast cell differentiation are reviewed. The relationship between ameloblast cytology, terminal differentiation and biochemical phenotype are discussed. The tissue-specific gene products characteristic of the ameloblast phenotype as well as their possible functions in formation of the enamel matrix are analyzed as well as

the role of maturation-stage ameloblast cells in controlling enamel biomineralization. Finally, pathological conditions in which alterations in the ameloblast or specific gene products result in an abnormal enamel phenotype are reviewed. Clearly, the scientific progress achieved in the last few years concerning the molecular determinants involved in tooth development has been remarkable. However, there remains considerable lack of knowledge regarding the precise mechanisms that control ameloblast differentiation and enamel biomineralization. Anticipated progress continues to require increased international cooperation and collaborations as well as increased utilization of structural biology investigations of enamel extracellular matrix proteins.

KEY WORDS: *epithelial-mesenchymal interactions, amelogenesis, enamel, tooth development, transcriptional factors, growth factors and receptors, amelogenin, tuftelin, enamel proteases, nanospheres*

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