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The Developmental Expression in Rat of Proteases Furin, PC1, PC2, and Carboxypeptidase E: Implications for Early Maturation of Proteolytic Processing Capacity

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The genes encoding mammalian subtilisin-like endoproteases furin, PC1, and PC2 have been isolated and are implicated in endoproteolytic cleavage of precursor molecules, which is a key step in posttranslational maturation of proproteins and neuropeptide precursors. Following endoproteolytic cleavage, the carboxyl-terminal basic amino acid residues are removed by carboxypeptidase E (CPE). We have examined the expression of these genes during rat development by *in situ* hybridization and compared their expression patterns to those of potential substrates. In the primitive streak stage of embryogenesis (e7) furin is expressed in both endoderm and mesoderm. This overall expression pattern is maintained until e10, when a distinctly higher level of furin expression is observed in the heart and liver primordia. In mid- and late gestational stages furin is broadly expressed in the peripheral tissues, and, therefore, may contribute to the proteolytic processing of numerous fetal proproteins, such as the precursors for natriuretic factors in heart and IGF-II throughout the embryo. In contrast, the expressions of PC1 and PC2 are initiated much later (e13) and are mainly confined to the developing nervous system, but with distinct spatial distributions. At midgestational ages, PC1 mRNA is mainly expressed in the hypothalamus and peripheral ganglia, while PC2 is expressed not only in these tissues but also in the thalamus, midbrain, pons, medulla oblongata, cortical plate, and spinal cord. Besides neuropeptide precursor processing in the nervous system, PC1 and PC2 may also be involved in the proteolytic processing in additional regions as evidenced by the finding that both PC1 and PC2 mRNAs are expressed in the embryonic pituitary and pancreas. CPE mRNA is expressed in both neural tissues and some non-neural tissues. In the developing nervous system, the expression of CPE encompasses all the regions where PC1 and PC2 are expressed and in fact includes most brain regions as neurogenesis proceeds. CPE mRNA is also expressed in some peripheral tissues, such as the embryonic heart and cartilage primordia, and in some cases its expression overlaps with furin expression. Thus, CPE may functionally collaborate during development with the subtilisin family of endoproteases in the completion of proteolytic processing of neuropeptide precursors in the nervous system and proproteins in the peripheral tissues. In the pituitary, the endoproteolytic processing of polyfunctional precursor proopiomelanocortin (POMC) occurs in a developmentally regulated manner. We have shown that while PC2 mRNA is predominately expressed in the intermediate lobe in the adult, we observed an increased expression of PC2 mRNA in developing rat anterior lobe, peaking at early postnatal stages. The expressions of PC1 and PC2 in early postnatal pituitary overlap with POMC expression and the levels of PC1 and PC2 expression in pituitary parallel changes in POMC endoproteolytic processing pattern, suggesting regulation of endoprotease expression may account for differential processing of proproteins in development.

[Key words: furin, PC1, PC2, carboxypeptidase E,* in situ *hybridization, ontogeny, neuropeptide processing, prohormone processing]

Most neuropeptides and peptide hormones are initially synthesized as inactive protein precursors, which require various posttranslational modifications to generate bioactive products. One of the crucial steps of this process is proteolytic cleavage. This usually involves endoproteolytic cleavage of precursors at the carboxyl side of specific dibasic residues, followed by exoproteolytic removal of the terminal basic residues from the peptide (Douglass et al., 1984; Mains et al., 1990). The exopeptidase carboxypeptidase E (CPE), which is responsible for the removal of exposed C-terminal basic amino acid residues following endoproteolytic cleavage, has previously been identified and characterized (Fricker, 1988). Until recently, the identity of the endoproteases performing endoproteolytic cleavage had remained uncertain despite intensive biochemical and enzymatic studies. The yeast functional homolog of mammalian endoprotease, kex2, has been shown to perform accurately endoproteolytic cleavages of several mammalian proproteins in gene transfer experiments, implying the possible evolutionary conservation of the endoprotease gene family (Thim et al., 1986; Thomas et al., 1988). In recent years several mammalian endoproteases, including

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furin (PACE), PC1 (PC3), PC2, PC4, PC5 (PC6), and PACE4 have been isolated by virtue of sequence homology with the subtilisin-like catalytic domain of kex2 (Lusson et al., 1993; Nakagawa et al., 1993; Van de Ven et al., 1993). Since many cell lines are capable of processing heterologous prohormones and neuropeptide precursors at correct sites in gene transfer experiments (Thomas and Thorne, 1988), it is possible that this relatively limited number of proteases is sufficient to proteolytically process a large number of proproteins. Among the newly isolated endoproteases, furin, PC1, and PC2 have been shown to be capable of correctly processing multiple protein precursors at physiologically utilized sites (Bresnahan et al., 1990; Wise et al., 1990; Benjannet et al., 1991; Misumi et al., 1991; Thomas et al., 1991; Smeekens et al., 1992). Furin, PC1, and PC2, therefore, may act as a core set of processing enzymes and could be expected to be broadly distributed to meet the proteolytic demand.

All cells intrinsically possess a constitutive secretory pathway, in which secretory proteins and membrane-associated receptors are proteolytically processed from precursors in a Golgi-related compartment. A wide variety of proproteins are processed through this pathway, including the precursors of growth and trophic hormones (e.g., NGF, brain-derived neurotrophic factor, platelet-derived growth factor, TGFβ, IGF-II), receptors (insulin receptor, IGF-I receptor, E-cadherin, N-cadherin), and plasma proteins (albumin, blood coagulation factors, complement factors) (Barr, 1991; Bresnahan et al., 1993). The processing products are released constitutively without apparent intracellular storage and regulation (Burgess and Kelly, 1987). Alignment of the sequences around the cleavage site of their precursors reveals that besides dibasic residues, an Arg residue usually exists at position −4. The common motif deduced, Arg-X-Lys/Arg-Arg, seems to be the preferential recognition site for the endoproteases that reside in the constitutive pathway (Watanabe et al., 1992). This notion is substantiated by mutagenesis analysis of the renin precursor (Hatsuzawa et al., 1990; Oda et al., 1991; Watanabe et al., 1992). When the nonbasic residue at position −4 was substituted by Arg, the mutated prorenin is processed efficiently in CHO cells, which only have the constitutive pathway and normally secrete prorenin synthesized from a transfected prorenin substrate. Coexpression of furin enhanced the proteolytic conversion of mutated prorenin, but not native prorenin. Other coexpression studies have shown that furin is able to cleave the von Willebrand factor precursor (Wise et al., 1990), proNGF (Bresnahan et al., 1990), proalbumin and complement pro-C3 (Misumi et al., 1991), which strongly suggest that furin is involved in precursor maturation within the constitutive pathway. In agreement with this, furin is found in the adult to be expressed in all tissues and cell types examined (Hatsuzawa et al., 1990) and is predominantly localized in the Golgi apparatus (Misumi et al., 1991).

In addition to the constitutive secretory pathway, neuroendocrine cells also possess a regulated secretory pathway. The proteases involved in the endoproteolysis in this pathway usually reside in a post-Golgi compartment and cleave preferentially the dibasic sites (Lys-Arg or Arg-Arg) or sometimes a monobasic site (Arg) of the precursor. These sites are mostly present on prohormones (e.g., POMC, proinsulin, prosomatostatin, proglucagon, progastrin, procalcitonin) and neuropeptide precursors (proenkephalin, prodynorphin, provasopressin, proCRH, proCGRP, prosubstance P) (Douglass et al., 1984; Eipper et al., 1986). The processed peptide products are stored in the secretory granules, the content of which is released upon appropriate stimulation (Burgess and Kelly, 1987). PC1 and PC2 have been shown to cleave preferentially the pure dibasic sites and potentially monobasic sites in the secretory granule compartment (Benjannet et al., 1991; Thomas et al., 1991; Nakayama et al., 1992). Consistent with this notion, expressions of PC1 and PC2 in adult thus far have been found to be largely restricted to neuroendocrine tissues (Seidah et al., 1990, 1991).

A wide variety of proproteins are synthesized in development that require proteolytic processing to generate peptide products possessing either mitogenic or growth-prohibiting activities or important in establishing cellular function. As a direct example, the *C. elegans* homolog of furin (*bli-4*) has been shown to be involved in cuticle formation and early larval development (Peters et al., 1991). In *Drosophila* the furin homolog dKLIP-1 has been shown to be expressed in developing oocytes and multiple embryonic tissues (Hayflick et al., 1992). Here we report the developmental expression profile of the mRNAs encoding proprotein convertases furin, PC1, PC2, and CPE in mammalian embryo using *in situ* hybridization histochemistry. Emphasis was placed on comparing their patterns of expression with each other and with potential substrates. We found that furin RNA is accumulated in broad regions of embryonic tissues, whereas expressions of PC1, PC2, and CPE are largely restricted to the developing neuroendocrine system. These data suggest that these proteases may act as a core set of enzymes in development and play distinct roles in the proteolytic maturation of proproteins and neuropeptide precursors.

Materials and Methods

Tissue preparation. All studies were conducted in accordance with the principles and procedures outlined in the NIH Guide for the Care and Use of Laboratory Animals. For *in situ* hybridization, embryos, fetuses, and neonates from 32 timed-pregnant Sprague–Dawley female rats were used. At least one litter for each age group from which data is reported (e7, e10, e12, e13, e14.5, e16, e18) was used with at least two embryos examined at each age. In addition, at least one embryo from multiple other intervening ages (e8, e9, e9.5, e10.5, e11, e11.5, e13.3, e13.5, e14, and e15) was also examined and exhibited no major qualitative differences compared to the prior stage discussed in detail. The animals were killed by decapitation and the uteri (for embryos e7–e9.5), whole embryos (e10–e15), or whole fetuses or fetal head and trunk (e16–e18) were collected. Embryos and fetuses were staged according to the morphology of their limb buds in reference with the criteria established by Wanek et al. (1989) for mouse. Precise age of the embryos earlier than e9.5 was determined by the subsequent microscopic examination. Embryos younger than e12 were fixed by immersion in 4% paraformaldehyde for 12 hr and equilibrated in 20% sucrose solution before embedding. Older embryos or fetuses (e12.5–e18) were freshly frozen and embedded directly in OCT compound without prior fixation. Both sagittal and transverse cryostat sections were prepared as described (Lugo et al., 1989). Rat adult and postnatal pituitaries (p1, p5, p10, p15) were dissected, freshly frozen embedded, and sectioned as above.

Probes. ^{35}S-labeled cRNA transcripts were synthesized *in vitro* from the plasmid vectors harboring the corresponding cDNA sequences using Riboprobe Gemini Systems from Promega (Madison, WI). Plasmid pD8/rfurin contains a 1245 base pair (bp) rat furin cDNA fragment (corresponding to nucleotides 691–1935; Schafer et al., 1993), linearized with NcoI (generating 320 nt cRNA transcripts). pR8/rPC1 contains a 325 bp rat PC1 cDNA fragment (nt 715–1039; Day et al., 1992), linearized with BamHI (325 nt). pD24/rPC2 contains a 753 bp rat PC2 cDNA fragment (nt 559–1326; Day et al., 1992), linearized with NcoI (372 nt). pSP65-46 contains a 1200 bp rat CPE cDNA fragment (nt 150–1350; Fricker et al., 1989), linearized with HindIII (320 nt). p10D contains a 220 bp rat POMC cDNA (exon 1 and exon 2). cRNA probes were purified on Sephadex G-50 columns (Boehringer-Mannheim, Indianapolis, IN) and used for *in situ* hybridization experiments without hydrolysis. Digoxigenin UTP-labeled cRNA probes were synthesized

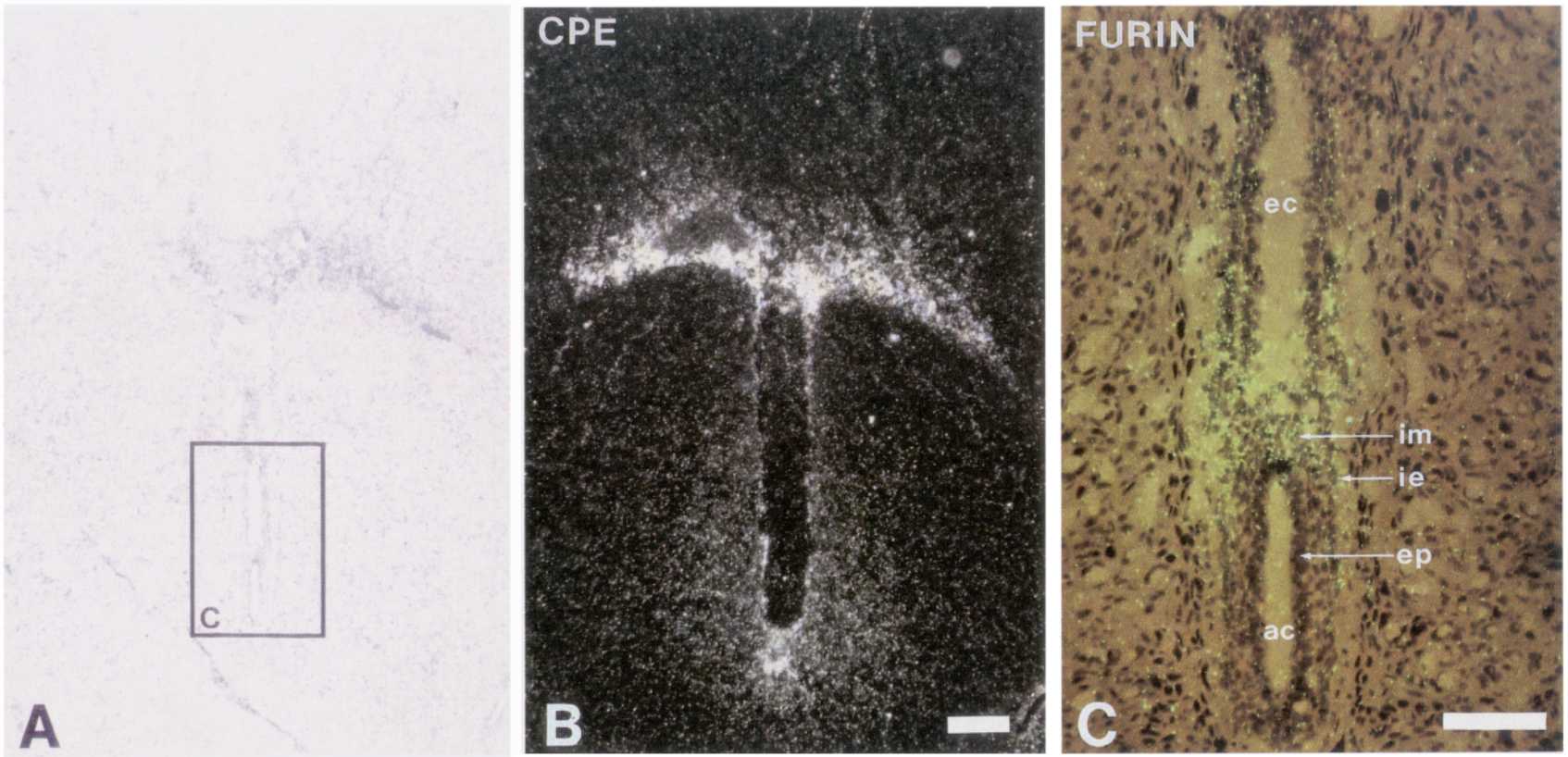


Figure 1. Patterns of furin and CPE gene expression at the primitive streak stage (e7). *A*, Bright-field micrograph showing a sagittal section of e7 embryo with surrounding uterine tissue. *B*, Dark-field image of an adjacent section hybridized with CPE cRNA; *white areas* represent positive hybridization. *C* is the section hybridized with furin cRNA and represents the area within the *rectangle* in *A*, photographed under epifluorescent illumination. A relatively high level of furin expression is observed in both the primitive and parietal endoderms, as well as the emerging intraembryonic mesoderm surrounding the primitive steak. Low level of furin expression is also observed in the extraembryonic ectoderm. Embryonic ectoderm lacks detectable furin expression. Although the embryo proper lacks CPE mRNA, a high level of CPE expression is detected in the decidual cells surrounding the implantation site. *ac*, amniotic cavity; *ec*, ectoplacental cavity; *ep*, epiblast; *ie,* intraembryonic endoderm; *im*, intraembryonic mesoderm. Scale bars: *B*, 1 mm; *C*, 100 μm.

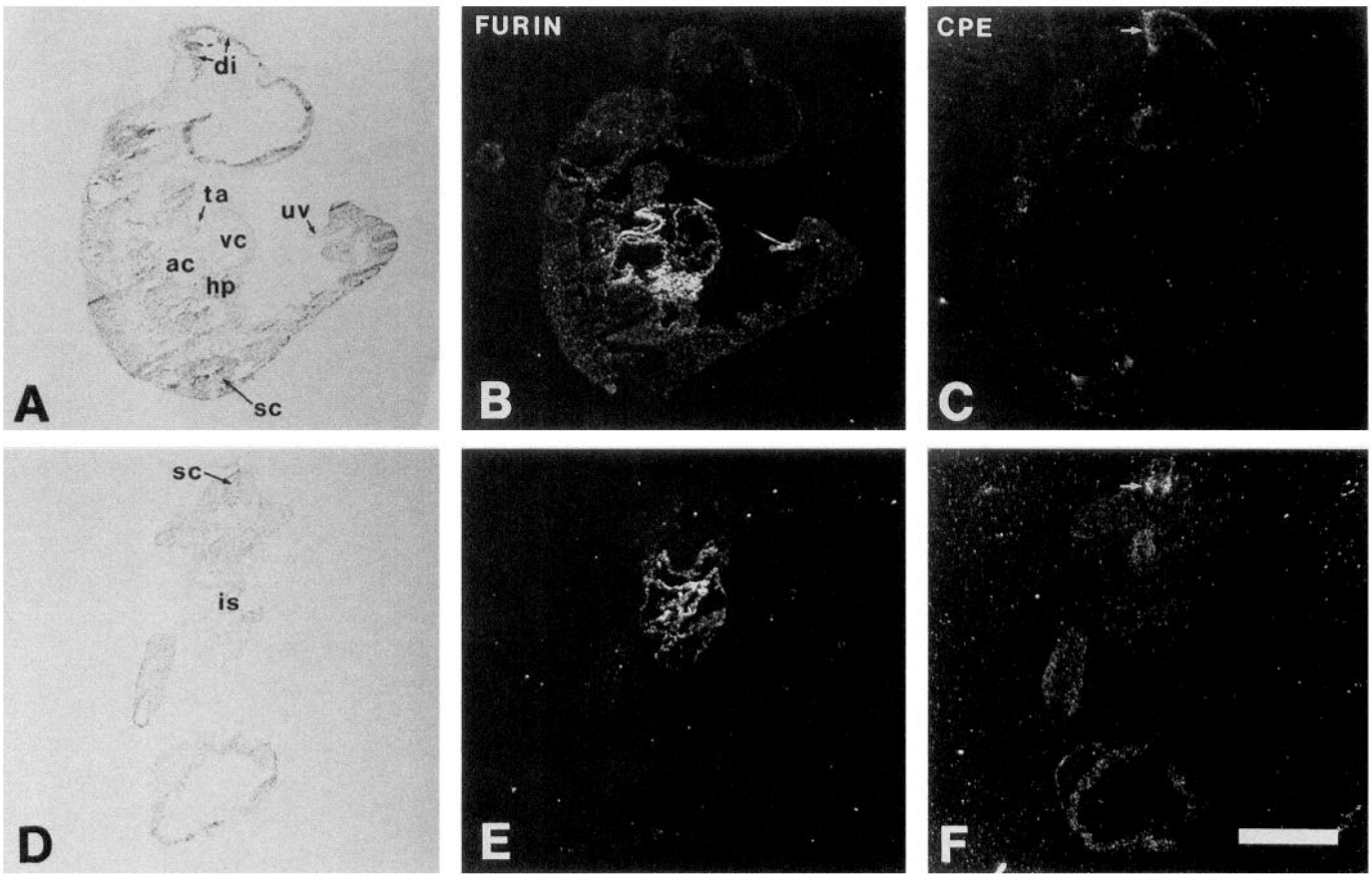


Figure 2. Localization of furin and CPE transcripts in e10 embryo. *A* and *D*, Bright-field illumination. *B, C, E,* and *F*, Dark-field illumination. Sagittal (*A–C*) and frontal sections (*D–F*) are hybridized with furin probe (*B, E*) and CPE probe (*C, F*), respectively. The highest level of furin expression is observed in myocardial walls of both the common atrial and ventricular chambers (*B*). The liver primordium embedded in the septum transversum also begins to express furin at the stage (*B*). In contrast, CPE expression is detected mainly in the mantle layer of the neuroepithelium containing differentiated neurons. CPE expression in the diencephalon (*C*) and spinal cord (*F*) are illustrated by *arrows*. *ac*, (common) atrial chamber; *di*, diencephalon; *hp*, hepatic primordium; *is*, interventricular septum; *sc*, spinal cord; *ta*, truncus arteriosus; *uv*, umbilical vein; *vc*, (common) ventricular chamber. Scale bar, 1 mm.

using an *in vitro* transcription system (Boehringer-Mannheim, Indianapolis, IN).

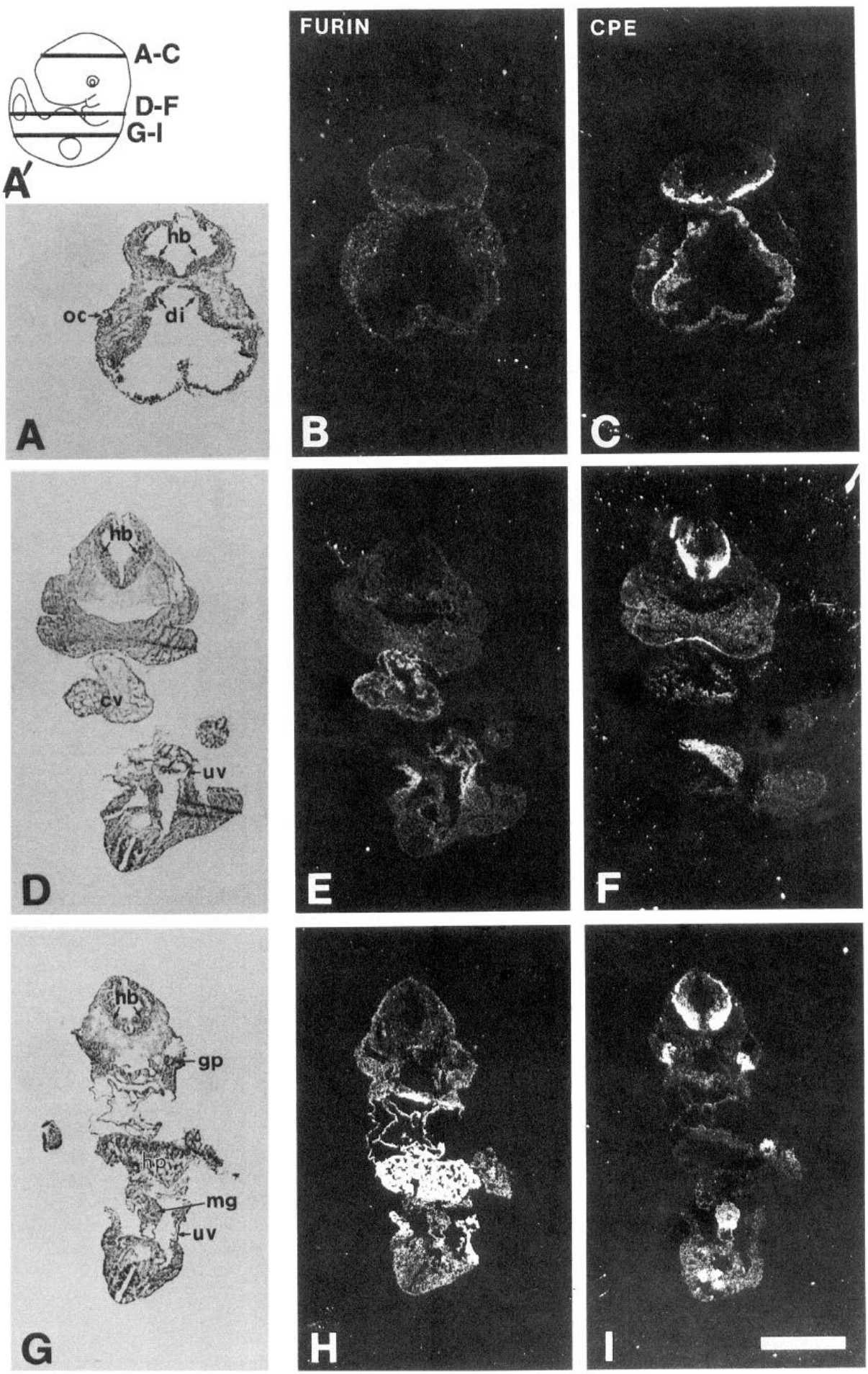
In situ *hybridization. In situ* hybridizations were performed using a protocol modified from Lugo et al. (1989) and have been detailed elsewhere (Zheng and Pintar, in press). Autoradiography was carried out at 4°C using 1:1 dilution of Kodak NTB2 emulsion (Eastman Kodak, Rochester, NY) for 6 weeks, unless otherwise indicated. In all cases hybridization with control (sense) RNA in adjacent sections yielded only low background. Dual labeling *in situ* hybridization using both ^{35}S-labeled cRNA and digoxigenin UTP-labeled cRNA was performed as described (Zheng and Pintar, in press).

Results

Furin expression encompasses both mesoderm- and endoderm-derived tissues

In contrast to the widespread expression of furin in the adult (Hatsuzawa et al., 1990; Barr et al., 1991), we observed a highly localized pattern of furin expression in the early postimplantation embryo, followed by distinct spatial and temporal changes in its expression in later development. Furin RNA is first detected at e7 in the late primitive streak stage of the embryo, when both the embryonic and extraembryonic mesoderm have appeared. At this stage the hybridization signal for furin is present in both embryonic and extraembryonic endoderm and mesoderm (Fig. 1*C*). In contrast, the embryonic ectoderm that overlays the embryonic mesoderm and endoderm noticeably lacks detectable furin expression.

Furin RNA remains uniformly expressed in the endoderm and mesoderm until e10, when significantly higher levels of furin expression are first observed in the cardiovasculature (Fig. 2*B,E*). At this stage, the heart is mainly divided into a common atrial chamber receiving blood from the sinus venosus and a common ventricular chamber pumping blood into the outflow tract of the truncus arteriosus and aortic sac. Furin expression is detected in the myocardial walls of both truncus arteriosi, the common atrial chamber, and the common ventricular chamber of the heart primordium. In addition, prominent hybridization also exists in the adjacent aortic sac, sinus venosus, and umbilical vein (Fig. 2*B,E*). At this stage the hepatic primordium embedded in the septum transversum also begins to express furin transcripts (Fig. 2*B*). By e12 a high level of furin expression is still observed in the vascular system, in both embryonic heart atria and ventricles, as well as major vasculature, such as the umbilical veins (Fig. 3*E,H*). At this stage, however, the liver primordium expresses the highest level of furin transcripts in the embryo (Fig. 3*H*). Other regions of the embryo, such as the cephalic and trunk mesenchyme, only express mid- to low levels of furin transcripts (Fig. 3*B,E,H* and data not shown). In contrast, the developing nervous system, which is derived from the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neuroectoderm, does not express furin RNA at any significant level.

By midgestational stages (e13–e16) the extent of mid- to high furin expression includes most, if not all, tissues outside of the nervous system (Figs. 4*D*, 5*D*, 6*L*). At these stages furin transcripts are expressed disseminatedly in facial and trunk regions. Especially intense labeling for furin is observed in the myotomes of the trunk. Furin is abundantly expressed in all precartilaginous bodies in skeletogenic centers, such as the cartilage primordium of cephalic bones, vertebral bodies, rib, and femur. The heart and the developing vasculature throughout the embryo remain positive for furin, but the highest level of furin expression is observed in liver. In late gestational stages, the expression pattern of furin RNA is unaltered but the overall level of hybridization, under otherwise identical conditions, has decreased (Fig. 7*D*). This is especially apparent for the myotomes and cartilage primordia. Relatively high level of furin expression is restricted to liver, lung, and mucosa of the gut. A low level of furin RNA is also expressed in the adrenal gland and kidney, while the developing pancreas noticeably lacks detectable furin expression (Fig. 7*D*). The nervous system remains negative for furin expression throughout the period examined (e7–e18) except in choroid plexus (Fig. 5*D* and data not shown). Finally, furin is transiently expressed in Rathke's pouch region from e13 to e18 (Figs. 4*D*, 5*D*, 8*F,J*; and data not shown).

PC1 and PC2 are distinctly expressed in the developing neuroendocrine system

PC1 and PC2 transcripts have a different temporal and spatial expression profile from that of furin. PC1 and PC2 expression is first detected at a low level at e13, which is several days later than furin is first expressed (Fig. 4*A,B*). At this age, PC1 and PC2 are expressed only in the developing nervous system; thus, the general domains of their expression are complimentary to that of furin. We found that the expression patterns of PC1 and PC2 are distinct from each other and that, in general, the expression of PC2 is more widespread than that of PC1. PC1 is first detected in part of the diencephalon corresponding to the anterior hypothalamus (Fig. 4*A*), while PC2 is expressed not only in the diencephalon but also in the midbrain, hindbrain, and ventral spinal cord (Fig. 4*B*). By e14.5 PC1 transcripts are detected in the hypothalamus (postoptic area) and ventral region of subthalamus, as well as all of the major cranial ganglia, dorsal root ganglia, and sympathetic ganglia examined (Fig. 5*A* and data not shown). A low level of PC1 expression is also detected in the dorsal thalamus and a subregion of hindbrain. At these stages PC2 RNA is also detectable in these regions. In addition, widespread PC2 expression is detected at a relatively higher level in the telencephalon (cortical plate), thalamus, midbrain, pons, medulla oblongata, spinal cord, and olfactory epithelium (Fig. 5*B* and data not shown). By e16 both PC1 and PC2 transcripts are detected at relatively high levels in the anterior hypothalamus, a region already active in peptide biosynthesis, whereas only PC2 expression domain extended dorsally to the posterior hypothalamic region (compare Fig. 8*C,D*). At this stage PC2 transcripts are observed throughout the cortical plate, thalamus, hypothalamus, cerebellum, midbrain, pons, olfactory bulb, medulla, and both dorsal and ventral horns of the spinal cord (Fig. 6*C,F,J* and data not shown). The expression level is particularly high in the dorsal thalamus (especially the medial habenula), hippocampus, the reticular and reticulotegmental nuclei of the pons, and the mantle layer of the midbrain. In the PNS, both PC1 and PC2 RNAs are expressed at a relatively high level, particularly evident in cranial and spinal ganglia. Expression of PC2 transcripts in both spinal cord and spinal ganglia contrasts to the expression of PC1 transcripts, which are detectable only in the spinal ganglia (compare Fig. 6*I,J*). PC2 probe also weakly labels the olfactory epithelium (Fig. 6*F*). PC1 RNA, but not PC2 RNA, is expressed in brachial plexus (compare Fig. 6*I,J*). At e18 PC1 and PC2 RNA expression are also observed in the developing pancreas (Fig. 7*A,B*).

CPE is expressed in all regions of the developing nervous system and some peripheral tissues

At the earliest stage examined (e7), CPE expression is observed in the spongy layer of endometrial tissues surrounding the implantation site, which represents the inner zone of the extensive decidual reaction (Fig. 1*B*). In contrast to furin, neither embryonic nor extraembryonic tissues express CPE at this stage. Expression of CPE in the embryo proper was first detected at a low level at e10. At this stage, the expression of CPE RNA is mainly restricted to the mantle layer of neuroepithelium and is essentially absent from the ventricular layer (Fig. 2*C,F*).

Serial transverse sections were examined for furin and CPE expression in order to determine whether they exhibit similar patterns of expression at different stages of development, which would imply the possible collaboration of the cognate enzymes in the completion of proteolytic processing of common substrates. Figure 3 illustrates the results of experiments performed with serial transverse sections at the embryonic stage e12. Although both furin and CPE transcripts are expressed at this stage, their expression domains only partially overlap. CPE RNA is found throughout the length of the neural tube, which is the only region not expressing any detectable level of furin RNA. Expression of CPE transcripts is particular high in the mantle layer of telencephalon, diencephalon, myelencephalon, and spinal cord neuroepithelium, but is not detectable in the floorplate (Fig. 3*C,F,I*). Peripheral ganglia, such as the trigeminal, glossopharyngeal, and spinal ganglia, are heavily labeled with CPE probe. The inner layer of the optic cup, which will form the future neural retina, also begins to express CPE (Fig. 3*C*). CPE RNA begins to be detected in the olfactory epithelium at this

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Figure 3. Serial transverse sections of e12 rat embryo, comparing furin and CPE expression. *A'*, Schematic drawing of an e12 embryo showing the approximate planes of section of other panels. Left column (*A, D, G*) contains bright-field photographs, each corresponding to the adjacent dark-field photographs of center column (*B, E, H*; hybridized with furin probe) and right column (*C, F, I*; hybridized with CPE probe). High level of hybridization for furin is observed in the hepatic primordium (*H*), heart (*E*), and umbilical veins (*E, H*), while lower level of hybridization is observed in mesenchymal tissues throughout the rest of the embryo (*B, E, H*). The developing nervous system at all levels noticeably lacks detectable furin expression (*B, E, H*). In contrast, strong hybridization for CPE is observed in mantle layer of neuroepithelium (*C, F, I*), peripheral ganglia (*I*), trabeculae carneae within the walls of ventricles (*F*), covering epithelium of branchial arch (*F*), and the mesenchyme surrounding the midgut mesentery (*F, I*). *cv*, common ventricle; *di*, diencephalon; *gp*, glossopharyngeal ganglion; *hb*, hindbrain; *hp*, hepatic primordium; *mg*, midgut; *oc*, optic cup; *uv*, umbilical vein. Scale bar, 1 mm.

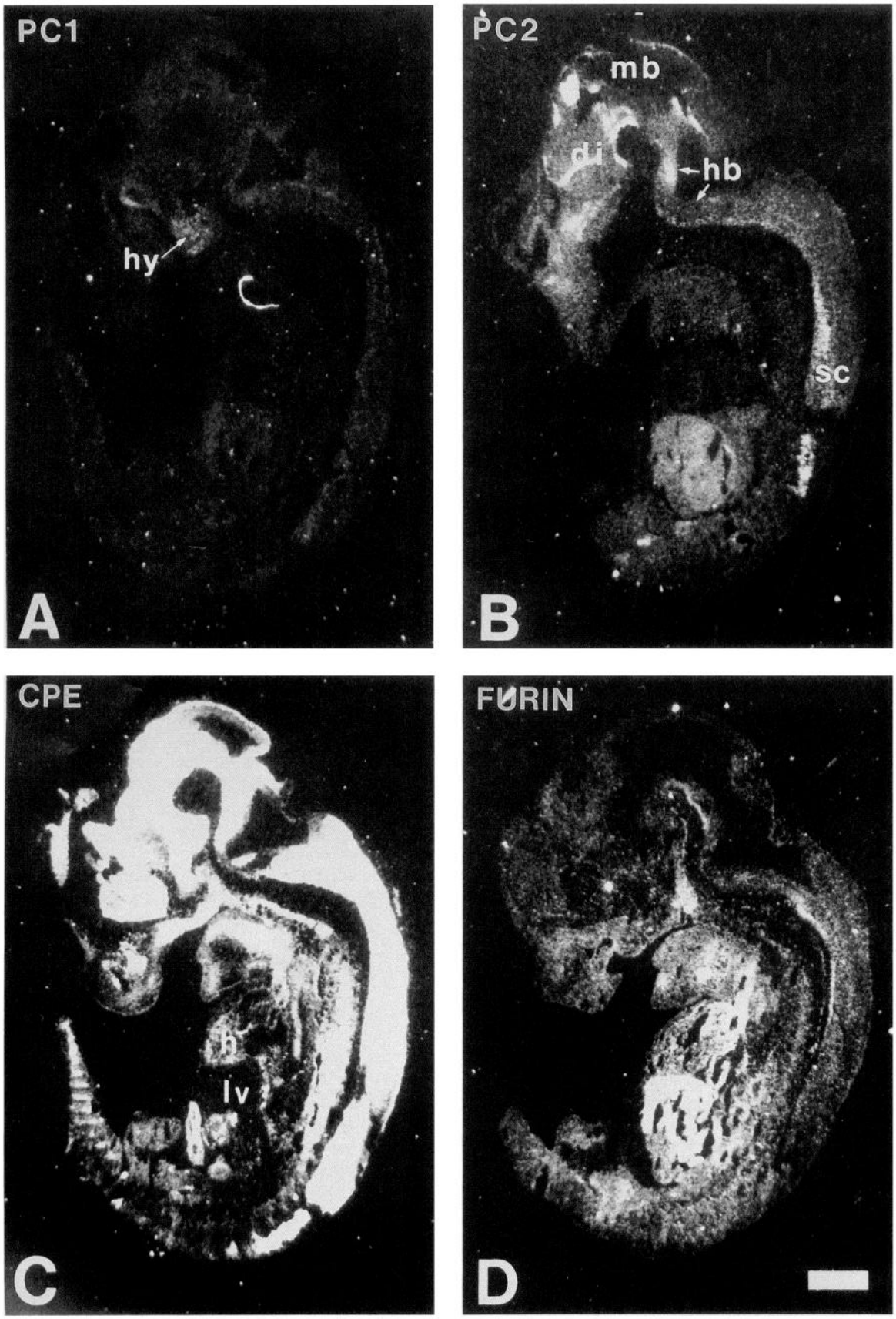

Figure 4. Comparative distributions of proteolytic processing enzymes in e13 embryos. Adjacent sagittal sections were hybridized separately with cRNAs for PC1 (*A*), PC2 (*B*), CPE (*C*), and furin (*D*). The expression of PC1 and PC2 transcripts is restricted to the developing nervous system, with the expression of PC2 more widespread than that of PC1. The expression of furin is complementary to those of PC1 and PC2, encompassing most of the peripheral tissues and organs. The expression domain of CPE covers all regions where PC1 and PC2 are expressed and some of the regions where furin are expressed, but is noticeably absent from the liver. See Results for details. *di*, diencephalon; *h*, heart; *hb*, hindbrain; *hy*, hypothalamus; *lv*, liver; *mb*, midbrain; *sc*, spinal cord. Scale bar, 1 mm.

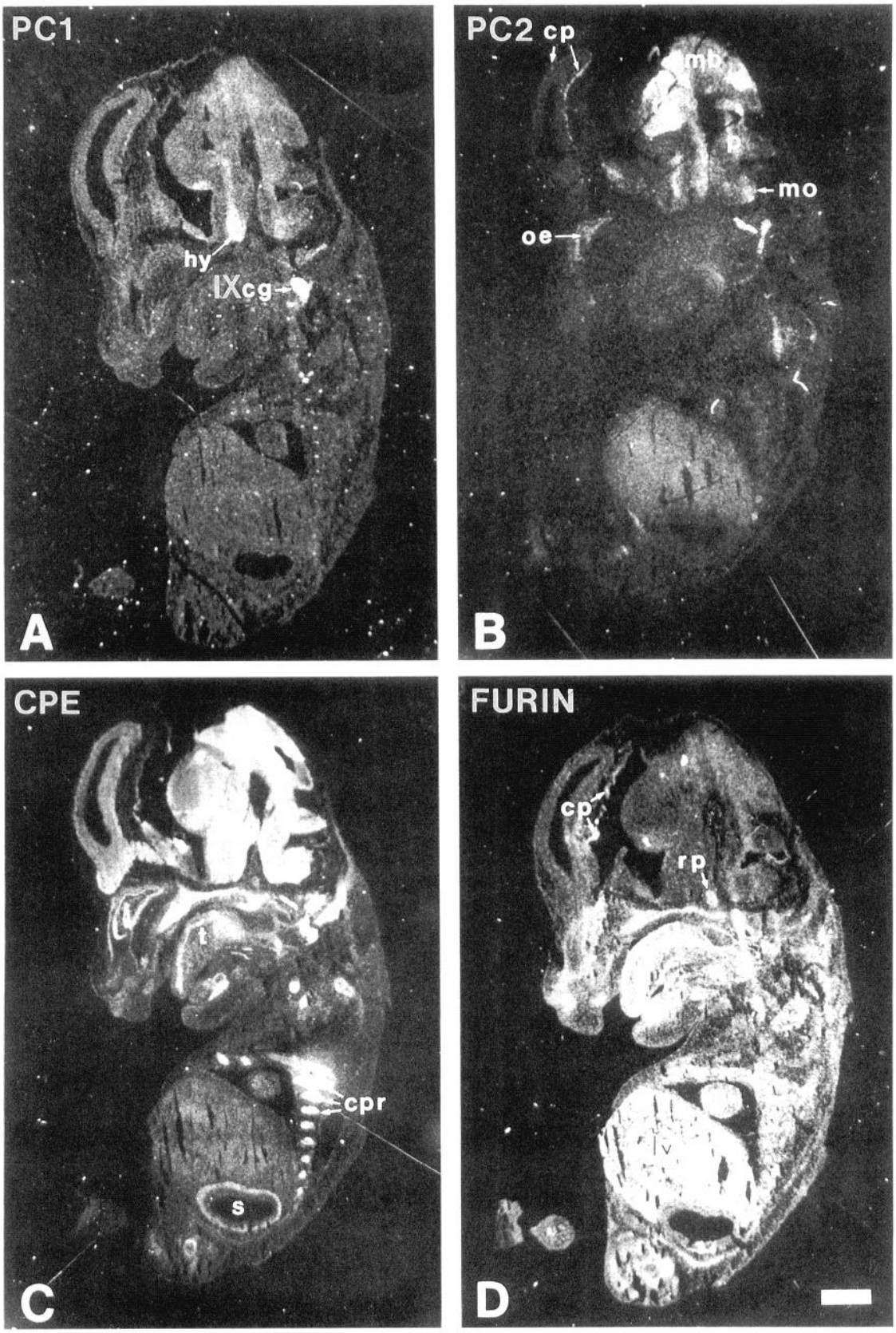

Figure 5. Comparative distributions of proteolytic processing enzymes in e14.5 embryos. Adjacent sagittal sections were hybridized separately with cRNAs for PC1 (*A*), PC2 (*B*), CPE (*C*), and furin (*D*). *IXcg*, IX cranial ganglia (glossopharyngeal ganglia); *cp*, cortical plate; *cpr*, cartilage primordium of ribs; *cp*, choroid plexus; *h*, heart; *hy*, hypothalamus; *lv*, liver; *mb*, midbrain; *mo*, medulla oblongata; *oe*, olfactory epithelium; *p*, pons; *rp*, Rathke's pouch; *s*, stomach; *t*, tongue; *th*, thalamus. Scale bar, 1 mm.

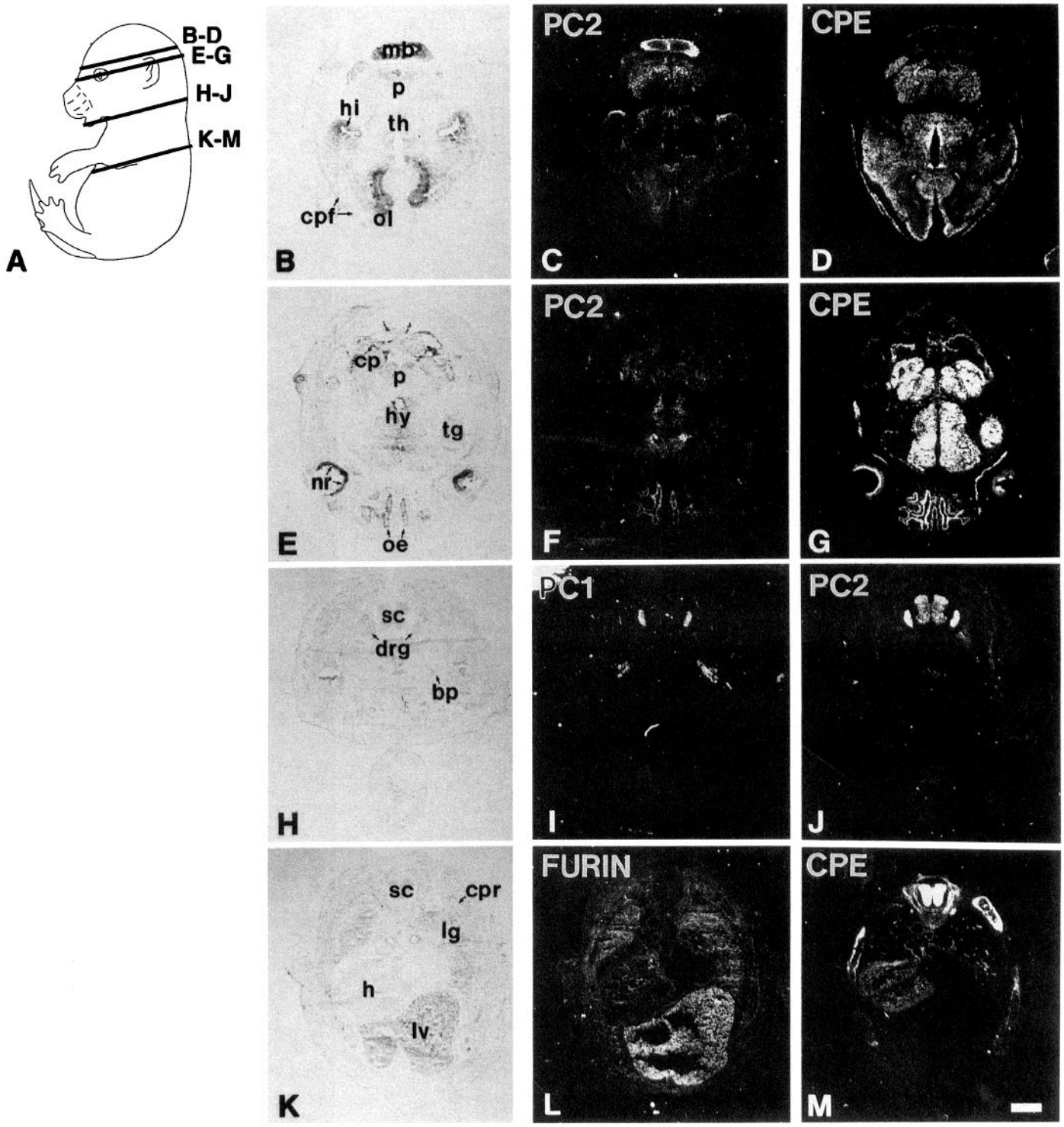

Figure 6. Comparative distributions of proteolytic processing enzymes in e16 embryo, with the approximate planes of transverse section indicated in *A*. *B, E, H,* and *K*, Bright-field micrographs of H&E-stained sections. Hybridizations were carried out on adjacent sections for PC2 (*C, F, J*), CPE (*D, G, M*), PC1 (*I*), and furin (*L*). See Results for details. *bp*, brachial plexus; *cp*, cerebellar primordium; *cpf*, cartilage primordium of basisphenoid bone; *cpr*, cartilage primordium of rib; *drg*, dorsal root ganglia; *h*, heart; *hi*, hippocampus; *hy*, hypothalamus; *lg*, lung; *lv*, liver; *p*, pons; *mb*, midbrain; *nr*, neural retina; *oe*, olfactory epithelium; *ol*, olfactory lobe; *sc*, spinal cord; *tg*, trigeminal ganglia; *th*, thalamus. Scale bar, 1 mm.

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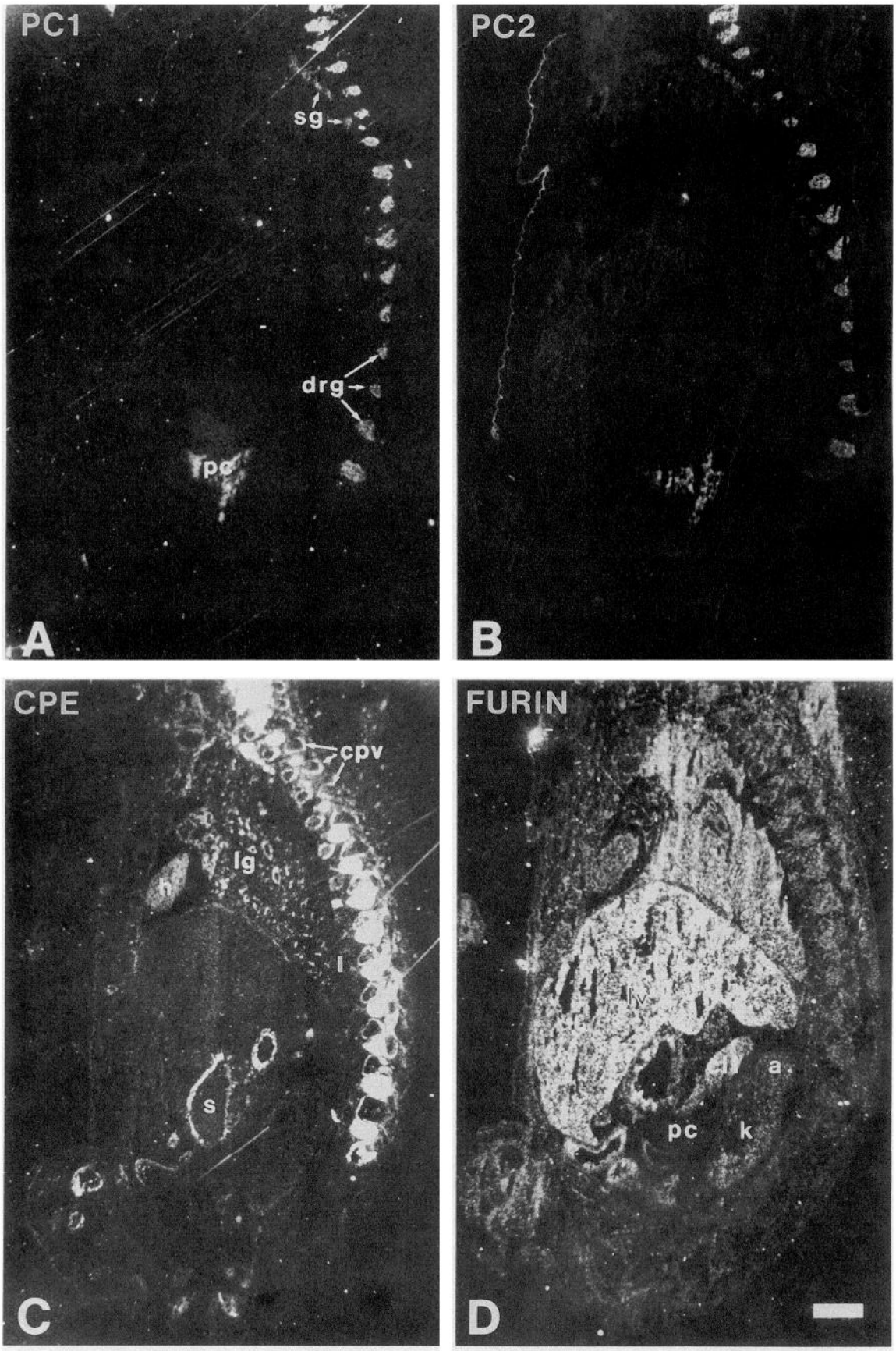
Figure 7. Patterns of proteolytic processing enzyme gene expression in late gestational stage (e18). Adjacent parasagittal sections of the trunk are shown, hybridized separately with PC1 (*A*), PC2 (*B*), CPE (*C*), and furin (*D*). Note that the developing pancreas expresses both PC1 and PC2, whereas no significant levels of either furin or CPE are detected. *a*, adrenal gland; *cll*, caudate lobe of liver; *cpv*, cartilage primordium of vertebra; *h*, heart; *k*, kidney; *lg*, lung; *lv*, liver; *pc*, pancreas; *s*, stomach; *sg*, sympathetic ganglia. Scale bar, 1 mm.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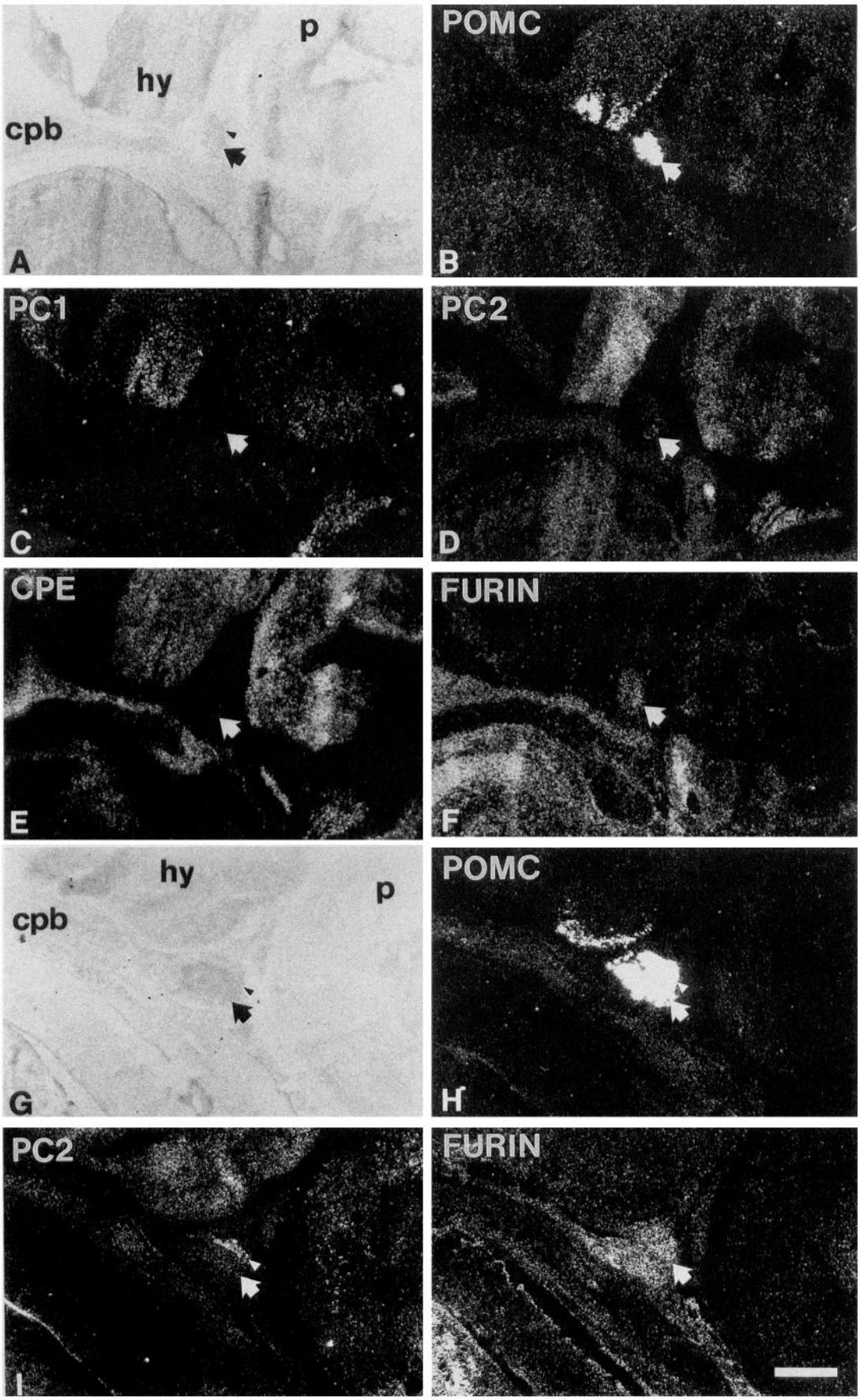
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stage (data not shown). In peripheral tissues, both furin and CPE transcripts are detected at mid- to low levels in heart, maxillary and mandibular components of first branchial arch. Even in regions where both furin and CPE are detected, relative levels of expression vary significantly. High levels of furin, for instance, are observed in the body wall surrounding the bilateral umbilical veins. In contrast, CPE transcripts are detected in the adjacent mesentery surrounding the midgut, where only a low level of furin RNA is observed (compare Fig. 3, *E* and *F*, *H* and *I*). In the embryonic heart, CPE probes mainly label interventricular septum and the trabeculae of the ventricle, while furin probes more heavily label atrial and ventricular myocardial walls (compare Fig. 3*E,F*).

At middle and late gestational stages, a fairly high level of CPE is expressed throughout both the CNS and PNS (Figs. 4*C*; 5*C*; 6*D,G,M*; 7*C*). High levels of CPE are observed throughout the mantle layers of forebrain, midbrain, hindbrain, and both dorsal and ventral parts of the spinal cord. All cranial and spinal ganglia invariably express a high level of CPE transcripts, as does the neural retina. CPE probe labels the neural crest cell populations widespread in the peripheral tissues, such as those distributed in the submucosa of the gut (Figs. 5*C*, 7*C*). Comparison of PC2 and CPE RNA expression in adjacent sections at e16 reveals that PC2 expression is restricted to part of CPE expression domain within the nervous system (Fig. 6*C,D,F,G*). In contrast to the low, variable level of PC2 RNA expression in different regions of the brain, a high level of CPE RNA is expressed rather uniformly in nearly all regions of the brain. Like furin, relatively high levels of CPE are also expressed in the choroid plexus (Figs. 5*C*, 6*G*). In contrast to PC1 and PC2, CPE is also abundantly expressed in certain peripheral tissues, such as the embryonic heart, cartilage primordia of cephalic bones and vertebral bodies, dorsum of tongue, and olfactory epithelium (Figs. 4*C*, 5*C*, 6*D,G,M*). On the other hand, the expression of CPE in peripheral tissues is not as widespread as furin. Expression of CPE in tongue, for instance, is restricted to its dorsal surface, whereas furin expression encompasses the intrinsic muscle of tongue as well (compare Figs. 4, *C* and *D*; 5, *C* and *D*). CPE is not expressed in the liver at any significant level in all the stages tested.

Furin, PC1, PC2, and CPE exhibit distinct ontogenetic pattern of expression in the pituitary gland

To evaluate the possible contributions of furin, PC1, PC2, and CPE in the proteolytic processing of proopiomelanocortin (POMC), we followed their expression in the pituitary gland from prenatal stages to adult. The results are summarized in Figures 8 and 9. Both PC1 and PC2 have been shown to cleave selectively on the dibasic sites on POMC in gene transfer experiments (Benjannet et al., 1991; Thomas et al., 1991). In adult pituitary, PC1 RNA is abundantly present in both anterior lobe (AL) and intermediate lobe (IL), the latter of which exhibits an apparently higher level of expression, which is probably due to its dense cellularity (Fig. 9*B*). PC2 RNA, on the other hand, is primarily expressed in IL, with only a very low level of signal detected in AL (Fig. 9*C*). Both AL and IL are rather uniformly labeled with CPE probe, indicating that most, if not all, cells in these two lobes express CPE transcripts (Fig. 9*D*). The posterior lobe is consistently negative for PC1 and PC2 probes in all *in situ* hybridization experiments, although prolonged exposure reveals that some posterior lobe pituicytes express a low level of CPE (data not shown). Furin mRNA is only detected at an extremely low level in the adult AL and IL (data not shown).

In embryogenesis, PC2 labeling first appears at e14.5 in Rathke's pouch, which is the progenitor for adult pituitary AL and IL (Fig. 8*D*). In particular, the labeling is present in the region corresponding to the presumptive AL. At this stage the expression of POMC is also initiated and is restricted to AL (Fig. 8*B*; see Lugo et al., 1989). Although both PC1 and PC2 transcripts are detected in the neighboring hypothalamus (Fig. 8*C,D*), no discernible level of PC1 RNA is observed in the Rathke's pouch at this stage (Fig. 8*C*). CPE RNA is also present at a low level in Rathke's pouch at this age (Fig. 8*E*). Interestingly, and in contrast to the result in adult, furin RNA is also abundantly expressed in the pituitary primordium at this stage (Fig. 8*F*). By e16 PC2 RNA is expressed in a band of cells corresponding to IL, as well as disseminatedly expressed at a low level in AL (Fig. 8*I*). At this stage POMC is expressed in both AL and IL (Fig. 8*H*; see Lugo et al., 1989). Compared to the more discrete pattern of PC2 expression, furin RNA is diffusely present in the Rathke's pouch region, with no discernible difference in level between AL and IL (Fig. 8*J*). In early postnatal stages (p1–p15) PC1 transcripts highlight patches of cells in AL but is essentially absent in IL. This is distinct from the more broad pattern of its expression in both AL and IL in the adult pituitary (compare Fig. 9*B,F*). In IL, PC2 hybridization achieves high density comparable to that in adult. Interestingly, we consistently observed groups of cells in the AL that express PC2 RNA. The comparably higher level and larger proportion of AL cells expressing PC2 compared to adult are particular evident at p15 (compare Fig. 9*C,G*). Double labeling *in situ* hybridization shows that at this stage expressions of both PC1 and PC2 in AL colocalize with POMC (Fig. 10). CPE RNA is uniformly expressed in both AL and IL at early postnatal stages, similar to that of adult (compare Fig. 9*D,H*). Furin only gives a very low level of labeling at various postnatal stages tested (not shown).

Discussion

We have used *in situ* hybridization to determine the spatial and temporal changes in the expression of proteolytic processing enzymes during prenatal rat development. Furin expression was the first of the processing enzyme genes to be expressed and was detected at the earliest stage examined (e7). Expression of furin in the embryonic endoderm and mesoderm coincides with the time insulin-like growth factor II (IGF-II) is first expressed (Lee et al., 1990). Since proIGF-II possesses multiple Arg-X-Lys/Arg-Arg motifs in the E domain (Gammeltoft, 1989), it appears likely that furin is active in proIGF-II processing at this stage.

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Figure 8. Ontogeny of the proteolytic processing enzymes in prenatal pituitary. *A* and *G*, Bright-field micrographs of H&E-stained fresh-frozen postfixed sections. Comparative distributions of processing enzymes with that of POMC in pituitary gland primordium (Rathke's pouch) in e14.5 (*A–F*) and e16 (*G–J*) are shown. Hybridizations are carried out for POMC (*B, H*), PC1 (*C*), PC2 (*D, I*), CPE (*E*), and furin (*F, J*). Note the distinct expressions of the proteolytic processing enzymes in the anterior lobe (*arrows*) and intermediate lobe (*arrowheads*). Specifically, expression of PC2 in e14.5 AL can be detected (*D*) and furin in e14.5 and e16 can be detected (*F, J*). *cpb*, cartilage primordium of basisphenoid bone; *hy*, hypothalamus; *p*, pons. Scale bar, 500 μm.

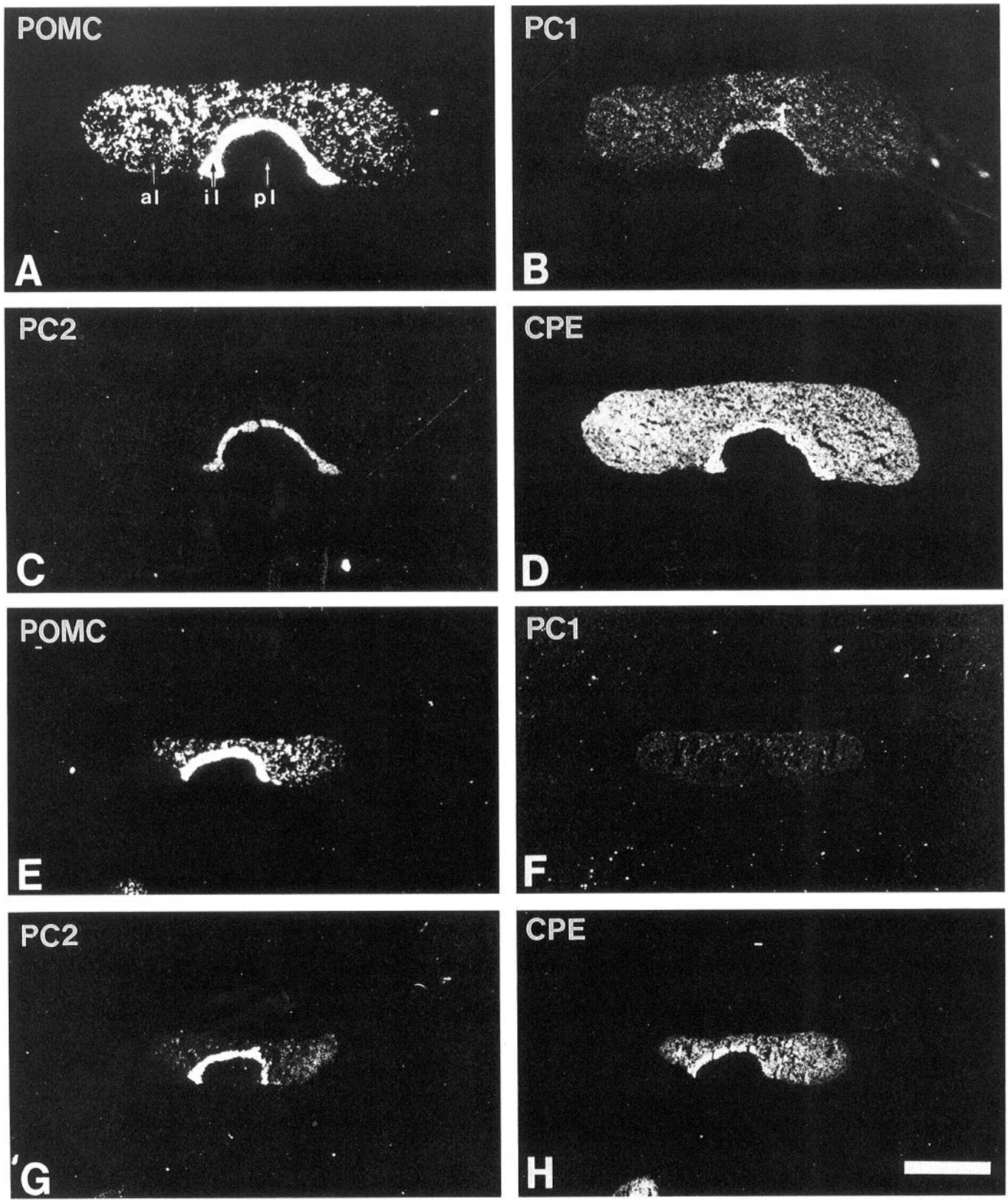

Figure 9. Frontal sections of adult pituitary (*A–D*) and p15 pituitary (*E–H*), taken under dark-field illumination. Lobe-specific expressions of PC1 (*B, F*), PC2 (*C, G*), and CPE (*D, H*) are compared with POMC expression (*A, E*). *al*, anterior lobe; *il*, intermediate lobe; *pl*, posterior lobe. Scale bar, 1 mm.

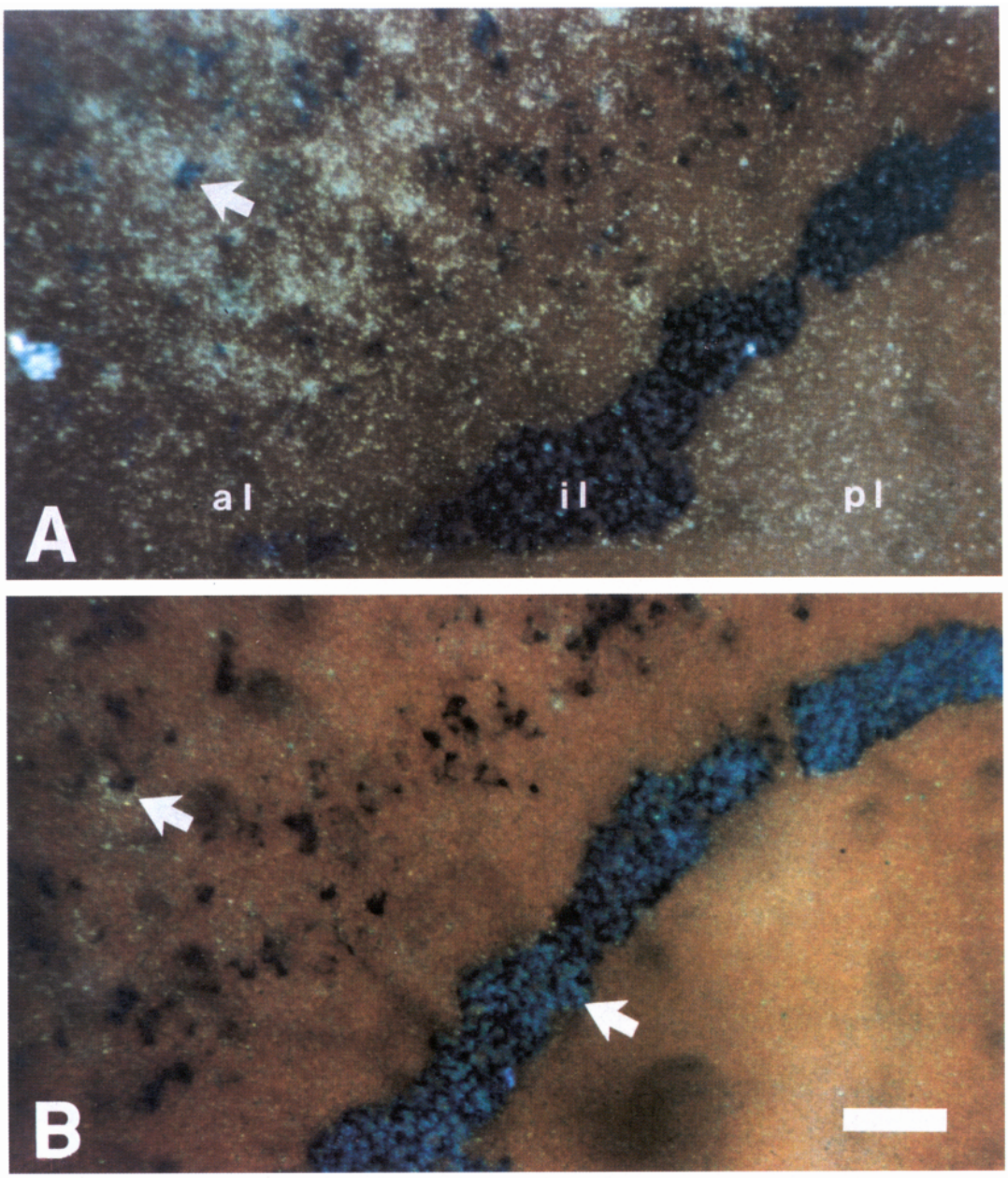

Figure 10. Colocalization of PC1 and PC2 with POMC in p15 pituitary. Frontal sections of p15 pituitary were hybridized simultaneously with digoxigenin UTP-labeled POMC probe and ^{35}S-UTP–labeled PC1 probe (*A*) or ^{35}S-UTP–labeled PC2 probe (*B*). Positions of POMC transcripts are revealed by areas of bluish chromogen deposits, mainly outlining the cytoplasm of POMC-expressing cells. Nearly all parenchymal cells in IL and scattered cells in AL express POMC. Cells expressing PC1 or PC2 are outlined by scattered epifluorescent grains. Some aggregates of autoradiographic grains in AL directly overlap with the chromogen deposits (*arrows*), indicating the coexpression of PC1 or PC2 with POMC. PC2 is expressed at nearly all cells in IL (compare with PC1 expression in *A*, which is not expressed in the IL at this stage), which overlaps POMC expression. Scale bar, 100 μm.

As the first organ system established in embryogenesis, the heart is well developed and functional as early as e10 in the rat. High levels of localized furin expression at this stage suggest a role for this gene during cardiac organogenesis. Intriguingly the gene encoding the precursor for atrial natriuretic factor (pro-ANF) begins to be expressed in the myocardial cells of both atria and ventricle at embryonic day 8 in mouse, reaches a peak at e9, and declines in subsequent stages (Zeller et al., 1987). The high level of furin expression in heart at e10 in rat is suggestive of its involvement in the proteolytic processing of fetal ANF or related natriuretic peptides in heart. Expression of furin in the heart also overlaps with other proproteins. For example, the gene encoding the opioid precursor proenkephalin is also expressed in a subpopulation of myocardial cells at e10 and subsequent stages (Zheng and Pintar, unpublished observations). In addition, the gene coding peptidylglycine α-amidating monooxygenase (PAM) is also expressed in heart at this stage (Zhang, Eipper, and Pintar, unpublished observations). The prominent presence of multiple proproteins and processing enzymes indicates that in addition to circulating blood the heart may also serve as a major secretory organ at the early stage of embryogenesis.

Starting from e12 the region expressing the highest level of

furin transcripts shifts from heart to liver, which is the site actively synthesizing multiple types of fetal proproteins, such as the precursors for albumin (Murakami et al., 1987), IGF-II (Stylianopoulou et al., 1988; Bondy et al., 1990), and TGF-β1 (Wilcox and Derynck, 1988). Expression of furin in other peripheral tissues overlaps with many possible substrates, such as IGF-II; in fact the expression pattern of furin at midgestational stages is strikingly similar to that of IGF-II (Stylianopoulou et al., 1988). Expression of furin in the cardiovascular system in midgestational stages overlaps with NGF expression (Scarisbrick et al., 1993), which can be correctly cleaved by furin (Bresnahan et al., 1990). The developing nervous system is largely devoid of furin expression, although it is possible that a low level of furin expression beyond detection may exist. The only site of furin expression in the fetal CNS appears to be the choroid plexus, which also expresses IGF-II RNA at this stage and is believed to have trophic role in the development of CNS (Ichimiya et al., 1988; Stylianopoulou et al., 1988; Bondy et al., 1990). In late gestational stages we observed an overall decrease in the level of furin RNA expressed, which is a temporal change paralleling a drop in the expression of some fetal growth factors and growth factor receptors gene expression (Stylianopoulou et al., 1988; Wilcox and Derynck, 1988). Therefore the high level of furin expression in the early and midgestational stages corresponds to a phase of transient surge in the expression of multiple types of growth factors and growth factor receptors in this critical period of embryogenesis.

Expression of both PC1 and PC2 begins later than furin (e13), which indicates their lack of involvement in proteolysis in earlier embryogenesis. Spatially, PC1 and PC2 occupy an overall expression domain roughly complimentary to that of furin, with the expression of PC1 and PC2 RNA restricted to nervous and endocrine systems throughout later stages. In the developing CNS, both PC1 and PC2 are expressed at relatively high levels in the hypothalamic region starting at midgestation. The high level of endoprotease mRNA expressed suggests a role in the early functional maturation of the hypothalamus, through which peptidergic mediators represents the central neural control of the endocrine and autonomic functions. In contrast to PC1, however, PC2 is also expressed at a significant level in extrahypothalamic regions of the brain, such as dorsal thalamus, midbrain, pons, medulla oblongata, and the spinal cord. Thus, the broader distribution of PC2 compared to PC1 that has been recently described in the adult (Schafer et al., 1993) is established during fetal life. In the telencephalon, the expression of PC2 RNA is restricted to the cortical plate, probably reflecting the onset of neuronal differentiation in this region. The expression of PC2 transcripts in the brain overlaps extensively with a number of neuropeptides known to be synthesized at these stages. For instance, at e16 high level of PC2 expression in medial habenular nucleus and thalamic nucleus overlaps with substance P (Sakanaka, 1992), the expression in ventral tegmental area overlaps with cholecystokinin (Cho and Joo, 1992), the expression in supraoptic nuclei overlaps with vasopressin (Buijs, 1992), and the expression in the medulla oblongata overlaps with proenkephalin (Zheng and Pintar, unpublished observations).

Both PC1 and PC2 are expressed at fairly high levels in the cranial and spinal ganglia. Prenatal and neonatal expressions of several neuropeptides, such as substance P and CGRP (Castrignano et al., 1990), somatostatin and VIP (Epstein and Poulsen, 1991), and cholecystokinin (De et al., 1990) have been documented in the peripheral ganglia, indicating a general involvement of proteolytic processing activities. Although the expressions of both PC1 and PC2 in the nervous system are believed to be confined exclusively to the neuronal cell populations in adult (Schafer et al., 1993), PC1 transcripts are detected in the glia elements constituting the brachial plexus. In peripheral tissues we observed a high level of PC1 and PC2 expression in the maturing pancreas at late gestational stages. Here PC1 and PC2 may be involved in the proteolytic processing of progastrin (Brand and Fuller, 1988), proinsulin (Gittes and Rutter, 1992), and the precursor for peptide-YY (Krasinski et al., 1991), all synthesized in pancreas at this time. In general, PC1 and PC2 transcripts are mainly detected in the specific regions of the developing neuroendocrine system and the level of which is generally in agreement with the peptide biosynthesis activity in development. Although both PC1 and PC2 are subtilisin family of endoproteases and are structurally closely related (Seidah et al., 1990, 1991), they exhibit noticeable difference in the spatial expression in development. PC2 RNA is comparably more broadly distributed than that of PC1. While PC2 RNA is expressed in both CNS and PNS, significant expression of PC1 RNA is largely restricted to the PNS, with the exception of the hypothalamus. Although both PC1 and PC2 cleave precursors at dibasic sites, they exhibit distinct cleavage preferences. The dibasic site on prorenin, for instance, is cleaved by PC1, but not PC2 (Benjannet et al., 1992); when multiple dibasic sites are available, they may be cleaved distinctly by PC1 and PC2 (Benjannet et al., 1991; Thomas et al., 1991). These observations, taken together with the distinct expression of PC1 and PC2 in development as described above, imply that distinct spatial distribution of PC1 and PC2 may be involved in tissue-specific (such as in pituitary) or regional-specific (such as in brain) proprotein processing. Discordant regulation in the ratio of PC1 and PC2 expressed may be an important way to generate peptide diversity required in development. This is probably best exemplified by the possible contributions of PC1 and PC2 in mediating developmentally regulated POMC proteolytic processing in pituitary (see below). Similar mechanism may also account for the differential proteolytic processing of other proproteins (Dickerson and Noel, 1991).

Synthesis of bioactive peptides usually requires sequential actions of endoproteases and exopeptidases. Since peptides containing C-terminal basic residues are generally not observed, the exoproteolytic removal of these residues presumably occurs rapidly and completely after endoproteolytic cleavage (Fricker, 1992). This is consistent with the comparably higher level of CPE gene expression observed than those of endoproteases, such as PC1 and PC2. In embryogenesis CPE gene expression is first detected in the maternal uterine tissues surrounding the early postimplantation embryo. Implantation of the embryo may have induced the immediate surrounding decidual cells to express high level of CPE transcripts. In the embryo proper, expression of CPE is initiated at a later stage (e10) and is predominantly distributed in the developing nervous system. From e10 to e14.5, we observed a rapid increase in the level of CPE expression, which is coincident with the period of neuronal cell differentiation and synaptogenesis. Expression of CPE covers all regions where PC1 and PC2 are expressed. CPE, therefore, may functionally collaborate with PC1 and PC2 in the completion of proteolytic processing of same neuropeptide precursors in development. Compared to the distinctive tissue expression of PC1 and PC2, however, CPE transcripts are distributed rather uniformly in nearly all part of the developing nervous system.

This probably reflects the fact that CPE is not enzymatically specific for any particular precursors, in contrast to selective proteolytic cleavage capacities of endoprotease, such as PC1 and PC2 (Benjannet et al., 1991; Thomas et al., 1991). At midgestation stages abundant CPE transcripts are detected in regions where no apparent PC1 or PC2 expression is observed, such as the dorsal tegmental neuroepithelium and basal telencephalon. Since expression of CPE should reflect the peptidergic potentiality, low level of PC1 or PC2 expression below the threshold of detection by *in situ* hybridization, or additional types of endoproteases (such as other newly isolated subtilisin-like endoproteases) may exist in these regions. In fact, at these stages nearly all regions of the CNS and PNS are expressing fairly high level of CPE, which is contrast to the comparably more restricted and variable pattern of CPE expression in adult (Birch et al., 1990; MacCumber et al., 1990). Expression of CPE, therefore, seems to represent one of the intrinsic properties of the developing nervous system. CPE RNA is also expressed in selective areas of the peripheral tissues. Expression of CPE in the embryonic heart is indicative of its involvement in the proteolytic maturation of proANF, as has previously been postulated (Lynch et al., 1988). We have noted, however, that not all tissues thought to require exopeptidytic processing express CPE RNA. This is best exemplified by the only partial overlapping temporally and spatially in the expression of CPE with furin. In the early postimplantation stages (e7–e9), when the expression of furin is initiated and maintained, no CPE is detected in the embryo proper. In addition, repeated *in situ* hybridization experiments failed to detect any CPE RNA in liver, a region expressing the highest level of furin RNA in mid- and late gestational stages. It is possible that a low level of CPE RNA may be expressed in these tissues, which is not detected in our experiments. Even though this may be the case, the low level of CPE expressed is certainly not accordant with the presumed proteolytic processing activity, as indicated by the multiple fetal proproteins produced and the high level of furin RNA expressed. Alternatively, other exopeptidase(s) may be employed to perform exopeptidytic cleavage in these tissues.

POMC is synthesized as a precursor to multiple hormonal peptides by corticotrophs in AL and melanotrophs in IL, as well as the arcuate neurons of the hypothalamus and at a low level by several additional cell types (Lundblad and Roberts, 1988; Smith and Funder, 1988). In AL cleavage of POMC mainly yields adrenocorticotropin (ACTH) and β-lipotropin (βLPH). In IL processing proceeds further so that smaller peptides, such as α-melanocyte–stimulating hormone (αMSH), γMSH, corticotropin-like intermediate lobe peptide (CLIP), γLPH, and β-endorphin (βEP) are released from POMC (Eipper and Mains, 1980; Smith and Funder, 1988). The selective usage of the dibasic sites flanking these peptides is responsible for the observed lobe-specific production of POMC-derived peptides. In agreement with reported results (Seidah et al., 1990, 1991; Day et al., 1992), we have shown by *in situ* hybridization, as well as RNase protection assay (Zheng and Pintar, in press), that PC1 RNA is expressed in both AL and IL, while PC2 RNA is mainly expressed in IL. In gene transfer experiments PC1 was shown to perform only limited cleavage on POMC, generating mainly ACTH and βLPH (Benjannet et al., 1991; Thomas et al., 1991). PC2, on the other hand, was shown to perform additional cleavages inside ACTH and βLPH. The results from localization studies thus agree closely with those of gene transfer experiments, suggesting that distinct distribution of PC1 and PC2 may be responsible for directing tissue-specific differential proteolytic processing of POMC in adult pituitary.

POMC is expressed at an early stage in pituitary development (e13 in rat; see Lugo et al., 1989) and is largely processed to give rise to several peptides that have been suggested as important in modulating fetal growth and development. In prenatal and neonatal AL, POMC is extensively proteolytically cleaved; thus βEP and MSH-sized peptides, as opposed to βLPH and ACTH, appear early in embryogenesis (Khachaturian et al., 1983; Allen et al., 1984; Sato and Mains, 1986; Pintar and Lugo, 1987). In postnatal pituitary, IL cells proteolytic process POMC in the same way as in adult IL cells. The βEP produced is not acetylated and may have trophic and/or tropic effects on somatic growth and brain maturation (Zagon and McLaughlin, 1983; Zagon and McLaughlin, 1987). Our results have shown that PC1 and PC2 are expressed in the developing pituitary in a manner clearly distinct from their expression in adult. In midgestational stages when Rathke's pouch has just begun to differentiate and the expression of POMC is restricted to AL, expression of PC2 RNA, but not PC1 RNA, is first initiated in AL. Expression of PC2 in AL increases steadily and in early postnatal stages, a higher level of PC2 RNA expression than in adult is observed. A similar observation for mouse pituitary has also been reported recently (Marcinkiewicz et al., 1993), although less is known about the POMC processing pattern during development in mouse. Dual labeling *in situ* hybridization has shown that PC2 expressed in AL and IL at postnatal day 15 (p15) overlaps with POMC-expressing cells. In addition, by e16 in midgestation and in early postnatal IL, the expression of PC2 has already reached high level, which may account for the early maturation of proteolytic processing capacity of this lobe toward POMC (Allen et al., 1984; Sato and Mains, 1986; Pintar and Lugo, 1987). Taken together, these results raise the possibility that expression of PC2 in pituitary may account for both the early maturation of proteolytic processing capacity of IL and for the transiently enhanced proteolytic processing of POMC in AL in development. Therefore, our *in situ* hybridization results are in general consistent with the notion that differential expression of PC1 and PC2 may be responsible for the observed developmentally regulated POMC proteolytic processing in pituitary. Thus, pituitary neuroendocrine cells may rely on manipulating the stoichiometry of processing enzymes in presenting desired profile of peptide products during development.

It should be noted that in the double labeling *in situ* hybridization not all AL cells expressing PC1 or PC2 at p15 also express POMC. It is possible that PC1 and PC2 expressed at this stage may be involved in the prohormone processing in non-POMC-expressing cells. Surprisingly, we found little if any PC1 is expressed in IL at prenatal and early postnatal stages. The lack of PC1 expression suggests that, in contrast to the adult, the proteolytic processing of POMC in IL may not require PC1 at these stages. A final surprising finding is that furin RNA is also detected in the Rathke's pouch at prenatal stages, but is not detected at any significant level in the adjacent hypothalamus or in postnatal and adult pituitaries. Furin RNA is not expressed in a lobe-specific manner and its level does not change in parallel with the increase in POMC expression and augment of POMC proteolytic processing. Instead, expression of furin is coincident with transient expression of some fetal proproteins in this structure, such as IGF-II (Stylianopoulou et al., 1988), which also exhibits a temporal decrease in abundance similar to furin. Based on the proteolytic cleavage selectivity of furin, it is more likely

that furin present in the pituitary rudiment is involved in the constitutive processing of those fetal proproteins, such as IGF-II, that possess an Arg-X-Lys/Arg-Arg motif (Gammeltoft, 1989). Thus, it seems that the biosynthesis of prohormone substrates and proteolytic processing enzymes during development may be coordinately regulated and that to accompany changes in the major types of protein precursors produced in development (i.e., growth factor precursors to prohormones), there is a switch in the predominant forms of processing enzymes expressed.

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