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Original Article

Tenascin expression in developing, adult and regenerating caudal spinal cord in the urodele amphibians

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ABSTRACT **Tenascin (Tn) protein and transcripts were analyzed in developing, adult and regenerating caudal spinal cord (SC) of *Pleurodeles waltl*. A polyclonal antibody (PAb) against *Xenopus* Tn and a newt Tn cDNA probe were used. In Western blots, anti-Tn PAb recognized Tn polypeptides of 200-220 kDa in tail regenerate extracts, but also the homolog of Tn/Cytotactin/J1 in brain and SC of adult newt. Immunofluorescence studies showed some reactivity around ependymoglial cells and strong labeling in the nervous tracts, in the developing as well as in the regenerating SC or adult SC. Immunogold electron microscopy revealed the presence of Tn throughout the ependymoglial cells, particularly near and along the plasma membrane of radial processes surrounding axons, especially growth cones. Tn could be more precisely found within rough endoplasmic reticulum and Golgi structures, or again in the surrounding extracellular space. This suggested that Tn was at least produced by radial glial profiles forming axonal compartments in which axons grew. Using the DNA probe for Tn, expression of Tn mRNA was also examined by Northern blot and RNAase protection analyses and by *in situ* hybridization, respectively. The levels of transcripts, barely detectable in adult tail, increased in regenerates from 3 days through 4-8 weeks post-amputation. *In situ* Tn mRNA were mainly localized in the mesenchyme, especially at the epithelial-mesenchymal interface, and in the developing cartilage, at the early regeneration stages, whereas high amounts of transcripts were seen not only at these stages, but also later, in the regenerating SC. Our main results supported the view that, in the caudal SC of newts, Tn, synthesized by radial ependymoglial cells, was similarly expressed during regeneration as well as larval development, and exhibited a sustained high accumulation level in the adult SC. On the basis of the multifunctional properties of Tn, the putative roles played by Tn as a substrate for neuronal pathfinding and boundary shaping were discussed.**

KEY WORDS: *tenascin, regeneration, spinal cord, urodele amphibians*

Introduction

Urodele amphibians are characterized by their epimorphic regeneration capacity to replace their amputated appendages (limbs and tail). During tail regeneration, these animals are able to differentiate a spinal cord (SC) within the regenerate (Holtzer, 1956). Chronologically, this differentiation involves the extension from the injured SC of an ependymal tube directing axon growth in a rostrocaudal direction and the subsequent differentiation in neuronal and supportive cells of ependymal cells (Egar and Singer, 1972; Nordlander and Singer, 1978; Arsanto *et al.*, 1992). Histological studies have suggested that ependymoglial and astroglial cells which emerge from the amputation level of the SC have the capacity to divide and build within the regenerate a cytoarchitecture providing a guiding substrate for axon outgrowth and cell positioning. The ependymal tube of the regenerate, acting in these adult animals like the embryonal neuroepithelium, is able to reproduce a segmented pattern with ventral root emergences and spinal ganglia formation, from which the PNS is reorganized (Géraudie *et al.*, 1988; Thouveny *et al.*, 1991, 1993). Among the vertebrates, this biological system provides one of the rare examples of SC regeneration whose cell and molecular mechanisms can be analyzed.

It is now well established that during the development of the nervous system, the extracellular matrix (ECM) is implicated as an important extrinsic cue that influences neuronal migration, axonal

Abbreviations used in this paper: SC, spinal cord; CNS, central nervous system; PNS, peripheral nervous system; Tn, tenascin; ECM, extracellular matrix; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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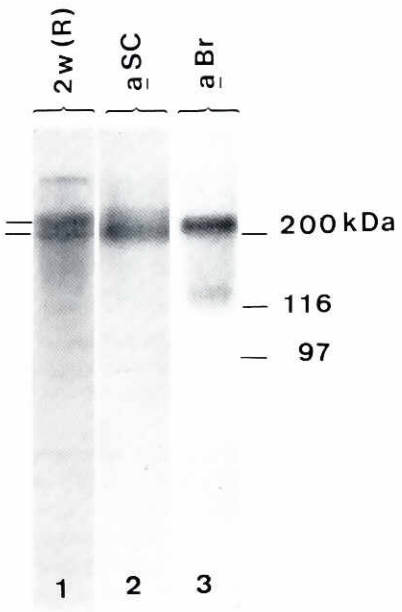

Fig. 1. Western blots of Tenascin (Tn) polypeptides. *Homogenates of 2 week-old tail regenerate* **(lane 1)**, *adult SC* **(lane 2)** *and adult brain* **(lane 3)** *from Pleurodeles waltl were resolved on 7% SDS/PAGE and then transferred to nitrocellulose. The blots were probed with anti-Tn polyclonal antibody. In regenerate extract, Tn clearly appears as two polypeptides of 200 and 220 kDa. In adult SC extract* **(lane 2)**, *a major band at 200 kDa is observed but only a faint band around 220 kDa is recognized by the antibody. In adult brain extract, only a large band around 220 kDa can be seen* **(lane 3)**. *Molecular mass standards: myosin (200 kDa), galactosidase (116 kDa) and phosphorylase b (97 kDa).*

growth, synaptogenesis and glial differentiation (for reviews, see e.g., Sanes, 1989; Hynes and Lander, 1992). Among the ECM molecules, the glycoprotein Tenascin (Tn) (Chiquet-Ehrismann *et al.*, 1986), also called Cytotactin (Grumet *et al.*, 1985) or Hexabrachion (Erickson and Taylor, 1987) in the chicken, J1 in the mouse (Faissner *et al.*, 1988) and Neuronectin in the human CNS (Rettig *et al.*, 1989), has been considered as a potential modulator of cell-ECM interactions during developmental processes (for reviews, see Edelman, 1986; Chiquet, 1989; Riou *et al.*, 1992; Erickson, 1993). In the CNS, Tn is expressed throughout the development. It can modify the functional properties of nerve cell surface and is involved in morphogenetic events such as cell distribution and axonal outgrowth (Chuong *et al.*, 1987; Bartsch *et al.*, 1992; Husmann *et al.*, 1992). In the PNS, Tn is accumulated along developing nervous tracts and penetrating axons in the limb bud (Wehrle and Chiquet, 1990; Martini and Schachner, 1991; Wehrle-Haller *et al.*, 1991; Wehrle-Haller and Chiquet, 1993). It is also secreted by Schwann cells in their basal lamina where it could act to maintain the growing axons in the endoneurial tube (Mège *et al.*, 1992). Thus, its wide distribution in embryonic brain compared to its restricted localization in the adult (Crossin *et al.*, 1986; ffrench-Constant *et al.*, 1986; Rieger *et al.*, 1986; Rettig *et al.*, 1989; Prieto *et al.*, 1990), and the regulation of its expression level in the differentiating or regenerating PNS (Sanes *et al.*, 1986) initially make this molecule one of the possible candidates for determining cell positioning pattern, nerve pathways (Chiquet, 1989; Sanes, 1989; Faissner and Kruse, 1990; Husmann *et al.*, 1992) and boundaries of anatomical pattern in the nervous system (Crossin *et al.*, 1989, 1990; Steindler *et al.*, 1989, 1990; Grierson *et al.*, 1990; Chiquet *et al.*, 1991; Jhaveri *et al.*, 1991; Perez and Halfter, 1993; Steindler, 1993). In the CNS and the PNS, Tn is expressed by glial and satellite (Schwann cell precursor) cells (see for review, Chiquet *et al.*, 1991), but mesenchyme-derived cells (fibroblasts) may also express this molecule in the periphery after denervation (Sanes *et al.*, 1986; Daniloff *et al.*, 1989; Erickson and Bourdon, 1989; Gatchalian *et al.*, 1989; Martini *et al.*, 1990).

In vitro Tn modulates glia-neuron interaction and neuritic outgrowth (Grumet *et al.*, 1985; Grierson *et al.*, 1990; Lochter *et al.*, 1991; Perez and Halfter, 1993). However, in various experimental conditions, it displays a more complex function according to the presence of adhesive or anti-adhesive specific domains on the molecule (see e.g. Spring *et al.*, 1989; Wehrle-Haller and Chiquet, 1993). It is now clear that the response of the cell to the Tn substrate is dependent upon the cell type, the expression of specific receptors and the coexpression of other adhesion molecules (Chiquet-Ehrismann *et al.*, 1988; Lotz *et al.*, 1989; Rathjen *et al.*, 1991; Zisch *et al.*, 1992). For instance, Tn can be a low adhesive substrate for some cells (neural crest cells) and can inhibit cell spreading and cell migration (Tan *et al.*, 1987; Mackie *et al.*, 1988; Halfter *et al.*, 1989) when offered as a substrate in combination with other ECM molecules (Chiquet-Ehrismann *et al.*, 1988; Spring *et al.*, 1989; Faissner and Kruse, 1990; Riou *et al.*, 1990). In spite of all these studies, the role of Tn *in vivo* is still under debate (see e.g., Erickson, 1993), particularly since the work of Saga *et al.* (1992) reporting that no defects of development were observed in mutant mice lacking a functional Tn gene.

Using a polyclonal antibody anti-chicken fibroblast Tn (a generous gift of M. Chiquet), we have previously shown that Tn expression sharply increased in the ECM of the blastemal mesenchyme during tail regeneration of *Pleurodeles waltl* (Arsanto *et al.*, 1990).

In this paper, using a polyclonal antibody against Tn of *Xenopus laevis*, prepared by some of us (Riou *et al.*, 1991), and which has been shown to recognize the molecule in the nervous system of *Pleurodeles* as well as *Xenopus* — (which was not the case for the antibody used in our previous work) — we have been able to analyze the expression of Tn protein in the regenerating caudal SC of *P. waltl*, in comparison with that present in the SC of larval and adult newts. The expression of Tn transcripts in the same system could be also investigated by using a newt Tn molecular probe isolated and characterized by Onda *et al.* (1991).

Tn expression detected during ependymal tube formation might be considered as one of the prerequisite events for SC regeneration, a biological phenomenon which characterizes the amphibian urodeles.

Results

Tn protein expression: Western blot analysis

Homogenates of tail regenerates (Fig. 1, lane 1) and CNS tissue, i.e. SC (Fig. 1, lane 2) and brain (Fig. 1, lane 3) from adult *P. waltl* were analyzed by immunoblotting with anti-Tn polyclonal antibody. In regenerates, e.g. a 2 week-old regenerate (Fig. 1, lane 1), Tn appeared as two polypeptides of relative molecular weight 200 and 220 kDa. In adult SC extracts (Fig. 1, lane 2), a major band at 200 kDa was observed but only a minor band at 220 kDa was present. In adult brain extract (Fig. 1, lane 3), only a large band around 200-210 kDa could be seen. In 2 week-old regenerate, a component around 300 kDa was detected (Fig. 1, lane 1).

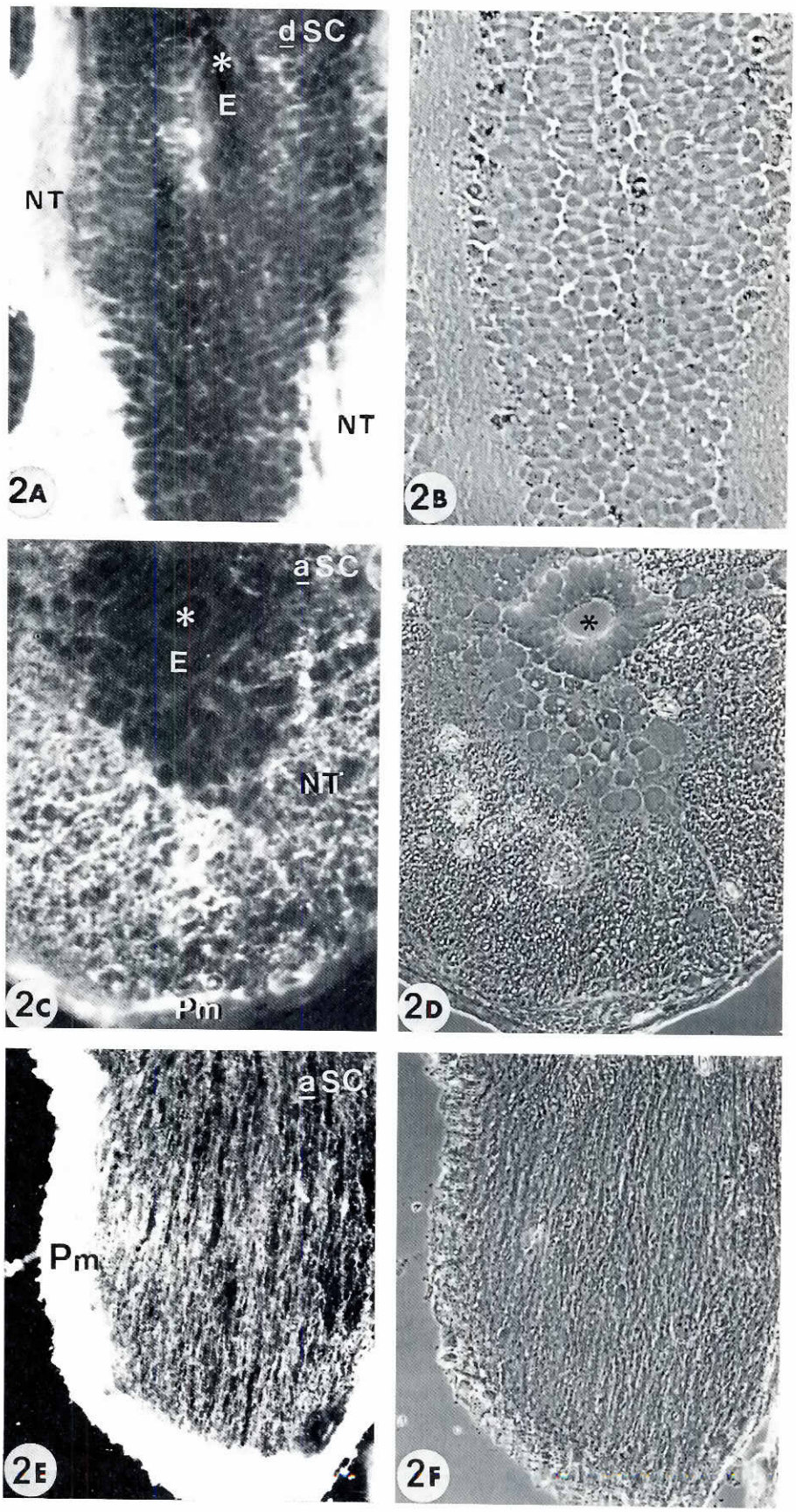

Fig. 2. Tn immunoreactivity in developing and adult caudal SC of *P. waltl*. **(A)** *Parasagittal section through developing caudal spinal cord (dSC). A faint reaction is observed around the cells of ependymoglia (E); the nervous tracts (NT) are strongly reactive. Asterisk: ependymal central canal.* **(B)** *Phase-contrast image of* **(A).** *x150.* **(C-D)** *Adult caudal spinal cord (aSC) in transverse sections.* **(C)** *A strong reactivity can be observed in the nervous tracts (NT), but a weaker staining is seen around the cells of the periependymal region. Ependymal cells (E) around the central canal (asterisk) show no labeling.* **(D)** *Phase-contrast image of* **(C).** *x150.* **(E-F)** *Part of a parasagittal section through the white matter of adult caudal spinal cord (aSC).* **(E)** *An intense staining is observed around the axons, viewed here in longitudinal section. Note also the bright reactivity of the pia mater (Pm).* **(F)** *Phase-contrast image of* **(E).** *x150.*

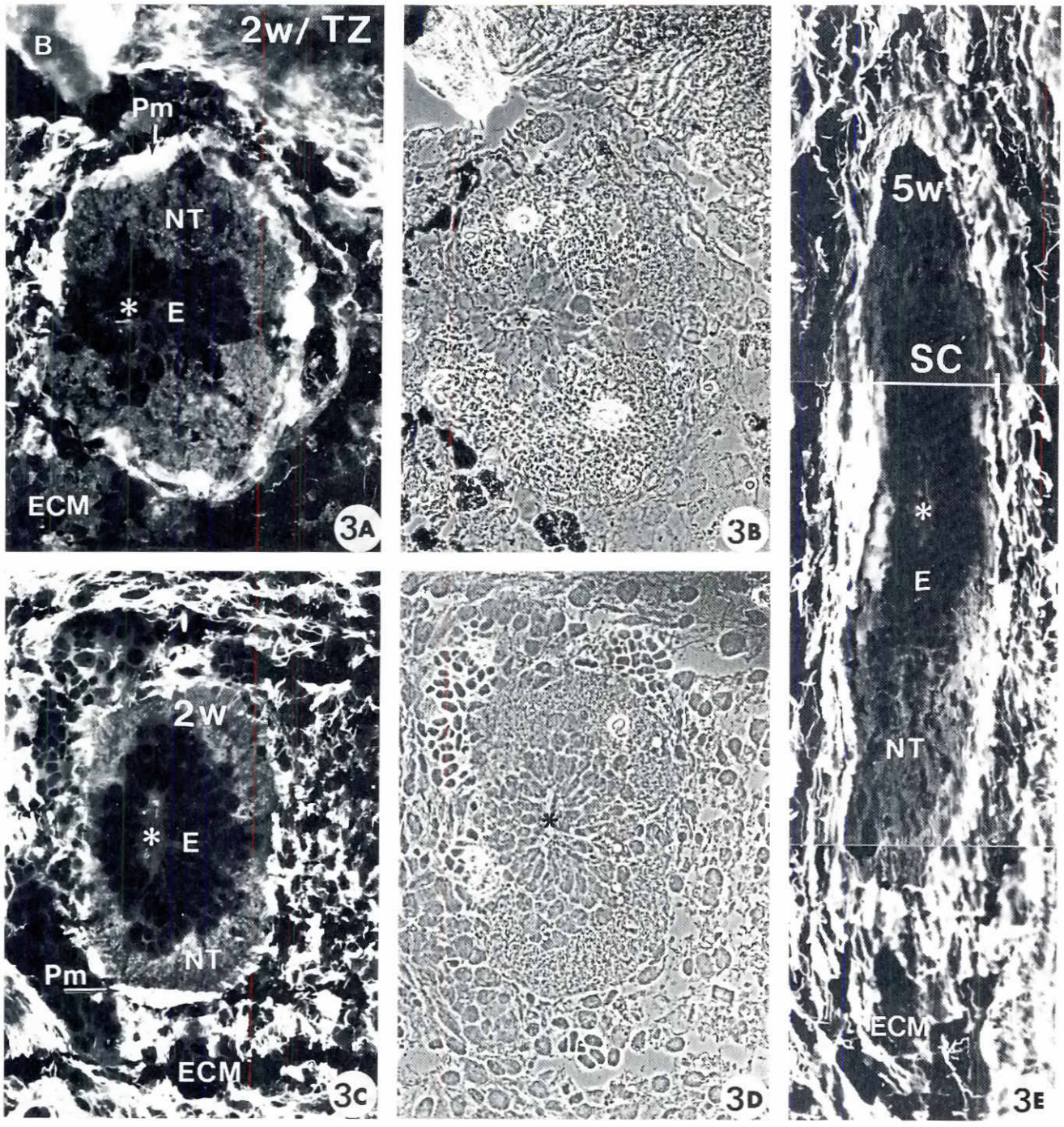

Fig. 3. Tn expression in regenerating caudal SC of adult *P. waltl*. **(A)** *Transverse section view of the SC in the stump transition zone (TZ) close to a 2 week-old tail regenerate. Intense immunoreactivity is seen in the nervous tracts (NT), in the pia mater (Pm) and in the neighboring extracellular matrix (ECM). Asterisk: Ependymal canal. B: bony fragment of the vertebra.* **(B)** *Phase-contrast image of* **(A)**. *x150.* **(C)** *Transverse section through a 2 week-old regenerating caudal SC. Intense staining is observed throughout the axonal compartments, and around, in the pia mater (Pm) and in the extracellular matrix (ECM). Only a weak labeling is seen at the ependymoglial (E) cell surfaces.* **(D)** *Phase-contrast image of* **(C)**. *x150.* **(E)** *Oblique section through a 5 week-old regenerating caudal SC. Immunoreactivity is nearly restricted to the nervous tracts (NT), ependymoglia (E) showing no or only a faint labeling. x140.*

Tn protein expression: immunohistochemical localization

In developing SC of *P. waltl* (Fig. 2A/B), a strong Tn immunoreactivity was observed in the nervous tracts, whereas a far less intense labeling could be seen within and/or around the ependymal and glial cells.

A similar pattern of Tn expression was observed in the caudal SC of adult newts (Fig. 2C/D and 2E/F). Indeed, at the level of fluorescence microscope, an intense staining appeared located in the SC white matter (Fig. 2C/D and 2E/F), whereas only a little, if any, labeling was seen in and/or

around the ependymal, periependymal or glial cells (Fig. 2C/D).

In the regenerating SC, e.g., in that of 2 or 5 week-old regenerates (Fig. 3C/D and 3E, respectively), as well as in the SC of the adjacent stump transition zone (Fig. 3A/B), a high level of Tn expression restricted to the nervous tracts and the pia mater was again observed, the ependymoglial cells showing only a faint expression.

Tn protein expression: structural features and immunoelectron gold localization

It has been demonstrated that regenerating tails of urodele amphibians show a rostrocaudal developmental gradient (see Géraudie *et al.*, 1988; Arsanto *et al.*, 1992 and references cited therein). However, 2 or 3 weeks after amputation, the organization of the regenerating SC appeared nearly reestablished within the neural tube in the rostral as well as in the caudal parts of the regenerate (Fig. 4A), except in the caudalmost tip (Arsanto, data not shown). Fig. 4B shows the cytoarchitectural features of the regenerating SC in a cross section through the caudal part of a 3 week-old newt tail regenerate. The ependymoglial palisade organization could be seen clearly. Ciliated ependymoglial cell bodies lining the central canal (Fig. 4B) developed radial profiles (Fig. 4B,C,D) extending in marginal endfeet where they contacted at the glia limitans (Fig. 4D). These radial glial processes arranged in register defined thereby longitudinal compartments (called "channels" or "tunnels" by Singer *et al.*, 1979) in which axons grew. In some cross sections (Figs. 4D, 5B), growth cones could be seen in axonal compartments where they often appeared close to the radial glial appendages (Fig. 5B)

Using anti-Tn PAb, immunoelectron gold staining studies on Lowicryl-embedded tissue sections through regenerating caudal SC were performed. Gold immunolabeling was seen throughout the radial extensions of the ependymoglial cells (Fig. 5A), especially near and along the plasma membranes, but it was in general difficult to specify in precisely what cytoplasmic infrastructures gold particles were located. However, in some favorable ultrathin sections, immunogold staining could be found within organelles, including distended saccules of rough endoplasmic reticulum (Fig. 5D), or cisternae (Fig. 5D) and vesicles (Fig. 5C) presumed to belong to the Golgi apparatus. Gold labeling was also sometimes observed in the extracellular space between radial glial processes and axons (Fig. 5E).

Tn mRNA expression

In this study we used a newt Tn cDNA molecular probe (NvTN1) isolated and characterized by Onda *et al.* (1991) from the related species *Notophthalmus viridescens* (Nv). The authors have shown that the sequence of 1.9 kb spanned 11 EGF-like repeats and three and one-half Fibronectin type III repeats, which are characteristic of the structure of Tn polypeptides. The aminoacid sequence identity between newt and chicken is over 90% if conserved substitution is allowed.

NvTN1 (1.9 kb) was hybridized, on Northern blots, with RNA from 3 day- and 8 week-old regenerates. A strong signal was observed at the level of a band corresponding to a transcript size of 8 kb (Fig. 6A). This is similar to that reported for chicken Tn, mouse Tn and *Xenopus* Tn mRNA (Pearson *et al.*, 1988; Jones *et al.*, 1989; Weller *et al.*, 1991; Umbhauer *et al.*, 1992). Comparison between 3 day-old regenerate RNA and 8 week-old regenerate RNA has shown a strong difference in the relative accumulation of the transcripts in the total RNA. Tn mRNA are rapidly transcribed in wound healing tissues after amputation. Detection of mRNA encoding Tn was carried out by RNase protection analysis. A 378 base T3-promoted anti-sense RNA probe was transcribed from NvTN1 (see Onda *et al.*, 1991). A 135 base T7-promoted anti-sense RNA probe transcribed from GAPDH insert, as a typical "housekeeping" gene (Fort *et al.*, 1985), was included in each hybridization (Fig. 6B) to normalize the amount of Tn mRNA to that of GAPDH mRNA. Fig. 6B shows that hybridization with total RNA led to the protection of about 313 bases for the Tn probe and 117 bases for the GAPDH probe. These fragments are absent from control with tRNA alone. Analyses of total RNA for Tn and GAPDH transcripts during regeneration were performed at different stages. Results showed that Tn mRNA was first detected in the 3 day-old blastema. This early Tn mRNA transcription appeared to be an immediate reaction to the trauma and Tn transcripts remained at a high level up to 30 and 60 day-old regenerate stages in comparison with the very faint expression level observed in adult tail tissues after a long exposure of the autoradiograms. Autoradiograms of three different experiments were scanned by densitometer and the relative intensity of the Tn band was normalized to the density of the GAPDH band in each lane (Fig. 6C). The results shown in this figure largely agree with our previous results on the expression of Tn polypeptides during tail regeneration in *Pleurodeles waltl* (Arsanto *et al.*, 1990).

In order to find out the tissue distribution of these Tn transcripts, we undertook *in situ* hybridization analyses with anti-sense probe. In Fig. 7A/B, Tn transcripts are present throughout the distal part of the mesenchyme and in the inner part of the wound healing epidermis in a 3 week-old regenerate. It is noteworthy that this accumulation of Tn transcripts in this region might be correlated to an epithelio-mesenchyme interaction as proposed for other biological systems by Chiquet-Ehrismann *et al.* (1986). These results agree with those obtained by Onda *et al.* (1991), during early stages of newt limb regeneration. In Fig. 7C/D, Tn mRNA hybridization with anti-sense probe was seen in the median part of a 3 week-old regenerate where accumulation of the Tn transcripts was found all along the ependymal tube located in the middle part of the mesenchyme of the regenerate. The distal regeneration vesicle of the ependymal tube was also strongly labeled although this structure was composed of loosely adherent glial cells with few axonal endings as previously described (Arsanto *et al.*, 1992). In 8 week-old regenerate (Fig. 7E), Tn transcripts were always intensely expressed in the regenerating SC. At this stage, differentiation of the vertebral body was effective and Tn RNA transcripts were strongly expressed in the perichondrium, but weakly expressed in the cartilaginous matrix. No labeling could be seen in controls with sense probe (Fig. 7F/G).

Discussion

In the present paper, by using immunohisto- and cytochemistry, Western and Northern blot analyses, RNAase protection assays and *in situ* hybridization, the expression patterns of Tenascin (Tn) protein and transcripts were investigated in the developing (larval), adult and regenerating caudal SC of the urodele *Pleurodeles waltl*.. Our main findings may be summarized and discussed as follows:

Immunoblotting results obtained on the CNS structures of *Pleurodeles waltl* with the polyclonal antibody to Tn of *Xenopus laevis* are consistent with data previously found in the nervous system of adult amphibians (Riou *et al.*, 1988, 1990; Onda *et al.*,

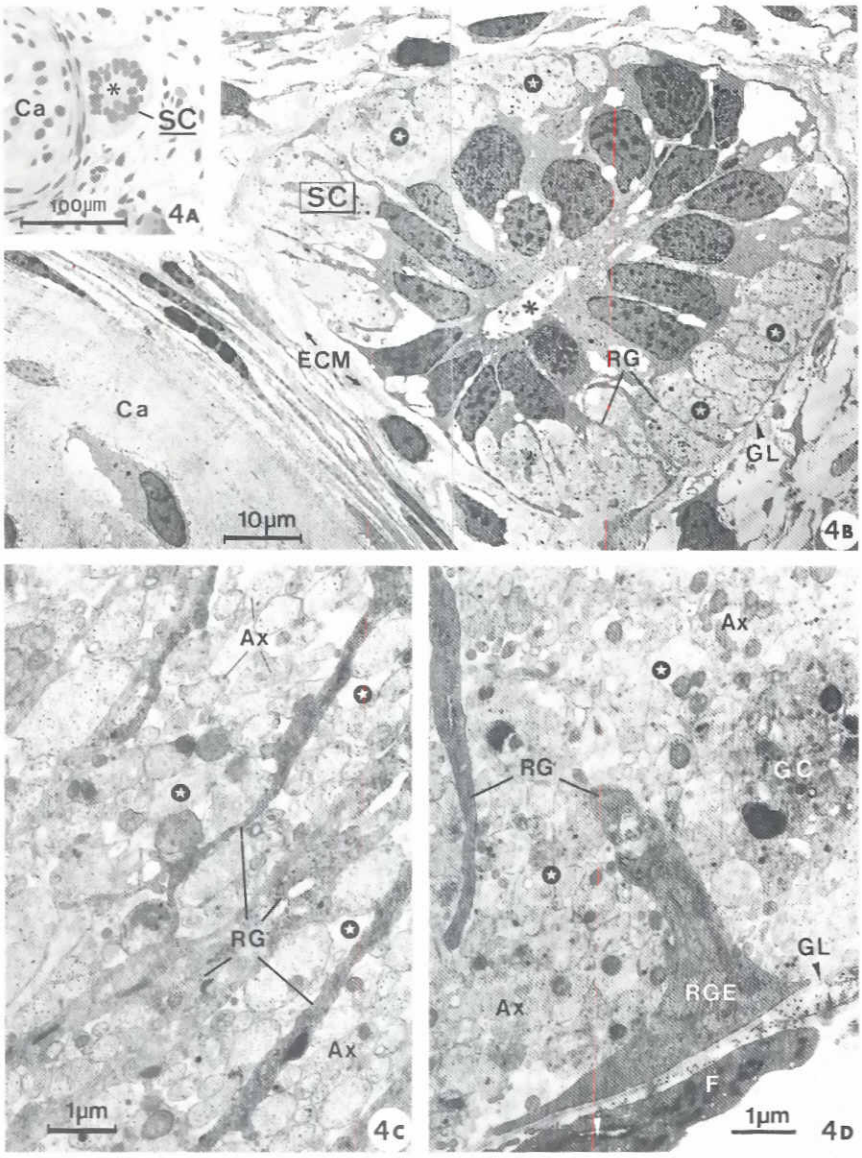

Fig. 4. Structural features of the regenerating SC in a 3 week-old tail regenerate (A) *Light micrograph showing the regenerating SC in the vicinity of the cartilaginous rod (Ca). Asterisk, ependymal canal. x20.* **(B)** *Electron micrograph equivalent to picture shown in* **(A)**. *The ependymoglial cytoarchitecture of the regenerating SC can be seen clearly. Ciliated ependymoglial cell bodies, lining the central canal (asterisk), extend radial glial processes (RG), at the glia limitans (GL) in marginal endfeet that define compartments (stars) occupied by growing axons. Ca, cartilage; ECM, extracellular matrix. x1800.* **(C)** *Enlarged part of* **(B)** *showing axonal compartments (stars) formed by thin elongated radial glial processes (RG). Ax: axons. x12600.* **(D)** *Another enlarged area of* **(B)** *to show that RG profiles form marginal endfeet (RGE), at the glia limitans (GL). Axonal compartments (stars) are here occupied by numerous sections of axons, and even that of a growth cone (GC). Note also, near the SC, the presence of an ECM-secreting fibroblast (F) in which can be seen collagen fibrils (arrowhead) in a deep cytoplasmic recess. x12500.*

1990; Mège *et al.*, 1992) and higher vertebrates (Chiquet and Fambrough, 1984; Grumet *et al.*, 1985). Tn is expressed in the SC and brain of adult *P. waltl* as a major band corresponding to a molecular weight around 200-210 kDa, thereby confirming that this *Xenopus* anti-Tn antibody suitably cross-reacts with Tn polypeptides present in the CNS of *P. waltl*.

Using this antibody against Tn, immunohistochemical study has shown that a similar pattern of Tn expression is seen in the regenerating as well as in the developing caudal SC. At this observation level, Tn appeared mainly accumulated in the SC white matter, a much weaker staining being found around the ependymoglial cells. Interestingly, we observed that a high Tn accumulation level in the white matter was sustained in the SC of adult newts, which prompts some delicate questioning the role that Tn may play at this developmental stage.

At the electron microscope level, immunogold staining provided interesting detailed informations regarding the source of Tn, during caudal SC regeneration, by revealing that this molecule is largely, if not exclusively, elaborated within organelles of radial ependymoglial processes defining axonal compartments. Furthermore, in our preparations, Tn appeared largely distributed along the plasma membrane, especially on the surface of these cells, but not very frequently in the extracellular space between them and axons. Therefore, the extensive Tn immunoreactivity seen in the SC white matter, under the fluorescence microscope, might be due to the abundance of Tn-positive radial processes in close association with axons rather than to an especially heavy accumulation of Tn in the axonal compartments themselves. It is noteworthy that these findings agree with immunoelectron peroxidase (Steindler *et al.*, 1989) and *in situ* hybridization (Prieto *et al.*, 1990) studies which have led the authors to the two following conclusions: (1) a subpopulation of glial cells produces most of the Tn/J1/Cytotactin found in the CNS structures of chicken or mouse respectively; (2) the protein may attach to the glial cell surfaces, after its release into the extracellular space, which was consistent with observations that glia can bind Tn (Friedlander *et al.*, 1988). It is also interesting to recall that Tn/J1/Cytotactin is detectable both in the supernatant and on the cell surface of cultured astrocytes, and mediates

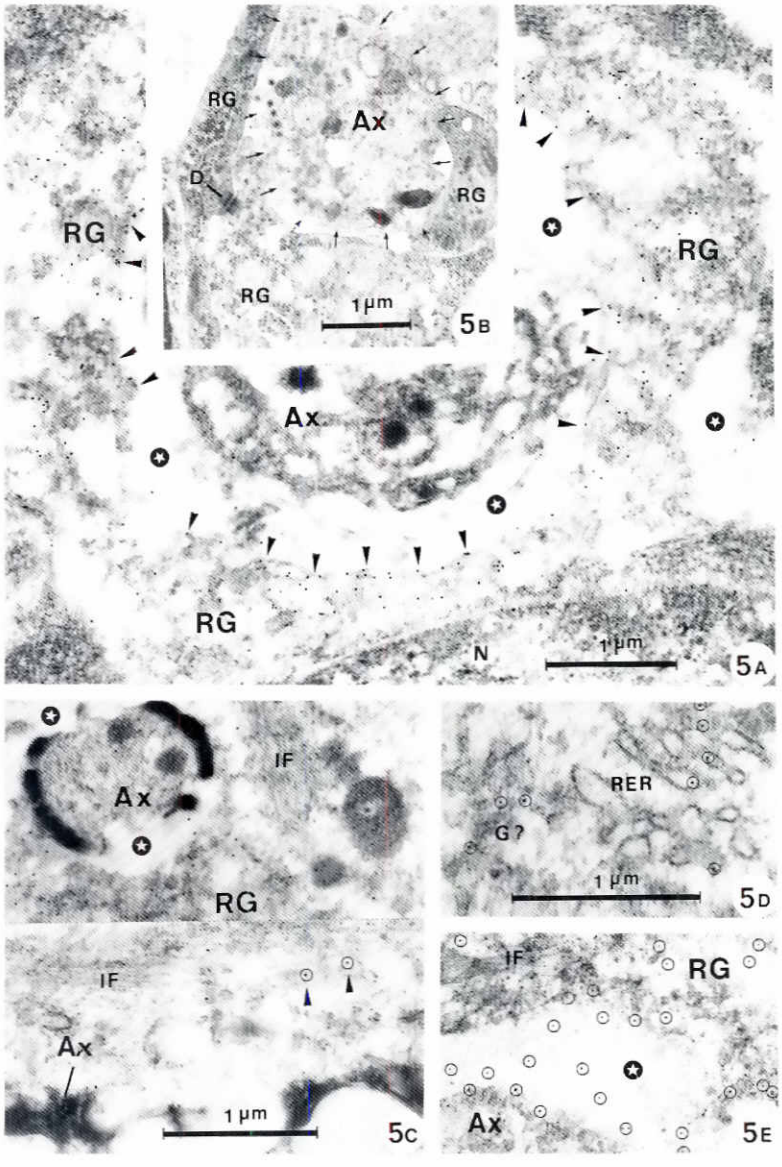

Fig. 5. Immunoelectron gold localization of Tn in 6 to 10 week-old regenerating caudal SC. (A) *Gold particles are seen throughout the cytoplasmic matrix of radial glial (RG) processes surrounding an axonal (or growth cone) section (Ax and arrows). Gold immunostaining is especially observed near and along the glial membrane (arrowheads). Stars, axonal compartments; N, nucleus. x16000.* **(B)** *Electron microscopic view similar to* **(A)**, *for a better interpretation of immunogold staining in* **(A)**. *RG, radial glial processes; Ax, axonal section; D, desmosome. x25000.* **(C)** *Part of another radial glial (RG) extension in close association with axons (Ax), which occur here myelinated. Gold particles (in circles) can be clearly localized within large vesicles (arrowheads). IF, intermediate filaments, i.e., here, Glial Fibrillary Acidic Protein filaments. x30000.* **(D)** *Enlarged part of radial glial cell showing gold particles within distended rough endoplasmic reticulum (RER) and putative Golgi cisternae (G). x37000.* **(E)** *Immunogold particles can be observed here, not only in the radial glial cell (RG), especially at its surface, but also in the extracellular space (star) between this cell and the neighboring axon (Ax). Note that some gold particles appear also located on the neuronal membrane. IF, intermediate filaments. x25000.*

neuron-glia adhesion *in vitro* (Grumet *et al.*, 1985; Kruse *et al.*, 1985; Faissner *et al.*, 1988) However, because it is important to specify how Tn may play a role in the CNS, especially in caudal SC regeneration in newts, further investigation, including new studies on fine localization of Tn, is needed.

Northern blot and RNase protection analyses showed a global increase of Tn transcripts during regeneration compared with corresponding normal tissues of the tail. These molecular data are consistent with our previous study on the pattern of Tn expression, increasing especially in the mesenchyme of the regenerating tail (Arsanto *et al.*, 1990). In the present work, *in situ* hybridization experiments performed during the early stages of regeneration (3 weeks after amputation) have shown that the glial cells expressed Tn transcripts all along the regenerating ependymal tube. An accumulation of Tn transcripts was also observed in the distal part of the mesenchyme, in the inner layer of the wound epidermis and in regions of cartilage condensation. These observations agree with those of Onda *et al.* (1991) in regenerating newt limb. Our observations show that the expression of Tn is associated with the newt tail SC regeneration in accordance with the suspected role of this molecule in glial and neuronal cell interactions during development of higher vertebrates (Grumet *et al.*, 1985; Kruse *et al.*, 1985; Crossin *et al.*, 1986; Tan *et al.*, 1987; Epperlein *et al.*, 1988; Mackie *et al.*, 1988; Lotz *et al.*, 1989, Grierson *et al.*, 1990, Bartsch *et al.*, 1992; Erickson, 1993; Wehrle-Haller and Chiquet, 1993). The plasticity of the SC in *Pleurodeles waltl* might involve the same interaction processes considered to be essential for spatial positioning of neurons (migration and attachment), neuronal process outgrowth and synaptic differentiation (for review, see e.g., Hynes and Lander, 1992) since regeneration reproduces the normal structure. However, our observations could not specify the exact involvement of Tn in adhesion mechanism during this epimorphic process in respect to *in vitro* studies on higher vertebrate cell systems (Tan *et al.*, 1987; Mackie *et al.*, 1988; Halfter *et al.*, 1989; Spring *et al.*, 1989; Faissner and Kruse, 1990; Grierson *et al.*, 1990; Perez and Halfter, 1993) which have demonstrated the dualistic

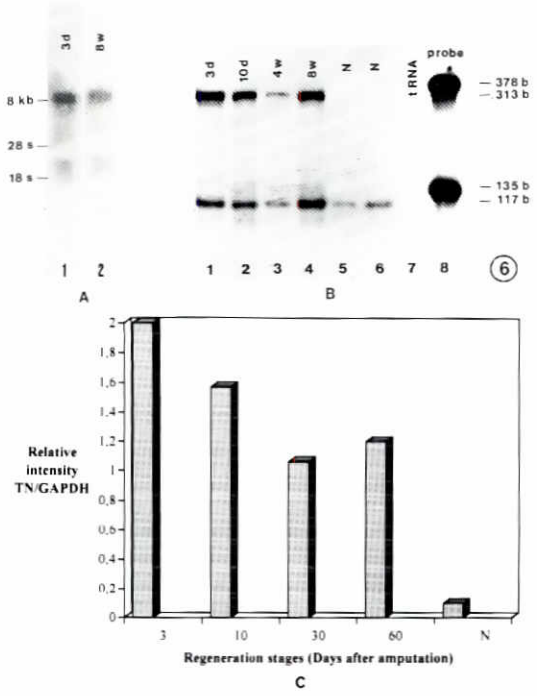

Fig. 6. Tn mRNA expression. (A) *Northern blot analysis with NvTN1 DNA probe. The probe was hybridized to Northern blots of total RNA from P. waltl tail regenerates of 3 days (lane 1) and 8 weeks (lane 2). A strong signal corresponding to a transcript size of 8 kb is seen. The levels of 18S and 28S RNA are indicated.* **(B)** *Detection of Tn transcripts during tail regeneration by RNAase protection analysis. For each regeneration stage, total RNA was hybridized with the Tn and GAPDH probes. Lanes 1-4, tail regenerates of 3 days, 10 days, 4 and 8 weeks after amputation, respectively. Tn transcripts are detected in high amounts from 3 days (lane 1) to 8 weeks (lane 4) regeneration stages (overnight exposure). Lanes 5-6, normal tail of adult newt. Only a faint signal is observed in adult newt tail extract after long exposure of the autoradiograms. Lane 7, tRNA negative control with tRNA alone. Lane 8, probes alone.* **(C)** *Histogram of the relative amount of Tn mRNA/GAPDH mRNA in different stages of tail regeneration. Autoradiograms were scanned by densitometer and the relative intensity of Tn bands was normalized to the density of GAPDH bands in each lane. TN mRNA were more transcribed in tissues of the regenerate than in normal tissues of the tail. Increase of Tn mRNA transcription is an early process induced by trauma.*

property of Tn (for review see e.g,. Wehrle-Haller and Chiquet, 1993). However, it would be reasonable to suspect a role in shaping the neural boundaries and anatomical pattern as proposed for Tn in different areas of brain and SC (Crossin *et al.*, 1989; Steindler *et al.*, 1989, and Steindler, 1993, for review). Indeed, *in vivo* distribution of Tn has been correlated with developmental growth, with the maintenance of neuronal fiber tracts (Rieger *et al.*, 1986; Tan *et al.*, 1987; Mackie *et al.*, 1988; Crossin *et al.*, 1989) and with nerve regeneration (Sanes *et al.*, 1986; Martini *et al.*, 1990; Mège *et al.*, 1992). During newt tail regeneration, while all the structures are progressively re-established within the regenerate, the regenerated ependymal tube acts as a primordial axial structure which allows the growth of axonal tracts from the stump, the positioning of newly differentiated motoneurons and the initiation of the PNS formation: ventral root emergence and spinal ganglia (Arsanto *et al.*, 1992). Tn expression in the rostrocaudal "channels" formed by the radial expansions of the glioependymal cells could give a biochemical support to primary tridimensional structure of the SC according to the blueprint hypothesis formulated by Singer *et al.* (1979). The sustained expression of Tn and its up-regulation during regeneration in the SC of *Pleurodeles waltl*, as well as that of Tn transcripts in glioependymal cells, might be considered as a "prerequisite property of the ependymal program expected in this non-neuronal cellular scaffolding of the SC" (Cooke, 1980).

Expression of Tn in the SC glioependymal tube may play a role in growth and regeneration following axotomy, as proposed for adhesion molecules such as polysialylated N-CAM during development (for review, see Edelman, 1986), remodeling processes in mammal adult brain (for references, see Bonfanti *et al.*, 1992) and regeneration, in newts, either of the retinotectal system (Becker *et al.*, 1993) or of the caudal SC (Caubit *et al.*, 1993).

Materials and Methods

Animal surgery procedure

The urodelean amphibia used in this study were larvae and adults of the European newt, *Pleurodeles waltl.*. These animals were obtained from the C.N.R.S. Amphibian Farm, Centre de Biologie du Développement, Université Paul Sabatier, Toulouse, France. Newts were reared in groups of 10-12 and maintained in circulating tap water thermostated at 18-20°C; the water was completely renewed twice a week. *P. waltl* were fed twice a week with beef heart or liver. Larvae were obtained from egg layings in the laboratory. Before surgery, adult animals were anesthetized with 1:1000 MS 222 (tricaine methane sulfonate, Sigma). Amputations were performed in the third rostral part of the tail, at the level of an intervertebral junction. Operated animals were observed daily and staged according to Iten and Bryant (1976) and our previous work (Thouveny *et al.*, 1991; Caubit *et al.*, 1993). After appropriate periods of regeneration, the blastema were harvested by reamputation. Samples covering the five stages of tail regeneration were used in this study.

Histology

For histological study, newt tail regenerates were cut off along with a few millimeters of the stump and fixed in Carnoy's fluid, dehydrated with ethanol and toluene, and embedded in 56°C paraffin. Serial sections (6 µm) were stained with groat hematoxylin, eosin, and the aniline blue technique.

Electron microscopy

Tissue samples were fixed for 2.5 h in 2.5 % glutaraldehyde, 4% formaldehyde in 0.1 M cacodylate buffer (pH 7.4), postfixed for 1 h in osmium tetroxide in the same buffer, dehydrated in acetone and embedded in araldite. Ultrathin sections were stained with uranyl acetate and lead citrate and examined with Hitachi H 600 electron microscope.

Antibodies

In this study a polyclonal antibody directed against Tenascin of the anuran amphibian *Xenopus laevis* was used. The production and specificity of IgGs have been reported previously (Riou *et al.*, 1991). Cross-reactivity of this antibody with urodele amphibian antigens was controlled by Western blots.

SDS/PAGE and Immunoblot analysis

Immediately after dissection, samples of *P. waltl* tissues (brain, spinal cord and tail regenerates) were homogenized in 7 volumes of 20 mM Tris-HCl, 10 mM EDTA, 0,2% Triton X100, 1 mM Phenylmethyl sulfonyl fluoride (PMSF), 1µg/ml antipain and pepstatin, 15 µg/ml benzamidine pH 8 (antipain, pepstatin and benzamidine were first solubilized in DMSO). The homogenization was carried out on ice in a Dounce homogenizer with a glass pestle for brain, spinal cord and young regenerates, and older regenerates were crushed in an Ultra-Turax T25 tissue grinder for 15-20 s before being homogenized. The samples were then sonicated and centrifuged at 10,000 g for 10 min at 4°C. Proteins present in the supernatant were resolved by electrophoresis on 7% or 5-13% SDS- PAGE (Laemmli, 1970). Proteins were then transferred onto nitrocellulose membrane according to Burnette (1981).

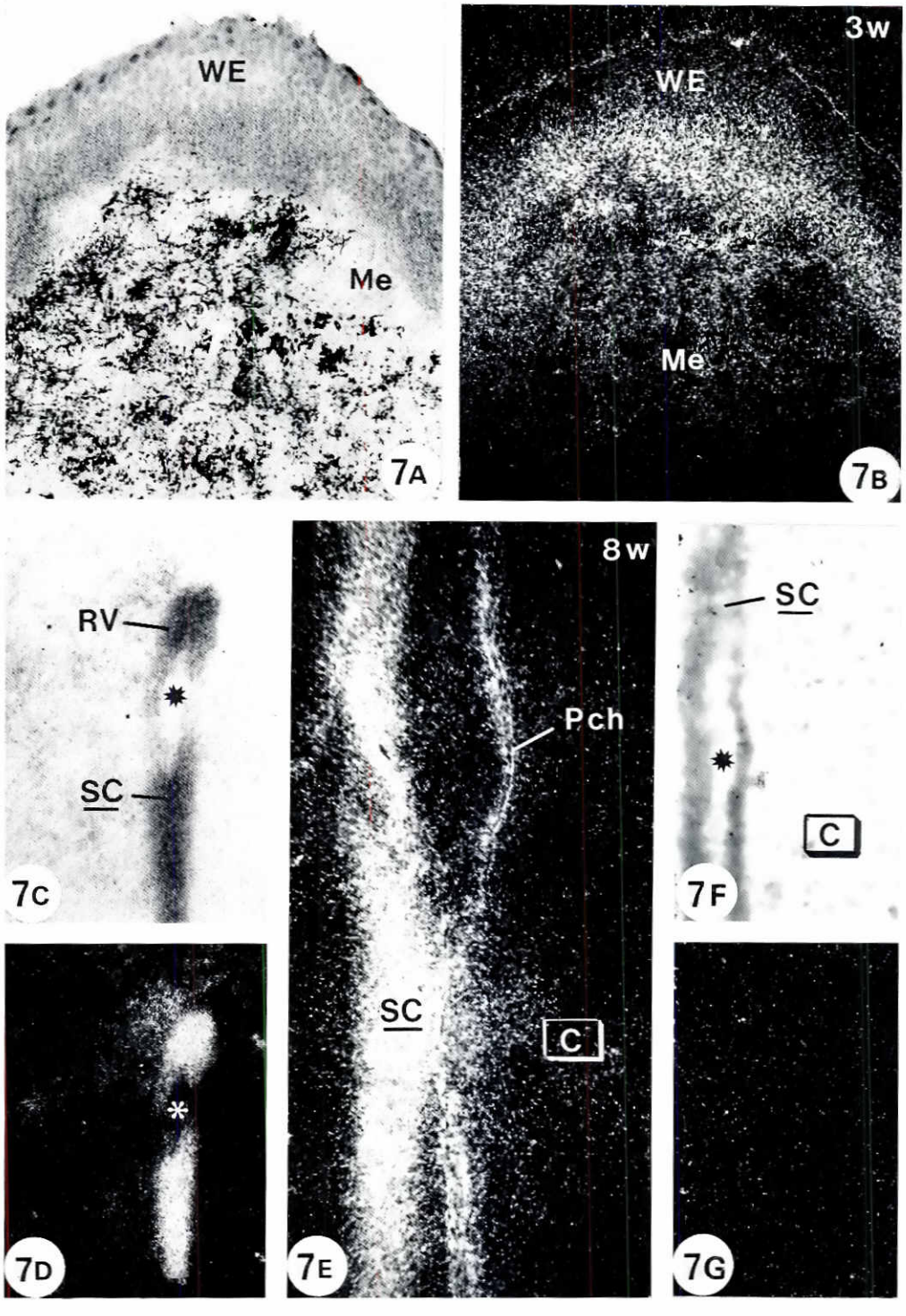

Fig. 7. *In situ* hybridization of Tn mRNA in sections through newt tail regenerates. *Note that all pictures have the same orientation according to the rostrocaudal axis, i.e., from bottom to top.* **(A-B).** *White* **(A)** *and dark* **(B)** *field views of the distal part of a 3 week-old regenerate. A high density of Tn transcripts is seen both in the inner layer of the wound epithelium (WE) and in the mesenchyme underlying the epidermal basal lamina. In* **(A),** *a large network of unlabeled melanocytes is observed in the mesenchyme. x10. White* **(C)** *and dark* **(D)** *field views of the median part of a 3 week-old regenerate. A high accumulation of Tn mRNA is found in the regenerating SC, especially at the level of the distal regenerating vesicle (RV). Asterisk, ependymal canal. x15.* **(E)** *Dark field view of the median part of a 8 week-old regenerate. Tn transcripts are intensely expressed in the regenerating SC and in the perichondrium (Pch) of the developing vertebral body whereas they are either weakly or not expressed at this stage in the cartilaginous matrix (C). x30.* **(F)** *and* **(G).** *White and dark field views of hybridization with labeled sense RNA as controls. x10. Asterisk, ependymal canal.*

Indirect immunofluorescence

Samples were directly embedded unfixed in OCT and frozen in liquid nitrogen. Longitudinal and cross sections of 15 µm were cut in a cryostat at -22°C. They were collected on gelatined slides and stored at -20°C. They were washed 1 h in PBS+1% BSA, then incubated 1 h with primary antibody. FITC-conjugated anti-rabbit IgG was used at 1:100 or 1:200 as secondary antibody. Washed slides were mounted in moviol and observed with epifluorescence Zeiss microscope and photographed on Tri-X pan (Kodak). Controls were made by omitting the first antibody.

Immunocytochemistry

Tissue preparation

To retain the antigenicity of the material, we performed immunocytochemistry on tissues fixed in cold for 1.5 h with Periodate-Lysine-

Formaldehyde (PLP) fixative according to McLean and Nakane (1974) and embedded at low temperature in the water-miscible resin Lowicryl K4M. They were then washed three times for about 10 min in PBS 0.1 M pH 7.4. Tissue processing for Lowicryl was performed according to Roth *et al.* (1981). The pieces were dehydrated in a graded ethanol series and gradually infiltrated at -20°C with Lowicryl resin. Polymerization at -20°C was brought on by ultraviolet irradiation for about 48 h.

Immunogold staining (IGS)

Ultrathin sections obtained from Lowicryl-embedded tissue were attached to Formvar-coated copper grids for colloidal gold immunolabeling. Grids were floated for 5 min on a large drop of 0.5% BSA in PBS, and washed with PBS. The grids were then transferred onto a drop of the specific anti-Tn polyclonal antibody at a 1:10 dilution with 0.5% BSA in PBS for 2 h at room temperature. After washing in PBS, the grids were incubated for 1 h at room temperature with protein A coupled to 15 nm colloidal gold (Tébu, Biocell Research Lab.), diluted 1/20 in 0. 5% BSA in PBS.

Ultrathin sections were subsequently stained with uranyl acetate and lead citrate, and were then viewed in the electron microscope. Control sections were obtained by omitting the first antiserum in this staining procedure. Control grids coated with preimmune serum were also prepared.

Northern-blot analysis and RNAase protection assay

RNA was isolated from frozen tissue by guanidium isothiocyanate extraction followed by CsCl gradient centrifugation (Chirgwin *et al.*, 1979; Sambrook *et al.*, 1989).

For Northern blot analysis, 10 µg of total RNA was fractionated on a 1% agarose/formamide gel and transferred to hybond N+ nylon membranes. The molecular probe used in this study was NvTN.1 previously described by Onda *et al.* (1991). Probe was radiolabeled with α^{32}P-dCTP using a random primer labeling kit (Amersham, UK) following the manufacturer's recommendations. The filter was hybridized with 1x106 cts/min/ml of radiolabeled probe at 42°C in 50% formamide; 5x SSPE; 0.5% SDS; 5x Denhardt's solution; 100 mg/ml salmon sperm DNA. Stringent washes were carried out in 2x SSC; 0.1% SDS at 58°C for 2x20 minutes. The filter was exposed to Kodak XAR film at -70°C with intensifying screens.

RNAase protection assays were carried out according to Krieg and Melton (1987) with minor modifications. A 350-base "Antisense" riboprobe labeled with (α^{32}P)UTP of NvTN1 was obtained as follows: pBluescript plasmid containing the Tn probe was transcribed by T3 RNA polymerase after having been linearized with Bst EII. A homologous probe of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Shi *et al.*, 1992) was used for control of RNA equivalent. The plasmid containing GAPDH insert has been linearized by Sty I and transcribed by T7 RNA polymerase. A 135-base "anti-sense" riboprobe was labeled under the same conditions. Full length probes were purified from a 0.4 mm thin polyacrylamide gel by elution at 37°C in 0.3 M Na acetate, 0.5% SDS, 2 mM EDTA, 20 µg/ml tRNA. Hybridization was carried out in the presence of 80% formamide, 0.4 M NaCl, 40 mM PIPES and 1 mM EDTA a 50°C for 36 h. The samples were digested by 1.75 µg RNAse T1 at 30°C for 1 h followed by proteinase K at 37°C for 15 min. After phenol/chloroform extraction and ethanol precipitation, the protected fragments were resolved by electrophoresis on a 5% polyacrylamide gel, and exposed to Kodak X-OMAT AR film with intensifying screens at -80°C.

In situ hybridization

Constructions for sens and antisens riboprobes were obtained according to the procedure described by Onda *et al.* (1991). Linearized template was transcribed with the appropriate RNA polymerase according to the manufacturer's protocol (Stratagene) in the presence of (α^{32}P)UTP. Transcribed products were isolated by ethanol precipitation. A protocol for *in situ* hybridization was adapted from *"Current Protocols in Molecular Biology"* (Ausubel *et al.*, 1987) with minor modifications. Regenerates of different stages were dissected and immediately frozen in OCT compound (Miles, Elkart, IN, USA). 10 µm cryostat sections were mounted on gelatin-coated slides. Prior to hybridization, sections were fixed with 4% paraformaldehyde, treated with proteinase K (10 µg/ml) for 15 min at 37°C, acetylated by immersing slides in 0.25% acetic anhydride in 0.1 M triethanolamine buffer, pH 8, for 10 min, and dehydrated through graded ethanol solutions. The hybridizations were carried out at 45°C in hybridization MIX (50% formamide, 0.6 M NaCl, 10 mM Tris-HCl, 1X Denhardt's solution, pH 7,5) with either sens or antisens riboprobes at concentration of $1x10^7$ cts/min/ml for 20 h. Slides were washed three times in 50% formamide, 2 SSC for 15 min at 50°C and treated with RNAse T1 (2 µg/ml) for 30 min at 37°C. Slides were further washed twice in 0.1 SSC for 30 min each at 50°C. Dehydrated sections were dipped in Kodak NTB-2 emulsion diluted 1:1; they were exposed 10-15 days at 4°C and developed in D19b, then stained in Giemsa diluted 1:10 in PBS.

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