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Cellular distribution of the new growth factor Pleiotrophin (HB-GAM) mRNA in developing and adult rat tissues

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Summary. Pleiotrophin (PTN), also known as HB-GAM, belongs to an emerging cytokine family unrelated to other growth factors. We report here the first comprehensive study using in situ hybridization on the cellular distribution of this new heparin-binding growth factor mRNA in rat tissues. PTN mRNA was developmentally expressed in many – but not all – neuroectodermal and mesodermal lineages, whilst no PTN mRNA was detected in endoderm, ectoderm and trophoblast. PTN mRNA was found in the nervous system throughout development, with a post-natal peak of expression. In the adult nervous system, significant expression persisted in hippocampal CA1 pyramidal neurons and in cortical neurons, but also in different non-neuronal cells types in various locations (olfactory nerve, cerebellar astrocytes, pituicytes, Schwann cells surrounding the neurons in sensory ganglia). PTN mRNA was also found during development in the mesenchyme of lung, gut, kidney and reproductive tract, in bone and cartilage progenitors, in dental pulp, in myoblasts, and in several other sites. Expression was differently regulated in each location, but usually faded around birth. In the adult, PTN mRNA was still present in the meninges, the iris, the Leydig cells of the testis and in the uterus. PTN mRNA was also strongly expressed in the basal layers of the tongue epithelium, which is the only epithelium and ectodermal derivative to express PTN mRNA, and this only after birth. PTN is known to be a growth factor for perinatal brain neurons and a mitogen for fibroblasts in vitro. Recently, trophic effects on epithelial cells and a role as a tumour growth factor have been reported. The mechanisms of regulation and the functions of PTN are however still uncertain. Its expression pattern during development suggests important roles in growth and differentiation. Moreover, the presence of PTN mRNA in several adult tissues and the up-regulation of PTN mRNA expression in the gravid uterus indicate that PTN also has physiological functions during adulthood.

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Key words: Development – Gene expression – *in situ* Hybridization – Mesoderm – Neuroectoderm

Introduction

Pleiotrophin (PTN) is a new heparin-binding protein with both neurite outgrowth-promoting activity on embryonic rat brain neurons in culture (Rauvala 1989) and mitogenic activity toward rat and mouse fibroblasts (Milner et al. 1989). It was initially isolated from early postnatal rat brain (Rauvala 1989) and bovine uterus (Milner et al. 1989). PTN has previously been known as p18 (Rauvala 1989), HBGF-8 (Milner et al. 1989) or HB-GAM (Merenmies and Rauvala 1990). The term Pleiotrophin was subsequently proposed to account for its suspected widespread role (Li et al. 1990).

PTN cDNA has recently been cloned in rat (Merenmies and Rauvala 1990), bovine and human cells (Li et al. 1990). The predicted amino acid sequence of PTN is highly conserved between these three species (Li et al. 1990) and contains a signal sequence suggesting that the protein is secreted (Merenmies and Rauvala 1990). PTN binds strongly to the extracellular matrix in vitro (Li et al. 1990) and an autocrine or paracrine action has been suggested (Rauvala 1989).

Although PTN shares some chemical similarities with the family of fibroblast growth factors (FGF) (Rauvala 1989), namely the heparin-binding ability, it does not belong chemically to the FGF family as formerly thought (Milner et al. 1989). The deduced amino acid sequence of PTN is distinct from any other known growth factor family (Merenmies and Rauvala 1990; Li et al. 1990). An almost 50% sequence homology was only found with the predicted amino acid sequence of the MK1 gene product (Merenmies and Rauvala 1990), a retinoic acid (RA)-induced differentiation factor isolated from mouse embryonal carcinoma cells (Kadomatsu et al. 1988). This factor is also expressed during normal mouse embryogenesis and its heparin-binding ability

has been demonstrated (Tomomura et al. 1990). On the basis of these data, the existence of a new gene family of developmentally regulated Pleiotrophins has been proposed (Li et al. 1990). This concept has been confirmed recently since expression of another member (called Ri-HB) of this new family has been reported in myoblasts and fibroblasts from early chick embryos (Raulais et al. 1991). After analyses by the Western and Northern blot techniques, developmental regulation of PTN expression has been reported in postnatal rat brain and in some peripheral tissues (Merenmies and Rauvala 1990; Li et al. 1990). Despite the fact that PTN mRNA expression has been reported as early as embryonic day 13 (Li et al. 1990), these publications have focused on the period just before and a few weeks after birth. Histological data are not available yet, and expression at different stages of embryonic life and at adulthood has also not been assessed in detail.

We report here the cellular localization of PTN mRNA, using in situ hybridization histochemistry (ISHH) during embryonic and postnatal development as well as in adult rat organs.

Materials and methods

Wistar rats were purchased from IFFA-CREDO (Brussels, Belgium) and housed in our University facility under standard conditions. Virgin females (weight 220–250 g) were presented to males overnight. Presence of a vaginal plug or spermatozoa in the vaginal smear in the morning determined day 0 of gestation (EO). Embryos were obtained at E11, E13, E15, E17, E19 (one litter each). Development was staged according to gestational age and checked for external developmental criteria (Christie 1964). A minimum of two embryos, randomly chosen, were used for each stage. Two newborns (day of birth = P0), P7, P14 and P21 pups and four adult (> 3 months) were also used. Postnatal animals were decapitated after ether anaesthesia, and embryos were killed by cold exposure. Tissues were quickly collected and properly oriented, embedded in OCT compound (Tissue-Tek, Miles), frozen in dry-ice-cooled 2-methylbutane and stored at −80° C. Cryostat sections (14 µm) were mounted on 0.1% poly-L-lysine (Sigma)-coated slides and stored at −20° C until use. ISHH was performed according to a previously published method (Schiffmann and Vanderhaeghen 1991). Briefly, slides were fixed in a 4% paraformaldehyde solution for 30 min, rinsed twice in 1 × PBS (phosphate-buffered saline), dehydrated and delipidated through a graded series of ethanols (60–100%) and chloroform and then air dried. Sections were incubated overnight at 42° C with 8×10^5 cpm per section of [^{35}S]-labelled probe ($4–8 \times 10^8$ cpm/µg) in hybridization buffer consisting of 50% formamide, 4 × SSC (1 × SSC = 0.15 M NaCl/0.015 M sodium citrate; pH 7.4), 0.02 M sodium phosphate at pH 7.4, 1 × Denhardt's solution, 1% sarcosyl, 10% dextran sulphate, 50 µg/ml yeast tRNA, 100 µg/ml denaturated salmon sperm DNA and 60 mM dithiothreitol. After hybridization, the sections were rinsed for 4 × 15 min in 1 × SSC at 55° C, dried and autoradiographed using Hyperfilm beta-Max film (Amersham) for 3–14 days, and thereafter dipped in Kodak NTB3 emulsion and developed after 3–8 weeks. Sections were then stained with haematoxylin and examined on an Axiophot Zeiss microscope under bright- and dark-field conditions. Structures were identified according to the corresponding developmental stages of mouse development (Theiler 1989; Rugh 1990). Materials from the different ages were processed simultaneously, and relative expression was assessed by visual comparison of the darkening on films and of the number of silver grains on emulsion-dipped slides without further quantification.

To ascertain the specificity of the labelling obtained, RNAse pretreatment (RNAse A type I-AS (Sigma), 50 µm g/ml, 1 h at 37° C) and competition with a 100-fold excess of unlabelled probe were performed. In addition, a 48-base oligomer of similar base composition corresponding to nucleotides 571–614 of the rat Neuromodulin/GAP-43 (Karns et al. 1987) was used as an unrelated positive control. Neuromodulin/GAP-43 is a neuronal growth cone protein expressed by neurons during axonal growth (Karns et al. 1987) and by enteric neurons from embryonic stages (Baetge et al. 1990) until adulthood (Sharkey et al. 1990).

Oligonucleotide probes were synthesized on an Applied Biosystem 381A DNA synthesizer, labelled with alpha ^{35}S dATP (> 1000 Ci/mmol, New England Nuclear) in the presence of terminal deoxynucleotidyl transferase (Gibco BRL) to a specific activity of $4–8 \times 10^8$ cpm/µg. Two 48-base rat PTN probes (5'GGT-TTG-TTT-GCA-CTC-GGC-ACC-AGT-GCG-AGT-GCC-CTC-CCG-GGT-GCC-TAG3' and 5'TTG-AGG-CTT-GGG-CTT-GGT-GAG-TTT-GCC-ACA-GGG-CTT-GGA-GAT-GGT-GAC3') and a 48-base human PTN probe (5'CCA-CTG-GTG-GGC-ACA-CAC-ACA-CTC-CAC-TGC-CAT-TCT-CCA-CAG-TCA-GAC3') were synthesized. They are respectively complementary to nucleotides [459–506] and [672–719] of the rat PTN mRNA (Merenmies and Rauvala 1990) and to nucleotides [382–430] of the human PTN mRNA (Li et al. 1990). When the human PTN probe was used on rat tissues, the stringency of the hybridization procedure was reduced by lowering the hybridization and washing temperatures respectively to 37° and 42° C.

RNA blot hybridization was performed according to standard procedures (Sambrook et al. 1989). Briefly, equal amounts of poly(A)+ purified RNA extracted from whole brains of E15, P0 and adult rats were separated on a formaldehyde-containing 1% agarose gel, blotted onto a nitrocellulose filter, prehybridized for 4 h in a solution containing 50% formamide, 4 × SSC, 5 × Denhardt's, 5 mM EDTA, 0.1% SDS, 250 µg/ml yeast tRNA and 100 µg/ml denaturated salmon sperm DNA, and hybridized overnight at 42° C in the same buffer containing [^{35}S]-labelled rat PTN probe (6×10^6 cpm/ml, 6×10^6 cpm/µg). The filter was then rinsed 6 × 15 min in 1 × SSC/0.1% SDS at 55° C and exposed to Hyperfilm beta-Max film for 14 days.

Results

PTN Probes characterisation and signal specificity

Hybridization with the three PTN probes yielded similar and reproducible results with satisfactory labelling (Fig. 1 A). RNAse pretreatment (Fig. 1 B) as well as competition in the presence of an 100-fold excess of the same unlabelled PTN probe totally abolished the labelling. Hybridization with the labelled Neuromodulin/GAP-43 probe yielded a totally distinct pattern of expression (Fig. 1 C). Moreover, addition of a 100-fold excess of unlabelled Neuromodulin/GAP-43 probe, or of either of the two other PTN probes, did not modify the hybridization of the labelled PTN probes. Northern Blotting analysis of poly(A)+ RNA extracted from rat brain showed a single band, of 1.8–1.9 kb, hybridizing to the PTN probes (Fig. 1 D).

PTN expression in rat tissues

Table 1 summarizes the embryological origin of the cell lineages expressing PTN transcripts during development and at maturity. PTN mRNA expression was found dur-

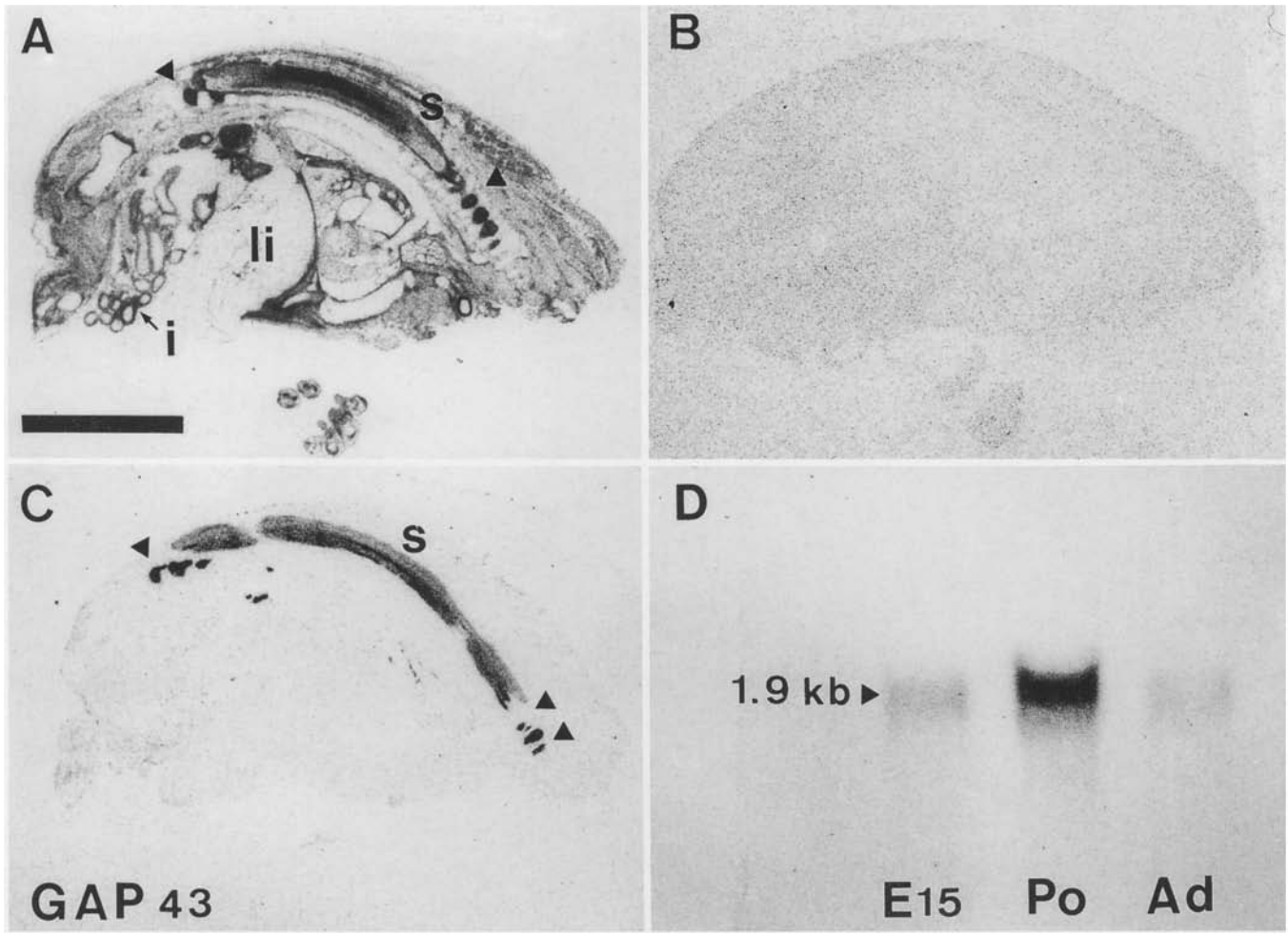

Fig. 1 A–D. Specificity of the hybridization labelling of the PTN probe. Sections (**A, B, C**) and Northern Blotting filter (**D**) were hybridized with the ^{35}S-labelled PTN probe (**A, B, D**) or GAP-43/Neuromodulin probe (**C**) and exposed to autoradiographic film for 7 days (**A, C**) or 14 days (**B, D**). **A, B, C** Adjacent parasagittal sections of a decapitated E17 rat embryo. **A** Labelling obtained with the PTN probe. **B** Abolition of labelling after RNAse pretreatment. **C** Labelling obtained with the GAP-43/Neuromodulin probe. *Arrowheads* indicate dorsal root ganglia. **D** Northern Blot of equal amounts of mRNA extracted from whole brain of E15 embryo (*E15*), neonate (*P0*) and adult (*Ad*) rat. The PTN probe hybridizes with a single band of 1.8–1.9 Kb. The intensity of the labelling indicates the relative amount of PTN transcripts in whole brain during development. Bar (**A, B, C**) 5 mm. *s*, spinal cord; *li*, liver; *i*, intestine

ing development in many – but not all – neuroectodermal and mesodermal lineages. No PTN mRNA expression was detected in trophoblastic, endodermal and ectodermal derivatives with one exception: the postnatal tongue epithelium was found to express PTN mRNA strongly in its basal layers.

The complex distribution pattern of PTN mRNA is detailed below by systems or organs.

Central nervous system. By Northern Blot, the hybridization was most intense in the newborn brain. Moderate labelling was present in the E15 embryonic brain and only a trace was detected in the adult brain (Fig. 1 D).

By ISHH, the mRNA was already detected in the neural tube of the E11 embryo (which was the first stage considered) and occupied the germinal layer throughout embryonic life (Fig. 2). The burst of expression in the newborn revealed by Northern Blot analysis was confirmed by ISHH, which showed a larger number of PTN mRNA-positive cells and a stronger labelling per cell in the newborn and during the two first postnatal weeks (Figs. 4, 5, 6). The ISHH persisted slightly in the adult brain (Fig. 3), with strengthening in the olfactory bulb, in the cerebral cortex – including the hippocampus – and in the cerebellum.

In the adult cortex (Fig. 3 A), the majority of the large neurons, including the pyramidal cells, contained moderate mRNA levels. A larger number of positive neurons was present in the superficial layers. Slight hybridization was also detected in the subcortical white matter, visible only macroscopically and under darkfield microscopic examination.

In the adult hippocampus (Figs. 3 A, 4 C), high mRNA levels were selectively found in the pyramidal cells of the CA1 sector of the Ammon's horn. CA2 to CA4 sectors and the granule cells of the Dentate Gyrus were strikingly less positive (Fig. 3 A). Before being selectively localized in the CA1 sector of the adult Ammon's

Table 1. Germ layer derivatives expressing PTN mRNA

	Development	Adulthood
Surface ectoderm		
Oral epithelium	−	+
Neuroectoderm		
Neural tube		
Central nervous system	+	+
Optic vesicle	Iris: +	Iris: +
Posterior pituitary	+	+
Pineal gland	+	+
Neural crest		
Cranial sensory ganglia	+	+
Dorsal root ganglia	+	+
Sympathetic ganglia	+	+
Head mesenchyme (face and mouth)	+	Leptomeninges: +
Mesoderm		
Head mesoderm		
Head mesenchyme (dorsal and caudal)	+	−
Dorsal/para-axial (=somites)		
Sclerotome (vertebrae and ribs)	+	−
Myotome (striated muscles)	+	−
Dermotome (dermis)	+	−
Intermediate		
Urogenital system (ducts, gonads, glands)	+	Uterus: + Leydig cells: +
Lateral		
Somatic (pleura, pericardium, peritoneum, limb buds)	+	−
Splanchnic (connective and smooth muscle of viscera and blood vessels)	+	−
Endoderm	−	−
Trophoblast	−	

Only the germ layer derivatives expressing PTN mRNA during development and/or at adulthood are listed.
+, −* :indicates the presence or absence of PTN transcripts. Specific structures are indicated only when expression is restricted to some definited part of a lineage. Structures indicated + may actually include various cell types of the same embryological origin, some of them containing PTN mRNA and others not. See text for details

horn, PTN transcripts were mainly found in the hippocampal germinal layer and CA future dendritic layers during perinatal development (Fig. 4A, D).

In the adult cerebellum (Figs. 3B, 5C), moderate mRNA levels were found in cells concentrated between the negative Purkinje cells. During development, in addition to these cells, PTN transcripts were also strongly expressed in numerous cells in the cerebellar white matter (Fig. 5A, B).

In the adult olfactory bulb (Fig. 6C), very high labelling was observed in the olfactory nerve layer and in the vomero-nasal nerve layer. Before being selectively localised in the nerve layer, the labelling was diffusely found in the different layers of the olfactory bulb, with the exception of the mitral cell layer (Fig. 6A, B).

Elsewhere in the adult CNS, slight mRNA levels were found in the majority of neurons in the basal ganglia, especially in the striatum; in the basal area of the forebrain, especially in the lateral septum; in the thalamus and to a lesser degree in the hypothalamus, in the brain stem and in the spinal cord.

In the hypophysis, cells of the pars neuralis moderately expressed PTN transcripts in the viciny of the Rathke's pouch at E15 (Fig. 7A). PTN mRNA was also present in the posterior hypophysis of older embryos, and a strong hybridization persisted in the pituicytes of the adult neurohypophysis (Fig. 7B). Some clusters of cells of the adult pituitary intermediate lobe also expressed PTN mRNA, but Rathke's Pouch and adenohypophysis were not labelled at any age studied.

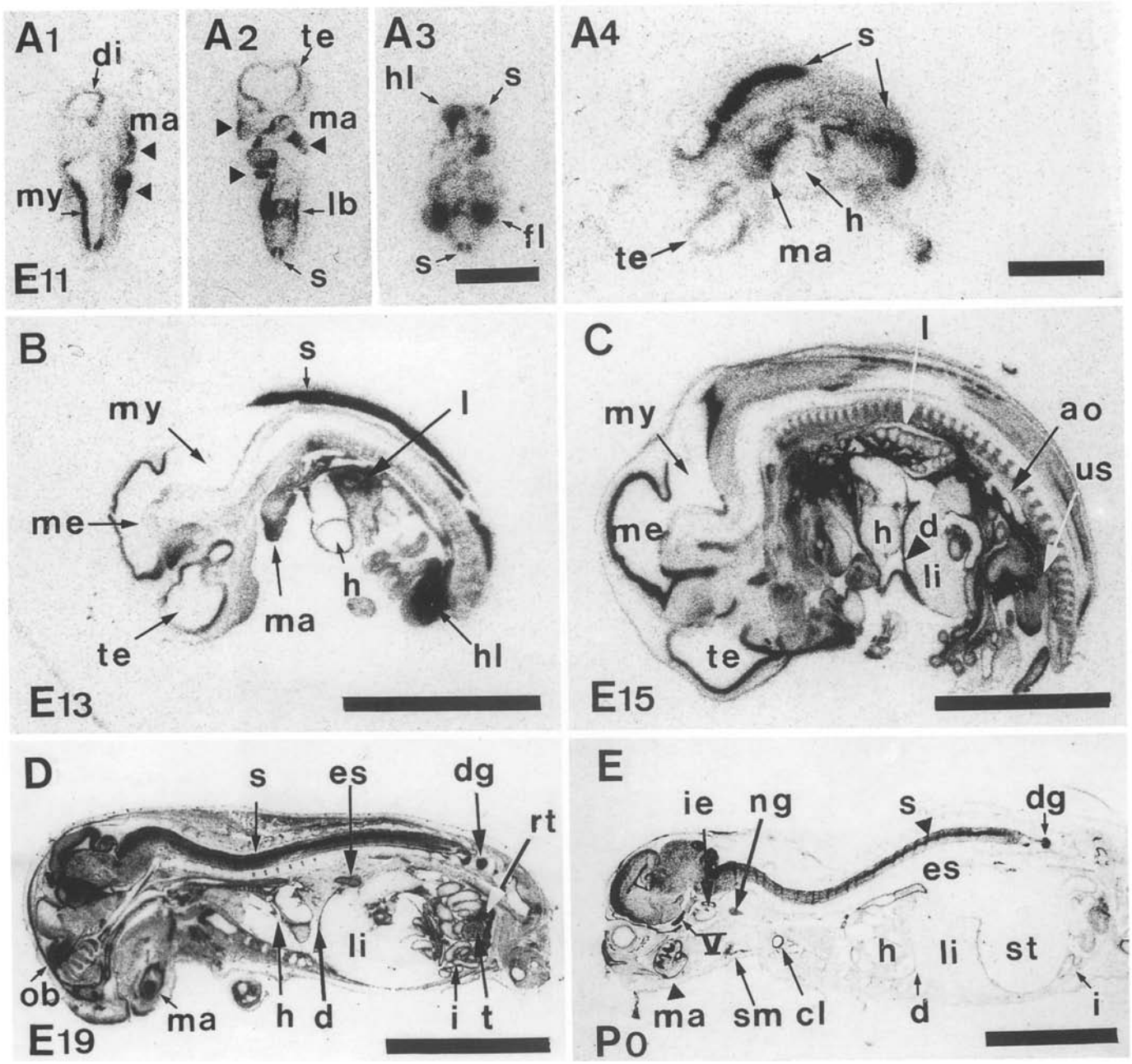

Fig. 2A–E. Macroscopic pattern of PTN gene expression in rat embryos. Autoradiographic films exposed for 7 days. **A1** to **A3** Transverse sections, 450 μm apart; **A4** Parasagittal section of E11 embryo. **B, C, D, E** Parasagittal sections of E13, E15, E19 embryos and newborn (P0) respectively. PTN transcripts are already abundant at E11, both in the neural tube and in various differentiating mesenchymes: branchial arches (*arrowheads*), lung bud and limb buds. The hybridization remains high in the nervous system by the end of gestation. In contrast, the intense PTN mRNA expression observed in the connective tissues tends to fade as development progresses. At birth (P0), PTN transcripts are detected only in a few locations in the mesenchyme: ocular sclera, mandibula, submandibular glands, clavicula, oesophagus and gastric wall. Note the disappearance of labelling in the diaphragm between E19 and P0. Bars: 2 mm in **A1** to **A4**; 5 mm in **B** and **C**; 1 cm in **D** and **E**. *di*, diencephalon; *my*, myelencephalon; *ma*, mandibular arch or mandibula; *te*, telencephalon; *l*, lung or lung bud; *s* spinal cord; *fl*, forelimbud; *h*, heart; *hl*, hindlimb bud; *me*, mesencephalon; *d*, diaphragm; *li*, liver; *ao*, aorta; *ob*, olfactory bulb; *es*, oesophagus; *i*, intestine; *rt*, retroperitoneum; *t*, testis; *dg*, dorsal root ganglia; *V*, trigeminal ganglia; *sm*, submandibular gland; *cl*, clavicula; *ng*, nodose ganglia; *st*, stomach; *us*, urogenital sinus

In the adult pineal, high PTN mRNA levels were found in a minor subpopulation of cells.

Meninges and choroïd plexus. PTN mRNA was moderately expressed in a majority of cells of the leptomeninges from E17 to P0 and was still detected in the adult. In contrast, no labelling was seen in the choroïd plexus at any of the ages studied.

Sensory organs. The optic placode as well as the optic vesicle, and later the lens and retina, were negative during development and in the adult. In young embryos,

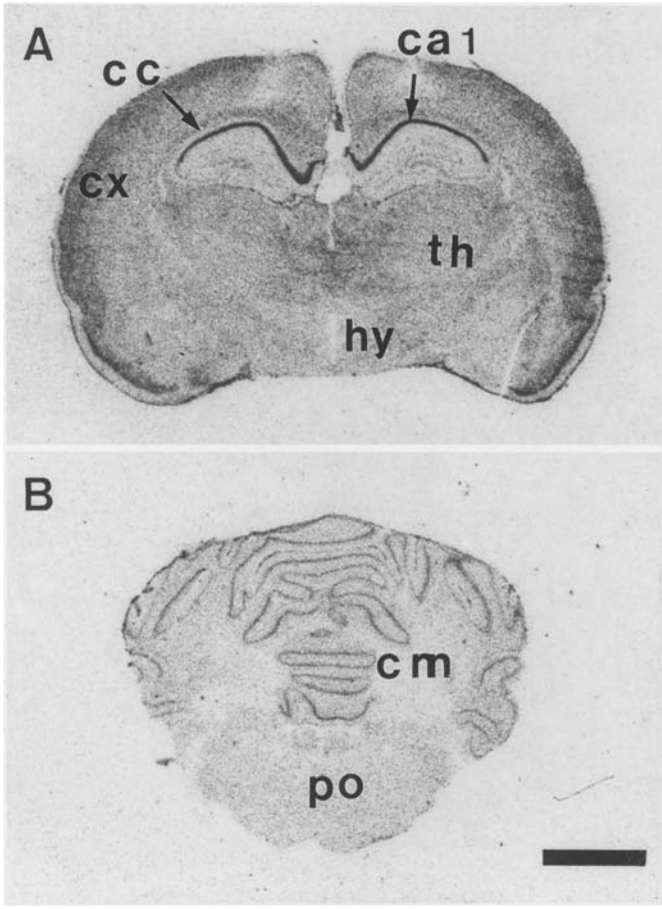

Fig. 3A, B. Macroscopic pattern of PTN mRNA expression in the adult brain, at the level of the hippocampus (**A**) and cerebellum (**B**). Bar 2.5 mm. *CA1*, first subfield of the Ammon's Horn of hippocampus; *cx*, cortex; *hy*, hypothalamus; *th*, thalamus; *cm*, cerebellum; *po*, pons; *cc*, corpus callosum

the mesenchyme surrounding the eyeball presented some strengthening of the diffuse PTN mRNA expression found in the head mesenchyme (see below). At E19 (Fig. 7E) significant PTN mRNA expression was confined to the iris, palpebrae and sclera. No expression was present in the ciliary process. At P0 (Fig. 7D), only the iris, sclera and the mesenchyme around the lacrymal glands were still positive. In the adult eyeball, PTN mRNA expression was detected only in the iris.

The olfactory placode, and later the olfactory neuroepithelium, did not display significant hybridization.

The optic placode did not show any PTN mRNA expression, but by the end of gestation intense PTN mRNA expression was detected in the inner ear (Fig. 7C, D). The labelling was localized in the mesenchyme just beneath the auditory epithelium (Fig. 7C).

Sensory ganglia. Dorsal root ganglia (DRG) were diffusely labelled as early as E11. Virtually all the ganglionic cells, expressed PTN mRNA intensely from E13 until birth (Fig. 8A). During the first few weeks after birth, the hybridization faded in the neurons but not in the surrounding Schwann cells. This pattern of PTN mRNA expression restricted to the cells around the neurons of the DRG was clearly observed at P21, and persisted at adulthood (Fig. 8B). Similar findings were noted at cervical, dorsal and lumbar levels.

Schwann cells of the spinal roots were only moderately labelled in embryos but negligible hybridization was detected after birth.

Strong labelling of nearly all cells of the trigeminal and nodose ganglia was also observed from E13 until birth (Fig. 2E) but the postnatal development of their PTN mRNA expression pattern was not assessed in the present study.

Sympathetic chain and ganglia (cervical and coeliac). The sympathetic chain and ganglia showed a diffuse PTN mRNA expression from E15 until adulthood where only coeliac ganglia were examined. This expression was always lower than that exhibited by the DRG cells at the same stage. In the adult coeliac ganglia, PTN transcripts were detected mainly in the large, neuronal, cells.

Adrenal. Little labelling was seen either in the medulla or in the cortex from E15 to adulthood. However, the retroperitoneal mesenchyme surrounding the adrenal gland strongly expressed PTN mRNA from E15 to birth. This disappeared later and no labelling was observed in the adult retroperitoneal tissue and lymph nodes.

Peripheral nerves. There was no labelling in the newborn and adult sciatic nerve.

Face and oral cavity. Branchial arches displayed strong PTN mRNA expression at E11 (Fig. 2A). Later, transcripts were detected in the mesenchyme of the face (Fig. 2B, C, D) especially in the zones of rapid development such as the jaw, maxillary and palatial processes. PTN mRNA expression in the facial bones is reported below with the other osteo-cartilaginous structures.

At E13 and E15 the mesenchyme of the tongue and lip (Fig. 9A) was intensely labelled, but this faded at older ages. The oral epithelium was not labelled until birth (Fig. 9C) and was not studied in postnatal stages. In contrast, the tongue epithelium, which was not labelled until E19, began to express PTN transcripts weakly after birth. The hybridization increased at P7 and was strong at adulthood (Fig. 9D). This expression was limited to the basal layers of the keratinized epithelium of the tongue (Fig. 9D). No labelling was observed in the adult tongue musculature.

The dental anlage did not express PTN mRNA (Fig. 9A) but the dental pulp strongly expressed PTN transcripts at a later stage of tooth development. At E 19 the first inferior molar pulp strongly expressed PTN mRNA (Fig. 9B). At P0, the expression in the dental pulp of the first molar had faded but now appeared strongly in the second molar (Fig. 9C).

A significant PTN mRNA expression was noted in the mesenchyme around the submandibular glands at E19 and P0 (Fig. 2D, E) but not in their epithelial component. This pattern was similar to that encountered around the lacrymal glands (see above and Fig. 7D).

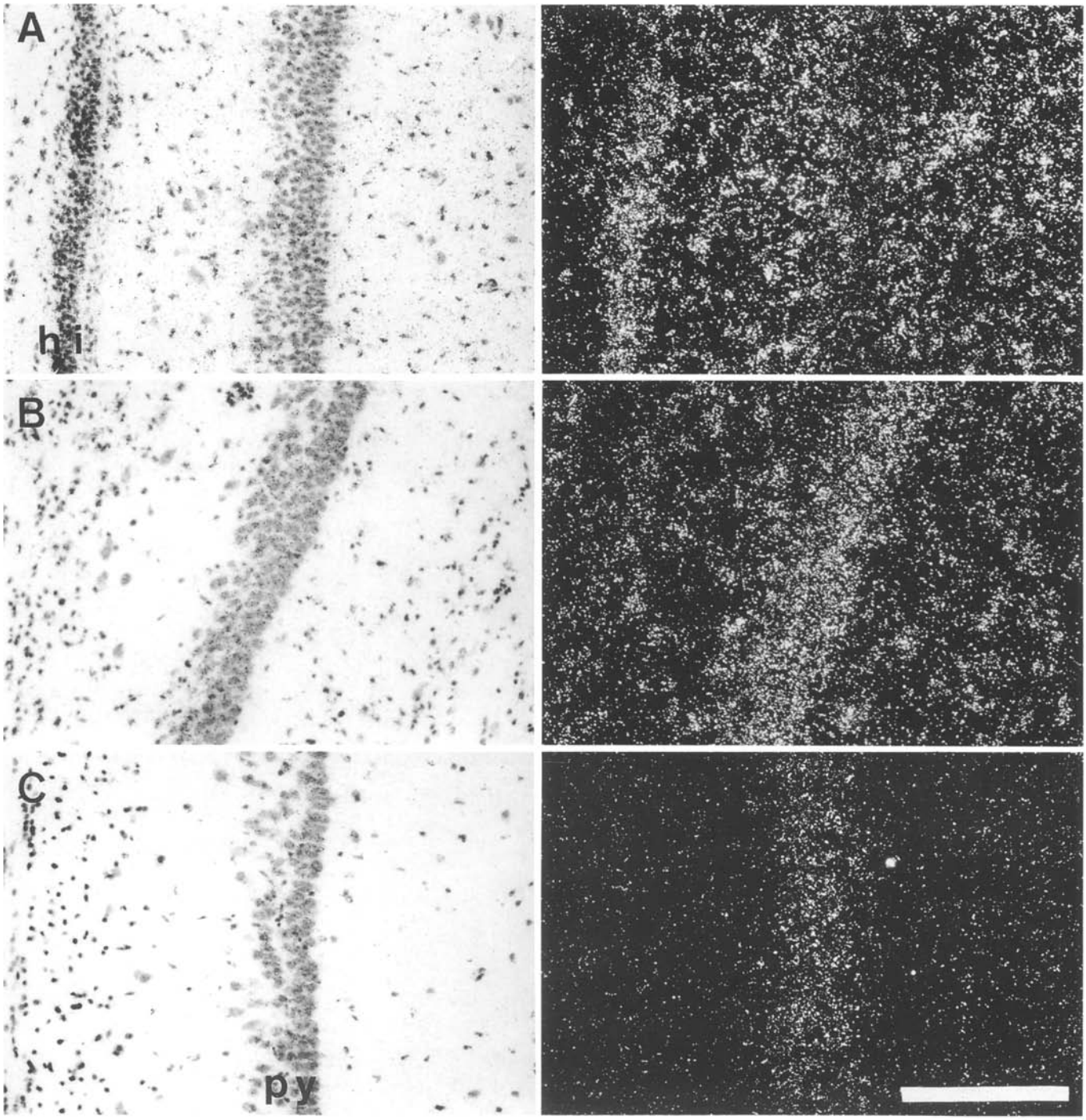

Fig. 4A–C. PTN mRNA expression in the postnatal first sector of the Ammon's Horn (CA1) of the hippocampus. Pairs of bright- and darkfield micrographs. **A, B** Respectively one and two weeks after birth. **C** Adult. Bar 250 μm. *py*, pyramidal cell layer; *hi*, hippocampal formation neuroepithelium

Neck and thoracic wall. The mesenchyme of the neck and thoracic wall was strongly labelled from E13 to E19 (Fig. 2B, C, D) but the labelling had already vanished at birth (Fig. 2E).

The thyroid and thymus remained unlabelled.

Skin. A moderate and diffuse PTN mRNA expression was observed in the dermis between E17 and E19. Some increase in the hybridization in the dermal sheet of the whiskers and, occasionally, around some hair follicles was noted at E19 and P0, but the epidermis, hair and other cutaneous adnexae did not display any significant hybridization at any age studied.

Cardiovascular and respiratory systems. Results are shown in Table 2.

PTN mRNA expression was also observed in the mesenchyme around the extraembryonic umbilical vessels

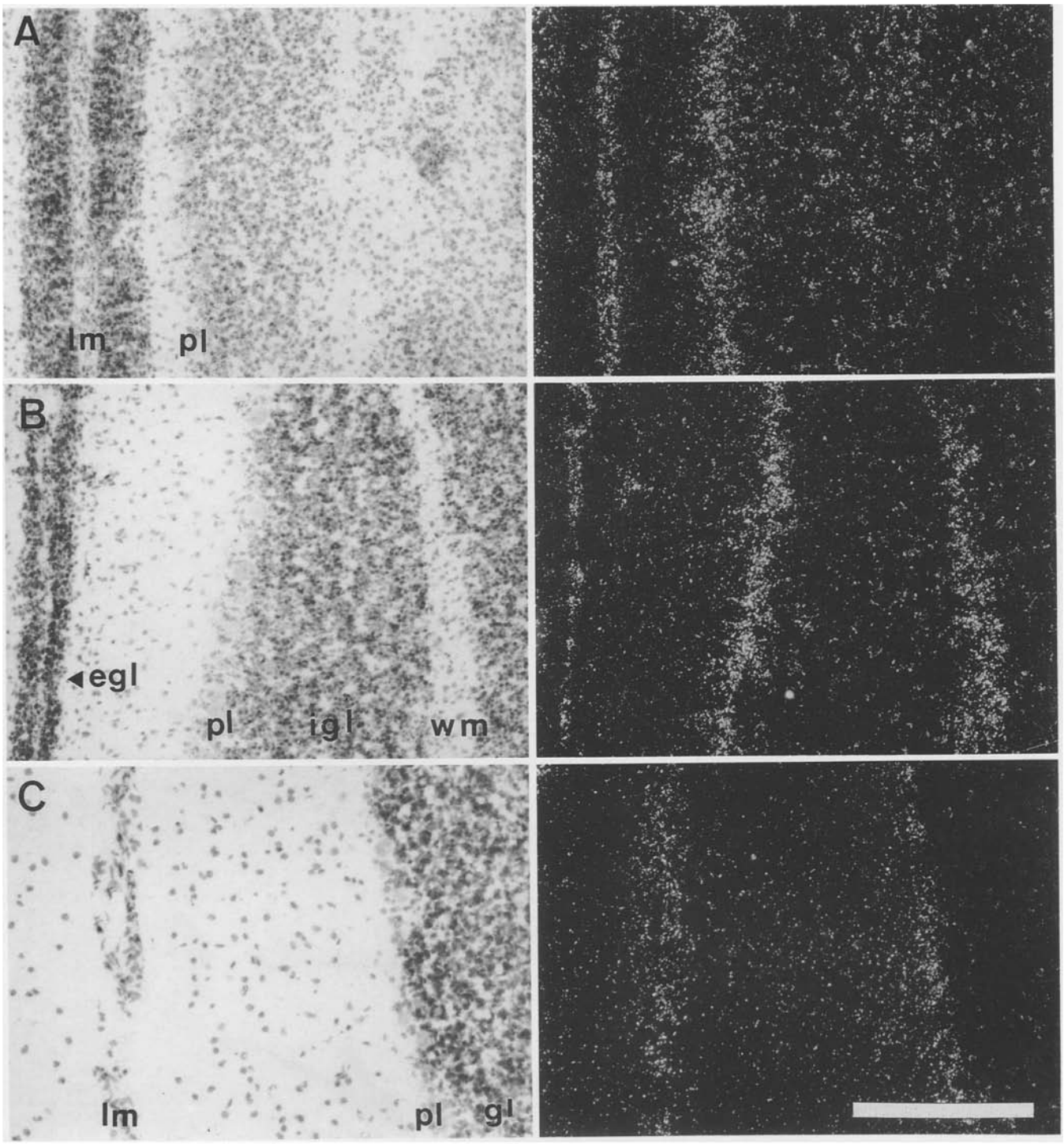

Fig. 5A–C. PTN mRNA expression in the postnatal cerebellum. Pairs of bright- and darkfield micrographs. **A, B** Respectively one and two weeks after birth. **C** Adult. Bar 250 μm. *lm*, leptomeninge; *wm*, white matter; *egl*, external granular layer; *igl*, internal granular layer; *gl*, granular layer; *pl*, Purkinje cell layer

at E11 (Fig. 2A) and E13 but this was not studied in later stages.

Digestive tract. PTN mRNA expression in the gut wall is summarized in Table 3.

There was no difference in the PTN hybridization between the embryonic enteric nervous system (ENS) and the surrounding tissues (Fig. 11B). In contrast, GAP-43/Neuromodulin clearly identified the embryonic enteric nervous system at the same age (Fig. 11C).

The mesentery also strongly expressed PTN mRNA at E15 (Fig. 2C), E17 (Fig. 11A) and E19 and expression faded subsequently.

The digestive epithelium, liver, spleen and pancreas were unlabelled at all the stages studied (Fig. 2).

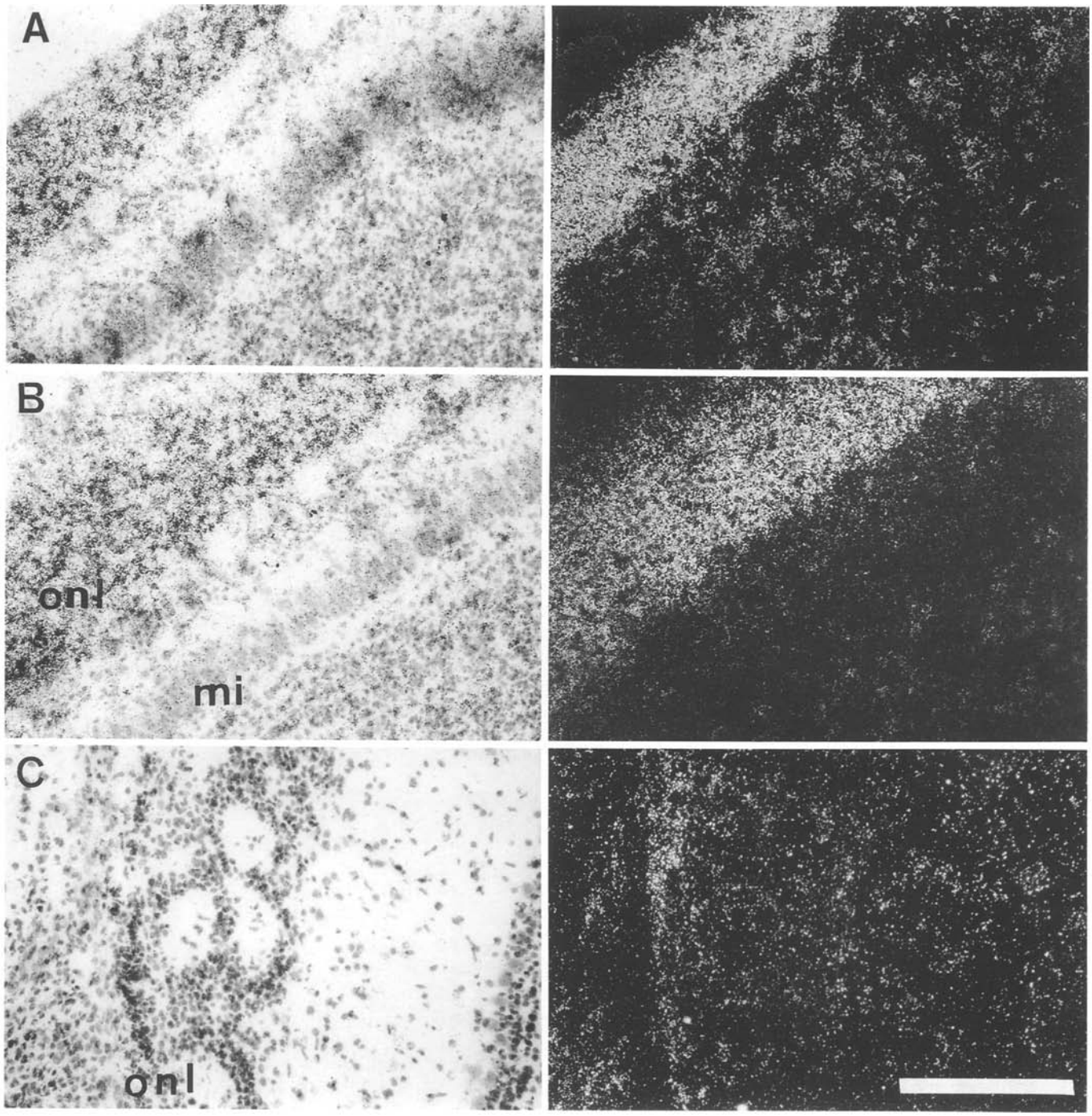

Fig. 6A–C. PTN mRNA expression in the postnatal olfactory bulb. Pairs of bright- and darkfield micrographs. **A, B** Respectively one and two weeks after birth. **C** Adult. Bar 250 μm. *onl*, olfactory nerve layer; *mi*, mitral cell layer

Urogenital system. The nephritic blastema and genital ridges were moderately labelled at E13. At E15, the mesenchymes of the gonad, metanephros and urogenital sinus were intensely labelled. At E17, the metanephritic mesenchyme strongly expressed PTN mRNA, with increases around the pelvis and the ureter. At that time an increasing medullocortical gradient of PTN mRNA expression became apparent (Fig. 12A). At E19, the mesenchyme around the pelvis became negative (Fig. 12B) and at P0 the labelling was only found in the outermost cortical mesenchyme (Fig. 12C). No expression was observed in the nephrons themselves (Fig. 12D). No significant expression was observed in the urinary bladder at E19, P0 and adult.

At E17 and E19 (Figs. 2D, 12E), the testicular capsule (tunica albuginea) and the intertubular mesenchyme of the testis moderately expressed PTN mRNA. The mesenchyme around the Wolffian ducts also presented a moderate labelling, which was more pronounced in their dorsal portion. At P0, a moderate expression was

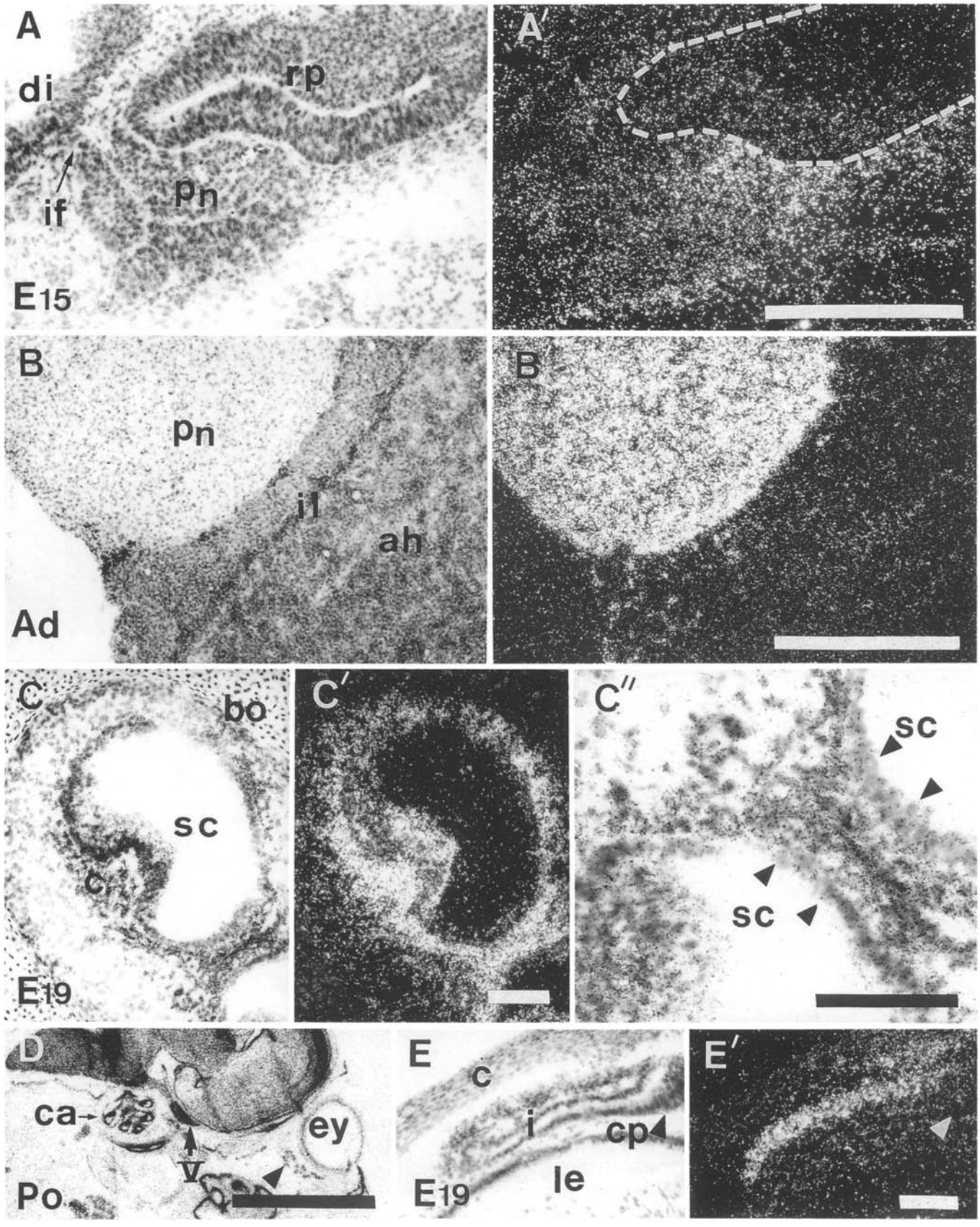
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Fig. 7A–E

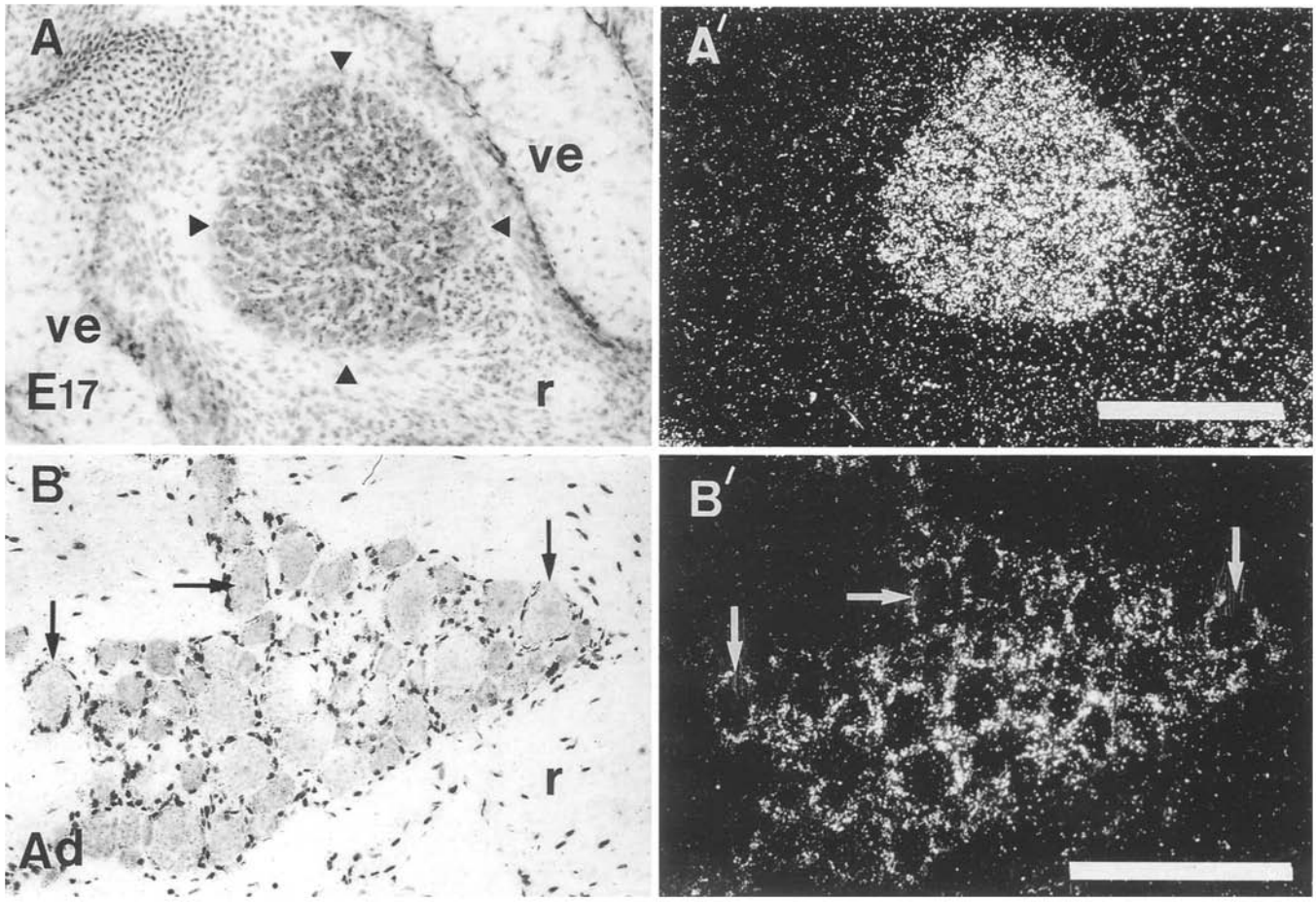

Fig. 8A, B. PTN expression in fetal and adult DRG. Pairs of bright- and darkfield micrographs. **A** E17 embryo DRG: intense PTN mRNA expression in most cells of the DRG (*arrowheads*). Weak labelling is also present in the dorsal root cells. No PTN mRNA expression is detected in or around the vertebrae. **B** Adult DRG: PTN mRNA expression is confined to the Schwann cell satellites of the DRG neurons (some neurons are indicated by *arrows*). The Schwann cells of the nerve bundles did not express PTN mRNA. Bar 200 μm. *ve*, vertebra; *r*, dorsal root

Fig. 7A–E. PTN mRNA expression in the pituitary gland, ear and eye. **A, B, C, C″, E** Brightfield micrographs. **A′, B′, C′, E′** Darkfield images corresponding to **A, B, C, E** respectively. **D** Autoradiogram. **A, A′** E15 embryo pituitary: PTN mRNA is present in the pars neuralis (neurohypophysis) and in adjacent mesenchyme (*bottom right*). The floor of the diencephalon, the infundibulum and the Rahtke's pouch (*dotted line*) are unlabelled. **B, B′** Adult pituitary: intense PTN mRNA expression persists in the pituicytes of the neurohypophysis. Some clusters of cells expressing PTN transcripts are also present in the pars intermedia. No hybridization is observed in the adenohypophysis (anterior lobe). **C, C′** E19 embryo ear: PTN mRNA expression in a semicircular canal. **C″** Transcripts are present in the mesenchyme surrounding the semicircular canal but not in the epithelium (*arrowheads*). **D** Newborn (P0). Presence of PTN transcripts in the inner ear (cochlea), trigeminal ganglia, sclera of eyeball and lacrymal gland mesenchyme (*arrowhead*). **E** E19 embryo eye. PTN mRNA is present in the iris while the ciliary process (*arrowhead*), cornea and lens are unlabelled. Bars 250 μm in **A**; 500 μm in **B**; 100 μm in **C** and **E**; 5 mm in **D**. *di*, diencephalon; *rp*, Rahtke's Pouch, *ah*, adenohypophysis; *pn*, pars neuralis (posterior hypophysis); *il*, intermediate lobe; *if*, infundibulum; *bo*, bone; *cr*, crista ampullaris; *sc*, semicircular canal; *cc*, cochlea; *ey*, eye; *co*, cornea; *le*, lens; *ir*, iris; *V*, trigeminal ganglia; *cp*, ciliary process

Table 2. PTN mRNA expression in the cardiovascular and respiratory systems

	E15	E17	E19	P0	P7	Adult
Pericardium (Figs. 1A, 2C, D, E)	+	+	−	−	−	
Myocardium	−	−	−	−	−	−
Vascular endothelium	−	−	−	−	−	−
Vascular musculature (Fig. 2A, E)	+	+	+	−	−	−
Pulmonary blastema (Figs. 2A, 10A)	+					
Lung parenchyma		−	−	−	−	−
Bronchid wall (Fig. 10B, C, D)		+	+	+	+	−
Tracheal mesenchyme[a]		+	+	+	+	−
Respiratory epithelium	−	−	−	−	−	−

[a] Cartilaginous rings of the trachea were not labelled

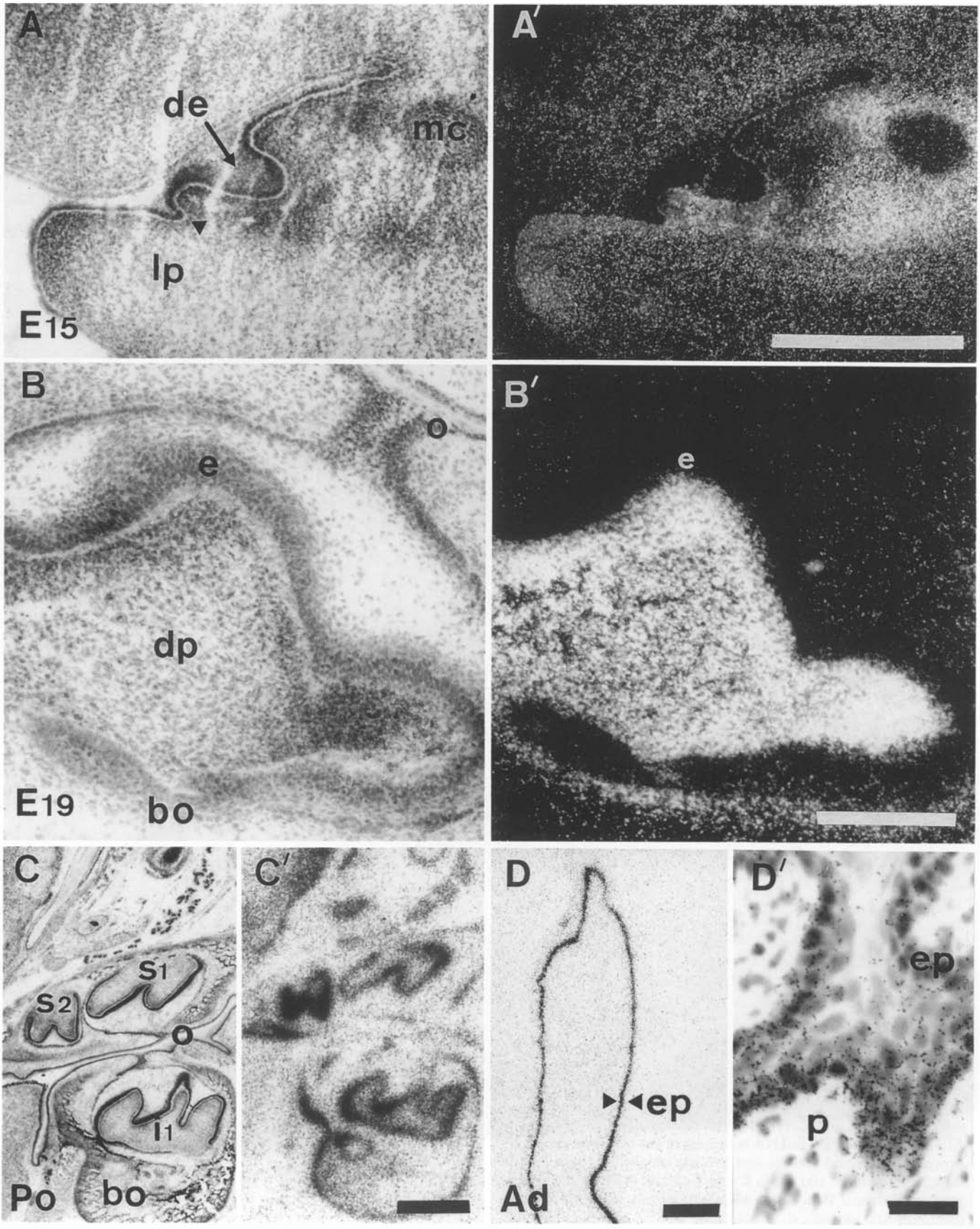

Fig. 9 A–D

Table 3. PTN mRNA expression in the gut wall

	E13	E15	E17	E19	P0	P7	Adult
Gut mesenchyme (Fig. 11A, B)	+	+	+				
Gut musculature			−	−	−	−	−
Serosa of:							
Oesophagus				+	+	+	−
Stomach				+	+	−	−
Intestine				+	−	−	−
Colon				+	−	−	−
Mucosa	−	−	−	−	−	−	−

still observed in the testicular intertubular mesenchyme and in the connective tissue around the epididimis, but disappeared in the tunica albuginea. Leydig cells of the testicular interstitium displayed a persistent moderate PTN mRNA expression at adulthood (Fig. 12F). In contrast, no PTN transcript was detected after birth in the ovary.

Intense PTN mRNA expression was observed during the development of the urogenital sinus (Fig. 2C).

In the adult virgin uterus, PTN mRNA expression was moderate and diffuse in the myometrium, with some increase in labelling beneath the decidual zone. Gravid uterus from E11 to birth showed two distinct zones of strong PTN mRNA expression: in the myometrium and in the compact decidua, just beneath the implantation zone (Fig. 13). Conversely, the endometrium and glands as well as the trophoblast and placenta at these ages were not labelled.

No labelling was observed in any epithelial structures along the entire urogenital system at any of the ages studied.

Fig. 9A–D. PTN mRNA expression in the developing oral cavity. **A, B, D′** Brightfield micrographs. **A′, B′** Darkfield images corresponding to **A** and **B** respectively. **C** Macrophotography of a section stained with haematoxylin. **C′, D** Autoradiograms. **C′** corresponds to **C**. **A, A′** E15 embryo; parasagittal section through the oral cavity. PTN mRNA expression is intense around the Meckel's cartilage and in the mesenchyme around the first dental anlage. Expression is also accentuated in the lip mesenchyme. Oral epithelium is not labelled. *Arrowhead* indicates the lip furrow band. **B, B′** E19 embryo; parasagittal section through the first lower molar: intense PTN mRNA expression is present in the dental papilla (pulp) and around the mandibular bone. Oral epithelium (*right upper corner*) is not labelled. **C, C′** Newborn (P0); parasagittal section at the level of the first and second molars. PTN mRNA expression in the first molar pulp had faded while it remains high in the second molar pulp. **D, D′** Adult tongue; intense PTN mRNA expression is present in the periphery of the tongue. Expression is localized in the basal layers of the tongue epithelium (**D′**). No expression is observed in the underlying mesenchyme. Bars 500 μm in **A**; 100 μm in **B**; 1 mm in **C**; 2 mm in **D**; 25 μm in **D′**. *de*, dental anlage; *mc*, Meckel's cartilage; *lp*, lip; *o*, oral cavity; *dp*, dental pulp (papilla); *bo*, mandibular bone; *sI* and *sII*, first and second superior molars respectively; *il*, first inferior molar; *e*, enamel organ; *ep*, tongue epithelium; *p*, papilla of the tongue

Bones and cartilages. The mesenchyme of the branchial arches was intensely positive at E11 (Fig. 2A). At E15, PTN mRNA expression in the jaw mesenchyme seems to precede the differentiation of the future mandibular structure (Fig. 9A). Later, the cartilaginous and bony structures of the face displayed a peripheral increase of the diffuse PTN mRNA expression of the head mesenchyme, and this persisted after its extinction in the mesenchyme (Fig. 2C, D, E). The pattern of expression in the skull was similar to that observed in the other bones and cartilaginous structures.

At E13 and E15, the sclerotomes between the vertebral primordia were strongly positive (Fig. 2B, C). No further PTN mRNA expression was detected in the vertebrae or intervertebral disc after E17 (Figs. 1A, 8A) but expression in the periosteum of the ribs persisted, although fading, at least until P7.

Notochord remnants did not show labelling at any age.

PTN transcripts were extremely abundant in the entire limb buds at E11 (Fig. 2A) and E13 (Fig. 2B). At E15, strong PTN mRNA expression was detected in the mesenchyme of the hindlimb, but transcripts were less abundant beneath the skin, and the developing skeletal components were not labelled (Fig. 14A). At E17, PTN mRNA expression was limited to the periosteum and to tendinous structures of the paw (Fig. 14B). PTN mRNA expression was present at least until P0 at the periphery of the long bones of the hindpaw and of the clavicula (Figs. 2E, 14C).

In the skeleton, the expression of PTN mRNA was always confined to osteoblasts, which were localized some rows outside the matrix apposition zone, and PTN transcripts were not detected inside bone or cartilage itself (Fig. 14C). PTN mRNA expression was not studied in calcified bone.

Striated muscles. The somites were moderately labelled at E11 and E13. The parietal musculature of the trunk displayed moderate labelling around E15 (Fig. 2C) but this subsequently decreased. From E17, the PTN mRNA labelling in the striated muscle bundles of the limbs appeared no higher than the background. However, moderate labelling was present in some interfascicular connective tissues and in tendons (Fig. 14B).

The PTN mRNA expression in the diaphragm was much stronger than in other muscles, and lasted longer, as it was detected from E15 until E19 (Figs. 2C, D, 10E) and disappeared only at P0 (Fig. 2E).

Discussion

This is, to our knowledge, the first study using ISHH on the cellular PTN mRNA expression in the rat tissues. The specificity of the hybridization obtained with the three oligomers used was established, since it was prevented by RNAse pretreatment, and by competition with an excess of homologous unlabelled probe, but not with an excess of a different probe. Moreover, the unrelated GAP43/Neuromodulin probe, which had the same

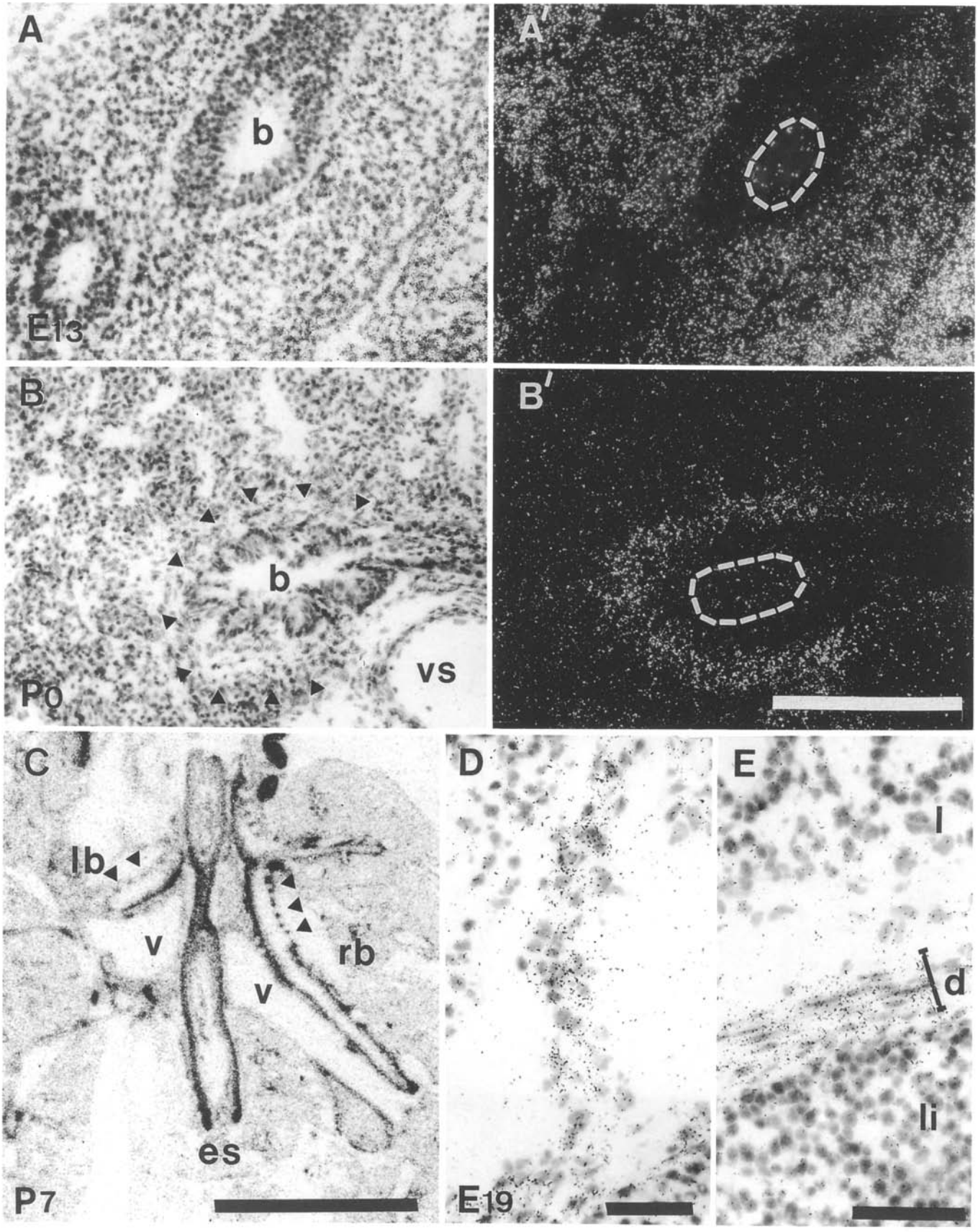

Fig. 10A–E

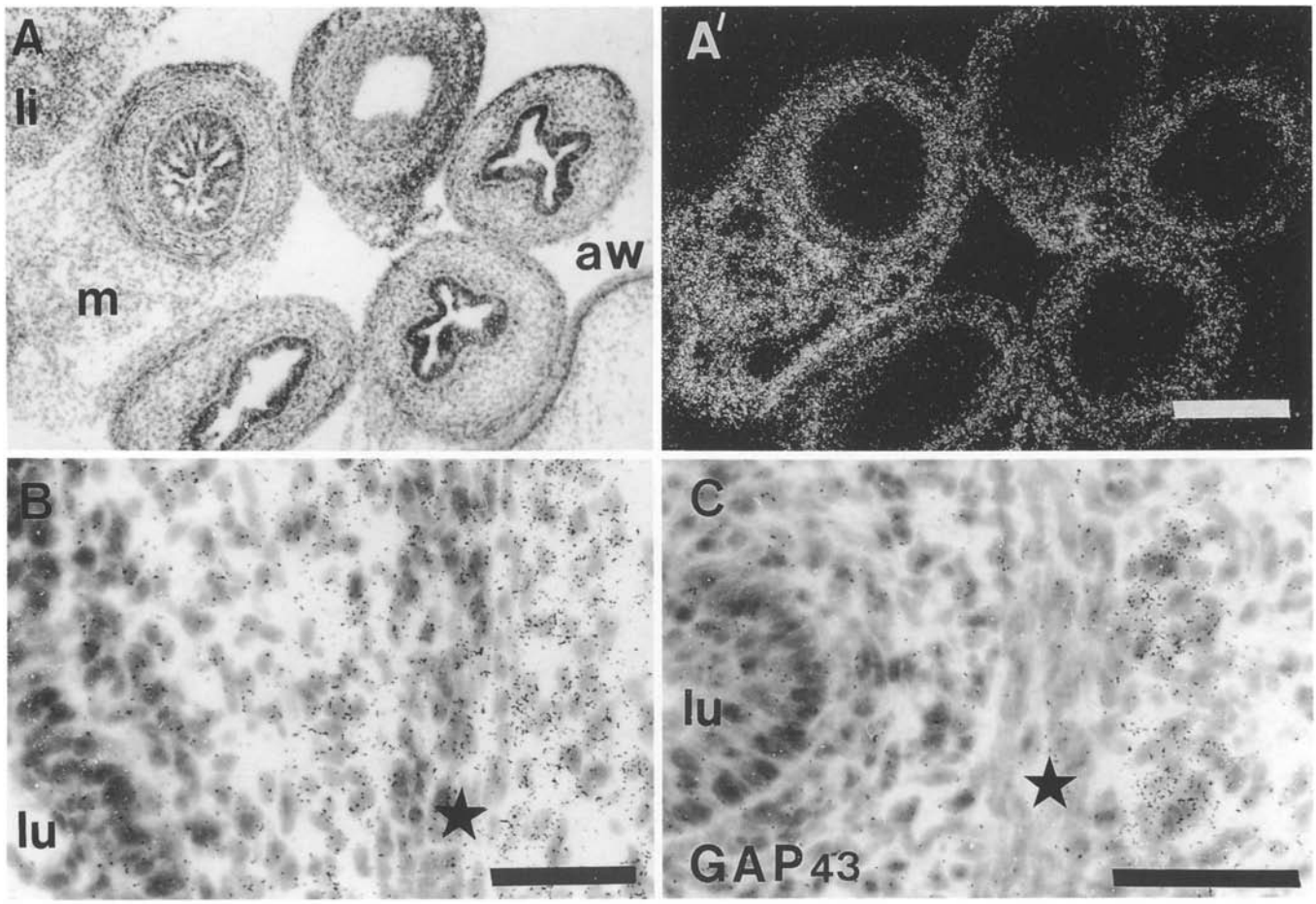

Fig. 11A–C. PTN mRNA expression in the embryonic gut wall. **A, B, C** Brightfield micrographs. **A′** Darkfield image corresponding to **A**. **A, A′** E17 embryo; intestinal loops in the extraembryonic coelom. Double ring pattern of hybridization in the gut wall. PTN mRNA is also present in the mesentery. No label is observed in the liver or in the abdominal wall. **B** Higher power view of the same section. The differentiating circular smooth musculature (*) presents a lower PTN mRNA expression than the submucosa and the serosa. PTN mRNA expression in the enteric nervous system is not different from that of the surrounding mesenchyme. **C** Intestine of the same E17 embryo on an adjacent section; the GAP43/Neuromodulin probe specifically labels the ENS neurons located at the outer part of the differentiating circular smooth musculature (*). Bars 200 μm in **A**; 50 μm in **B** and **C**. *li*, liver; *aw* abdominal wall; *ms*, mesentery; *, circular smooth musculature; *lu*, intestinal lumen

Fig. 10A–E. PTN mRNA expression in the developing lung. **A, B, D, E** Brightfield micrographs. **A′, B′** Darkfield images corresponding to **A** and **B** respectively. **C** Autoradiogram. **A, A′** E13 embryo: diffuse PTN mRNA expression in the mesenchyme of the pulmonary blastema. Absence of labelling in the epithelium (*dotted line*, bronchic lumen). **B, B′** Newborn (P0). Expression is now confined in the bronchic wall (*arrowheads*). Specific labelling is absent in the lung parenchyma and vessel wall. **C** P7 pup: posterior view of a transverse section through the mediastinum. PTN mRNA is detected only in the wall of the trachea and main bronchi. The cartilaginous rings are not labelled (*arrowheads*). The oesophageal musculature also displays strong PTN expression. (Note: the dark paratracheal dot at right is an artifact). **D** E17 embryo. PTN transcripts expressed by the mesenchyme cells of an interlobar septum of the lung. **E** E17 embryo. PTN mRNA expression in the differentiating myoblasts of the diaphragm. In the diaphragm, PTN mRNA expression faded between E17 and E19, and was no longer detectable at P0 (see Fig. 2 D, E). Bars: 200 μm in **A** and **B**; 5 mm in **C**; 50 μm in **D** and **E**. *b*, bronchic lumen; *rb*, right main bronchus; *lb*, left main bronchus; *v*, blood vessel; *es* esophagus; *li*, liver; *l*, lung; *d*, diaphragm

length and similar guanosine-cytosine content, yielded a totally distinct pattern of hybridization, in agreement with published data (Baetge et al. 1990). Finally, under conditions similar to those used for ISHH, our probes hybridized with a single band of expected size (Merenmies and Rauvala 1990) on Northern blot of poly(A)+ RNA extracted from rat brain.

The present study confirms the Northern blot studies already published (Merenmies and Rauvala 1990; Li et al. 1990) on the perinatal PTN mRNA expression. In the CNS, a postnatal burst of PTN mRNA expression was observed both by Northern blotting and ISHH. In other tissues where PTN transcripts are present, the time course of the ISHH followed the same general postnatal decrease as reported with Northern Blotting.

Comparison between ISHH and Northern Blot data should however be cautious, as both methods have different methodological limitations which may led to some apparent discrepancies. For example, if tissue expression

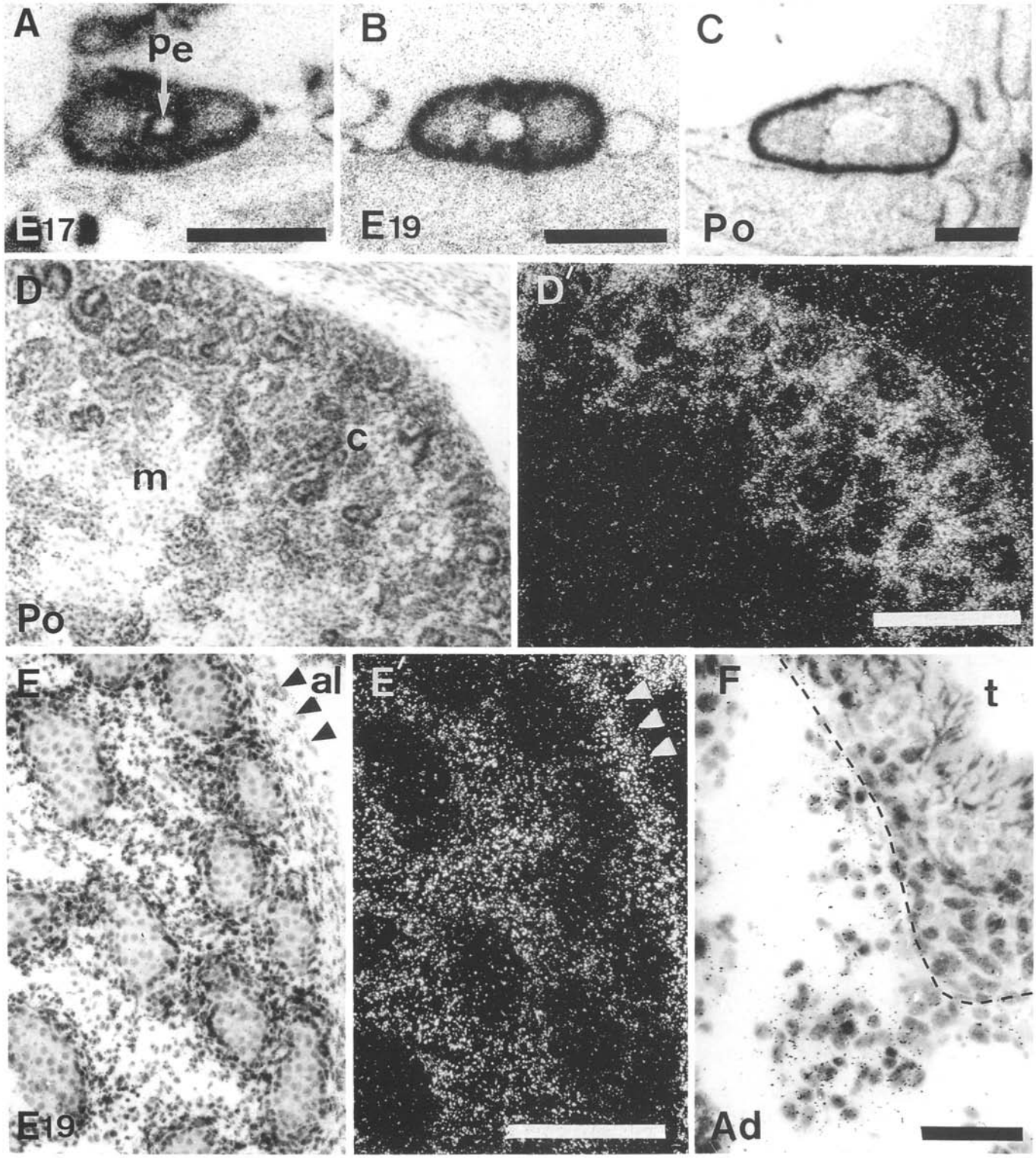

Fig. 12A–F. Developmental expression of PTN mRNA in the rat kidney and testis. **A, B, C** Autoradiographs of parasagittal sections through the renal pelvis. **D, E, F** Brightfield micrographs. **D,′ E′** Darkfield images corresponding to **D** and **E** respectively. **A** E17 embryo; intense PTN mRNA expression in the nephritic mesenchyme and around the pelvis. **B** E19 embryo: fading of the label around the pelvis and in the medulla. **C** Newborn (P0); no labelling is detected in the medulla and around the pelvis. It is restricted to the outer cortex. **D, D′** Newborn (P0) kidney. PTN mRNA expression in the cortical mesenchyme surrounding the nephrons. **E, E′** E19 testis. PTN mRNA expression is present in the testicular interstitium and albuginea (*arrowheads*) but not in the testis cords. **F** Adult testis; persistence of PTN mRNA expression in the testicular interstitium (Leydig cells) and absence of PTN transcript in the seminiferous tubes (*dotted line*). Bars 2 mm in **A, B** and **C**; 200 μm in **D** and **E**; 50 μm in **F**. *pe*, pelvis; *m*, medullary; *c*, cortex; *t*, siminiferous tubes (testis cords in embryos); *al*, albuginea

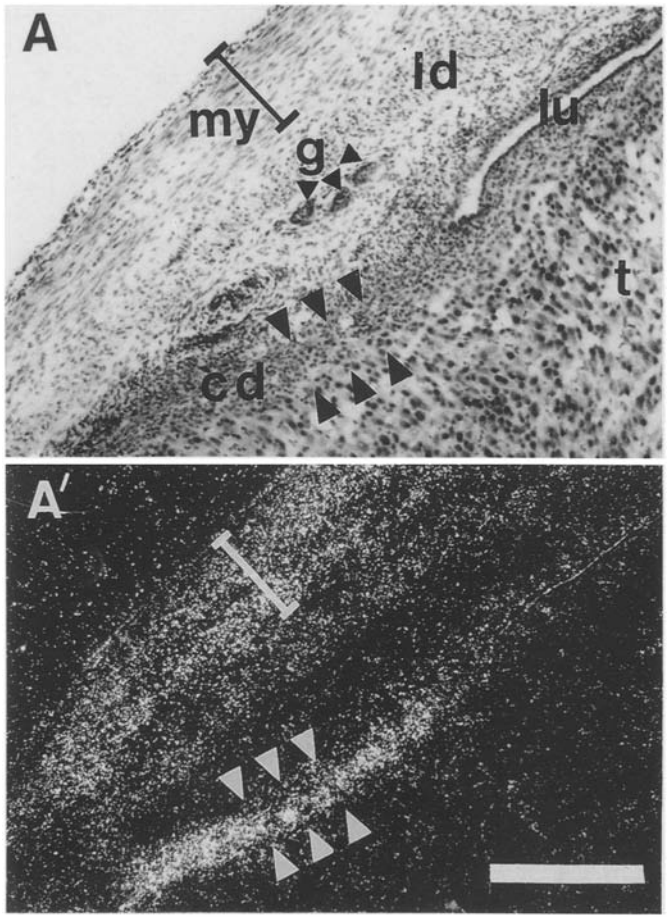

Fig. 13. PTN mRNA expression in the gravid uterus. Pair of bright- and darkfield images of a longitudinal section through the implantation side of a E11 embryo. PTN transcripts are abundant in the myometrium (*bar*) and in the compact decidua (*arrowheads*) beneath the implantation zone. Bar 250 μm. *my*, myometrium; *cd*, compact decidua; *ld*, loose decidua; *t*, trophoblast; *lu*, uterine lumen; *g*, uterine glands

is weak and diffuse, as may occur with some growth factors, discrimination of the ISHH labelling from background may be difficult, and Northern Blotting may then be more sensitive (see e.g. Perkins et al. 1991). This problem appeared to be of limited importance with the PTN mRNA signal which was usually strong, especially during development. The weak ISHH in the dermis accounts for the faint expression reported in the skin at E21 and P1 (Li et al. 1990). The weak PTN mRNA expression detected in the postnatal lung parenchyma contrasts with the strong labelling seen in Northern Blot analysis (Li et al. 1990). PTN transcripts were found to be progressively confined around the main bronchi and the trachea during prenatal development. This may be the source of the labelling found on Northern Blot analysis of RNA extracted from perinatal lung if lung parenchyma has not been carefully dissected. We also did not detect the moderate expression reported in the heart at P6 (Merenmies and Rauvala 1990) nor that in striated muscles reported at P1 and P8 (Li et al. 1990). Alternatively, we observed moderate labelling in tendons and interstitial connective tracts of the striated muscles. This may be the actual source of postnatal muscular PTN mRNA found on Northern blot, as no ISHH labelling was observed in the muscular bundles at these ages but only in early stages of myoblast differentiation. On the other hand, ISHH may be more efficient than Northern Blot at detecting an expression limited to a small number of cells (Nakanishi et al. 1990). Indeed, we provided evidence that the faint signal on Northern blot of adult rat whole brain actually accounts for a weak and diffuse PTN mRNA expression and also for a high expression in discrete subpopulations representing a very small percentage of the total brain cells. The sequential ISHH study of development in whole embryo sections until birth, and of numerous postnatal organs and the identification of most of the cellular types which express transcripts within the organs, allowed us to refine and extend the data obtained by Northern Blot analysis.

Developmental aspects of PTN mRNA expression

PTN transcripts were already abundant at the first stage studied (E11), and our data summarized in Table 1 indicate that PTN mRNA is expressed in many – but not all – neuroectodermal and mesodermal lineages. In contrast, PTN mRNA expression was not observed in the trophoblast nor in endodermal and ectodermal derivatives, with the exception of the postnatal tongue.

PTN has been immunolocalized in dispersed cells from newborn brain and was reported to be present in the neuron-like cells but not in the GFAP-positive astrocytes (Rauvala 1989). In contrast, our ISHH data indicate that, if PTN transcripts are actually found in some neuronal subpopulations, they are also present, both in the central and peripheral nervous system, in populations of non-neuronal cells (olfactory nerve layer, pituicytes, Schwann cells of the DRG). Localization of PTN mRNA in astrocytes remains to be definitively demonstrated, but is already suggested by the presence during development of numerous PTN transcripts in cells in the white matter and in cells concentrated between the cerebellar Purkinje neurons, which mimic the distribution of GFAP mRNA-containing astrocytes (Landry et al. 1990). Moreover, preliminary data on the presence of PTN-like immunoreactivity in the Bergmann glia of the developing rat cerebellum have been recently presented (Wewetzer et al. 1991).

In contrast to the intense and sustained expression in the germinal layer of the nervous system throughout embryonic life, PTN transcripts were only transiently expressed during development in the connective tissues and declined in most locations already by the end of gestation. PTN mRNA expression was detected in the nephrogenic mesenchyme, in bone and cartilage progenitors, in early stages of striated muscle development, in differentiating mesenchyme of the lung, gut and vascular bed and in several other locations. This expression appears to be specific to certain tissue types rather than to their embryonic origins, as a similar expression pattern was observed in bones and cartilages of the head irrespective of their neuroectodermal or mesodermal ori-

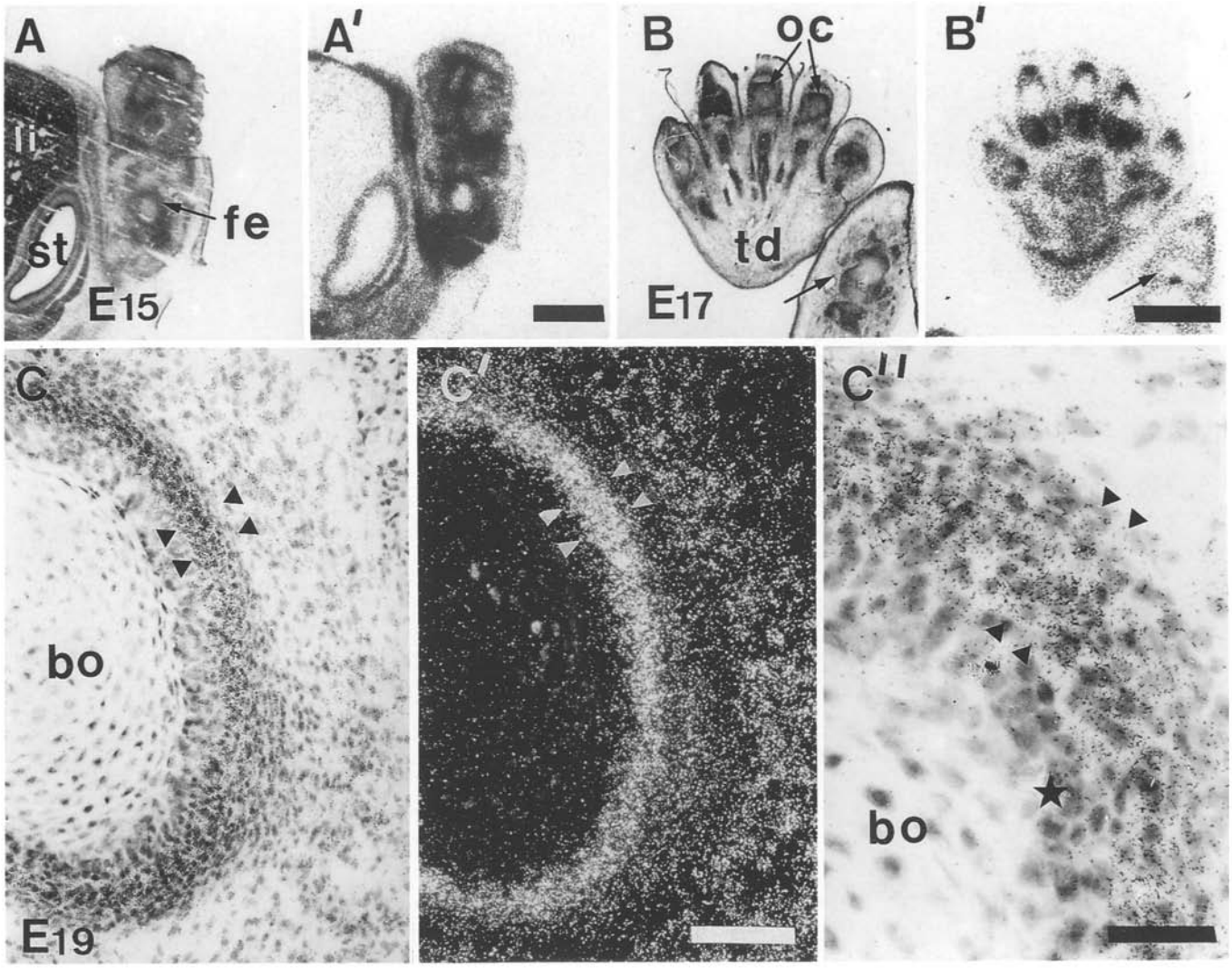

Fig. 14A–C. PTN mRNA expression in fetal bones. **A, B** Macrophotographs of haematoxylin-stained sections. **A′, B′** Respectively, autoradiographs of the same sections. **C, C″** Brightfield micrographs. **C′** Darkfield image corresponding to **C**. **A, A′** E15 embryo; longitudinal section through the hindlimb. Intense PTN mRNA expression in the limb mesenchyme but not in the differentiating skeletal structures. **B, B′** E17 embryo: hindpaw and tail: PTN transcripts are present in the tendinous and bony structures of the paw but not in the ossifications centres of the metatarsals. In comparison, PTN mRNA expression around the tail vertebrae (*thin arrow*) is far less intense than around the bones of the paw. **C, C′** E19 embryo clavicle: PTN transcripts are present in a peripheral ring (*arrowheads*). Transcripts are also present in adjacent musculo-tendinous structure (**C′**). **C** Higher magnification of the same section showing PTN mRNA expression in pre-osteoblasts localized some rows outside the matrix apposition zone (*). Bars 1 mm in **A** and **B**; 100 μm in **C** and **C′**; 50 μm in **C″**. *li*, liver; *st*, stomach; *fe*, femur; *os*, ossification centre; *td*, tendons; *bo*, bone matrix

gin. The time-course of PTN mRNA expression seems to be developmentally regulated in different ways for each system and for different parts within each system (for example: the differential expression in the oesophagus and intestine in the perinatal period). Although we did not address, in the present study, the comparison of PTN mRNA expression with known markers of tissue differentiation (as actins or myosin for example, see e.g. Sawtell and Lessard 1989; Lyons et al. 1990), this point clearly deserves further studies.

PTN mRNA expression in adult tissues

High PTN mRNA expression persists in different cell types (see above) of several discrete structures of the adult nervous system: hippocampus, olfactory nerve, neurohypophysis, cerebellum, sensory ganglia). Moderate amounts of PTN transcripts were also detected in the leptomeninges, in the iris and in the intertubular mesenchyme (Leydig cells) of the adult testis. The intense PTN mRNA expression in the basal layers of the adult tongue epithelium appears as an exception to the general pattern of PTN mRNA expression, as it is the only site where expression appears postnatally. It is also the only epithelial structure and the only ectodermal derivative to express PTN mRNA. This observation is currently unexplained, as is the expression of the Nerve Growth Factor (NGF) in the submandibular gland of the adult male mouse (Levi-Montalcini and Bookes 1960; Scott et al. 1983).

The presence of PTN transcripts in the adult uterus has been reported previously (Milner et al. 1989), and the present study indicates that this expression is diffuse throughout the myometrium with some strengthening beneath the decidua. Interestingly, this expression was found to be considerably increased during gestation, and a physiological role for PTN in the modifications of the uterus during the reproductive cycle appears therefore highly probable.

Functional significance of the PTN mRNA expression

The high conservation of the amino acid sequence of PTN among mammalian species (Li et al. 1990) strongly suggests that PTN plays important roles. PTN was first characterized as a neurite outgrowth-promoting factor in the culture of embryonic rat neurons (Rauvala 1989) and as a mitogen for rat and mouse fibroblasts in vitro (Milner et al. 1989). Moreover a so-called osteoblast-specific protein OSF-1 isolated from neonatal mouse calvaria was found after cloning to be identical to PTN (Tezuka et al. 1990). The present data on the PTN mRNA expression indicate that many cell types may actually be involved.

PTN is probably secreted (Rauvala 1989; Merenmies and Rauvala 1990) and an autocrine or paracrine action is quite probable (Rauvala 1989). PTN also binds strongly to the extracellular matrix (Li et al. 1990) which is known to be essential in the mesenchyme/epithelium interactions (Beaulieu et al. 1991). PTN mRNA is not expressed in normal epithelial structures (with the exception of the postnatal tongue epithelium) but it is consistently expressed in mesenchyme surrounding the epithelia at some stages of differentiation. The intense and persistent PTN mRNA expression around the lacrymal and submandibular glandular epithelia at a time where PTN mRNA expression has already vanished from the nearby mesenchyme also raises the hypothesis that PTN might possibly be involved in the developmental mesenchyme/epithelium interactions.

During the preparation of this manuscript, characterization of a PTN receptor of mouse fibroblastic NIH 3T3 cells has been reported (Kuo et al. 1992) and the action of PTN as an epithelial growth factor has been confirmed (Wellstein et al. 1992). Moreover PTN has been identified as a tumour growth factor and, interestingly, the expression of PTN mRNA has been shown to be induced by malignant transformation in breast epithelial cells and melanocytes (Wellstein et al. 1992). PTN mRNA has been found in a human osteosarcoma cell line (Tezuka et al. 1990) and different PTN mRNA expression in meningioma subtypes has also been observed (Mailleux et al., in preparation).

Our data on PTN mRNA expression in normal adult tissues, specially the up-regulation of PTN mRNA expression observed in the gravid uterus, indicate that PTN is also a cytokine involved in physiological mechanisms during maturity. Further work will probably reveal other modifications of the PTN mRNA expression under physiological and pathological conditions.

The Pleiotrophin family

PTN belongs to an emerging new family of developmentally regulated cytokines, referred to below as the Pleiotrophin family (Li et al. 1990; Raulais et al. 1991). Two other members are known so far: the MK1 gene product (Kadomatsu 1988) and Ri-HB (Raulais et al. 1991), respectively in mouse and chick. The pattern of mRNA expression reported for these two factors appears to be quite different and more restricted than that of PTN mRNA. The expression of both factors is regulated by RA (Kadomatsu 1988; Raulais et al. 1991). No data concerning the influence of RA on PTN expression have been published yet (Raulais et al. 1991). PTN appears to be involved – among the many other features cited above – in the development of bone and cartilage (Tezuka et al. 1990). Therefore, if RA should have the same action on the regulation of PTN expression as it has on the two other known members of the PTN family, this would possibly bring new insights towards understanding the already complex action of RA (see e.g. Tabin 1991).

PTN, MK1 and Ri-HB have been cloned in different species and their counterparts probably exist in other species. This Pleiotrophin family is just emerging, but they already appear to be important cytokines involved in growth and differentiation of many (essentially) neuroectodermal and mesodermal normal lineages and in neoplasia. Further studies on the regulation of their expression, their receptors and their modes of action seem therefore to be extremely promising.

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