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ARTICLES

Evidence for Regulation of Amelogenin Gene Expression by 1,25-Dihydroxyvitamin D_3 In Vivo

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Abstract The unique hereditary enamel defect clearly related to the disturbance of one enamel matrix protein is X-linked amelogenesis imperfecta (AI), in which several mutations of amelogenin gene have been identified. The clinical phenotype of many of these subjects shows similarities with enamel defects related to rickets. Therefore, we hypothesized that rachitic dental dysplasia is related to disturbances in the amelogenin pathway. In order to test this hypothesis, combined qualitative and quantitative studies in experimental vitamin D-deficient (−D) rat model systems were performed. First, Western blot analysis of microdissected enamel matrix (secretion and maturation stages) showed no clear evidence of dysregulation of amelogenin protein processing in −D rats as compared with the controls. Second, the ultrastructural investigation permitted identification of the internal tissular defect of rachitic enamel, the irregular absence of intraprismatic enamel observed in −D animals, suggesting a possible link between prism morphogenesis and vitamin D. In addition, the steady-state levels of amelogenin mRNAs measured in microdissected dental cells was decreased in −D rats and up-regulated by an unique injection of 1,25-dihydroxyvitamin D_3 (1,25$(OH)_2D_3$). The present study shows evidences that amelogenin expression is regulated by vitamin D. This is the first study of an hormonal regulation of tooth-specific genes. J. Cell. Biochem. 76:194–205, 1999.

Key words: vitamin D; ameloblast; amelogenin; bone; matrix proteins; osteocalcin

Vitamin D plays an important role in calcium and phosphate homeostasis during skeletal development [DeLuca and Schnoes, 1976]. Vitamin D regulates the gene expression of proteins involved in the formation of mineralized tissues [Yoshizawa et al., 1997]. The genomic pathway requires binding of the main active metabolite 1,25$(OH)_2D_3$ to the vitamin D receptor (VDR), heterodimerization with a retinoid X receptor (RXR), and interaction with specific vitamin D-responsive element (VDRE) consensus sequences of target genes [Colnot et al., 1995; Haussler et al., 1998]. Rickets in humans and animals have been instructive models for the investigation of vitamin D action [Berdal et al., 1993; Matkovits et al., 1995; Glorieux, 1997; Haussler et al., 1997]. These pathologies may be the result of mutations of genes involved in vitamin D pathway or secondary to nutritional deficiency [Glorieux, 1997; Haussler et al., 1997]. Hereditary vitamin D-resistant ricket (HVDRR) is an autosomal recessive disorder caused by defects in the gene encoding for the VDR [Haussler et al., 1997]. Clinically, HVDRR results in a phenotype characterized by severe bowing of the lower extremities, short stature, alopecia, and enamel hypoplasia. Serum analysis shows hypocalcemia, hypophosphatemia, elevated alkaline phosphatase, secondary hyperparathyroidism, and increased 1,25$(OH)_2D_3$.

Abbreviations used: AI, amelogenesis imperfecta; HVDRR, hereditary vitamin D-resistant ricket; VDRE, vitamin D-responsive element; VDR, vitamin D receptor; RXR, retinoid X receptor; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; ERE, estrogen-responsive element; GRE, glucocorticoid-responsive element.

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The pathophysiology of rickets has been investigated in nutritionally vitamin D-deficient rats [Berdal et al., 1993; Matkovits and Christakos, 1995]. This experimental system, which mimics the HVDRR phenotype, was used in the present study in order to investigate the role of vitamin D in odontogenesis.

By contrast to bone, dental mineralized tissues are formed by two types of secretory units [Slavkin et al., 1992]. These units are composed of epithelial and mesenchymal cells derived from cephalic neural crests [Thesleff et al., 1995]. Odontogenic cells are characterized by expression of specific matrix proteins, including dentin sialophosphoprotein gene products [MacDougal et al., 1997] for odontoblasts and amelogenins for ameloblasts [Sasaki and Shimokawa, 1995], respectively. Furthermore, they share the expression of various proteins with other mineralized tissues (e.g., osteocalcin for bone/cementum/dentin, calbindin-D_{28k}, and calbindin-D_{9k} for bone/cartilage/enamel/dentin) [Bailleul-Forestier et al., 1996; Berdal et al., 1996; Davideau et al., 1996]. Most of these proteins, commonly present in dental mineralized tissues, appear to be the target of $1,25(OH)_2D_3$ [Berdal et al., 1993]. Indeed, VDR has been shown to be expressed and functional in osteoblasts, odontoblasts, and ameloblasts [Berdal et al., 1993; Bailleul-Forestier et al., 1996; Davideau et al., 1996].

Amelogenesis is a multistage process involving presecretion, secretion, and maturation stages [Smith and Nanci, 1995] that generate a fully mineralized layer of enamel on the crowns of teeth. This process is controlled by ameloblasts that secrete an extracellular protein matrix [Deutsch et al., 1995] that provides a gel scaffold that supports apatitic crystal growth [Fincham and Simmer, 1997]. They may also coordinate the influx of calcium and phosphate ions required for growth of the enamel crystals [Robinson et al., 1998].

Amelogenins are the major (90%) matrix proteins of forming enamel [Sasaki and Shimokawa, 1995]. They are encoded by a single gene located on the X chromosome in rat and mouse [Lau et al., 1989; Nakahori et al., 1991]. Two different amelogenins, located on the X and Y chromosomes, exist in other species, including bovoids and humans [Nakahori et al., 1991; Salido et al., 1992; Chen et al., 1998]. However, X amelogenin produce 90% of the total produced mRNA in humans [Salido et al., 1992]. Amelogenin contains at least 7 exons and produces several alternatively spliced forms. Alternative splicing encodes for translated proteins with established differences in their primary amino acid sequence [Gibson et al., 1991; Bonass et al., 1994a; Simmer et al., 1994; Li et al., 1995]. Together with extensive extracellular processing, these modifications generate a complex spectrum of amelogenin proteins in developing enamel [Brookes et al., 1996]. Ultimately, all these amelogenin proteins are completely degraded and removed from the tissue during the transition and maturation stages [Robinson et al., 1998; Smith, 1998].

The phenotype of X-linked AI resulting from amelogenin mutations suggests that this group of proteins plays a key role in enamel morphogenesis and biomineralization [Lench and Winter, 1995]. Dysmorphic teeth in AI [Wright et al., 1997] and rachitic patients [Berdal, 1997] are similar. Our aim is to discover whether there is a potential link among vitamin D, amelogenin, and amelogenesis.

MATERIALS AND METHODS

Animals and Diets

For tissue distribution studies, 56 day-old female Sprague-Dawley rats (n = 20; CERJ, le Genest-Saint-Isle, France), fed a standard diet, were studied by in situ hybridization and Northern blotting.

For developmental studies in vitamin D-deficient rats, Sprague-Dawley rats (n = 140) were raised from vitamin D-deficient mothers, housed in a dark room, and fed ad libitum with a vitamin D-deficient diet (−D second-generation animals), as previously described [Berdal, 1997]. Animals were randomly sacrified at 1, 2, 3, 4, 6, 9, and 12 days postnatally for ultrastructural studies and 56 days postnatally for biochemical studies. In addition, Sprague-Dawley rats (n = 20) raised from normal mothers, fed a standard diet, were used as controls.

For molecular studies, 3 week-old Sprague-Dawley female rats (n = 60) were kept for 5 weeks under ultraviolet (UV) light-free conditions and fed a vitamin D-deficient diet (−D rats), as previously described [Berdal et al., 1993]. −D animals were randomly sorted into three groups. First and second group animals were injected intraperitoneally with $1,25(OH)_2D_3$ (650 pmol/100 g BW) in 0.1 ml 10% ethanol-90% propylene glycol and killed 8 h (n = 20) and 24 h (n = 20) later (+8-h, +24-h rats). The

third group (n = 20) was injected intraperitoneally with only the vehicle (ethanol) and sacrified 8 h later (−D). In addition, Sprague-Dawley female rats (n = 20), fed a standard diet, were used as controls (C). All the animals were anesthetized and killed by decapitation in accordance with the French rules governing laboratory experiments.

In Situ Hybridization

Amelogenin sense and antisense RNA probes were prepared, from full-length cDNA subcloned into Bluescript plasmid [Bonass et al., 1994b] and labeled with [^{35}S]-UTP (Amersham, les Ulis, France), by in vitro transcription using T7 or T3 RNA polymerases, or both (Boehringer, Meylan, France).

For in situ hybridization experiments, animals (n = 20) were perfused with 4% paraformaldehyde, 10% sucrose in phosphate-buffered saline (PBS), for 15 min. Mandibles were then dissected out and fixed by immersion in the same fixative for 1 h at 4°C, and rinsed in 15% sucrose overnight at 4°C. Cryostat sections were then made and in situ hybridization performed as previously described [Hotton et al., 1995]. Briefly, sections were fixed in a 4% paraformaldehyde solution, washed in 2× SSC, pretreated with proteinase K (Sigma, le Verpillière, France), acetylated, and hybridized with the labeled probes, diluted to about 60,000 cpm/µl, in a humid chamber overnight at 50°C for 16 h. Afterward, sections were washed under high-stringency conditions and treated with RNase A (Sigma). The slides were dipped into LM1 emulsion (Amersham) and exposed for 10 days at 4°C. After development, sections were stained with hematoxylin (Sigma), dehydrated, and mounted with DePex medium (OSI, Paris, France). Sections were then observed and photographed with a Leitz Orthoplan photomicroscope.

Biochemical Analysis

Incisors were dissected free of the jaws and wiped clean of adhering enamel organ with a moist paper tissue. After 1 min, a white opaque zone appeared on the enamel surface. Secretory stage enamel (enamel apical to this zone) and maturation-stage enamel (enamel incisal to this zone) were removed and extracted in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (5µg enamel/µl SDS-PAGE sample buffer) by grinding with a fine glass rod. Samples loaded at 5 µl per lane were subjected to SDS-PAGE on 12.5% gels. Gels were either fixed and stained in 12.5% trichloroacetic acid (TCA) containing 0.1% Coomassie Brilliant Blue R250 or electroblotted onto nitrocellulose. The nitrocellulose was blocked by overnight incubation in blocking buffer (0.9% NaCl, 10 mM Tris, 4% soya flour, pH 7.4) and then incubated with rabbit antirecombinant amelogenin (kindly provided by Dr. A. Fincham, Center for Craniofacial Molecular Biology, University of Southern California) diluted 1:500 in blocking buffer for 1.5 h at room temperature. Excess antibody was removed by washing in Tris-buffered saline containing 0.05% Tween 20. The nitrocellulose was incubated with anti-rabbit IgG peroxidase conjugate (Sigma) diluted 1:500 in blocking buffer for 1.5 h at room temperature, and washed as described above. Cross-reactivity was visualized by incubating the blot in 10 mM sodium acetate, pH 5, containing 0.015% H_2O_2 and 0.04% 3-amino-9-ethylcarbazole. Control blots were probed directly with anti-rabbit IgG peroxidase conjugate without prior incubation with anti-amelogenin antibody.

Electron Microscopy

Animals for electron microscopy (n = 140) were perfused with 4% paraformaldehyde plus 2,5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.3. Mandibles were dissected out and fixed by immersion in the same fixative for 90 min at 4°C, rinsed in 0.1 M cacodylate buffer, and postfixed in the same buffer containing 3% osmic acid for 1 h. Molars were dissected out and processed for embedding in epon. Sections (80 nm) were cut on an LKB Ultrotome III ultramicrotome and mounted onto 300-mesh nickel grids. Finally, the sections were stained in aqueous uranyl acetate and lead citrate and observed with a Philips CM12 (80-kV) transmission electron microscope.

Northern Blotting

For Northern blotting experiments, mandibles were removed and incisors microdissected out, as previously described [Berdal et al., 1993], in order to separate dental epithelium and mesenchyme. Total RNA was isolated from established vitamin D target tissues [Berdal et al., 1993; Matkovits and Christakos, 1995; dental mesenchyme, dental epithelium and kid-

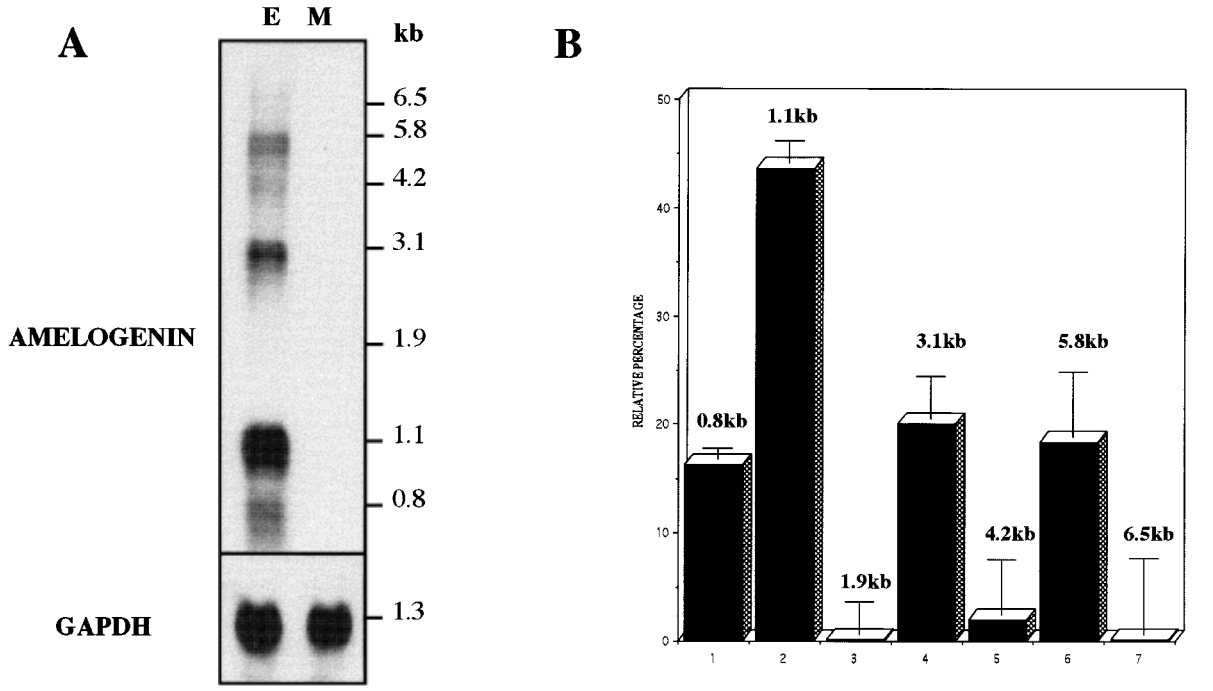


Fig. 1. Amelogenin alternative polyadenylation/cleavage sites. **A:** Seven different amelogenin transcripts are found in rat enamel organ (E). In contrast, GAPDH mRNA is found in both tissues. Nonamelogenin transcripts are shown in pulp cells (M). Relative size of amelogenin mRNAs was calculated by comparing their electrophoretic mobility to known molecular weights (0.8, 1.1, 1.9, 3.1, 4.2, 5.8, and 6.5 kb). **B:** Amelogenin transcripts were quantitated by densitometric scanning and relative optical densities compared. Values are expressed as relative percentages. Minor 1.9- and 6.5-kb transcripts are not quantitated.

ney) using a guanidium thiocyanate-phenol chloroform procedure (Euromedex, France). Total RNA (10 µg) was electrophoretically fractionated on a 1% agarose-formaldehyde gel and transferred onto a nylon membrane (Amersham). Membranes were prehybridized and then hybridized with a rat amelogenin cDNA probe [Bonass et al., 1994b] ^{32}P (Amersham) labeled by random priming (Boeringer). All membranes were stripped by washing in 50% formamide and 10 mM phosphate buffer, pH 6.5, for 30 min at 65°C and then rehybridized with glucose glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA probe. The hybridized blots were autoradiographed using Kodak film (Amersham). All the experiments were repeated four times. The optical density of the bands was quantitated by computer-assisted video densitometry (Genomic, Visio-Mic II). The relative optical densities obtained using the amelogenin probe were divided by the relative optical density obtained after probing with the GAPDH probe to normalize for sample variation. Results are expressed as the mean ±SE, and significance was determined by Student's *t*-test.

RESULTS

Differential Tissue Distribution of Amelogenin mRNA

Northern blotting of the microdissected enamel organ showed seven different transcripts present in different amounts (Fig. 1A). By contrast, amelogenin mRNA was not detected in dental papilla (Fig. 1A). The relative size of amelogenin mRNA was calculated by comparing electrophoretic mobility (Fig. 1A). Finally, amelogenin transcripts were quantitated by densitometric scanning and relative optical densities were compared (Fig. 1B).

Amelogenesis may be followed through three developmental stages: presecretion, secretion, and maturation. Amelogenin mRNA distribution was studied by in situ hybridization in rat incisors. More precisely, mRNA was highly expressed in differentiated ameloblasts, essentially during the secretion stage but also during the maturation stage (Fig. 2B,C). In addition, amelogenin mRNA was also detected in apparent smaller amounts in pre-ameloblasts located near the apical loop (Fig. 2A). Labeling was much more intense during enamel secretion

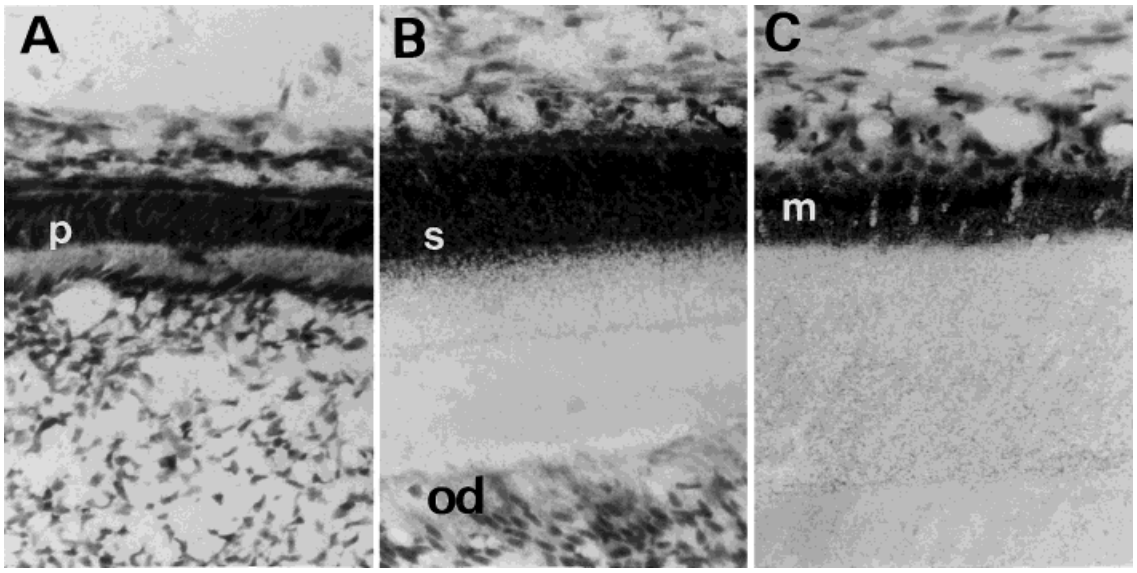


Fig. 2. Amelogenin mRNA tissue distribution. **A–C:** Amelogenin transcripts are shown in pre-ameloblasts (p), secretory ameloblasts (s), and mature ameloblasts (m), by in situ hybridization. Odontoblasts (od) are negative. ×640.

but persists even after ameloblast maturation. Odontoblasts in contact with dentin were negative (Fig. 2B). Sense amelogenin probe gave a negative signal (not shown).

Enamel Matrix in Vitamin D-Deficient and Control Animals

Secretion and maturation-stage enamel of control and −D animals was compared by SDS-PAGE and Western blot probing with anti-amelogenin antibodies. In rachitic incisor the proportion of presecretion, secretion, and maturation ameloblast stages was slightly different, in relation to the known distances in +D rats. From these data, control and −D rats appeared to have the same basic profiles with respect to amelogenins in the overall matrix (Fig. 3). Blots were also probed directly with anti-rabbit IgG peroxidase conjugate without prior incubation with primary anti-amelogenin antibody. Lack of immunoreactive bands indicates that there is no nonspecific cross-reactivity between the IgG peroxidase conjugate and enamel proteins or any endogenous peroxidase activity in the enamel samples (not shown).

Vitamin D-deficient animals were also analyzed by ultrastructural methods. The developmental events appeared delayed in −D rats. The overal sequence was maintained, albeit with major cellular and extracellular alterations. Deposition of enamel had started in 6-day-old rats. The detailed analysis of the distribution of the prismatic structures (in 6–12 days postnatal −D rats) showed enamel dysmorphogenesis, related to elongated apical Tomes processes in −D rats (Fig. 4). More specifically, the intraprismatic regions of the enamel prisms were almost absent in enamel from −D rats (Fig. 4). Furthermore, the secreted matrix was reduced, compared with controls, using the same corresponding anatomical region for the −D rat teeth, as previously descibed [Berdal et al., 1987].

Time Course and Quantitative Analysis of Amelogenin Transcripts After Single $1,25(OH)_2D_3$ Injection in −D Rats

Striking differences were observed between control and vitamin D-deficient rat enamel organs. The steady-state mRNA levels were different with or without a 5-week-vitamin D depletion (Fig. 5A). Amelogenins mRNA expression appeared to be dramatically reduced in −D rats when compared with control (C) rats (Fig. 5A).

Vitamin D-deficient animals were given one intraperitoneal injection of $1,25(OH)_2D_3$, sacrified 8 (+8 h) and 24 h (+24 h) after administration and compared with vehicle-injected animals (−D). Quantitative analysis of four separate sets of pooled enamel organs (6/lane) showed repeated differences between −D/+8-h and −D/+24-h groups (Fig. 5B,C). More precisely, 8 h after a single injection of $1,25(OH)_2D_3$ in −D rats, up-regulation of amelogenin mRNA

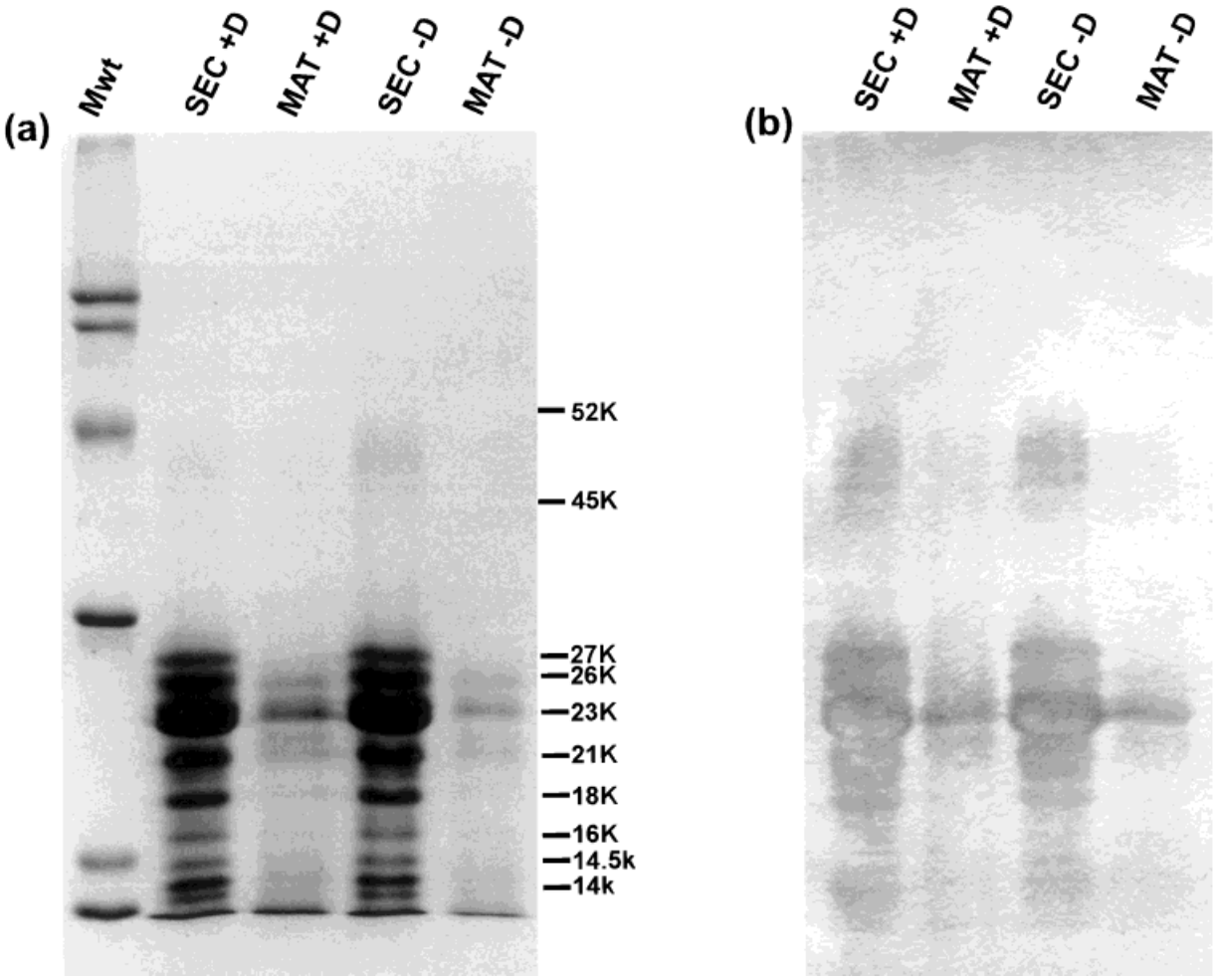


Fig. 3. Comparative analysis of amelogenin proteins during secretion and maturation stages in vitamin D-deficient and control animals. Coomassie blue-stained proteins showed the same basic electrophoretic profiles with respect to amelogenins (20–27 and 40–50 kDa) in control and vitamin D-deficient animals at the secretion (SEC+D vs SEC−D) and maturation (MAT+D vs MAT−D) stages, respectively **(a)**. Immunodetected amelogenins appeared more abundant in enamel at the secretion (SEC+D, SEC−D) compared with maturation stage (MAT+D, MAT−D), in both control and vitamin D-deficient animals **(b)**.

levels was observed (Fig. 5B,C). At 24 h after a single injection of $1,25(OH)_2D_3$ in −D rats, mRNAs showed maximal steady-state levels (Fig. 5B,C). Expression of mRNA was significantly ($P < 0,05$) induced by $1,25(OH)_2D_3$ at 8 h and 24 h (Fig. 5C). The steady-state levels of the four main transcripts (0.8, 1.1, 3.1, and 5.8 kb) varied identically under vitamin D action (not shown).

The validity of hormonal effects on enamel organ cells was checked by the action of $1,25(OH)_2D_3$ in a classically established vitamin D target organ, the kidney [Matkovits and Christakos, 1995]. The modulation of the steady-state levels of the three transcripts (1.9, 2.8, and 3.2 kb) of calbindin-D_{28k} were examined in kidney of the same groups of animals. Renal calbindin-D_{28k} mRNA was undetectable in −D rats, significant stimulation was observed at 8 and 24 h after a single $1,25(OH)_2D_3$ injection (Fig. 6A,B). The results described are representative of those obtained in four separate experiments and average rates of mRNA expression were compared with the GAPDH mRNA expression (Figs. 5, 6).

DISCUSSION

Amelogenesis occurs in three distinct stages: the pre-secretion, secretion, and maturation stages [Deutsch et al., 1995; Smith and Nanci,

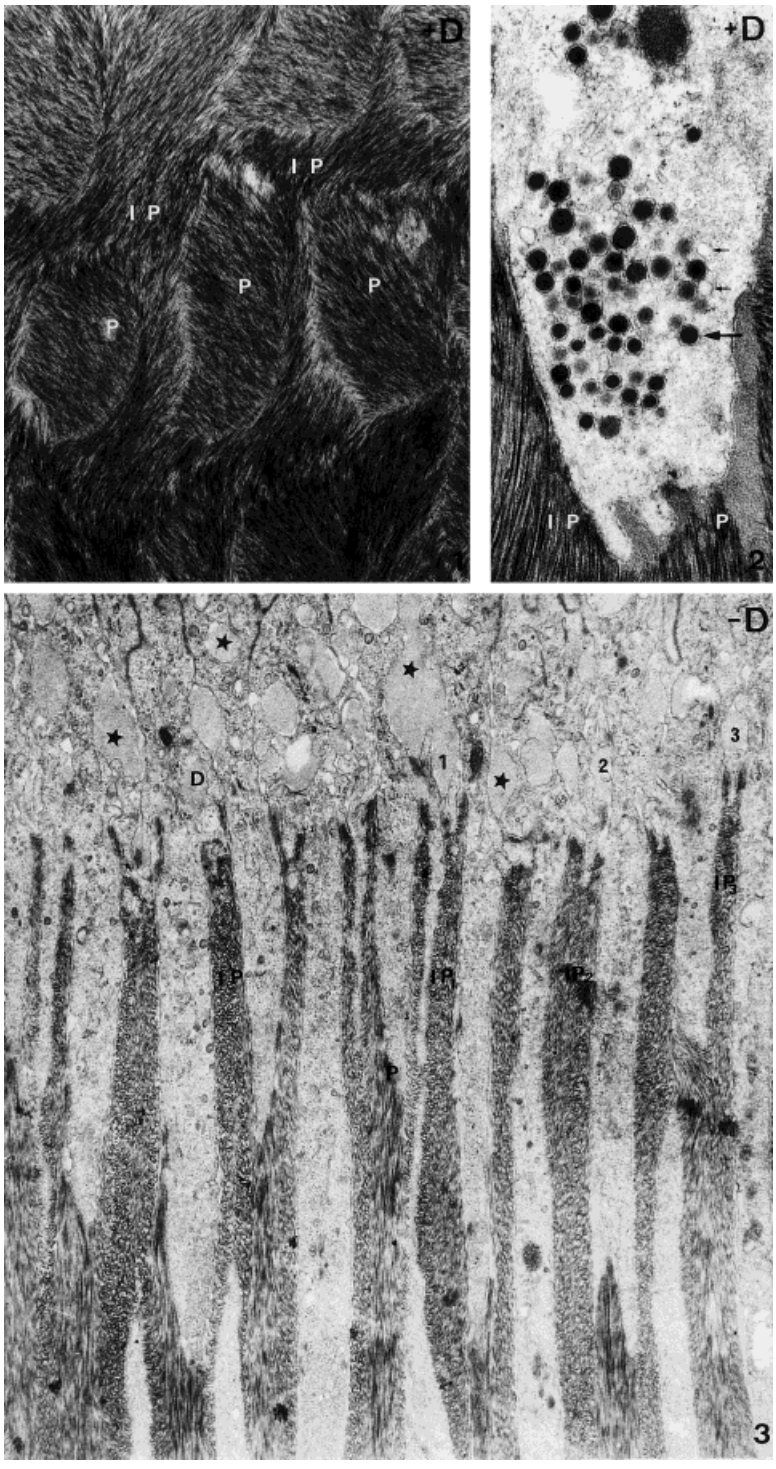
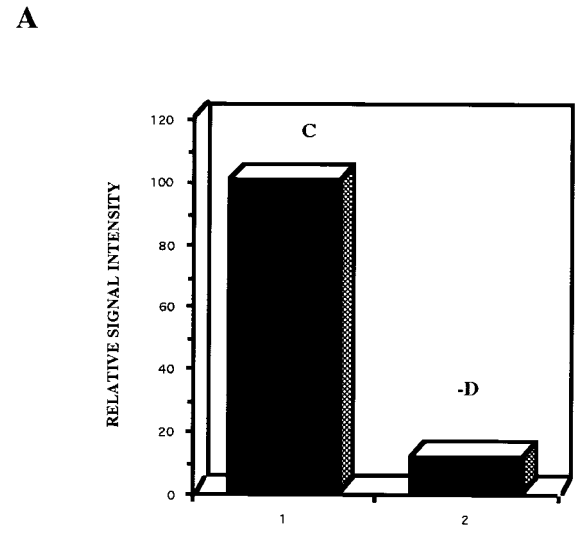
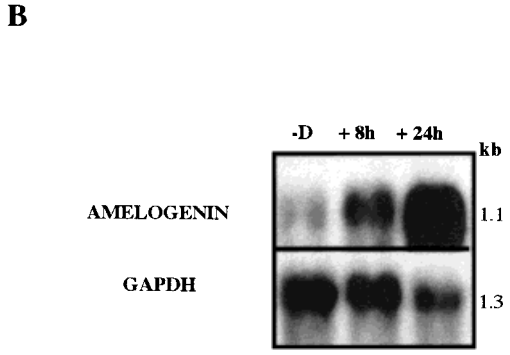
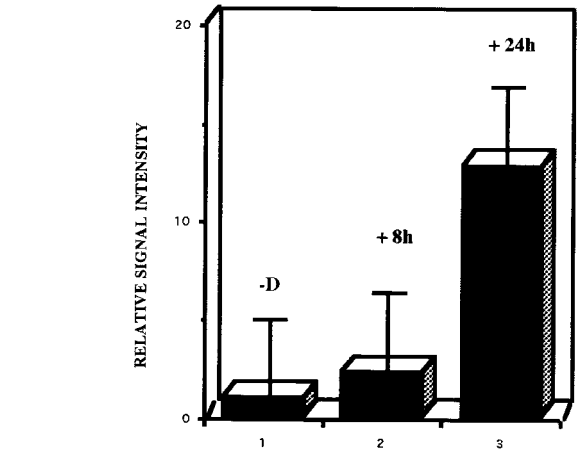


Fig. 4. Comparative analysis of enamel ultrastructure in 9 day-old control (+D) and vitamin D-deficient (−D) animals. Enamel matrix is composed of intraprismatic (P) and interprismatic (IP) areas characterized by the differential crystal orientation (**1**). ×30,000. Tomes process and the external prismatic layer of enamel are shown in a +D rat (**2**). ×30,000. The secretory pole contain numerous secretory vesicles of different sizes and electron densities (arrows, 2). Intraprismatic region (P) of enamel prism is almost absent in −D rats, By contrast with numerous interprismatic (IP_{1-3}) areas (3). ×20,000. In addition, Tomes process appear elongated compared with +D rats (**3**). Accumulation of stippled material (stars; $_{1-3}$) between the distal aspects of ameloblasts (D) is also found in large amounts in −D samples (3).

1995]. Each stage exhibits characteristic properties in terms of associated ameloblast morphology and the extracellular matrix biochemistry [Fincham and Simmer, 1997; Robinson et al., 1998]. During normal development, in situ investigations have shown that enamel matrix protein mRNAs are expressed in the polarized secretory ameloblasts during the bell stage [Wurtz et al., 1996]. In this study, amelogenin mRNA expression was detected very early (e.g., in young pre-ameloblasts). These findings are in agreement with previous work based on different methods [Couwenhoven and Snead, 1994; Inage et al., 1996; Zeichner-David et al., 1997].







Specifically, amelogenin mRNA has been detected in cap stages by reverse transcription-polymerase chain reaction (RT-PCR), in which ameloblasts are not polarized [Couwenhoven and Snead, 1994; Zeichner-David et al., 1997]. Our data may be related to the use of cryostat sections, for which no decalcification procedures have been applied [Hotton et al., 1995]. By contrast, production of amelogenin mRNA was observed not only during secretion, but during the maturation stage as well. This finding is in accordance with previous studies [Zeichner-David et al., 1997; Robinson et al., 1998] and may suggest that amelogenin has a function in the maturation stage, as well as in secretion-stage enamel. However, nascent amelogenin protein is virtually undetectable in maturation-stage enamel; any extracellular function at this stage may involve amelogenin acting as a signal molecule, rather than as a structural molecule. Alternatively, maturation-stage amelogenin expression may represent residual amelogenin activity as the ameloblast alters its function from a matrix secreting role to a matrix absorbing role.

Alternative splicing of amelogenin produces several mRNAs that encode for several translated proteins having differences in their primary amino acid sequence [Gibson et al., 1991; Bonass et al., 1994a; Simmer et al., 1994; Li et al., 1995]. These variations are believed to play a role in modulating the assembly of the enamel organic matrix [Gibson et al., 1991; Bonass et al., 1994a; Simmer et al., 1994; Li et al., 1995]. Our Northern blot analysis of microdissected enamel organs revealed the presence of seven different rat amelogenin transcripts. The 1.1-kb

Fig. 5. Amelogenin mRNAs regulation by $1,25(OH)_2D_3$. **A:** Female vitamin D-deficient rats (−D) are compared with control animals (C) concerning the main amelogenin mRNA (1.1 kb). The quantity of the corresponding transcript was evaluated by comparing the relative optical density after GAPDH probing.**B:** Vitamin D-deficient animals were given one interperitoneal injection of $1,25(OH)_2D_3$, sacrified 8 (+8 h) and 24 h (+24 h) after administration and compared with vehicle-injected animals (−D). The quantity of the main amelogenin transcript (1.1 kb) was evaluated by comparing the relative optical density after GAPDH probing. **C:** Up-regulation (2.3-fold) of amelogenin mRNA levels was observed 8 h after a single injection of $1,25(OH)_2D_3$ in −D rats (+8 h). At 24 h after a single injection of $1,25(OH)_2D_3$ in −D rats, amelogenin mRNAs showed maximal steady-state levels (+24 h). Expression of amelogenin mRNA was significantly ($P < 0.05$) induced by $1,25(OH)_2D_3$ at 8 h (+8 h) and 24 h (+24 h).

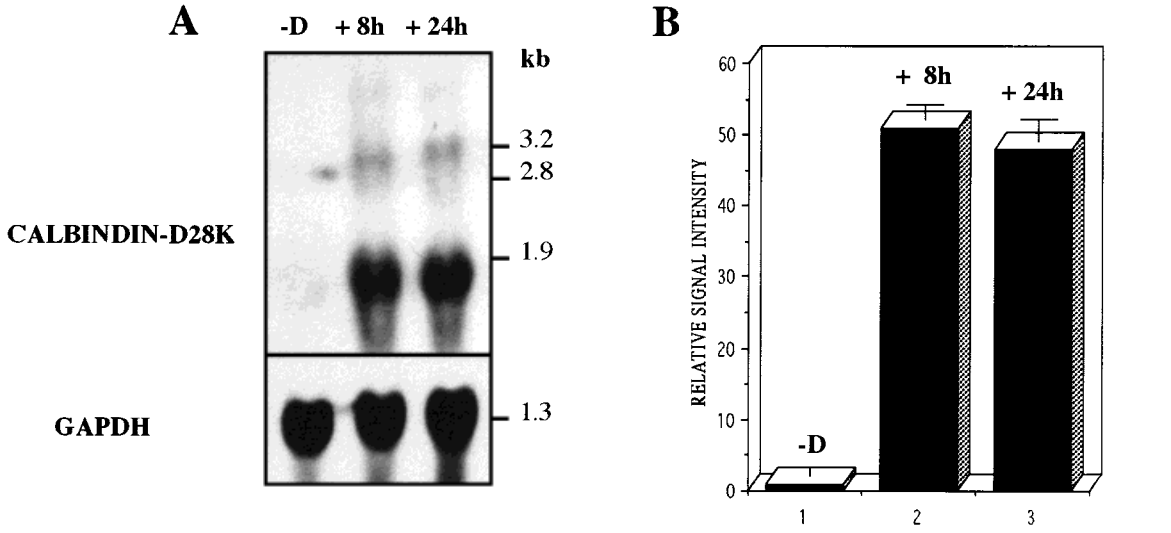


Fig. 6. Calbindin-D_{28k} regulation by $1,25(OH)_2D_3$ in kidney. **A:** Total RNAs from kidney organ of the same animals used for amelogenin expression studies were hybridized for calbindin-D_{28k} (12-h exposure), and GAPDH (12-h exposure), sequentially. Significant stimulation is observed at 8 h (+8 h) and 24 h (+24 h). **B:** The relative optical density obtained using calbindin-D_{28k} probe was divided by the relative optical density obtained after probing with the GAPDH probe to normalize sample variation. Expression of calbindin-D_{28k} mRNA was significantly ($P < 0.05$) induced by $1,25(OH)_2D_3$ at 8 h (+8 h) and 24 h (+24 h).

and 0.8-kb bands correspond to the long and short mRNA after addition of poly(A) tails as previously shown [Gibson et al., 1991; Bonass et al., 1994a; Simmer et al., 1994; Li et al., 1995]. We also observed bands at 5.8 kb and 3.1 kb that were previously reported by other investigators after longer membrane exposure [Li et al., 1995; Wurtz et al., 1996]. In addition, three other transcripts of 1.9, 4.2, and 6.5 kb were identified in our studies. All these additional bands may indicate minor amounts of primary transcripts and/or splicing intermediates of amelogenin mRNA and/or hnRNA, as previously suggested [Li et al., 1995; Wurtz et al., 1996]. Interestingly, two additional exons have been recently identified in the 3′ region of rat amelogenin cDNA [Li et al., 1998].

Tooth is a target of vitamin D action [Berdal et al., 1995; Hotton et al., 1995; Berdal, 1997]. Cells devoted to enamel and dentin formation have been shown to express VDR and thus to be potentially under the control of the main metabolite, $1,25(OH)_2D_3$ [Berdal et al., 1993; Bailleul-Forestier et al., 1996; Davideau et al., 1996; this study]. Required heterodimers with RXR may be formed, as RXR transcripts are present in both dental epithelial and mesenchymal cells [Mark et al., 1995]. Matrix proteins of mineralized tissues studied so far have been shown to be sensitive to vitamin D action, such as osteocalcin [Demay et al., 1990; Ozono et al., 1990; Breen et al., 1994]. Therefore, the main dental specific matrix proteins were hypothetized to be under the influence of vitamin D action. The continuously erupting rat incisor was used as experimental model. In this model, the life cycle of enamel forming cells is orderly recapitulated along the dental axis from the early stages until the ending of amelogenesis [Smith and Nanci, 1995]. Thus, the observed modulations of gene expression, as established previously for calbindin-D_{28k} [Hotton et al., 1995; Berdal et al., 1996], may reflect the effects of hormones on the overall stages of enamel formation and mineralization [Davideau et al., 1996]. Our data suggest that the amelogenin is sensitive to the hormonal control of $1,25(OH)_2D_3$, presumably via a VDR genomic pathway as shown previously for calbindin-D_{28k} in tooth [Berdal et al., 1993] and in other systems [Matkovits and Christakos, 1995]. In the same animals, the observed variations of calbindin-D_{28k} mRNA induced by $1,25(OH)_2D_3$ in kidneys validated our observations in teeth. Indeed, calbindin-D_{28k} is accepted as a target-gene for $1,25(OH)_2D_3$, as two different VDREs have been shown to be functional in mouse kidney [Gill and Christakos, 1993; Takeda et al., 1994]. A non-VDRE consen-

sus sequence has been identified in mouse amelogenin gene [Chen et al., 1998].

Preliminary experiments were realized on secretion and maturation-stage enamel from control and −D second-generation animals using SDS-PAGE and Western blotting. In this model, the spectrum of amelogenin proteins appeared to be similar in control and −D animals, during both stages, suggesting that no differences in the amount of amelogenins secreted in control and vitamin D-deficient rats could be the result of a feedback mechanism in which low levels of matrix amelogenin proteins stimulate increased translation from each mRNA, as previously suggested in other models [Saini et al., 1990; Bag and Wu, 1996]. Alternatively, it may be the degradation of the extracellular proteins instead of their production that mediates the dysplasia caused by vitamin D deficiency. Minor enamel proteins, such as enamelin, tuftelin, and ameloblastin, could also be responsible for the enamel dysplasia. Other methods of investigations are required to provide a conclusion on amelogenin proteins such as metabolic studies of amelogenin synthesis and quantitative electron microscopic investigation.

Interestingly, morphological studies in the same animal model identified ultrastructural enamel dysplasia involving a selective decrease in intraprismatic enamel. Furthermore, the total thickness of enamel was reduced in these animals as clinically observed in the enamel of HVDRR patient. In conclusion, our data suggest that specific dysplasia of rachitic enamel (decrease of intraprismatic enamel) is secondary to vitamin D dysregulation of amelogenin expression.

The function of amelogenins in enamel has been evidenced both in vitro and in vivo. Laboratory experiments that block or disturb amelogenin expression result in distinctive enamel disruptions [Diekwisch et al., 1993; Lyngstadaas et al., 1995]. Amelogenins observed in ameloblasts and enamel of human tooth germ appeared to be produced by X chromosome amelogenin activity (the X amelogenin generates 90% of the total amelogenin mRNA [Salido et al., 1992]). X-linked AI is caused by mutations of the amelogenin [Lench and Winter, 1995]. Prism disturbances, similar to those observed in rachitic enamel, have been reported in AI patients [Wright et al., 1997]. All these data support the notion that amelogenins contribute to enamel prism morphogenesis and mineralization. In vitamin D-deficient rats, dysplastic enamel is one of the main morphological traits and correspond to the human clinical situation seen in rickets [Laufer et al., 1987; this study]. Rachitic patients presenting features of pathological vitamin D status also exhibit dysplastic enamel [Laufer et al., 1987].

Enamel dysplasia is a phenotypic trait commonly found in genetic diseases and also as clinical data in other systemic disturbances than vitamin D deficiency. Our study suggests that $1,25(OH)_2D_3$ up-regulates the transcription of rat amelogenin gene and that amelogenin plays a key role in the control of amelogenesis by vitamin D in rats and in a broader view, presumably in the genetic and hormonal regulation of enamel formation in vivo.

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