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Differential Expression of Thrombospondin 1, 2, and 3 During Murine Development

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ABSTRACT **Thrombospondin 1 is a secreted, trimeric glycoprotein that mediates interactions between cells and extracellular matrix and exhibits cell-specific effects on migration and proliferation. Recently, two additional thrombospondin genes (thrombospondin 2 and 3) have been identified. To study the functions of these proteins, we have used in situ hybridization and RNAse protection assays to compare the expression of the genes encoding thrombospondin 1, 2, and 3 during murine embryogenesis. Thrombospondin mRNAs were associated with ossification, neuronal organogenesis, and lung development, although transcripts were differentially expressed. Thrombospondin 1 was predominant from days 10 to 13. During this period, high but transient levels of expression were observed in the neural tube, head mesenchyme, and cardiac cushions. In contrast, a more constant level of thrombospondin 1 mRNA was apparent in resident megakaryocytes of the liver, as well as in circulating megakaryocytes; neither thrombospondin 2 nor 3 was detected in these cells. Thrombospondin 1 was also produced by cells of the developing kidney and gut. The expression of thrombospondin 2 was confined principally to organized connective tissue that included pericardium, pleura, perichondrium, periosteum, meninges, ligaments, and reticular dermis. Thrombospondin 2 was also produced by differentiating skeletal myoblasts and by cells of the kidney and gut. Moreover, high levels of expression were detected in blood vessels. Thrombospondin 3 mRNA was restricted to brain, cartilage, and lung. Although thrombospondin 1, 2, and 3 belong to a family of structurally related genes, the differences observed in the spatiotemporal distribution of the corresponding mRNAs indicate unique functions for these secreted proteins.** © 1993 Wiley-Liss, Inc.

Key words: Thrombospondin, Gene expression, Extracellular matrix, Chondrogenesis, Neurogenesis, Embryogenesis, Development

INTRODUCTION

Thrombospondin (TSP) was initially described in platelet alpha granules (Lawler et al., 1978) as a large, trimeric glycoprotein (M_r420,000) that functioned in platelet aggregation and clot formation (for a review see Asch and Nachman, 1989; Mosher, 1990; Frazier, 1991). Further investigation established that TSP was also secreted by a variety of cultured cells that include endothelial cells (McPherson et al., 1981; Mosher et al., 1982; Canfield et al., 1990), smooth muscle cells and fibroblasts (Raugi et al., 1982; Jaffe et al., 1983), keratinocytes (Wikner et al., 1987), macrophages (Jaffe et al., 1985), and glial cells (Asch et al., 1986). These findings, coupled with reports describing several cell-surface receptors for the protein (Roberts et al., 1987; Taraboletti et al., 1987; Lawler et al., 1988; Kaesberg et al., 1989; Frazier, 1991; Prater et al., 1991; Guo et al., 1992; Leung et al., 1992), indicated a broader spectrum of functions than that initially recognized in coagulation.

Indeed, TSP has been shown to modulate endothelial cell attachment (Murphy-Ullrich and Höök, 1989), proliferation of both smooth muscle and endothelial cells (Majack et al., 1986; Bagavandoss and Wilks, 1990; Taraboletti et al., 1990), neurite outgrowth by several types of neurons and ganglion cells (Neugebauer et al., 1991; Osterhout et al., 1992; O'Shea et al., 1991), and chemotaxis/haptotaxis of melanoma cells (Taraboletti et al., 1987). Furthermore, TSP is able to affect cell-matrix interactions by its acknowledged ability to bind to several proteins of the extracellular matrix, such as collagen, fibronectin, plasminogen activator, heparan sulfate proteoglycan, and SPARC (Frazier, 1987; Sage and Bornstein, 1991). TSP also binds to TGF-β1 (Murphy-Ullrich et al., 1992) and might therefore regulate the response of cells to this cytokine through extracellular sequestration or by presentation of the cytokine to cells.

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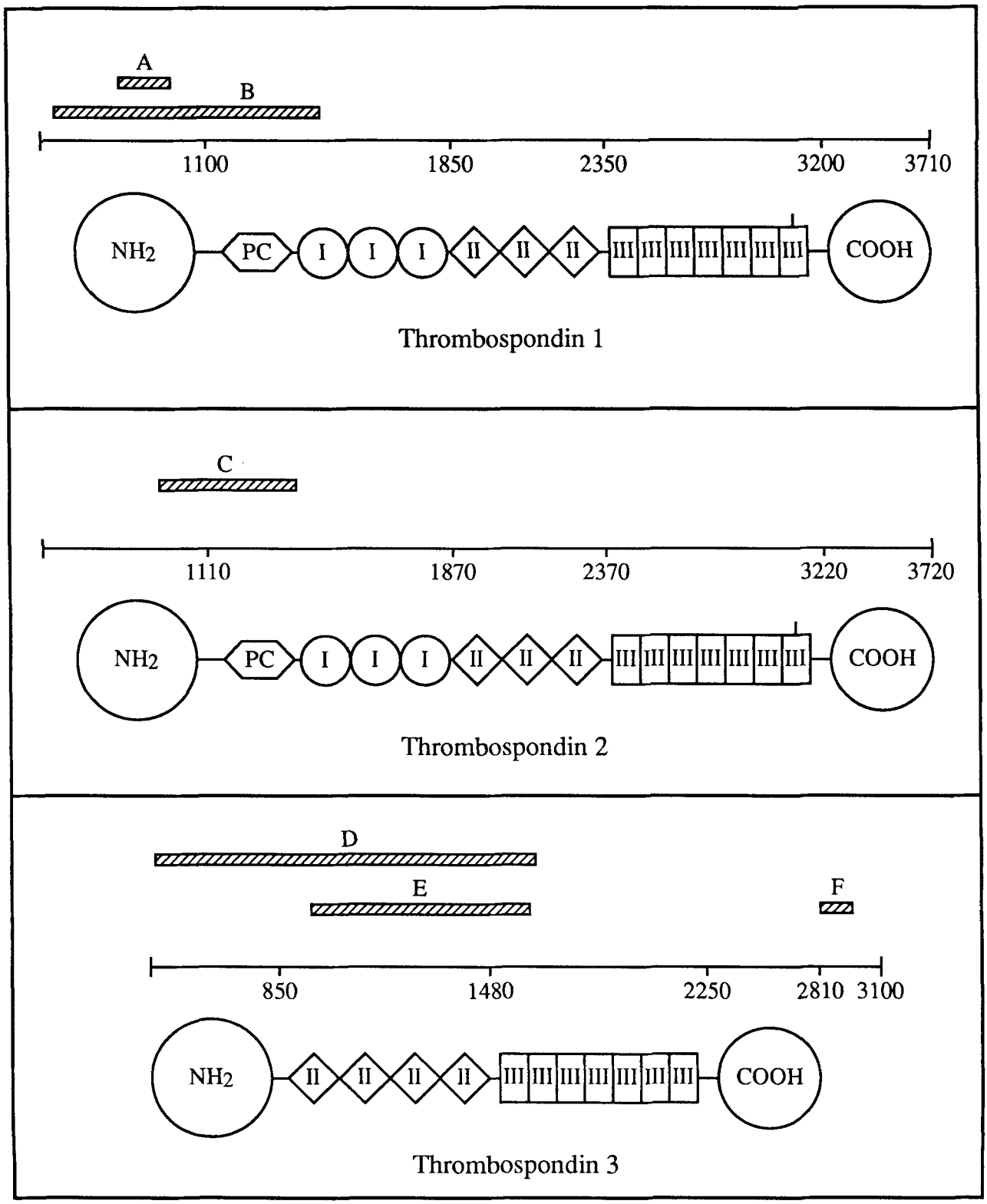


Fig. 1. Location of cDNA probes relative to the TSP cDNA sequence and to a schematic representation of the corresponding protein structure of TSP 1, 2, and 3. For TSP 1 and TSP 2 the solid line represents the cDNA sequence, with nucleotide 1 as the start of transcription. For TSP 3, nucleotide 1 represents the first base of the translation start codon, since the transcription start site is not known. Shown are the NH_2 and COOH terminal domains, the procollagen (PC) region, and the type I, II, and III repeats. **A:** 294 bp mouse TSP 1 (MTSP 1) probe generated by reverse PCR. **B:** 1.3 kb human TSP1 cDNA probe (Kobayashi et al., 1986). **C:** 514 bp mouse TSP 2 (MTSP 2) probe generated by reverse PCR. **D:** 1550 bp mouse TSP 3 (MTSP 3) cDNA probe (phage 4, Bornstein et al., 1993). **E:** 680 bp MTSP3 probe generated by reverse PCR; F: 147 bp *AluI*/*AluI* genomic DNA fragment from the 3′ UTR of MTSP3 (Vos et al., 1992).

The multiplicity of functions attributed to TSP reflects its macromolecular features. TSP can be subdivided into several sequential regions that include a) an amino-terminal, globular, heparin-binding domain; b) an interchain disulfide knot; c) a cysteine-rich domain homologous to the α1 chain of type I procollagen; d) three TSP or properdin (type I) repeats; e) three EGF-like (type II) repeats; f) seven Ca^{+2}-binding (type III) repeats, and g) a carboxy-terminal cell-binding region (Mosher, 1990; Bornstein, 1992). Many of the functions attributed to TSP, as well as the receptor-binding sites, have been mapped to specific regions within these domains. Although these discrete domains, and the existence of several receptors, could account for the variety of functions attributed to TSP, reports of two additional homologous genes (TSP 2 and TSP 3) (Bornstein et al., 1991a,b; Lawler et al., 1991; LaBell et al., 1992; Vos et al., 1992; Laherty et al., 1992) have revealed a new dimension of complexity in the structure/function relationship of these proteins. It could be hypothesized, for example, that many of the functions initially attributed to TSP 1 could be shared by other members of the family, contingent on cell- or site-specific expression. The significant degree of sequence conservation among these proteins (Bornstein, 1992) supports this possibility. For example, it is reasonable to assume that certain antibodies raised against platelet TSP (TSP 1) would also recognize TSP 2 and 3. This point is especially relevant since many functional and structural questions have been addressed through the use of antibodies. Therefore, it seems important for our understanding of TSP function to determine the specific patterns of expression for TSP 1, 2, and 3 by a method that provides clear discrimination among the three proteins.

Analysis of protein distribution during embryonic and fetal development can often elucidate potential functions during morphogenesis. In addition, mechanisms similar to those observed in embryogenesis, such as migration and proliferation, are frequently reiterated during tissue repair in the adult. Thus, information obtained from embryonic development can, to a certain extent, be used to interpret cellular responses to pathological conditions, in which ectopic expression of regulatory molecules is frequently observed. We have generated a number of TSP type-specific riboprobes that were used, in this study, to address tissue/cell-specific distribution of TSP mRNA during murine development, and have characterized the temporal and spatial expression of three distinct TSP gene transcripts during organogenesis in the mouse. The expression of mRNAs corresponding to TSP 1, 2, and 3 differed in all organs studied. Although the three genes were expressed in brain, lung, and cartilage, the pattern of expression for each transcript was cell-specific and, in some cases, temporally distinct. Despite the occurrence of homologous domains, which often imply related functions, we found marked differences in the location of transcripts for TSP 1, 2, and 3. We therefore predict that TSP 1, 2, and 3 subserve overlapping but distinct functions during development and, possibly, during tissue repair.

RESULTS

The distribution of TSP mRNA transcripts was determined on mouse embryos from 10 to 18 days postconception and in the neonatal mouse brain. For direct comparison among transcripts, hybridization with TSP 1, 2, and 3 anti-sense probes was performed in parallel on 5 μm serial sections, which provided internal controls for probe specificity and extent of background radioactivity. Controls with sense-oriented probes, which were included in each experiment, were always negative and exhibited minimal background radioactivity. Since each anti-sense probe displayed a distinct pattern of hybridization in most cases, our data indicate that the hybridization reactions were specific and that the three TSP mRNAs were differentially expressed during development. We used several probes to examine TSP 1, 2, and 3 mRNAs. This precaution enhanced our confidence with regard to the specificity of the probes. Figure 1 illustrates the orientation, length, and location of the DNA templates used for the riboprobes relative to the primary structure of each of the TSPs.

Fig. 2. Distribution of TSP 1 transcripts during murine development. All figures show hybridization to the murine anti-sense TSP 1 probe (probe A in Fig. 1). **A:** Head mesenchyme of a day 10 mouse embryo; mesenchymal cells exhibit strong hybridization signal. **B:** Bright-field image of A, head mesenchyme (hm), blood vessel (v). **C:** Bright-field image of a small blood vessel (arrow) containing a circulating megakaryocyte positive for TSP 1 (arrow) (day 11). **D:** Transverse section of a 12 day brain and adjacent mesenchyme; signal can be detected in the head mesenchyme and in the neuronal cells adjacent to the lumen. **E:** TSP 1 RNA can be detected in day 16 brain in the connective tissue; arrow indicates expression in the connective tissue and presumptive choroid plexus. **F:** Presence of TSP 1 transcripts in the meninges (m) can be observed as early as day 12. **G:** Sagittal section of a 13 day heart; arrows indicate hybridization signal in the cardiac cushions. **H:** Sagittal section of a 14 day heart; transcripts for TSP 1 are prevalent in the cushions (asterisks). **I:** In the 16 day heart, a hybridization signal is evident in the parietal pericardium (p). **J:** TSP 1 mRNA expression in megakaryocytes of the liver (12 day) (arrows). **K:** TSP 1 transcripts are also seen in megakaryocytes (arrows) at day 17, despite the reduction in the number of these cells at this stage of development. **L:** Kidney of a 16 day embryo; expression of TSP 1 is concentrated at the interface between the cortical and medullary areas. **M:** Low magnification view of the abdominal organs of a 16 day embryo; TSP 1 mRNA is present in the liver, kidney (k), and the developing smooth muscle layer of the stomach (s). **N:** TSP 1 transcripts are present in the smooth muscle layer of the stomach (s) (16 day embryo). **O:** Transverse section of the small intestine (si) shows TSP 1 mRNA in the muscular layer (16 day embryo). **P:** Transverse section of several vibrissae (vi), showing hybridization signal throughout the connective tissue and in the sheath that surrounds each vibrissa (16 day embryo). **Q:** Sagittal section of the cerebral cortex (cc); low levels of hybridization are apparent throughout the brain, but a strong signal is still present in the meninges (m) (17 day embryo). **R:** TSP 1 mRNA is abundant in the developing palate and cochlea (c), signal is also present in the epithelium of the cochlea (17 day embryo). c, cochlea; cc, cerebral cortex; hm, head mesenchyme; m, meninges; p, pericardium; k, kidney, s, stomach, si, small intestine; v, vessel; vi, vibrissae. Bar, 100μm.

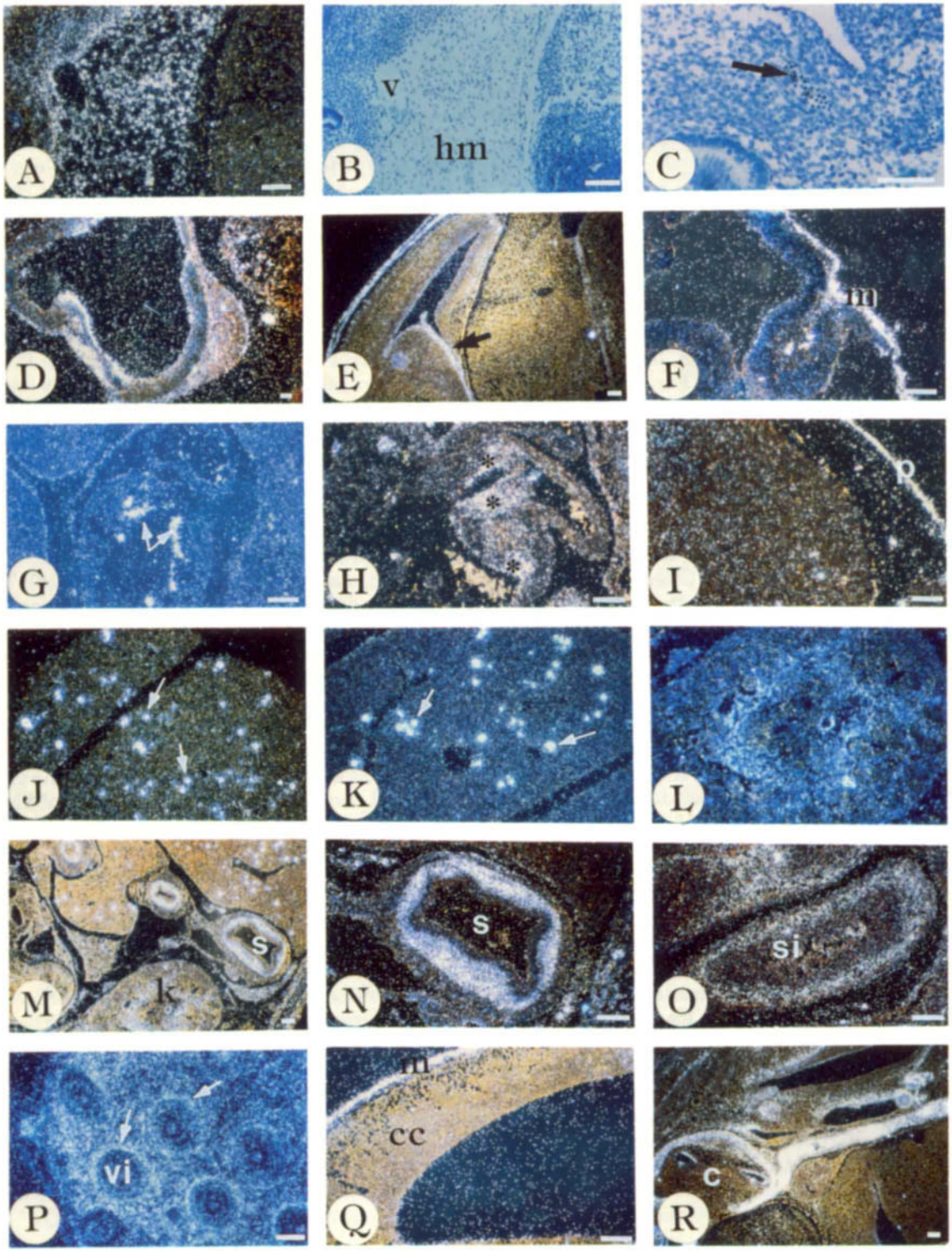


Fig. 2.

Although the probes had similar specific activities, we did not attempt to make quantitative comparisons from in situ hybridization. For this purpose we used RNAse protection analysis and quantitation by phosphor-imaging on four different organs: heart, liver, brain, and anterior limb.

Expression of TSP 1 mRNA

Significant levels of TSP 1 transcripts were detected as early as day 10. Mesenchymal cells expressing TSP 1 mRNA were found scattered throughout the head mesenchyme (Fig. 2A,B) at 10–12 days postconception. The number of positive cells after day 12 decreased significantly and no signal was detected in mesenchymal cells after day 14. In addition, large circular cells in the lumen of blood vessels (Fig. 2C) exhibited a strong hybridization signal. A cell with similar morphology was seen in the connective tissue of several organs and in the chambers of the developing heart. These mononuclear cells appear to be of hematopoietic origin and are tentatively identified as circulating megakaryocytes. Indeed, hybridization of TSP 1 in megakaryocytes could be detected in the liver as early as day 12 and as late as day 17 (Fig. 2J,K). The presence of TSP 1 in the liver was also demonstrated by RNAse protection analysis. High steady-state levels of mRNA were observed on days 11–13, with a significant decrease thereafter (Figs. 3D, 4D). From in situ hybridization data we can identify megakaryocytes as the source of TSP 1 transcripts in the liver; the intensity of the hybridization signal within the cells did not appear to change over time. In addition, the number of megakaryocytes in the embryonic liver did not change significantly between days 13 and 16. The apparent discrepancy between data from the RNAse protection and the in situ hybridization can be explained by the dilution effect of megakaryocytes relative to the parenchyma during a period in which the mass of the liver is increasing rapidly. Thus the differential growth patterns between megakaryocytes and other liver cells is asymmetric, and in general, quantitation of transcript levels by this procedure must be interpreted with caution. After day 17, however, the reduced number of megakaryocytes in the liver accounts for the low levels observed by RNAse protection assay. In the adult, TSP 1 transcripts were also detected in megakaryocytes from the spleen and bone marrow (data not shown).

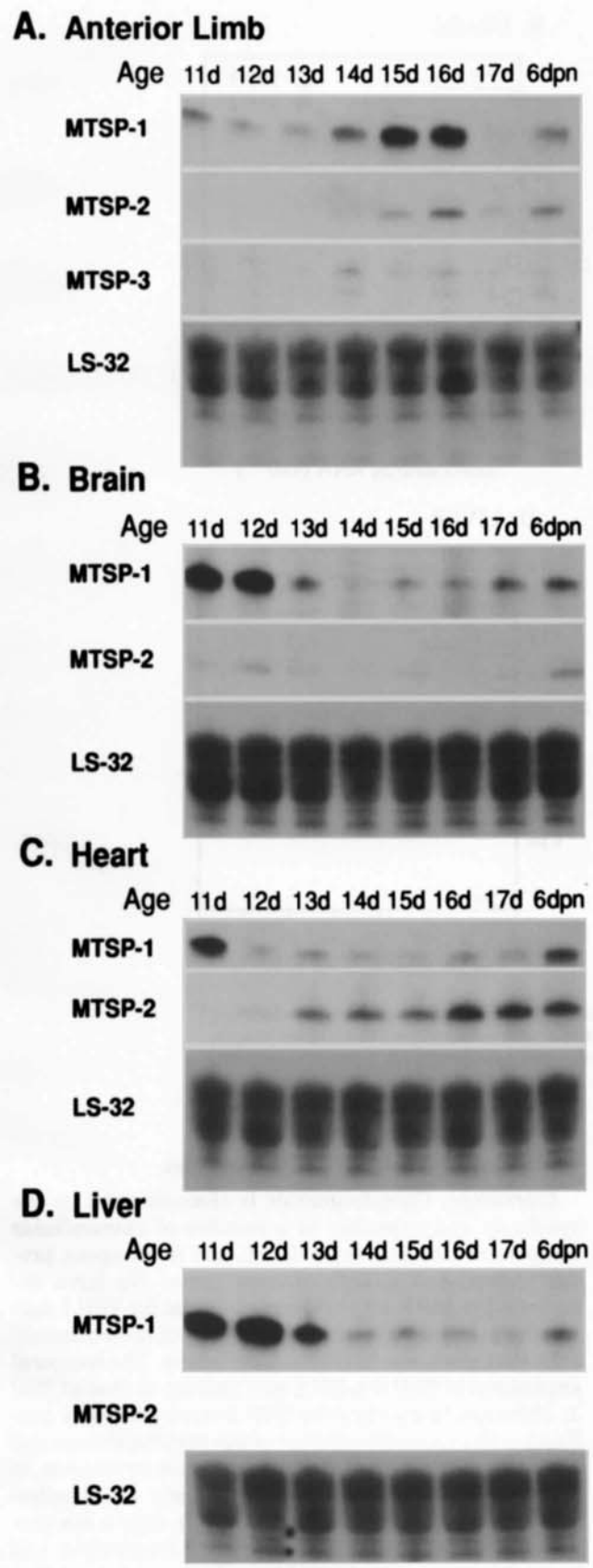
High levels of signal for TSP 1 mRNA were detected in the developing brain as early as day 10 (Fig. 2D,E) and were present until day 13. Similar findings were obtained by RNAse protection assays (Fig. 3B). Quantitation of these data showed a peak of expression on days 11–12 and a significant decrease thereafter (Fig. 4B). These findings are consistent with the role proposed for TSP in neurite outgrowth (Neugebauer et al., 1991; O'Shea et al., 1991; Osterhout et al., 1992), since proliferation of neurons, accompanied by significant extension of neuronal processes, occurs predominantly at this time. After day 13, the hybridization signal was significantly lower and most of the transcripts for TSP 1 in the brain were confined to connective tissue, vessels, and choroid plexus. TSP 1-positive cells within these tissues are probably responsible for the relatively low, but detectable signal observed by RNAse protection assay after day 12 (Figs. 3B, 4B). TSP 1 mRNA was also detected in the meninges from day 12 to 18 (Fig. 2F) and in neonatal mouse (data not shown).

Expression of TSP 1 in the heart was coincidental with the initiation of septation (day 11). The process of septation is dependent on the migration of epithelial cells (cardiac endothelium) into the cardiac jelly or myocardial basement membrane and their differentiation into mesenchyme (Mjaatvedt and Markwald, 1989). TSP 1 transcripts were first detected in the cardiac endothelium and were subsequently confined to the mesenchyme of the cardiac cushions until morphogenesis of the valves was complete (Fig. 2G,H). A positive signal was also detected in the parietal but not in the visceral pericardium (Fig. 2I). This pattern of expression was corroborated by RNAse protection analysis (Fig. 3C). In these experiments, a peak of TSP 1 expression was detected at day 11 (Figs. 3C, 4C). At the fetal phase of heart morphogenesis (day 16), TSP 1 expression diminished significantly. We encountered variability in the RNA samples from heart, as can be observed from the standard deviations in Figure 4. After evaluation of a large number of in situ hybridization experiments, we noticed a marked difference in the number of megakaryocytes present in the chambers of the heart. It is conceivable that fluctuations in the volume of blood were responsible for the variability observed, since organs with high levels of blood (such as heart and liver) generally showed the highest standard deviations.

During the late embryonic and early fetal period, expression of TSP 1 was also apparent in the developing kidney (Fig. 2L,M), particularly at the interface between the medullary and the cortical areas. At this time, expression of TSP 1 was also observed in the smooth muscle layer of the intestine (Fig. 2O). Although rarely present in connective tissue during fetal development, TSP 1 mRNA was detected 1) in the external sheath of vibrissae (Fig. 2P), 2) in the mesenchyme during intramembraneous ossification, particularly in the calvaria, and 3) in cartilaginous tissues that included the cochlea (Fig. 2R). A surprising result was the detection of TSP 1 mRNA in several epithelia, including the neuroepithelium of the cochlea (Fig. 2R) and the epithelium of the lung (Fig. 7A,D).

Expression of TSP 2 mRNA

Transcripts for TSP 2 were detected as early as day 11 in the dorsal aorta (Fig. 5A). The association of TSP 2 mRNA with the endothelium of large and small vessels was a constant finding, and TSP 2 mRNA was often associated with marked angiogenic activity, as seen, for example, in the meninges and choroid plexus (Fig. 5B,C). However, expression of TSP 2 transcripts



was also detected in large vessels of the neonatal mouse (Fig. 5D).

In the embryonic and fetal brain, we were not able to detect TSP 2 transcripts, although the meninges were positive from days 12 to 18 (Fig. 5E). Low levels of TSP 2 mRNA in the brain were observed by RNAse protection at days 11–12 (Figs. 3B, 4B). From several in situ hybridization experiments on embryos of this age, we attributed most of the signal to small vessels and to the connective tissue from the meninges.

A general conclusion that emerges after analysis of a large number of embryos is the close association of TSP 2 with tissues derived from mesenchyme, such as connective tissue, cartilage, and bone. However, unlike TSP 1, TSP 2 transcripts were only detected in connective tissue cells after day 13. TSP 1 was associated with "early" mesenchymal cells, whereas TSP 2 was expressed by later generations of mesenchymal cells. This temporal comparison is summarized in Table 1. The peak of TSP 2 expression coincided with the organization of connective tissue surrounding organs, bone, or cartilage that occurs on days 14–16. TSP 2 mRNA in connective tissue cells appeared concentrated in areas of demarcation within and between tissues. For example, frequent hybridization was observed in connective tissue sheaths separating groups of myotubes in developing muscle, and in fascia between different tissues, such as that between muscle and dermis.

In the heart, high levels of TSP 2 mRNA were evident from days 13–18 in the visceral and parietal layers that form the pericardium (Fig. 5H). Low levels of hybridization were also detected in the cardiac cushions (data not shown). RNAse protection assays showed that expression of TSP 2 in the heart followed that of TSP 1 (Figs. 3C, 4C). Although a great proportion of TSP 2 signal could be attributed to the connective tissue of the pericardium and blood vessels, TSP 2 was also observed in cardiac valves and in cardiomyocytes.

From day 15 onward, TSP 2 transcripts were detected predominantly in dense connective tissue (Fig. 5G), such as the reticular dermis (Fig. 5N) and vibrissae (Fig. 5J), ligaments (Fig. 5K), perichondrium, and periosteum (Fig. 6B,E,H). In the tooth germ, an epithelio-mesenchymal organ surrounded by mesenchyme, morphogenesis of the periodontal ligament, as well as the alveolar bone, was associated with significant levels of TSP 2 mRNA (Fig. 5I).

At 16–18 days, a strong hybridization signal was detected in the central mesenchyme of the kidney (Fig.

Fig. 3. Differential expression of thrombospondins in organs of embryonic mice. Ribonuclease protection assays were performed with total RNA from isolated organs at different times during development. **A:** Anterior limb. **B:** Brain. **C:** Heart. **D:** Liver. As a control for RNA concentration, a probe for ribosomal protein L32 (L32) mRNA was included in all assays. Hybridization of MTSP 3 is shown only in limb, since no detectable TSP 3 levels were observed in brain, heart, and liver at these developmental ages.

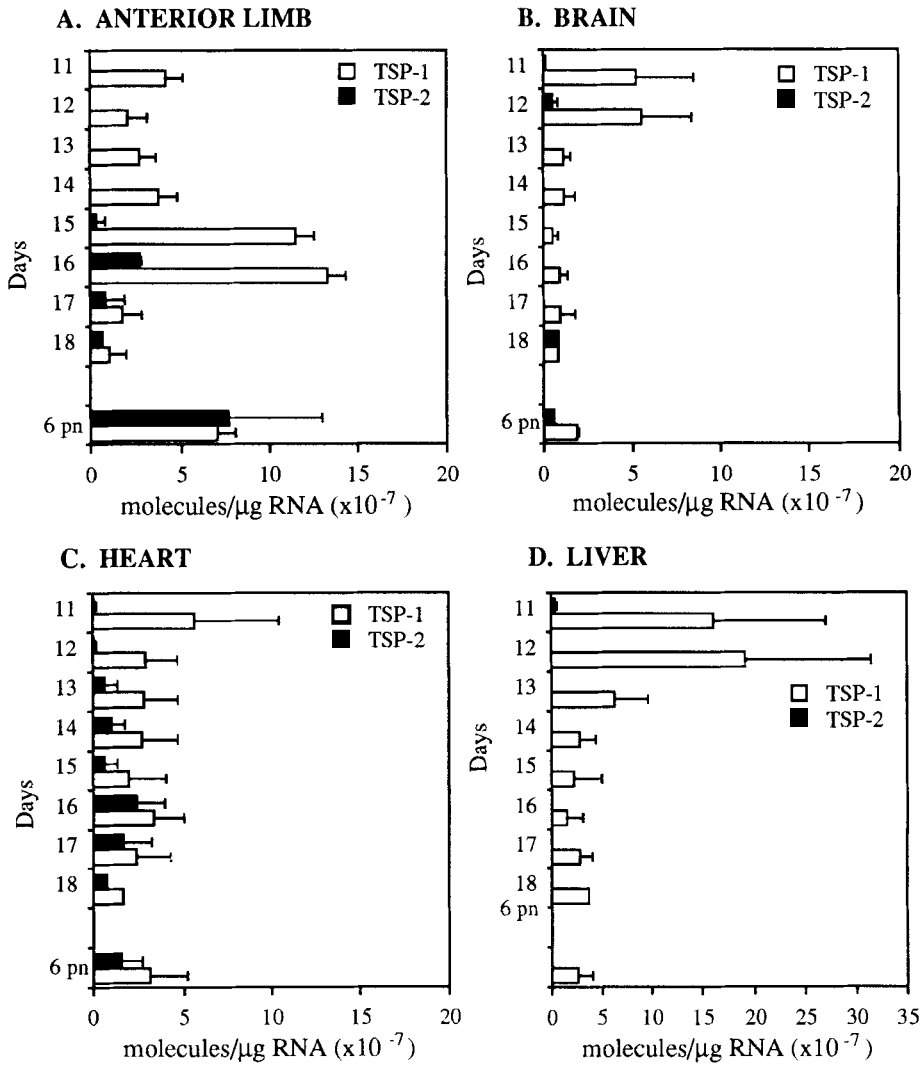


Fig. 4. Relative abundance of mRNA for TSP 1, 2, and 3 in organs of embryonic mice during development. **A:** Anterior limb. **B:** Brain. **C:** Heart. **D:** Liver. RNase protection assays were analyzed by phosphor imaging and signals were normalized to those of L32. Mean ±s.d. from 2–4 experiments are shown as molecules/ μg RNA ($\times 10^7$).

5M) and in the smooth muscle layer of some organs of the digestive tract (Fig. 5L). The outermost caudal regions of the developing gut appeared to be negative. No expression was detected in the liver (Fig. 3D), with the exception of Glisson's capsule.

Expression of TSP 3 mRNA

Expression of TSP 3 was observed from day 15 onward. Since we were unable to detect a hybridization signal before day 15 (Table 1), expression of TSP 3 is more likely to be minimal or absent until later stages of murine embryogenesis. Since the distribution of TSP 3 transcripts is confined to lung, cartilage, and brain, which are also sites of expression for TSP 1 and TSP 2, we will discuss our findings in the context of a direct comparison among these three transcripts.

Co-Expression of Thrombospondins

Cartilage. Chondrogenesis is characterized by the synthesis and assembly of a number of extracellular components, such as types II, IX, and X collagens, proteoglycans, and specific glycoproteins. We have detected a low mRNA hybridization signal for TSP 1 during the precartilaginous condensation of mesenchymal cells that gives rise to ribs and vertebrae. The temporal expression of TSP 2 mRNA was similar to that of TSP 1, although transcripts for TSP 2 were generally confined to the connective tissue of the perichondrium and periosteum (Fig. 6B,H,K,N), with scant expression in chondrocytes (Fig. 6B). Whereas nearly all chondrocytes expressed TSP 1 (Fig. 6A,G,J,M), only a low percentage of these cells contained TSP 3 transcripts, and hybridization was restricted to mature and fully differ-

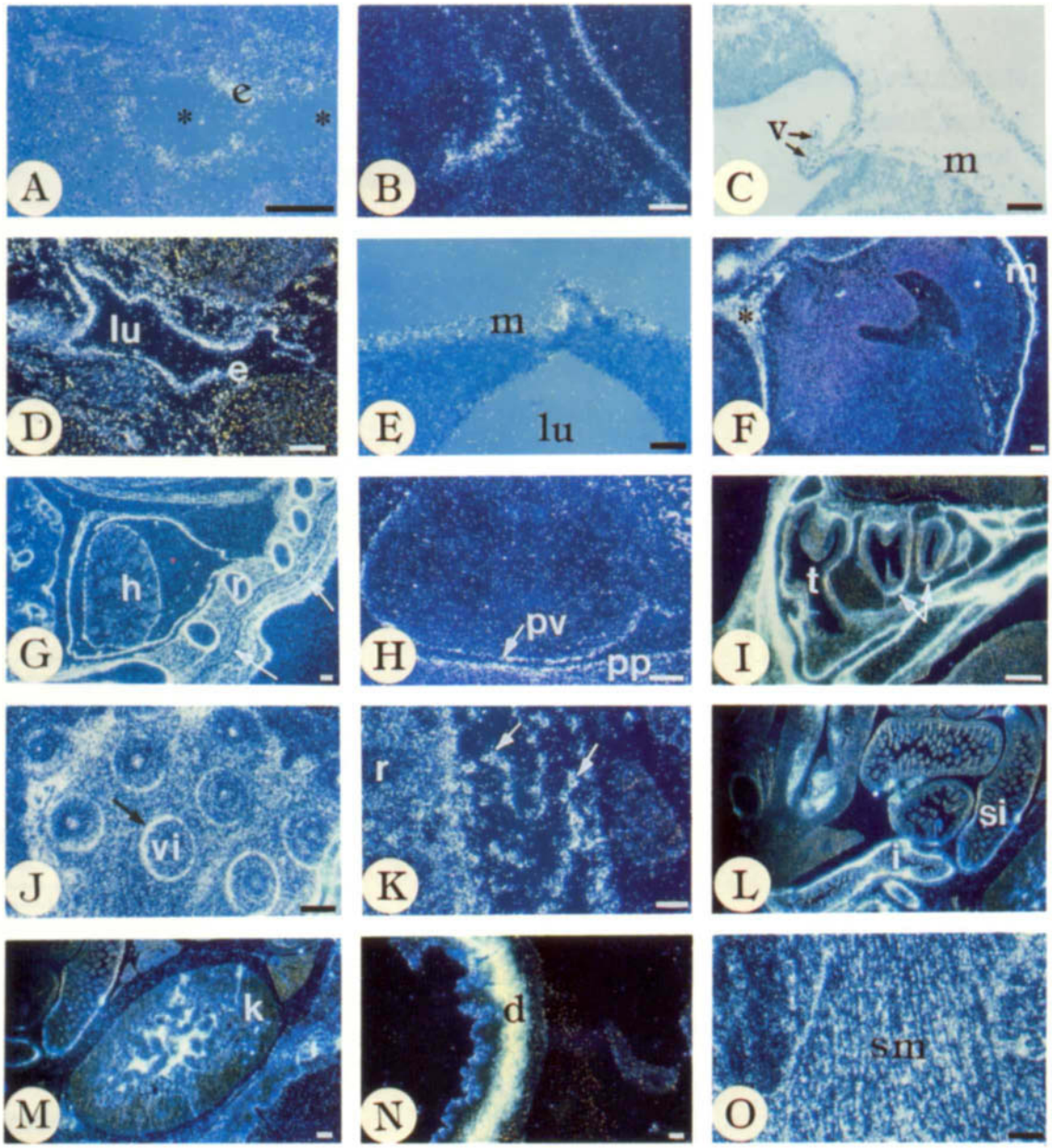
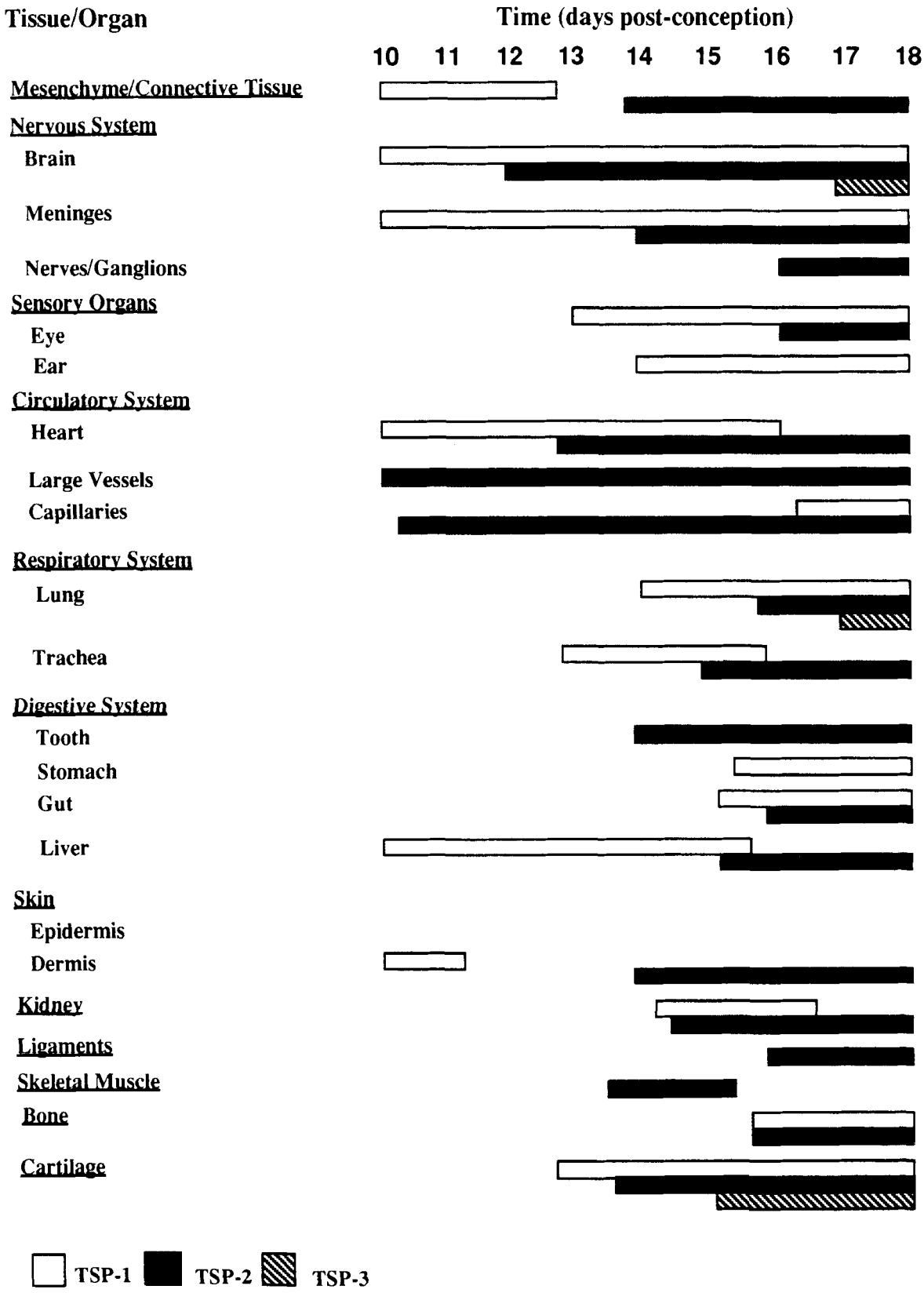


Fig. 5. Expression of TSP 2 transcripts in murine development. All figures represent hybridization with the murine anti-sense TSP 2 probe (probe C in Fig. 1). **A:** Transverse section of the dorsal aorta of an 11 day mouse embryo; hybridization signal is observed in the endothelium (e) lining the lumen (asterisks). **B:** TSP 2 transcripts are present in both the capillaries and in the connective tissue of the meninges (12 day embryo). **C:** Bright-field image of B; meningial vessels (v) (arrows) and blood vessels of mesenchyme (m) are indicated. **D:** TSP 2 is present in blood vessels of the neonatal mouse, especially in the endothelium (e). **E:** Transverse section of a 12 day brain; hybridization signal is concentrated in the meninges (m). **F:** TSP 2 transcripts in the meninges (m) of an 18 day fetus, as well as in the connective tissue of the head (asterisk). **G:** Sagittal section through the thorax of a 15 day embryo; TSP 2 transcripts are observed in connective tissue (e.g., dermis (arrows)), perichondrium of the ribs (r), and pericardium; **H:** TSP 2 is present in both visceral (pv) and parietal pericardium (pp) of a 16 day embryonic heart. **I:** Sagittal section of developing teeth (t); strong hybridization signal is observed in the dental mesenchyme of both cuspid and pulp (arrows), and in the alveolar bone (16 day embryo). **J:** Transverse section of vibrissae (vi) showing TSP 2 mRNA in the connective tissue that surrounds each vibrissa (arrow) (16 day embryo). **K:** TSP 2 transcripts are expressed by differentiating fibroblasts (arrows) adjacent to the vertebral column (16 day embryo). **L:** Low magnification view of the abdominal area of a 16 day embryo; TSP 2 mRNA is present in the smooth muscle layer of some (but not all) segments of the intestine (i). **M:** In the kidney (k), expression of TSP 2 is restricted to the medullary zone (16 day embryo). **N:** Expression of TSP 2 is significant in the reticular dermis (d) (16 day embryo). **O:** TSP 2 transcripts can be observed in differentiating skeletal muscle (sm) (16 day embryo). d, dermis; e, endothelium; h, heart; i, large intestine; lu, lumen; m, meninges, mesenchyme; pp, parietal pericardium; pv, visceral pericardium; k, kidney, r, rib; si, small intestine; sm, skeletal muscle; t, tooth; v, vessel; vi, vibrissae. Bar 100 μm.

Table 1. Expression of TSP Transcripts During Marine Development



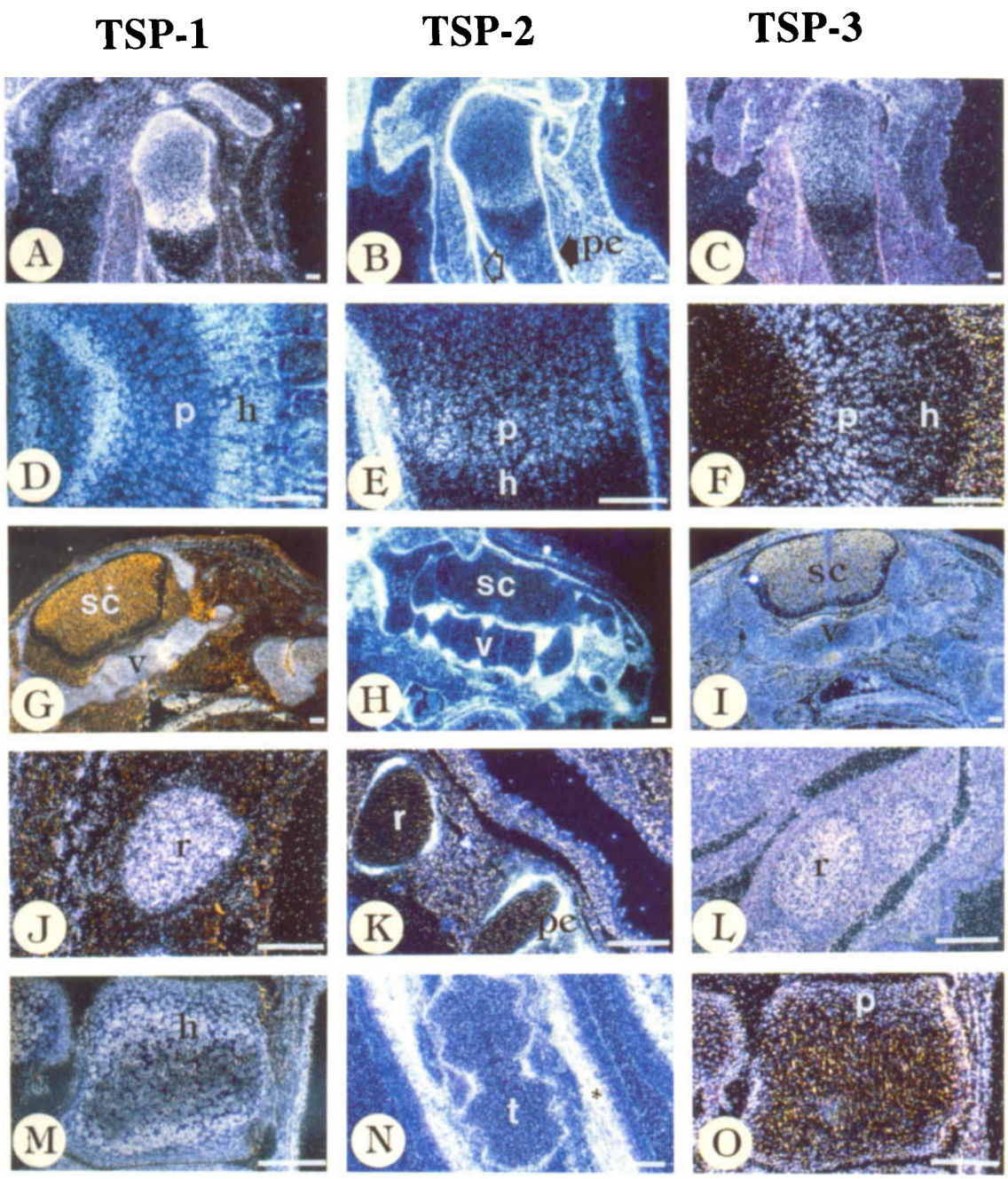


Fig. 6. Distribution of TSP 1, 2, and 3 transcripts in cartilage and bone of embryonic mice. Localization of TSP 1 (A,D,G,J,M), TSP 2 (B,E, H,K,N), and TSP 3 (C,F,I,L,O) mRNA by in situ hybridization. **A–C:** Long bone of a 18 day fetus; in B, closed and open arrows indicate signal in perichondrium (pe) and tendon, respectively. **D–F:** High magnification view of a cartilage growth plate during endochondral ossification. **G–I:** Transverse section of spinal cord (sc) and vertebrum (v) of a 17 day fetus. **J–L:** Transverse section of rib (r), 16 day embryo. **M–O:** Tail (t) of a 17 day fetus. h, hypertrophic zone; p, proliferating zone; pe, perichondrium; r, rib; sc, spinal cord; t, tail; v, vertebrum; asterisk, dense connective tissue. Bar, 100 μm.

entiated chondrocytes (Fig. 6I,L,O). In the limb, the highest levels of expression for TSP 1, 2, and 3 were correlated with overt chondrogenesis, i.e., days 14–16 (Fig. 3A). Analysis of relative steady-state mRNA levels between TSP 1 and 2 revealed that TSP 1 was 5-fold increased over TSP 2, although both transcripts exhibited the highest levels at day 16 (Fig. 4A). A comparison of the temporal expression of the three TSP mRNAs is presented in Table 1.

In the growth plate, a precise temporal and spatial control of proliferation, differentiation, migration, and matrix synthesis occurs. Here the expression of TSP transcripts differed. TSP 1 mRNA was expressed to a high degree in the resting and hypertrophic zones (Fig.

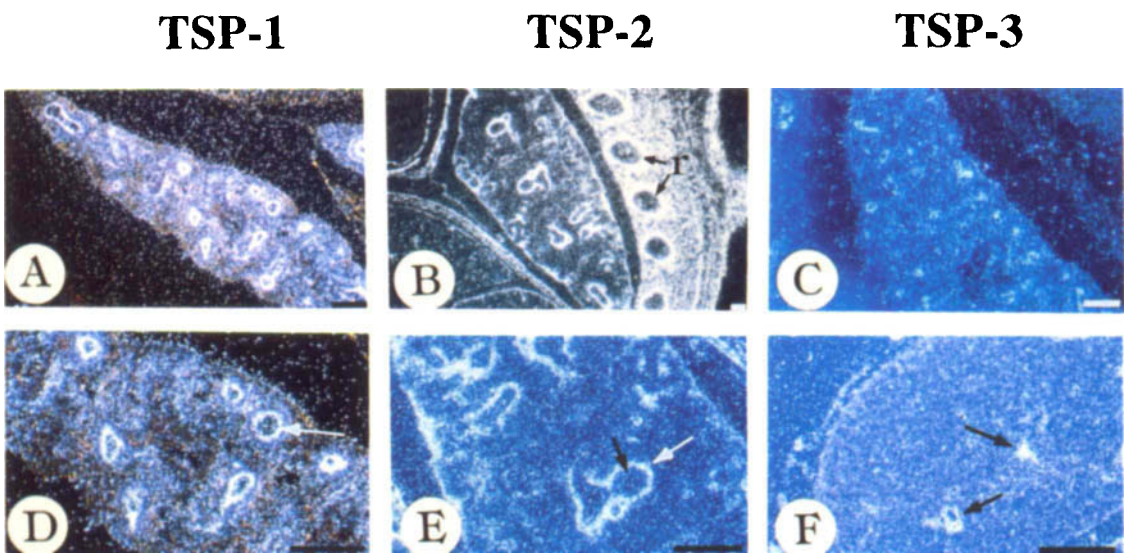


Fig. 7. Expression of TSP 1, TSP 2, and TSP 3 in developing lung. Distribution of TSP 1 (A and D), TSP 2 (B and E), and TSP 3 (C and F) by in situ hybridization of embryonic and fetal mouse tissue. **A:** Transverse section of a 14 day lung. **B:** Sagittal section of the thoracic region of a 16 day embryo. Hybridization signal is detected in connective tissue, e.g. the perichondrium of the ribs (arrows), visceral and parietal pleura, and in the mucosa of the developing bronchi. **C:** Sagittal section of a 17 day fetal lung. Hybridization signal is present in small bronchi. **D:** High magnification view of a sagittal section of a 15 day embryonic lung. Arrow indicates hybridization signal in the epithelium of the bronchi. **E:** High magnification of panel B. Signal is concentrated in the connective tissue that surrounds the bronchi (white arrow); unlike TSP 1, TSP 2 is not present in the epithelium (black arrow). **F:** In the 18 day lung, TSP 3 mRNA was detected in terminal bronchi and alveoli (arrows). Bar, 100 μm.

6A,D), whereas low levels of TSP 2 were detected in the late proliferative zone (Fig. 6B,E), and TSP 3 transcripts were observed in the early proliferative zone (Fig. 6C,F).

Most of the osteoblasts associated with newly-deposited osteoid exhibited a strong signal for TSP 1 transcripts (Fig. 6A). In contrast, low levels of hybridization for TSP 2 (Fig. 6B) and TSP 3 (Fig. 6C) mRNAs were observed in these cells.

Lung. In the mouse, the lung first develops as an endodermal evagination of the foregut (days 8 and 9) and thereafter proceeds by repetitive monochotomous and dichotomous branching during the glandular stage (9 to 16 days) (Ten Have-Opbroek, 1981). TSP 1 was detected in the lung initially at day 14 (Table 1) and was restricted to the columnar epithelium that lines the presumptive bronchial tree (Fig. 7A,D). This expression was greatest on days 15 and 16 and persisted until day 18. At the beginning of the canalicular phase, the embryonic lung is invaded by capillaries (day 16). Expression of TSP 2 mRNA was observed during this period (Fig. 7B,E, Table 1). High levels of TSP 2 remained until day 18 in cells beneath the epithelium of bronchi and bronchioles. Due to limits in the resolution of the light microscope, we could not determine whether the expression of TSP 2 in the mesenchyme around the epithelial branches was exclusive to vessels. The last phase of lung development, the alveolar stage (after day 17), is characterized by the formation of numerous alveoli and an increase in the number of blood vessels. Only at this point was TSP 3 detected in the epithelia of small alveolar spaces (Fig. 7C,F). Table 1 illustrates the expression of each TSP during lung morphogenesis.

Brain. Although TSP 1 mRNA was abundant on days 10–12 in neuroepithelial cells (Fig. 2D), this level of expression diminished abruptly and was absent by day 13. Similar results were obtained by RNAse protection assay. High levels of expression were detected on days 11–12 (Figs. 3B, 4B), with a significant decrease thereafter. A similar pattern was seen with TSP 2 mRNA (Figs. 3B, 4B). In contrast, TSP 3 was not detected in the brain until day 17, when low levels of transcripts were observed in the fetal hippocampus (data not shown). Table 1 shows a temporal comparison of TSP transcripts in the brain. This table, however, does not depict the oscillations in the expression of each TSP throughout development. After birth, expression of all three TSP genes in the brain was generally enhanced, particularly that of TSP 3. Although the three genes were apparently transcribed at similar times, a pattern of cell-specific expression was evident for each TSP transcript. These results indicate that the TSP mRNAs are transcribed independently by selected populations of neurons.

An interesting pattern of TSP expression was observed in the olfactory bulb. TSP 1 was present in a subset of neurons from the ventricular olfactory bulb (Fig. 8D,G), whereas no signal above background was observed for TSP 2 (Fig. 8E,H). Strong signal reflecting TSP 3 mRNA was localized to the accessory olfactory bulb (Fig. 8F,I).

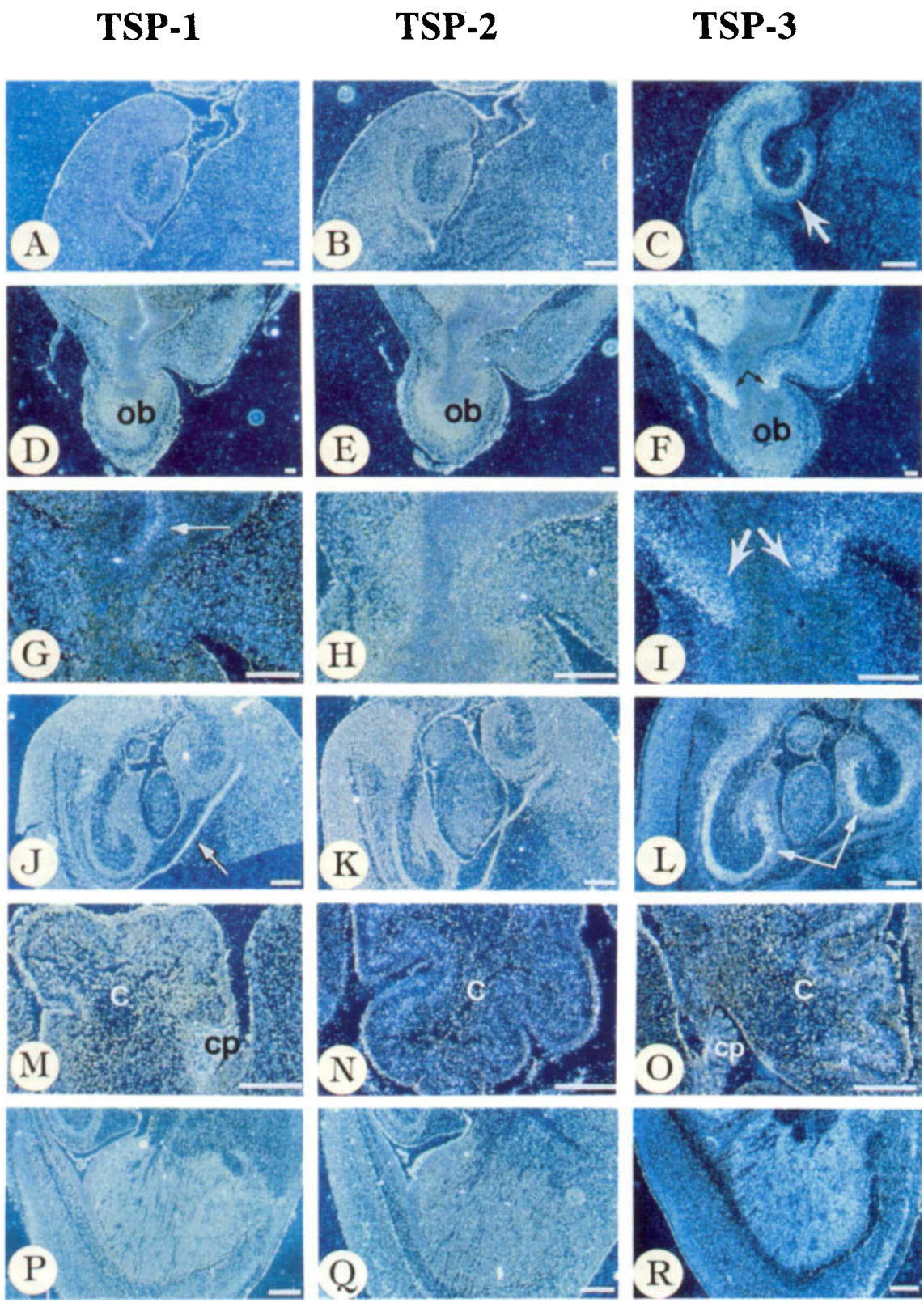


Fig. 8. Expression of TSP 1, TSP 2, and TSP 3 in the neonatal murine brain. Localization of transcripts for TSP 1 (A,D,G,J,M,P), TSP 2 (B,E-,H,K,N,Q), and TSP 3 (C,F,I,L,O,R) in sagittal sections of neonatal brain by in situ hybridization. **A–C:** Dentate gyrus hilus is indicated by arrow in C. **D–F:** Sagittal section of the olfactory bulb (ob), small arrows in F indicate signal in the accessory olfactory bulb. **G–I:** Higher magnification view of panels D–F; arrows indicate areas of hybridization with the respective probes. **J–L:** Hippocampus; arrow in J indicates hybridization in connective tissue and arrows in L indicate hybridization in the hippocampus. **M–O:** Sagittal sections of the cerebellum (c); in M and O, the choroid plexus (cp) is also present. **P–R:** Section through the diencephalon. c, cerebellum; cp, choroid plexus; ob, olfactory bulb2. Bar, 100 μm.

Although low levels of TSP 1 mRNA were present in the cerebellum, transcripts could be identified in the choroid plexus (Fig. 8M). In contrast, TSP 2 transcripts were observed primarily in granular cells (Fig. 8N). TSP 3 mRNA was also abundant in the granular layer of the developing cerebellum (Fig. 8O). These observations are consistent with previous findings of TSP protein in the cerebellum by immunocytochemistry (O'Shea et al., 1990b). A hybridization signal unique to TSP 3 was detected in the hippocampus (Fig. 8C,L) and in the diencephalon (Fig. 8R); background levels of radioactivity were observed in sections of the same region hybridized with the anti-sense TSP 1 or 2 probe (Fig. 8A,B,P,Q).

DISCUSSION

In addition to its well-established role in platelet aggregation, several functions have been attributed to the extracellular glycoprotein TSP. TSP has been reported both to stimulate and inhibit cell growth in a variety of cell types (Majack et al., 1986; Bagavandoss and Wilks, 1990), to promote cell attachment, to dissociate focal contacts (Murphy-Ullrich and Höök, 1989), and to inhibit angiogenesis (Good et al., 1990; Iruela-Arispe et al., 1991). In the past, these multiple and diverse effects on cell behavior have been attributed to one or more of the structural domains present in TSP, or to the presence of several proposed receptors (Roberts et al., 1987; Lawler et al., 1988; Kosfeld et al., 1991; Yabkowitz and Dixit, 1991; Prater et al., 1991; Guo et al., 1992; Leung et al., 1992). Although these elements could account for the multiple activities, the existence of similar TSP genes brings yet another source of complexity into the analysis of TSP function. Because early studies were conducted prior to the discovery of other TSP genes, it is now necessary to clarify which TSP is produced by a specific cell type and which protein is responsible for a particular activity.

To characterize the pattern of expression of TSPs during development and to provide insight into cell-specific functions, we have performed an analysis of TSP 1, 2, and 3 transcripts in the mouse embryo from days 10–18 and in the neonatal brain. Results indicate that each TSP transcript has a temporally and spatially independent distribution. In some instances, the expression of all three TSP genes was induced concomitantly with the differentiation of specific tissues.

Comparison of the primary structures of the TSPs revealed that several of the domains characteristic of platelet-derived TSP (TSP 1) have been conserved in TSP 2 and TSP 3 (Bornstein, 1992). All three proteins share the type III repeats (Ca^{2+}-binding) and all contain at least 3 type II (EGF-like) repeats. TSP 1 and TSP 2 are more similar to each other than each is to TSP 3. TSP 3 lacks the procollagen and the type I (TSP or properdin) repeats, which are common to both TSP 1 and TSP 2. Given the high degree of conservation in many of the structural domains found in TSP 1 and 2, one might initially predict that these proteins would share similarities in function or could even organize into heterotrimers, particularly since two cysteine residues (Cys 252 and Cys 256 in TSP-1) responsible for disulfide linkage are conserved in TSP 1 and 2. Indeed, formation of TSP 1-TSP 2 heterotrimers has been reported to occur in cultured Swiss 3T3 cells (O'Rourke et al., 1992). During the development of the mouse embryo, however, two points argue against a predominance of heterotrimeric chains: 1) If indeed heterotrimeric forms of TSP 1 and 2 formed a major part of the synthetic repertoire of cells, one could expect that the distribution of transcripts for TSP 1 and 2 would overlap and not be as clearly distinguishable as observed in this study, and 2) Although the organization of heterotrimeric forms of TSP protein could occur in the extracellular space, the clear temporal differences in expression of TSP 1 and 2 (Table 1) would argue against this possibility. In the adult, however, formation of heterotrimers cannot be excluded in cases of spatio-temporal co-expression.

Transcripts for TSP 1, 2, and 3 were expressed in the lung, although their tissue distribution and time of expression differed significantly. TSP 1 transcripts were restricted to the epithelium of large bronchi, whereas TSP 2 was localized in the connective tissue of the same bronchi. TSP 3 was not detected in the lung until day 17, when it was expressed in small terminal bronchioles and alveoli, but never in larger elements of the respiratory tree. Distinct patterns of expression were also seen in the brain, in which transcripts for all three genes were expressed in different areas by what appeared to be distinct cell types.

In cartilage, all three TSPs were expressed by chondrocytes. However, levels of transcripts appeared to change with the stage of proliferation or differentiation. Significant levels of TSP 1 mRNA were associated with condensing mesenchyme prior to overt chondrogenic differentiation. TSP 1 transcripts were low in proliferating chondrocytes but high in hypertrophic cells. In contrast, TSP 2 mRNA was only detected at low levels in proliferating chondrocytes and at significant levels in the perichondrium, and its chondrogenic layer, whereas TSP 3 was observed only in differentiated chondrocytes. Therefore, it appears that, in chondrocytes, the expression of each TSP gene is under unique spatio-temporal regulation. The coincidental expression of all three genes by the same cell type, however, makes chondrocytes an attractive system to study the regulation of TSPs. As a correlate, TSPs could become important markers of chondrocyte differentiation. The presence of TSP in cartilage has been previously reported in the adult cartilage of the intervertebral disc and meniscus (McDevitt and Webber, 1990; Miller and McDevitt, 1991; Hedbom et al., 1992) and by O'Shea and Dixit (1988) in embryonic chondrocytes. Comparison of the pattern of expression between our results and those described by earlier studies suggests that the latter were probably detecting a mixture of TSP 1, 2, and 3.

Expression of TSP 1 and TSP 2 has also been described in vitro during the differentiation of MC3T3 osteoblasts (Sherbina and Bornstein, 1992). In that study, a substantial difference in the levels of TSP 1 and 2 transcripts was observed during the process of osteoblast differentiation. TSP 1 mRNA levels increased, whereas TSP 2 levels remained constant. Our analysis of TSP 1 expression in developing bone appears to support these conclusions. Strong hybridization signal for TSP 1 was detected in differentiated osteoblasts; in contrast, these cells exhibited low levels of TSP 2 and 3.

Although TSP 1 is a secreted protein, its pattern of mRNA expression was not typical of other extracellular matrix genes. Constitutive expression of TSP 1 mRNA was observed in megakaryocytes. Transcripts were initially identified in circulating megakaryocytes of the early embryo, subsequently in resident megakaryocytes of the embryonic and fetal liver, and finally in the bone marrow and spleen of the adult mouse. These data corroborate previous results which reported the presence of TSP in adult megakaryocytes (Jaffe et al., 1985; Beckstead et al., 1986). Expression in megakaryocytes was unique to TSP 1, since neither TSP 2 nor TSP 3 was identified in this cell type. The presence of TSP 3 transcripts was also poorly correlated with sites of extensive ECM deposition. TSP 3 was abundant in the brain of the late fetus and neonatal mouse at a time when less proliferation and pronounced differentiation and migration occurs. Conversely, the pattern of TSP 2 expression seemed more typical of an extracellular matrix protein. Strong expression was associated with the differentiation of the connective tissue sheaths that occurs from day 14–16 in all organs (Table 1).

During development and wound repair, TSP 1 has often been described as a protein associated with areas in which cells are actively undergoing proliferation and migration. In general, expression of TSP 1 has been thought to decline with differentiation. However, in addition to its synthesis by megakaryocytes, TSP 1 appears to be expressed at very high levels by nonproliferative chondrocytes and osteocytes, and occasionally, by endothelial cells of large blood vessels during later stages of embryogenesis (Sherbina and Bornstein, 1992; O'Shea and Dixit 1988; present report). In contrast, TSP 2 is expressed by endothelial cells during the early stages of vessel formation, in areas of fibroblast proliferation, and by proliferating chondrocytes. Indeed an overview of data from previous studies and from this report indicates that TSP 2 is more likely associated with proliferation and migration than TSP 1. Although the primary structures of TSP 1 and 2 are largely conserved, our data provide strong evidence that functional differences between the two proteins might be significant.

An important difference between TSP 1 and TSP 2 is the regulation of these transcripts by growth factors. Indeed, significant variability is observed when the TSP 1 and 2 promoters are compared (Bornstein et al., 1991a). The two genes respond differently to stimulation by serum and PDGF (Bornstein et al., 1991b; Laherty et al., 1992; Framson and Bornstein, 1993). Differences in the response of individual genes to growth factors might be reflected in the expression of these genes during development, a period when tight regulation by a combination of growth stimulators and inhibitors occurs. Indeed, our in situ hybridization analysis revealed differences in the temporal expression of TSP 1, 2, and 3. Transcripts for TSP 1 were observed in the earliest embryos (day 10) examined. In contrast, the onset of TSP 2 expression occurred concomitantly with the organization of connective tissue after day 13. TSP 3 was not detectable until day 15, in cartilage (Table 1).

Comparison of our data obtained by in situ hybridization with those of previous reports on the immunolocalization of TSP protein (Wight et al., 1985; O'Shea and Dixit, 1988; O'Shea et al., 1990a and 1990b) suggests that those studies identified all three TSPs. However, immunolocalization data obtained with a monoclonal antibody directed against the amino-terminal, heparin-binding domain of TSP 1 (Corless et al., 1992) shows a greater degree of similarity to the findings described here for the localization of TSP 1 transcripts. It is interesting that the expression of TSP 1 overlaps considerably with the expression reported for TGF-β1. This growth factor has been described in a variety of murine tissues in which we have also detected TSP 1: the osteogenic growth plate (Thorp et al., 1992), brain during early development (Flanders et al., 1989), megakaryocytes and developing kidney (Ellingsworth et al., 1986), blood vessels (Wilcox and Derynck, 1988), and connective tissue, cartilage, bone, hair follicles, teeth, and cardiac cushions (Heine et al., 1987). This pattern was more similar to TSP 1 than to the other two TSPs. It is also peculiar that in the adult, as with TGF-β1 (Assoian et al., 1983; Seyedin et al., 1985), the level of TSP 1 in bone is only exceded by that in platelets. A recent report has demonstrated high-affinity binding of platelet TSP (TSP 1) to TGF-β1 (Murphy-Ullrich et al., 1992). Although the biological significance of this interaction is not yet clear, it has been suggested that the binding might protect TGF-β1 from inactivation in the extracellular space, and/or that TSP could act as a modulator of TGF-β1 activity. In any case, the co-expression of TSP 1 and TGF-β1 mRNA suggests that both genes could share common regulatory features. This regulation would be of considerable interest if TSP 1 acted as a carrier for TGF-β1 (Murphy-Ullrich et al., 1992).

We have reported significant differences in the cellular and temporal synthesis of gene transcripts for three TSP genes, a result that indicates diverse and independent functions for these proteins during development. These studies set the stage for future investigations which will expand our current understanding of the developmentally regulated, cell-specific regula-

tory mechanisms that control the expression and function of the TSP genes. In this regard, the development of chain-specific antibodies will greatly improve our ability to trace the location and understand the function of these related gene products.

EXPERIMENTAL PROCEDURES

Animals and Preparation of Tissue

Swiss-Webster females were mated with males and checked daily for a vaginal plug, the appearance of which represented day 0. Females were sacrificed daily between 10 and 18 days after conception. For RNA extraction, heart, liver, anterior limbs, and brain were dissected in sterile Dulbecco's modified Eagle's medium with the aid of a dissecting microscope. For in situ hybridization studies, embryos were fixed by immersion for 12–16 hours in 3% paraformaldehyde in 0.1 M sodium phosphate (pH7.6) containing 1% sucrose at 4°C. Embryos of 15–18 days were sectioned sagittally to ensure appropriate fixation. The age of the embryos was confirmed by morphological characteristics according to Rafferty (1970). Embryos were embedded in paraffin and sagittal sections of 5 μm were mounted on Probe-on-slides™ (Fisher, PA).

Construction of Riboprobe Vectors and Probe Synthesis

To avoid the possibility of cross-hybridization between TSPs, regions of low homology were selected to generate transcript-specific probes. Riboprobe vectors were constructed by standard recombinant techniques, as follows: A) a 294 bp *Bgl*II/*Pst*I mouse TSP 1 cDNA, generated by polymerase chain reaction (PCR), was cloned into pBSM13$^+$ in an antisense orientation relative to the T3 promoter; B) a 1.3 kb *Eco*RI/*Eco*RI human TSP 1 cDNA fragment was cloned in an antisense orientation relative to the SP6 promoter into the vector pGEM2; C) a 514 bp *Pst*I/*Pst*I mouse TSP 2 cDNA, generated by PCR, was cloned into the vector pBS M13$^+$ in an antisense orientation relative to the T7 promoter; D) a 1550 bp *Eco*RI/*Eco*RI mouse TSP 3 cDNA, isolated from a murine lung library, was cloned into pBSK$^-$ in an antisense orientation relative to the T7 promoter; E) a 680 bp *Sal*I/*Sal*I mouse TSP 3 cDNA, generated by PCR, was cloned into pBS M13$^+$ in an antisense orientation relative to the T7 promoter, and F) a 147 bp *Alu*I/*Alu*I fragment of TSP 3 was cloned into pBSM13$^+$ in an antisense orientation relative to the T7 promoter. Figure 1 illustrates the location of these probes in relation to a schematic representation of the protein sequence of each of the three TSPs.

Riboprobes were labeled with dUTP α-[^{35}S] (Amersham, IL) and the specific RNA polymerase (Promega, WI). The specific activity of the probes ranged from 80 Ci/mmol to 180 Ci/mmol (approximately 10^9 cpm/μg). Prior to use in hybridization reactions, probes larger than 200 bp were partially hydrolyzed by the procedure of Cox et al. (1984).

RNA Extraction and RNase-Protection Assays

RNA was isolated by the procedure described by Chomczynski and Sacchi (1987), as modified by Puissant and Houdebine (1990). Total RNA was resuspended in DEPC-treated water and was monitored on agarose gels. RNase protection assays were performed as previously described (Liska et al., 1990). Specific riboprobes are as follows: for mouse TSP 1 (MTSP1), a 272 bp genomic fragment (nucleotides +3916 to +4148) that protects 181 bp of exon 5 (Bornstein et al., 1990); for mouse TSP 2 (MTSP2), a 267 bp genomic fragment that protects 142 bp of exon 5 (Bornstein et al., 1991a); for mouse TSP 3 (MTSP3), a genomic fragment that protects 147 bp of exon 22 (Vos et al., 1992). Antisense and sense probes for MTSP1 were prepared with the T3 and T7 RNA polymerases, respectively. Antisense and sense probes to MTSP2 and MTSP3 were prepared with the T7 and T3 RNA polymerases, respectively. A riboprobe that detects L32 was used to normalize RNA in the assays (Dudov and Perry, 1984). RNAse protection assays were repeated with RNA isolated from 2–4 different litters.

Quantitation was achieved by hybridization of the antisense riboprobes to known amounts of sense RNA to generate a standard curve. Autoradiographic analyses were performed by the Phosphorimager Facility of the Markey Molecular Medicine Center at the University of Washington (Seattle, WA).

In Situ Hybridization

In situ hybridization was performed as described by Holland et al. (1987) with slight modifications. Briefly, paraffin sections were hydrated, fixed in 4% paraformaldehyde (pH 7.4) for 5 minutes, washed in PBS, and digested with 20 μg/ml of proteinase K for 5 minutes. Sections were washed and post-fixed in 4% paraformaldehyde (pH 7.4). Subsequently, sections were immersed for 10 minutes in 0.1 M triethanolamine (pH 8.0) containing 0.25% acetic anhydride, rinsed in DEPC-treated water, and dried. Sections were then treated for 1–4 hours at room temperature in prehybridization solution (0.6 M NaCl, 0.12 M Tris-HCl (pH 8.0), 0.008 M EDTA, 0.02% Ficoll 400, 0.02% polyvinylpyrrolidone, 0.1% BSA, 500 μg/ml salmon sperm DNA, 600 μg/ml yeast total RNA, 50% deionized formamide). Hybridization was performed at 53°C for 12–16 hours in an humidified chamber containing filter paper saturated with 4 × SSC and 50% formamide (hybridization solution is equivalent to the prehybridization solution containing 10% dextran sulfate, 0.1% SDS, 10 mM DTT, and 3×10^6 cpm- [S^{35}]-labeled probe/2 slides). The sections were washed with formamide buffer (50% formamide, 4 × SSC, and 5 mM DTT) for 30 minutes at 53°C, and were incubated 30 minutes in 3.5 × SSC at room temperature. The slides were incubated with 20 μg/ml of RNAse A in 3.5 × SSC for 30 minutes, and were subsequently washed at high stringency in 0.1 × SSC for 2 hours at 65°C. The high

stringency hybridization and washing conditions resulted in low background. Subsequently, the sections were dehydrated in a graded series of ethanol/water mixtures containing 0.3 M ammonium acetate. The sections were air-dried, coated with NTB2 nuclear emulsion (Eastman Kodak Co, NY), and exposed in light-excluded chambers at 4°C for 2–4 weeks. After development, the sections were counterstained with 1% toluidine blue.

To ensure appropriate sampling, embryos from each developmental stage were collected from at least three females and subjected to in situ hybridization. In the case of TSP 3, four independent litters were analyzed. In addition to sections from mice of embryonic and fetal ages, serial sections of neonatal mouse brain were used to examine the distribution of the TSP transcripts.

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