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Original Article

Expression of the intermediate filament nestin during rodent tooth development

CATHARINA TERLING[1,2], ALON RASS[1,3], THIMIOS A. MITSIADIS[1], KAJ FRIED[4], URBAN LENDAHL[1] and JOANNA WROBLEWSKI[1,3]*

[1]*Department of Cell and Molecular Biology, Medical Nobel Institute, Karolinska Institutet, Stockholm,* [2]*Center for Oral Biology, Novum, Karolinska Institutet, Huddinge,* [3]*Department of Oral Diagnostics, School of Dentistry, Karolinska Institutet, Huddinge and* [4]*Department of Neuroscience, Karolinska Institutet, Sweden*

ABSTRACT The developing tooth represents a suitable model for understanding the molecular mechanisms involved in induction, morphogenesis and differentiation of organs. It is conceivable that the developmental changes could be reflected in the distribution of different cytoskeletal components and in this report we analyze the expression of the intermediate filament nestin during rodent tooth development at the protein and mRNA levels (by immuno light and electron microscopy, and by *in situ* hybridization). Nestin is expressed at all stages of tooth development, but the expression levels increase after birth in both ectodermal and ectomesenchymal derivatives. The shift in nestin distribution, from the proliferating dental lamina to the dental mesenchyme, indicates that nestin may be involved in inductive phenomena. At early stages of mineralization, nestin is seen within the apical parts of the presecretory ameloblasts. Nestin is also expressed in odontoblasts, both during odontogenesis and after tooth eruption. The increase in nestin expression from early to late developmental stages and sustained expression in a differentiated cell type contrasts with previously observed patterns of nestin expression during nerve and muscle development. This suggests that nestin could be used as a specific marker for the odontoblast.

KEY WORDS: *development, intermediate filaments, nestin, odontoblasts, odontogenesis*

Introduction

The ability of cells to adopt a variety of shapes and to carry out coordinated and directed movements depends on the cytoskeleton, a complex network of protein filaments that extends throughout the cytoplasm. This dynamic structure is formed by three classes of filamentous assemblies: microfilaments, intermediate filaments (IF) and microtubules. Many tissues undergo a sequential remodelling of IF component during development such that one set of IFs is expressed at early stages followed by expression of another set at later stages. Nestin is a recently characterized IF that has been shown to be expressed at early phases of CNS (central nervous system) and muscle development and to be replaced by neurofilaments and GFAP (glial fibrillary acidic protein) in nervous tissue and by desmin in muscle (Lendahl *et al.*, 1990; Sejersen and Lendahl, 1993; Dahlstrand *et al.*, 1995). Nestin and the neurofilaments belong to one branch of the evolutionary tree of IF genes, while for example vimentin, GFAP and desmin belong to the other branch (Weber *et al.*, 1991; Albers and Fuchs, 1992; Dahlstrand *et al.*, 1992b). Despite the relatively large evolutionary distance, nestin appears to copolymerize with vimentin, desmin (Sjöberg *et al.*, 1994b), and GFAP (Redies *et al.*, 1991).

To learn whether the sequential IF expression always includes nestin as an early-expressed IF we have analyzed nestin expression in the developing rodent tooth. The developing tooth provides us with a valuable model of development; combined in a single organ system are the phenomena of spatial organization, symmetry, acquisition of complex form and organ-specific cytodifferentiation. Odontogenesis is governed by sequential and reciprocal interactions between the epithelium and mesenchyme (Thesleff and Hurmerinta, 1981; Lumsden, 1988). Tissue recombination experiments suggest that the presumptive dental epithelium induces the underlying ectomesenchyme, which then condenses and differentiates. During advanced stages of tooth development some epithelial cells differentiate to ameloblasts, secreting enam-

Abbreviations used in this paper: IF, intermediate filaments; CNS, central nervous system; GFAP, glial fibrillary acidic protein; BMP, bone morphogenetic protein; E, embryonal day; IDE, inner dental epithelium; SI, stratum intermedium; p.n., post natal; PNS, peripheral nervous system; PA, paraformaldehyde; PBS, phosphate buffered saline; BSA, bovine serum albumin.

***Address for reprints:** Department of Cell and Molecular Biology, Medical Nobel Institute, Karolinska Institutet, S-171 77 Stockholm, Sweden. FAX: 46.8.345429.

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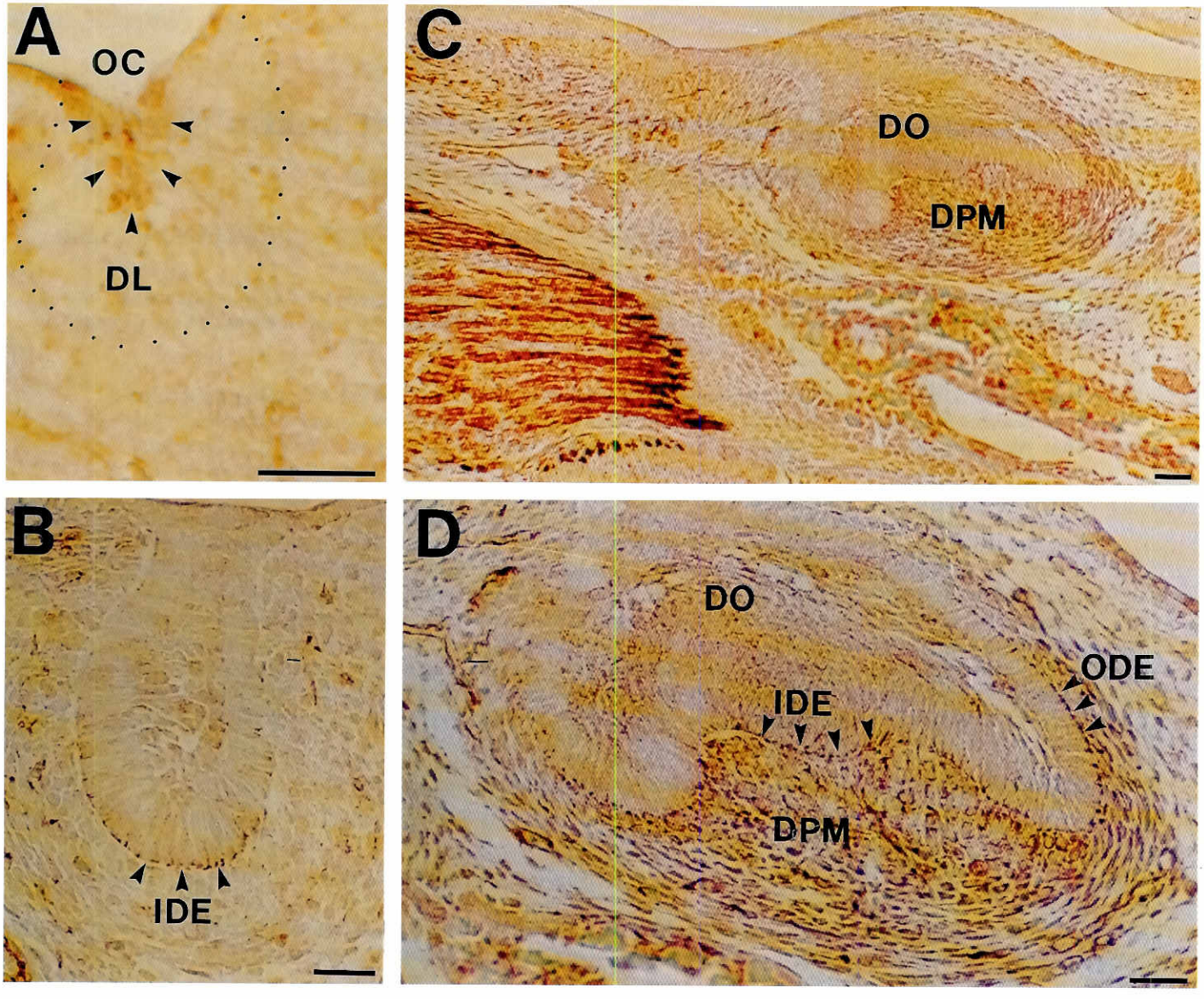


Fig. 1. Nestin distribution in the developing first lower molar. *(DL: dental lamina, DPM: dental papilla mesenchyme, IDE: inner dental epithelium, ODE: outer dental epithelium, DO: dental organ, OC: oral cavity).* **(A)** *At E13, immunoreactivity is seen within the central part (arrows) of the proliferating dental lamina (dotted line).* **(B)** *At bud stage (E14), the epithelial derivatives and the surrounding condensing ectomesenchyme show weak immunostaining. Note the more distinct staining within the basal membrane of the future inner dental epithelium (arrows).* **(C,D)** *At cap stage (E15), nestin remains in the dental organ. The strongest immunolabeling is seen within the cells of the dental papilla mesenchyme which are lined up along the inner dental epithelium. The basal membrane of the inner dental epithelium and parts of the outer dental epithelium show distinct immunoreactivity (arrows). Bars, 100 μm.*

el matrix, and some of the ectomesenchymal cells into odontoblasts, responsible for dentin matrix formation. Several molecules which play important roles in these processes have been identified. Bone morphogenetic protein (BMP-4) is expressed early in the forming dental lamina and then expression shifts to the condensing ectomesenchyme (Vainio *et al.*, 1993). Similar expression patterns have been observed for transcription factors of the homeobox type, e.g. *msx-1*, *msx-2*, and *Egr-1* (Hill *et al.*, 1989; MacKenzie *et al.*, 1991a,b, 1992; Vainio *et al.*, 1993). In this report we analyze the expression of nestin during different stages of odontogenesis. Our data reveal a strict temporospatial pattern of nestin expression during tooth development. In contrast to the nerve and muscle development, nestin expression becomes more prominent at later stages of tooth development, and serves as a specific marker for differentiated cell type in the adult tooth.

Results

In this report we have analyzed the expression of nestin during the different stages of tooth development. Our data show that

the expression changes in a temporally and spatially specific manner.

Embryonic tooth development

In the tooth buds of the mouse embryo (E13), nestin immunoreactivity was first detected in the central core of the proliferating dental lamina (Fig. 1A). Later, nestin was seen in the future internal dental epithelium (Fig. 1B). Nestin immunostaining could also be noted within the condensing ectomesenchyme in the forming dental papilla (Fig. 1B).

At cap stage (E15), nestin expression was seen both in the developing enamel organ and in the underlying condensing ectomesenchyme (Fig. 1C,D). The stellate reticulum was weakly stained while the developing dental papilla displayed strong immunoreactivity. The intensity of the staining in the dental papilla was high close to the inner dental epithelium (IDE) (Fig. 1C,D). Distinct immunolabeling could be detected at the borderline between the papilla (the odontoblast layer) and the IDE (Fig. 1D). The basal membrane of the outer dental epithelium was also nestin positive (Fig. 1D).

When the molars had reached the bell stage (E18), the dental follicle, the stratum intermedium (SI) and the pulp showed distinct nestin immunoreactivity (Fig. 2A). At this stage SI displayed the highest levels of epithelial immunoreactivity observed in the course of our experiments. The staining of the pulp was more intense in the pulp horns, and that of the SI in the bottom of the developing fissures (Fig. 2A). We were also interested to see how the levels of nestin protein corresponded to nestin mRNA levels. Sections from the same stage were therefore analyzed by *in situ* hybridization. The same divergence between epithelial and mesenchymal nestin expression could be followed at mRNA level (Fig. 2B).

Postnatal tooth development

During cytodifferentiation, in postnatal (p.n) day 3 (Fig. 3A-C), nestin expression was found in the ameloblasts, SI and in the odontoblastic cell layer, where the signal was most intense. In the cusps, both the SI cells and the ameloblasts were positive for nestin, the latter mostly in their apical parts (Fig. 3B). In the developing fissures, only the SI showed nestin immunolabeling (Fig. 3C). At day 5 p.n. (Fig. 4A-C), a reduction in nestin expression was observed in the central part of the pulp. The staining was now confined to the odontoblastic cell layer and their processes, extending into the full width of the dentin (Fig. 4B). Some of the looped and wire like structures next to the odontoblastic cells and their processes, possibly nerve cells, were positive for nestin (Fig. 4C).

At day 12 (Fig. 5A,B) and day 28 (Fig. 5C), the odontoblastic cell layer in the still unerupted mandibular molars remained strongly nestin positive together with the SI cells. In fully erupted teeth derived from 5.5-month-old adult rats, only the odontoblasts and their processes showed distinct nestin immunoreactivity (Fig. 6).

Immunogold electron microscopy

To achieve a more detailed understanding of nestin distribution we applied immuno electron microscopy. In molars from rats aged 5 days p.n., nestin immunoreactivity was detected in the ameloblasts, where a moderate immunogold labeling was noted

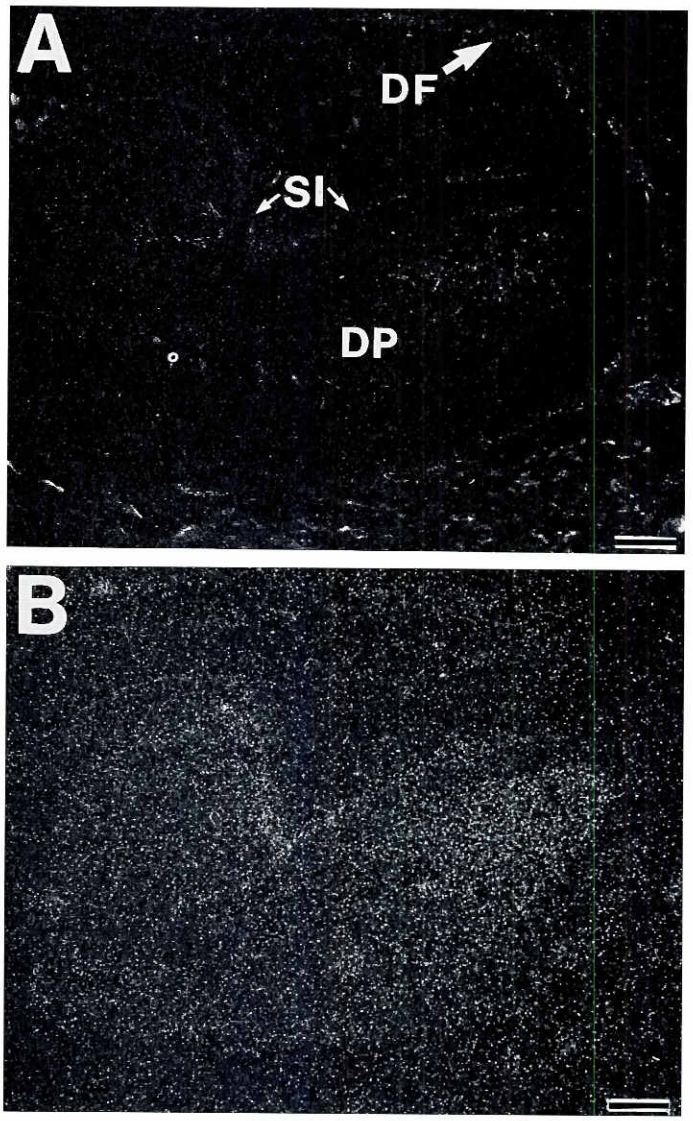
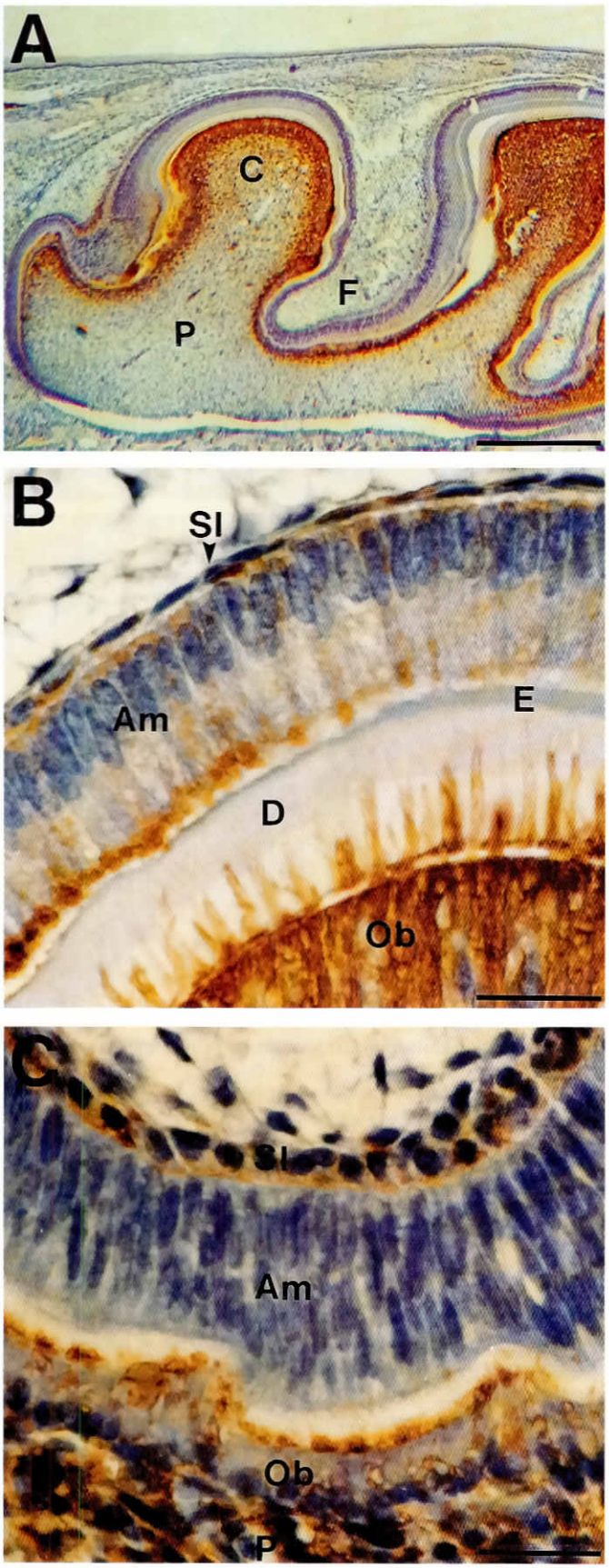


Fig. 2. Nestin distribution at (A) protein and (B) mRNA levels in the first lower molar at E18. *(DF: dental follicle, SI: stratum intermedium, DP: dental papilla).* **(A)** *At bell stage, immunoreactivity (detected with TRITC-conjugated secondary antibody) is seen within the cells of the dental follicle (arrow) and stratum intermedium (small arrows). Staining in the dental papilla is stronger in the forming pulp horns while the stratum intermedium shows the strongest nestin staining in the forming fissures.* **(B)** *Corresponding section processed for in situ hybridization. The nestin mRNA distribution corresponds well to the protein staining. Bars, 200 μm.*

in the basal part of the cells, while labeling appeared to be somewhat stronger in the apical region (Figs 7A-B). A robust nestin immunoreactivity was also present in the elongated cells of the stratum intermedium (Fig. 7C). In the pulp of the early postnatal molar, immunogold nestin staining was marked in both cell bodies and processes of the odontoblasts (Fig. 8). In addition, distinct nestin immunoreactivity was seen repeatedly both in cells of the subodontoblast region, and in connective tissue cells of the pulp proper. In the adult molar, nestin immunoreactivity was still prominent in the odontoblasts, and strongly labeled odontoblast



processes were seen extending through the predentine and into the dentine (Fig. 9A). As seen also in the immature pulp, nestin-positive mesenchymal cells which varied in morphological appearance were scattered both in the subodontoblast zone and in deeper pulpal regions (Fig. 9B). However, the density of such cells appeared to be lower than in the 5-day-old molar. In addition, nestin immunoreactivity was seen in the cytoplasm of Schwann cells ensheathing unmyelinated – but not myelinated – nerve fibers (Fig. 9C).

Discussion

Expression of the intermediate filament nestin has previously been analyzed during nerve and muscle development. In both cases nestin expression is largely observed during early phases of development (Lendahl *et al.*, 1990; Sejersen and Lendahl, 1993). The picture of nestin expression during tooth development that emerges from this study is somewhat different: while nestin is also expressed during early stages, expression levels increase during odontogenesis in certain cell types and nestin continues to be expressed in a differentiated cell population, the odontoblasts, in the adult animal.

Different nestin expression profiles in the ectomesenchymal and epithelial derivatives in the tooth

Nestin exhibits complex expression patterns during tooth development and the expression profiles differ in epithelial and mesenchymal cells. In epithelial cells the expression was generally low and maximal mRNA and protein levels were found at bell stage of tooth development in ameloblasts and cells of SI. Nestin expression was to a certain extent down regulated at later stages and nestin immunoreactivity disappeared from the ectodermal derivatives after tooth eruption. In contrast, mesenchymal derivatives revealed a more distinct nestin expression pattern and maximal levels were recorded later in development.

In other tissues, CNS and muscle, nestin is expressed at early stages of histogenesis. In the early CNS, the expression is predominantly confined to the progenitor cells (Hockfield and McKay, 1985; Frederiksen and McKay, 1988; Dahlstrand *et al.*, 1995). In myogenesis nestin is expressed from myoblasts into the myotube stages, suggesting that post-mitotic cells may express nestin (Sjöberg *et al.*, 1994b). In the present study we observed expression of nestin in odontoblasts and their processes, which would seem to be at odds with the nervous system and muscle expression patterns. It should however be remembered that odontoblasts normally are quiescent when differentiated, but maintain synthetic potential, which can be triggered by several noxious processes (trauma, caries, temperature fluctuations,

Fig. 3. Nestin distribution in the first lower molar at p.n. day 3. *(P: pulp, C: cusp, F: fissure, D: dentin, E: enamel, Am: ameloblasts, Ob: odontoblasts, SI: stratum intermedium).* **(A)** *Nestin is widely distributed within the pulp where the staining is concentrated to the cusps and fissures.* **(B)** *Higher magnification of a cusp. Nestin is seen within the cells of stratum intermedium, in the apical parts of the ameloblasts and in the odontoblasts and their processes, extending into the forming dentin.* **(C)** *The odontoblasts lining the fissures display a lower level of immunoreactivity than the odontoblasts in the cusps. The stratum intermedium is immunopositive while the ameloblasts are negative. Bars: A, 1000 μm; B,C, 100 μm.*

etc.). Induction of nestin in response to external factors has also been observed in nervous tissue and muscle. Thus, nestin is expressed in CNS (Dahlstrand *et al.*, 1992a; Tohyama *et al.*, 1992) and PNS (Florenes *et al.*, 1994) tumors as well as in cell lines immortalized from early CNS (Frederiksen and McKay, 1988; Redies *et al.*, 1991). Nestin upregulation is also observed in response to CNS injury (Frisén *et al.*, unpublished observation). Similarly, nestin expression is observed in muscle fibers from patients with Duchenne's and Becker's muscular dystrophy (Sjöberg *et al.*, 1994a). It is thus possible that the unique characteristic of the odontoblasts, i.e. retaining the capacity to regenerate after trauma as long as the odontoblasts are viable, requires nestin. Odontoblasts are strongly polarized with basally located nuclei and apical secretory and junctional complexes. Maintenance of the polarized state requires the cytoskeleton integrity (Ruch *et al.*, 1982), but the question remains, to what extent nestin expression in odontoblasts plays a role for the cytoskeletal organization. It will be interesting to analyze nestin distribution after trauma.

It has previously been shown that other IFs, i.e. vimentin and keratins, are expressed during tooth development (see Table 1). During both CNS and muscle development, nestin expression is intimately associated with vimentin expression, and it may be possible that the apparent lack of phenotype in mice in which the vimentin gene has been inactivated by homologous recombination (Colucci-Guyon *et al.*, 1994) could, at least in part, reflect a functional replacement by nestin. The data presented here suggest that nestin is expressed in at least one region where vimentin is absent, namely in preameloblasts and cells of SI (see Table 1), showing that nestin is not always coexpressed with vimentin. It is also interesting to observe that nestin expression appears to be followed by expression of keratins in differentiated ameloblasts, since keratins and nestin most likely do not copolymerize. In both muscle and CNS development, the ensuing IFs can polymerize with nestin.

Little is yet known about factors controlling nestin expression. Regulatory elements for the early CNS and muscle expression patterns have recently been identified (Zimmerman *et al.*, 1994), but no tooth expression was observed with the reporter gene. This may suggest that expression in tooth is regulated by a different set of response elements located outside the mapped regions. It is noteworthy that nestin expression pattern during early odontogenesis strongly resembles the expression of mRNA coding for bone morphogenetic proteins, BMP-4 and BMP-6, members of the transforming growth factor beta family (Vainio *et al.*, 1993). BMP-4 has been proposed to have a role in early tooth morphogenesis and BMP-6 is suggested to be involved in odontoblast differentiation and functional maturation (Heikinheimo, 1994).

In conclusion, our analysis of nestin expression during tooth development provides further evidence for a dynamic cytoskele-

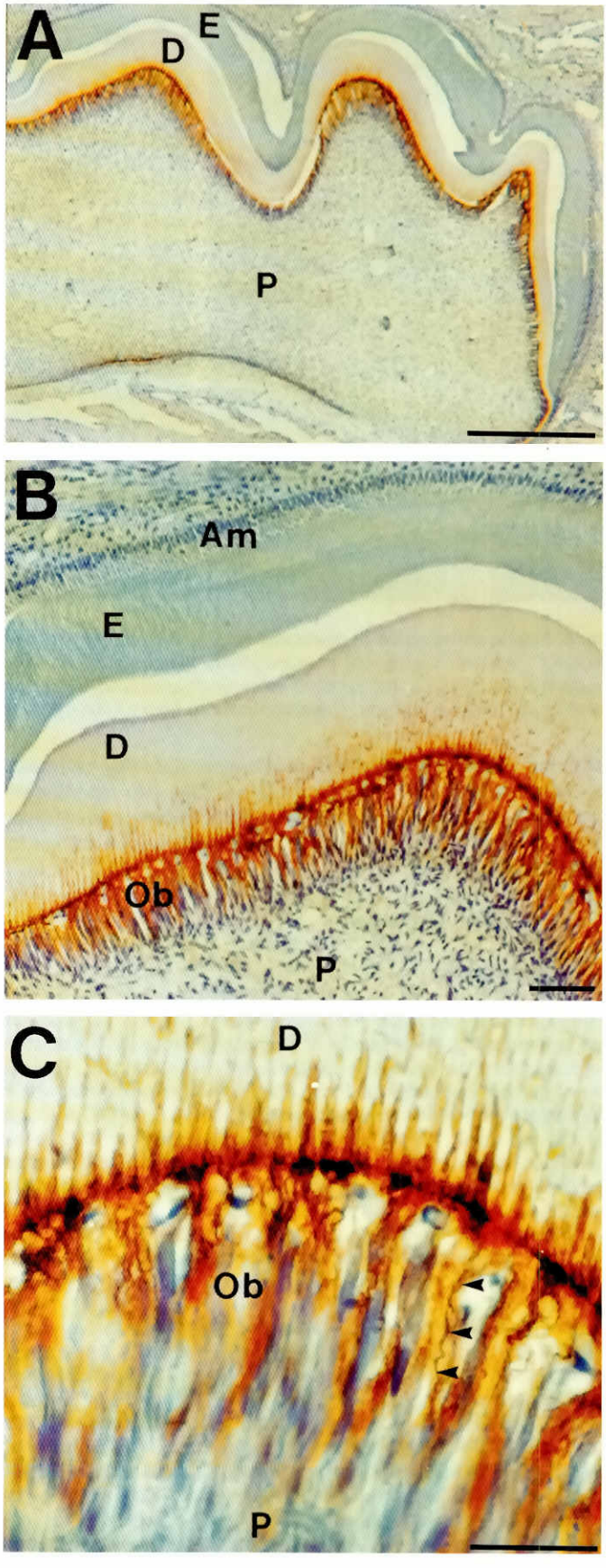


Fig. 4. Nestin distribution in the first lower molar at p.n. day 5. *(P: pulp, D: dentin, E: enamel, Am: ameloblasts, Ob: odontoblasts).* **(A)** *The nestin distribution is exclusively located within the odontoblastic cells of the pulp.* **(B)** *Nestin positive odontoblasts along a cusp.* **(C)** *Higher magnification showing the odontoblasts and their processes, followed by wire like structures (arrows), extending from the pulp into the dentin. Bars: A, 1000 μm; B,C, 100 μm.*

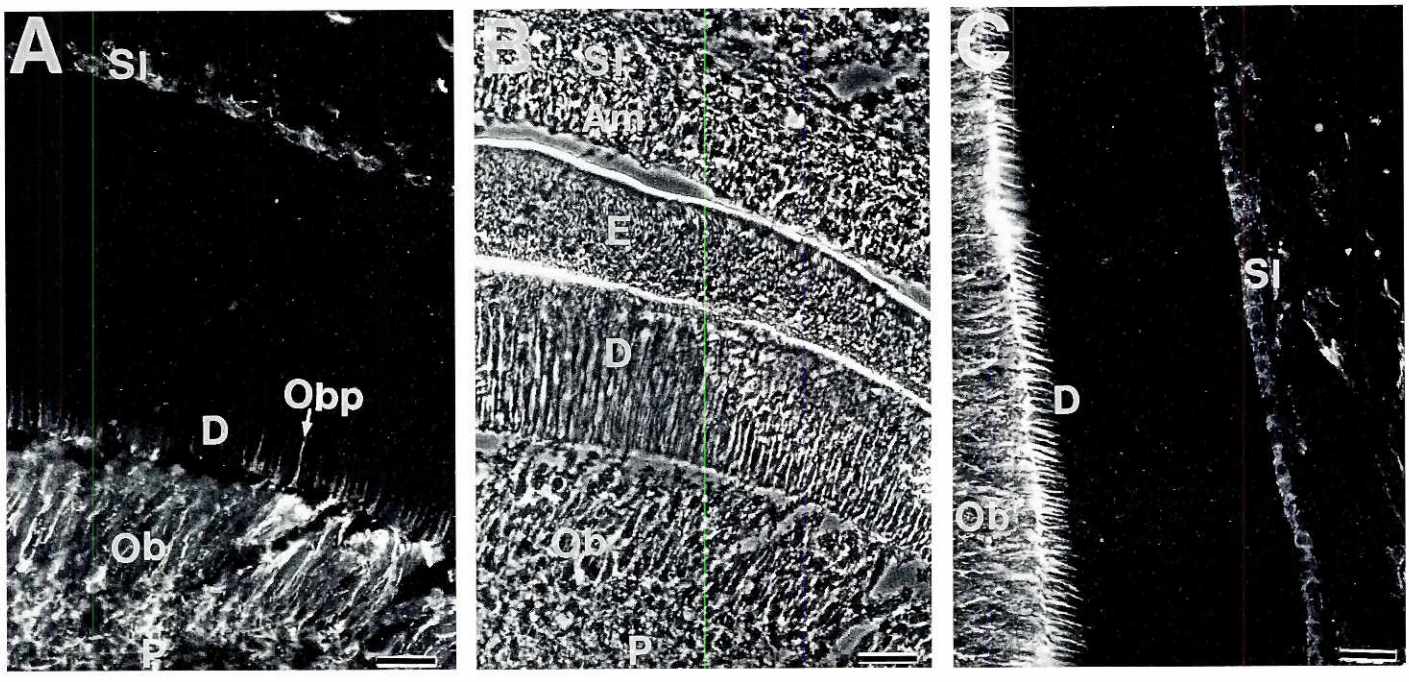


Fig. 5. Nestin distribution in postnatal stages. *(SI: stratum intermedium, Ob: odontoblasts, Obp: odontoblastic processes, Am: ameloblasts, D: dentin, E: enamel, P: pulp).* **(A)** *Immunolabeled (fluorescent) odontoblasts and processes (small arrow) at p.n. day 12. Note that the stratum intermedium also shows distinct immunoreactivity. Phase-contrast micrograph* **(B)** *of the same section.* **(C)** *Day 28, the odontoblasts and stratum intermedium are distinctly stained. The nestin positive odontoblastic processes are clearly extending into the dentin. Bars, 100 μm.*

ton in this organ and shows that nestin is permanently expressed in odontoblasts. To date, no genes that are specifically expressed in odontoblasts throughout development have been identified and nestin may thus be a potentially useful marker for this cell type.

Materials and Methods

Tissue specimens

Balb-C mice were obtained from Bomholt, Denmark. The animals were kept and mated at the local animal facility. The onset of gestation was determined by the presence of vaginal plugs (day 0). The animals were sacrificed on embryonic days 13, 14, 15, 17 and 18. Fetuses were dissected out of the fetal membranes and fixed for 5 h at 4°C in 4% paraformaldehyde/phosphate buffered saline (PA/PBS) solution, pH 7.4, and either 1) immersed in 10% sucrose/PBS for 12 h before freezing in liquid nitrogen, or 2) dehydrated in graded ethanol, cleared in xylene and embedded in paraffin. The heads of the frozen fetuses were mounted in Tissue-Tek (Miles, Naperville, IL, USA), 7 μm-thick frontal and sagittal sections were cut on a cryostat microtome. Both cryo- and paraffin-sections were collected on silanized histological glass slides. The cryosections were stored at -70°C until further processing.

Sprague-Dawley rats were obtained from BK Universal (Stockholm, Sweden) and kept and mated at the local animal facility. The animals were sacrificed either by cervical dislocation (postnatal (p.n.) day 3 and 5), CO_2 asphyxiation (12, 20, 28 p.n. days and 5.5 months old) or, after chloral hydrate anesthesia, transcardial perfusion with tyrode's solution followed by 4% PA/PBS (5 days and 4 months, used for EM immunohistochemistry). The lower jaws of 3- and 5-day-old rats were dissected and fixed in 4% PA/PBS, dehydrated and paraffin-embedded. The jaws were sectioned in the parasagittal plane (7 μm thick). Serial sections were collected on silanized slides. The jaws from rats older than p.n. day 5 were fixed for 16 h in 4% PA/PBS, decalcified in cacodylate-buffered EDTA solution (Bjurholm *et al.*, 1989) and immersed in 10% sucrose/PBS for

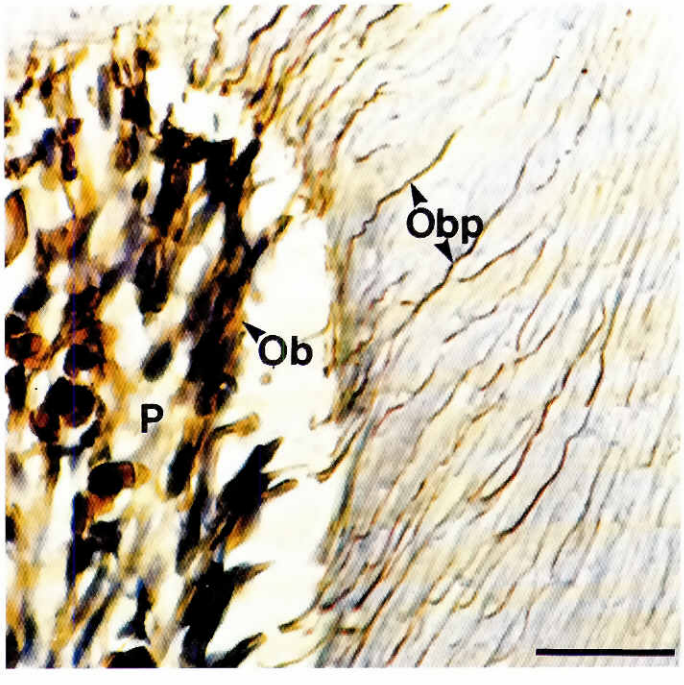


Fig. 6. Nestin distribution in adult molar. *(Ob: odontoblasts, Obp: odontoblastic processes, P: pulp). Nestin remains within the odontoblasts and their processes (arrows) extending into the mature dentin. Bar, 100 μm.*

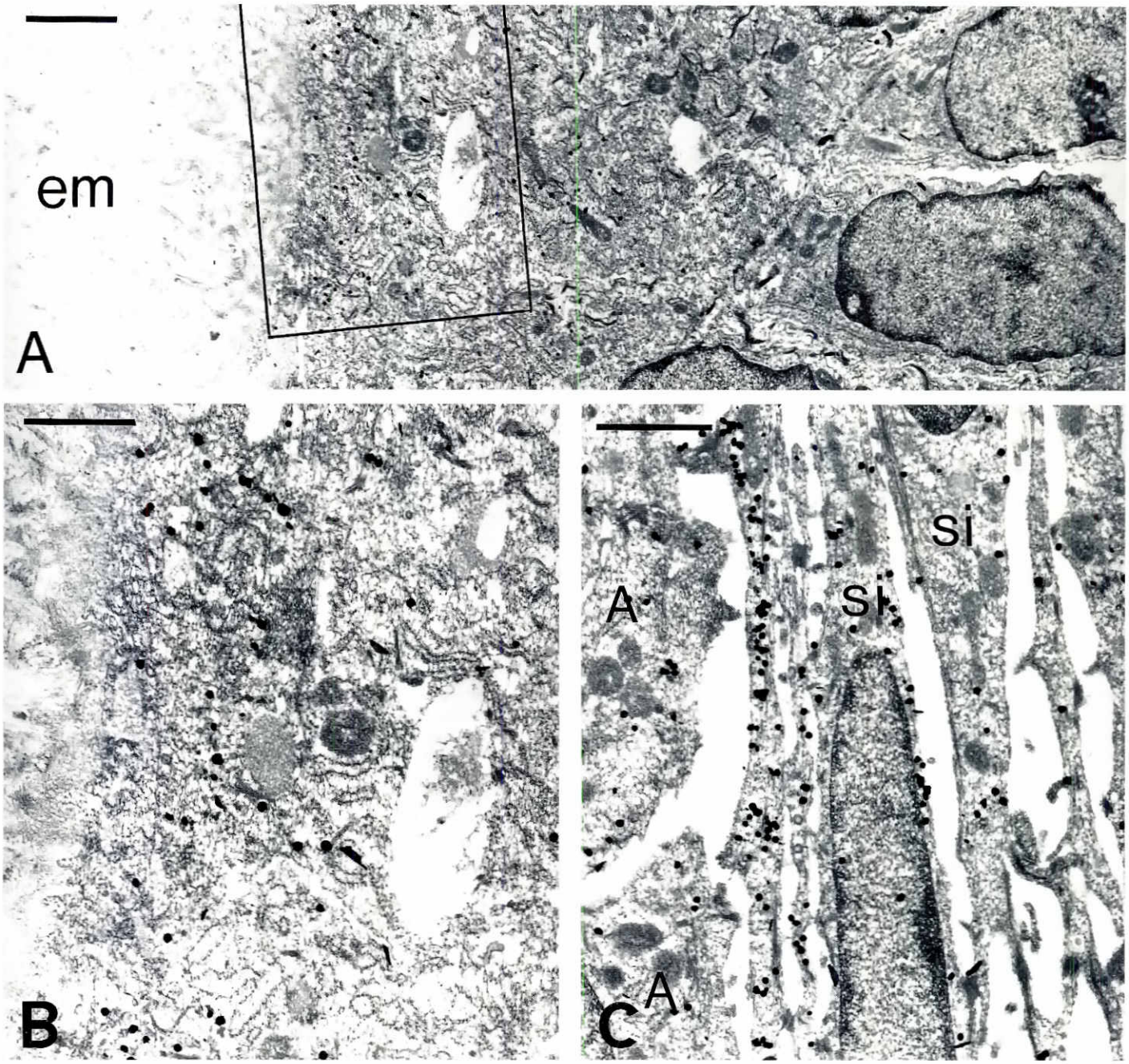


Fig. 7. Electron micrographs from a 5 day-old molar after nestin immunogold-silver labeling. (A) *Immunogold labeling in the ameloblast layer. em: enamel matrix. Boxed region is enlarged in* **(B),** *which shows an accumulation of gold particles in the apical parts of two ameloblasts.* **(C)** *Inner part of the stratum intermedium (SI), where gold labeling is evident in cell bodies and slender cellular processes. A: ameloblast. Bars: A, 2 μm; B,C, 1 μm.*

12 h before freezing in liquid nitrogen. The specimens were sectioned as described above.

Antibody

For detection of nestin, a rabbit anti-nestin antiserum No 130 (Sjöberg *et al.*, 1994b) diluted 1:500 in PBS (pH 7.4) was used. This antiserum, which was raised against the C-terminal region of rat nestin, specifically identifies nestin in immunocytochemistry and in Western blots (Dahlstrand *et al.*, 1992a; Sejersen and Lendahl, 1993) and does not crossreact with other known intermediate filaments (Sjöberg *et al.*, 1994a,b). Furthermore, the No 130 antiserum generates the same pattern in immunocytochemistry and Western blots as the monoclonal anti-nestin antibody Rat.401 (Hockfield and McKay, 1985) and a new polyclonal antiserum against human nestin (#4350) (Lendahl, unpublished observations).

Immunohistochemistry

Cryo- and paraffin-sections of the mouse embryos were incubated with the primary antibody for 3 h at room temperature, washed in PBS and incubated for 1 h with the secondary antibody (rhodamine-conjugated

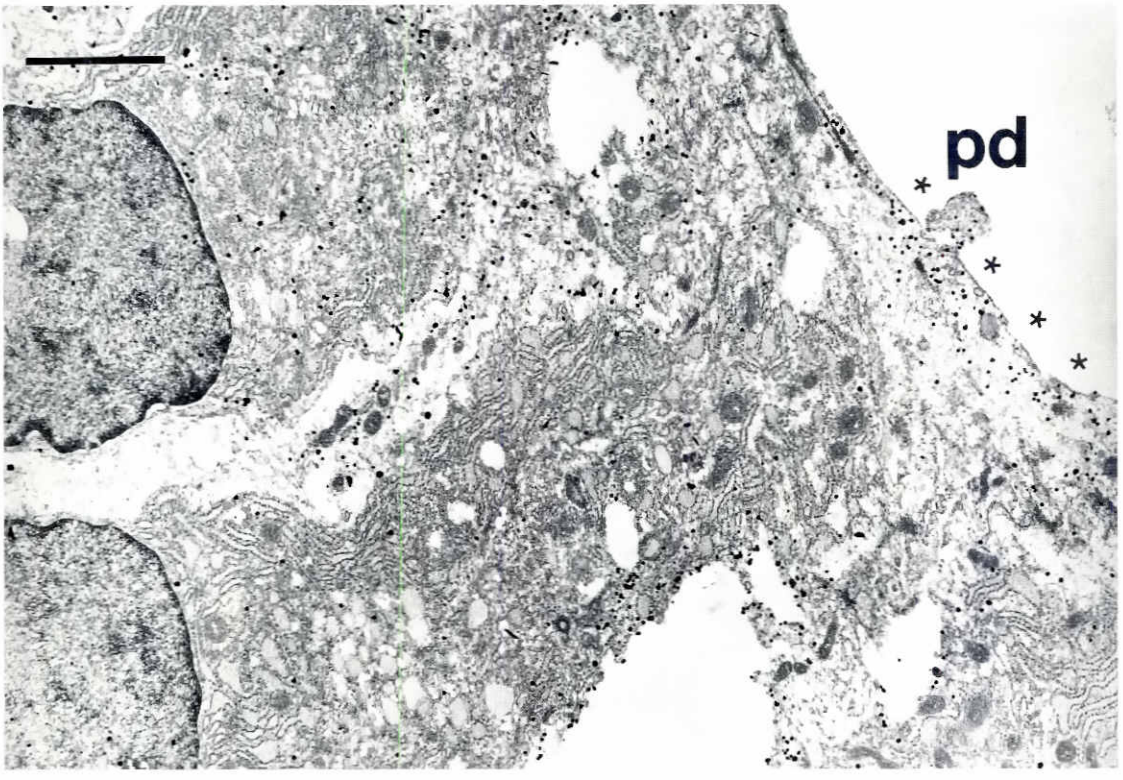


Fig 8. Electron micrographs from a 5 days old rat molar after nestin immunogold-silver labeling. *Nestin-immunoreactive odontoblasts at the pulp-predentin border. Gold particles are scattered through the cytoplasms with focal accumulations, especially in the short odontoblast processes (*) which extend into the predentin (pd). Bar, 2 µm.*

swine anti-rabbit immunoglobulins, – Dakopatts A/S, Glostrup, Denmark – diluted 1:50 in PBS). The sections were mounted in PBS/glycerol 1:1 and analyzed by epifluorescence microscopy. All paraffin sections used were deparaffinized in xylene, rehydrated in PBS and permeabilized with 0,2% Triton X-100 in PBS for 3 min. The specificity of the staining was controlled by omitting incubation with the primary antibody.

Immunofluorescence staining of 12, 20, 28 p.n. days and 5.5 month-old rat mandibles was also carried out. As a positive control, developing skeletal muscle of the E18 mouse limb was used. The unspecific binding of the antibodies was blocked with 3% bovine serum albumin (BSA)/PBS for 30 min before the primary antibody was added and sections were incubated at 4°C overnight. After washing in PBS, the sections were then incubated for 1 h with the secondary antibody (biotinylated swine anti rabbit immunoglobulins – Dakopatts A/S, Glostrup, Denmark – diluted 1:300 in PBS). The immunosignal was detected with a ABC-horseradish peroxidase kit (Dakopatts, Denmark) and diamino benzidine tetrahydrochloride as a substrate. Some of the sections were counterstained with hematoxylin eosin and photographed in a Nikon UFX II Labphot microscope.

Immunogold electron microscopy

For pre-embedding immunogold electron microscopy, molars from 5 day- and 4 month-old animals were cut into 50 µm-thick cryostate sections, rinsed in PBS and immersed in nestin antiserum as described above. Subsequently, sections were rinsed, incubated with 0.8 nm gold particle-conjugated goat anti-rabbit antibodies (Aurion), rinsed again, and osmicated by a modification of Marchi method (Hildebrand and Aldskogius, 1986). Finally, sections were intensified by use of a silver enhancement reaction (Intense M, Janssen Biotech.) and embedded in Vestopal W between transparent acetate foils. This permitted examinations at the light microscopical level, and selected regions were then sectioned by use of an LKB Ultrotome to 600 Å, contrasted with uranyl acetate and lead citrate and examined in a Philips CM12 electron microscope.

In situ hybridization

A 48-mer antisense oligonucleotide probe to mouse nestin: 5´-GGT CCC TGG GAA TCC TGG ATT TCT TCT GTG TCC AGA CCA CTT TCT TGT-3´, corresponding to a sequence encoding the carboxy-terminal region (Dahlstrand *et al.*, 1995) was purchased from Scandinavian Gene

TABLE 1

PRESENCE AND ABSENCE OF VARIOUS CYTOSKELETON PROTEINS IN DIFFERENT DENTAL TISSUES

DENTAL TISSUE	CYTOSKELETON PROTEINS							
	intermediate filaments					micro-filaments		micro-tubules
	desmin	vimentin	nestin	pre-keratin	keratin	actin	α-sm actin	tubulin
dental papilla	+ (3)	+ (1, 3)	+		- (3)	+(1, 3)	+ (3)	
odontoblasts	- (3)	+ (1, 3)	+				- (3)	
odontoblastic processes	- (3)	+ (2)	+			+ (2)	- (3)	+ (2)
pulp	+ (3)	+ (1, 3)	+	- (1)	- (3)		+ (3)	
endothelium (vessel walls)	+ (3)					+(1, 3)	+ (3)	
enamel organ	- (3)	- (1, 3)	+	+ (1)	+ (3)	+(1, 3)	- (3)	
ameloblasts			-	+ (1)		+ (1)		
stratum intermedium		+						

The presence (+) and absence (-) of various cytoskeleton proteins in the different dental tissues according to: (1) Lesot *et al.*, 1982; (2) Sigal *et al.*, 1985; (3) Lombardi *et al.*, 1992 and the current study on nestin.

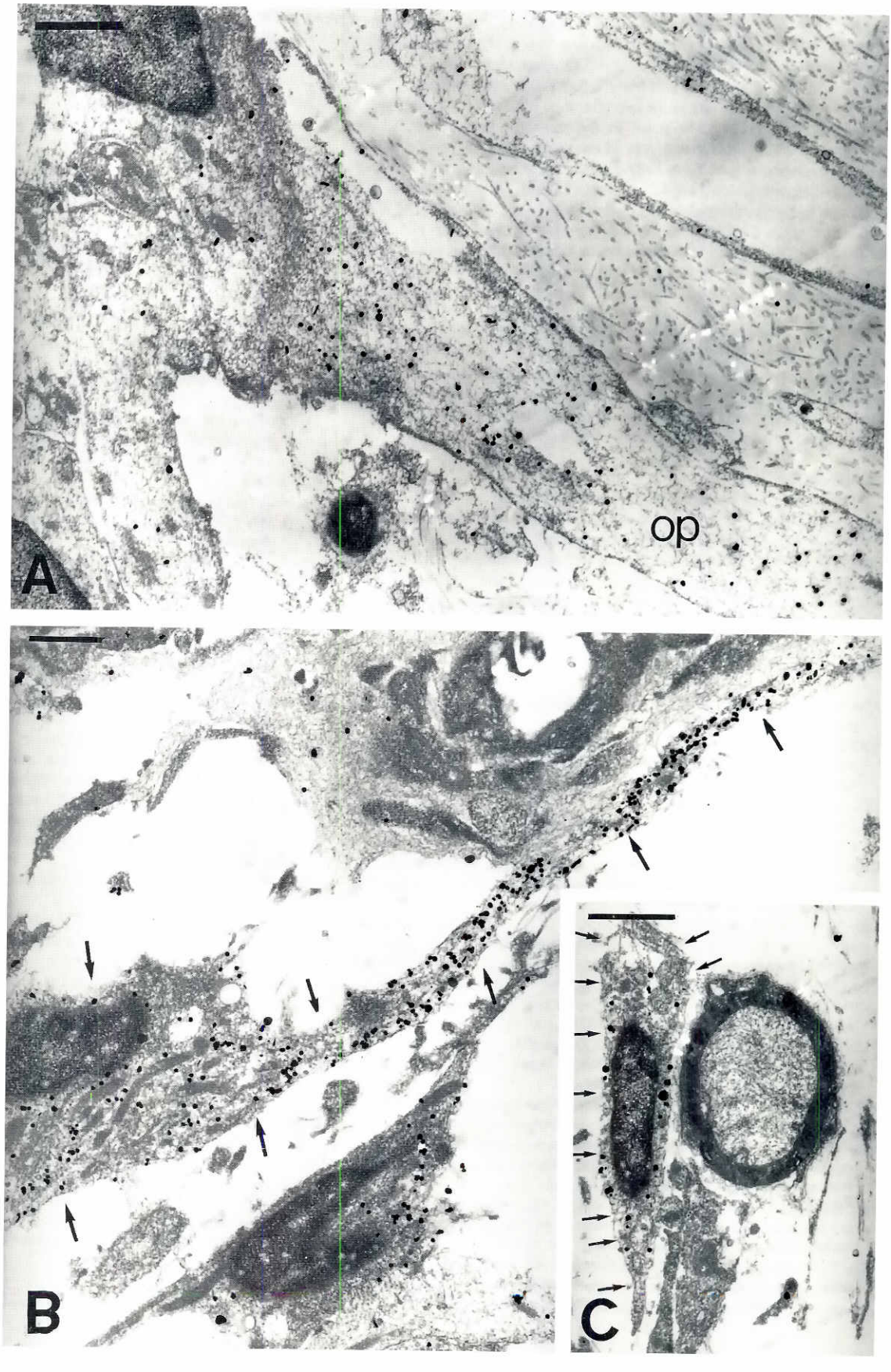


Fig. 9. Electron micrographs from an adult (4 month-old) rat molar after nestin immunogold-silver labeling. (A) *An odontoblast with a marked nestin-positive odontoblast process (op) which continues through the predentin into the dentin.* **(B)** *Cell body and long slender process of a heavily nestin labeled connective tissue cell (arrows) located in the pulpal mesenchyme central to the subodontoblast zone.* **(C)** *is from the same region as* **(B)** *and shows* **(A)** *nestin-labeled Schwann cell (arrows) which ensheaths an unmyelinated axon. The Schwann cell that surrounds the adjacent myelinated nerve fiber appears to be unlabeled. Bars, 1 μm.*

Synthesis (Köping, Sweden). The probe was labeled at the 3´-end using α^{35}S-dATP (NEN, Boston, MA, USA) and terminal deoxynucleotidyl transferase (IBI, New Haven, CO, USA). Cryosections from 16, 17 and 18 day-old mouse embryos and from 3, 5, 12, 20, 28 postnatal days as well as 5.5 month-old rats were hybridized at 42°C for 16 h with radio labeled probe ($1x10^6$ CPM/slide) in hybridization solution (50% formamide, 4xSSC, 1xDenhardt, 1% N-laurylsarcosine (Sigma), 0.02 M sodium phosphate (pH 7.0), 10% dextran sulphate, 250 μg/mg yeast tRNA, 500 μg/ml sheared salmon sperm DNA and 200 mM dithiothreitol). After hybridization, the slides were washed four times in 1xSSC at 55°C and two times in 0.5xSSC (15 min per wash). In the last wash the slides were allowed to cool to room temperature, rinsed in distilled water, dehydrated in graded ethanol, air dried and exposed to X-ray film (Hyperfilm ß-max, Amersham). After exposure for 8 days at -20°C the film was developed in D19 (Kodak developer). The slides were dipped in liquid film emulsion (NTB2, Kodak), developed after 6 weeks exposure at 4°C in D19 and the autoradiographs were examined in a light microscope equipped for epipolarization and dark field analysis (Nikon UFX II). Developing skeletal muscle was used as a positive control of hybridization and as negative control, a collagen type II probe, of the same length and specific activity as nestin probe, was used.

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