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Proenkephalin A is expressed in mesodermal lineages during organogenesis

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Proenkephalin A (PEA) encodes several neuropeptides with an opioid activity, as well as other peptides with as yet unknown functions. As an initial step toward finding possible roles for PEA gene products in non-neuronal tissues, we have determined sites of PEA expression during mouse embryonic development, employing *in situ* hybridization. We report here the unexpected observation that in addition to its abundance in brain, PEA RNA is expressed in non-differentiated mesodermal cells of diverse lineages in the process of their development into several adult tissues and organs; it drops to undetectable levels upon terminal differentiation of these tissues. In a particular example of differentiating mesoderm, the developing kidney, the transient expression of PEA mRNA and of its encoded peptide Met-enkephalin was demonstrated by both *in situ* and Northern blot hybridizations, as well as by a radioimmunoassay. These findings suggest a novel role for PEA-derived peptide(s) in mesoderm growth or differentiation during organogenesis.

Key words: proenkephalin A/gene expression/mouse development/mesoderm/*in situ* hybridization

Introduction

Enkephalins are naturally occurring peptides exhibiting opiate-like activity. Since their discovery by Hughes *et al.* in 1975, >18 different peptides and opioid activity have been isolated and characterized. All of these peptides are processed products of one of three precursor proteins. The precursor proteins are, in turn, each encoded by a single copy gene: proenkephalin A (PEA), proenkephalin B (PEB) and pro-opiomelanocortin (POMC) (Douglass *et al.*, 1984). PEA is the precursor of the opioid peptides Met-enkephalin, Leu-enkephalin, Met-enkephalin-Arg-Phe, Met-enkephalin-Arg-Gly-Leu and Met-enkephalin-Arg-Arg-Val (Comb *et al.*, 1982; Gubler *et al.*, 1982; Noda *et al.*, 1982). PEA is also the precursor of peptides with no demonstrable opioid activity like amidorphin 8-26 and synenkephalin (Liebisch *et al.*, 1986; Liston *et al.*, 1983). On the basis of the prevalence of enkephalins in the central nervous system (CNS) as well as on the basis of extensive physiological and pharmacological studies, it is widely accepted that enkephalins function as neurotramsmitters, neuromodulators or neurohormones (Akil *et al.*, 1984; Imura *et al.*, 1985). Recent reports have shown, however, that PEA mRNA and encoded peptides are also present in non-neuronal tissues within the brain, as well as in a number of other organs (Kilpatrick *et al.*, 1985; Howells *et al.*, 1986; Zurawski *et al.*, 1986; Vilijn *et al.*, 1988). These observations suggested that PEA-encoded peptides might play a role in non-neuronal tissues that is distinct from their role in the CNS. One possibility, not yet explored, is that PEA-derived peptides function during development.

Results and Discussion

In order to identify developmental processes in which PEA mRNA might be involved we searched for PEA mRNA in all tissues throughout embryonic development. We reasoned that a search for PEA mRNA might be more informative than the analysis of any specific PEA-encoded peptide for two reasons: unlike mRNA, peptides are likely to be transported from their site of synthesis, and mRNA is a more general probe in the sense that it can detect the capacity to encode as yet unidentified peptides. *In situ* hybridization methodology was employed in order to allow tracing of particular cell lineages expressing PEA mRNA, as well as to determine their differentiation stage.

A 417-bp-long PEA DNA fragment, fully contained within the main coding exon (exon 3) of rat PEA gene, served as the source of PEA-specific sequences. ^{35}S-Labelled antisense RNA was synthesized *in vitro* and was used as an hybridization probe. Ten-micrometre-thick frozen sections were prepared from BALB/c mouse embryos at ages 10–19 days of gestation, and were processed and hybridized under conditions that favor formation of RNA–RNA hybrids (Hogan *et al.*, 1986).

During development, PEA mRNA was detected in various compartments of the central nervous system. For example, in a 12.5-day-old embryo PEA mRNA was predominantly detected in the basal plate of the brain stem (Figure 1A and D). In later stages of embryonic development, PEA mRNA was detected in additional brain regions such as the thalamus, pons, the choroid plexus and in scattered enkephalinergic cells in the spinal cord (data not shown). Studies concerning the distribution of PEA in the developing CNS, however, are beyond the scope of this report. Rather, this study focuses on the expression of PEA mRNA in the less expected locations outside the CNS.

As organogenesis progresses, increasingly higher levels of PEA mRNA are found in a number of mesodermal tissues. In the section of the 15.5-day-old embryo, shown in Figure 1C and F, the highest levels of PEA mRNA were detected in the cartilagenous and kidney primordia. To ascertain that the signals observed in the *in situ* hybridizations indeed represent an authentic PEA mRNA, control hybridizations were carried out employing a 'sense probe' (i.e. a riboprobe synthesized from the same PEA plasmid template but in the

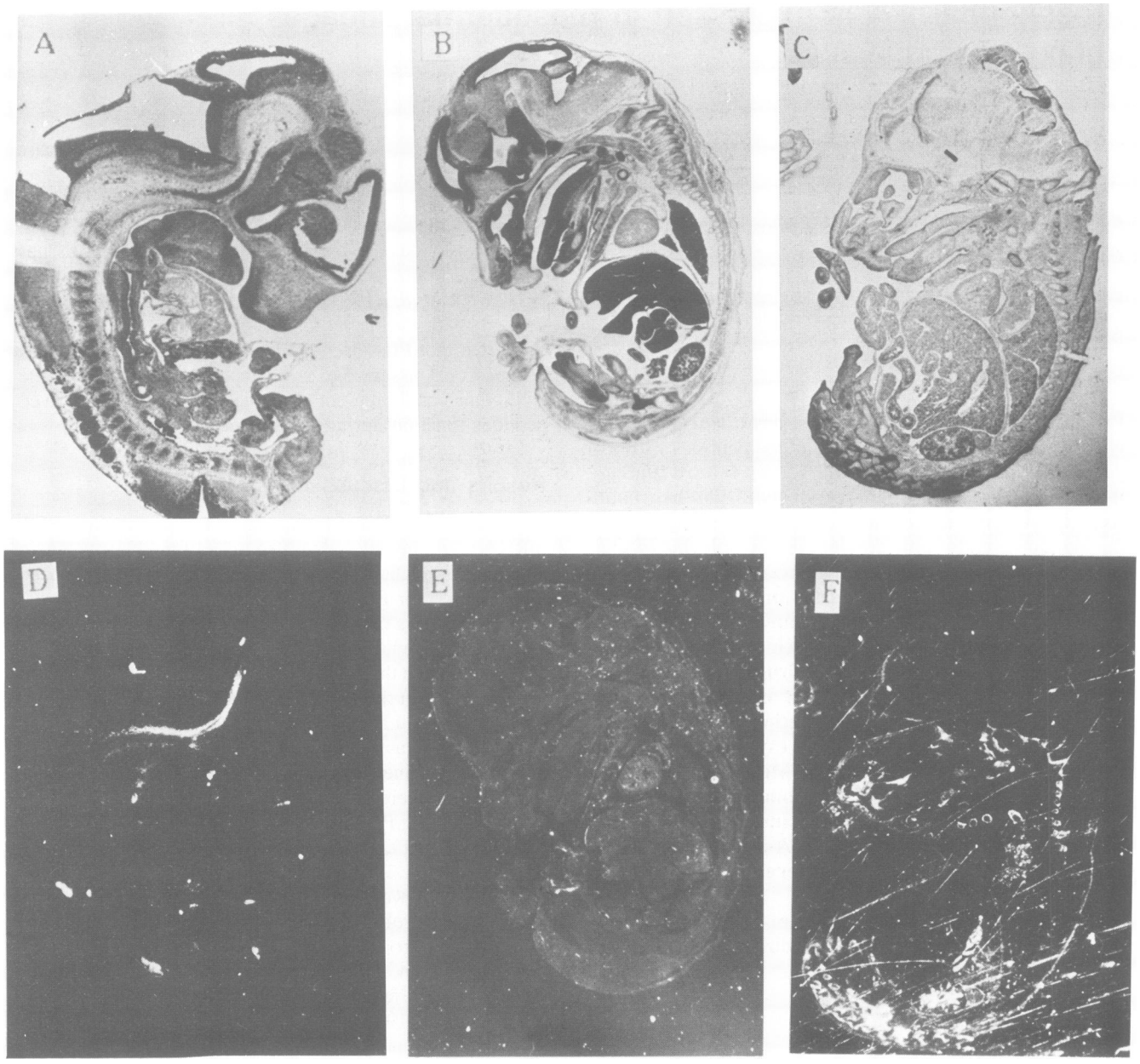

Fig. 1. *In situ* hybridization of embryonic sections with PEA-specific probe. (**A, D**) 12.5-day-old embryo. (**B, C, E, F**) 15.5-day-old embryos. Top figures were photographed under bright-field illumination, while bottom figures show dark-field images of the same sections. Hybridization was with an antisense PEA probe (**A** and **D**, **C** and **F**) or with a sense PEA probe (**B** and **E**). Following 4 days of autoradiographic exposure, sections were stained with Giemsa stain and photographed (magnification in A and D is twice the magnification of the 15.5-day-old embryos shown).

sense orientation). In all cases no hybridization signals were detected. (See Figure 1B and E for an example of a 'sense' control).

Overall, PEA mRNA was detected in mesenchymal tissues during the process of differentiation into cartilage, bone, dermis, kidney tubules and choroid of the eyes. Examples of these observations at a resolution that allows further identification of expressing cell types are highlighted in Figures 2–4. Figure 2 shows different body areas of a single 15.5-day-old embryo: a section through spinal vertebrae (Figure 2A), a section through the larynx (Figure 2B), a section through the eye (Figure 2C), and a section through the kidney (Figure 2D). High levels of PEA mRNA were detected in the perichondrium of all cartilaginous primordia—the vertebrae (Figure 2A), the laryngeal cartilage (Figure 2B), the cartilage of the ribs (Figure 3B and D), as well as the cartilage of the long bones of the limbs and skull (data not shown). Intense hybridization over the cartilaginous primordia was especially prominent on days 14–17 of gestation, was gradually reduced at later stages, and was undetectable in fully differentiated cartilage.

Embryo sections provide an opportunity to distinguish between cells that are at progressive stages of their differentiation in a single tissue or organ primordium. For example, differentiation of perichondrium cells into mature chondrocytes can be followed by comparing cells in a single section of a developing rib (Figure 3B). As can be seen in Figure 3B and D PEA mRNA is abundant in the perichondrium but undetected in mature non-proliferating chondrocytes, enforcing the notion that PEA is down-

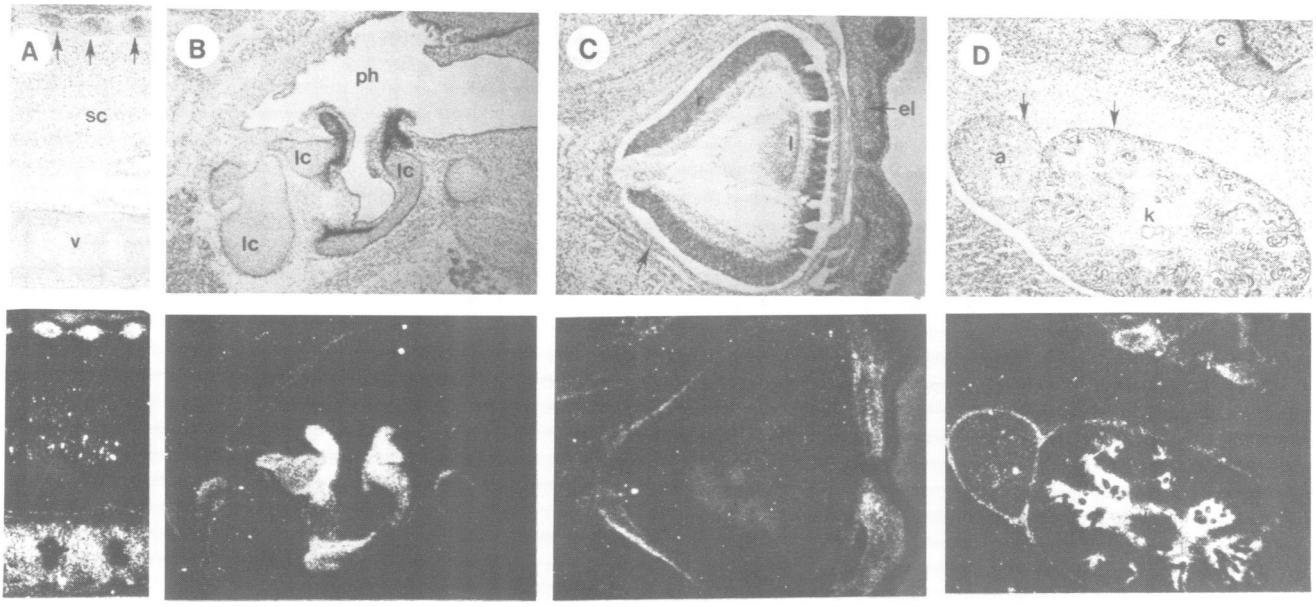

Fig. 2. *In situ* hybridization of 15.5-day-old embryo sections with PEA-specific probe. (**A**) Section through vertebrae. (**B**) Section through the larynx. (**C**) Eye section. (**D**) Kidney section. Following 4 days of autoradiographic exposure, sections were stained with Giemsa stain and were photographed (at ×125 magnification) under bright-field (top figures) and dark-field (bottom figures) illumination. (**A**) V, vertebrae; sc, spinal cord; arrows point to vertebrae processes. (**B**) Ph, pharynx; lc, laryngeal cartilage. (**C**) r, retina; l, lens; el, eyelids; arrow points to the choroid. (**D**) k, kidney; a, adrenal gland; c, cartilage.

regulated upon terminal differentiation of these cells. Similar patterns of restricted PEA expression, i.e. only in cells preceding the conversion to mature chondrocytes, were found in all other cartilaginous primordia examined (data not shown). The developing kidney is another organ where both non-differentiated and differentiated cells of the same mesenchymal lineage can be viewed in a single section. The differentiation of mesenchymal cells in the kidney starts in the periphery in cell clusters around the tips of the ureter epithelium. As a result, the non-differentiated mesoderm occupies the inner parts of the kidney. As can be seen in Figure 2D, in the kidney of the 15.5-day-old embryo PEA transcripts are detected predominantly in the kidney medulla but are undetected in the renal tubuli and glomeruli at the periphery of the kidney (where differentiation has already occurred). PEA mRNA was also detected in the mesoderm-derived connective tissue of the renal and adrenal capsules. In order to allow the better identification of PEA expressing cells in the developing kidney, we provide higher magnification images of the *in situ* hybridization (Figure 4). As can be clearly seen in Figure 4, PEA expression is detectable only in the loose non-differentiated mesenchyme. Interestingly, PEA-expressing cells are mostly distributed in clusters around the epithelial cells of the tubuli. The question whether the proximity to epithelial cells plays any role in activation of PEA in the non-differentiated mesenchyme remains to be determined. Kidneys of an adult mouse (8 weeks of age) contained no detectable PEA mRNA by an identical *in situ* hybridization analysis (data not shown), indicating that PEA expression in the embryonic kidney does not reflect a role of enkephalins in a specialized kidney function, but more likely is associated with a particular step(s) of kidney development.

PEA expression was also found in at least two other cell types of mesodermal origin: the mesenchyme beneath the epidermis of the skin (Figure 3B and D), and the choroid and sclera of the eye (Figure 2C, and for more details see Figure 3A and C). Together, the tissues shown above to express PEA mRNA represent three major mesodermal lineages: head mesenchyme, dorsal mesoderm and intermediate mesoderm.

In order further to establish that the signals observed in the *in situ* hybridizations are indeed authentic PEA mRNA, as well as to compare the molecular species of PEA mRNA in embryonic tissues to its respective message in brain, we performed Northern blot analysis employing the same PEA-specific probe. It should be pointed out that rat and mouse PEA sequences are >95% homologous. This high degree of sequence conservation enabled its use as an hybridization probe against the heterologous RNA under stringent hybridization conditions, as previously used by others (Kilpatrick and Millette, 1986). Nevertheless, we repeated both *in situ* and Northern hybridization experiments with the respective mouse PEA probe that we recently recloned with the aid of the polymerase chain reaction (PCR) methodology (see Materials and methods). Essentially identical results were obtained. As can be seen in Figure 5A a single band of 1.4 kb was detected in both hypothalamus (our reference for PEA mRNA in the CNS) and each of the embryonic tissues examined (eyes, appendages, skin, muscle and kidney), suggesting that the same molecular species of mRNA encodes PEA peptides in both the CNS and in non-neuronal embryonic tissues. Note that the levels of PEA mRNA in certain tissues (e.g. legs) are higher than the respective levels in the brain at this particular stage of embryonic development.

In rodents, the development of several organs continues after birth. This includes the kidney, where final differentiation proceeds during the first few days of postnatal development. We compared, therefore, the levels of PEA mRNA in embryonic, early, late postnatal and adult kidney. As shown in Figure 5, we detected the 1.4 kb PEA transcript

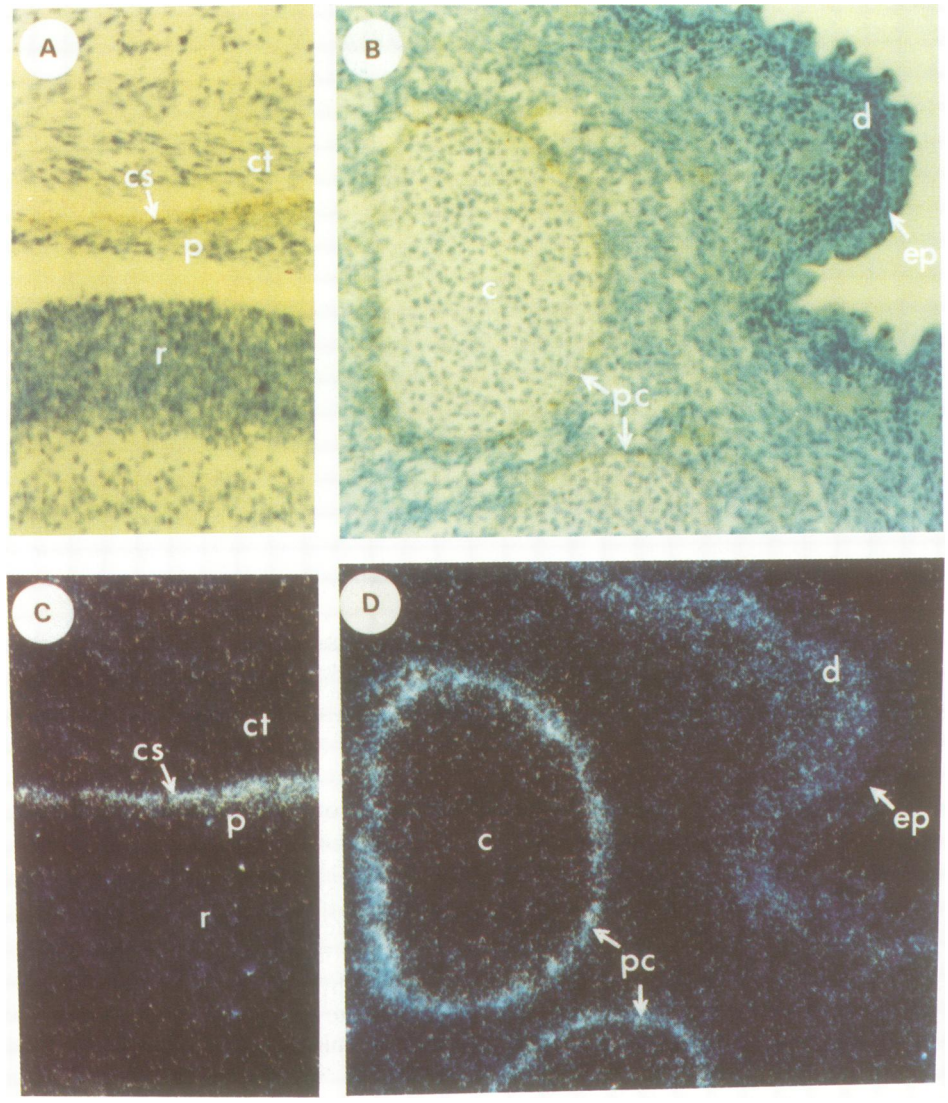

Fig. 3. *In situ* hybridization of 15.5-day-old embryo sections with a PEA-specific probe. (**A**, **C**) Bright- and dark-field photography respectively of an enlarged area from Figure 1C, r, retina; p, pigmented epithelium of the retina; cs, choroid sclera; ct, connective tissue around the eyeball. (**B**, **D**) A section through ribs and skin. Bright- and dark-field photography respectively. c, cartilage; pc, perichondrium; d, dermis; ep, epidermis. Sections were photographed at ×250 magnification. Experimental conditions were the same as those used in the experiment shown in Figure 1.

before birth, shortly after birth, but not in the adult kidney, consistent with the notion that PEA expression is down-regulated upon completion of kidney development. The kidney system was also chosen in order to demonstrate the transient presence of PEA-encoded peptides. This organ can be obtained from embryonic sources, safely free of contaminating tissues, in sufficient amounts to be used in a radioimmunoassay (RIA). As can be seen in Table I, low, but significant amounts of immunoreactive Met-enkephalin were reproducibly detected in the embryonic kidney just before birth and shortly after birth (1 day), but were undetected in the adult kidney (day 60). A procedure was also applied that allowed the distinction between free and cryptic Met-enkephalin. All Met-enkephalin was present in its fully processed form (Table I). Interestingly, in rat heart and in the C6 glioma cell line, where levels of PEA mRNA and Met-enkephalin were compared, it was found that despite the abundance of PEA mRNA only a minute amount of Met-enkephalin was detected. This disparity may be due to the release of peptides to some distal sites. However, it may also reflect a tissue-specific translational control or (as indeed suggested in these studies by the presence of larger proteins) a differential proteolytic processing in different tissues (Kilpatrick and Millette, 1986; Yoshikawa and Sabol, 1986; Kilpatrick *et al.*, 1987; Yoshikawa and Aizawa, 1988). The relatively low levels of Met-enkephalin in the developing kidney presumably reflect, among other possibilities, the situation that only a small fraction of cells express PEA at this developmental stage (see Figure 4 for comparison). Although preliminary, our peptide data, and in particular, the down-regulation of Met-enkephalin upon completion of kidney development, which parallels our *in situ* and Northern hybridization data, further substantiates the mRNA data and enforce the notion that a PEA-encoded peptide plays a role in this developmental process.

When the same RNA preparations were hybridized with probes specific to the two other genes encoding opioid peptides—PEB and POMC—no hybridization was detected

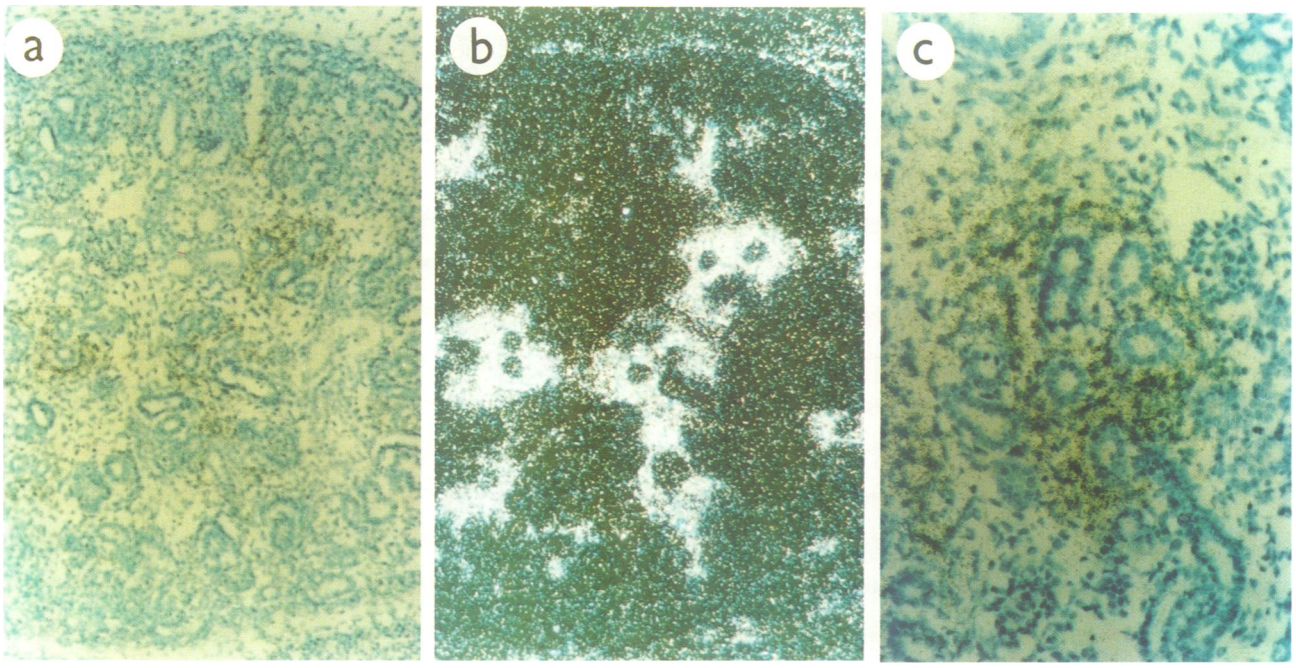

Fig. 4. *In situ* hybridization of a kidney section of a 17.5-day-old mouse embryo. Experimental conditions were the same as before. **a** and **b** are photographs of the same section under bright- and dark-field respectively. Magnification in **c** is two times higher than in a and b. Note in c that autoradiographic grains are predominantly in loose mesenchyme clustered around epithelial cells.

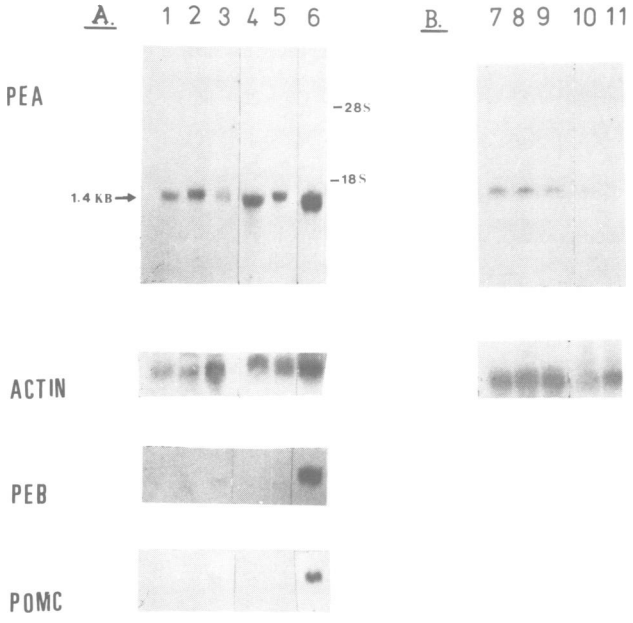

Fig. 5. Northern blot analysis of PEA-specific RNA. Total RNAs were from: **1**, eyes of a 17-day-old mouse embryo (15 μg); **2**, legs of a 17-day-old mouse embryo (15 μg); **3**, brain of a 17-day-old mouse embryo (20 μg); **4**, skin of a 21-day-old rat embryo (20 μg); **5**, muscle of a 21-day-old rat embryo (20 μg); **6**, hypothalamus of an adult rat (10 μg). **Lanes 7–11**, rat kidney of a 18- and 21-day-old embryo and at days 1, 28 and 60 after birth respectively (20 μg). 28S and 18S markers correspond to the position of the rRNA bands in the gel. The filter hybridized with the PEA probe was rehybridized with the actin probe and a duplicate filter hybridized with the PEB probe was rehybridized with the POMC probe. Exposure times were: PEA probe, **lanes 1–6**: 15 h; **lanes 7–11**: 36 h. β-Actin probe: 6 h. PEB probe: 30 h. POMC probe: 15 h.

Table I. Met-enkephalin in the developing kidney

	Age		
	E-21	P-1	P-60
Met-enkephalin	145 ± 13	110 ± 15	<2
Met-enkephalin after proteolytic digestion	130 ± 12	100 ± 10	<2

Between 10 and 20 rat kidneys of the indicated age were pooled. Extracts were prepared and analyzed as described in Materials and methods. With or without a prior digestion with trypsin and carboxypeptidase B to release the cryptic forms of Met-enkephalin. The values of immunoreactive Met-enkephalin are expressed in fmol/g wet tissue and are representative of three independent experiments. E-21, embryonic 21 days p.c.; P-1, day of birth; P-60, 60 days after birth.

in the non-neuronal embryonic tissues examined (Figure 4a). Consistent with these results we could not detect hybridization signals with PEB and POMC over the developing cartilages, dermis and kidneys by *in situ* hybridization analysis using sections from the same series used in the respective PEA experiments shown above. As a positive control for these negative *in situ* hybridization results, we could readily detect POMC RNA in the embryonic pituitary gland (data not shown). These results indicate that expression in non-differentiated mesoderm cells is not a common feature of genes encoding opioid peptides but it is a feature specific to PEA.

The function, if any, of PEA-encoded peptides in embryonic development remains to be elucidated. A putative dual role of PEA-encoded peptides as both neuropeptides and as growth or differentiation factors, if established, will

follow precedents set by other neuropeptide-encoding polyprotein systems. For example, substance P, processed from its preprotachykinin A precursor, functions both as a neurotransmitter and also stimulates growth of connective tissue and affects the production of inflammatory cytokines (Nilsson *et al.*, 1985). Also, proteolytic processing of POMC yields a peptide that possesses an opioid activity (β-endorphin) and another peptide (ACTH) that stimulates the growth of all cells of the adrenal cortex (Douglass *et al.*, 1984), and is a specific mitogen for mammalian myogenic cells (Cossu *et al.*, 1989). Similar growth-promoting activities have not yet been shown for PEA. In a recent report, Vilijn *et al.* (1988) showed a 3-fold difference in PEA mRNA levels between brain astrocytes that have been cultured from embryonic brain and cultures established from neonatal brain. On the basis of this finding, they suggested that PEA may be down-regulated during brain development. A specific developmental process in which PEA may play a role has not yet been identified, and certainly not in a developmental process outside the central nervous system.

Mesoderm differentiation is a major process in embryonic development that involves a succession of inductive processes. It is likely that several molecules acting either in concert or in a cascade fashion regulate these complex cellular interactions (Gurdon *et al.*, 1989). Two major features of the PEA expression during mesoderm differentiation render PEA as a good candidate for a player in this process. First is the specificity for mesodermal lineages and the generality for several major mesodermal lineages, at the same time. Second is the transient nature of PEA expression in each lineage preceding its terminal differentiation. This suggestion is experimentally testable, for example, by the use of available cell culture systems that reproduce mesoderm growth and differentiation *in vitro*.

Materials and methods

In situ hybridization analysis

Sections were derived from BALB/c mouse embryos. Embryos were aged as '0.5 day postcoitum' at noon of the day on which the vaginal plug was found. *In situ* hybridization was performed essentially as described by Hogan *et al.* (1986). Briefly, 10-μm-thick frozen sections were collected on poly-L-lysine-coated glass slides, refixed in 4% paraformaldehyde and dehydrated in graded ethanol solutions. Before hybridization, sections were pretreated successively with 0.2 N HCl, 2 × SSC, 0.125 mg/l pronase, 4% paraformaldehyde and acetic anhydride in triethanolamine buffer. Hybridization was carried out at 50°C overnight in 50% formamide, 0.3 M NaCl containing 10% dextransulfate, 1 × Denhardt's solution, 1 mg/ml carrier tRNA, 10 mM DTT, 5 mM EDTA and 2 × 10^8 c.p.m./ml ^{35}S-labelled riboprobe. Post-hybridization washing was performed under stringent conditions that included an incubation at 50°C for >4 h in 50% formamide/0.3 M NaCl and a 30 min incubation at 37°C in 20 μg/ml RNaseA. Autoradiography was performed using Kodak NTB-2 nuclear track emulsion.

Isolation and blot analysis of RNA

Total RNA was extracted by homogenization in guanidine thiocyanate followed by centrifugation through CsCl according to the method of Chirgwin *et al.* (1979). Total RNA was denatured in glyoxal and subjected to electrophoresis on a 1.5% agarose gel in 10 mM phosphate buffer. The RNA was transferred to a nylon-based membrane (Genescreen, NEN) by the capillary blot procedure and hybridized with the indicated probe as described (Yoshikawa and Sabol, 1986). The membranes were washed under stringent conditions which included washes at 55°C in 0.2 SSC and 0.1% SDS, 0.1% sodium pyrophosphate.

Hybridization probes

Hybridization probes were as follows (i) Rat PEA: a 417-bp *Pst*I fragment that begins 21 bp downstream from the start of exon III of the rat PEA gene that was isolated from pREK-9 plasmid (Rosen *et al.*, 1984). (ii) Mouse PEA: a mouse PEA subclone was recloned in our laboratory directly from mouse genomic DNA by the polymerase chain reaction (PCR) methodology and the use of PEA-specific oligonucleotides synthesized on the basis of the previously sequenced Ty 3.9 PEA cDNA clone (Zurawski *et al.*, 1986). Briefly, the oligonucleotides (GCAGATCT)ATGTACAAAGACAGCA-GCAA and (AGAAGCTT)TCTTGTTGGTGGCTGTCTTT were synthesized. These oligonucleotides each contain a 20mer corresponding to PEA DNA sequences on opposite strands and a 5′ 8mer linker that contain a restriction site for *Bgl*II and *Hin*dIII respectively. Upon PCR amplification, a 294 bp fragment was produced that contains a 278 bp PEA sequence derived from exon III and is bracketed by *Bgl*II and *Hin*dIII sites. The purified fragment was digested with *Bgl*II and *Hin*dIII and was cloned into the *Bam*HI and *Hin*dIII sites of the PBS polylinker. The identity of the insert was confirmed by DNA sequencing. (iii) Rat PEB: a 0.6 kb *Hin*dIII fragment of the genomic PEB clone, pλRD2-19 (Civelli *et al.*, 1985). This fragment is derived from the third exon of PEB and was subcloned in a PBS vector. (iv) Mouse POMC: a 0.9 kb *Eco*RI–*Hin*dIII fragment was isolated from the POMC cDNA clone pMKSU-16 (Uhler *et al.*, 1983) and subcloned in a PBS vector.

Following subcloning into the vector PBS (Stratagene), the constructs were linearized by digestion with the appropriate restriction endonuclease to allow synthesis of a ^{35}-labeled RNA probe in either antisense or sense orientation respectively (using T3 or T7 polymerases). These RNA probes were used in the *in situ* hybridization analysis. For RNA blot analysis, ^{32}P-nick-translated probes were prepared.

Isolation and analysis of peptides

Peptide extraction and radioimmunoassay were performed as described elsewhere (Liston and Rossier, 1984; Zurawski *et al.*, 1986). Before RIA for Met-enkephalin (Amersham), aliquots of acidified extracts were purified directly on a C18 octadecyl Amprep minicolumns in a non-polar extraction procedure in accordance with the manufacturer's recommendations (Amersham). Other aliquots of the acidified extract were subjected to a sequential digestion by trypsin and carboxypeptidase B prior to column purification and were also assayed for immunoreactive Met-enkephalin. RIA was carried out with enkephalin antiserum (rabbit) and [^{125}I]Met-enkephalin (Amersham, RIA grade). Reaction mixture contained antiserum, [^{125}I]Met-enkephalin (30 000 c.p.m.), Met-enkephalin standards or extract samples, 50 mM sodium phosphate buffer, pH 7.4, 0.2% gelatin and 10 mM EDTA. Reaction mixtures were incubated at 4°C for 24 h and the bound label was precipitated using a second antibody.

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