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Original Article

In situ hybridization with non-radioactive digoxigenin-11-UTP-labeled cRNA probes: localization of developmentally regulated mouse tenascin mRNAs

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ABSTRACT An improved method for *in situ* hybridization was developed in order to identify the tissue-specific expression of messenger RNA (mRNA) for the novel extracellular matrix glycoprotein, tenascin, during mouse development. Non-radioactive RNA probes were generated by incorporating digoxigenin-11-UTP instead of conventional isotopic labels. Hybridization of anti-sense probes to complementary mRNAs was detected by a chromogenic staining reaction catalyzed by an anti-digoxigenin antibody-alkaline phosphatase conjugate. Markedly improved enhancement of staining was achieved by expanding the complexity of probes and strictly controlling the degree of proteolytic digestion of paraformaldehyde-fixed tissue sections. Six different complementary RNA (cRNA) probes representing most of the tenascin mRNA sequence were prepared. Very weak signals were obtained after single applications of each probe, but strong specific signals were present when all six probes were mixed together. In either case, no signal was found without prior proteolytic digestion of tissue sections with proteinase K. Treatment with increasing concentrations of proteinase K initially resulted in increased sensitivity of signal detection, but extensive digestion resulted in histological sections of poor quality for light microscopy. Optimal conditions varied according to the tissue type examined. In lung, *in situ* hybridization detected tenascin mRNA in the relatively large cells lining alveolar walls adjacent to type I pneumocytes. In cerebellum, glial cells of the Purkinje cell layer contained tenascin mRNA, but Purkinje cells did not. In both cases, hybridization signals were confined to the cytoplasm of cells, and no extracellular staining was observed. This method provides a promising new tool for analysis of spatio-temporal regulation of tenascin gene expression during embryogenesis and oncogenesis.

KEY WORDS: *in situ hybridization, digoxigenin-11-UTP-labeled cRNA probes, extracellular matrix, mouse tenascin, embryogenesis*

Introduction

In situ hybridization histochemistry has become a powerful tool for analysis of spatio-temporal regulation of specific mRNAs in embryonic and adult tissues of many species. Three major reasons account for its increasing importance in cell biology research: 1) there is often a significant lag between the time of mRNA transcription and protein translation of genes during organismal development; 2) in some cases only complementary DNA (cDNA) probes are available for studies of gene expression; 3) secreted proteins such as those of the extracellular matrix (ECM) have tissue distributions that are different from those of their mRNAs. Previous methods of *in situ* hybridization employed radioactive isotopes (Brady and Finlan, 1990) that were hazardous, labor intensive, and used autoradiography rather than direct visualization of histological sections for interpretation of results. In addition, autoradiographic emulsions required long exposure times amounting to weeks or even months. Because silver grains, formed as a result of radioactive disintegrations, were located in the photographic emulsion above the plane of section of the tissue, the resolution of the technique was insufficient to localize signals within single cells. Recently, non-

Abbreviations used in this paper: mRNA, messenger RNA; cRNA, complementary RNA; cDNA, complementary DNA; ECM, extracellular matrix; DIG, digoxigenin-11-UTP; TN, tenascin; FN, fibronectin; LN, laminin; kDa, kilodalton; EGF, epidermal growth factor; FNIII repeat(s), fibronectin type III like repeat(s); kb, kilobase(s); P, postnatal day; MTN, mouse tenascin; AE, alternative exon; DEPC-ddH_2O, diethylpyrocarbonate-treated, autoclaved double-distilled water; RT, room temperature; NSS, normal sheep serum; anti-DIG-AP, anti-digoxigenin antibody-alkaline phosphatase conjugate.

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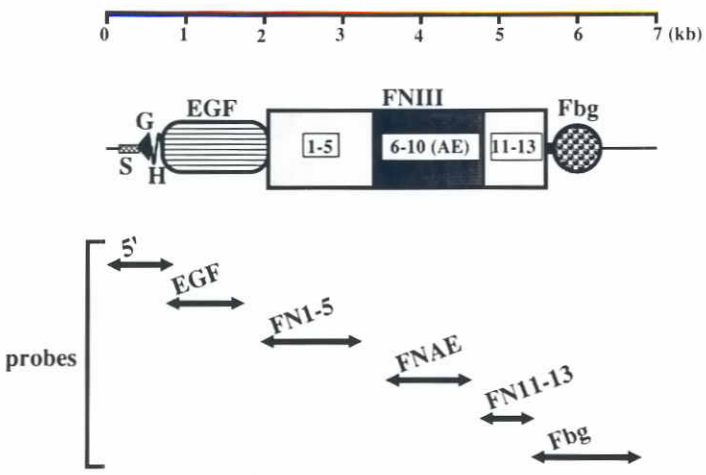


Fig. 1. Structural model of mouse tenascin and alignments of RNA probes. *TN has a signal peptide (S), a sector of central globule (G) and an a-helical structure (H) at the N-terminus. 14 1/2 EGF-like repeats (EGF) and 13 FN type III-like repeats (FNIII) follow. 6th-10th FNIII are alternatively spliced exons (AE). At the C-terminus, there is a fibrinogen homologous domain (Fbg). Thin line represents non-coding region. Digoxigenin-11-UTP-labeled cRNA probes correspond to each domain including the 5' (5' end to 1st EGF-like repeat), EGF (1st-11th EGF-like repeats), FN1-5 (1st-5th FNIII repeats), FNAE (6th-10th FNIII repeats), FN11-13 (11th-13th FNIII repeats) and Fbg (fibrinogen homologies to 3' end) domain. Six different sense and anti-sense probes were mixed and used for hybridization.*

radioactive *in situ* hybridization methods have been developed in order to circumvent these problems. These methods are comparatively rapid, simple, and make use of non-toxic chromogenic reagents for direct histochemical examination of tissues. Among several alternatives (Burns *et al.*, 1985; Nakane *et al.*, 1987; Baldino and Lewis, 1989; Hemmati-Brivanlou *et al.*, 1990), we chose a method of *in situ* hybridization employing digoxigenin-11-UTP (DIG)-labeled cRNA probes (Hemmati-Brivanlou *et al.*, 1990), which has the advantages of high sensitivity, specificity, and low background, for localization of the ECM glycoprotein, tenascin.

Tenascin (TN), is believed to play an important role as a substrate adhesion molecule for migrating cells during embryogenesis and organogenesis (Edelman, 1988). TN is deposited in the intercellular microenvironment along with other ECM components such as collagen, fibronectin (FN), laminin (LN) and proteoglycans. TN is a six-armed multidomain macromolecule composed of disulfide-linked subunits of 190-240 kDa. The primary structure of TN protein has been predicted from the nucleotide sequences of chicken, mouse, and human cDNAs. It contains signal peptides, epidermal growth factor (EGF)-like repeats, FN type III-like (FNIII) repeats, and fibrinogen homologous sequences (Fig. 1). TN mRNA isoforms of two different lengths (5.5 kilobases (kb) and 7 kb) are known to exist in the mouse, and they may be generated by alternative splicing of several FN III repeats (Saga *et al.*, 1991; Weller *et al.*, 1991). Within the FN III domain between the first five and the last three constitutive repeats, there are three additional insertional repeats in chicken (Spring *et al.*, 1989), five in mouse (Saga *et al.*, 1991; Weller *et al.*, 1991) and seven in human mRNAs (Gulcher *et al.*, 1989). TN expression normally occurs transiently during embryonic development (Chiquet and Fambrough, 1984; Chiquet-Ehrismann *et al.*, 1986; Aufderheide *et al.*, 1987; Mackie *et al.*, 1987b), and it disappears in most adult tissues. However, under some pathological conditions such as those associated with tumorigenesis (Mackie *et al.*, 1987a), wound healing (Mackie *et al.*, 1988) and regeneration of tissue (M. Kusakabe and T. Sakakura, personal communication), TN may be re-expressed in adults. Some of its developmental roles may include participation in cell adhesion and migration, epithelial shedding and renewal, epithelio-mesenchymal interface formation, organogenesis, and somatic growth regulation. Integral cell surface receptors for TN may also be involved in these diverse biological processes.

Future investigations of TN function and the regulatory mechanisms of its expression during embryogenesis and oncogenesis will require experimental methods for specific identification of TN mRNAs in various tissues. This study describes detailed methods of *in situ* hybridization with non-radioactive DIG-labeled cRNA probes that detect TN expression in single cells. Optimal conditions for demonstrating TN mRNAs in newborn mouse lung and cerebellum also were determined.

Results and Discussion

Tissue-specific expression of tenascin transcripts

TN is known to be expressed in both neural and non-neural tissues in embryonic and early postnatal development of the mouse. Before *in situ* hybridization to localize the tissue-specific expression of TN mRNAs, we examined TN mRNA levels in extracts of various tissues by Northern blotting. The results reveal the existence of two distinct mRNA isoforms with sizes of 7 kb and 5.5 kb (Fig. 2). These isoforms are thought to be generated by alternative splicing of common RNA precursor molecules (Spring *et al.*, 1989; Saga *et al.*, 1991; Weller *et al.*, 1991). In the P2 mouse, brain, skeletal muscle and stomach expressed both isoforms. Lung and kidney contained only the 7 kb isoform, thymus only the 5.5 kb form. Neither form was found in heart or liver. In brain, the major mRNA band was the larger isoform, while in the skeletal muscle and stomach, it was the smaller form. Lung and cerebellum, which showed high levels of TN mRNA expression, were selected for analysis by *in situ* hybridization.

Optimization of in situ *hybridization methodology*

The most important step of the *in situ* hybridization procedure is proteolytic digestion of tissue sections before application of the probes. Many different types of pre-treatments of sections have been tried in order to improve sensitivity and reduce non-specific background staining of *in situ* hybridization. Some of these include acid treatment, heat treatment, proteolytic digestion, and acetylation (Ingham *et al.*, 1985; Hogan *et al.*, 1986). We found that digestion of sections with proteinase K was critical for success. Sections of P2 mouse cerebellum (Figs. 3A-F) and lung (Figs. 3G-L) were subjected to different concentrations of proteinase K ranging from 0 to 63 μg/ml for 10 min at room temperature (RT). Then they were hybridized with the anti-sense probe mixture, MTN-. Intensities of the resulting hybridization signal increased in proportion to the concentration of proteinase K within the range 8 to 32 μg/ml (Figs. 3A-C, G-I). The strongest signal was obtained at 32 μg/ml (Figs. 3C,I). Above this concentration, however, proteolytic digestion resulted in excessive tissue degradation and poor quality histology, especially for cerebellum (Figs. 3D,J). Under these latter conditions, even positive signals were weak in cerebellum. Consequently,

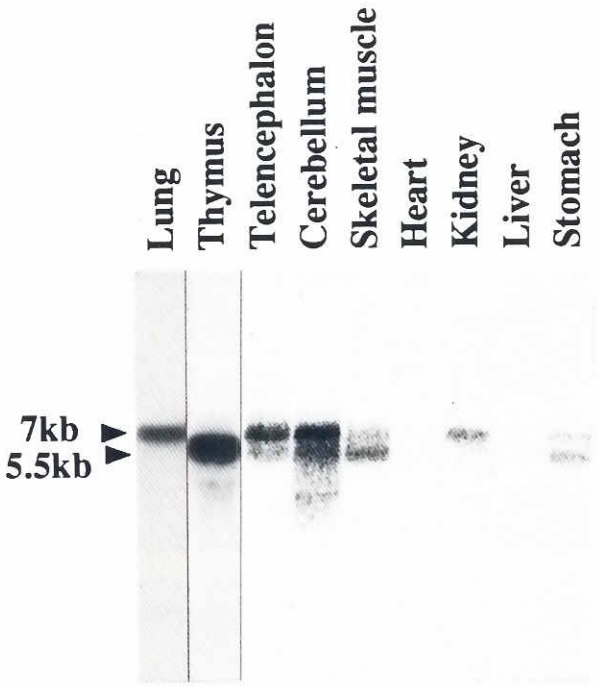


Fig. 2. Northern blot illustrating tissue-specific expression of TN mRNA isoforms. *10 μg of total RNAs from P2 mice were electrophoresed in formaldehyde agarose gels, transferred to a nitrocellulose membrane, and hybridized. Two major transcripts (7 kb and 5.5 kb) were observed.*

whenever a new lot of proteinase K was used, titration of proteolytic activity was required to determine the optimal concentration for detecting strong signals with good histological preservation (Hemmati-Brivanlou *et al.*, 1990). When 1% Triton X-100, a non-ionic detergent, was substituted for proteinase K, no signal was detected. Heat treatment of sections by incubation in 2x SSC (1x SSC=150 mM sodium chloride, 15 mM sodium citrate, pH 7.0) at 70°C for 30 min (Hogan *et al.*, 1986) was also ineffective for improving sensitivity. The low background obtained with DIG-labeled cRNA probes made an acetylation step (Ingham *et al.*, 1985) unnecessary. In negative controls, sections pre-treated with 32 μg/ml proteinase K and hybridized with the sense probe, MTN+, showed very low background (Figs. 3E,K). Proteinase K concentration, reaction time and reaction temperature were varied in order to establish optimum conditions. Incubation of sections in 1 μg/ml at 37°C for 20-30 min was best for lung, 15-20 min for cerebellum. These conditions were used for further experiments.

Signal intensity depends on probes

DIG-labeled cRNA probes were generated using the transcription vector, pGEM-1, which contains unique RNA polymerase promoters on either side of a polylinker sequence with many different restriction sites. At first, a probe corresponding to the 5' region of TN mRNA (1.0 kb) was generated and used for hybridization, but no signals were detected. In order to increase the signal intensity, five more probes containing different parts of TN mRNA (EGF, FN1-5, FNAE, FN11-13, and Fbg) were prepared (Fig. 1). When each of them was used independently, no hybridization signal was observed for either the 5', FN11-13 or the Fbg probe (Fig. 4A). Signal was barely detectable from the EGF, FNAE (Fig. 4B), and FN1-5 probes (Fig. 4C). However, when these probes were combined, strong specific signals were obtained (Fig. 4D). It is possible that secondary structure of mRNA or tightly associated binding proteins may prevent hybridization of probes in some segments of TN mRNA. Therefore, combining several probes that hybridize with different regions of mRNA may give better results for *in situ* hybridization.

Distribution of tenascin mRNAs in lung and cerebellum

As shown in Figs. 3 and 4, TN mRNA was localized in the P2 lung and cerebellum under optimum conditions for *in situ* hybridization. TN mRNA was detected in the relatively large cells lining the alveolar walls adjacent to type I pneumocytes as well as within cells at the apex of the protrusion of the alveolar septa (Fig. 4D). In cerebellum, TN mRNA was present in Bergmann radial glial cells in the developing Purkinje cell layer, a similar distribution to that of chicken (Prieto *et al.*, 1990). In contrast, neither Purkinje cells nor glial cells in the external granular cell layer were stained, but a few scattered glial cells in the internal granular layer were positively stained (Fig. 3C). These findings were consistent with those obtained by Northern blotting (Fig. 2) in which only a 7 kb mRNA isoform was observed in lung, while both (7 kb and 5.5 kb) isoforms were revealed in cerebellum. One interesting possibility is that the two isoforms may be expressed separately in different cell types in the cerebellum. Further investigations employing probes that are specific for these isoforms are needed to resolve this point.

In conclusion, *in situ* hybridization with non-radioactive DIG-labeled cRNA probes was shown to be a rapid, highly sensitive alternative to radiolabeled probes for identification of TN mRNAs in single cells. In combination with immunohistochemistry, this method offers a promising new approach for elucidation of the morphoregulatory roles of TN during normal development and tumorigenesis.

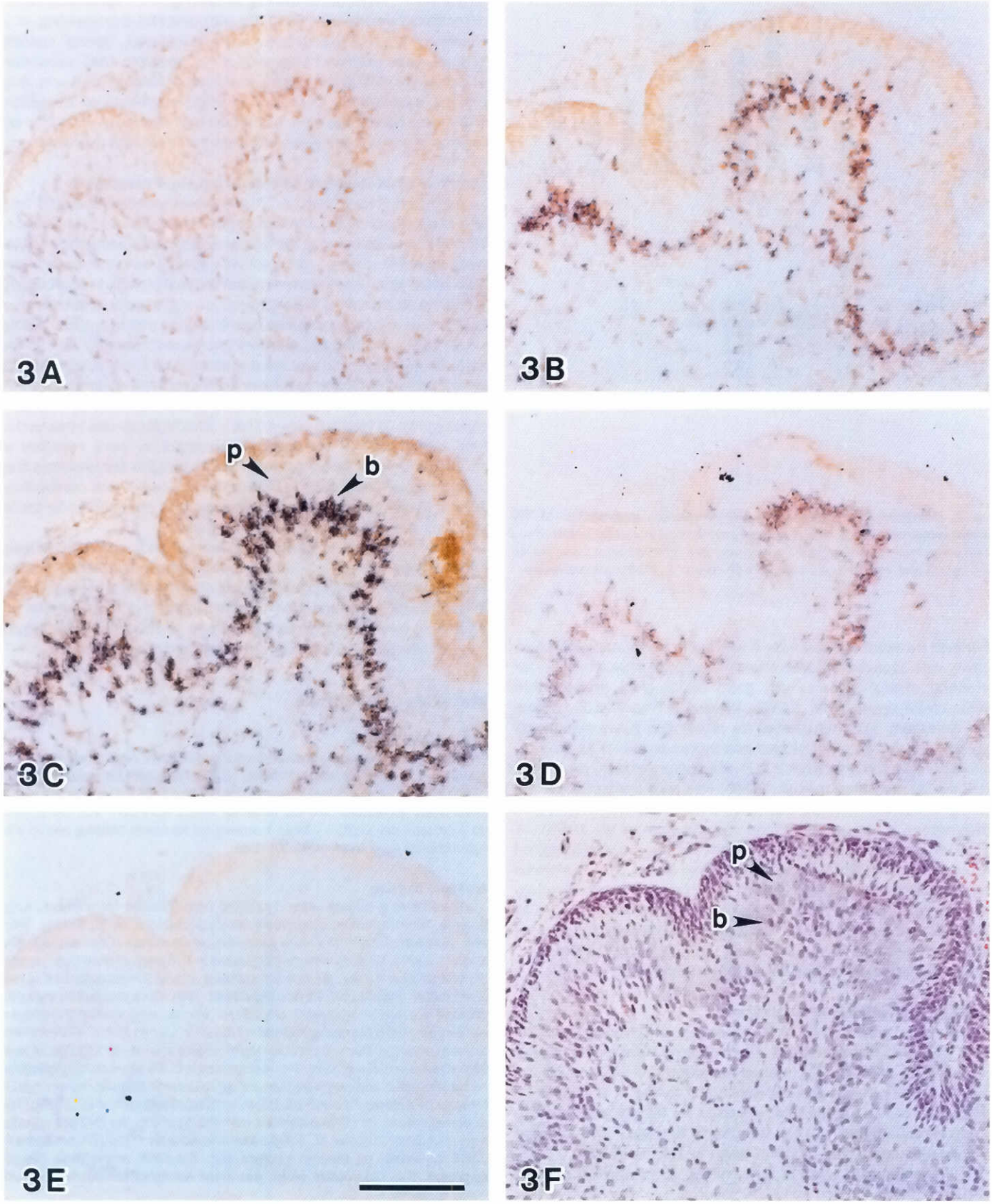
Materials and Methods

Animals and tissues

C3H/HeN mice were obtained from Charles River Japan, and they were maintained in the Animal care facility at the Tsukuba Life Science Center (RIKEN). Mice were housed in plastic cages, fed CRF-1 (Oriental Bio.), and given water *ad libitum*. The day on which litters were born was designated as postnatal day 0 (P0). Tissue samples for Northern blotting and *in situ* hybridization were taken from P2 mice.

Northern blotting

The following tissues were dissected from P2 mice for analysis: lung, thymus, telencephalon, cerebellum, skeletal muscle, heart, kidney, liver, and stomach. Total RNAs were prepared as described (Chomczynski and Sacchi, 1987). Tissues were homogenized in 4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7; 0.5% sarcosyl, 0.1 M 2-mercaptoethanol and 2 M sodium acetate, pH 4 then was added. RNA was extracted by vigorous shaking in a mixture of phenol, chloroform, and isoamyl alcohol and phases were separated by centrifugation at 8,000 *g* for 15 min at 4°C. RNA present in the aqueous phase was precipitated in isopropanol at -20°C. 10 μg of total RNAs from each tissue were electrophoresed in 0.8% agarose-formaldehyde denaturing gels, and they were transferred to Gene Screen membranes (NEN Research Products, Chads Ford, PA) by blotting (Sambrook *et al.*, 1989). The region of mouse TN cDNA extending from the 5' end to the FN2 constitutive repeat (2.5 kb) (Saga *et al.*, 1991) was labeled with ^{32}P-dCTP (Amersham, 3000 Ci/mmol) by random primers with the DNA polymerase Klenow fragment. This radioactive probe was used for hybridization detection of RNAs on blots.


3A
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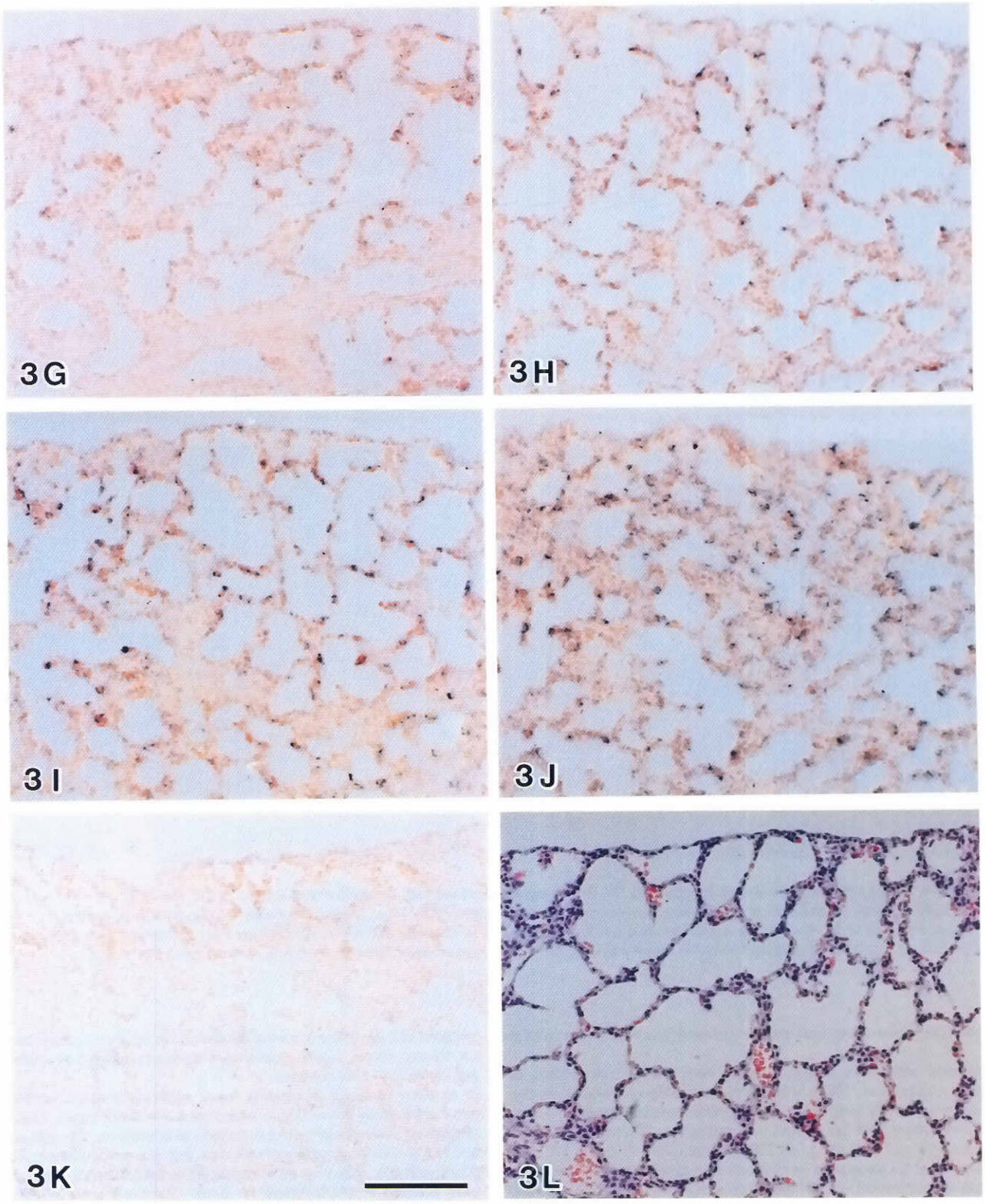

Fig. 3. Signal intensity depends on proteinase K digestion. *Sections of P2 cerebellum* **(A-F)** *and lung* **(G-L)** *were digested with 8* **(A, G)**, *16* **(B, H)**, *32* **(C, I)** *and 63* **(D, J)** *μg/ml proteinase K at room temperature for 10 min and were hybridized with anti-sense probe, MTN-. Sense probe, MTN+* **(E, K)** *also was hybridized to sections digested with 32 μg/ml proteinase K as negative controls. Sections were stained with hematoxylin-eosin for histological examination* **(F, L)**. *Arrowheads indicate Purkinje cells (p) and Bergmann radial glial cells (b). Bar: 100 μm.*

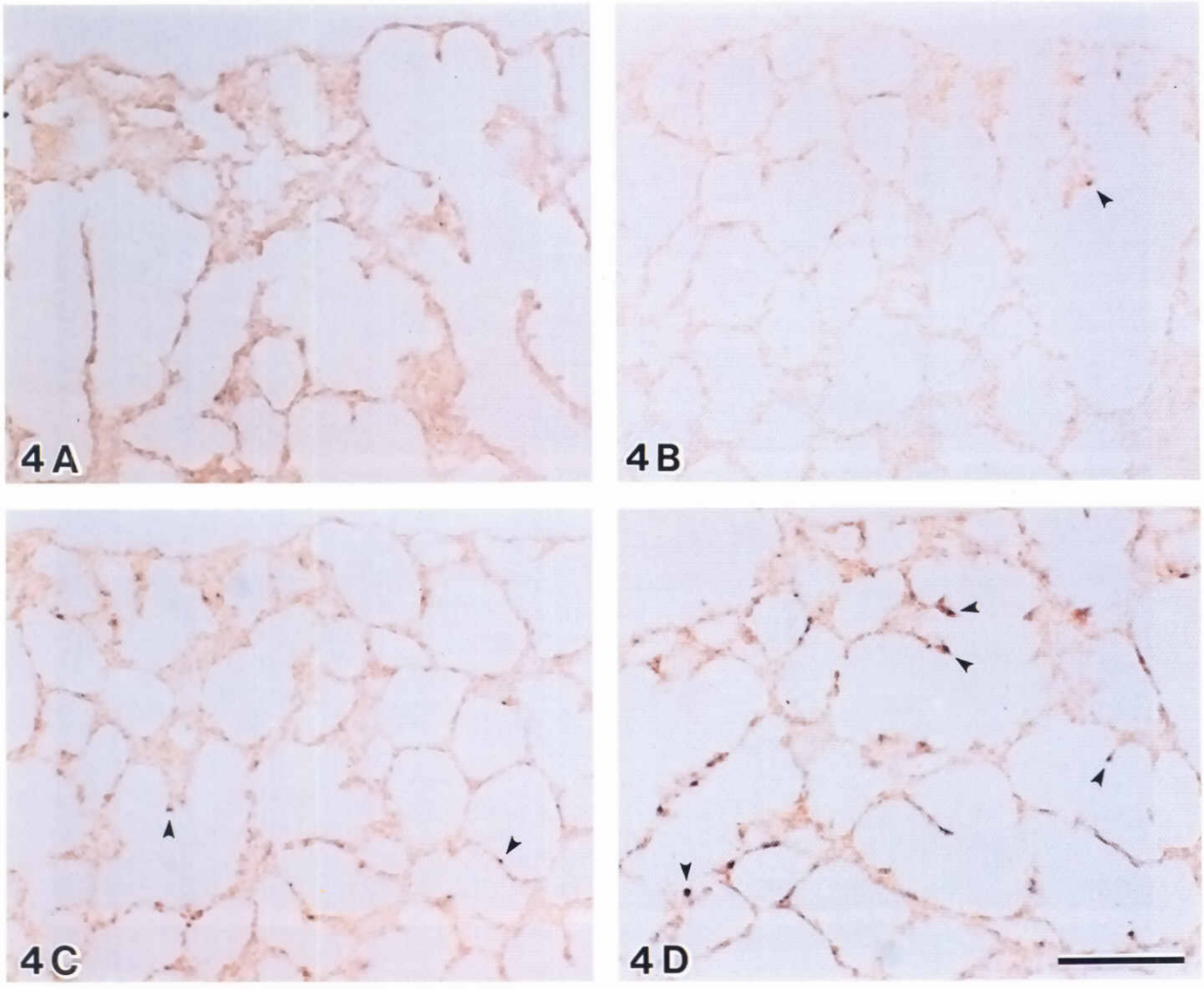

Fig. 4. Signal intensity depends on probe length and TN cDNA region represented. *Six different probes (5', EGF, FN1-5, FNAE, FN11-13, and Fbg) were tested individually in hybridization reactions, and a mixture of these probes, MTN-, was tested separately. No signal was observed with either the 5', FN11-13 or Fbg probe* **(A)**. *A weak signal was barely detected with the EGF, FNAE* **(B)**, *and FN1-5* **(C)** *probes. Drastic improvement of signal intensity without loss of specificity was obtained with the probe mixture, MTN-* **(D)**. *Arrowheads indicate positively stained cells. Bar: 100 μm.*

Tissue fixation, embedding, and section pre-treatment for* in situ *hybridization

Preparation and pre-treatment of tissue sections were essentially as described by Hogan *et al.* (1986) with some modifications as follows. Briefly, tissue samples from P2 mice were excised and were fixed overnight with 4% paraformaldehyde in PBS (phosphate buffered-saline; 130 mM NaCl, 7 mM Na_2HPO_4 and 3 mM NaH_2PO_4, pH 7.0). Tissues were rinsed in PBS and were dehydrated by sequential immersion in a graded ethanol series of increasing concentration in 1 hr incubation steps (70% x 2, 90% x 2, and 100% x 2 ethanol). Then they were incubated for 1 hr in each of the following: once in 50% ethanol+50% xylene; twice in 100% xylene; once in 25% paraffin (Wako pure chemical industries, Osaka, Japan)+25% paraplast (Monoject, MO)+50% xylene (60°C); and twice in 50% paraffin+50% paraplast (60°C). Tissues were embedded into blocks in the latter paraffin mixture. Microtome sections of 5 μm thickness were applied to Vectabond (Vector laboratories, CA) treated slides. Finally, slides were air-dried and were incubated for 30 min at 56°C to affix sections.

In order to begin processing for *in situ* hybridization, sections were deparaffinized by immersing in xylene for 5 min three times. Tissues were rehydrated in decreasing concentrations of ethanol (100%, 90% and 70%) and lastly diethylpyrocarbonate-treated, autoclaved double-distilled water (DEPC-ddH_2O). Then they were incubated for 20 min in 0.2 M HCl at RT and were washed in DEPC-ddH_2O for 5 min. Sections were equilibrated in Proteinase buffer (50 mM Tris-Cl, 5 mM EDTA, pH 7.5) at RT for 10 min prior to proteolytic digestion with 8-63 μg/ml proteinase K (Merck, Darmstadt, Germany) at RT for 10 min. Alternatively, proteinase K digestion was performed at 37°C for 10-30 min at 1 μg/ml. Reactions were terminated by rinsing in 0.2% glycine in PBS at RT for 1 min. After two 3 min washes in PBS,

TABLE 1

CONTROL EXPERIMENTS FOR *IN SITU* HYBRIDIZATION WITH DIGOXIGENIN-LABELED cRNA PROBES

1.	Northern blot analyses
2.	*In situ* hybridization with sense probe
3.	Inappropriate probe for the tissue of interest
4.	Inappropriate tissue for the probe of interest
5.	Pre-hybridization with nonlabeled probe
6.	Multiple probes coding for different regions of the same gene
7.	Pre-treatment of sections with RNase
8.	Check endogenous alkaline phosphatase activity of tissues

tissues were post-fixed with 4% paraformaldehyde for 15 min at RT, washed in PBS for 3 min, and dehydrated with increasing concentrations of ethanol (70%, 90% and 100%) before drying in air.

Preparation of digoxigenin-11-UTP-labeled cRNA probes

The amino acid sequence of mouse TN deduced from eleven cDNA clones (Saga *et al.*, 1991) is more than 99% homologous to that recently derived from twelve independent clones (Weller *et al.*, 1991). cDNA inserts derived from six different parts of mouse TN cDNA were subcloned into pGEM-1 (Promega Corporation, Madison, WI). These were as follows: 1) 5' region (5'), an 850-basepair (bp) *Eco* RI-*Pvu* II insert; 2) EGF like repeats (EGF), a 1000-bp *Dde* I insert; 3) a 1st-5th FN III repeats (FN1-5), a 1299-bp *Pst* I-*Pvu* II insert; 4) alternatively used FN III repeats (FNAE), 1091-bp *Sma* I-*Sac* II insert; 5) 11th-13th FN III repeats (FN11-13), a 679-bp *Pvu* II-*Bam* HI insert; 6) fibrinogen homology region (Fbg), a 1305-bp *Bam* HI-*Eco* RI insert. Each cDNA was linearized by digestion with appropriate restriction endonuclease (Melton *et al.*, 1984). Digoxigenin-11-UTP (Boehringer Mannheim, Mannheim, Germany) labeled single-stranded sense (+) and anti-sense (-) complementary RNA (cRNA) probes were prepared according to the manufacturer's instructions. Briefly, 1 µg of linearized template DNAs were incubated for 2 hr at 37°C in a 20 µl mixture containing 1 mM amounts of ATP, CTP, and GTP, 0.65 mM UTP, 0.35 mM digoxigenin-11-UTP, 40 units of the appropriate RNA polymerase (SP6 or T7), and 20 units of RNase inhibitor. The reaction was stopped by adding 2 µl of 0.2 M EDTA, pH 8.0 to chelate magnesium ions, and 20 units of DNase I were added for 15 min at 37°C to remove the template. Labeled RNAs were precipitated in 75 µl pre-chilled ethanol plus 2.5 µl 4 M LiCl at -70°C for 30 min. RNA precipitates were pelleted by centrifugation, and supernatants were discarded. Pellets were redissolved in 50 µl DEPC-ddH_2O.

Labeled RNAs were subjected to limited alkaline hydrolysis in order to reduce the average size to ca. 0.1 kb. This treatment improves the ability of probes to penetrate tissue sections and subsequently hybridize with tissue mRNAs. RNAs were incubated in hydrolysis buffer (80 mM $NaHCO_3$, 120 mM Na_2CO_3) for *x* min at 60°C. *x* was calculated by the formula:

$$x\ (\text{min}) = \frac{L_0 - L_{AH}}{0.11 \times L_0 \times L_{AH}}$$

(L_0=length of labeled cRNA, L_{AH}= length of alkaline-hydrolysed RNA [kb]), proposed by Cox *et al.* (1984). To stop hydrolysis, 100 µl Neutralization buffer (0.3 M sodium acetate pH 6.0, 1% glacial acetic acid) was added to the reaction. Hydrolysed RNAs were precipitated in 500 µl pre-chilled ethanol containing 20 µl 3 M sodium acetate, pH 5.2. Precipitates were collected as before and were redissolved in 20 µl DEPC-ddH_2O. 1 µl samples of each RNA were diluted 1:100, and absorbances were measured at 260 nm. Probe concentrations then were adjusted to 100 ng/µl and were stored at -20°C. Six different sense and anti-sense probes were combined in equal amounts (sense: MTN+, anti-sense: MTN-), and the mixture was used for *in situ* hybridization at a final concentration of 600 pg/µl in hybridization buffer.

Transcription products and alkaline-hydrolysed RNAs were checked by Northern blotting and detection with the same anti-digoxigenin antibody-alkaline phosphatase conjugate (anti-DIG-AP) (Boehringer Mannheim, Mannheim, Germany) as that used for *in situ* hybridization.

In situ *hybridization*

Hybridization reactions were carried out as described (Hogan *et al.*, 1986; Holland *et al.*, 1987; Nomura *et al.*, 1988) with some modifications. Sections were circled with a waterproof pen (PAP pen, Daido Sangyo, Japan) and were incubated at 42°C overnight without coverslips in 40 µl of freshly prepared hybridization buffer (50% formamide, 10 mM Tris-Cl pH 7.5, 1 mM EDTA, 600 mM NaCl, 10 mM DTT, 1x Denhardt's solution (0.02% ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin (Pentax fraction V)), 0.25% SDS, 10% Dextran sulfate, and 200 mg/ml *E. Coli* tRNA with 600 pg/µl alkaline-hydrolysed DIG-labeled probes). Probes and *E. Coli* tRNA were denatured at 80°C for 2 min and were rapidly chilled on ice prior to use.

After hybridization, slides were washed as described (Holland *et al.*, 1987; Nomura *et al.*, 1988). Briefly, sections were rinsed in 4x SSC at 42°C, and were washed in 50% formamide, 2x SSC at 42°C for 15 min. Then they were incubated in 1x STE (10 mM Tris-Cl, 500 mM NaCl, 1 mM EDTA, pH 7.5) at 37°C for 15 min. In order to remove excess unhybridized RNA, slides were treated with 10 µg/ml RNase A in 1x STE at 37°C for 30 min. Thereafter, successive washing steps were performed as follows: once in 1x STE at 37°C for 15 min, twice in 2x SSC at 42°C for 15 min, and twice in 0.2x SSC at 42°C for 15 min.

In situ hybridization signals were detected by immunohistochemical staining with anti-DIG-AP. Slides were soaked in 100 mM Tris-Cl, 150 mM NaCl, pH 7.5 (Buffer #1) for 2 min. Sections were covered with blocking solution (250 µl of 2% normal sheep serum (NSS), 0.3% Triton X-100 in Buffer #1) for 30 min at RT. Blocking solution was aspirated, and anti-DIG-AP diluted 1:500 in 1% NSS, 0.3% Triton X-100 in Buffer #1 (100 µl/section) was applied for 2 hr at RT. Sections were washed twice in Buffer #1 for 15 min at RT, soaked in 100 mM Tris-Cl, 100 mM NaCl, 50 mM $MgCl_2$, pH 9.5 (Buffer #3) for 2 min, and were covered with color-substrate solution (500 µl of 337.5 µg/ml nitroblue tetrazolium salt, 175 µg/ml 5-bromo-4-chloro-3-indolyl phosphate toluidinium salt, and 1 mM levamisole in Buffer #3) for 2-3 hr at RT. Incubation for longer than this time resulted in an increase in non-specific background staining. Color development was monitored by light microscopy. When a satisfactory signal was obtained, slides were washed in 10 mM Tris-Cl, 1 mM EDTA, pH 7.5, dehydrated in a graded ethanol series to xylene as before, and mounted with coverslips using Malinol mounting medium (Muto pure chemicals, Tokyo, Japan). Photomicrography was performed with an Olympus BH2 microscope.

Control experiments are indispensable for confirming positive signals

Control experiments were performed to confirm specificity of hybridization between probes and target mRNAs. These are summarized in Table 1. The sense strand probe was used as a control, because it has an identical number of nucleotides as well as an identical guanine/cytosine (GC) content to the anti-sense probe (Figs. 3E, K). A separate trial of the anti-sense probes, FNAE and FN1-5, corresponding to a non-overlapping region of the target mRNA, confirmed specificity of the hybridization reaction (Figs. 4B, C). Because FNAE and FN1-5 showed the same pattern of hybridization, it is likely that they recognize the same mRNA (Giaid *et al.*, 1989; McCabe and Pfaff, 1989; Siegel, 1989; Höfler, 1990).

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References

AUFDERHEIDE, E., CHIQUET-EHRISMANN, R. and EKBLOM, P. (1987). Epithelial-mesenchymal interactions in the developing kidney lead to expression of tenascin in the mesenchyme. *J. Cell Biol. 105:* 599-608.

BALDINO, Jr., F. and LEWIS, M.E. (1989). Nonradioactive *in situ* hybridization histochemistry with digoxigenin-deoxyuridine 5'-triphosphate-labeled

oligonucleotides. In *Gene Probes* (Ed. P.M. Conn). Methods in Neurosciences, Vol. 1. Academic Press, San Diego, pp. 282-292.

BRADY, M.A.W. and FINLAN, M.F. (1990). Radioactive labels: autoradiography and choice of emulsions for *in situ* hybridization. In *In Situ Hybridization. Principles and Practice* (Eds. J.M. Polak and J.O'D. McGee). Oxford University Press, Oxford, pp. 31-57.

BURNS, J., CHAN, V.T.-W., JONASSON, J.A., FLEMING, K.A., TAYLOR, S. and McGEE, J.O'D. (1985). A sensitive method for visualizing biotinylated probes hybridized *in situ:* rapid sex determination on intact cells. *J. Clin. Pathol. 38:* 1085- 1092.

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-EHRISMANN, R., MACKIE, E.J., PEARSON, C.A. and SAKAKURA, T. (1986). Tenascin: an extracellular matrix protein involved in tissue interactions during fetal development and oncogenesis. *Cell 47:* 131-139.

CHOMCZYNSKI, P. and SACCHI, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem. 162:* 156-159.

COX, K.H., DeLEON, D.V., ANGERER, L.M. and ANGERER, R.C. (1984). Detection of mRNAs in sea urchin embryos by *in situ* hybridization using asymmetric RNA probes. *Dev. Biol. 101:* 485-502.

EDELMAN, G.M. (1988). Morphoregulatory molecules. In *Topobiology. An Introduction to Molecular Embryology.* Basic Books, New York, pp. 86-125.

GIAID, A., GIBSON, S.J., STEEL, J.H. and POLAK, J.M. (1989). *In situ* detection of peptide messenger RNA using complementary RNA probes. In *Gene Probes* (Ed. P.M. Conn). Methods in Neurosciences, Vol. 1. Academic Press, San Diego, pp. 150-164.

GULCHER, J.R., NIES, D.E., MARTON, L.S. and STEFANSSON, K. (1989). An alternatively spliced region of the human hexabrachion contains a repeat of potential N-glycosylation sites. *Proc. Natl. Acad. Sci. USA 86:* 1588-1592.

HEMMATI-BRIVANLOU, A., FRANK, D., BOLCE, M.E., BROWN, B.D., SIVE, H.L. and HARLAND, R.M. (1990). Localization of specific mRNAs on *Xenopus* embryos by whole-mount *in situ* hybridization. *Development 110:* 325-330.

HÖFLER, H. (1990). Principles of *in situ* hybridization. In *In Situ Hybridization. Principles and Practice* (Eds. J.M. Polak and J.O'D. McGee). Oxford University Press, Oxford, pp. 15-29.

HOGAN, B.L.M., CONSTANTINI, F. and LACY, E. (1986). Techniques for visualizing genes and gene products. In *Manipulating the Mouse Embryo.* Cold Spring Harbor Laboratory, Cold Spring Harbor, pp. 219-244.

HOLLAND, P.W.H., HARPER, S.J.,McVEY, J.H. and HOGAN, B.L.M. (1987). *In vivo* expression of mRNA for the Ca^{++}-binding protein SPARC (Osteonectin) revealed by *in situ* hybridization. *J. Cell Biol. 105:* 473-482.

INGHAM, P.W., HOWARD, K.R. and ISH-HOROWICZ, D. (1985). Transcription pattern of the *Drosophila* segmentation gene *hairy. Nature 318:* 439-445.

MACKIE, E.J., CHIQUET-EHRISMANN, R., PEARSON, C.A., INAGUMA, Y., TAYA, K., KAWARADA, Y. and SAKAKURA, T. (1987a). Tenascin is a stromal marker for epithelial malignancy in the mammary gland. *Proc. Natl. Acad. Sci. USA 84:* 4621-4625.

MACKIE, E.J., HALFTER, W. and LIVERANI, D. (1988). Induction of tenascin in healing wounds. *J. Cell Biol. 107:* 2757-2767.

MACKIE, E.J., THESLEFF, I. and CHIQUET-EHRISMANN, R. (1987b). Tenascin is associated with chondrogenic and osteogenic differentiation *in vivo* and promotes chondrogenesis *in vitro. J. Cell Biol. 105:* 2569-2579.

McCABE, J.T. and PFAFF, D.W. (1989). *In situ* hybridization: a methodological guide. In *Gene Probes* (Ed. P.M. Conn). Methods in Neurosciences, Vol. 1. Academic Press, San Diego, pp. 98-126.

MELTON, D.A., KRIEG, P.A., REBAGLIATI, M.R., MANIATIS, T., ZINN, K. and GREEN, M.R. (1984). Efficient *in vitro* synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. *Nucleic Acids Res. 12:* 7035-7056.

NAKANE, P.K., MORIUCHI, T., KOJI, T., TANNO, M. and ABE, K. (1987). *In situ* localization of mRNA using thymine-thymine dimerized cDNA. *Acta Histochem. Cytochem. 20:* 229-243.

NOMURA, S., WILLS, A.J., EDWARDS, D.R., HEATH, J.K. and HOGAN, B.L.M. (1988). Developmental expression of 2ar (Osteopontin) and SPARC (Osteonectin) RNA as revealed by in situ hybridization. *J. Cell Biol. 106:* 441-450.

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.

SAGA, Y., TSUKAMOTO, T., JING, N., KUSAKABE, M. and SAKAKURA, T. (1991). Mouse tenascin: cDNA cloning, structure and temporal expression of isoforms. *Gene* (In press).

SAMBROOK, J., FRITSCH, E.F. and MANIATIS, T. (1989). Northern hybridization. In *Molecular Cloning. A Laboratory Manual (2nd ed.).* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, pp. 7.39-7.52.

SIEGEL, R.E. (1989). Localization of neuronal mRNAs by hybridization histochemistry. In *Gene Probes* (Ed. P.M. Conn). Methods in Neurosciences, Vol. 1. Academic Press, San Diego, pp. 136-150.

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.

WELLER, A., BECK, S. and EKBLOM, P. (1991). Amino acid sequence of mouse tenascin and differential expression of two tenascin isoforms during embryogenesis. *J. Cell Biol. 112:* 355-362.

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