SPC4, SPC6, and the Novel Protease SPC7 Are Coexpressed with Bone Morphogenetic Proteins at Distinct Sites during Embryogenesis

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Abstract. In the present study, we screened for subtilisin-like proprotein convertases (SPCs) that potentially regulate the activation of known growth factors during embryonic development. We isolated a novel protease, SPC7, as well as several known SPCs. SPC7, like SPC1, is expressed ubiquitously throughout development. In contrast, SPC4 and SPC6 exhibit dynamic expression patterns. SPC4 transcripts were initially detected in the granulosa cells of secondary follicles. Shortly after implantation, SPC4 transcripts are localized to extraembryonic cell populations, and at later stages are detected in discrete tissues including the primitive gut, heart, neural tube, and limb buds. Within the limb buds, SPC4 mRNA is most abundant in the apical ectodermal ridge (AER). At later stages of limb development, SPC4 mRNA is strongly expressed in cartilage and in the interdigital mesenchyme. In contrast, high SPC6 mRNA levels are detected in somites, the dorsal surface ectoderm, and in vertebral cartilage primordia. In limb buds, SPC6 is strongly expressed in the AER, and at later stages in dorsal mesenchyme. A comparison of these expression patterns with those of several bone morphogenetic proteins (BMPs) indicates that processing of these growth factors may be limited by the local availability of SPCs.

THE growth, differentiation, and patterning of embryonic tissues largely depends on cell–cell interactions mediated in part by secreted growth factors. Since most peptide growth factors are synthesized as inactive precursors that require proteolytic maturation, it seems likely that their availability during development is locally controlled by the activities of specific proteases. For example, in the early *Drosophila* embryo, a gradient of the signaling molecule spätzle is generated by a zymogenic cascade of proteases active in the ventral region of the perivitelline space. This gradient of spätzle activity is required for specifying the fates of blastoderm cells along the dorsoventral axis (for review see Hecht and Anderson, 1992). Genetic studies in *Drosophila* also suggest that the metalloprotease tolloid acts upstream of *decapentaplegic (dpp)*, a member of the transforming growth factor-β (TGFβ) family, to locally enhance dpp activity (Shimell et al., 1991; Ferguson and Anderson, 1992).

Furthermore, in fertilized *Xenopus* oocytes, localized proteolytic activation of the TGFβ-related signaling molecule Vg-1 is thought to be necessary for establishing the primary body axis of the embryo (Thomsen and Melton, 1993). However, the protease(s) responsible for the maturation of Vg-1 remain(s) to be identified.

A family of proteases involved in propeptide processing was recently identified in multicellular organisms based on their similarity to the yeast convertase kexin (for review see Steiner et al., 1992; Van de Ven et al., 1993; Seidah et al., 1994). In mammals, six members of this family of serine proteases have been characterized to date, including furin, PC2, PC1/3, PACE4, PC4, and PC5/6. Since their catalytic domains closely resemble that of subtilisin, these proteases have been designated subtilisin-like proprotein convertases (SPC)[1] SPC1 to SPC6, respectively (Steiner et al., 1992). Individual SPC family members exhibit distinct but overlapping substrate specificities (Benjannet et al., 1991; Breslin et al., 1993; Creemers et al., 1993). As a general rule, they hydrolyze peptide bonds adjacent and distal to clusters of multiple basic residues that are situated in a favorable context (Brennan and Nakayama, 1994; Rholam et al., 1995). The SPC1 consensus cleavage site is after the sequence motif R-X-K/R-R (Hosaka et al., 1991), although cleavage after R-X-X-R has also been reported (Molloy et al., 1992). Such multibasic cleavage sites have been identified in the precursors of many secreted growth

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1. *Abbreviations used in this paper*: AER, apical ectodermal ridge; BMP, bone morphogenetic protein; dpc, days post coitum; SPC, subtilisin-like proprotein convertase.

factors, including hepatocyte growth factor, insulin-like growth factors, and members of the TGFβ supergene family (for review see Barr, 1991). Coexpression experiments have shown that SPC1 can process TGFβ1 and the related molecule activin (Roebroek et al., 1993; Dubois et al., 1995). Moreover, a purified soluble form of SPC1 partially cleaves the TGFβ1 precursor (Dubois et al., 1995), arguing that SPC1 or a closely related convertase is responsible for TGFβ1 maturation. However, it remains to be determined whether other TGFβ-related growth factors such as bone morphogenetic proteins (BMPs) also are activated by SPC1 or other SPC family members.

Interestingly, localized expression of SPCs has been described in both *Drosophila* and *C. elegans* embryos (Hayflick et al., 1992; Roebroek et al., 1993; Thacker et al., 1995). Likewise, at least some mammalian SPC family members show tissue-specific expression patterns (Seidah et al., 1990; Nakayama et al., 1992; Beaubien et al., 1995; Dong et al., 1995). For example, in both embryos and adults SPC2 and SPC3 expression is largely confined to neuroendocrine tissues, suggesting that these proteases may be specifically required for the maturation of neuropeptides (Seidah et al., 1990; Zheng et al., 1994). However, the expression patterns of other SPCs during mammalian embryogenesis have not yet been analyzed, and thus their candidate substrates have remained unknown. In particular, it has not been determined whether SPCs are colocalized with BMPs and thus are possibly involved in the processing of these potent regulators of development (for review on BMPs see Kingsley, 1994).

In the present study, we used a PCR-based strategy to isolate SPC family members expressed in early mouse embryos. Furthermore, the expression patterns of individual SPCs were examined by in situ hybridization. We found that SPC1 and SPC7, a novel family member identified in our screen, are ubiquitously expressed throughout development. In contrast, expression of both SPC4 and SPC6 is highly regulated and partially overlaps with that of a number of BMPs. Finally, the expression domains of SPC4 and SPC6 were compared in more detail with those of BMP2, BMP4, and BMP7 during limb development. Based on these results, potential interactions of SPCs and BMP-related growth factors are discussed.

Materials and Methods

Reverse PCR Screening and cDNA Cloning

Embryos were collected from CD-1 females at 6.5 days post coitum (dpc) and dissected free of extraembryonic tissues. PolyA RNA was isolated (Micro-FastTrack mRNA isolation kit, Invitrogen), and cDNA was synthesized using Superscript reverse transcriptase (GIBCO BRL, Gaithersburg, MD). SPC sequences were amplified using the degenerate primers 5′-AACGAATTCATHTAYAGYGCMWSCTGGGG-3′ (upper), and 5′-AACTCTAGATGYTGYABRTCBCKCCAGGT-3′ (lower), encoding the conserved sequence motifs, IYSASWGP and LTWRDM/LQH, respectively, within the catalytic domains of mammalian SPCs. PCR was performed in 10 mM Tris·HCl at pH 7.5 containing 50 mM KCl, 0.8 mM dNTP, 2.5 U Amplitaq (Perkin Elmer Cetus, Norwalk, CT), 2 μM upper primer, and 3 μM lower primer. After an initial denaturation step (5 min at 94°C) and three initial cycles (1 min 49°C, 2 min ramp to 72°C, 45 s 72°C, 1 min 94°C), samples were subjected to 27 amplification cycles (1 min 68°C, 1 min 72°C, 1 min 94°C). The amplified product of ~450 bp was subcloned into the EcoRV site of pBS-KSII (Stratagene LTD, La Jolla, CA). Individual clones were analyzed by colony hybridization following standard procedures (Sambrook et al., 1989). Clones which did not hybridize to cDNA fragments of mouse SPC1, -2, -3, -4, or -6 were sequenced (Sequenase kit, USB, Cleveland, OH). One of these clones was used to screen 10^6 plaques of an 8.5-dpc mouse embryonic cDNA library (Fahrner et al., 1987) as described (Sambrook et al., 1989). A single clone carrying a 1.36-kb insert was sequenced and used to rescreen the same library.

RACE PCR

The 5′ end of the SPC7 cDNA was recovered by Rapid Amplification of cDNA Ends (RACE) using polyA RNA from 16.5 dpc embryos (Marathon kit, Clontech Inc., Palo Alto, CA). RACE PCR was performed according to the manufacturer using the gene specific primer 5′-GTAGGCTGGACAAAGAGGTAGTGC-3′, and the adapter primer #1 (Clontech), and a 2:1 mixture of Amplitaq (Perkin Elmer Cetus Inc.) and DeepVent polymerase (New England Biolabs Inc., Boston, MA). Conditions for amplification were 1 min at 94°C, 1 min at 65°C, and 1 min at 72°C (30 cycles). Nested PCR was performed under the same conditions using the adapter primer #2 (Clontech Inc). The amplified product (500 bp) was subcloned into pBS-KSII and sequenced. Identical sequences were obtained for both cDNA strands from two independent RACE experiments. The RACE product was fused to the remainder of the cDNA at a unique BsmI site.

In Situ Hybridization

Pregnant CD-I mice were killed by asphyxiation with halothane. Embryos were fixed overnight at 4°C in PBS containing 4% paraformaldehyde. After dehydration through a graded ethanol series, embryos were cleared in xylenes and embedded in paraffin wax consisting of a 1:1 mixture of Paraplast Plus and Tissue Prep 2 (Fisher Scientific, Pittsburgh, PA). Sections (6 μm) were collected on Tespa-treated glass slides. In situ hybridization was performed as described (Jones et al., 1991). To synthesize an antisense mRNA probe specific for SPC4, a cDNA fragment comprising nucleotides 829 to 1231 (Hosaka et al., 1994) in pBluescript was transcribed with T3 RNA polymerase following linearization with EcoRI. Similarly, a probe detecting all known splice variants of SPC6 was generated that comprised nucleotides 740-2086 of the mouse SPC6 cDNA (Lusson et al., 1993). The SPC7-specific probe was transcribed from a 1.36-kb cDNA template spanning nucleotides 1042 to 2407. The probes specific for BMP2 (Lyons et al., 1989), BMP4, (Jones et al., 1991), and BMP7 (Dudley et al., 1995) have been described. Sense mRNA probes were used as negative controls. All probes were labeled with [α-^{35}S]UTP (DuPont New England Nuclear, Boston, MA).

RNase Protection Assay

Total RNA was prepared from embryos dissected free of extraembryonic membranes. Guanidinium lysates of whole embryos were extracted with a mixture of phenol and choloroform, and RNA was precipitated with isopropanol (Chomczinsky and Sacchi, 1987). T7 RNA polymerase was used to transcribe a 220-bp PstI–BamHI fragment of the murine Sp1 cDNA (kindly provided by P. Mitchell, Zürich) subcloned into pBS-KSII. A PCR-derived SPC7 cDNA fragment (see above) comprising 453 bp was blunt-end ligated into the EcoRV site of pBS-KS. Upon linearizing this plasmid with EcoRI, T3 RNA polymerase was used to synthesize an SPC7 antisense mRNA probe. 20 μg of total RNA was hybridized at 63°C for 12 h to [α-^{32}P]UTP-labeled antisense riboprobes (Williams et al., 1988). To digest single-stranded RNA, the hybridization reactions were chilled on ice and incubated at 37°C with 4 μg/ml RNase A and 8 U/ml RNase T1 for 30 min (Boehringer Mannheim Corp., Indianapolis, IN). Ribonuclease-resistant fragments were subjected to electrophoresis and visualized by exposing the gel with an intensifying screen to an X-ray film at −80°C for 3 d (SPC7) or for 1 d (Sp1), respectively.

Results

A Novel SPC-related Gene Expressed in Early Mouse Embryos

Several members of the BMP subgroup of the TGFβ family including BMP2, BMP4, and nodal are known to be essential during mouse gastrulation (Conlon et al., 1991; Zhou et al., 1993; Winnier et al., 1995; Bradley, A., per-

sonal communication). To identify subtilisin-like proteases potentially involved in processing these growth factors, polyA RNA from early gastrulation stage embryos (6.5–7.5 dpc) was subjected to reverse PCR using degenerate primers complementary to highly conserved sequences within the catalytic domains of known mammalian SPCs. The amplification products were analyzed by colony hybridization using probes specific for known mouse SPCs. Of 145 clones analyzed, 127 corresponded to SPC1 or to the unrelated gene tum-P91A (GenBank accession No. M25149). Of the remaining clones, thirteen corresponded to SPC4, two encoded SPC3, and two clones represented SPC2. We also identified a single clone corresponding to a novel gene which was designated SPC7. Using this PCR fragment to screen an 8.5-dpc mouse cDNA library, we recovered a single partial cDNA (1.36 kb). Rescreening the library with this larger fragment yielded a 2.8-kb clone encompassing the original 1.36-kb cDNA fragment and containing a polyA tail. A comparison to known SPC sequences suggested that this clone lacked at least 100 nucleotides of coding information at its 5′end. This region was isolated by Rapid Amplification of cDNA Ends (RACE) to generate a full-length cDNA.

In the resulting composite SPC7 cDNA, an open reading frame was identified which starts at position 180 and encodes for a protein of 770 amino acid residues. The sequence flanking the start codon of this open reading frame is TTCTGATGC and does not resemble a Kozak consensus sequence. Moreover, it is preceded by two additional short open reading frames starting at position 115 and 126, respectively. The predicted amino acid sequence of SPC7 is shown in Fig. 1. The protein structure closely resembles that of other SPC family members, comprising a proregion

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  1  MPKGRQKVPHLDAHLGLPICLWLELAIFFLVPQVMGLSEAGGLDILGTGG
 51  LSWAVHLDSLEGERKEESLTQQADAVAQAAGLVNAGRIGELQGHYLFVQP
101  TGHRQAMEVEAMRQQAEAVLARHEAVRWHSEQTLLKRAKRSIHFNDPKYP
151  QQWHLNNRRSPGRDINVTGVWERNVTGRGVTVVVVDDGVETHRQDIAPNY
201  SPEGSYDLNSNDPDPMPHPDEENGNHHGTRCAGEIAAVPNNSFCAVGVAY
251  GSRIAGIRVLDGPLTDSMEAVAFNKHYQINDIYSCSWGPDDDGKTVDGPH
301  QLGKAALQHGVMAGRQGFGSIFVVASGNGGQHNDNCNYDGYANSIYTVTI
351  GAVDEEGRMPFYAEECASMLAVTFSGGDKMLRSIVTTDWDLQKGTGCTEG
401  HTGTSAAAPLAAGMIALMLQVRPCLTWRDVQHIIVFTAIQYEDHHADWLT
451  NEAGFSHSHQHGFGLLNAWRLVNAAKIWTSVPYLASYVSPMLKENKAVPR
501  SPHSLEVLWNVSRTDLEMSGLKTLEHVAVTVSITHPRRGSLELKLFCPSG
551  MMSLIGAPRSMDSDPNGFNAWTFSTVRCWGERARGVYRLVIRDVGDEPLQ
601  MGILQQWQLTLYGSMWSPVDIKDRQSLLESAMSGKYLHDGFTLPCPPGLK
651  IPEEDGYTITPNTLKTLVLVGCFSVFWTIYYMLEVCLSQRNKASTHGCRK
701  GCCPWAPRRQNSKDAGTALESMPLCSSKDLDGVDSEHGDCTTASSFLAPE
751  LDCPPHQPPDLLQGKSGQIC
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Figure 1. Amino acid sequence of a novel subtilisin-like proprotein convertase, SPC7. The putative signal sequence (Heijne, 1986; No. 212) is overlined in bold. The subtilisin-like catalytic domain distal from a multibasic precursor cleavage site is boxed, and the P-box is underlined. Asterisks denote the catalytic residues. The exceptional sequence RGS which in other SPCs is RGD is double underlined. The GenBank accession number of SPC7 is U48830.

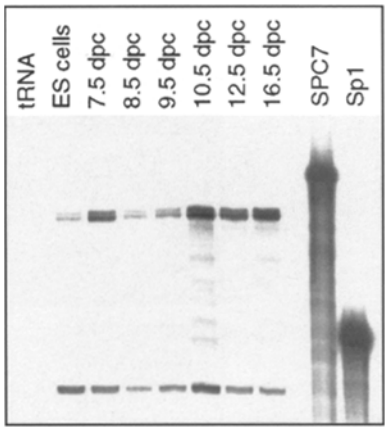

Figure 2. Ribonuclease protection analysis of SPC7 mRNA expression during mouse embryogenesis. Total RNA was from undifferentiated embryonic stem (*ES*) cells or from mouse embryos collected at the indicated stages (*dpc*, days post coitum); SPC7 and Sp1 probes were 508 and 265 nucleotides in size, respectively. Yeast tRNA served as a negative control.

separated from the catalytic domain by a multibasic cleavage site, and a conserved region distal to the catalytic domain referred to as the P-box (Fig. 1). In contrast to all other known family members, SPC7 lacks an RGD motif within the P-box. Such RGD motifs may mediate binding of secreted forms of SPCs to the extracellular matrix protein integrin (Seidah et al., 1991). Finally, the carboxy-terminal domain of SPC7 is serine-, threonine-, and cysteine-rich, but it lacks the characteristic cysteine repeats found in SPC4, SPC6, and SPC1 (Roebroek et al., 1992). In addition, a stretch of hydrophobic residues was identified in position 664-687 which may represent a transmembrane domain analogous to that found in SPC1 (Roebrock, 1986).

SPC1 and SPC7 Are Widely Expressed during Mouse Embryogenesis

Previous studies have shown that SPC1 is ubiquitously expressed in the developing rat embryo (Zheng et al., 1994). Similarly, our in situ hybridization experiments indicated that SPC1 and SPC7 are both expressed at constitutive levels throughout postimplantation stage embryos (data not shown). Also, by RNase protection assays, SPC7 expression was detectable at all developmental stages examined (Fig. 2), and in all adult tissues analyzed, including brain, lung, muscle, heart, liver, kidney, spleen, and thymus (data not shown). No significant changes in the relative abundance of SPC7 mRNA were observed between gastrulation and mid-gestation.

SPC4 and SPC6 mRNA Expression at Early Developmental Stages

In the adult ovary, high levels of SPC4 mRNA are restricted to the granulosa cells of secondary follicles (Fig. 3, *A–C*). During embryogenesis, SPC4 expression appears to be tightly regulated from the earliest stage examined onwards. At 6.5 dpc, SPC4 transcripts are abundant in the maternal decidual cells surrounding the implantation site,

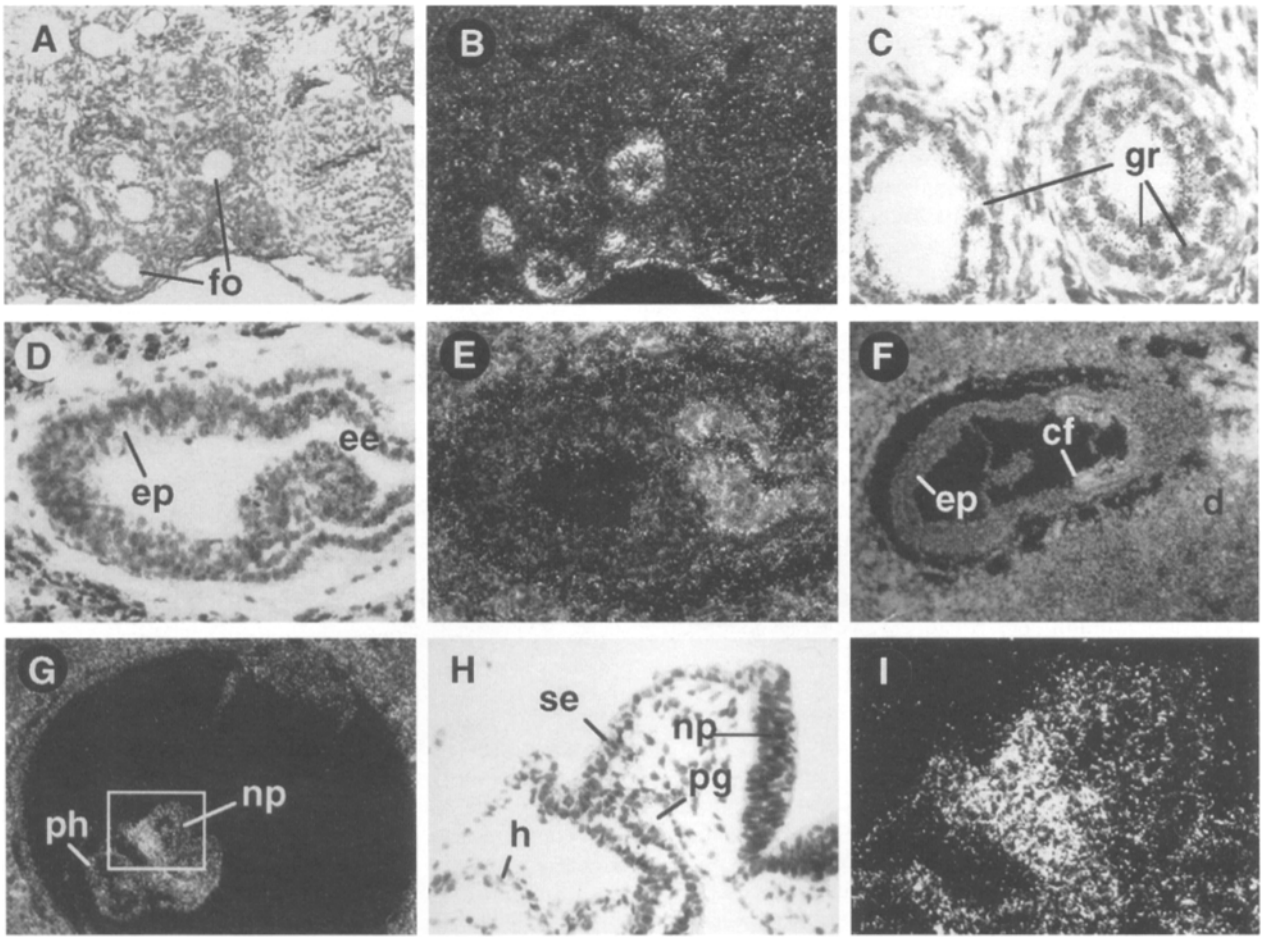

Figure 3. SPC4 mRNA expression at early stages of mouse development. (*A* and *B*) Adult ovary, showing expression in granulosa cells surrounding secondary follicles (bright-field and dark-field illumination, respectively); (*C*) higher magnification view of granulosa cells; (*D* and *E*) sagittal section through a pregastrula stage embryo (6.5 dpc); (*F*) sagittal section at 7.5 dpc; (*G*) postgastrula stage embryo (8.5 dpc) sectioned transversely at the level of the primitive heart; (*H* and *I*) magnification from boxed area in *G* showing expression in and laterally from the primitive gut. In *D–F*, anterior is at the top. *fo*, follicles; *gr*, granulosa cell layers; *ee*, extraembryonic region; *ep*, epiblast; *cf*, chorionic folds; *d*, decidua; *np*, neural plate; *ph*, primitive heart; *pg*, primitive gut; *se*, surface ectoderm.

and in the extraembryonic ectoderm (Fig. 3, *D–E*). During gastrulation (7.5 dpc), elevated levels of SPC4 mRNA are restricted to the chorion (Fig. 3 *F*). At 8.5 dpc, the highest level of expression is observed in and laterally to the primitive gut (Fig. 3, *G–I*). In addition, SPC4 mRNA is transiently expressed at high levels in all tissues of the developing heart between 9.5 and 10.5 dpc (Fig. 4, *A* and *B*, and data not shown). At 11.5 dpc, high levels of SPC4 mRNA are detected in the floor plate of the neural tube and the apical ectodermal ridge (AER) of developing limb buds (Fig. 4, *C–E*).

Like SPC4, SPC6 is weakly expressed throughout the embryo, but markedly upregulated at discrete sites during development. Prominent expression is observed in the decidua at the site of fusion of the two decidual lobes (data not shown). Within the 7.5-dpc embryo, the hybridization signal was most intense in the extraembryonic primitive endoderm, the amnion (Fig. 5, *A* and *B*), and the nascent mesoderm located in the distal region of the primitive streak (Fig. 5, *A–D*). At 8.5 dpc, SPC6 is most abundantly expressed in the somites and in the yolk sac (Fig. 5 *E*). As the somites mature, the intense SPC6 hybridization signal is confined to the dermamyotome compartment (Fig. 5 *F*). Between 9.5 and 11.5 dpc, SPC6 mRNA is abundantly expressed in the AER of limb buds (Fig. 6 *D*, and data not shown). By 12.5 dpc, the SPC6 expression pattern in developing limbs becomes more complex (see below). At this stage, SPC6 expression is also upregulated in the vertebral and facial cartilage primordia and in the intrinsic muscle of the tongue (Fig. 5, *G–I*).

SPC4 and SPC6 mRNA Expression in Developing Limbs Overlaps with that of Bone Morphogenetic Proteins

Interestingly, both SPC4 and SPC6 were found to be abundantly expressed in a stripe of thickened ectodermal cells along the anteroposterior margin of developing limb buds. These cells, referred to as the AER, are essential for axis formation, outgrowth, and patterning of the limb (Saunders, 1948; Summerbell, 1974). Several members of the BMP family including BMP2, -4, and -7 are known to be

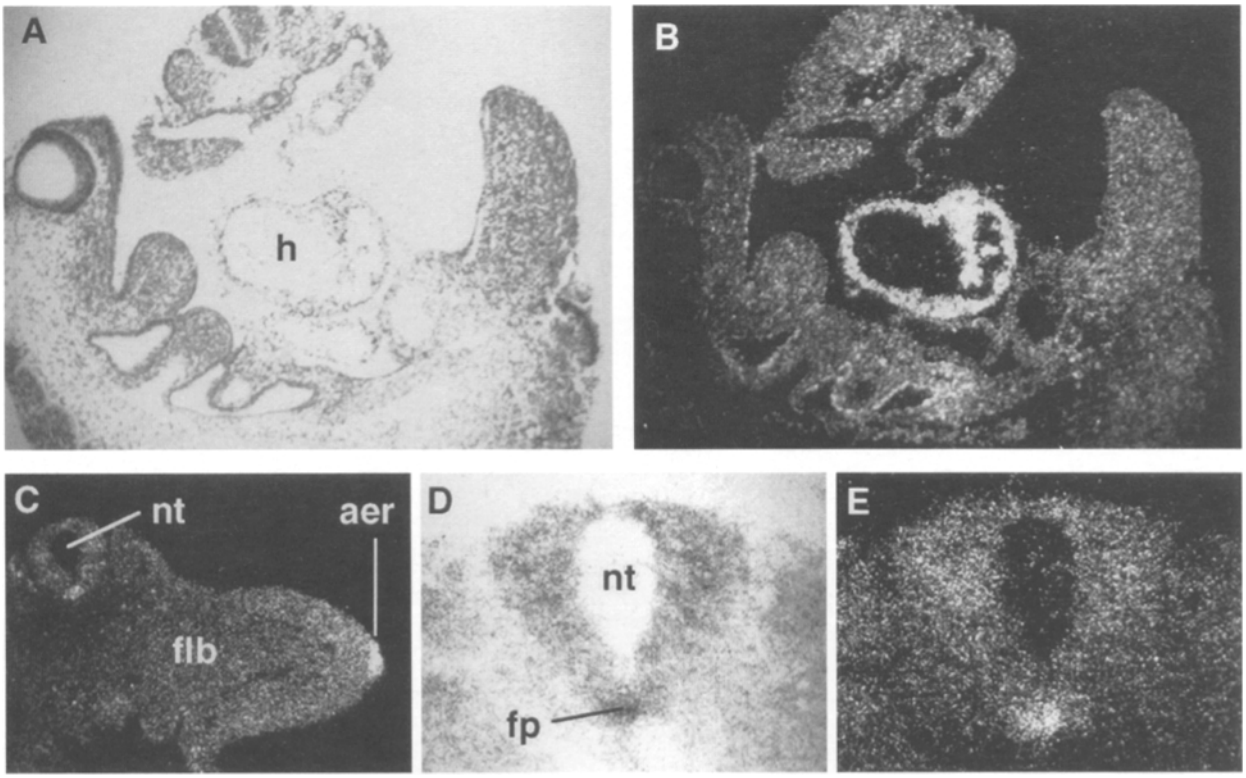

Figure 4. SPC4 mRNA expression in the developing heart, limb, and neural tube. (*A* and *B*) 9.5 dpc embryo showing SPC4 expression in the developing heart; (*C*) forelimb bud of an 11.5-dpc embryo; (*D* and *E*) transverse section through the neural tube at 11.5 dpc. Note the elevated level of SPC4 expression in the floor plate. *h*, primitive heart; *flb*, forelimb bud; *aer*, apical ectodermal ridge; *nt*, neural tube; *fp*, floor plate.

expressed in the AER and in distinct regions of the underlying mesenchyme. Specifically, BMP4 mRNA is localized to a group of posterior cells in the limb bud mesenchyme (Jones et al., 1991; Fig. 6, *A* and *B*), whereas BMP7 expression is more uniform (Lyons et al., 1995; Fig. 6 *F*). BMP2 is very weakly expressed in the mesenchyme (Lyons et al., 1990, 1995; Fig. 6 *D*). Since these BMPs represent candidate SPC substrates, it was important to examine the potential overlap in their expression domains with those of SPC4 and SPC6. Adjacent sections through limb buds were hybridized with probes specific for SPC4, SPC6, and a panel of BMPs. As shown in Fig. 6, high levels of BMP2, BMP4, and BMP7 transcripts are coexpressed with SPC4 and SPC6 mRNA in the AER. Additionally, SPC4 and SPC6 transcripts are detected also in the underlying mesenchyme, although at significantly lower levels than in the AER (Fig. 6, *C* and *E*).

During later stages of limb development, SPC4 and SPC6 exhibit unique and distinct expression patterns. At 12.5 dpc, SPC4 mRNA is detected in cartilage primordia of the long bones (Fig. 7, *A* and *B*) and in mesenchymal cells at the distal tip of the limb, but is absent from the overlying ectodermal cells (Fig. 7, *C* and *D*). As seen in transverse and longitudinal sections, the SPC4 signal observed in the distal tip of the developing limb reflects expression in the interdigital mesenchyme and in the cartilage primordia of the future digits (Fig. 7, *H* and *I*). In contrast to SPC4, SPC6 expression is confined to the condensing mesenchymal cells surrounding the cartilage (Fig. 7, *E–F*). Furthermore, in the distal region of the developing limb, SPC6 transcripts are more abundant in dorsal compared to ventral mesenchyme (Fig. 7, *E–G*). Similarly, hybridization with probes specific for BMP2, -4, and -7, indicated that these BMPs all are strongly expressed in interdigital mesenchyme, but not in cartilage primordia (Fig. 7, *J–M*; data for BMP4 not shown).

SPC4 and SPC6 mRNA Expression at Later Stages of Mouse Development

At 16.5 dpc, SPC4 is strongly upregulated in the primary ossification centers of the long bones and the digits (Fig. 8, *A–D*, and data not shown). At this stage, SPC4 mRNA is almost undetectable in resting and proliferating chondrocytes at the periphery of the cartilage. However, SPC4 mRNA is very abundant in hypertrophic chondrocytes within the ossification centers. These results, together with data showing SPC4 expression in early cartilage primordia, suggest that SPC4 may be involved in bone formation. Note that at 16.5 dpc, SPC4 mRNA is also detected in the epidermis (Fig. 8, *A* and *B*). In the intestine, SPC4 expression is localized to the mesenchymal cells of the villi (Fig. 8, *E* and *F*). In striking contrast, SPC6 mRNA in the developing gut is abundant in the epithelial cells of the villi but not in the mesenchyme (Fig. 8, *G* and *H*).

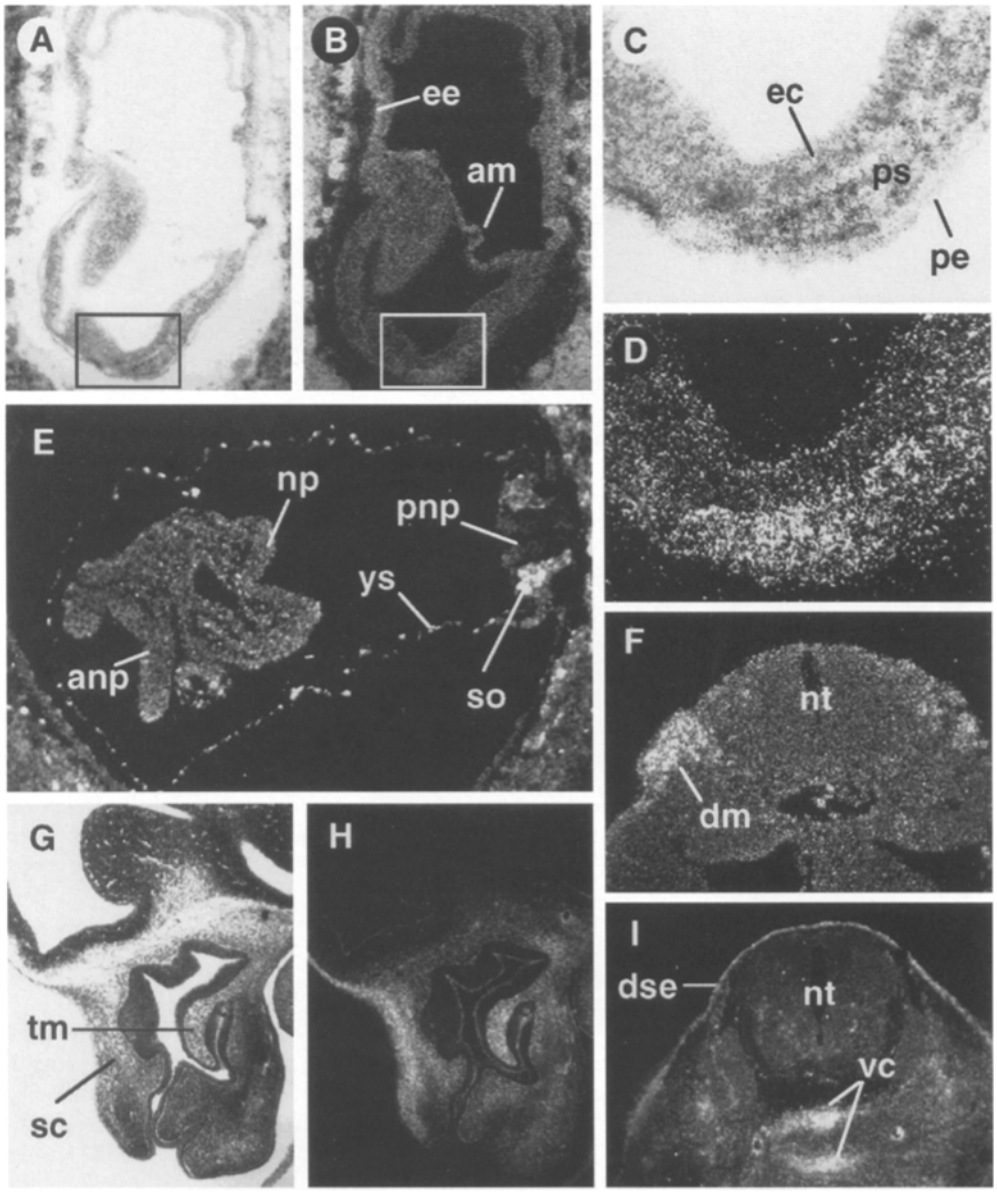

Figure 5. SPC6 expression at early stages of development. (*A* and *B*) Gastrulation stage embryo (7.5 dpc) under bright-field (*A*) and dark-field illumination (*B*), respectively; (*C* and *D*) higher magnification view of boxed area in *A* and *B* shows SPC6 expression at the distal tip of the primitive streak. (*E*) Elevated SPC6 expression in somites and the yolk sac at 8.5 dpc. (*F*) SPC6 expression in the neural tube at 9.5 dpc; (*G* and *H*) parasagittal section through the head of a 12.5-dpc embryo; (*I*) 12.5 dpc spinal cord sectioned at the level of the forelimbs. *ee*, extraembryonic region; *am*, amnion; *ec*, embryonic ectoderm; *ps*, primitive streak; *pe*, primitive endoderm; *anp*, anterior neural plate; *np*, neural plate; *pnp*, posterior neural plate; *ys*, yolk sac; *so*, somite; *nt*, neural tube; *dm*, dermamyotome; *tm*, tongue muscle; *sc*, skull cartilage; *dse*, dorsal surface ectoderm; *vc*, vertebral cartilage primordium.

Discussion

To evaluate their possible developmental function(s), we have analyzed the expression of individual members of the subtilisin-like propeptide convertase gene family during mouse embryogenesis. In a screen for SPCs expressed in early mouse embryos, we isolated cDNA fragments corresponding to all known SPCs with the exception of SPC5. Interestingly, we also recovered a cDNA encoding a novel family member, SPC7. The catalytic domain of SPC7 is 50–54% identical to those of other family members. Like other SPCs, SPC7 contains a signal sequence and a proregion extending to a conserved consensus SPC cleavage site, followed by the subtilisin-related catalytic domain and the P-box. Given these structural similarities to other SPCs, SPC7 probably requires proteolytic maturation to become active. Moreover, activation may be autocatalytic as described for SPC1, -2, and -3 (Leduc et al., 1992; Matthews et al., 1994; Shennan et al., 1995). It should be noted that SPC7 is distinct from the PC7 described by Tsuji and colleagues (Tsuji et al., 1994), since the latter represents the rat homologue of SPC4/PACE4 (Johnson et al., 1994). However, SPC7 identified in our screen seems to be homologous to a human leukemia proprotein convertase sequence (accession number U33849) deposited in the Genbank database during the preparation of this manuscript. SPC7 mRNA was found to be ubiquitously expressed at all developmental stages examined, suggesting that SPC7 is constitutively active. Alternatively, its enzymatic activity may be controlled at the level of proteolytic maturation of the SPC7 precursor, or at the level of translation given that the translation initiation site of SPC7 is not in a favorable context.

In contrast to SPC1 and SPC7, SPC4 and SPC6 were found to display dynamic expression patterns throughout development. In early postimplantation stage embryos (6.5 dpc), SPC4 expression is restricted to the extraembryonic ectoderm. By 7.5 dpc, high levels of SPC4 expression are detected in the developing chorion, an essential ex-

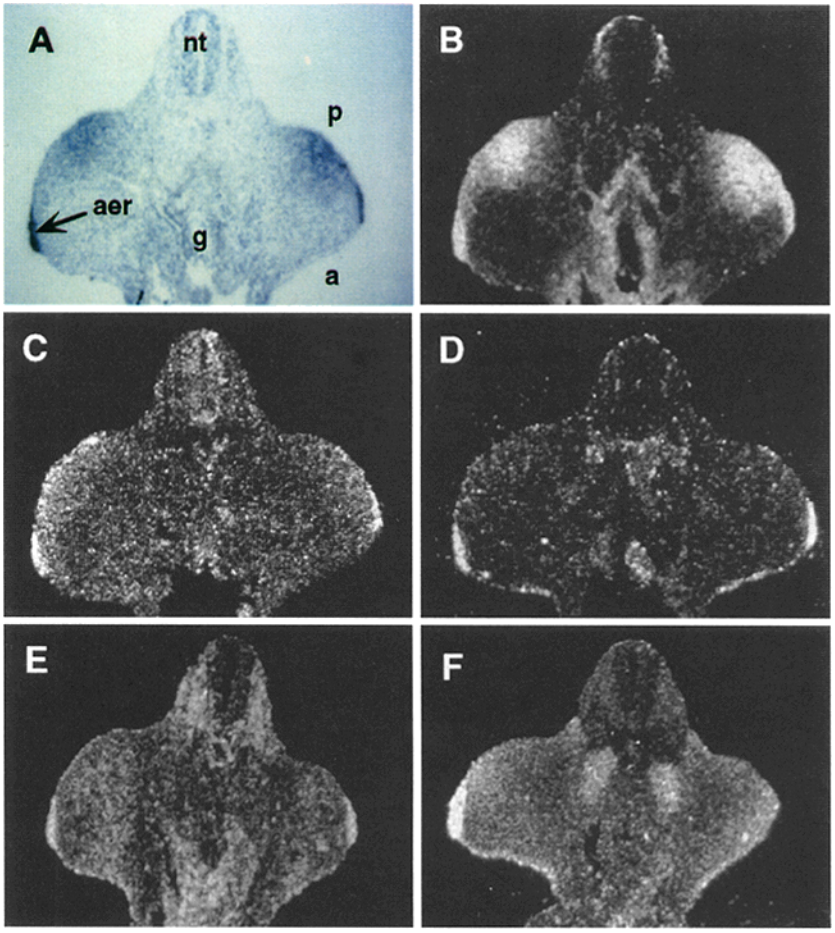

Figure 6. Comparison of SPC4 and SPC6 expression domains with those of bone morphogenetic proteins (BMPs) at an early stage of limb development. Adjacent tissue sections through the hind limb buds of an 11.5-dpc embryo were hybridized with probes specific for (*A* and *B*) BMP4, (*C*) SPC4, (*D*) BMP2, (*E*) SPC6, or (*F*) BMP7. The plane of sectioning almost paralleled the anteroposterior axis to ensure that AER cells were represented on all sections, and to allow simultanous visualization of the graded expression of BMP4 in the limb bud mesenchyme. *nt*, neural tube; *aer*, apical ectodermal ridge; *a*, anterior; *p*, posterior.

traembryonic tissue that contributes to the developing placenta. The function of SPC4 in these specific cell populations is unknown, but it is of interest that BMP2 and -4 are both abundantly expressed in the same extraembryonic cell populations during gastrulation (Winnier et al., 1995; and Constam, D.B., unpublished observation). Moreover, loss of BMP2 or BMP4 activity has been shown to cause embryonic failure. Given the overlap in their expression domains, we suggest that SPC4 and SPC6 may be involved in processing of one or both of these BMPs.

At later stages of development (11.5 dpc), SPC4 is expressed in the neural tube in a pattern suggesting a possible role in dorsoventral patterning. Neural tube patterning along the dorsoventral axis requires dorsalizing signals which specify cells in the dorsal region of the neural tube to become commissural neurons. Such signals are believed to derive from the surface ectoderm, and recent experiments suggest that these may be mediated in part by BMP4 and BMP7 (Liem et al., 1995). In contrast, the notochord and floor plate provide opposing ventralizing signals which specify ventral cells to become motor neurons (Yamada et al., 1993). Ventralizing activities identified to date include sonic hedgehog (Shh), a signaling molecule expressed in notochord and floor plate cells (Echelard et al., 1993; Marti et al., 1995). Additionally, floor plate cells express high levels of BMP6 at 11.5 dpc (Dudley, A.T., and E.J. Robertson, unpublished observation). Therefore, our observation that SPC4 expression is upregulated in floor plate cells at this developmental stage raises the possibility that SPC4 may be responsible for BMP6 maturation. Similarly, SPC6 is strongly expressed in the dorsal surface ectoderm and might therefore activate dorsalizing molecules such as BMP4 and BMP7.

In early limb buds, three signaling centers have been identified that are critical for axis formation and patterning: (*1*) A group of posterior mesenchymal cells designated the zone of polarizing activity (ZPA) that specifies the anteroposterior axis, (*2*) the dorsal surface ectoderm which determines the dorsoventral axis, and (*3*) the apical ectodermal ridge essential for limb outgrowth and patterning along the proximodistal axis (for review see Tabin, 1995). Recent data argue that these signaling centers do not function independently, but rather are intimately linked (Laufer et al., 1994; Niswander et al., 1994; Yang and Niswander, 1995). The exact role played by individual BMPs in this signaling network remains to be determined.

A particularly important question is why several BMPs including BMP2, -4, and -7 are all strongly expressed in the AER. Interestingly, we find that BMP expression within the AER is closely associated with very high levels of SPC4 and SPC6 expression. This suggests that SPC4 or SPC6 may be involved in the formation or maintenance of the AER. It is also possible that combined activities of these convertases regulate BMP maturation within this tissue. Further evidence that SPC4 and SPC6 may be involved in this pathway comes from the finding that SPC4

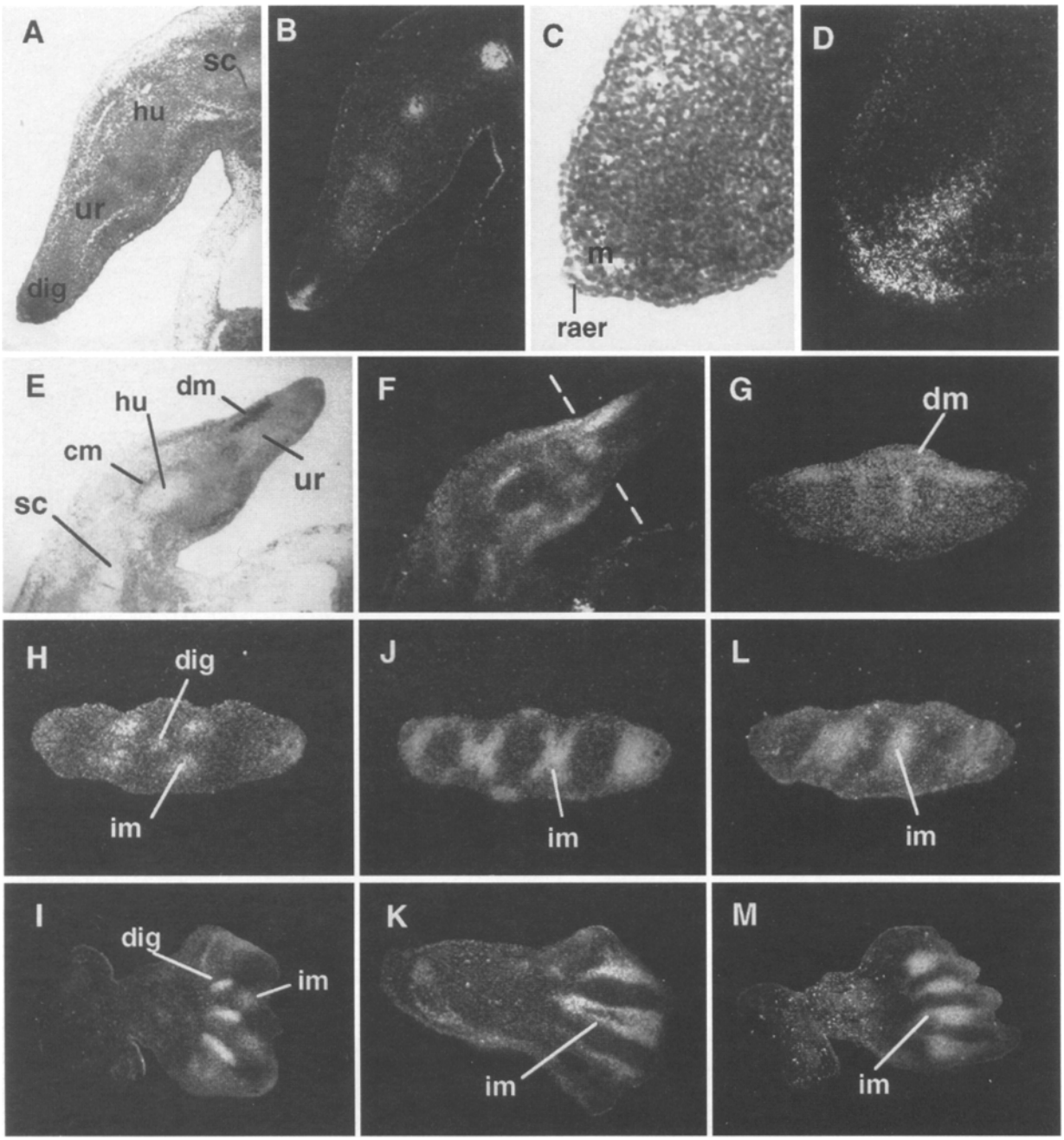

Figure 7. SPC4 and SPC6 expression at later stages of limb development partially overlaps with that of several BMPs. (*A* and *B*) SPC4 expression in the forelimb at 12.5 dpc; (*C* and *D*) higher magnification view of *A* and *B* showing SPC4 expression at the distal tip of the limb. (*E* and *F*) Longitudinal and (*G*) transverse section of a 12.5-dpc limb hybridized to SPC6 probe. The stippled line in *F* indicates where the section in *G* was obtained. (*H*, *J*, and *L*) Transverse and (*I*, *K*, and *M*) longitudinal sections through the distal tips of 12.5 dpc limb buds hybridized to (*H* and *I*) SPC4, (*J* and *K*) BMP2, and (*L* and *M*) BMP7. *sc*, *hu*, *ur*, and *dig*: cartilage primordia of scapula, humerus, ulna/radius, and digits, respectively; *m*, mesenchyme; *raer*, remnant of the apical ectodermal ridge; *cm*, condensing mesenchyme; *dm*, dorsal mesenchyme; *im*, interdigital mesenchyme.

and SPC6 transcripts also colocalize with BMP mRNAs at more advanced stages of limb development. In particular, BMP2, -4, and -7 expression overlaps with that of SPC4 and SPC6 in interdigital and dorsal limb mesenchyme, respectively. The subtle differences in their expression patterns suggest that these proteases may act combinatorially to locally modulate BMP activities and to pattern distinct limb structures.

Endochondral bone formation is initiated by the condensation of mesenchyme, followed by proliferation and differentiation of chondroblasts into chondrocytes and deposition of cartilaginous matrix. Upon further differentiation, chondrocytes hypertrophy, degenerate, and are calcified while osteogenic cells invade and deposit bone matrix. Endochondral bone formation involves the coordinated action of BMPs, a TGFβ subfamily whose members were pu-

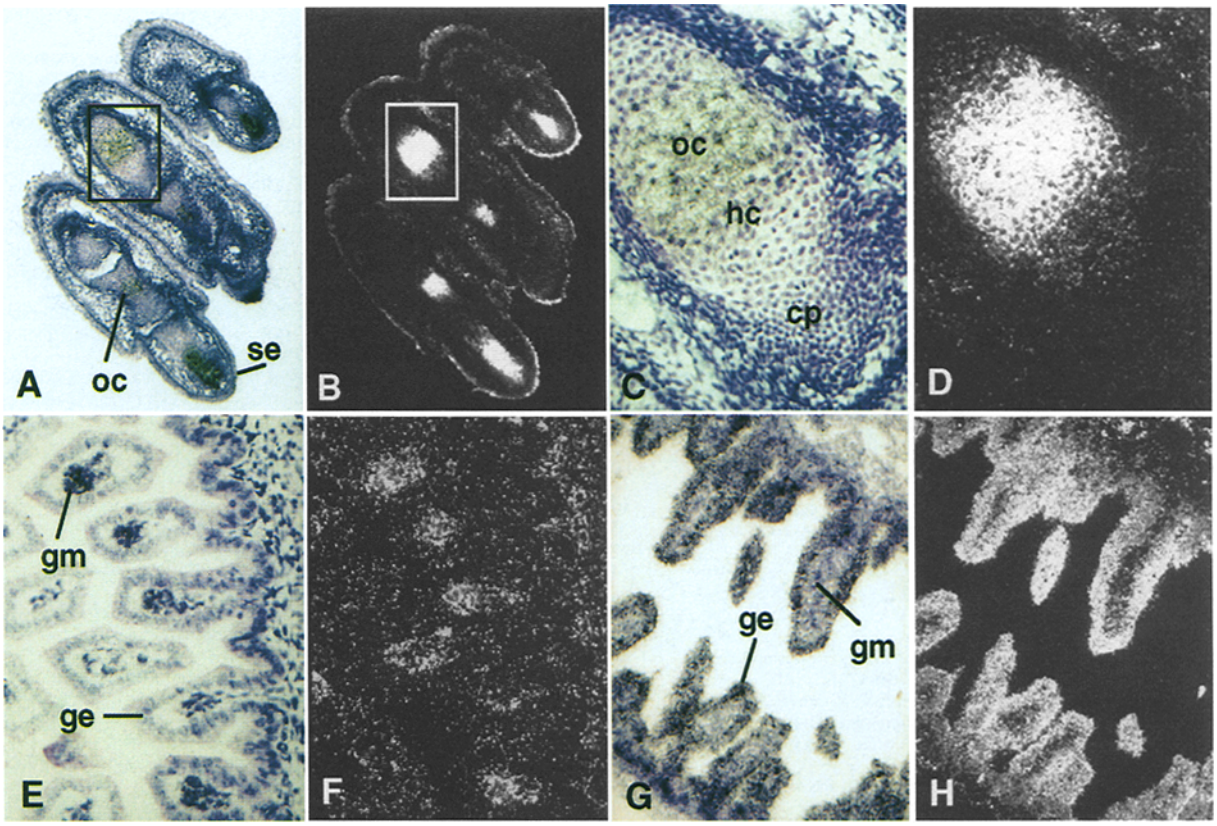

Figure 8. Distinct patterns of SPC4 and SPC6 mRNA expression at 16.5 dpc. (*A* and *B*) SPC4 expression in the foot; (*C* and *D*) higher magnification view of the primary ossification center of metacarpal bone boxed in *A* and *B*; (*E* and *F*) SPC4 expression in the villi of the intestine is restricted to mesenchymal cells; (*G* and *H*) SPC6 expression confined in the intestinal villi to epithelial cells. *se*, surface ectoderm; *oc*, primary ossification center; *hc*, hypertrophic chondrocytes; *cp*, cartilage primordium; *gm*, gut mesenchyme; *ge*, gut epithelium.

rified and cloned based on their capacity to induce ectopic cartilage and bone in rat muscles (Wozney et al., 1988; Celeste et al., 1990; Wang et al., 1990). The expression of BMPs during skeletal development is consistent with a role in regulating chondrocyte differentiation (Lyons et al., 1989; Jones et al., 1992). Thus, BMP2 is thought to promote the condensation and differentiation of mesenchymal cells into chondroblasts. By contrast, BMP6 has been suggested to control the recruitment or terminal differentiation of hypertrophic chondrocytes. Direct evidence for an involvement of BMPs in skeletal development is provided by the *short ear* mutation. In these mice, loss of BMP5 function leads to abnormal size, shape, and number of early mesenchymal condensations and ultimately results in multiple skeletal defects in the sternum, ribs, and in vertebral processes (Kingsley et al., 1992). Likewise, a loss of function mutation in the BMP-related gene GDF5 reduces the length of long bones and the number of digital bones (Storm et al., 1994).

The present findings demonstrate that SPC4 and SPC6 are differentially regulated during bone formation. Thus, SPC6 transcripts were most abundant in the cartilage primordia of the vertebrae, the facial area of the skull, and in condensing limb mesenchyme. In contrast, SPC4 expression was detected in the cartilage primordia that eventually give rise to the long bones and digits of the limbs. Later during skeletogenesis, SPC4 expression is very strongly induced in hypertrophic cartilage but remains virtually undetectable in chondroblasts and undifferentiated chondrocytes. Therefore, SPC4 and SPC6 may have distinct functions during bone formation and are possibly involved in BMP signaling pathways. For example, the prominent expression of SPC4 in hypertrophic cartilage suggests that this protease may control the terminal differentiation of chondrocytes or activate growth factors that act on invading osteogenic cells. Since hypertrophic chondrocytes express high levels of BMP6 (Lyons et al., 1989; Bitgood and McMahon, 1995), it will be interesting to determine whether the convertase responsible for BMP6 maturation is SPC4.

Overall, the present data demonstrate that the novel protease SPC7, like SPC1 (Zheng et al., 1994), is ubiquitously expressed at constitutive levels during embryogenesis. In contrast, SPC4 and SPC6 expression is highly regulated in discrete tissues. Therefore, SPC4 and SPC6 may be required for local activation of proproteins not susceptible to cleavage by the ubiquitous convertases SPC1 and SPC7. Alternatively, since the substrate specificities of individual SPCs significantly overlap (Creemers et al., 1993; Rehemtulla et al., 1993; Hosaka et al., 1994), we suggest that SPC4 and SPC6 may act in concert with SPC1 and SPC7 to locally enhance the maturation of common substrates. In the future, gene targeting experiments will be important to assess in vivo the specific contributions of individual SPCs to proprotein maturation.

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