

## The in situ localization of tenascin splice variants and thrombospondin 2 mRNA in the avian embryo

Richard P. Tucker

Department of Neurobiology and Anatomy, Neuroscience Program, Bowman Gray School of Medicine, Wake Forest University, Winston-Salem, NC 27157-1010, USA

### SUMMARY

Tenascin and thrombospondin belong to the growing family of extracellular matrix glycoproteins believed to have an anti-adhesive function during development. Immunohistochemistry has been used to identify these proteins in the developing central nervous system, in the matrix surrounding peripheral neurons, and in connective tissue. The antibodies used in most of these studies, however, could not distinguish between different splice variants (tenascin) nor different genetic forms (thrombospondin). For this reason, we used the reverse transcriptase polymerase chain reaction to generate DNA probes that are specific to the transcripts of high  $M_r$  tenascin and thrombospondin 2. These probes were then used for an in situ hybridization study to determine the cellular origins of specific tenascin and thrombospondin forms throughout the development of the chick. The mRNA encoding high  $M_r$  tenascin was found associated with motile cells and in tissues undergoing dynamic modeling: migrating glia, epithelial glia used as a substratum for migrating neurons, the growing tips of lung

buds, and during osteogenesis. In contrast, the mRNAs of low  $M_r$  tenascin were concentrated in areas of cartilage deposition and chondrocyte proliferation. Thrombospondin 2 mRNA was not detected in the developing central nervous system at any time during development by in situ hybridization. In contrast, it was found in embryonic mesenchyme, perichondrium, epimysium, and endothelial cells. Thrombospondin 2 mRNA was detected in poly(A) RNA isolated from embryonic spinal cord and cerebellum by polymerase chain reaction, though it was not detected in poly(A) RNA from the avascular retina. Thus, thrombospondin 2 mRNA may be present in the developing brain at low levels in endothelial cells or blood cells. These data support the notion that tenascin splice variants have distinct roles during development, and that thrombospondin 2 is more likely to be playing a role associated with the morphogenesis of connective tissue than neuronal development.

Key words: in situ hybridization, tenascin, thrombospondin

### INTRODUCTION

Tenascin and thrombospondin are multimeric glycoproteins with restricted distributions in the embryonic extracellular matrix (for review see Chiquet-Ehrismann, 1991; Erickson and Bourdon, 1989; Frazier, 1991; Sage and Bornstein, 1991). Immunohistochemistry has revealed that tenascin and thrombospondin are particularly abundant in the developing central and peripheral nervous systems as well as in areas of connective tissue morphogenesis (Grumet et al., 1985; Mackie et al., 1987; Mackie et al., 1988; O'Shea and Dixit, 1988). In vitro studies have demonstrated that these glycoproteins provide a favorable substratum for promoting neurite outgrowth and the motility of a variety of cell types (Chuong et al., 1987; Halfter et al., 1989; Wehrle and Chiquet, 1990; O'Shea et al., 1991; Neugebauer et al., 1991). As some cells fail to form focal adhesions on tenascin and thrombospondin, and the addition of these glycoproteins can cause cells that have already spread on adhesive glycoproteins to lose their strong attachments (Chiquet-

Ehrismann et al., 1988; Spring et al., 1989; Halfter et al., 1989; Lotz et al., 1989; Murphy and Höök, 1989; Murphy-Ullrich et al., 1991), it is generally believed that both tenascin and thrombospondin may promote cell motility by acting as anti-adhesive extracellular matrix components.

Three alternative splice variants of tenascin have been identified in the chick. The largest ( $M_r$   $230 \times 10^3$ ) splice variant has 11 fibronectin-type III repeats, whereas the smaller forms ( $M_r$   $200 \times 10^3$  and  $190 \times 10^3$ ) have 9 or 8. Immunohistochemistry with splice variant-specific antibodies has shown that the high  $M_r$  form of tenascin is found in a subset of the matrix stained with tenascin antibodies that recognize all tenascin forms (Matsuoka et al., 1990; Kaplony et al., 1991), and high  $M_r$  tenascin was shown to be less resistant to proteolysis than the low  $M_r$  variants (Chiquet et al., 1991). These data indicate that different tenascin variants may have distinct functions and turnover rates in the embryonic extracellular matrix.

In contrast to tenascin, which is encoded by a single gene, different forms of thrombospondin are encoded by two or

more closely related genes. The different gene products have very similar putative functional domains and can share up to 76% of their amino acid sequence (LaBell et al., 1992). The best characterized of these, thrombospondin 1 and thrombospondin 2, have been shown by northern blot analysis of murine tissues to have distinct distributions and patterns of developmental regulation (Laherty et al., 1992). For example, thrombospondin 2 mRNA was more abundant than thrombospondin 1 mRNA in developing mouse brain, whereas thrombospondin 1 mRNA was relatively abundant in embryonic liver.

We have used the reverse transcriptase polymerase chain reaction (PCR) to generate probes to high  $M_r$  tenascin and chick thrombospondin 2 mRNA. These probes were used to identify the transcripts encoding these glycoproteins in the developing avian embryo by in situ hybridization. The mRNA of high  $M_r$  tenascin was found in a subset of the tissues expressing tenascin, including glia and regions of osteogenesis. In contrast, thrombospondin 2 mRNA could not be detected in the central nervous system by in situ hybridization, though it was abundant in embryonic mesenchyme condensing to form cartilage as well as endothelial cells. Thus, it is unlikely that thrombospondin 2 is playing a major role in neural morphogenesis. These data indicate that different tenascin splice variants and different thrombospondin forms may have distinct roles in embryogenesis.

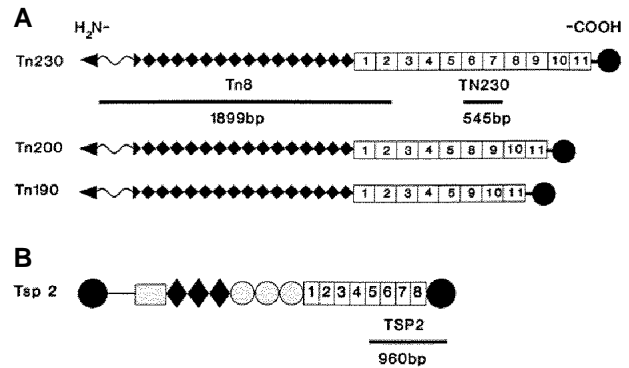
## MATERIALS AND METHODS

### PCR cloning of tenascin and thrombospondin 2

Reverse transcriptase PCR was used to generate high  $M_r$  tenascin and thrombospondin 2 cDNAs. Total RNA was made from confluent cultures of the chick fibroblast cell line SL-29 using methods detailed by Ausubel et al. (1988). This RNA was copied into DNA using a reverse transcriptase PCR kit following instructions provided by the manufacturer (Perkin Elmer Cetus). Sense and anti-sense oligonucleotide primers were designed to generate either a 545 bp PCR product spanning the 6th and 7th fibronectin-type III repeats of chick tenascin (Spring et al., 1989) or a 960 bp PCR product corresponding to the carboxy terminus of chick thrombospondin 2 (amino acid residues 834 to 1154, Lawler et al., 1991; see Fig. 1A,B). Although the part of thrombospondin corresponding to the clone is highly conserved at the amino acid level between different thrombospondin forms in mouse (81%; Laherty et al., 1992), at the nucleic acid level there is significant divergence that ensures specific hybridization to chick thrombospondin 2 mRNA. PCR products were analyzed by agarose gel electrophoresis and restriction mapping. Both the high  $M_r$  tenascin probe (TN230) and the thrombospondin 2 probe (TSP2) were the predicted size and were cut at the predicted sites by *EcoRI*, *HindIII*, and *BglI*.

### Probe labelling and in situ hybridization

Whole chick embryos (Hubbard Farms) or appropriate, freshly dissected body regions were fixed on embryonic day (E) 3, 5, 7, 10, 14, or 18 by immersion in ice-cold 4% paraformaldehyde in phosphate-buffered saline (PBS) for periods ranging from 2 hours (E3) to overnight (E18). Specimens were then rinsed in PBS, cryoprotected overnight in 25% sucrose in PBS, then rapidly frozen in OCT compound. Serial 12- $\mu$ m frozen sections were collected on poly-L-lysine-subbed slides and processed for in situ hybridization using techniques detailed previously (Tucker and McKay, 1991).



**Fig. 1.** Schematic representations of chick tenascin (A) and chick thrombospondin 2 (B) showing the relationship of the probes used for in situ hybridization to each molecule. cTn8 spans the portion of tenascin conserved in each of the 3 known avian splice variants. TN230 corresponds to the 6th and 7th fibronectin type III repeats, which are unique to high  $M_r$  tenascin (Tn230). TSP2 hybridizes to the sequence encoding the 5th through 8th thrombospondin type III repeats as well as most of the globular terminal domain.

In brief, thawed sections were rinsed in a prehybridization solution ( $5 \times$  SSC,  $5 \times$  Denhardt's, 100  $\mu$ g/ml salmon sperm DNA, 20 mM  $\beta$ -mercaptoethanol; Sigma) for 1 hour and dehydrated in ethanol. Probes (including the 1899 bp chick tenascin cDNA cTn8, a generous gift from Dr R. Chiquet-Ehrismann) were purified with GeneClean II (Bio101), labelled with  $^{35}$ S-dCTP using a random primer-based kit (Promega), and the unincorporated nucleotides were removed with a Sephadex G-50 mini-spin column (Worthington). 20  $\mu$ l of hybridization buffer (prehybridization buffer with 50% deionized formamide, 20 mM Tris, 0.1% lauryl sulfate and 1% dextran sulfate) with  $5 \times 10^6$  cts/minute of boiled probe was then added to each section and incubated overnight at 42°C. A similarly purified and labelled 955 bp double-stranded pUC19 *TaqI/Sau3AI* restriction fragment was used to control for spurious hybridization. Following incubation with the probe, the slides were rinsed in  $1 \times$  SSC both at room temperature and at 42°C. To account for differences in the size and concentration of the tenascin cDNA probes, autoradiographic exposures were adjusted so that the density of silver grains in the E14 spinal cord following hybridization with cTn8 and TN230 were similar (see below). The spinal cord was chosen to normalize exposures because immunoblots revealed high levels of high  $M_r$  tenascin relative to smaller forms between E10 and E18 (Tucker, 1991b). Thus, most of the tenascin transcripts in the E14 spinal cord should encode high  $M_r$  tenascin (i.e. TN230 and cTn8 signals would be nearly identical in the spinal cord if it was possible to give the two probes identical hybridization properties). Amersham Hyperfilm- was exposed to the slides for 2 (cTn8) to 5 (TN230, TSP2 and the control) days for low-resolution analysis of the hybridization pattern. The slides were then dipped in Kodak NTB2 nuclear track emulsion and exposed for 3-7 days.

In order to compare differences in tenascin mRNA concentrations in neuronal (spinal cord) and connective (vertebral body) tissue with a given probe, as well as assist in normalizing exposures, silver grains were counted in one or more 9900  $\mu$ m<sup>2</sup> areas using an image processing system (NIH Image). For simplicity, densities are expressed as grains per 100  $\mu$ m<sup>2</sup>. The entire E10 ependymal layer fell within one sampling region. However, it was possible to sample both E14 ventral funiculi, as well as up to 4 regions within each vertebral body. Statistical differences were calculated using a Student's *t* test.

## Immunohistochemistry

Sections adjacent to those processed for in situ hybridization were stained with the mouse monoclonal tenascin antibody M1 (Chiquet and Fambrough, 1984), with secondary antibody alone as a control, or with hematoxylin and eosin to observe general tissue organization. Some sections were also stained with alkaline phosphatase (Sigma) to identify bone spicules. Methods for immunohistochemistry have been detailed previously (e.g. Tucker, 1991a). Secondary antibodies were tagged either with TRITC (rabbit anti-mouse IgG; Sigma) or with horseradish peroxidase (goat anti-mouse IgG; BioRad) and colored with chloronaphthol as chromogen. All slides (dark-field, bright-field illuminated, and immunofluorescence) were photographed with a Nikon Labophot microscope.

## Reverse transcriptase PCR to identify mRNAs in tissues

To identify the transcripts of high  $M_r$  tenascin and thrombospondin 2 in tissues, poly(A) RNA was made from E14 spinal cord, neural retina, and cerebellum using the Micro FastTrack kit (Invitrogen). 400 ng of poly(A) RNA from each sample was used for reverse transcriptase PCR (see above) using the high  $M_r$  tenascin and thrombospondin 2 oligonucleotide primers. PCR products were analyzed and compared with TN230 and TSP2 from SL-29 cell total RNA by slab gel electrophoresis.

## RESULTS

### Embryonic day 3 to embryonic day 7

The earliest stage examined by in situ hybridization was E3. In cross sections through the trunk at axial levels just caudal to the anterior to posterior wave of neural crest cell migration there was no significant hybridization with cTn8, TN230 or TSP2 (Fig. 2A-C) over background levels (not shown). There was no anti-tenascin staining in adjacent sections (not shown). When sections through the E3 head were incubated with anti-tenascin, staining was particularly prominent around the recently invaginated lens vesicle (Fig. 3A). In situ hybridization revealed tenascin transcript within the lens vesicle at points near the edge of the optic cup. Both cTn8 and TN230 hybridized in this region (Fig. 3B,C), but the former probe, which recognizes the mRNAs of both high and low  $M_r$  tenascin, generated a more robust signal. The TSP2 hybridization signal in the E3 lens vesicle and central nervous system (Fig. 3D) was not significantly different from background hybridization (not shown).

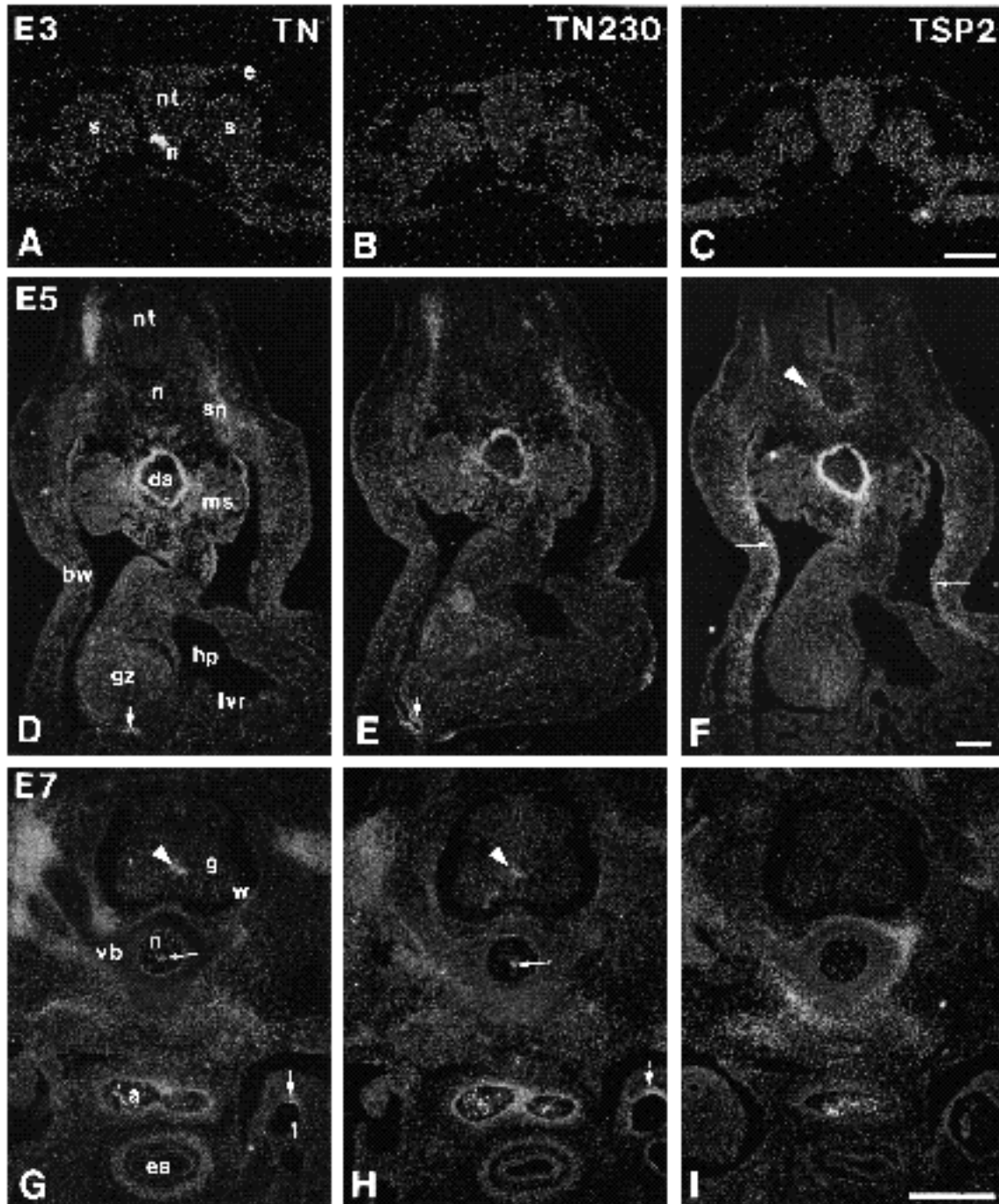
In contrast to E3, a strong signal was seen in cross sections through the mid-trunk region with all 3 probes at E5 (Fig. 2D-F). The strongest signal was found in the endothelial cells of the dorsal aorta, which were labelled with cTn8, TN230 and particularly intensely by TSP2. The cTn8 and TN230 probes gave similar staining patterns at this age, indicating that the high  $M_r$  splice variant of tenascin is found in many tissues at the outset of their morphogenesis. The strongest signals were found in spinal nerves, the most medial mesenchyme of the mesonephric kidney, cells scattered throughout the gizzard and the mesoderm-derived lining of the liver at sites where lobes are forming. In addition to the endothelial cells of the dorsal aorta, TSP2 hybridized within mesenchyme surrounding the notochord and mesenchyme in the medial portion of the body wall. The neural tube was unlabelled by all 3 probes.

At E7 the distribution of the mRNA encoding high  $M_r$  tenascin is still similar to the pattern of hybridization seen with the universal tenascin probe (Fig. 2G,H). Both hybridized within the smooth muscle cells of the dorsal aorta and, somewhat more weakly, within the smooth muscle cells of the esophagus. A subset of cells within the notochord were also labelled. Hybridization with TN230 and cTn8 was particularly intense in the endoderm-derived epithelium at the growing tip of lung bronchioles, as well as within a limited portion of the spinal cord ependymal layer just dorsal to the ventral floor plate. By E7, TSP2 no longer hybridized in dorsal aorta endothelium (Fig. 2I). Lung, smooth muscle, and the spinal cord were also unlabelled. The strongest hybridization was seen in mesenchymal cells surrounding the notochord participating in vertebral morphogenesis.

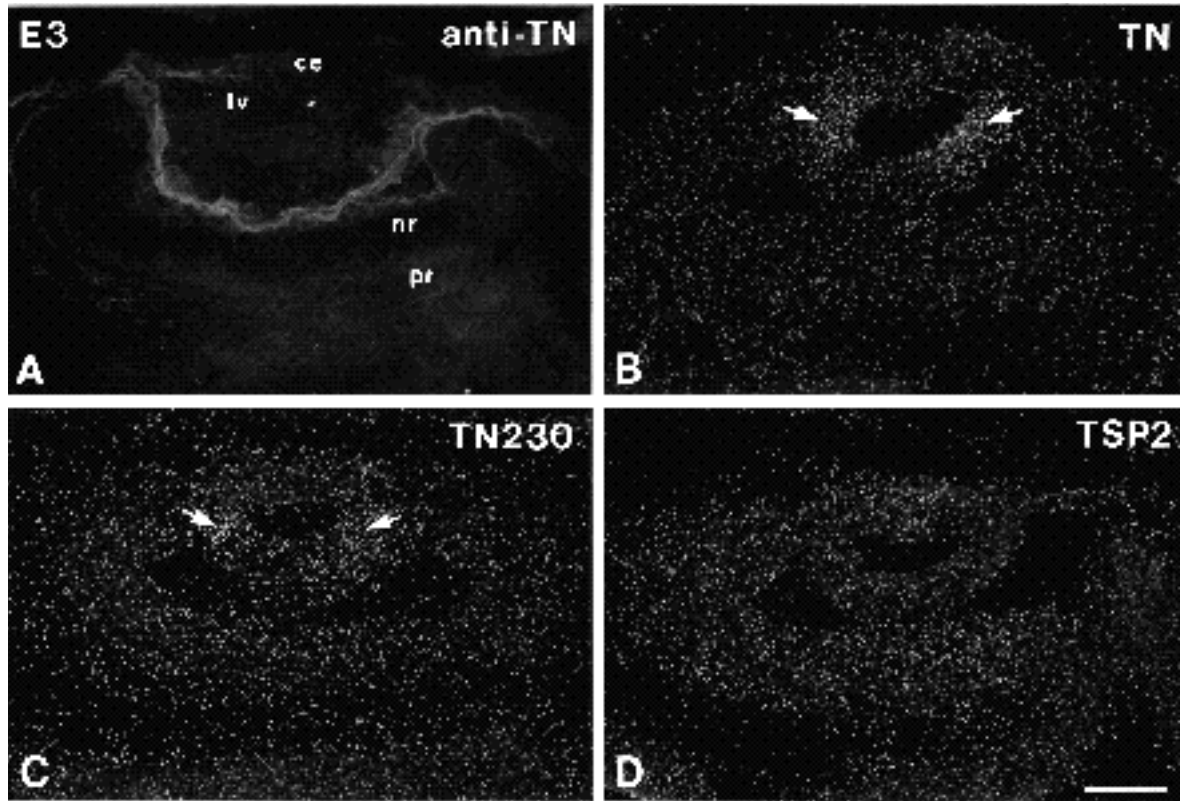
### Embryonic day 10 to embryonic day 18

In older and larger embryos X-ray film overlays were useful for studying the crude distribution of transcripts as well as changes in the relative intensity of hybridization over time (Fig. 4). At E10, cTn8 hybridizes within areas undergoing chondrogenesis, the medial and ventral spinal cord and the proventriculus (crop; Fig. 4A). TN230 hybridizes within the same regions as cTn8, but when the film exposure is adjusted so the intensity of the signal within the spinal cord with both tenascin probes is similar (see Materials and methods), it is clear that the mRNA of high  $M_r$  tenascin is concentrated in the developing central nervous system (Fig. 4B). The hybridization signal with TSP2 at E10 is intense and restricted to endothelial cells and condensing mesenchyme (Fig. 4C). By E14 the differences in the signals with cTn8 and TN230 are even more pronounced (Fig. 4E,F). Low  $M_r$  tenascin transcripts are abundant in tissues surrounding the cartilaginous vertebrae. The mRNA of high  $M_r$  tenascin is found at the tips of vertebral laminae in regions of bone spicule development (see below), as well as in spinal cord white matter. Like low  $M_r$  tenascin mRNAs, thrombospondin transcripts are abundant near areas of chondrogenesis (Fig. 4G). TSP2 also hybridizes strongly within the epimysium, the connective tissue sheath surrounding groups of muscles. At E18 the hybridization signals are similar to those seen at E14: transcripts encoding low  $M_r$  tenascin are associated with the margins of cartilaginous tissues, whereas the mRNA of high  $M_r$  tenascin is found in spinal cord white matter and regions undergoing osteogenesis (Fig. 4I,J). The TSP2 signal is much weaker at E18 than at earlier ages, and is limited to the lining of the cartilaginous, but not ossified, vertebrae. No signal could be detected in striated muscle with tenascin or thrombospondin 2 probes at any time in development, nor was thrombospondin 2 detected in the spinal cord at any time from E3 to E18. pUC was used to show background hybridization (Fig. 4D,H,L).

The distribution of tenascin and thrombospondin 2 transcripts were also studied following autoradiography, and compared to adjacent sections incubated with tenascin monoclonal antibodies or histological stains (Fig. 5). In the E10 chick trunk at the level of the brachial plexus tenascin immunoreactivity is found in the ventral spinal cord, tendons and perichondrium (Fig. 5B). Background hybridiz-



**Fig. 2.** Dark-field micrographs of frozen sections through the trunks of E3 (A-C), E5 (D-F) and E7 (G-I) chick embryos following in situ hybridization with cTn8 (TN; A,D,G), the high  $M_r$  tenascin mRNA-specific probe (TN230; B,E,H), or TSP2 (C,F,I). No signals were detectable in the mid-trunk at E3 with any of the probes. At E5, each of the probes hybridized strongly within dorsal aorta endothelial cells. Both tenascin probes hybridized within the mesodermal portion of the liver (arrow in D,E), in spinal nerves, and in kidney mesenchyme. TSP2 hybridized within mesenchyme surrounding the notochord (arrowhead) and in mesenchyme lining the body wall (small arrows in F). At E7, the tenascin probes hybridized within a portion of the spinal cord ependymal layer just dorsal to the ventral floor plate (arrowhead in G,H). A subset of notochord cells (small arrows in G,H) and the epithelial lining of growing bronchioles (arrows in G,H) were also strongly labelled. The tenascin probes and TSP2 hybridized in different sets of mesenchymal cells associated with the vertebrae chondrogenesis. Abbreviations: a, aortic arch; bw, body wall; da, dorsal aorta; e, ectoderm; es, esophagus; g, grey matter; gz, gizzard; hp, hepatic portal; l, lung; lvr, liver; ms, mesonephric kidney; n, notochord; nt, neural tube; s, somite; sn, spinal nerve; vb, vertebral body; w, white matter. Bar, 100  $\mu$ m (A-C; D-F); 500  $\mu$ m (G-I).

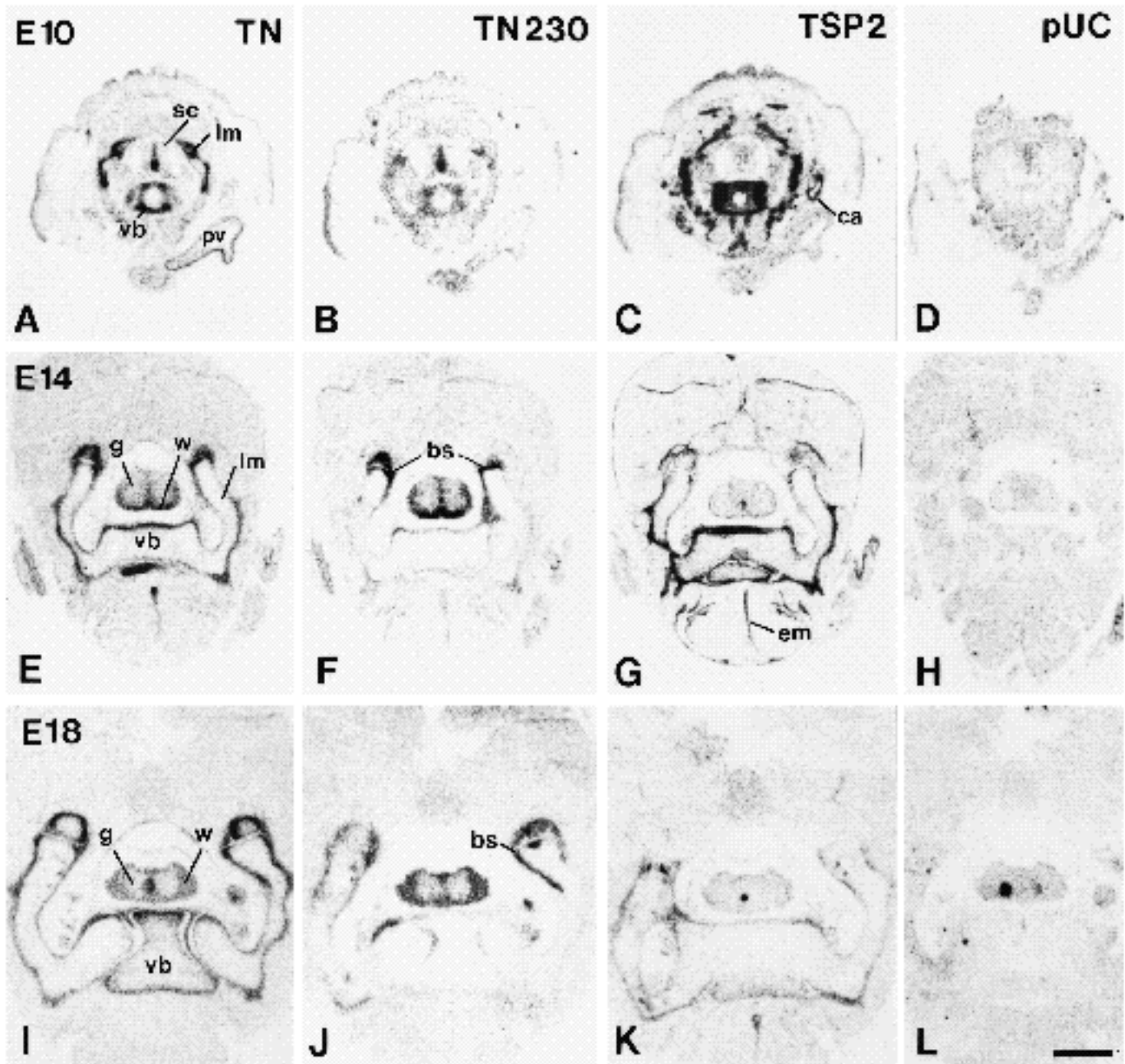


**Fig. 3.** Frontal sections through the E3 retina and lens vesicle following anti-tenascin staining (A) and in situ hybridization with cTn8 (B), TN230 (C) and TSP2 (D). Tenascin immunoreactivity is seen surrounding the lens vesicle, especially between the retina and the lens vesicle. Tenascin mRNA is concentrated within the lens vesicle vis-à-vis the margins of the optic cup (arrows in B,C). No TSP2 mRNA could be detected in the lens or retina at E3. Abbreviations: ce, corneal epithelium; lv, lens vesicle; nr, neural retina; pr, pigmented retina. Bar, 100  $\mu$ m.

ation with pUC was minimal at this age (Fig. 5C). Within the spinal cord, the pattern of hybridization with cTn8 and TN230 were indistinguishable: both probes generated a strong signal within the ependymal layer (but not the floor plate) and within cells in the ventral horn and ventral funiculus. This indicates that high  $M_r$  tenascin is being synthesized by a subpopulation of proliferating and migrating spinal cord glia (Tucker and McKay, 1991), and is consistent with previous anti-tenascin western blots of E10 chick spinal cord homogenates (Tucker, 1991b). No thrombospondin 2 mRNA could be detected in adjacent E10 spinal cord sections (Fig. 5F). In the vertebral body, all three probes hybridized in distinct patterns. Low  $M_r$  tenascin mRNAs (i.e. areas where cTn8 hybridizes, but not TN230) are concentrated in areas undergoing chondrogenic differentiation, but not in hypertrophic cartilage adjacent to the notochord. High  $M_r$  tenascin mRNA is detected only at the outermost margin of the developing vertebral body. Similarly, the TSP2 signal is intense at the outermost margins of the E10 vertebral body, although TSP2 also hybridizes in areas of precartilage mesenchyme undergoing the earliest stages of condensation. Both TN230 and TSP2 hybridized within notochord cells at E10. Another region of overlap in high  $M_r$  tenascin and thrombospondin 2 expression is in the endothelial cells of major blood vessels (Fig. 6A-C): cTn8, TN230, and TSP2 all hybridized within the endothelial cells of the carotid

artery. The TSP2 hybridization signal was also intense in 'spots' scattered between the carotid artery and the vertebral body (arrows in Fig. 6C). The cells responsible for this signal are unknown, but the distribution of small capillaries in nearby sections stained with hematoxylin and eosin is similar. cTn8 also hybridizes intensely in smooth muscle cells surrounding the proventriculus. A somewhat weaker but reproducible signal was seen in the endoderm-derived lining of the proventriculus. TN230 hybridized in these same regions, but the signal was weaker than that seen with cTn8 when the autoradiographic exposure was normalized to make the signals with the two probes in the spinal cord indistinguishable (see Fig. 5D,E). No signals were seen in spinal or cranial nerves at E10 with any of the probes, demonstrating the transient nature of tenascin expression in Schwann cells.

At E14 the mRNA of high  $M_r$  tenascin is still readily detectable in spinal cord glia and in a subset of the regions of the developing vertebrae hybridized with cTn8 (Fig. 5G,H). Transcripts encoding low  $M_r$  tenascin are found in the thin, innermost proliferative portion of the perichondrium, whereas the mRNA of high  $M_r$  tenascin is seen in regions where bone spicules are first appearing (these were identified on adjacent sections stained with hematoxylin and eosin or alkaline phosphatase) and in the inner layer of perichondrium at sites where tendons and ligaments are attach-

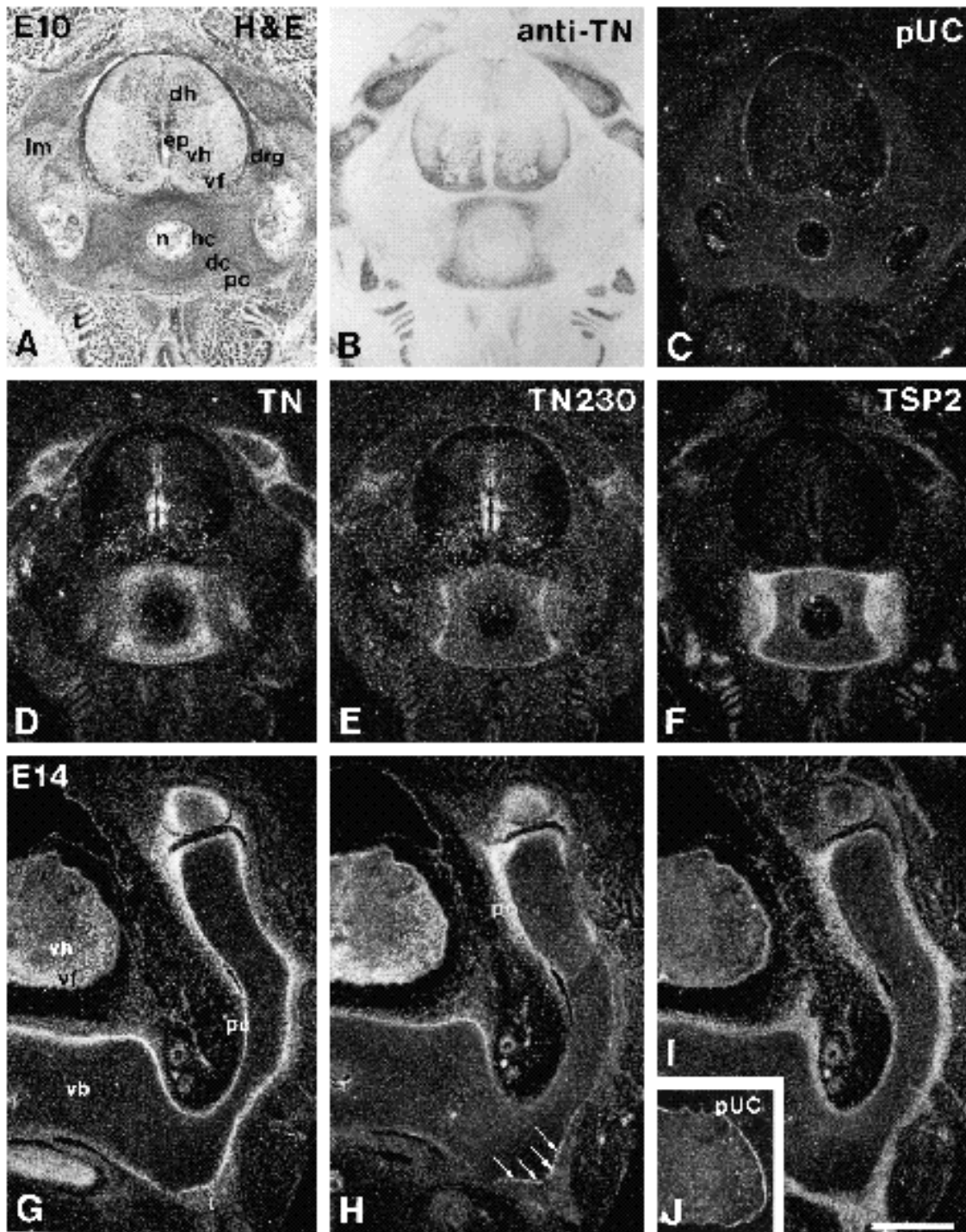


**Fig. 4.** X-ray film images of cross sections through the brachial spinal cord, axial skeleton and musculature of E10 (A-D), E14 (E-H) and E18 (I-L) chick embryos following hybridization with cTn8 (TN; A,E,I), TN230 (B,F,J), TSP2 (C,G,K) or the pUC control (D,H,L). Tenascin mRNA is found in chondrogenic mesenchyme, perichondrium, the lining of the proventriculus and in spinal cord white matter. High  $M_r$  tenascin transcripts are concentrated in regions of osteogenesis and in spinal cord white matter. The TSP2 hybridization signal is intense in the lining of the carotid artery at E10, and is associated with chondrogenesis and the perichondrium, but not osteogenesis. Thrombospondin 2 mRNA is also found in the epimysium, the connective tissue that ensheathes muscles. Abbreviations: bs, bone spicules; ca, carotid artery; em, epimysium; g, grey matter; lm, vertebral lamina; pv, proventriculus; sc, spinal cord; vb, vertebral body; w, white matter. Bar, 1 mm.

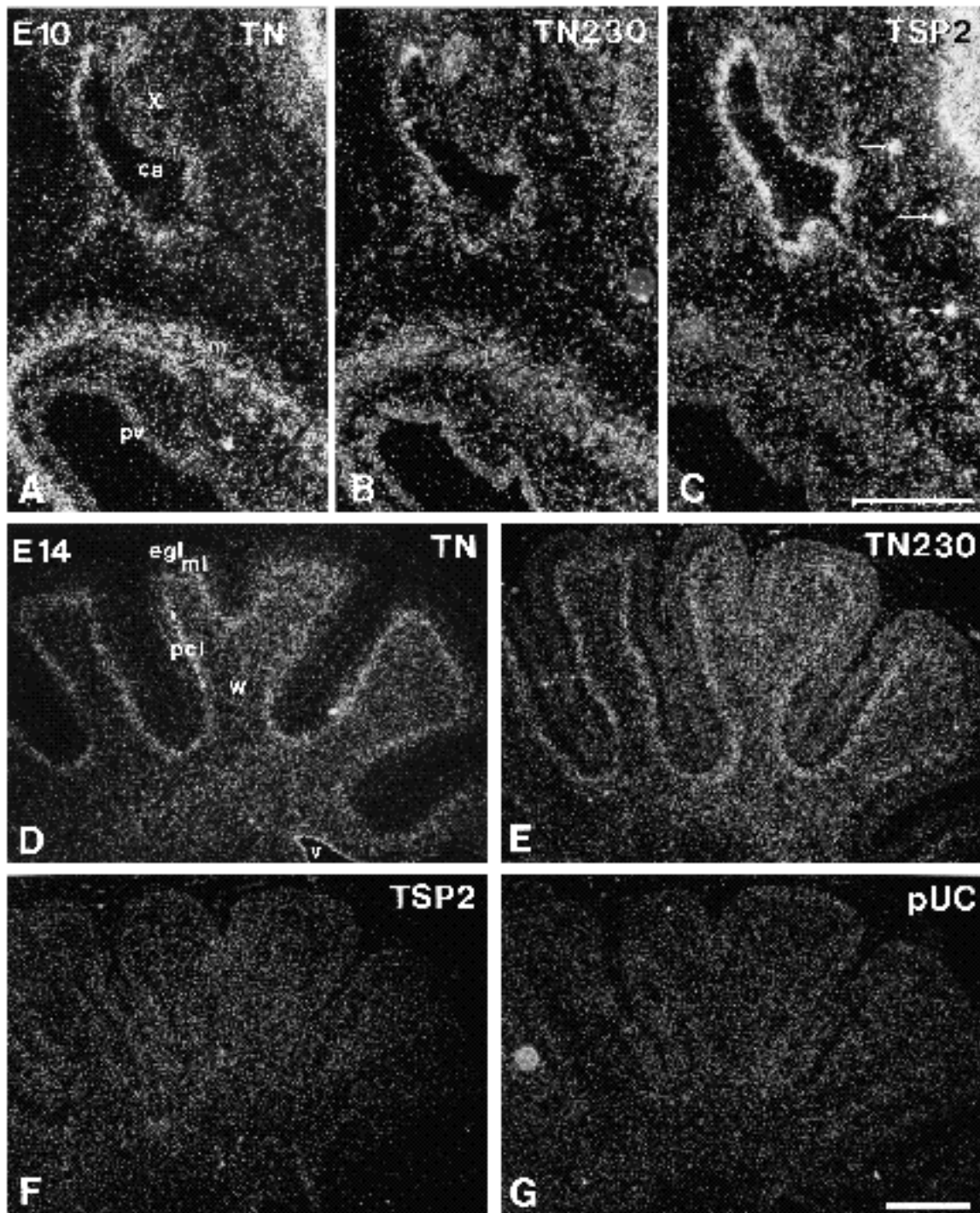
ing to the vertebrae. Thrombospondin 2 mRNA was detected in the loose mesenchymal portion of the perichondrium that ensheathes the cartilaginous vertebrae. TSP2 also hybridized in ligaments, tendons and epimysium (Fig. 5I). TSP2 hybridization in the E14 spinal cord was indistinguishable from background levels (Fig. 5I,J).

Silver grains were counted using an image processing system to compare the concentrations of tenascin transcripts in neuronal and non-neuronal tissues (Table 1). Following in situ hybridization with cTn8, there was less than a 2-fold difference between the density of silver grains over the ependymal layer and the vertebral body at E10. In contrast,





**Fig. 5.** Cross sections through the E10 (A-F) and E14 (G-J) brachial spinal cord and axial skeleton stained with hematoxylin and eosin (A), anti-tenascin (B), or hybridized with pUC (C,J), cTn8 (TN; D,G), TN230 (E,H) or TSP2 (F,I). At E10, the cTn8 hybridization signal is similar to staining with anti-tenascin: cells within the ventral spinal cord, ependymal layer, and in regions of chondrogenesis are labelled. High  $M_r$  tenascin mRNA is more abundant in the spinal cord than in developing connective tissue. Thrombospondin 2 transcripts, in contrast, are abundant at the margins of chondrogenic tissues and cannot be detected in the spinal cord. At E14 tenascin transcripts are concentrated in the glia of the ventral funiculus and in the thin, innermost layer of the perichondrium. High  $M_r$  tenascin mRNA is found in periosteum (bone spicules were identified in adjacent sections stained with hematoxylin and eosin or alkaline phosphatase), and in perichondrium at regions where tendons insert on the vertebra (thin arrows in H). Thrombospondin 2 mRNA is concentrated in tendons and in the thicker, outermost mesenchymal perichondrium at E14. Hybridization with TSP2 in the E14 spinal cord is indistinguishable from pUC. Abbreviations: dc, differentiated cartilage; dh, dorsal horn; drg, dorsal root ganglion; ep, ependymal layer; hc, hypertrophic cartilage; Im, vertebral lamina; n, notochord; pc, perichondrium; po, periosteum; t, tendons; vb, vertebral body; vf, ventral funiculus; vh, ventral horn. Bar, 500  $\mu$ m.



**Fig. 6.** Dark-field micrographs of adjacent cross sections through the E10 chick trunk (A-C) and adjacent mid-sagittal sections through the E14 chick cerebellum (D-G) following in situ hybridization with cTn8 (TN), TN230, TSP2, or pUC19 control DNA (pUC). cTn8, TN230 and TSP2 hybridize within carotid artery endothelial cells. Smooth muscle cells that surround the proventriculus are labelled by both cTn8 and TN230, and are unlabelled by TSP2. There is an intense hybridization signal with TSP2 in small, unidentified groups of cells near the carotid artery (arrows, C). The hybridization signals with cTn8 and TN230 are similar in the E14 cerebellum. Cells within the Purkinje cell layer (but not the Purkinje cells themselves, which could be identified by their characteristic nuclei), glia scattered in the cerebellar white matter and cells lining the ventricle are labelled. TSP2 hybridization was indistinguishable from the pUC19 control. Abbreviations: ca, carotid artery; egl, external granular layer; ml, molecular layer; pcl, Purkinje cell layer; pv, proventriculus; sm, smooth muscle; w, white matter; X, vagus nerve. Bar, 250  $\mu$ m (A-C); 500  $\mu$ m (D-G).



**Table 1. Silver grain densities in neuronal and non-neuronal tissues**

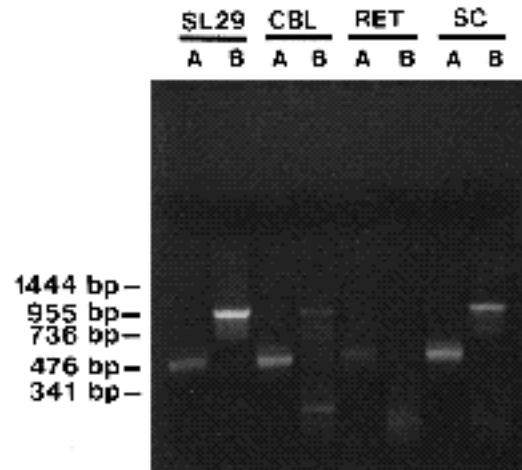
Age and tissue ( <i>n</i> )	cTn8	TN230	pUC
E10 spinal cord ependymal layer (1)	89*	41	9
E10 vertebral body (4)	56	6	2
E14 spinal cord ventral funiculus (2)	72±2	77±8	5
E14 vertebral body perichondrium (4)	84±12	28±13	4

\*Numbers indicate silver grains per 100 μm<sup>2</sup>. Where appropriate this number is the mean of multiple regions (±s.d.). The number of regions sampled in a given tissue is indicated by (*n*).

there was a 7-fold difference in the density of silver grains in the ependymal layer and vertebral body following hybridization with TN230. Similar differences were seen at E14, when an increased area of tenascin expression within the spinal cord (the ventral funiculi) permitted enough samples to be counted for statistical comparison. There was a significant difference ( $P < 0.01$ ) in silver grain densities in the spinal cord ventral white matter compared to the vertebral body perichondrium following hybridization with TN230, but silver grain densities in these regions were similar ( $P > 0.1$ ) following hybridization with cTn8.

As others have shown that thrombospondin 2 mRNA appears transiently in the developing mouse brain (Laherty et al., 1992), and anti-thrombospondin stains the developing mouse cerebellum (O'Shea et al., 1990), probes were hybridized to mid-sagittal sections through the E14 cerebellum (Fig. 6D-G); a time when neurite outgrowth, neuroblast proliferation, and neuronal cell body migration are taking place. Both cTn8 and TN230 hybridized within Bergmann glial cells, the cell bodies of which are interspersed with the large perikarya of Purkinje cells (identified by counterstaining the sections with the fluorescent Hoechst nuclear dye), and other glia scattered throughout cerebellum white matter. Thrombospondin 2 mRNA, in contrast, was not detected by in situ hybridization in the E14 cerebellum.

Thrombospondin 2 mRNA could be present in the developing chick central nervous system at levels below the detection limits of in situ hybridization. To test this possibility, poly(A) RNA from E14 spinal cord, cerebellum and retina was used as the source of template for reverse transcriptase PCR with the TN230 and TSP2 oligonucleotide primers. Resulting PCR products were compared with TN230 and TSP2 from SL-29 cell total RNA, the source of RNA used to construct probes for in situ hybridization (Fig. 7). The 545 bp TN230 PCR product was readily detectable in samples derived from spinal cord or cerebellum poly(A) RNA. TN230 was detectable, albeit at lower levels, in retina poly(A) RNA-derived samples as well. This crude quantification of the mRNA encoding high  $M_r$  tenascin in different central nervous system regions reflects the relative abundance of tenascin in these regions as previously determined by western blotting (Tucker, 1991b). The 960 bp TSP2 product was seen in the spinal cord and cerebellum preparations, but not in the retina. Two funda-



**Fig. 7.** Reverse transcriptase PCR with TN230 oligonucleotide primers (A) and TSP2 oligonucleotide primers (B) using total RNA from SL-29 chick embryo fibroblasts or the same amount of poly(A) RNA from E14 cerebellum (CBL), retina (RET) and spinal cord (SC). The 545 bp TN230 PCR product and 960 bp TSP2 PCR product are clearly seen in the samples derived from SL-29 total RNA. TN230 was amplified from each of the RNA samples. In contrast, TSP2 was amplified from the cerebellum and spinal cord poly(A) RNA preparations, but not from the retina poly(A) RNA sample. The bands found near the bottom of the lanes with cerebellum and retina TSP2 reactions are unincorporated primers.

mental differences in the cellular organization of the retina and the other brain regions could account for the absence of thrombospondin 2 mRNA in the E14 retina: the avian retina is avascular and lacks astrocytes. Thus, thrombospondin 2 mRNA may be present in the developing central nervous system at levels below the limits of in situ hybridization detection in endothelial cells, blood cells or astrocytes.

## DISCUSSION

In previous studies we and others used cTn8 (Pearson et al., 1988), a chick tenascin cDNA that hybridizes to all identified tenascin variants, to identify total tenascin mRNA in the developing embryo (Tucker 1991a,b,c; Tucker and McKay, 1991; Koch et al., 1991; Wehrle-Haller et al., 1991). Here, we used a novel probe, TN230, that specifically hybridizes to the transcript of  $230 \times 10^3 M_r$  tenascin. Regions where the mRNAs of the low  $M_r$  variants of tenascin ( $M_r$   $200 \times 10^3$  and  $190 \times 10^3$ ) are concentrated was determined indirectly by comparing the cTn8 and TN230 hybridization patterns (i.e. areas where cTn8 hybridizes, but not TN230, are enriched for the low  $M_r$  splice variants). This is different from the approach used in a previous study (Prieto et al., 1990) where a cDNA probe that recognized the mRNAs of all forms of tenascin was compared to that of a probe that did not hybridize to the transcript encoding the lowest relative molecular mass form ( $190 \times 10^3$ ) of tenascin. We chose to study the origins of the high  $M_r$  splice variant of tenascin because recent data (Chiquet et al., 1991;

Murphy-Ullrich et al., 1991; see below) indicate possible functional differences only between  $M_r$   $230 \times 10^3$  tenascin and the smaller ( $M_r$   $200 \times 10^3$  and  $190 \times 10^3$ ) forms, and not between the smaller forms themselves.

From E3 to E7, the patterns of tenascin expression identified with the universal probe, cTn8, and the high  $M_r$  tenascin probe, TN230, were very similar. This indicates that the mRNA encoding high  $M_r$  tenascin is relatively abundant at the outset of morphogenesis. In older embryos (E10-E18) these differences became more distinct, reflecting the increased segregation of the expression of tenascin splice variants over time in specific tissues. In some cells, especially spinal cord glia, Bergmann glia, endoderm-derived epithelial cells at the growing tips of lung bronchioles, the endothelial cells of major vessels and in regions of osteogenesis, the hybridization signals with cTn8 and TN230 were still similar, indicating that the mRNA of high  $M_r$  TN was probably the most abundant species being expressed in these tissues. In other cells, the cTn8 hybridization signal was strong, but the TN230 hybridization signal was weak or absent altogether. This is particularly evident during bone and cartilage morphogenesis. At E10, low  $M_r$  tenascin mRNAs were abundant in regions of cell proliferation and cartilage deposition surrounding the notochord, whereas the transcript encoding high  $M_r$  tenascin is detected in only the peripheral-most subset of this tissue. At later embryonic stages, cTn8 hybridized intensely in the thin layer of perichondrium immediately adjacent to vertebral cartilage, where the TN230 signal was very weak or absent.

A variety of factors make it difficult to compare directly the *in situ* hybridization silver grain densities with the two tenascin probes in a quantitative fashion: minor differences in probe length (both were labelled with random primers to minimize this problem), as well as differences in probe concentration, GC/AT ratios, secondary structure, mRNA secondary structure and section thickness can effect the final density of silver grains. For this reason, we have chosen to normalize autoradiographic exposures using a region expected to have predominantly high  $M_r$  tenascin transcript: the E14 spinal cord (Tucker, 1991b). This technique leads us to conclude that in regions where cTn8, but not TN230, generates a strong hybridization signal, the low  $M_r$  tenascin mRNAs must be relatively abundant. To demonstrate this, silver grain densities were determined in different regions of the same section hybridized with a given probe. Significant differences exist between silver grain densities in the spinal cord and connective tissue following hybridization with TN230 but not cTn8. Thus, low  $M_r$  tenascin mRNAs predominate in perichondrium.

These data support the notion that the different tenascin splice variants are likely to be playing distinctive roles during embryogenesis. In most cases, high  $M_r$  tenascin is expressed in regions of dynamic tissue remodelling and cell motility (e.g. the growing tips of lung bronchioles), whereas low  $M_r$  tenascin expression is associated with cell proliferation and differentiation (e.g. the inner layer of embryonic perichondrium). This is particularly evident in the spinal cord, where high  $M_r$  tenascin is being made by migrating spinal cord glia, as well as in the cerebellum, where the cells that act as the substratum for granule cell migration,

the Bergmann glia, make high  $M_r$  tenascin. Recent studies by others support the hypothesis that high  $M_r$  tenascin may selectively promote motility. When primary cultures of chick fibroblasts are transformed with polyomavirus middle-T, the secretion of high  $M_r$  tenascin, but not low  $M_r$  tenascin, is induced (Matsuoka et al., 1990). Immunohistochemistry with a high  $M_r$  tenascin-specific antibody showed this form to be abundant in the corneal matrix in regions where cell motility was taking place (Kaplony et al., 1991). Finally, Murphy-Ullrich et al. (1991) recently showed that a recombinant tenascin fragment corresponding to the portion of high  $M_r$  human tenascin that is selectively spliced from the low  $M_r$  forms retains the ability of intact tenascin to disrupt focal adhesions *in vitro*.

Previous studies have shown that thrombospondin immunoreactivity is present throughout the embryonic murine extracellular matrix (e.g. O'Shea and Dixit, 1988). This staining was particularly intense in the developing cerebellum (O'Shea et al., 1990). As purified thrombospondin can promote spinal cord and dorsal root ganglion neurite outgrowth (O'Shea et al., 1991), as well as retinal ganglion cell neurite outgrowth (Neugebauer et al., 1991), it has been hypothesized that thrombospondin is playing an important role in neuronal morphogenesis. Recently, it has become evident that thrombospondin is encoded by multiple genes (e.g. Laherty et al., 1992; LaBell et al., 1992). Two of these, thrombospondin 1 and thrombospondin 2, were shown by northern blot analysis of developing mouse tissues to have distinct patterns of expression (Laherty et al., 1992). In blots of whole embryo total RNA, thrombospondin 2 appeared later in development than thrombospondin 1. Thrombospondin 2 was also more abundant than thrombospondin 1 in total RNA samples isolated from developing mouse brain. Our data indicate that chick thrombospondin 2 mRNA (Lawler et al., 1991; Laherty et al., 1992) is abundant at the outset of mesenchyme condensation during cartilage morphogenesis, in epimysium and in endothelial cells. The expression of thrombospondin 2 in each of these tissues is developmentally regulated and peaks between E7 and E14. In contrast, thrombospondin 2 mRNA was never detected by *in situ* hybridization in the peripheral or central nervous system. Special attention was given to the spinal cord and retina (regions where cells have been isolated by others to study thrombospondin-growth cone interactions; O'Shea et al., 1991; Neugebauer et al., 1991) as well as the cerebellum (anti-thrombospondin will arrest granule cell migration in explants of cerebellar cortex; O'Shea et al., 1990). Thus, it is likely that thrombospondin 2 is playing an important role in connective tissue development, but other forms (thrombospondin 1 or other, yet uncharacterized forms) are functioning during nervous system development. It is important to recognize that thrombospondin 2 mRNA is present at levels below detection by *in situ* hybridization in the developing brain: E14 spinal cord and cerebellum (but not retina) poly(A) RNA preparations could be used to provide template for cloning TSP2. The apparent absence of thrombospondin 2 mRNA in the E14 retina provides some clues as to the source of thrombospondin 2 mRNA in spinal cord and cerebellum: the chick retina is free of vasculature, pia mater and other investing sheaths, as well as astrocytes. Although one or all

of these cells and tissues may make low-levels of thrombospondin 2, it seems likely that endothelial cells, which contain this transcript outside the nervous system, are the source of 'brain' thrombospondin 2 mRNA.

Tenascin and thrombospondin share many characteristics: they are abundant in the stroma of certain tumors and are made by tumor cell lines (e.g. Varani et al., 1987; Natali et al., 1991), they are particularly abundant in the extracellular matrix of embryonic connective tissue and brain (e.g. Mackie et al., 1988; O'Shea and Dixit, 1988) and both share properties *in vitro* that indicate they may act as anti-adhesive matrix glycoproteins that promote cell motility (e.g. Chiquet-Ehrismann et al., 1988; Murphy and Höök, 1989). Though these molecules may be found together and potentially interact with each other in the extracellular matrix, their cellular origins are distinctive and with rare exceptions non-overlapping. Moreover, the mRNA of high  $M_r$  tenascin is consistently found in regions of cell motility and dynamic tissue modelling, whereas low  $M_r$  tenascin mRNAs are found in regions of tissue condensation. Thus, the expression of tenascin splice variants and thrombospondin forms are probably regulated through distinctive mechanisms, implying unique functions for these similar glycoproteins during development.

I thank Drs J. Adams, J. Hammarback and E.J. Mackie for comments on the manuscript, and Dr R. Chiquet-Ehrismann for helpful discussions. I would also like to thank K. Anstrom, D. Jenrath, S. McKay and P. Poss for their assistance with various aspects of this project. This work was supported by a grant from the National Science Foundation (BNS-9021124) and an Academic Research Initiation Grant from the North Carolina Biotechnology Center.

## REFERENCES

- Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. and Struhl, K. (1988). *Current Protocols in Molecular Biology* New York: John Wiley & Sons.
- Chiquet-Ehrismann, R. (1991). Anti-adhesive molecules of the extracellular matrix. *Curr. Opin. Cell Biol.* **3**, 800-804.
- Chiquet-Ehrismann, R., Kalla, P., Pearson, C. A., Beck, K. and Chiquet, M. (1988). Tenascin interferes with fibronectin action. *Cell* **53**, 383-390.
- Chiquet, M. and Fambrough, D. M. (1984). Chick myotendinous antigen. I. A monoclonal antibody as a marker for tendon and muscle morphogenesis. *J. Cell Biol.* **98**, 1926-1936.
- Chiquet, M., Vrucinic-Filipi, N., Schenk, S., Beck, K. and Chiquet-Ehrismann, R. (1991). Isolation of chick tenascin variants and fragments. *Eur. J. Biochem.* **199**, 379-388.
- Chuong, C.-M., Crossin, K. L. and Edelman, G. M. (1987). Sequential expression and differential expression of multiple adhesion molecules during the formation of cerebellar cortical layers. *J. Cell Biol.* **104**, 331-342.
- Erickson, H. P. and Bourdon, M. A. (1989). Tenascin: an extracellular matrix protein prominent in specialized embryonic tissues and tumors. *Ann. Rev. Cell Biol.* **5**, 71-92.
- Frazier, W. A. (1991). Thrombospondins. *Curr. Opin. Cell Biol.* **3**, 792-799.
- Grumet, M., Hoffman, S., Crossin, K. L. and Edelman, G. M. (1985). Cytotactin, an extracellular matrix protein of neural and non-neural tissues that mediates glia-neuron interaction. *Proc. Natl. Acad. Sci. USA* **82**, 8075-8079.
- Halfter, W., Chiquet-Ehrismann, R. and Tucker, R. P. (1989). The effect of tenascin and embryonic basal lamina on the behavior and morphology of neural crest cells *in vitro*. *Dev. Biol.* **132**, 14-25.
- Kaplony, A., Zimmermann, D. R., Fischer, R. W., Imhoff, B. A., Odermatt, B. F., Winterhalter, K. H. and Vaughan, L. (1991). Tenascin  $M_r$  220 000 isoform expression correlates with corneal migration. *Development* **112**, 605-614.
- Koch, M., Wehrle-Haller, B., Baumgartner, S., Spring, J., Brubacher, D. and Chiquet, M. (1991). Epithelial synthesis of tenascin at tips of growing bronchi and graded accumulation in basement membrane and mesenchyme. *Exp. Cell Res.* **194**, 297-300.
- LaBell, T. L., Milewicz, D. J. M., Distech, C. M. and Byers, P. H. (1992). Thrombospondin II: partial cDNA sequence, chromosome location, and expression of a second member of the thrombospondin gene family in humans. *Genomics* **12**, 421-429.
- Laherty, J. C., O'Rourke, K., Wolf, F. W., Katz, R., Seldin, M. F. and Dixit, V. M. (1992). Characterization of mouse thrombospondin 2 sequence and expression during cell growth and development. *J. Biol. Chem.* **267**, 3274-3281.
- Lawler, J., Duquette, M. and Ferro, P. (1991). Cloning and sequencing of chicken thrombospondin. *J. Biol. Chem.* **266**, 8039-8043.
- Lotz, M. M., Burdsal, C. A., Erickson, H. P. and McClay, D. R. (1989). Cell adhesion to fibronectin and tenascin: quantitative measurements of initial binding and subsequent strengthening response. *J. Cell Biol.* **109**, 1795-1806.
- Mackie, E. J., Thesleff, I. and Chiquet-Ehrismann, R. (1987). Tenascin is associated with chondrogenic and osteogenic differentiation *in vivo* and promotes chondrogenesis *in vitro*. *J. Cell Biol.* **105**, 2569-2579.
- Mackie, E. J., Tucker, R. P., Halfter, W., Chiquet-Ehrismann, R. and Epperlein, H. H. (1988). The distribution of tenascin coincides with pathways of neural crest cell migration. *Development* **102**, 237-250.
- Matsuoka, Y., Spring, J., Ballmer-Hofer, K., Hofer, U. and Chiquet-Ehrismann, R. (1990). Differential expression of tenascin splicing variants in the chick gizzard and in cell cultures. *Cell Diff. Dev.* **32**, 417-424.
- Murphy, J. E. and Höök, M. (1989). Thrombospondin modulates focal adhesions in endothelial cells. *J. Cell Biol.* **109**, 1309-1319.
- Murphy-Ullrich, J. E., Lightner, V. A., Aukhil, I., Yan, Y. Z., Erickson, H. P. and Höök, M. (1991). Focal adhesion integrity is downregulated by the alternatively spliced domain of human tenascin. *J. Cell Biol.* **115**, 1127-1136.
- Natali, P. G., Nicotra, M. R., Bigotti, A., Botti, C., Castellani, P., Risso, A. M. and Zardi, L. (1991). Comparative analysis of the expression of the extracellular matrix protein tenascin in normal human fetal, adult and tumor tissues. *Int. J. Cancer* **47**, 811-816.
- Neugebauer, K. M., Emmett, C. J., Venstrom, K. A. and Reichardt, L. F. (1991). Vitronectin and thrombospondin promote retinal neurite outgrowth: developmental regulation and role of integrins. *Neuron* **6**, 345-358.
- O'Shea, K. S. and Dixit, V. M. (1988). Unique distribution of the extracellular matrix component thrombospondin in the developing mouse embryo. *J. Cell Biol.* **107**, 2737-2748.
- O'Shea, K. S., Rheinheimer, J. S. T. and Dixit, V. M. (1990). Deposition and role of thrombospondin in the histogenesis of the cerebellar cortex. *J. Cell Biol.* **110**, 1275-1283.
- O'Shea, K. S., Liu, L.-H. J., Kinnunen, L. H. and Dixit, V. M. (1991). Role of the extracellular matrix protein thrombospondin in the early development of the mouse embryo. *J. Cell Biol.* **111**, 2713-2723.
- Pearson, C. A., Pearson, D., Shibahara, S., Hofsteenge, J. and Chiquet-Ehrismann, R. (1988). Tenascin: cDNA cloning and induction by TGF- $\beta$ . *EMBO J.* **7**, 2977-2981.
- Prieto, A. L., Jones, F. S., Cunningham, B. A., Crossin, K. L. and Edelman, G. M. (1990). Localization during development of alternatively spliced forms of cytotactin mRNA by *in situ* hybridization. *J. Cell Biol.* **111**, 685-698.
- Sage, E. H. and Bornstein, P. (1991). Extracellular proteins that modulate cell-matrix interactions. SPARC, tenascin, and thrombospondin. *J. Biol. Chem.* **266**, 14831-14834.
- Spring, J., Beck, K. and Chiquet-Ehrismann, R. (1989). Two contrary functions of tenascin: dissection of the active sites by recombinant tenascin fragments. *Cell* **59**, 325-334.
- Tucker, R. P. (1991a). The distribution of J1/tenascin and its transcript during the development of the avian cornea. *Differentiation* **48**, 59-66.

- Tucker, R. P.** (1991b). The distribution of tenascin and its transcript in the developing avian central nervous system. *J. Exp. Zool.* **259**, 78-91.
- Tucker, R. P.** (1991c). The sequential expression of tenascin mRNA in epithelium and mesenchyme during feather morphogenesis. *Roux's Arch. Dev. Biol.* **200**, 108-112.
- Tucker, R. P. and McKay, S. E.** (1991). The expression of tenascin by neural crest cells and glia. *Development* **112**, 1031-1039.
- Varani, J., Carey, T. E., Fligel, S. E. G., McKeever, P. E. and Dixit, V. M.** (1987). Tumor type-specific differences in cell-substrate adhesion among human tumor cell lines. *Int. J. Cancer* **39**, 397-403.
- Wehrle, B. and Chiquet, M.** (1990). Tenascin is accumulated along developing peripheral nerves and allows neurite outgrowth in vitro. *Development* **110**, 401-415.
- Wehrle-Haller, B., Koch, M., Baumgartner, S., Spring, J. and Chiquet, M.** (1991). Nerve-dependent and -independent tenascin expression in the developing chick limb bud. *Development* **112**, 627-637.

(Accepted 1 October 1992)