

Differential expression of type I and type III collagen genes during tooth development

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Summary

Collagen gene expression during mouse molar tooth development was studied by quantitative *in situ* hybridization techniques. Different expression patterns of type I and type III collagen mRNAs were observed in the various mesenchymal tissues that constitute the tooth germ. High concentration for pro-alpha 1(I) and pro-alpha 2(I) collagen mRNAs were found within the osteoblasts. We found that the cellular content of type I collagen mRNAs in the odontoblasts varies throughout the tooth formation: whereas mRNA concentration for pro-alpha 1(I) collagen decreases and that of pro-alpha 2(I) increases, during postnatal development. Moreover, different amounts of pro-alpha 1(I) and pro-alpha 2(I) collagen mRNAs were observed in crown and root

odontoblasts, respectively. Type III collagen mRNAs were detected in most of the mesenchymal cells, codistributed with type I collagen mRNAs, except in odontoblasts and osteoblasts.

Finally, this study reports differential accumulation of collagen mRNAs during mouse tooth development and points out that type I collagen gene expression is regulated by distinct mechanisms during odontoblast differentiation process. These results support the independent expression of the collagen genes under developmental tissue-specific control.

Key words: collagen genes, tooth development, odontoblast differentiation.

Introduction

Collagenous proteins constitute the main component of the mammalian extracellular matrix and are made of genetically distinct polypeptide chains. For example, type I collagen consists of two alpha 1(I) and one alpha 2(I) chains, while type III collagen comprises three alpha 1(III) chains (Linsenmayer, 1983).

The mechanisms by which collagen gene expression is regulated are not well understood at this time (Bornstein and Sage, 1989). Most evidence indicates a coordinated expression of the genes for pro-alpha 1(I) and pro-alpha 2(I) chains of type I collagen. The mRNAs coding for pro-alpha 1(I) and pro-alpha 2(I) chains share similar start signal sequences and seem to be cotranslated on the same endoplasmic reticulum membrane (de Wet *et al.* 1983; Yamada *et al.* 1983; Veis *et al.* 1985; Veis and Kirk, 1989). Moreover, the steady-state ratio of these mRNAs remains generally near 2:1 (Hämäläinen *et al.* 1985; Olsen and Prockop, 1989). In contrast, there is also some evidence for an independent regulation of pro-alpha 1(I) and pro-alpha 2(I) genes (de Wet *et al.* 1983; Stephenson *et al.* 1985; Olsen and Prockop, 1989), and it has been suggested that the two genes, which are located on different chromosomes

(Sundaresan and Francke, 1989), are regulated by different promoters (Lichtler *et al.* 1989; Schmidt *et al.* 1986).

In most tissues, type III collagen is codistributed with type I collagen and they probably form copolymers (Keene *et al.* 1987). At the level of the DNA sequence, homologies have been reported between the 5' region of their genes, which may suggest the existence of common regulatory elements (Yamada *et al.* 1983). In cell culture systems, type I and type III collagen mRNA contents often change in a parallel manner (Liau *et al.* 1985; Uitto *et al.* 1985; Jimenez *et al.* 1986; Russell *et al.* 1989). However, *in vivo* observations showed that the steady-state ratio of type I and type III collagen mRNAs varies depending on the tissue (Sandberg *et al.* 1989).

In order to examine the tissue specificity of collagen gene expression, we have chosen the mouse tooth system as the development allows the investigation of mesenchymal cell differentiation and extracellular matrix formation during embryonal or postnatal morphogenesis (Thesleff and Hurmerinta, 1981; Andujar *et al.* 1985; Ruch, 1987). The sequence of events that leads mesenchymal cells to become terminally differentiated odontoblasts occurs in several precise stages (Thesleff

and Hurmerinta, 1981; Andujar *et al.* 1985; Ruch, 1987). During this differentiation process, a major functional aspect concerns the amplification of the synthesis of type I and type I trimer collagens (Lesot, 1981; Lesot *et al.* 1981; Ruch, 1987). It has been suggested that suppression of type III collagen production is associated with type I collagen accumulation (Ruch, 1987), but our own observations did not confirm this (Andujar *et al.* 1988).

The present study reports the existence of a differential expression of type I and type III collagen genes in relation to mouse molar tooth development.

Materials and methods

cDNA probes

cDNA clones for human alpha 1(I), alpha 2(I) and alpha 1(III) chains of procollagens were described elsewhere: plasmid HF677 carries the complementary nucleotide sequence of the pro-alpha 1(I) collagen mRNAs (Chu *et al.* 1982), plasmid HF32 contains a portion of insert complementary to the pro-alpha 2(I) collagen mRNAs (Myers *et al.* 1981), and plasmid pHFS3 contains a fragment of the pro-alpha 1(III) collagen mRNAs sequence (Sandberg *et al.* 1989). All the probes were prepared from insert-purified DNA. Briefly, recombinant plasmid DNA was subjected to restriction cutting, agarose gel separation of the insert, electroelution, concentration on ion-exchange column (Elutip-d, Schleicher and Schull Inc.), and quantification by spectrophotometry. Probes used corresponded to the 1.5 kb DNA insert from HF677 plasmid digested by *EcoRI*, the 1.6 kb fragment from HF32 plasmid digested with *EcoRI* and *XhoI*, and the 0.7 kb insert from pHFS3 plasmid digested with *EcoRI* and *XhoI*. The probes were prepared by random priming labeling with one (alpha ³²P-dCTP) or two (alpha ³²P-dCTP and dTTP, Amersham) labeled nucleotide precursors. Control experiments were performed by hybridizing sections labeled with pBR 322 or pUC 8 plasmid DNAs.

Preparation of tissue

Analyses were carried out on the first-lower molars from 10-day embryo to 10-day-old postnatal Swiss Wistar mice (Mérieux, France).

Fixation was performed by perfusion of a 4% paraformaldehyde in PBS, except for 10- to 15-day-old embryos, which were fixed by immersion in the same solution for 18 h at 4°C. Tissues were demineralized, embedded in Tissue Tek II medium (Miles-Yeda Laboratories) and frozen in liquid nitrogen-cooled isopentane. Cryostat sections (10 µm) were transferred to gelatin-subbed slides, air-dried and kept frozen until pretreatment. Sections from different developmental phases were analysed simultaneously on the same slide.

The slides were immersed in 50 mM Tris-HCl, 5 mM EDTA buffer (pH 7.6), then treated with 1 µg ml⁻¹ proteinase K (Boehringer Mannheim) in Tris-HCl/EDTA buffer for 30 min at 37°C. Sections were washed twice in PBS containing 0.2% glycine (5 min each), twice in PBS (10 min each), and were then fixed in 2% paraformaldehyde in PBS buffer for 15 min. Fixative was rinsed off by two washes in PBS (10 min each), followed by immersion in 0.1 M triethanolamine-HCl buffer (pH 8.0) for 5 min, before acetylation. This was carried out by immersing the slides in triethanolamine-HCl buffer, containing 0.25% acetic anhydride for 10 min. The slides were washed twice in 2×SSC buffer (10 min each), then

dehydrated in ethanol, and stored air dried at -80°C until use. All steps of the above procedure were carried out at room temperature unless mentioned.

Some pretreated slides were stained with Masson triple staining for histological purposes.

Hybridization procedure

The pretreated sections were prehybridized for 2 h at 37°C in a humid chamber with a mixture containing 50% deionized formamide, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.15 M NaCl, 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 250 µg denatured Herring sperm DNA, 500 µg ml⁻¹ yeast tRNA, 200 µg ml⁻¹ BSA and 10% dextran sulfate.

The sections were then covered with 20 µl of the same mixture including the radioactive probe, topped with siliconized coverslips, sealed with rubber cement and incubated for 12 h at 37°C in a humid chamber.

We took great care to hybridize the serial sections with ³²P-labeled alpha 1(I) and alpha 2(I) collagen probes of identical specific radioactivity (0.8–1.2×10⁹ disintegrations min⁻¹ µg⁻¹) and final concentration in the hybridization mixture (40–80×10⁶ disintegrations min⁻¹ ml⁻¹).

After hybridization, sections were washed for 3 h at 37°C in 50% formamide, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA and 0.15 M NaCl, with 3 changes over that period. This was followed by dehydration in ethanol and air-drying. Autoradiography was performed by dipping the slides into either Ilford K-5 (Ilford) or LM-1 (Amersham) emulsions, and by exposition for 3–15 days at 4°C in light-tight boxes containing activated silica gel. The emulsions were developed in Kodak D-19 developer, washed in 0.5% acetic acid and fixed in 30% ammonium thiosulfate. The slides were stained with 0.01% toluidine blue, air-dried and mounted with Entellan (Merck).

Quantification procedures

The area density of silver grains (grains/100 µm²) were computed by epi-polarization light with an image analyser (Samba 2002, TINT, France) coupled to a Zeiss Axioplan microscope equipped with a 40× planapochromat lens. Five sections for each developmental phase were analysed from four independent hybridization experiments. It should be noted that sections from different developmental phases were grouped on the same slide. For a given probe, twelve equivalent regions were chosen at random from each section. The grain density on the epithelial tissue was taken as the background value.

Results

To demonstrate that differential collagen gene expression takes place in the course of the mouse molar tooth development, we analysed two precise developmental phases: one corresponds to the overt cytodifferentiation that takes place at the terminal differentiation of the crown odontoblasts (19-day embryo, Fig. 1), and the other to the stage when the functional root odontoblasts are observed and the periodontal ligament starts to develop from the follicular mesenchymal cells (10-day postnatal, Fig. 5). Tooth germ is an epithelio-mesenchymal organ surrounded by several kinds of tissues, including the alveolar bone and the oral epithelium (Figs 1 and 5). The alveolar bone is made by highly specialized matrix-producing cells, the osteoblasts, which constitute a positive internal control for

type I collagen gene expression (Figs 2, 3, 6 and 7). The oral epithelium and the enamel organ represent the negative internal controls, because these epithelial cells do not express either type I or type III collagens (Figs 2–4 and 6–8). Thus, tissue heterogeneity constituted an advantage for the control of hybridization specificity.

It is difficult to obtain specific hybridization using collagen cDNA probes because of the high degree of homology of the nucleotide sequences encoding the different collagen types (Hayashi *et al.* 1986; Sandberg and Vuorio, 1987). Therefore, in order to avoid cross-hybridization, we used probes from human cDNA fragments coding for the region in between the triple helical domain and the COOH-propeptide and carried out stringent conditions of hybridization and washing. These probes are highly homologous to the same regions of the mouse collagen genes (Chu *et al.* 1985). The procedure's effectiveness is demonstrated by the low background observed on the epithelium (Figs 2–4 and 6–8) or on the control sections (data not shown). Moreover, odontoblasts and osteoblasts strongly express type I collagen genes (Figs 2, 3, 6 and 7), but not type III collagen gene (Figs 4 and 8).

Finally, the topological accumulation of the procollagen mRNAs was demonstrated by quantitative estimation of silver grains density (grains/100 μm^2 , Figs 9–11). The signal intensity reflects the cell concentration of the mRNAs, since the cellular density remains almost unchanged within the odontoblast layer and the dental pulp, during the experimental period.

Expression of pro-alpha 1(I) collagen gene

Our results show that differential accumulation of mRNAs coding for pro-alpha 1(I) collagen occurs during tooth morphogenesis. Before overt differentiation of the odontoblasts, only bone cells show a high signal with pro-alpha 1(I) probe (data not shown). The first differentiated odontoblasts are observed on the principal cusp of the molar tooth germ in 19-day embryos (Fig. 1), when they show a high content of pro-alpha 1(I) collagen mRNAs as do the osteoblasts. This is in contrast to pulp and follicular mesenchymal cells in which the signal was very faint (Figs 2 and 9). During postnatal development, 10-day-old mice are observed to have functional root odontoblasts and formation of the periodontal ligament takes place. At this stage, the cellular content of pro-alpha 1(I) collagen mRNAs in odontoblasts decreases (Figs 6 and 10). The periodontal ligament and the dental pulp cells account for 12% and 2%, respectively, of the signal detected in odontoblasts. Different levels of pro-alpha 1(I) collagen gene expression were observed between crown and root odontoblasts. Thus, the signal intensity in root odontoblasts was found to be approximately half the signal level detected in crown odontoblasts (Figs 6 and 11).

Expression of pro-alpha 2(I) collagen gene

The highest signal detected with the pro-alpha 2(I) collagen probe was observed in bone cells during embryonal or postnatal development. Before overt

Figs 1–4. Sections of 19-day embryo mice tooth germs show the first differentiated odontoblasts (arrow) on the top of the molar cusp, the pre-odontoblasts (Po) on the lateral and apical portions of the dental pulp (P). Enamel organ (E), pre-ameloblast (A), follicular mesenchyme (F), alveolar bone (B). Bar=100 μm .

Fig. 1. Morphological aspect of the pretreated section subjected to trichrome triple staining.

Fig. 2. Hybridization signal for pro-alpha 1(I) collagen mRNAs shows a strong positive reaction in both odontoblasts and osteoblasts.

Fig. 3. Hybridization signal for pro-alpha 2(I) collagen mRNAs shows a strong positive reaction in osteoblasts, however, the odontoblasts display a lower signal.

Fig. 4. Hybridization signal for pro-alpha 1(III) collagen mRNAs shows a positive reaction within the follicular mesenchyme. No silver grains are observed within odontoblasts or osteoblasts.

Figs 5–8. Sections of 10-day-old postnatal mice tooth germ showing the crown (arrow) and root (arrowhead) odontoblasts. Alveolar bone (B), periodontal ligament (L), dental pulp (P), dentine (D), ameloblast (A), Hertwig's epithelial sheath (H), incisor odontoblast (Io), incisor periodontal ligament (Li), oral epithelium (Ep), tendon (T). Bar=100 μm .

Fig. 5. Morphological aspect of the pretreated section subjected to Trichrome triple staining.

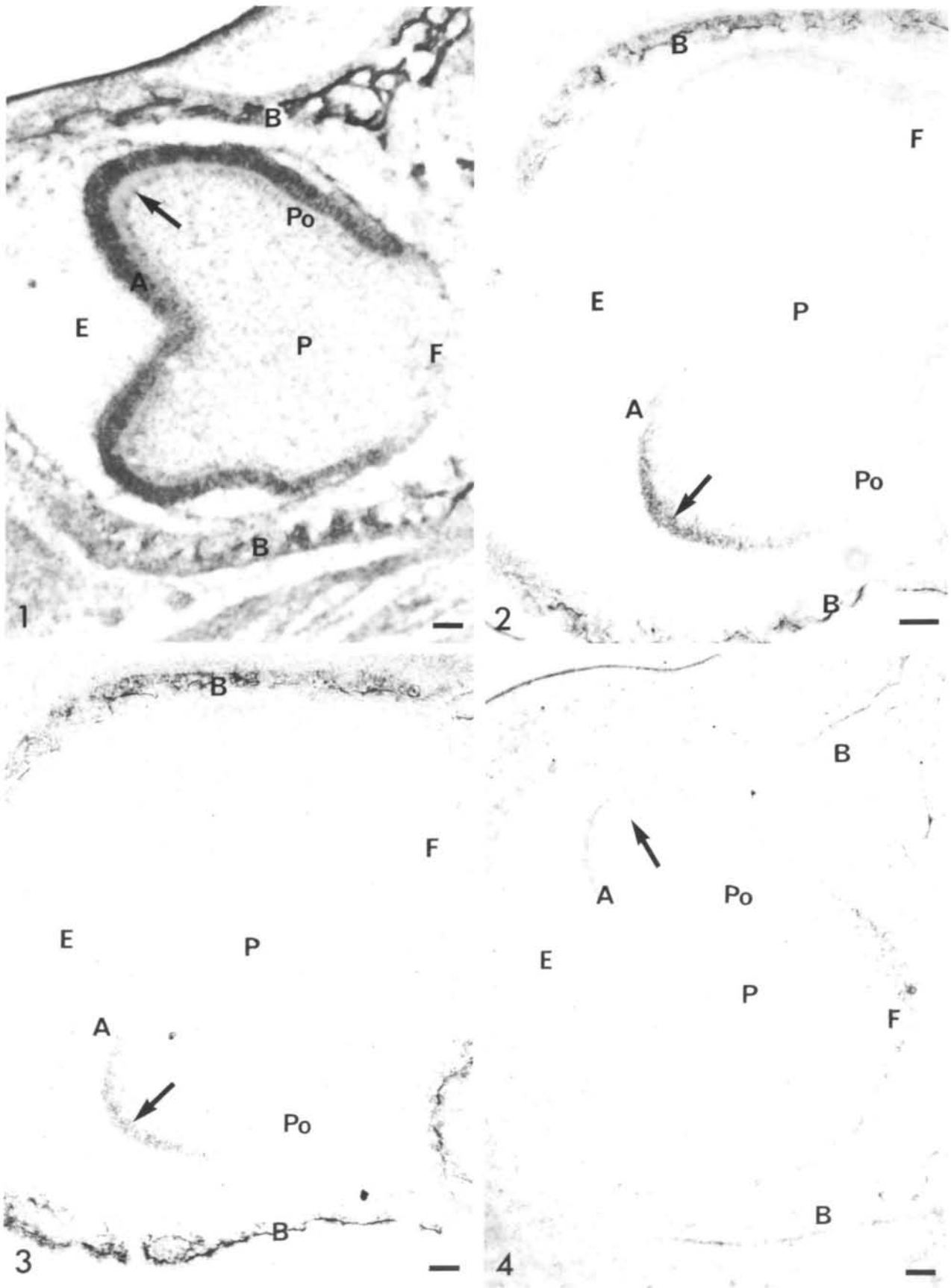
Figs 6 and 7. Hybridization signal for pro-alpha 1(I) (Fig. 6) and pro-alpha 2(I) (Fig. 7) collagen mRNAs showing a strong positive reaction within odontoblasts, bone cells and tendon.

Fig. 8. Hybridization signal for pro-alpha 1(III) collagen mRNAs showing a positive reaction within both molar and incisor periodontal ligament. No labelling above the background, is detected in odontoblasts or bone cells.

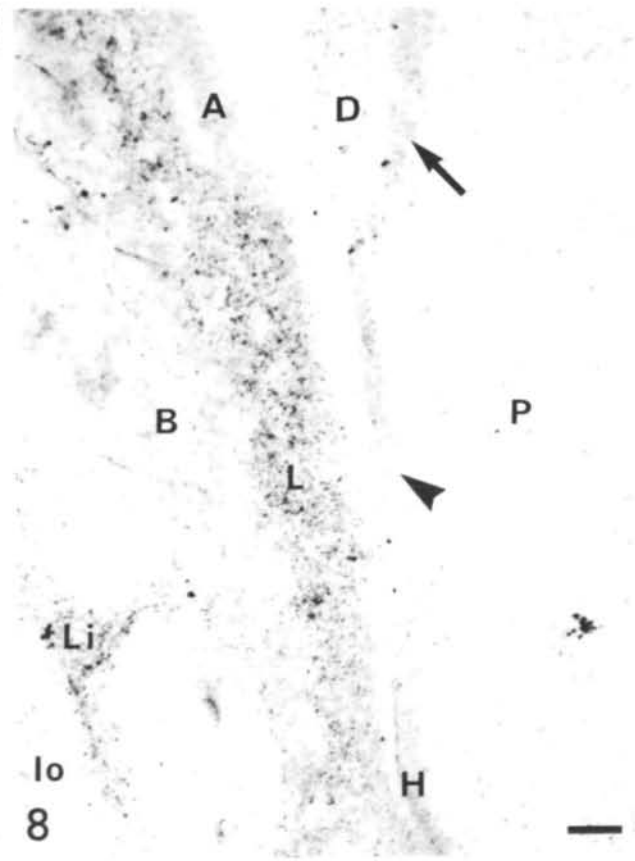
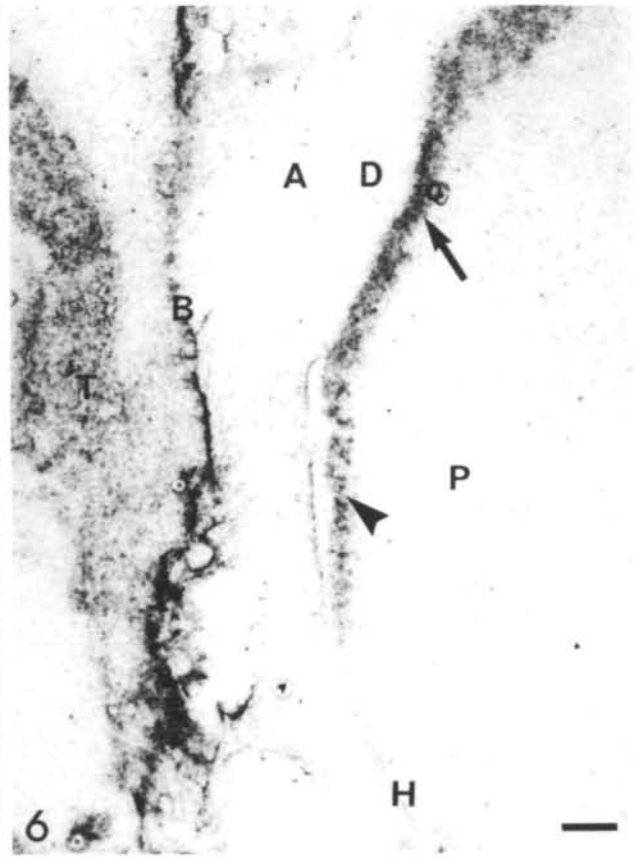
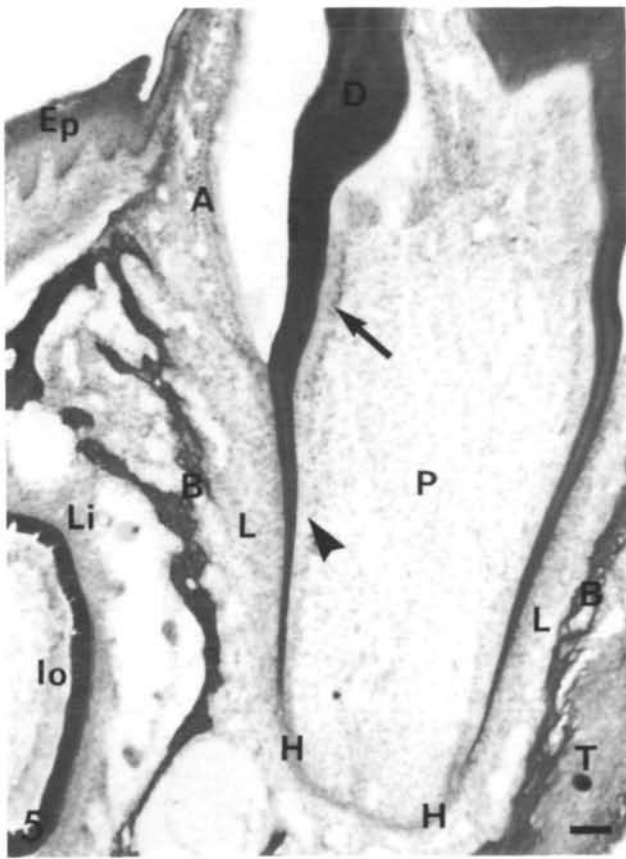
differentiation of odontoblasts, a very low signal intensity with pro-alpha 2(I) collagen mRNAs probe was detected within dental cells (data not shown). In 19-day embryos, the first overtly differentiated odontoblasts displayed 33% of the signal intensity observed with pro-alpha 1(I) collagen probe, while the follicular and pulp cells expressed only a faint signal (Figs 3, 9). The relative increase of pro-alpha 2(I) collagen mRNAs expression by odontoblasts was noted during postnatal development (Figs 7, 10). Discrete increase of pro-alpha 2(I) collagen mRNA signal was also noted at the stage when the follicular mesenchymal cells give rise to the periodontal ligament, while the signal pattern in dental pulp cells remained unchanged (Figs 7, 10). When crown and root odontoblasts were compared for the expression of alpha 2(I) collagen mRNAs, we noted that the root odontoblasts displayed lower mRNAs concentration than crown odontoblasts (Figs 7, 11).

Expression of pro-alpha 1(III) collagen gene

The expression pattern of pro-alpha 1(III) collagen gene did not vary significantly during the developmental phases studied. The highest pro-alpha 1(III) collagen mRNAs signal was detected in the follicular mesenchyme in 19-day embryos and in 10-day-old postnatal mice, when the follicular cells give rise to the periodontal ligament. The dental pulp cells showed



Figs 1-4. For legend see p. 693



Figs 5-8. For legend see p. 693

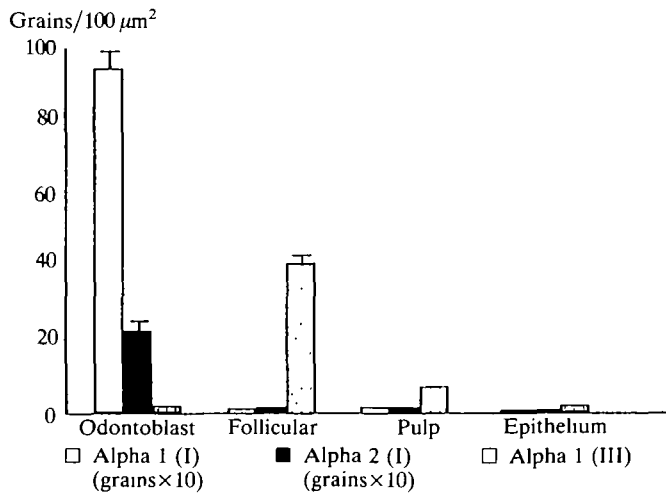


Fig. 9. Histogram showing the variations of the hybridization signal for procollagen mRNAs in 19-day embryo mice tooth germs. Values are expressed as the number of grains detected over a 100 μm² surface of the section, under an image analyzer. Results are the average counts of 60 equivalent areas. Note the differential signal intensity for alpha 1(I) mRNAs versus alpha 2(I) mRNAs in odontoblasts.

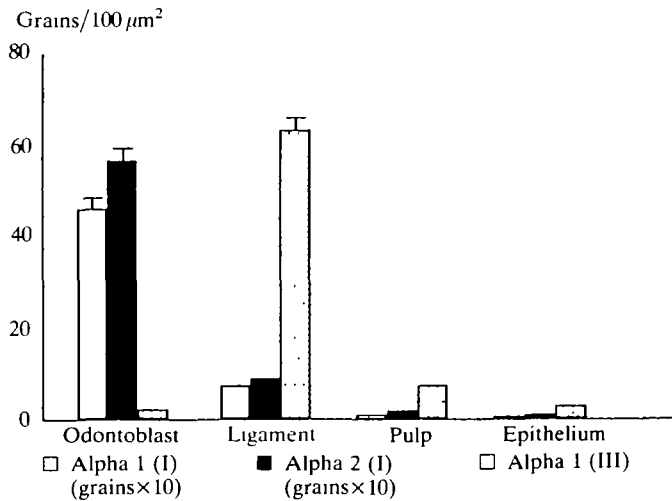


Fig. 10. Variations of the hybridization signal for procollagen mRNAs in 10-day-old postnatal mice tooth germs. Data were obtained as described in Fig. 9. Note the similar relative signal intensity for alpha 1(I) mRNAs and alpha 2(I) mRNAs in odontoblasts at this stage of development.

approximately 20% of the signal intensity detected in the follicular mesenchyme or periodontal ligament cells. No signal above the background level was observed in either odontoblasts or osteoblasts (Figs 4, 8, 9 and 10).

Discussion

The aim of this paper was to study collagen gene expression during mouse molar tooth development.

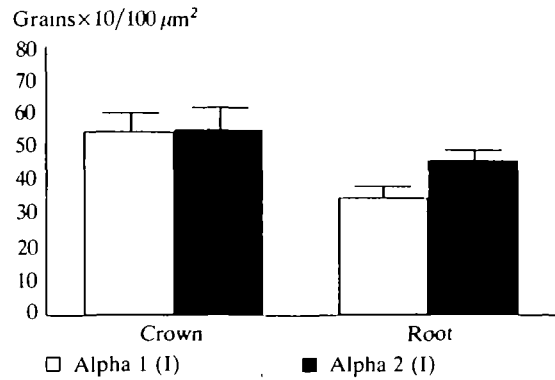


Fig. 11. Histogram comparing the hybridization signal for type I collagen mRNAs in crown and root odontoblasts of 10-day-old postnatal mice tooth germs.

Significant variations of expression of these genes were observed. The signal intensity of pro-alpha 1(I) collagen mRNAs at the onset of the odontoblast differentiation was high, but it decreased during postnatal development. In contrast, the signal intensity of pro-alpha 2(I) collagen mRNAs increased during the same period. Taken together, these data demonstrate the existence of independent regulation of these collagen mRNAs during the odontoblast differentiation.

Although the precise level of the collagen gene regulation remains to be elucidated (for review see, Bornstein and Sage, 1989), most experimental data suggest that the rate of procollagen synthesis is determined by the steady-state concentration of its mRNAs, therefore by a control exerted at the level of transcription (Rowe *et al.* 1978; de Wet *et al.* 1983; Hämmäläinen *et al.* 1985; Kravis and Upholt, 1985; Uitto *et al.* 1985; Jimenez *et al.* 1986; Kosher *et al.* 1986; Sandberg and Vuorio, 1987; Olsen and Prockop, 1989). During the early odontoblast differentiation process, we have detected the presence of high concentrations of pro-alpha 1(I) collagen mRNAs concomitantly with low concentrations of pro-alpha 2(I) collagen mRNAs. This shows that the rate of synthesis of both type I and type I trimer collagens are related to their specific mRNA concentration. Nevertheless, these results enhance the view that odontoblasts synthesize an excess of alpha 1(I) collagen chain (Munksgaard *et al.* 1978; Lesot, 1981; Lesot *et al.* 1981; Sodek and Mandell, 1982).

With our previous immunohistochemical data (Andujar *et al.* 1988), these data support the idea that the amplification of the synthesis of type I and type I trimer collagens is a significant step in the odontoblast differentiation process.

Earlier biochemical investigations have demonstrated the existence of a trimer of type I collagen in normal embryonic chick tendons and bone calvaria. This is a form that is less frequent in adult tissues (Jimenez *et al.* 1977). It has been shown also that adult odontoblasts *in vivo* or in organ culture, synthesize collagen type I trimer (Munksgaard *et al.* 1978; Sodek and Mandell, 1982). The use of calibrated probes with very similar size to the non repeated domain of collagen

alpha 1 and alpha 2 chains (1.5 and 1.6 kb respectively) and of identical specific radioactivity, allowed us to study the relative cellular pool of pro-alpha 1(I) and pro-alpha 2(I) mRNAs. In particular, we found that postnatal odontoblasts displayed similar cell concentrations of the two type I collagen mRNAs species. If postnatal odontoblasts are to synthesize collagen type I trimer, then translational and/or post-translational mechanisms should be involved in producing a higher ratio of alpha 1 to alpha 2 chains, but future studies will be needed to clarify this point.

Recently, functional differences have been reported between crown and root odontoblasts in terms of the quantity and quality of the phosphoproteins synthesized (Steinfert *et al.* 1989). The lower levels of pro-alpha 1(I) and pro-alpha 2(I) collagen mRNAs detected within the root odontoblasts may be interpreted as an additional phenotype characteristic of these cells. The high amounts of type I collagen mRNAs and the absence of type III collagen mRNAs in the osteoblasts confirm the metabolic activity of these cells as demonstrated elsewhere (Sandberg and Vuorio, 1987; Sandberg *et al.* 1988 and, 1989; Gerstenfeld *et al.* 1989).

The patterns of distribution of type I and type III collagen mRNAs within the various types of mesenchymal tissues studied agree with our previous intracellular immunolabeling analysis of the different collagen molecules (Andujar *et al.* 1988). Moreover, the absence of type III collagen mRNAs in odontoblasts strongly suggests that the corresponding gene is repressed and confirms a previous supposition that type III collagen synthesis does not occur during the odontoblast differentiation process (Andujar *et al.* 1988). Taken together, our data strengthen the idea for a specific transcriptional control of collagen genes in odontoblasts, which would be in agreement with previous observations on mutated pro-alpha 1(I) collagen gene expression in these cells (Kratochwil *et al.* 1989; Schwarz *et al.* 1990).

In summary, the mouse molar tooth development may constitute a suitable biological system to investigate expression of the genes coding for the extracellular matrix components for which tissue-specific controls and independent mechanisms for development exist.

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