**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 tenasin; AE, alternative exon; DEPC-ddH₂O, 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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probes

Fig. 1. Structural model of mouse tenasin 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. 14/2 EGF-like repeats (EGF) and 13 FN type III-like repeats (FNIII) follow. The 6th-10th FNIII are alternatively spliced exons (AE). At the C-terminus, there is a fibrinogen homologous domain (Fbg). Thin letters represent non-coding region. Digoxigenin-11-UTP-labeled cRNA probes correspond to each domain including the 5' EGF-like repeats (EGF), FN1-5 (1st-5th FNIII repeats), FNAE (6th-10th FNIII repeats), FN11-13 (11th-13th FNIII repeats) and Fbg (fibrinogen homologous 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, tenasin.

Tenasin (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 extracellular 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; Auferheide 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 tenasin 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,
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 (PO). 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 32P-dCTP (Amersham, 3000 Ci/mmole) by random primers with the DNA polymerase Klenow fragment. This radioactive probe was used for hybridization detection of RNAs on blots.
In situ hybridization of tenascin mRNAs

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.
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 weak signal was barely detected with the EGF, FNAE, and FN1-5 probes. Drastic improvement of signal intensity without loss of specificity was obtained with the probe mixture, MTN-. 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 Na₂HPO₄, 3 mM NaH₂PO₄, 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-ddH₂O). Then they were incubated for 20 min in 0.2 M HCl at RT and were washed in DEPC-ddH₂O for 5 min. Sections were equilibrated in Proteinase buffer (50 mM Tris-CI, 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,
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 PGM-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) 1st-5th FN III repeats (FN1-5), a 850-bp EcoRI-Pvu II insert; 4) alternatively used FN111 repeats (FNAE), 1091-bp Bgl II insert; 5) 11th-13th FNIII repeats (FNll-13), a 679-bp Pvu II - I-Pvu II insert; 6) fibrinogen homology region (Fbg), a 1305-bp Bgl II-EcoRI insert. Each cDNA was linearized by digestion with appropriate restriction endonucleases (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 µl of linearized template DNAs were incubated for 2 hr at 37°C in a 20 µl mixture containing 1 µM 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 TT), 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-dH2O.

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 NaClO3, 120 mM Na2CO3) for x min at 60°C. x was calculated by the formula:

\[ x(\text{min}) = \frac{L_p}{0.11xL_p} \]

(L_p = length of labeled cRNA, L_AU = length of alkaline-hydrolysed RNA [kb]), proposed by Coxx, 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-dH2O. 1 µl samples of each RNA were diluted 1:100, and absorbances were measured at 260 nm. Probe concentrations were adjusted to 100 mg/ml and were stored at -20°C. 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.


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