Original Article

Spatial and temporal activity of the dentin sialophosphoprotein gene promoter: differential regulation in odontoblasts and ameloblasts

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ABSTRACT **Dentin sialoprotein and dentin phosphoprotein are non-collagenous proteins that are cleavage products of dentin sialophosphoprotein (DSPP). Although these two protein products are believed to have a crucial role in the process of tooth mineralization, their precise biological functions and the molecular mechanisms of gene regulation are not clearly understood. To understand such functions, we have developed a transgenic mouse model expressing a reporter gene (lacZ) under the control of ~6 kb upstream sequences of *Dspp*. The transgenic fusion protein was designed to reside within the cells to facilitate the precise identification of cell type and developmental stages at which the *Dspp-lacZ* gene is expressed. The results presented in this report demonstrate: (a) the 6 kb upstream sequences of *Dspp* have the necessary regulatory elements to direct the tissue specific expression of the transgene similar to endogenous *Dspp,* (b) both odontoblasts and ameloblasts exhibit transgene expression in a differentiation dependent manner, and (c) a differential regulation of the transgene in odontoblasts and ameloblasts occurs during tooth development and mineralization.**

KEY WORDS: *transgene, dentinogenesis, teeth, gene expression, DSPP*

Introduction

Organogenesis during the development of the mammalian embryo is a multi-step process involving reciprocal signals between epithelium and underlying mesenchyme. These interactions result in the formation of a variety of organs such as cutaneous structures, gut organs, pharyngeal and respiratory organs, kidney, hair and teeth (Lumsden, 1988; Gurdon, 1991; Gilbert, 1997). Such a specific developmental program depends on several molecular signals and unique combinatorial interactions of morphogens and transcription factors (Neubuser *et al.*, 1997; Zhang *et al.,* 1997). Although different classes of transcription factors, growth factors and their receptors are expressed during these inductive interactions (Linde and Goldberg, 1993; Mass and Bei, 1997; Thesleff and Sharpe, 1997; Dassule and McMahon, 1998; Tucker *et al.*,1998), the precise functions of the candidate genes during these interactions have not been clearly defined. During tooth development, reciprocal interactions between oral epithelium and underlying mesenchyme result in the differentiation of ameloblasts and odon-toblasts. Epithelial derived ameloblasts secrete the enamel matrix and mesenchymal derived odontoblasts secrete the dentin matrix. Both of these matrices undergo subsequent mineralization (Lumsden, 1988; Eisenmann, 1989; Linde and Goldberg, 1993; Fincham *et al.*, 1999).

Dentin, a specialized connective tissue secreted by odonto-blasts, is comprised of both organic and inorganic components including collagenous (type I monomer, trimer, Type III, V and VI) and non-collagenous proteins such as osteonectin, ostiopontin, osteocalcin, bone sialoprotein, dentin matrix protein 1, dentin sialoprotein (DSP) and dentin phosphoprotein (DPP) also known as phosphophoryn (MacDougall *et al.*, 1985; Butler, 1987; Linde and Goldberg, 1993). DSP expression profile in differentiating and mature odontoblasts in the developing tooth has been reported earlier (D'Souza *et al.*, 1992, 1997; Bronckers *et al.*, 1993; Ritchie

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Abbreviations used in this paper: DSPP, dentin sialophosphoprotein; DSP, dentin sialoprotein; DPP, dentin phosphoprotein; LacZ, β-galactosidase.

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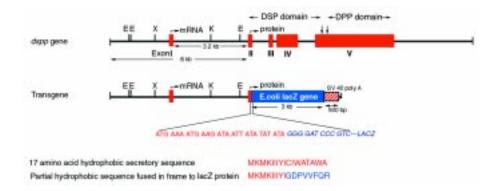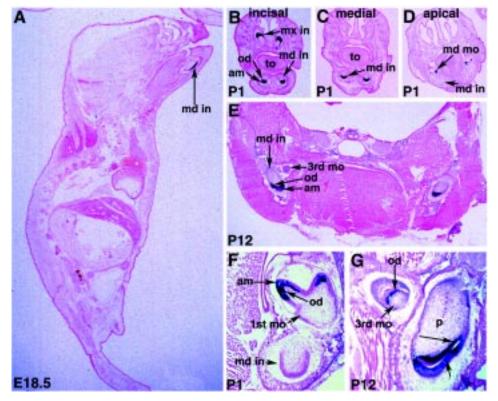
Fig. 1. Structure of wild type dentin sialophosphoprotein gene (*Dspp*) and *Dspp*-beta galactosidase transgene. *Transcription and translational initiation sites are shown within exon I and exon II. The predicted cleavages of DSPP into DSP and DPP are indicated with two arrows. The region between two arrows is the junction sequence. The transgene construct shows the in frame fusion between Dspp and lac-Z genes and the fusion disrupts the 17 amino acid hydrophobic sequence required for secretion.*

et al., 1996, 1997). Recently, we have reported the cloning and characterization of a cDNA and the corresponding genomic region coding for mouse DSP and DPP (MacDougall *et al.*, 1997b; Feng *et al.*, 1998). *Dspp* is transcribed as a 4.4 kb mRNA with a continuous open reading frame of 940 amino acids (DSPP) and later cleaved into amino terminal 370 amino acids (18-387) DSP and carboxy terminal 489 amino acids (452-940) as DPP peptides (MacDougall *et al.*, 1997b; Feng *et al.*, 1998). Promoter analysis using 5' progressive deletions mapped the basal promoter activity to –95bp and two potential enhancer and suppressor regions between –1447 and –791; -791 and –510bp respectively, within a 1447bp upstream *Dspp* gene sequence (Feng *et al.*, 1998).

The *Dspp* mRNA expression during mouse development is detected in a tissue specific manner in the odontoblasts as early as E17 day, and later transiently in secretory ameloblasts (Bronckers *et al.*, 1993; D'Souza *et al.*, 1997; Begue-Kirn *et al.*, 1998a,b; MacDougall *et al.*, 1998). In this communication, we report the

generation and analysis of beta-galactosidase reporter transgenic mice using the ~6 kb upstream sequences of the *Dspp* gene in order to characterize spatial and temporal expression during tooth development. This mouse model will allow us to gain insight into specific *in vivo* biological functions and mechanisms of *Dspp* gene regulation. The 6 kb upstream sequences include previously reported 1.4 kb promoter region (Feng *et al.*, 1998), an additional 1 kb 5' sequence, entire 3.2 kb of intron I, and exon II containing translational initiation codon. The E.coli *lacZ* (beta-galactosidase) gene coding region was fused in frame to the first 9 of 17 hydrophobic amino acids from DSPP exon II so that the protein will be retained in the cytoplasm as a DSPP-lacZ fusion protein. Most importantly, the presence of lacZ activity within the cell would facilitate the identification of specific cell type/s and stages of differentiation during tooth development. In this report, we demonstrate the presence of the regulatory sequences responsible for the tissue specific expression of *Dspp-lacZ*. We also show

Fig. 2. Tissue specific activity of DSPP-lacZ protein. (A) *Sagittal section of E18.5 day transgenic mouse showing mandibular incisor specific expression.* **(B,C,D)** *Frontal sections of newborn transgenic mouse at incisal, medial and apical regions showing expression in both maxillary and mandibular incisors as well as molars. Expression is prominent in both odontoblasts and ameloblasts.* **(E)** *Frontal section of a P12 day transgenic mouse showing the tooth specific expression.* **(F,G)** *Frontal section of a P1 and P12 day transgenic mouse showing the lacZ activity in molars and incisors. am, ameloblasts; md in, mandibular incisors; mo, molars; mx in, maxillary incisors; od, odontoblasts; to, tongue; p, pulp.*

correlation between the expression of *Dspp-lacZ* and the endogenous gene both in spatial and temporal fashion along with the identification of specific cell types that express *Dspp* during odontogenesis.

Results

Generation of Dspp-lacZ transgenic mice

Linearized transgenic construct consisting of 6 kb upstream *Dspp* sequence, E. coli lacZ gene and SV40 poly-adenylation signal (Fig. 1) was microinjected into fertilized FVB/N mouse eggs (Hogan *et al.*, 1994). Tail DNA Southern analysis was performed to identify transgene integration by standard methods and three independent mouse lines were established from the founder mice. Two transgenic mouse lines displayed a similar transgene expression profile as determined by lacZ activity.

Tissue specific expression of Dspp-lacZ transgene

The whole body sagittal sections of E18.5 day (Fig. 2A) and the frontal sections of P1 and P12 day mouse heads (Fig. 2B-G) clearly indicate the tooth restricted lacZ activity. The activity of the transgenic protein was first detected in the odontoblasts of E17.5 day and continued throughout all the developmental stages examined (data is shown in later Figures). Tissue specific expression, as determined by DSPP-lacZ activity in mandibular incisors of the transgenic embryo of E18.5 day, is shown in Figure 2A. The lacZ activity at this stage is found to be restricted only to the incisor odontoblasts. In subsequent serial sections of the same embryo, DSPP-lacZ activity is apparent in both the ameloblasts and the odontoblasts of maxillary and mandibular incisors. Mandibular molars of the *Dspp-lacZ* transgenic embryo also exhibited lacZ activity in the odontoblasts and very low levels in the ameloblasts (data shown in later figures). The frontal sections of the P1 day transgenic mouse displayed lacZ activity only in differentiating and mature odontoblasts and secretory ameloblasts of mandibular and maxillary incisors (Fig. 2B). The activity appears restricted to the labial surface of the mandibular and maxillary incisors both in the ameloblasts and the odontoblasts (Fig. 2B,C). At this developmental stage, mandibular first molars begin to show detectable levels of transgene activity (Fig. 2D). Interestingly, lacZ activity in the molars is initiated laterally on the mesial aspects towards the tongue in the oral cavity. The activity in the incisors is undetectable in the cross sections at the plane of the 1st molar tooth. This indicates that the odontoblasts at this plane are polarized but are not capable of expressing the transgene. The frontal sections of P12 day transgenic mice show tissue specific activity of the DSPP-lacZ (Fig. 2E). The third molar in the frontal section shows very low expression of transgene activity (Fig. 2E). In contrast to the observation made in the cross section of the P1 day transgenic mouse mandibular incisor at the 1st molar plane (Fig. 2F), the P12 day transgenic mouse incisor sections have shown strong lacZ activity at 3rd molar plane (Fig. 2G) and also further into the apical end. This expression profile parallels the active differentiation of odontoblasts towards the apical end of the incisors.
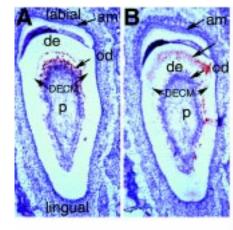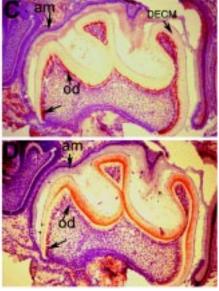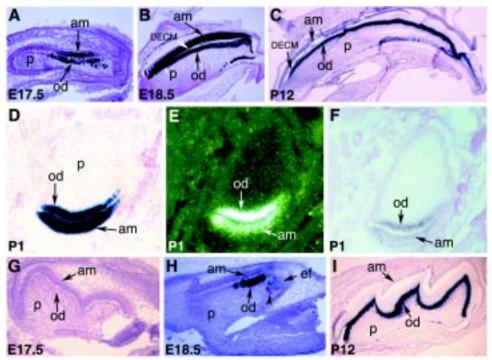

Fig. 3. Comparative analysis of DSPP-lacZ and DSP in incisors and molars of P12 day transgenic mice. *The expression of DSPP-lacZ and DSP by immunostaining is performed using polyclonal antibodies.* **(A)** *Expression of DSPP-lacZ and* **(B)** *DSP in the cross sections of maxillary incisors.* **(C,D)** *Sagittal sections of a molar tooth. Small arrow pointing at the base of the molars indicates the presecretory odontoblasts. am, ameloblasts; od, odontoblasts; d, dentin; p, pulp; DECM, dentin extracellular matrix.*

Comparative analysis of DSPP-lacZ and DSP protein expression in developing teeth

We have compared the expression patterns of both transgenic protein (DSPP-lacZ) and one of the endogenous *Dspp* gene products, DSP, by immunohistochemical analysis in *Dspp-lacZ* transgenic mice at different stages of tooth development. Figure 3 shows the immunolocalization of lacZ protein and DSP using rabbit antibodies to lacZ protein (5 prime -3 prime Inc., Boulder, CO) and polyclonal DSP antibodies generated to recombinant mouse dentin sialoprotein domain (rDSP) produced in the proEX E.coli expression system (MacDougall *et al.*, in preparation). Incisor sections of a P12 day *Dspp-lacZ* transgenic mouse show strong lacZ activity in polarized, differentiating and mature odontoblasts. LacZ activity is not detectable in the ameloblasts at all stages of differentiation (Fig. 3A). Expression of DSP protein is detected mostly in the dentin extracellular matrix (DECM) just above the secretory and mature odontoblasts (Fig. 3B) due to the secretion through the odontoblast processes (MacDougall *et al.*, 1985; Butler *et al.*, 1992; Rabie and Veis, 1995). Both DSPP-lacZ and DSP expression in the incisors is restricted mostly to the labial region. Expression of these two proteins in molars of the transgenic mouse is shown in Figure 3C and D. Similar to the expression in the incisors, DSPP-lacZ in the molars is localized to the odontoblasts and DSP mostly to the DECM. Little or no detectable levels of DSP staining are seen in the ameloblasts. DSP is also detected within the presecretory odontoblasts (indicated by an arrow in Fig. 3C and D) and later in the DECM as a result of subsequent differentiation into secretory and mature odontoblasts. As the DSP protein is known to be secreted at the mineralization front through odontoblast cell processes, both DSP-lacZ and DSP proteins are also detected in these processes projected into DECM. Due to lack of the functional secretory signal, the transgenic protein is seen accumulated in the odontoblast processes in contrast to the native DSP protein. Overall, the immunostaining data demonstrate the

Fig. 4. Developmental expression of transgene in the incisors and molars from E17.5, E18.5 and P12 day transgenic mice. (A,B,C) *Expression of the transgene activity in E17.5, 18.5 and P12 day incisors. Expression is first detected from E17.5 day and persists throughout development in the odontoblasts. Expression in the E18 day shown in panel B is dramatically increased. In P12 day incisors, the expression is continuous in the odontoblasts with little or no expression in the ameloblasts.* **(D,E,F)** *Cross sections of a P1 day transgenic mouse showing the lacZ activity (panel D), mRNA expression by a* [35]*S labeled riboprobe (panel E), and bright field image (panel F). The expression of transgene in developing molars is shown in* **(G,H,I)**. *In E17.5 sections, lacZ activity is undetectable (panel G). LacZ activity is detected in odontoblasts and relatively small amounts in ameloblasts (panel H). The expression in P12 day molar seems to include all the odontoblasts (panel I). am, ameloblasts; od, odontoblasts; p, dental pulp; ef, enamel free zone; DECM, dentin extracellular matrix.*

similar expression patterns for both DSPP-lacZ and endogenous DSP in a tissue-and cell-specific manner in the developing teeth.

The temporal activity of DSPP-lacZ protein in developing teeth

In order to establish the precise timing of DSPP-lacZ protein expression in the odontoblasts and the ameloblasts during tooth development, we examined lacZ activity in the incisors and molars of the transgenic mice at different developmental stages. DSPP-lacZ protein activity in the incisors and molars from E17.5, 18.5, and P12 day *Dspp-lacZ* transgenic mice is shown in Figure 4. LacZ activity is first detected in the incisors of transgenic embryos at 17.5 day (Fig. 4A). The activity is detected in the restricted layers of odontoblasts and ameloblasts from medial to incisal end (Fig. 4A). Later the DSPP-lacZ activity appears to extend along the single layer of cells more towards the apical end of the incisors (Fig. 4B,C), suggesting the recruitment of post mitotic cells into the layer of differentiating/ differentiated odontoblasts and ameloblasts. As tooth development progresses, lacZ activity is continued only in polarized odontoblasts and dramatically decreased in the secretory ameloblasts by P12 (compare the activity in the layer of ameloblasts in Fig. 4C versus 4B). This transient expression phenomenon of *Dspp* mRNA in the secretory ameloblasts of developing incisors and molars has been reported earlier (Bronckers *et al.*, 1993; D'Souza *et al.*, 1997; Begue-Kirn *et al.*, 1998a,b; MacDougall *et al.*,1998). The strong lacZ activity detected in the incisal end of the ameloblasts in E18.5 (Fig. 4B) and P1day transgenic incisors (Fig. 2B,C) could possibly be due to the cumulative enzyme activity as well as the stability of the lacZ protein. To test this possibility, we compared lacZ enzyme activity and mRNA expression in the frontal sections of a P1day transgenic mouse. Figure 4D,E and F show the relative comparison of the DSPP-lacZ activity and mRNA expression in odontoblasts and ameloblasts of mandibular incisor. The DSPP-lacZ activity seems to be high in ameloblasts and low in odontoblasts (roughly, twice in ameloblasts) (Fig. 4D), while its mRNA level is higher in odontoblasts as compared

to ameloblasts (Fig. 4E,F). These differences suggest that the presence of strong lacZ activity could be the result of cumulative enzyme activity rather than the transgenic mRNA expression.

The expression of the DSPP-lacZ protein is first detected in E18.5 mandibular molars (Fig. 4G,H). Similar to the expression in the incisors, the lacZ activity is seen mostly in the odontoblasts (Fig. 4H). The activity is restricted to the cuspal surface (lingual) and very low or no activity is detected along the opposite surface (Fig. 4H,I). The activity in the odontoblasts opposite to the enamel free zone (note the arrow head in Fig. 4H) appears to be less. These observations are very similar to the amelogenin gene expression reported earlier (Snead *et al.*, 1988). The transient expression phenomenon of DSPP-lacZ protein is also evident within the molars at later stages similar to the incisors (Fig. 4I).

Surprisingly, we detected significant DSPP-lacZ activity in the incisal ends of both maxillary and mandibular incisors of adult mice (Fig. 5Ab). To correlate the DSPP-lacZ activity with DSP expression, the sagittal sections of the decalcified mandibular incisors of a P60 day transgenic mouse were immunostained with lacZ and rDSP antibodies. Both DSPP-lacZ and DSP have shown a similar pattern of expression (Fig. 5B,C). Incisal ends of the incisors have shown relatively high immunostaining (arrow pointing the tips of incisors in Fig. 5B,C). Higher magnification of the incisor tips (sagittal and cross sections) exhibit more localized activity in the odontoblasts and odontoblast processes (Fig. 5D,E). The localized and abundant expression of DSPP-lacZ and DSP suggests that physical stress forces may induce this protein as the rodent incisors are continuously erupting due to normal wearing.

Differential regulation of DSPP-lacZ in odontoblasts and ameloblasts

The cytoplasmic localization of the transgene activity allowed us to further examine the precise developmental progression of DSPP-lacZ activity in the odontoblasts and the ameloblasts. The

activity of the transgene in serial cross sections of the incisors from the apical to the incisal end (Fig. 6A-D, E18.5; E-H, P1; and I-L, P12 day) show the sequence of transgenic protein activity in the odontoblasts and the ameloblasts. Initially, a low level of transgene activity is detected in the incisors of E17.5 day followed by a significant increase in E18.5 day as the differentiation of the odontoblasts and the ameloblasts progresses. We chose to examine the expression of the transgene from the apical to the incisal end to correlate with the polarization and morphological differentiation of the odontoblasts and the ameloblasts. We could detect the transgene activity first in the polarized and differentiating odontoblasts at the apical end and later in the secretory ameloblasts (Fig. 6B,F,J) in E18.5, P1 and 12 day transgenic mouse sections. This observation clearly demonstrates that the DSPP is synthesized first in the odontoblasts and later in the ameloblasts. Presence of high levels of the DSPP-lacZ activity in later stages of the ameloblasts differentiation (Fig. 6C,G,K) and significant decrease by P12 days (Fig. 6L) clearly demonstrate the transient expression pattern reported earlier for *DSPP* mRNA (Ritchie *et al.*, 1996, 1997; D'Souza *et al.*, 1997; Begue-Kirn *et al.*, 1998a,b; MacDougall *et al.*, 1998). Although initially, the transgene expression appears to be localized from the medial to the incisal region and later extend more towards the apical end, the activity of the DSPP-lacZ confirms the developmental stage dependent expression in the odontoblast and the ameloblast. The expression pattern correlates well with the morphologically mapped differentiation of the odontoblasts and the ameloblasts from apical (early) to incisal end (mature). However, presence of higher activity in ameloblasts at the incisal region of E18.5 and P1 incisors (Fig. 6G,H) could be explained as the cumulative enzyme activity of DSPP-lacZ fusion protein as discussed in the earlier section. The data obtained from cross sections of developing incisors indicate that the expression of the transgene in the ameloblasts may be mediated by odontoblasts. During later stages, the decreased expression could be due the onset of mineralization, which may prevent the odontoblasts mediated signals resulting in down regulation of DSPP in ameloblasts.

Discussion

Demonstration of tissue specific expression and utilization of such specific sequences would be valuable in understanding the precise functions and regulatory mechanisms of various genes. The *Dspp* has been mapped to human chromosome 4q21.3 within a region containing the gene loci for dentinogenesis imperfecta type II, dentin dysplasia type II and dentinogenesis imperfecta type III disorders (MacDougall *et al.*, 1997a, 1999a; MacDougall, 1998). Genomic and cDNA clones for mouse *Dspp* were identified and well characterized to understand the structure, strength of the basal promoter, enhancer and suppressor regions using progressive deletions of 5' upstream sequences *in vitro* using the odontoblast cell line, MO6-G3 (MacDougall *et al.*, 1997b; Feng *et al.*, 1998). The *Dspp* transcript of 4.4 kb encodes an open reading frame of 940 amino acids which is homologous to DSP at the amino and DPP at the carboxy terminus with 64 amino acids in between as linker region. DSP contains about 30% carbohydrate, 10% sialic acid and is believed to have a function during the differentiation of ameloblasts and odontoblasts. On the other hand, DPP has been identified as highly phosphorylated and confined to the dentin
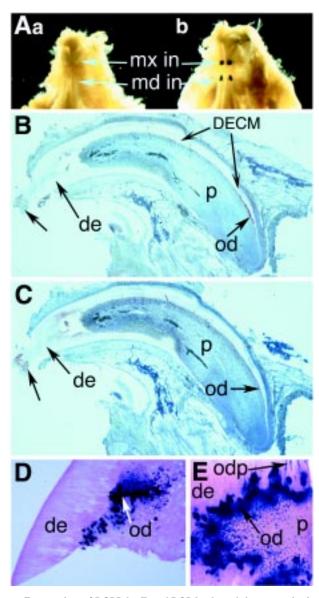

Fig. 5. Expression of DSPP-lacZ and DSP in the adult mouse incisors. *The activity is observed in the tips of both mandibular and maxillary incisors in a localized manner* **(Aa** *and* **Ab)**. **(A)** *P60 day wild type and transgenic mouse heads stained for the lacZ activity.* **(B,C)** *Immunostaining of P60 day decalcified transgenic mouse incisors with lacZ antibodies (panel B) and rDSP antibodies (panel C). Note the staining of incisor tips (arrows) along with the odontoblast specific staining.* **(D,E)** *Higher magnification of incisor tips. Sagittal section (panel D) shows the predominant staining towards the labial surface of the incisors and mostly restricted to mature odontoblasts. Cross section (panel E) shows a similar pattern and also staining in the odontoblast processes. md in, mandibular incisors; mx in, maxillary incisors; od, odontoblasts; de, dentin; p, pulp; odp, odontoblast process; DECM, dentin extracellular matrix.*

enamel junction and the mineralized dentin layer, suggesting its role as a key regulator of both enamel and dentin mineralization. Even though the modifications on DSP and DPP have been identified, the transcriptional and post-translational mechanisms have not yet been clearly understood. DNA sequence analysis of the *Dspp* upstream region identified consensus binding sites for
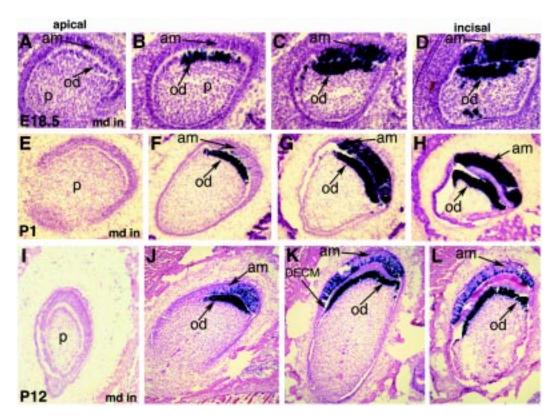
Fig. 6. Developmental expression pattern of transgene in odontoblasts and ameloblasts. (A-L) *Order of expression of the transgene in the odontoblasts and ameloblasts. Panels A-D, E-H and I-L represent serial cross sections from apical to incisal ends of E 18.5, 1 and 12 day transgenic mice incisors, respectively. The expression is first seen in the odontoblasts (panels B, F and J). Although, the lacZ activity seems to be continuous up to P1 day in odontoblasts and ameloblasts, a decreased activity is observed only in the ameloblasts in P12 day (panel L). am, ameloblasts; de, dentin; od, odontoblasts; p, dental pulp; DECM, dentin extracellular matrix.*

Msx-1, TCF-1, SRE and AP1, indicating their potential role in controlling the gene expression (Feng *et al.*, 1998). Interestingly, the DNA sequencing of further upstream sequences has revealed potential binding sites to CBFA1 of runt family transcription factors, suggesting a possible role in control of the gene transcription (MacDougall *et al.*, 1999b). Studies to determine the functional aspects of these transcription factors in *Dspp* gene regulation are currently being performed.

We have utilized ~6 kb upstream sequences covering all the cis-acting elements to identify the tissue specificity and temporal activity of the promoter in transgenic mice using beta-galactosidase as a reporter gene. In addition to the expression pattern, these mice also facilitate a better understanding of *Dspp* regulation *in vivo*. The reporter protein expressed under the control of the *Dspp* upstream sequence is not secreted into DECM due to the truncated secretory signal sequence. Localization of the protein activity within the cells facilitates precise identification of cell type and the differentiation stage. The odontoblast and the ameloblast specific expression of lacZ protein presented in this report is consistent with the endogenous gene expression pattern and indicate that the 6 kb *Dspp* upstream sequence includes all the necessary regulatory elements required to direct tissue specificity. The transgenic protein activity appeared in polarized maturing odontoblasts before the predentin layer is formed as indicated earlier (MacDougall *et al.*, 1985; Bronckers *et al.*, 1993). Interestingly, we have detected high levels of the transgene activity at the incisal end of adult incisors and the overlapping expression of endogenous DSP, suggesting additional mechanisms of gene induction. The presence of the activity first within the odontoblasts and later in the ameloblasts followed by the down regulated activity with the onset of dentin mineralization strongly supports the possibility of an odontoblast mediated signaling mechanism. We speculate that the signaling from odontoblasts could be the secreted molecule(s) which regulate the expression of *Dspp* in the odontoblasts by endocrine/autocrine and in the ameloblasts by juxtacrine/paracrine mechanisms. Targeted expression of such candidate genes in odontoblasts will further elucidate the "cross talk" between odontoblasts and ameloblasts.

Materials and Methods

Generation of Dspp-lacZ transgenic mice

The regulatory sequences of *Dspp* consisting of a 6 kb 5' upstream sequence, including 66bp exon I (transcriptional start site), the entire 3.2 kb of intron I and the first 45bp of the exon II (translation initiation codon ATG and truncated 9 of the 17 amino acid hydrophobic sequence) were fused in frame to bacterial beta-galactosidase (*lacZ*) gene (Fig. 1) to express in a tissue specific manner and to retain the fusion protein within the cytoplasm. The 9.5 kb transgene fragment was purified and microinjected into fertilized FVB/N mouse oocytes to generate transgenic mice as described (Hogan *et al.,* 1994).

Tissue preparation and lacZ activity determination

Timed pregnant wild type and transgenics of E15.5 to 18.5 and P1, 12 and 60 were assayed for the temporal and spatial activity of DSPP-lacZ fusion protein. The embryos/tissues were dissected and washed in PBS, fixed in 4% paraformaldehyde for 1 h at 4°C, washed in phosphate buffered saline (PBS) and incubated overnight for 4 days in lacZ staining reagent containing 0.02% NP-40, 0.01% sodium deoxycholate, 1 mM spermidine, 2 mM magnesium chloride, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide and 0.5 mg/ ml blu-o-gal (GibcoBRL, Gaithersburg, MD, USA) (Bieberich *et al.*, 1993). Frozen sections of the stained tissue were

collected, counterstained with hematoxylin and eosin and photographed. For the developmental expression, the tissue samples from neonates to adults up to 60 days were decalcified in 0.1M EDTA, 0.15M sodium phosphate buffer pH 7.4 at room temperature for 7-15 days and processed for frozen sectioning. The frozen tissue sections were stained for lacZ activity and counterstained with hematoxylin and/or eosin for general histology.

In situ hybridization

The decalcified tissues were processed and frozen sections of 10-12 micron thickness were collected and hybridized with ^{35}S-labeled (Sreenath *et al.*, 1996) *lacZ* specific sense and anti-sense riboprobes. The slides were washed and dipped in NTB-2 photographic emulsion (Eastman Kodak Co., Rochester, NY, USA) and exposed for 3-7 days at 4°C, developed and photographed in dark and bright fields.

Immunostaining

DSPP-lacZ and DSP were detected in the decalcified frozen sections of transgenic mouse molars and incisors using polyclonal antibodies. The tissue sections were processed and stained according to specifications (Zymed Laboratories, San Francisco, CA, USA). Briefly, the sections were washed with PBS and blocked for the endogenous peroxidase activity with 3% hydrogen peroxide in methanol for 15 min, washed thoroughly in PBS for 30 min. The tissue sections were treated with the blocking reagent for 10 min to minimize the non-specificity and incubated with the primary antibodies (lacZ 1:1000; DSP 1:400) overnight at 4°C. The tissue sections were washed in PBST and treated with secondary antibodies conjugated with peroxidase for 15 min. The presence of the proteins was detected by using AEC reagent as substrate to peroxidase. The slides were counterstained with hematoxylin or eosin, mounted with GVA aqueous mount, examined and photographed under light microscope.

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