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Effects of porcine 25 kDa amelogenin and its proteolytic derivatives on bone sialoprotein expression

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Background and Objective: Amelogenins are hydrophobic proteins that are the major component of developing enamel. Enamel matrix derivative has been used for periodontal regeneration. Bone sialoprotein is an early phenotypic marker of osteoblast differentiation. In this study, we examined the ability of porcine amelogenins to regulate bone sialoprotein transcription.

Material and Methods: To determine the molecular basis of the transcriptional regulation of the bone sialoprotein gene by amelogenins, we conducted northern hybridization, transient transfection analyses and gel mobility shift assays using the osteoblast-like ROS 17/2.8 cells.

Results: Amelogenins (100 ng/mL) up-regulated bone sialoprotein mRNA at 3 h, with maximal mRNA expression occurring at 12 h (25 and 20 kDa) and 6 h (13 and 6 kDa). Amelogenins (100 ng/mL, 12 h) increased luciferase activities in pLUC3 (nucleotides −116 to +60), and 6 kDa amelogenin up-regulated pLUC4 (nucleotides −425 to +60) activity. The tyrosine kinase inhibitor inhibited amelogenin-induced luciferase activities, whereas the protein kinase A inhibitor abolished 25 kDa amelogenin-induced bone sialoprotein transcription. The effects of amelogenins were abrogated by 2-bp mutations in the fibroblast growth factor 2 response element (FRE). Gel-shift assays with radiolabeled FRE, homeodomain-protein binding site (HOX) and transforming growth factor-beta1 activation element (TAE) double-strand oligonucleotides revealed increased binding of nuclear proteins from amelogenin-stimulated ROS 17/2.8 cells at 3 h (25 and 13 kDa) and 6 h (20 and 6 kDa).

Conclusion: These results demonstrate that porcine 25 kDa amelogenin and its proteolytic derivatives stimulate bone sialoprotein transcription by targeting FRE, HOX and TAE in the bone sialoprotein gene promoter, and that full-length amelogenin and amelogenin cleavage products are able to regulate bone sialoprotein transcription via different signaling pathways.

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During tooth morphogenesis, epithelial cells differentiate into secretory ameloblasts, which synthesize and secrete enamel matrix proteins (1–3). Immature enamel matrix consists of about 30% proteins by weight, almost all of which is removed from the tissue with increasing mineralization (4–7). Amelogenins are the major constituents

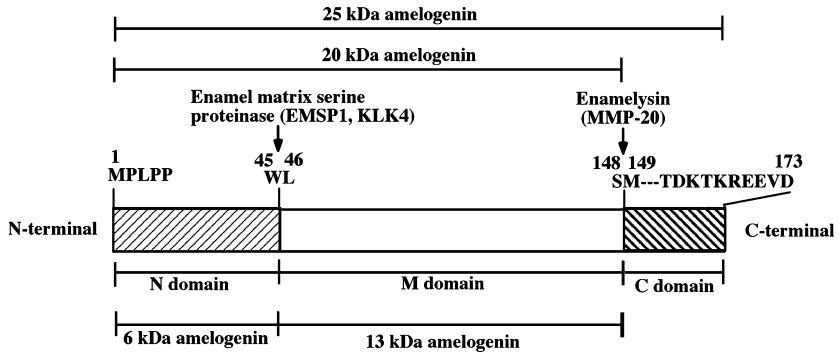

Fig. 1. Schematic structure of amelogenin. The original amelogenin in porcine developing enamel is 25 kDa amelogenin (1–173 amino acids). Enamelysin (MMP-20) cleaves (between amino acids 148 and 149) the carboxyl terminal peptide of 25 kDa amelogenin to convert it to 20 kDa (1–148 amino acids). This 20 kDa amelogenin is the most abundant amelogenin in the immature enamel of the secretory stage and is further degraded by enamel matrix serine proteinase 1 (EMSP1, KLK4) into 13 kDa (46–148; M domain) and 6 kDa (1–45; N domain) amelogenins.

existing in the developing enamel matrix, and an amelogenin with an apparent molecular weight of 20 kDa, derived from 25 kDa amelogenin by the action of enamelysin (MMP-20), is the major amelogenin in porcine secretory enamel (8–12). It has been shown that the complex mixture of amelogenins is generated by both the degradation of amelogenins and also by alternative RNA splicing. Osteoblasts, odontoblasts and bone marrow stromal cells also express the amelogenin gene (13–15), suggesting that osteoblasts and odontoblasts come into contact with full-length amelogenin and amelogenin cleavage products. Amelogenin is a cell adhesion protein (5), a potential regulator of cementum-associated genes, such as bone sialoprotein and type I collagen, and specific amelogenin gene splice products may be tissue-specific epithelial mesenchymal signaling molecules (6). Amelogenin gene null mutation mice display an amelogenesis imperfecta phenotype and show root resorption and reduced expression of bone sialoprotein (16–18). Enamel matrix derivative (EMD), the acid extract of porcine cheese-like enamel matrix, has been developed as a clinical treatment to promote periodontal regeneration (19–22). Approximately 90% of the protein in the EMD is amelogenins, and the remaining 10% consists of nonamelogenin enamel matrix proteins and growth factors (3,20,22).

Bone sialoprotein is a mineralized tissue-specific protein that is glycosylated, phosphorylated and sulfated (23–25). Bone sialoprotein nucleates hydroxyapatite crystal formation (24–26) and is expressed in several cancers where it is associated with the formation of ectopic hydroxyapatite microcrystals (27,28). To study the transcriptional regulation of bone sialoprotein, rat, human and mouse bone sialoprotein gene promoters were cloned, sequenced and characterized (29–32). These promoters have an inverted TATA box (33) overlapping a vitamin D response element (34), and an inverted CCAAT box (nucleotides −50 to −46) which is bound with the NF-Y transcription factor (35,36). A cyclic AMP (cAMP) response element (CRE) (37–39), a fibroblast growth factor 2-response element (FRE) (37,40), a pituitary-specific transcription factor-1 (Pit-1) motif that mediates the effects of parathyroid hormone (41) and a homeodomain protein-binding site (HOX) have been characterized (20,42,43). Further upstream in the rat bone sialoprotein gene promoter, a transforming growth factor-beta1 (TGF-β1) activation element (TAE) (20,43,44) and a glucocorticoid response element overlapping an activator protein 1 site have also been identified (45,46).

EMD is used clinically; however, little is known about the effects of amelogenins on periodontal regeneration. Therefore, we wished to determine the effect of amelogenins on bone sialoprotein transcription. The purpose of the present study was to evaluate the ability of porcine 25 kDa full-length amelogenin and its proteolytic derivatives to stimulate osteoblast-specific gene expression, such as bone sialoprotein. In this study we showed that amelogenins regulate bone sialoprotein transcription in osteoblast-like ROS 17/2.8 cells through FRE, HOX and TAE in the rat bone sialoprotein gene promoter.

Material and methods

Materials

Alpha minimal essential medium (α-MEM), fetal calf serum, Lipofectamine, penicillin and streptomycin, and TrypLE™ Express were obtained from Invitrogen (Carlsbad, CA, USA). The pSV-β-galactosidase control vector (β-Gal) and the pGL3-basic luciferase reporter vector were purchased from Promega (Madison, WI, USA). The protein kinase A (PKA) inhibitor (H89) and the protein kinase C (PKC) inhibitor (H7) were from Seikagaku Corporation (Tokyo, Japan). The tyrosine kinase inhibitor herbimycin A (HA) and guanidium thiocyanate were purchased from Wako Pure Chemical (Tokyo, Japan).

Cell culture

The rat osteoblast-like ROS 17/2.8 cells (47) were maintained in α-MEM containing 10% fetal calf serum. Cells were grown to confluence in 60-mm tissue culture dishes, then cultured in α-MEM without serum and incubated with or without amelogenin fractions for time-periods extending over 3–12 h. RNA was isolated from triplicate cultures at various time-points and analyzed for the expression of bone sialoprotein mRNA by northern hybridization, as described below.

Purification of amelogenins

Tooth germs of permanent incisors were dissected from fresh mandibles of

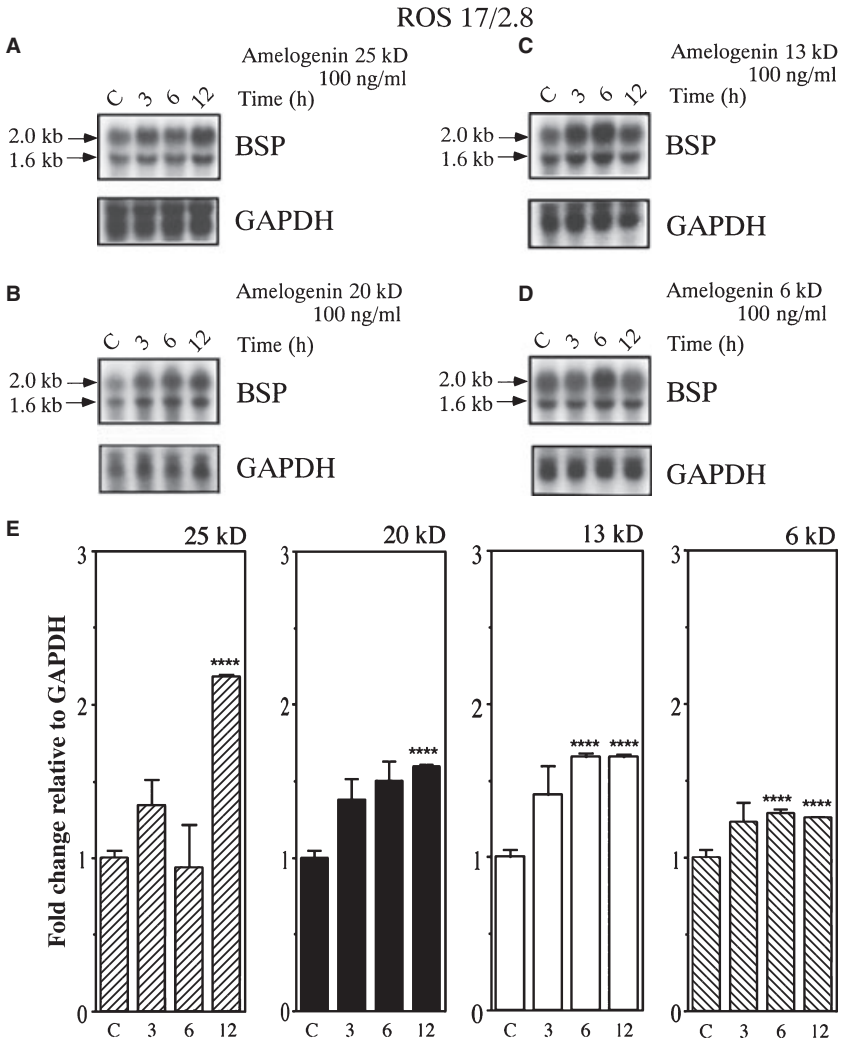

Fig. 2. Northern hybridization analysis of the effects of purified porcine amelogenins (25, 20, 13 and 6 kDa) on bone sialoprotein mRNA expression. (A) 25 kDa amelogenin. (B) 20 kDa amelogenin. (C) 13 kDa amelogenin. (D) 6 kDa amelogenin. A 12 h time-course experiment revealed an increase in bone sialoprotein mRNA after the administration of 100 ng/mL of amelogenin to ROS 17/2.8 cells. Total RNA was isolated from triplicate cultures harvested after incubation times of 3, 6 and 12 h, and used for northern hybridization analysis using a ^{32}P-labelled rat bone sialoprotein DNA probe and a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) DNA probe. One of three replicate analyses is shown. (E) Quantitative analyses of the triplicate data sets are shown with standard errors. Significant differences from control: ****$p < 0.01$.

pigs that were approximately 6 mo old. After the removal of the surrounding soft tissues and pulp, the tooth germs were washed in cold saline. A thin outer layer of enamel was scraped from the surface of the secretory-stage enamel using a razor blade. A pooled enamel sample was suspended in 10 volumes of 50 mM sodium carbonate-sodium bicarbonate buffer (pH 10.8) containing proteinase and phosphatase inhibitors (50 mM ϵ-aminocaproic acid, 5 mM benzamidine, 1 mM *p*-hydroxymercuribenzoic acid, 1 mM phenylmethylsulfonyl fluoride and 1 mM levamizole), and was homogenized using a Polytron homogenizer for 30 s and centrifuged for 15 min at 10,000 *g*. This extraction procedure was repeated three times. The supernatant was concentrated by ultrafiltration (YM-1 membrane; Millipore, Billerica, MA, USA) and applied to a column of Sephadex G-100 (4 × 100 cm) equilibrated with 50 mM sodium carbonate-sodium bicarbonate buffer (pH 10.8) containing the same inhibitors at one-tenth of the above concentrations. Enamel proteins extracted from the thin outer layer of the porcine secretory enamel were separated into four fractions. Amelogenins of 25 kDa and 20 kDa, the major amelogenins in the enamel of the very early secretory stage, were eluted in the second peak. Amelogenins of 13 kDa and 6 kDa were eluted in the third and fourth peaks (Fig. 1). These amelogenin samples were further fractionated by reverse-phase HPLC using a Pharmacia DfB HPLC system equipped with a TSK-gel ODS-120T column. The

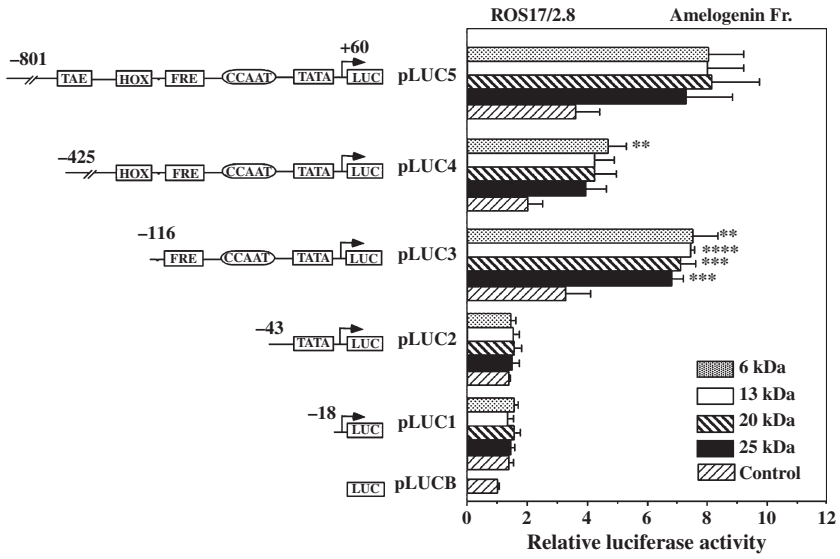

Fig. 3. Amelogenins up-regulate bone sialoprotein promoter activities. Transient transfections of ROS 17/2.8 cells, in the presence or absence of purified porcine amelogenins (25, 20, 13 and 6 kDa) (100 ng/mL) for 12 h, were used to determine transcriptional activities of chimeric constructs that included various regions of the bone sialoprotein promoter ligated to a luciferase reporter gene. The results of transcriptional activities obtained from three separate transfections with constructs are shown; pLUC basic (pLUCB) and pLUC1 to pLUC5 have been combined and the values are expressed with standard errors. Significant differences from control (relative luciferase activity of pLUCB): $^{**}p < 0.05$; $^{***}p < 0.02$; $^{****}p < 0.01$.

column was equilibrated with 0.05% trifluoroacetic acid and was eluted with a linear acetonitrile gradient (40–60%) containing 0.05% trifluoroacetic acid at a flow rate of 1.0 mL/min (11).

Northern hybridization

Aliquots (20 μg) of total RNA were fractionated on a 1.2% agarose gel and transferred onto a Hybond-N+ membrane. Hybridizations were performed at 42°C with either a ^{32}P-labeled rat bone sialoprotein or a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe. Following hybridization, the membranes were washed four times, for 5 min each, at 21°C in 300 mM sodium chloride, 30 mM trisodium citrate, pH 7.0, containing 0.1% sodium dodecyl sulfate. This was followed by two, 20-min washes at 55°C in 15 mM sodium chloride, 1.5 mM trisodium citrate, pH 7.0, 0.1% sodium dodecyl sulfate. The hybridized bands were scanned in a Bio-imaging analyzer and normalized to the expression of GAPDH (44).

Transient transfection assays

Exponentially growing cells were used for transfection assays. Twenty-four hours after plating, cells at 50–70% confluence were transfected using a Lipofectamine reagent. The transfection mixture included 1 μg of a luciferase (LUC) construct (45) and 2 μg of β-Gal vector as an internal control to normalize for individual transfection efficiencies. Two days post-transfection, cells were deprived of serum for 12 h, purified 25, 20, 13 and 6 kDa amelogenins (100 ng/mL) were added and the cells were cultured for a further 12 h before harvesting. The luciferase assay (Picagene, Toyoinki, Tokyo, Japan) was performed according to the supplier's protocol using a Luminescence reader (Acuu FLEX Lumi 400; Aloka, Tokyo, Japan) to measure the luciferase activities. The protein kinase inhibitors H89 (5 μM) and H7 (5 μM) were used to inhibit PKA and PKC, respectively. Herbimycin A (1 μM) was used to inhibit tyrosine kinase (35,36). Oligonucleotide-directed mutagenesis by PCR was utilized to introduce dinucleotide substitutions using the Quick Change Site-directed Mutagenesis Kit. All constructs were sequenced, as described previously, to verify the fidelity of the mutagenesis (40).

Gel mobility shift assays

Confluent ROS 17/2.8 cells in T-75 flasks incubated for 3, 6 and 12 h with purified 25, 20, 13 and 6 kDa amelogenins (100 ng/mL) in α-MEM without serum were used to prepare nuclear extracts, as described previously (41). Double-stranded oligonucleotides encompassing the inverted CCAAT (nucleotides −61 to −37, 5′-CCGTGAC CGTG**ATTGG**CTGCTGAGA), FRE (nucleotides −98 to −79, 5′-TTTTCT**G GTGAGAA**CCCACA), the HOX (nucleotides −204 to −179, 5′-TCCTCAGC CT**TCAATTAAA**TCCCACA) and the TAE (nucleotides −506 to −482, 5′-CAA AGCC**TTGGC**AGCCC**GGCTG**GCT) in the rat bone sialoprotein promoter were prepared commercially. For gel-shift analysis the double-stranded-oligonucleotides were end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. Nuclear protein extracts (3 μg) were incubated for 20 min at room temperature (= 21°C) with 0.1 pM radiolabeled double-stranded oligonucleotide in buffer containing 50 mM KCl, 0.5 mM EDTA, 10 mM Tris–HCl (pH 7.9), 1 mM dithiothreitol, 0.04% Nonidet P-40, 5% glycerol and 1 μg of poly (dI–dC). Following incubation, the protein–DNA complexes were resolved by electrophoresis on 5% non-denaturing acrylamide gels (38:2, acrylamide/bisacrylamide), run at 150 V at room temperature. Following electrophoresis, the gels were dried and autoradiograms were prepared and analyzed using a Bio-imaging analyzer.

Statistical analysis

Triplicate or quadruplicate samples were analyzed for each experiment, and experiments were replicated to ensure consistency of the responses to amelogenin fractions. Significant differences between the control and amelogenin treatments were determined using the unpaired Student's *t*-test.

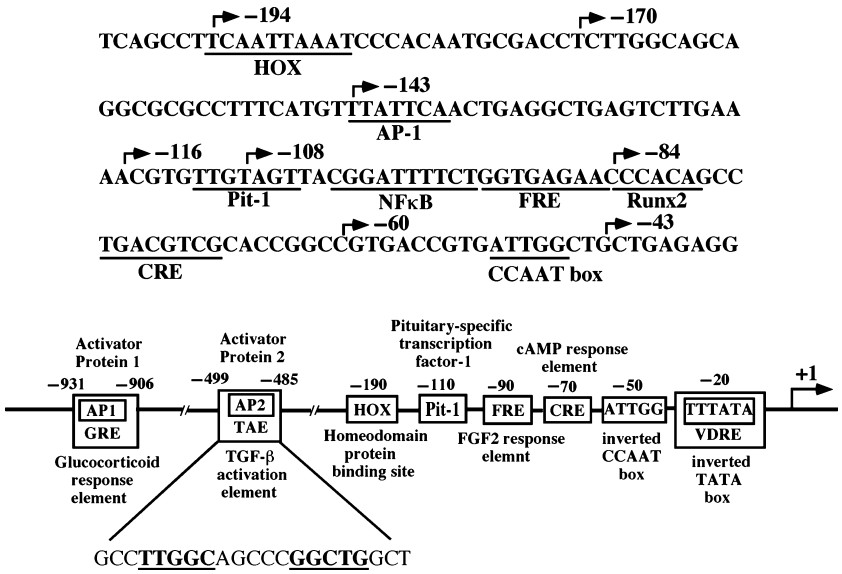

Fig. 4. Regulatory elements in the proximal rat bone sialoprotein promoter. Upper panel: the nucleotide sequence of the rat bone sialoprotein gene proximal promoter is shown from nucleotides −201 to −35. The inverted CCAAT box, cyclic AMP response element (CRE), Runx2, fibroblast growth factor 2-response element (FRE), nuclear factor-kappaB (NF-κB), pituitary-specific transcription factor-1 (Pit-1), activator protein 1 (AP-1) and homeodomain protein-binding site (HOX) are present. Lower panel: the positions of the inverted TATA and CCAAT boxes, a CRE, an FRE, a Pit-1, a HOX and a vitamin D response element (VDRE) that overlaps the inverted TATA box are shown in the proximal promoter region of the rat bone sialoprotein gene, and a transforming growth factor-beta activation element (TAE) overlapping an AP2 element and a glucocorticoid response element (GRE) overlapping the AP1 in the distal promoter. The numbering of nucleotides is relative to the transcription start site (+1).

Results

Stimulation of bone sialoprotein mRNA expression in ROS 17/2.8 cells

To study the regulation of bone sialoprotein expression by 25, 20, 13 and 6 kDa amelogenins (100 ng/mL), we performed northern hybridization analysis of total RNA extracted from osteoblastic ROS 17/2.8 cells. The 25, 20, 13 and 6 kDa amelogenins increased bone sialoprotein mRNA accumulations at 3 h, with maximal levels of bone sialoprotein mRNA obtained at 12 h (25 and 20 kDa) (Fig. 2A,B,E) and at 6 h (13 and 6 kDa) (Fig. 2C,D and E).

Transient transfection analysis of rat bone sialoprotein promoter constructs

To determine the sites of amelogenin-regulated transcription in the 5′-flanking region of the rat bone sialoprotein gene, various sizes of promoter constructs ligated to a luciferase reporter gene were transiently transfected into ROS 17/2.8 cells, and their transcriptional activities were determined in the presence or absence of 25, 20, 13 and 6 kDa amelogenins (100 ng/mL). The transcriptional activities of pLUC3, which encompasses nucleotides −116 to +60, were increased by 25, 20, 13 and 6 kDa amelogenins at 12 h. Only the 6 kDa amelogenin up-regulated the pLUC4 (−425 to +60) luciferase activity (Fig. 3). In shorter constructs (pLUC1, −18 to +60; pLUC2, −43 to +60), luciferase activities were not increased by amelogenins. Included within the DNA sequence that is unique to the pLUC3 construct (nucleotides −116 to −43) is an inverted CCAAT box (ATTGG; between nucleotides −50 and −46), a CRE (between nucleotides −75 and −68), a FRE (between nucleotides −92 and −85) and a Pit-1 motif (between nucleotides −111 and −105) (Fig. 4). HOX (TCAATTAAAT, nucleotides −194 to −185) and TAE (**TTGGC**AG-CCC**GGCTG**, nucleotides −499 to −485) are present in pLUC4 and pLUC5, which were identified as EMD response elements a the previous study (Fig. 4) (20). To determine more precisely the target sites in the bone sialoprotein promoter through which the amelogenin effects were being mediated, we prepared a series of 5′ deletion constructs between nucleotides −116 and −43. The luciferase activities of the promoter construct –116BSPLUC were increased by 25, 20, 13 and 6 kDa amelogenins (100 ng/mL, 12 h), whereas the luciferase activities of –108BSPLUC were up-regulated by amelogenin cleavage products (20, 13 and 6 kDa amelogenins; 100 ng/mL, 12 h) (Fig. 5). As protein kinases mediate amelogenin signaling, we also investigated the effects of the PKC inhibitor H7, the PKA inhibitor H89 and the tyrosine kinase inhibitor HA on amelogenin-mediated transcription. Although the induction of pLUC3 promoter activities by amelogenins (25, 20, 13 and 6 kDa) was inhibited by HA, no such effects were observed for the PKC inhibitor, indicating involvement of tyrosine kinase in the signaling pathway (Fig. 6). The PKA inhibitor only abolished the bone sialoprotein promoter activities induced by 25 kDa full-length amelogenin, suggesting that full-length amelogenin and amelogenin cleavage products regulate bone sialoprotein transcription via different signaling pathways (Fig. 6). Next we introduced mutations in the possible response elements encoded within nucleotides −116 to −43 of pLUC3, as shown in Fig. 7. Whereas mutations in the Pit-1 (M-PIT) had no effect on the stimulation of amelogenins, mutations in the FRE (M-FRE) significantly reduced the effects of amelogenins (25, 20, 13 and 6 kDa) on the transcriptional activities (Fig. 7). Mutations in the CRE (M-CRE) inhibited transcription of bone sialoprotein induced by 25 kDa full-length amelogenin, indicating involvement of cAMP in the signaling pathway induced by 25 kDa amelogenin. These results suggest that the FRE is required as a functional

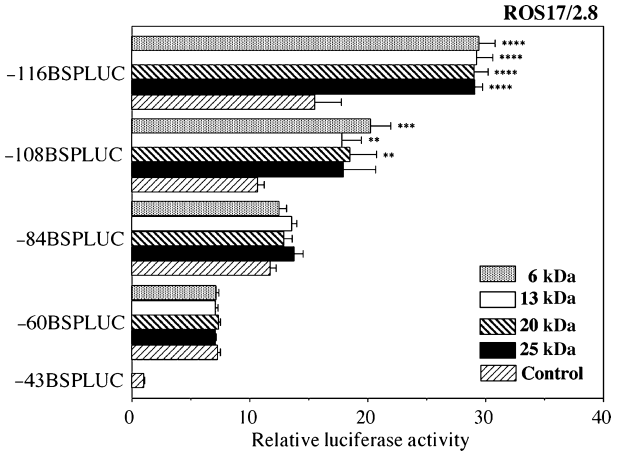

Fig. 5. Fine 5′-deletion mapping of nucleotides −116 to −43 in the bone sialoprotein promoter. A series of rat bone sialoprotein promoter 5′ deletion constructs were analyzed for relative promoter activity after transfection into ROS17/2.8 cells and examined for induction by purified porcine amelogenins (25, 20, 13 and 6 kDa) (100 ng/mL, 12 h). The results of transcriptional activity obtained from three separate transfections with the constructs −43 BSPLUC (−43 to +60), −60 BSPLC (−60 to +60), −84 BSPLUC (−84 to +60), −108 BSPLUC (−108 to +60) and −116 BSPLUC (−116 to +60) have been combined and the values expressed with standard errors. Significant differences from the control: $^{**}p < 0.05$; $^{***}p < 0.02$; $^{****}p < 0.01$.

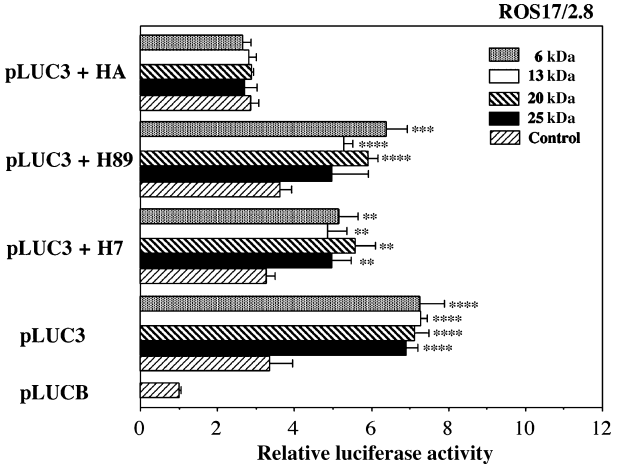

Fig. 6. Effect of kinase inhibitors on transcriptional activation by purified porcine amelogenins (25, 20, 13 and 6 kDa). Transient transfection analysis of pLUC3 treated with amelogenins (100 ng/mL) for 12 h in ROS 17/2.8 cells is shown together with the effects of the protein kinase C (PKC) inhibitor (H7; 5 μM), the protein kinase A (PKA) inhibitor (H89; 5 μM) and the tyrosine kinase inhibitor (herbimycin A; HA; 1 μM). The results obtained from three separate transfections were combined and the values are expressed with standard errors. Significant differences compared with controls are shown at the following probability levels: $^{**}p < 0.05$; $^{***}p < 0.02$; $^{****}p < 0.01$.

cis-element for the up-regulation of bone sialoprotein transcription by amelogenins and that CRE could be involved in full-length 25 kDa amelogenin-regulated bone sialoprotein gene expression.

Gel mobility shift assays

To identify nuclear proteins that bind to the FRE, HOX and TAE, double-stranded oligonucleotides were end-labeled and incubated with equal amounts (3 μg) of nuclear proteins extracted from confluent ROS 17/2.8 cells that were either not treated (Control) or treated with 100 ng/mL of 25, 20, 13 and 6 kDa amelogenins for 3, 6 and 12 h. When we used the inverted CCAAT sequence as a probe, the DNA–NF-Y protein complex did not change after treatment with 25, 20, 13 and 6 kDa amelogenins (Fig. 8–11, lanes 1–4). With nuclear extracts from confluent control cultures of ROS 17/2.8 cells, shifts of FRE, HOX and TAE DNA–protein complexes were evident (Fig. 8–11, lanes 5, 9 and 13). The 25 kDa amelogenin increased FRE–, HOX– and TAE–protein complexes at 3 h and returned to control levels at 6 h (Fig. 8, lanes 5–16). The 20 kDa amelogenin increased FRE–, HOX– and TAE–protein complexes at 6 h and returned to control levels at 12 h. (Fig. 9, lanes 5–16). The 13 kDa and 6 kDa amelogenins increased FRE–, HOX– and TAE–protein complexes at 3 h, reached maximal levels at 6 h and returned to the control level at 12 h (Fig. 10 and 11; lanes 5–16).

Discussion

These studies have shown that porcine 25 kDa full-length amelogenin and its proteolytic derivatives (of 20, 13 and 6 kDa) increased bone sialoprotein transcription in osteoblastic ROS 17/2.8 cells. Transduction of the signaling of amelogenins (25, 20, 13 and 6 kDa) was mediated through tyrosine kinase and 25 kDa amelogenin-induced bone sialoprotein transcription via the PKA pathway, which targets nuclear proteins that bind to FRE, HOX and TAE in the rat bone sialoprotein gene promoter.

The expression of amelogenins in osteoblasts, osteoclasts, bone marrow cells and odontoblasts, together with the accumulating data indicating amelogenin induction of osteogenesis (6,13–15), suggest that amelogenins can regulate bone metabolism. Amelogenin null mice are smaller than wild-type mice before weaning. The smaller size of amelogenin null mice could potentially be a result of the lack of leucine-rich amelogenin peptide (LRAP) expression, leading to a delay

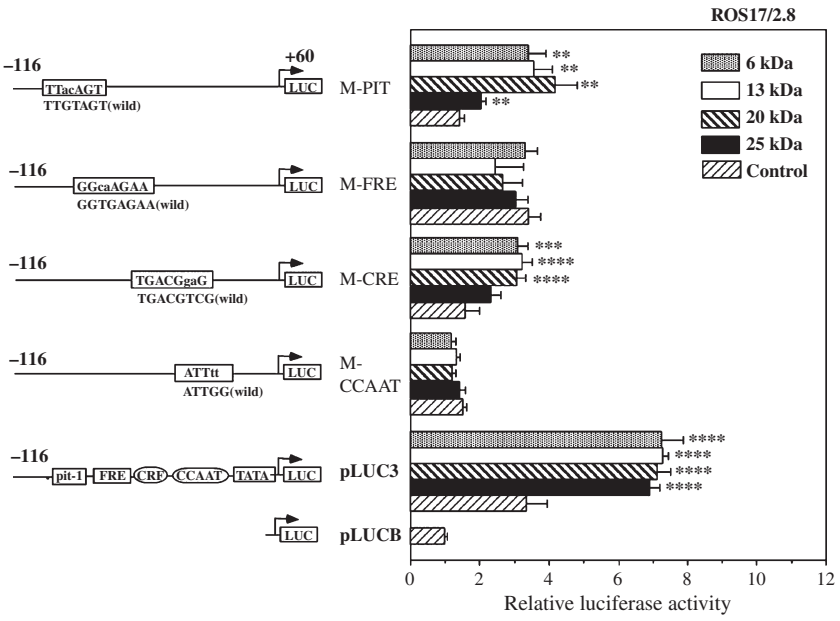

Fig. 7. Site mutation analysis of luciferase activities. Dinucleotide substitutions were made within the context of the homologous −116 to +60 (pLUC3) bone sialoprotein promoter fragments. Mutations in the CCAAT region (M-CCAAT) (ATT**tt**), mutations in the cyclic AMP response element (M-CRE) (**c**GACG**c**CG), mutations in the fibroblast growth factor 2-response element (M-FRE) (GG**ca**AGAA) and mutations in the pituitary-specific transcription factor-1 (M-PIT) (TT**ac**AGT) were analyzed for relative promoter activity after transfection into ROS 17/2.8 cells and examined for induction after treatment with amelogenins (100 ng/mL) for 12 h. The results of transcriptional activities obtained from three separate transfections with constructs were combined and the values are expressed with standard errors. Significant differences compared with controls are shown at the following probability levels: $^{**}p < 0.05$; $^{***}p < 0.02$; $^{****}p < 0.01$. The bold lowercase letters indicate mutation sequences.

in development (48). LRAP is an signaling molecule that enhances osteoblastic cell differentiation in mouse embryonic stem cells (49). LRAP increased as much as 4000-fold for bone sialoprotein mRNA expression, fivefold for Osterix mRNA expression, and sixfold for calcium accumulation in LRAP-treated ES cells. (49). Low-molecular-mass amelogenin polypeptide from bovine dentin induced chondrogenesis. (50). These data suggest that a low concentration of amelogenins in bone and dentin might have osteogenic function. Loss of enamel proteins during amelogenesis is caused by the action of proteinases that exist in the enamel matrix (8,9). The original amelogenin in porcine developing enamel is 25 kDa amelogenin (amino acids 1–173). Enamelysin (MMP-20) cleaved the carboxyl terminal peptide of 25 kDa amelogenin to convert it to 20 kDa (amino acids 1–148). Twenty kilodalton amelogenin is the most abundant amelogenin in the immature enamel of the secretory stage. Twenty kilodalton amelogenin is further degraded by the 34 kDa serine proteinase (enamel matrix serine proteinase 1; EMSP1) into 13 kDa (amino acids 46–148) and 6 kDa (amino acids 1–45) amelogenins (10–12,51) (Fig. 1). In secretory enamel, the mineral volume increases with depth, providing space for thickening existing crystallites by decreasing amelogenins. The sequential release by proteolysis of the C-terminal domain and 13 kDa peptides from 25 kDa amelogenin micelles provides the space needed for crystal growth in width and thickness. The released C-terminal and 13 kDa fragments are soluble in neutral solution and are reabsorbed by secretory stage ameloblasts. The resultant space is used for crystal growth. Because 13 kDa amelogenin has 11 residues of His in the molecule, it may carry the proton produced when $HPO_4^{2-}$is incorporated into the mineral. The hydrophobic sequences, MPLPP (amino acids 1–5), PMGGW (amino acids 41–45), HPVV (amino acids 51–55) and PPLPPMF (amino acids 141–147), were found to correspond to the N- and C-terminal sides of 6-, 13- and 20-kDa amelogenins, respectively, although the HPVV is not located at the N-terminal end of 13 kDa amelogenin (10–12).

Amelogenin gene null mutations exhibited hypoplastic and disorganized enamel, cementum defects and revealed a reduction of bone sialoprotein expression in cementoblasts and osteoblasts (16–18). Bone sialoprotein is a marker of early osteogenic differentiation that can regulate the formation of mineral crystals (23–26). Our results showed that 25, 20, 13 and 6 kDa amelogenins increased the steady-state level of bone sialoprotein mRNA (Fig. 2). Amelogenins (25, 20, 13 and 6 kDa) stimulated bone sialoprotein promoter activities in pLUC3 (nucleotides −116 to +60), and 6 kDa amelogenin increased pLUC4 (nucleotides −425 to +60) activity (Fig. 3). From transient transfection assays we initially located the amelogenin responsive region to the proximal promoter (pLUC3; nucleotides −116 to −43) of the rat bone sialoprotein gene (Fig. 3), which encompasses an inverted CCAAT box (nucleotides −50 to −46), a CRE (nucleotides −75 to −68), a Runx2 (nucleotides −84 to −79), a FRE (nucleotides –92 to –85), a nuclear factor-kappaB (NF-κB; nucleotides –93 to –102) and a Pit-1 (nucleotides −111 to −105) motif (Fig. 4). Moreover, HOX (nucleotides −194 to −185) and TAE (nucleotides −499 to −485) elements also exist in pLUC4 and pLUC5. Although mutation of the Pit-1 (M-PIT) was without effect, M-FRE completely abrogated the activities of amelogenins (25, 20, 13 and 6 kDa) and M-CRE abolished the effect of 25 kDa amelogenin (Fig. 7). While amelogenins increased -116BSPLUC activity, and amelogenin cleavage products (20, 13 and 6 kDa) induced −108BSPLUC activity, they did not enhance −84BSPLUC activity (Fig. 5). These results also support the results of

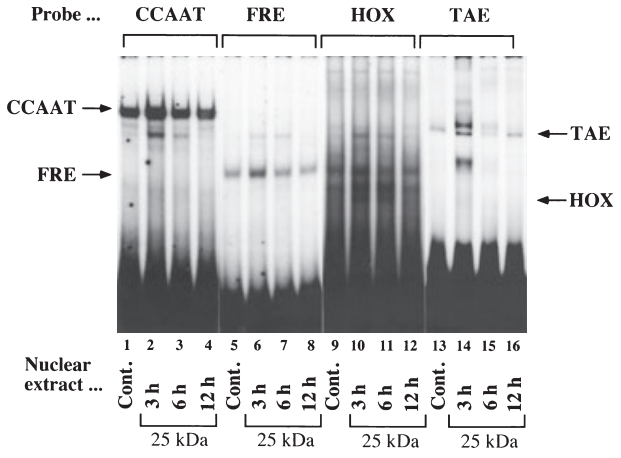

Fig. 8. Twenty-five kilodalton amelogenin increases the DNA-binding activity recognized by the fibroblast growth factor 2-response element (FRE), homeodomain protein-binding site (HOX) and transforming growth factor-beta activation element (TAE) sequences. Radiolabeled double-stranded inverted CCAAT (−61 CCGTGACCGTG**ATTGG**CTGCTGAGA −37; lanes 1–4), FRE (−98 TTTTCT**GGTGAGAA**CCCACA −79; lanes 5–8), HOX (−204 TCCTCAGCCT**TCAATTAAA**TCCCACA −179; lanes 9–12) and TAE (−506 CAAAGCC**TTGGC**AGCCC**GGCTGG**CT −482; lanes 13–16) oligonucleotides were incubated for 20 min at 21°C with nuclear protein extracts (3 μg) obtained from ROS 17/2.8 cells incubated without amelogenin (lanes 1, 5, 9 and 13) or with amelogenin (100 ng/mL) for 3 h (lanes 2, 6, 10 and 14), 6 h (lanes 3, 7, 11 and 15) and 12 h (lanes 4, 8, 12 and 16). DNA–protein complexes were separated on a 5% polyacrylamide gel in low-ionic-strength Tris–borate buffer, dried under vacuum and exposed to an imaging plate for quantification using a Bio-imaging analyzer. The bold letter indicates sequence of consensus transcription factor binding site.

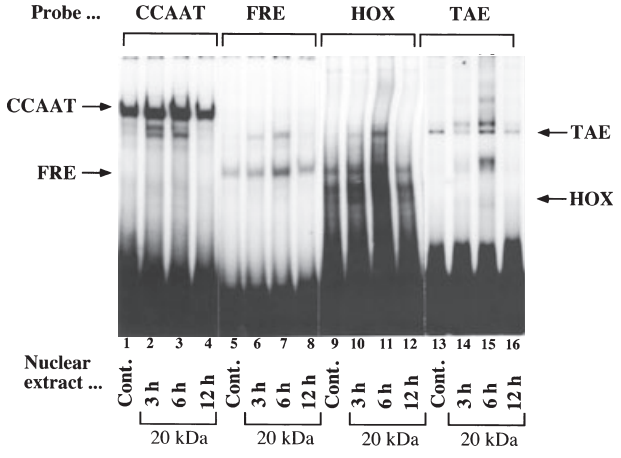

Fig. 9. The 20 kDa amelogenin increases the DNA-binding activity recognized by the fibroblast growth factor 2-response element (FRE), homeodomain protein-binding site (HOX) and transforming growth factor-beta activation element (TAE) sequences. Radiolabeled double-stranded inverted CCAAT (−61 CCGTGACCGTG**ATTGG**CTG CTGAGA −37; lanes 1–4), FRE (−98 TTTTCT**GGTGAGAA**CCCACA −79; lanes 5–8), HOX (−204 TCCTCAGCCT**TCAATTAAA**TCCCACA −179; lanes 9–12) and TAE (−506 CAAAGCC**TTGGC**AGCCC**GGCTGG**CT −482; lanes 13–16) oligonucleotides were incubated for 20 min at 21°C with nuclear protein extracts (3 μg) obtained from ROS 17/2.8 cells incubated without amelogenin (lanes 1, 5, 9 and 13) or with amelogenin (100 ng/mL) for 3 h (lanes 2, 6, 10 and 14), 6 h (lanes 3, 7, 11 and 15) and 12 h (lanes 4, 8, 12 and 16). The bold letters indicate sequence of consensus transcription factor binding site.

mutation luciferase assays (Fig. 7), because FRE exists between nucleotides −108 and −84. The involvement of the FRE, HOX and TAE elements is supported by gel shift analyses in which more complexes with the FRE, HOX and TAE elements were formed using nuclear extracts from amelogenin-stimulated cells (Fig. 8–11).

We previously reported that FRE-binding proteins are Smad1, Runx2 and Dlx5, and HOX-binding transcription factors are Smad1 and Dlx5 (42). While 3′-portions of the FRE (T**GGTGAGAA**CCCACAGC) and the HOX (T**TCAATTAA**ATCCCACAAT) were consensus Runx2-binding sites (Fig. 4), only FRE–protein complexes were supershifted by Runx2 antibody (42). The specific sequence in pLUC5 is between nucleotides −425 and −801, and the identified response element in this region is the TAE. The TAE was identified as the TGF-β1 activation element, containing the 5′-portion of the NF-1 canonical sequence (TTGGC) (44). The results of gel shift assays showed that TAE–protein complexes were increased by amelogenins (Fig. 8–11).

In our previous study, EMD regulated bone sialoprotein transcription through HOX and TAE elements in the rat bone sialoprotein gene promoter (20). Recombinant porcine amelogenin (rP172, full-length native porcine P173) enhanced bone sialoprotein transcription via FRE, HOX and TAE elements (43). Ninety per cent of the proteins in the EMD are amelogenins, and the remaining 10% are nonamelogenin enamel matrix proteins and growth factors such as TGF-β and bone morphogenetic protein (20). Discrepancy between the activities of EMD and recombinant porcine amelogenin on BSP transcription might be caused by the complex components of EMD.

In this study, we have shown that 25 kDa amelogenin and its proteolytic derivatives induced bone sialoprotein gene expression by targeting FRE, HOX and TAE in the bone sialoprotein gene promoter and that full-length amelogenin and amelogenin cleavage products could regulate bone sialoprotein transcription via different signaling pathways. Continued studies are necessary to investigate the effects of amelogenins on periodontal ligament-derived multipotent stem cells, which are a crucial cell population for periodontal regeneration.

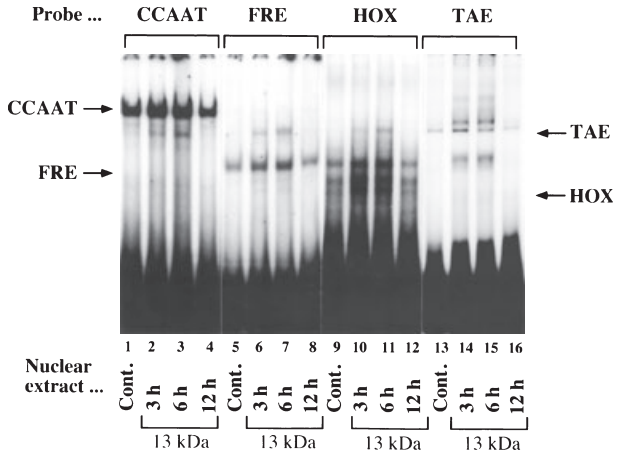

Fig. 10. The 13 kDa amelogenin increases the DNA-binding activity recognized by the fibroblast growth factor 2-response element (FRE), homeodomain protein-binding site (HOX) and transforming growth factor-beta activation element (TAE) sequences. Radiolabeled double-stranded inverted CCAAT (−61 CCGTGACCGTG**ATTGG**CTGCTGAGA −37; lanes 1–4), FRE (−98 TTTTCT**GGTGAGAA**CCCACA −79; lanes 5–8), HOX (−204 TCCTCAGCCT**TCAATTAAA**TCCCACA −179; lanes 9–12) and TAE (−506 CAAAGCC**TTGGC**AGCCC**GGCTG**GCT −482; lanes 13∼16) oligonucleotides were incubated for 20 min at 21°C with nuclear protein extracts (3 μg) obtained from ROS 17/2.8 cells incubated without amelogenin (lanes 1, 5, 9 and 13) or with amelogenin (100 ng/mL) for 3 h (lanes 2, 6, 10 and 14), 6 h (lanes 3, 7, 11 and 15) and 12 h (lanes 4, 8, 12 and 16). The bold letters indicate sequence of consensus transcription factor binding site.

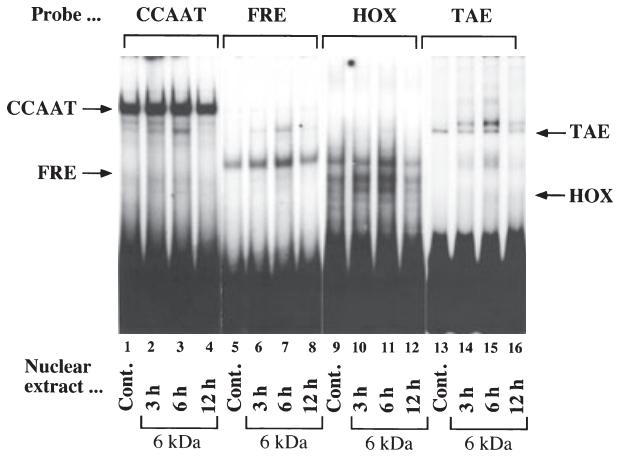

Fig. 11. The 6 kDa amelogenin increases the DNA-binding activity recognized by the fibroblast growth factor 2-response element (FRE), homeodomain protein-binding site (HOX) and transforming growth factor-beta activation element (TAE) sequences. Radiolabeled double-stranded inverted CCAAT (−61 CCGTGACCGTGATTGGCTGCTGAGA −37; lanes 1–4), FRE (−98 TTTTCTGGTGAGAACCCACA −79; lanes 5–8), HOX (−204 TCCTCAGCCT**TCAATTAAA**TCCCACA −179; lanes 9–12) and TAE (−506 CAAAGCC**TTGGC**AGCCC**GGCTG**GCT −482; lanes 13–16) oligonucleotides were incubated for 20 min at 21°C with nuclear protein extracts (3 μg) obtained from ROS 17/2.8 cells incubated without amelogenin (lanes 1, 5, 9 and 13) or with amelogenin (100 ng/mL) for 3 h (lanes 2, 6, 10 and 14), 6 h (lanes 3, 7, 11 and 15) and 12 h (lanes 4, 8, 12 and 16). The bold letter indicate sequence of consensus transcription factor binding site.

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