Identifying Promoter Elements Necessary for Enamelin Tissue-Specific Expression

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Key Words

Enam • *cis*-regulatory elements • Ameloblasts • Enamel proteins • Osteoblasts • Tissue-specific expression

Abstract

Enamel development requires the strictly regulated spatio-temporal expression of genes encoding enamel matrix proteins. The mechanisms orchestrating the initiation and termination of gene transcription at each specific stage of amelogenesis are unknown. In this study, we identify *cis*-regulatory regions necessary for normal enamel (*Enam*) expression. Sequence analysis of the *Enam* promoter 5'-non-coding region identified potentially important *cis*-regulatory elements located within 5.2 kb upstream of the *Enam* translation initiation site. DNA constructs containing 5.2 or 3.9 kb upstream of the *Enam* translation initiation site were linked to an LacZ reporter gene and used to generate transgenic mice. The 3.9-kb *Enam*-LacZ transgenic lines showed no expression in ameloblasts, but ectopic LacZ staining was detected in osteoblasts. In contrast, the 5.2-kb *Enam*-LacZ construct was sufficient to mimic the endogenous *Enam* ameloblast-specific expression pattern. Our study provides new insights into the molecular control of *Enam* cell- and stage-specific expression.

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Introduction

Dental enamel is produced through the secretions of highly specialized epithelial cells called ameloblasts [Smith, 1998], which create and maintain an extracellular milieu favorable for mineral deposition [Nanci, 2008]. Amelogenin, enamel and ameloblastin are the major secretory products of ameloblasts [Fincham et al., 1999]. Severe enamel phenotypes have been demonstrated in mice with defective amelogenin (*Amel*) [Gibson et al., 2001; Prakash et al., 2005], enamel (*Enam*) [Masuya et al., 2005; Hu et al., 2008] or ameloblastin (*Ambn*) [Fukumoto et al., 2004] genes. In humans, defects in *AMELX* cause X-linked amelogenesis imperfecta, while *ENAM* mutations cause autosomal dominant amelogenesis imperfecta [Hu and Yamakoshi, 2003; Stephanopoulos et al., 2005; Kim et al., 2006].

Abbreviations used in this paper

E18	embryonic day 18
TIC	translation initiation codon

Increasing evidence suggests that each enamel protein has specific properties that may correlate with unique functional roles. Amelogenin epitopes are relatively absent from the mineralization front where enamel crystals elongate [Orsini et al., 2001], while amelogenin 'nanospheres' [Fincham et al., 1994] appear to space enamel crystals and keep them in parallel arrays [Beniash et al., 2005; Margolis et al., 2006]. Ameloblastin (also called amelin or sheathlin) might maintain rod and interrod boundaries [Krebsbach et al., 1996; Hu et al., 1997b; Lyngstadaas, 2001] and contribute to ameloblast attachment to the enamel surface [Cerny et al., 1996; Fukumoto et al., 2004]. Intact (uncleaved) enamelin localizes to the mineralization front where it may be involved in crystal elongation, while its cleavage products accumulate in the deeper enamel and are thought to regulate crystal habit [Tanabe et al., 1990; Hu et al., 1997a]. This study focuses on enamelin.

Enamelin expression initiates in preameloblasts and continues throughout the secretory stage as demonstrated by in situ hybridization [Hu et al., 2001a], semiquantitative RT-PCR [Nagano et al., 2003] and X-gal histochemistry in the enamelin β -gal knockin mouse [Hu et al., 2008]. Enamelin expression is not detected in any other tissues during development. Execution of this spatially and temporally restricted pattern within ameloblasts is critical for the completion of amelogenesis, and perturbation of this expression results in abnormal enamel in mice and humans. However, it remains unknown how enamelin expression is initiated and maintained during ameloblast differentiation. The transcriptional regulation of amelogenin [Gibson et al., 1998; Gibson, 1999; Papagerakis et al., 1999; Zhou et al., 2000; Zhou and Snead, 2000; Papagerakis et al., 2003; Caton et al., 2005] has been investigated for a decade, but much less is known about the regulation of enamelin expression. Characterizing the enamelin *cis*-regulatory promoter region will give new insights into the molecular control of enamelin expression and will enhance our understanding of enamel formation under normal and pathological situations.

Methods

DNA Constructs and Detection of Enam-LacZ Activity in vitro

Two mouse *Enam* promoter fragments of different lengths were generated by PCR amplification using a previously isolated P1 clone as template [Hu et al., 2001b]. The following PCR primers used to generate the 3.9-kb fragment were forward 5'-GTC-GACGGATCCAAAACTTCTGCTCCAG-3' and reverse 5'-

TTCCCGGGCACCAAACTTTCATAAGCC-3'); the primers used to generate the 5.2-kb fragment were forward 5'-CAA-ACAGTCGACGTAAGTACTACTACCTTTGAGGGCGGTC-3' and reverse 5'-CAAACAGTCGACGTTTTACCAAACTTTCAT-AAGCC-3'. The amplification products were inserted into pLacZ [Dymecki, 1996] immediately 5' to the nuclear localization sequence- β -galactosidase code and were used for transgenic mouse production (fig. 1a).

The 2 *Enam-LacZ* constructs (3.9 and 5.2 kb) were transiently transfected into the ameloblast-like cell line LS8 (kindly provided by Dr. M. Snead, Center for Craniofacial Molecular Biology, USC, Los Angeles, Calif., USA) using a Lipofectamine 2000 transfection kit (Invitrogen, Carlsbad, Calif., USA) according to the manufacturer's protocol. Briefly, cells were plated in 6-well culture plates and transfection assays were performed when cells reached 70% confluence (1 μ g of either the 3.9- or 5.2-kb *Enam-LacZ* or the LacZ DNA constructs, mixed with 6 μ l of lipofectamine, was used for transfection within each separate well). Forty-eight hours after transfection, the media were removed, the cells were rinsed 3 times with PBS, fixed with 0.5% glutaraldehyde and assayed for β -galactosidase activity, as previously described [Yang et al., 2005]. Staining was evaluated and photographed using a dissecting microscope (SMZ1000; Nikon, Tokyo, Japan) and a digital camera (DXM1200; Nikon).

Generation of the 3.9- and 5.2-kb Enamelin-LacZ Hemizygotes Transgenic Mice

The 3.9- and 5.2-kb enamelin-LacZ DNA constructs were excised from the vector by restriction digestion, purified with a Qiaquick gel extraction kit (Qiagen, Germantown, Md., USA) and microinjected into fertilized CD1 eggs to generate transgenic mice. A total of 8 independent lines were generated at the Nathan Shock Aging Center at the University of Texas Health Science Center at San Antonio and at the Transgenic Animal Model Core at the University of Michigan. Founders were bred with C57BL/6 mice (Jackson Laboratory, Bar Harbor, Me., USA). Tail DNA was used for genotyping by PCR and/or Southern blot analysis. All experimental procedures involving mice were approved by the University of Texas Health Science Center at San Antonio and University of Michigan Committees on the Use and Care of Animals. The in vivo activity of the *Enam-LacZ* constructs was determined using a standard protocol to detect β -galactosidase activity by X-gal staining tissue sections from newborn to mice at postnatal day 21 [Lawson et al., 1999].

The genotype of the 3.9-kb *Enam-LacZ* transgenic mice was determined by PCR analysis of genomic DNA using the primer pair forward 5'-AAGTTTTGGGATTTGGCTCA-3' and reverse 5'-GTTGCACCACAGATGAAACG-3'. The 600-bp amplification product contained part of the enamelin promoter sequence, the coding sequence for the nuclear localization signal, and part of the bacterial β -galactosidase gene sequence.

Genotyping the 5.2-kb *Enam-LacZ* transgenic mice was accomplished by Southern blot hybridization using 100 μ g of mouse tail DNA, digested to completion with the restriction enzyme *Bgl*II (Stratagene, La Jolla, Calif., USA). The restricted genomic DNA was fractionated on 0.7% agarose gels and transferred to positively charged nylon membranes (No. 1-209-299; Roche, Indianapolis, Ind., USA). Randomly primed DNA probes were labeled with [α - 32 P]dCTP (Redivue deoxycytidine 5'-[α - 32 P] triphosphate, triethylammonium salt, AA0005, 10 μ Ci/ μ l, specific

Fig. 1. The 3.9- and 5.2-kb *Enam*-LacZ reporter constructs and their transgenic expression. **a** The smaller construct (3.9-kb *Enam*-LacZ) contained 3,931 bp of *Enam* 5' untranslated region extending from bases 1,555 to 5,485. The larger construct (5.2-kb *Enam*-LacZ) contained 5,217 bp of *Enam* from bases 269 to 5,485. Both *Enam* constructs contained the putative transcription initiation site at the start of exon 1 (at base 4,750) and the noncoding exons 1 and 2, as well as the beginning of exon 3 up to, but not including, the translation initiation site, which was supplied by the bacterial β -galactosidase cDNA (β -gal) fused to a nuclear localization sequence (NLS) and followed by a downstream polyadenylation sequence. **b** Transient transfection in an ameloblastic cell line (LS8). Both the 3.9- and 5.2-kb constructs were sufficient to drive the expression of β -galactosidase in the nucleus of the LS8 line. Cells transfected with a promoterless LacZ construct lacked β -galactosidase staining (left panel). Whole embryo (center) and paw (right panel) from postnatal day 1 transgenic and wild-type (WT) mice showed no β -galactosidase expression for the wild-type mice, tooth-specific expression for the 5.2-kb transgenic mice and ectopic bone expression for the 3.9-kb transgenic mice.

activity 3,000 Ci/mmol; Amersham, Piscataway, N.J., USA) using a Random Primed DNA Labeling Kit (No. 1-004-760; Roche) and purified by Quick Spin Columns (radiolabeled DNA purification kit, No. 1-273-922 or 1-273-949; Roche). Denatured probe was added to the buffer at 10^7 cpm and hybridized overnight. Low-stringency washes involved two 5-min washes at room temperature with $2\times$ SSC, 0.1% SDS followed by two 15-min high-stringency washes at 65°C in $5\times$ SSC and 0.1% SDS. The blots were exposed to X-ray film (BioMax MR, 894-1114; Kodak, Rochester, N.Y., USA) with intensifying screens at -70°C overnight.

Detection of *Enam*-LacZ Activity *in vivo*

Tissues from newborn mice as well as mice at postnatal days 1, 2, 3, 5, 6, 7, 9, 12, 14, 17 and 20 were collected immediately after sacrificing the animals. Whole bodies and/or heads were pro-

cessed for β -galactosidase staining as previously described [Lu et al., 2005], and β -galactosidase staining was compared to the endogenous enamel expression [Hu et al., 2000, 2001a].

For detection of the *Enam*-LacZ activity, tissue sections were postfixed for 5 min in 4% paraformaldehyde, washed in PBS ($3\times$ for 5 min each), and incubated in freshly prepared X-gal staining buffer (1 mg/ml X-gal, 100 mM HEPES, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 1 mM MgCl_2 , 2% Triton X-100 and 5 mM DTT, pH 8.0) at 45°C overnight. Tissue sections were then rinsed, counterstained with hematoxylin, and observed under a dissection microscope (SMZ1000; Nikon) or light microscope (Eclipse E600; Nikon) to determine the spatial and temporal activity of the enamel-LacZ reporter constructs. All images were captured using a digital camera (DXM1200; Nikon) and Act1 imaging software (Mager Scientific, Dexter, Mich., USA).

Results

Enamelin-LacZ Activity in vitro

Positive β -galactosidase activity was detected in both 3.9- and 5.2-kb *Enam*-LacZ transfected LS8 ameloblast cells 48 h following transfection, demonstrating that both fragments contain the necessary basal promoter elements (fig. 1b). Staining was restricted to the nuclei, indicating specific expression of the nuclear-localized reporter molecule. LS8 cells transfected with the promoterless LacZ vector (control) showed no β -galactosidase activity (fig. 1b).

The 3.9- and 5.2-kb Enamelin-LacZ Mice

For the 3.9-kb *Enam*-LacZ transgene, 3 of 5 founders were productive. For the 5.2-kb *Enam*-LacZ transgene, 5 of 9 founders were productive. Offspring of the productive founders were evaluated for β -galactosidase expression using embryonic day 18 (E18) whole mounts (fig. 1b) and tissue sections from newborn to postnatal day 20 mice (fig. 2).

The 3.9-kb Enam-LacZ Activity in vivo

Whole mounts of E18 wild-type mice were negative (fig. 1b), while the 3.9-kb transgenic mice at the same stages of development showed ectopic β -galactosidase expression (fig. 1b). Staining in the craniofacial region was localized to the mandible, nasal region and papilla of the hair bulbs (fig. 1b). Preosteoblasts in the alveolar bone surrounding the developing incisors and molars of 1- and 5-day-old mice were positive for β -galactosidase staining, but no positive staining was observed within the incisors or molars themselves (fig. 2a, b). In addition, histological sections at day 1 showed β -galactosidase staining within the long bones of the extremities, primarily in cells encapsulating developing bones and joints (fig. 2c). These results contrast with the ameloblast-specific endogenous *Enam* mRNA localization, as defined by previous studies [Hu et al., 2001a]. These findings suggest that the 3.9-kb *Enam* sequence was sufficient to promote reporter expression, but insufficient to direct an ameloblast-specific expression. Characterization of the 3 independent 3.9-kb *Enam*-LacZ transgenic lines showed similar expression patterns that differed only in the intensity of the β -galactosidase staining (data not shown).

The 5.2-kb Enam-LacZ Activity in vivo

The nondental localization of reporter activity observed in the 3.9-kb *Enam*-LacZ transgenics contrasted strongly with the reporter activity in the 5.2-kb *Enam*-

LacZ transgenics, which correlated well with endogenous *Enam* expression. Whole mounts of E18 5.2-kb mice were negative except for tooth and did not show staining in bone cells (fig. 1b). In postnatal tissue sections, β -galactosidase activity was initially detected in the secretory ameloblasts of day 1 incisors (fig. 2d, g), while the molars lacked reporter expression at this time. The 5.2-kb *Enam*-LacZ reporter activity was not detected in molar secretory ameloblasts until day 5 (fig. 2e, h). The 5.2-kb *Enam*-LacZ transgene expression in molars was strong in secretory and maturation stage ameloblast until postnatal day 14 (fig. 2i). β -Galactosidase staining was clearly detected within the nuclei of molar ameloblasts (fig. 2g, h), and persisted in a few ameloblasts until the molars erupted in 17-day-old mice (data not shown). No expression was detected in 20-day-old molars (data not shown). In addition, no staining was detected in any other tissues, like bone, liver and lung (fig. 2f). Compared with the *Enam* endogenous pattern [Hu et al., 2001a], the 5.2-kb construct directed tissue- and stage-specific expression of the reporter molecule that recapitulated *Enam* expression in vivo. The reporter activity varied depending upon which transgenic mouse line was characterized, but in the majority of mouse lines the expression of the reporter molecule clearly overlapped that of the endogenous enamel mRNA expression.

Discussion

Dental mineralized tissues comprise enamel, dentin and cementum. Dentin and cementum share a similar repertoire of extracellular matrix proteins as bone. In contrast, enamel proteins are not normally found in bone cells, suggesting that regulatory mechanisms might exist that prevent their expression outside of enamel. To determine the necessary *cis*-regulatory promoter elements necessary for the spatiotemporal expression of enamel in vivo, 3 transgenic lines were generated harboring 3.9 kb prior to the translation initiation codon (TIC) of the *Enam* linked to the reporter LacZ gene (3.9 kb). In addition, 5 independent transgenic lines were generated harboring 5.2 kb prior to the TIC (5.2 kb). The design of these 2 *Enam*-LacZ constructs (3.9 and 5.2 kb) was based upon sequence analysis of the human and mouse enamel genes that suggested that the major regulatory regions governing enamel expression are located within one or both of these *Enam*-regulatory regions. Our study reveals a *cis*-regulatory region that confers tooth-specific expression to the mouse *Enam* gene.

Fig. 2. Comparison of β -galactosidase activity expressed by the 3.9- and 5.2-kb *Enam*-LacZ transgenes. **a, b** β -Galactosidase expression was not detected in ameloblasts (Am) or dental pulp (DP) cells of day 1 incisors (**a**) or day 1 and 5 molars (**b**). In contrast, β -galactosidase activity was detected in alveolar bone (AB) surrounding the incisors and molars. **c** At postnatal day 1, staining of long bones of the extremities is also distinct; mostly from the cells that encapsulate the developing long bones and joints. **d-i** β -Galactosidase activity was detected in ameloblasts (Am) of postnatal day 1 incisor (**d, g**). In molars, β -galactosidase reporter

gene activity was not found in day 1 molars and it was first detected in the ameloblasts at postnatal day 5 (**e, h**). Positive staining of β -galactosidase activity specifically localized to the nuclei of the ameloblasts (**g, h**). The intensity of β -galactosidase activity peaked at postnatal day 9 and continued in maturation stage ameloblasts, being located within the nuclei until postnatal day 17 (**i**). Dental pulp (DP) and alveolar bone (AB) cells were devoid of staining. No β -galactosidase activity was detected in long bones of postnatal day 12 mice (**f**).

More specifically, in 3.9-kb mice, *Enam*-LacZ expression was dramatically downregulated in teeth, contrasting with the strong endogenous enamel mRNA expression detected by in situ hybridization. Instead, enamel- β -galactosidase activity was detected within preosteoblasts and chondrocytes, which were negative for endogenous *Enam* expression. Overall, the pattern exhibited by β -galactosidase activity was very different from endogenous *enamelin* expression, suggesting that the 3.9-kb fragment of the mouse *Enam* promoter lacks

important regulatory elements essential for the ameloblast-specific expression of this gene.

In contrast to the results obtained with the 3.9-kb fragment, LacZ reporter mice harboring 5.2 kb of the *Enam* gene prior to the TIC showed β -galactosidase expression exclusively in ameloblasts in all 5 transgenic lines generated. However, within 2 of the 5 lines examined, the intensity of expression was relatively low when compared with endogenous *Enam* expression. Chromosomal position effects of the transgene integration site,

and/or differences in the copy number of the transgene and/or the pattern of methylation of the CpG-rich regulatory sequences may cause variable expression levels between transgenic lines due to differences in chromatin structure modulation, as observed for other transgenes [Bestor and Tycko, 1996; Dobie et al., 1997]. Although we have not investigated in detail the exact reason for the observed differential intensity between the 5 independent transgenic lines, we are confident to conclude that the 5.2-kb reporter gene expression overlapped that of endogenous *enamelin* expression and well mimicked its tissue-specific pattern. Preservation of the essential aspects of enamel tissue-specific expression from early to late amelogenesis indicates that critical elements for enamel ameloblast expression are contained within the distal *cis*-regulatory region located between 5.2 and 3.9 kb upstream of the translation start site. Furthermore, this distal regulatory region may also contain negative regulatory elements that suppress enamel expression in bone cells. Preliminary analysis of the potential binding *trans*-regulatory factors within this distal regulatory region (between 5.2 and 3.9 kb upstream of the translation start site) indicated that Runx2 and Dlx/Msx transcription factors might be good candidates for regulating enamel expression during ameloblast differentiation. Runx2 and Msx/Dlx proteins are expressed by the ameloblasts concomitant with enamel expression, and knock-out mice and human genetics suggest that both Runx2 and Msx/Dlx proteins may play a role in controlling enamel formation [Price et al., 1998; D'Souza et al., 1999; Bei et al., 2004; Aioub et al., 2007].

While the molecular mechanisms governing tooth morphogenesis are being elucidated in detail [Thesleff and Sharpe, 1997], the genetic pathways controlling the specification of ameloblasts and enamel formation remain unclear. The terminal differentiation of ameloblasts starts during the late bell stage of tooth morphogenesis and is supposed to be regulated by reciprocal interactions between the epithelium and mesenchyme. Very little is known concerning the transcriptional mechanisms that regulate hard tissue formation in tooth. Our study provides new insights on the developmental- and tissue-specific expression of enamel within the enamel-forming ameloblasts.

In conclusion, the 5.2-kb sequence upstream of the translation initiation site may contain the regulatory binding sites necessary for the control of the temporal and spatial expression of mouse *Enam* gene. To determine what specific sequences and corresponding transcription factors are critical for the tissue-specific expression of enamel, *in vitro* promoter analysis and additional transgenic models are necessary. Nevertheless, our studies establish a framework for understanding the transcriptional regulation of enamel matrix proteins encoding genes within the ameloblasts.

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