

# Long and Short Splice Variants of Human Tenascin Differentially Regulate Neurite Outgrowth

Sally Meiners and Herbert M. Geller

Department of Pharmacology, UMDNJ-Robert Wood Johnson Medical School,  
Piscataway, New Jersey 08854

Tenascin-C has been implicated in regulation of neurite outgrowth both during development and after injury; however, its role as permissive vs inhibitory remains controversial. We report that different tenascin splice variants may have dramatically different impacts on neuronal growth. In a cell culture model, the largest and smallest splice variants (TN.L and TN.S) of human tenascin both promoted process extension when surface-bound. In contrast, soluble TN.S inhibited outgrowth, whereas soluble TN.L had no inhibitory effect. Perturbation experiments with antibodies, and outgrowth experiments with recombinant tenascin fragments, indicate that the differential properties of these molecules can be attributed to their distinctive array of FN-III repeats. Monoclonal antibodies were used to demonstrate at least two distinct neurite outgrowth promoting domains within the alternatively spliced region. These results suggest that the effect of tenascin on axon growth is a function of splice variants, as well as the form or conformation of those variants.

## INTRODUCTION

Interactions between neurons and extracellular matrix molecules have profound implications in both development and regeneration (reviewed in Letourneau *et al.*, 1994). Tenascin-C (hereafter referred to as tenascin) is a large extracellular matrix protein found in many embryonic tissues, including brain and spinal cord, and a few adult tissues (Crossin *et al.*, 1989; Erickson and Bourdon, 1989; Steindler *et al.*, 1989; Pindzola *et al.*, 1993; Zhang *et al.*, 1995a). In the central nervous system (CNS),<sup>1</sup> tenascin is transiently expressed by astrocytes

during embryonic and early postnatal development (Laywell *et al.*, 1992) and is reexpressed following injury (McKeon *et al.*, 1991; Pindzola *et al.*, 1993). However, functional studies *in vivo* and *in vitro* fail to provide a consistent picture of tenascin as either permissive or inhibitory to neuronal growth and regeneration.

The putative barrier-forming role of tenascin is supported by its localization at the boundaries of migratory pathways in the developing rat cortex, where it is hypothesized to direct neuronal targeting (Steindler *et al.*, 1989). However, it appears that neurites are found in these regions before tenascin, suggesting that other molecules may participate in the targeting process (Bartsch *et al.*, 1992) or alternatively, that neurites may provide an inductive signal for tenascin expression (O'Brien *et al.*, 1992). The role of tenascin after injury is also controversial. Recent work (Gates *et al.*, 1996) strongly suggests that tenascin inhibits neurite regeneration after injury. When living striatal scars from adult mice were removed before or after upregulation of tenascin and used as *in vitro* substrates for embryonic neurons, neurite outgrowth decreased as tenascin was upregulated. Furthermore, blocking tenascin with antibodies increased neurite growth. Other recent work (Zhang *et al.*, 1995b) supports the opposite hypothesis, that the molecule may stimulate axonal regeneration. In this study, regenerating CNS axons grew into a peripheral nervous system autograft transplanted into the thalamus, even though Schwann cells within the graft expressed much higher levels of tenascin than did surrounding brain tissue. These investigations raise the possibility that tenascin has different, and even opposing, effects on neurite regeneration depending upon the context of its presentation or the presence of distinct receptors for tenascin on different neurons.

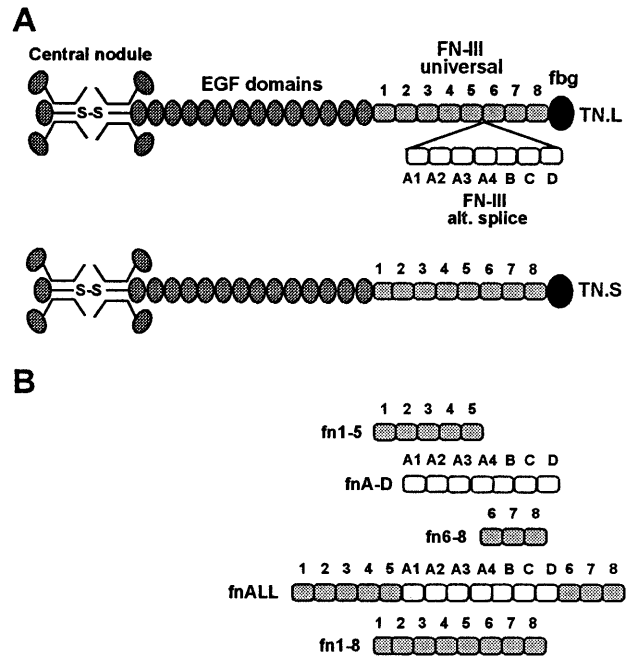
Results from functional experiments *in vitro* also lead to a conflicting picture of the role of tenascin. For

<sup>1</sup> Abbreviations used: CFDA, carboxy fluorescein diacetate; CNS, central nervous system; EGF, epidermal growth factor; fbg, fibrinogen; FN-III, fibronectin type III; fn, FN-III domain; mAb, monoclonal antibody; TN.L, large tenascin splice variant; TN.S, small tenascin splice variant.

example, tenascin has been reported to both promote and inhibit adhesion and outgrowth of several types of CNS neurons *in vitro* (Faissner and Kruse, 1990). The method of presentation also appears to affect its action: substrate bound tenascin promotes outgrowth (Wehrle and Chiquet, 1990; Lochter *et al.*, 1991; Wehrle-Haller and Chiquet, 1993; Chiquet and Wehrle-Haller, 1994), whereas soluble tenascin is inhibitory (Lochter *et al.*, 1991). However, when neurites are presented with a choice of matrix molecules, they generally avoid tenascin in preference for laminin or fibronectin (Faissner and Kruse, 1990). Our own work has shown that a subtype of type-1 cerebral cortical astrocytes which are high in tenascin impair neurite outgrowth (Meiners *et al.*, 1995); however, these astrocytes also express high levels of the inhibitory molecule chondroitin-6-sulfate proteoglycan, which may be more directly responsible for the restrictive phenotype.

The tenascin knockout mouse, with its largely normal phenotype (Saga *et al.*, 1992; Forsberg *et al.*, 1996), has further confounded the story, although recent reports indicate that the knockout mice are not totally normal. For example, knockout mice demonstrate a greater number of reactive astrocytes associated with stab wound lesions to the brain in comparison to wild-type mice (Steindler *et al.*, 1995). Furthermore, a recent study (Fukamaki *et al.*, 1996) has indicated that knockout mice exhibit motor coordination problems, which, as Crossin (1996) has pointed out, would be consistent with a role for tenascin in development of the cerebellar and cerebral cortices. Moreover, the absence of a morphological difference following elimination of a molecule does not imply that the molecule has no function when it is present.

Tenascin is structurally complicated, which may account for its conflicting activities. Tenascin is comprised of six arms; each arm is comprised of a linear array of epidermal growth factor (EGF) domains, fibronectin type III (FN-III) domains, and a single fibrinogen-like (fbg) domain. The number of domains depends on species and alternative RNA splicing; the multidomain structure of human tenascin is depicted in Fig. 1. Various functions have been mapped to specific domains; e.g., neurite outgrowth and cellular migration are apparently influenced through different FN-III domains (Husmann *et al.*, 1992). Other studies have demonstrated that tenascin has at least four independent cell binding regions, two of which appear to be adhesive (mapped to FN-III domain 3 and to the fbg domain) (Spring *et al.*, 1989; Prieto *et al.*, 1992; Aukhil *et al.*, 1993; Joshi *et al.*, 1993; Prieto *et al.*, 1993) and two of which



**FIG. 1.** Multidomain structure of human tenascin. (A) This diagram is adapted from Aukhil *et al.* (1993). The N termini of three arms are joined to form a trimer, and two trimers are connected via a disulfide bond to form a hexamer. Each arm consists of 14 EGF domains, 8–15 FN-III domains depending on alternative RNA splicing, and a single fibrinogen (fbg) domain. The universal FN-III domains (fn1–5 and fn6–8) are present in all tenascin splice variants. The large tenascin splice variant, TN.L, contains alternatively spliced FN-III domains designated fnA–D; these domains are missing from the small splice variant, TN.S. (B) Tenascin FN-III bacterial expression proteins used in this study are illustrated and correspond to universal FN-III domains 1–5 and 6–8 (fn1–5 and fn6–8), alternatively spliced FN-III domains A–D (fnA–D), or to the entire FN-III sequences of TN.L or TN.S (fnALL and fn1–8, respectively).

appear to be counteradhesive (mapped to the EGF domains and to FN-III domains 7–8) (Spring *et al.*, 1989; Prieto *et al.*, 1992). The fbg domain has also been implicated in heparin binding (Aukhil *et al.*, 1993).

To add to this complexity, tenascin occurs in multiple forms generated by alternative RNA splicing which differ in the number of FN-III domains (Jones *et al.*, 1989) (Fig. 1). Following the nomenclature of Aukhil *et al.* (1993), the universal FN-III domains (those present in all species and splice variants) are numbered 1–5 and 6–8; they are referred to in this study as fn1–5 and fn6–8. The alternatively spliced domains, which are inserted in between universal domains, are designated by letters. For example, the largest splice variant of human tenascin, human TN.L, has 7 alternatively spliced FN-III domains (designated A1, A2, A3, A4, B, C, and D, or fnA–D) that are missing in the shortest splice variant,

human TN.S (Fig. 1). Tissue distribution of the splice variants appears specific and under tight developmental control; e.g., in developing chick spinal cord, large tenascin isoforms are restricted to a subpopulation of glial cells within the cord, whereas small tenascin isoforms are restricted to surrounding connective tissue (Tucker, 1993). In developing chick cerebellum, only TN.L is expressed (Prieto *et al.*, 1990). This raises the possibility that different variants could serve different functions. Indeed, phases of increased cell migration and axon growth in the developing CNS have been closely correlated with expression of TN.L but not TN.S (Crossin *et al.*, 1989; Steindler *et al.*, 1989; Kaplony *et al.*, 1991; Bartsch *et al.*, 1992), suggesting that the alternatively spliced domains included only in TN.L might facilitate cell and neurite motility during embryogenesis.

While various functions have been attributed to the different domains of tenascin, these data have largely come from studies using purified tenascin substrates. Although these data are informative, such models are clear oversimplifications of the *in vivo* situation, where tenascin is only one of many different guidance molecules encountered by a neuron. Moreover, cells that produce tenascin are likely to secrete it into their environment (Mitashov *et al.*, 1995), which provides for yet another mechanism of neuronal guidance by the soluble molecule. To this point, soluble tenascin inhibited neurite outgrowth on substrate-bound tenascin, laminin, or fibronectin (Lochter *et al.*, 1991).

In this communication, we have therefore investigated the hypothesis that tenascin splice variants, expressed in the context of a cell, differentially regulate neurite growth. We utilized baby hamster kidney (BHK) cells transfected with TN.L or TN.S, which produce both cellular and secreted components of human tenascin. To determine whether the splice variants exerted their effects in a substrate-bound or a soluble form, TN.L and TN.S were purified and added to untransfected BHK cells. Both splice variants bound to the cell surface under these conditions. The role of the FN-III domains was investigated in neurite outgrowth studies by employing polyclonal antibodies to universal and alternatively spliced sequences, as well as bacterial expression proteins corresponding to defined FN-III regions. The contribution of alternatively spliced FN-III domains was further investigated in perturbation experiments with monoclonal antibodies specific for this region. Our results indicate that while both variants promoted outgrowth when bound, the inhibitory actions of soluble tenascin are due to TN.S; TN.L has no effect. Moreover, FN-III domains in both constitutively expressed and

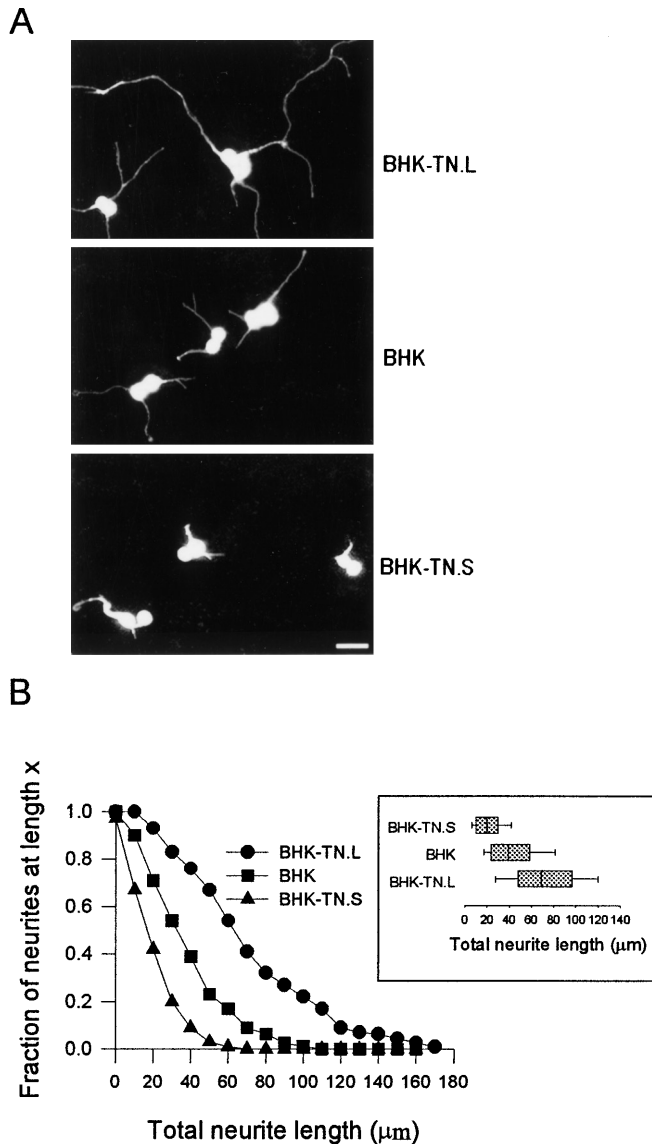
isoform-specific sequences are involved in regulation of neurite outgrowth, supporting a role for alternative splicing of tenascin in neurite growth and regeneration. Portions of this work have previously been presented in abstract form (American Society for Cell Biology, Washington, DC, 1995; International Meeting on Brain Extracellular Matrix, Ascona, Switzerland, 1995; Society for Neuroscience, Washington DC, 1996).

## RESULTS

### ***Neurite Outgrowth Is Enhanced on Cells Transfected with TN.L and Reduced on Cells Transfected with TN-S***

To explore the role of tenascin splice variants in the regulation of process outgrowth, dissociated E17 cerebral cortical neurons were plated onto monolayers of BHK cells transfected with TN.L or TN.S (BHK-TN.L or BHK-TN.S cells). After 24 h neurons were selectively labeled with CFDA (Petroski and Geller, 1994). The difference in appearance of neurites on BHK-TN.L and BHK-TN.S cells was striking: extensive neurite outgrowth occurred on BHK-TN.L cells in comparison to untransfected controls (Fig. 2A), whereas neurites were short and stubby on BHK-TN.S cells.

Neurite outgrowth assays were conducted to quantify process extension. Cumulative frequency histograms of total neurite length were constructed (Fig. 2B). Data are also presented in the form of a box-and-whisker plot (Fig. 2B, inset). Boxes enclose 25th and 75th percentiles of each distribution and are bisected by the median; whiskers indicate 5th and 95th percentiles. Outgrowth was significantly higher on BHK-TN.L cells as compared to BHK cells, and significantly lower on BHK-TN.S cells (Fig. 2B). Median total neurite lengths were 68, 39, and 20  $\mu\text{m}$  on BHK-TN.L, BHK, and BHK-TN.S cells, respectively. Polyclonal antibodies against full-length tenascin blocked both the permissive properties of BHK-TN.L cells and the inhibitory properties of BHK-TN.S (data not shown), indicating that the effects were due to tenascin and not to some other factor produced by the transfected cells. In other experiments, similar outgrowth values were obtained using E17 hypothalamic neurons (data not shown); hence the growth promoting properties of TN.L and the restrictive properties of TN.S are applicable to more than one type of CNS neuron. These data suggest that cells that make and secrete tenascin will have different effects on neurite outgrowth depending upon the ratio of tenascin isoforms produced.



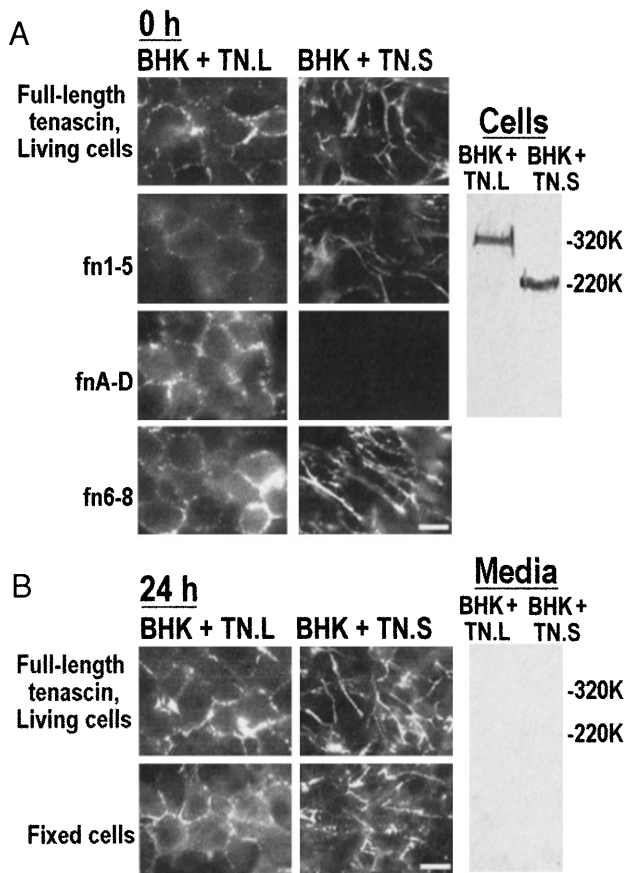
**FIG. 2.** Neurite outgrowth is enhanced on cells transfected with TN.L and reduced on cells transfected with TN.S. (A) Cerebral cortical neurons were allowed to extend neurites on BHK-TN.L, BHK, or BHK-TN.S cells for 24 h. Neurites were visibly longer on BHK-TN.L cells and visibly shorter on BHK-TN.S cells in comparison to controls. (B) Cumulative frequency histograms of the total neurite length were plotted as the fraction of neurites longer than a specific length measured in micrometers. A sample of 100 neurons was considered for each condition. One representative experiment of a total of 5 experiments is shown. Outgrowth was significantly greater on BHK-TN.L cells and significantly reduced on BHK-TN.S cells when compared to BHK cells ( $P < 0.05$ ; Kolmogorov-Smirnov test). Data are also presented in the form of a box-and-whisker plot (inset). Boxes enclose 25th and 75th percentiles of each distribution and are bisected by the median; whiskers indicate 5th and 95th percentiles.

### Bound and Soluble TN.L and TN.S Differentially Influence Neurite Outgrowth

Transfected BHK cells express tenascin on their surface (Chung *et al.*, 1995) and also secrete about  $3 \mu\text{g}/\text{ml}$  TN.L or TN.S into the media per 24-h period (data not shown; see also Aukhil *et al.*, 1993). To determine whether the splice variants exerted their effects on neurite outgrowth in a substrate-bound or a soluble form, TN.L and TN.S were purified from transfected cells and then added to untransfected cultures. After a 6-h incubation period at  $37^\circ\text{C}$ , bound tenascin was visualized on living cells using polyclonal antibodies against full-length tenascin, universal domains fn1-5 or fn6-8/fbg, or alternatively spliced domains fnA-D. BHK cells incubated with TN.L revealed punctate immunoreactivity with all four antibodies (Fig. 3A), indicating that some or all of the universal and alternatively spliced domains were exposed on the cell surface. BHK cells incubated with TN.S revealed intense fibrillar immunoreactivity with full-length tenascin, fn1-5, and fn6-8/fbg antibodies only. No staining was detected with the fnA-D antibody, which was to be expected since TN.S lacks the alternatively spliced domains A-D. Untransfected BHK cells did not show any staining with any of the antibodies (data not shown). The different patterns of immunoreactivity for bound TN.L and bound TN.S suggest that the variants adopt different conformations within a preformed matrix and were reminiscent of TN.L and TN.S localization reported in transfected BHK cells (Chung *et al.*, 1995). Differential affinities for fibronectin or other cell surface binding sites are very likely at the base of the differential staining patterns: the short splice variant binds preferentially to fibronectin fibrils on BHK cells, apparently via a primary site in fn3 and a weaker site in fn6-8 (Chung *et al.*, 1995), whereas the long splice variant binds preferentially to annexin II on BHK cells, via a site in fnA-D (S. Meiners, unpublished data).

BHK cells were also incubated with tenascin for 6 h at  $4^\circ\text{C}$  instead of  $37^\circ\text{C}$  due to the fact that a small amount of tenascin was internalized by the cells at  $37^\circ\text{C}$  (data not shown). The intensity of surface staining was indistinguishable at the two temperatures, but no internalization of protein occurred at  $4^\circ\text{C}$  as determined by immunocytochemical analysis of fixed cells (data not shown). This allowed examination of tenascin on Western blots, which revealed similar amounts of bound TN.L and bound TN.S (Fig. 3A).

TN.L or TN.S were bound to BHK cells, the medium was exchanged, and dissociated E17 cerebral cortical



**FIG. 3.** Binding of TN.L and TN.S to untransfected BHK cells. (A) Living cultures were immunostained with full-length tenascin, fn6-8/fbg, fn1-5, or fnA-D antibodies followed by fluorescein-conjugated secondary antibodies. BHK cells incubated with TN.L revealed punctate immunoreactivity with all of the antibodies; BHK cells incubated with TN.S revealed fibrillar immunoreactivity with full-length tenascin, fn6-8/fbg, and fn1-5 antibodies only. Cell extracts (25  $\mu$ g) were analyzed on Western blots probed with full-length tenascin antibody followed by alkaline phosphatase-conjugated secondary antibodies. Blots demonstrated similar amounts of TN.L and TN.S bound to BHK cells at the beginning of the experiment (0 h). Scale bar, 25  $\mu$ m. (B) TN.L and TN.S were bound to BHK cells, and cells were maintained at 37°C for 24 h. Living cultures stained with full-length tenascin antibody revealed that tenascin remained bound to the cells throughout the course of the experiment. Cells permeabilized with acetic acid/ethanol (95%/5%) revealed a small amount of internalized tenascin. Western blots probed with full-length tenascin antibody revealed no detectable tenascin in the media after 24 h.

neurons were plated onto the cultures. The extent of neurite outgrowth was determined at 24 h. Immunocytochemical studies with living cells at 24 h revealed that the proteins remained bound to the cell surface throughout the course of the experiment, and Western blots revealed no detectable tenascin in the media after 24 h (Fig. 3B). Staining of fixed cells revealed a small amount

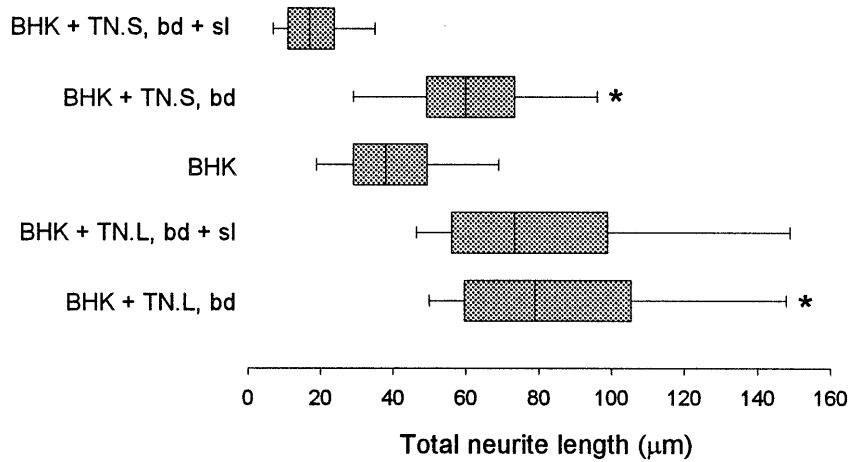
of internalized tenascin, which appeared as small fluorescent spots in the cytoplasm (Fig. 3B).

Both tenascin variants promoted neurite outgrowth in the bound form, suggesting the presence of a common outgrowth promoting site (or sites) (Fig. 4). The extent of neurite outgrowth on bound TN.L was always higher than on bound TN.S; the difference was significant ( $P < 0.05$ ; Kolmogorov-Smirnov test). Therefore, an additional neurite outgrowth promoting site (or sites) may reside within fnA-D, or, alternatively, a common outgrowth promoting site may be more exposed in TN.L than in TN.S due to conformational differences imposed by fnA-D. Polyclonal antibodies against full-length tenascin blocked the promotion of neurite outgrowth by both bound splice variants (data not shown), indicating that the effects on outgrowth were directly attributable to tenascin, as opposed to, for example, tenascin blocking some inhibitory molecule on BHK cells. Furthermore, polyclonal antibodies against fibronectin reduced process extension on BHK cells even in the presence of bound TN.S, suggesting that the diminished enhancement of neurite outgrowth on bound TN.S vs bound TN.L was not due to the blockade of neurite outgrowth promoting sites on fibronectin (data not shown).

The impact of soluble splice variants on neurite extension was assessed by first binding tenascin to the cell surface as above; cerebral cortical neurons were then cultured for 24 h on these cells in medium containing an excess of soluble tenascin. The addition of soluble TN.S impaired outgrowth on bound TN.S. (Fig. 4), whereas the addition of soluble TN.L had no effect. Antibodies to full-length tenascin blocked the impairment of outgrowth by soluble TN.S (data not shown), again suggesting direct involvement of tenascin. The effect of TN.S was dose-dependent with a tendency toward saturation at 10  $\mu$ g/ml, the amount used in all further experiments (data not shown). These results suggest that soluble TN.S is more inhibitory than bound TN.S is permissive, and that soluble TN.L does not influence neurite outgrowth. Hence, an inhibitory site may be exposed on soluble TN.S, but hidden on soluble TN.L due to conformational differences imposed by the alternatively spliced domains. Alternatively, an inhibitory site may be common to both soluble variants, but an additional permissive site (or sites) within the alternatively spliced domains may overcome its effects.

#### Mapping of Neurite Outgrowth Promoting and Inhibiting Domains Using FN-III Antibodies

TN.L and TN.S differ only in their number and arrangement of FN-III domains; other parts of the



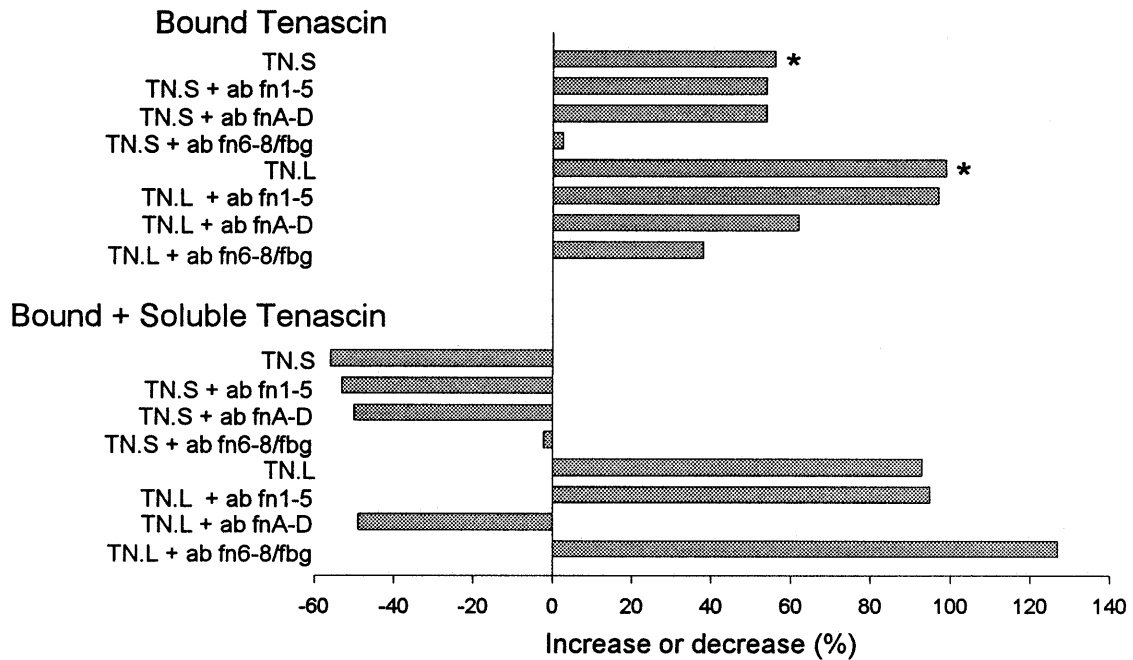
**FIG. 4.** Bound and soluble tenascin splice variants differentially affect neurite growth on a cellular substrate. TN.L and TN.S were bound to BHK cells, and cerebral cortical neurons were plated onto the cultures and allowed to extend processes for 24 h. A sample of 100 neurons was considered for each condition. One representative experiment of five is shown. Box-and-whisker plots for distributions of the total neurite length show that both molecules enhanced neurite outgrowth; outgrowth values were somewhat higher on TN.L even though BHK cells bound similar amounts of both variants. Asterisks indicate that distributions on bound TN.L and bound TN.S were significantly different from each other ( $P < 0.05$ ; Kolmogorov-Smirnov test). In contrast, soluble TN.S negated the permissive effects of bound TN.S and inhibited outgrowth, whereas soluble TN.L had no effect.

molecule are identical. Therefore, we investigated whether the FN-III domains contribute to the differential regulation of neurite outgrowth by substrate-bound and soluble splice variants. First, polyclonal antibodies against full-length tenascin, fn1-5, fnA-D, or fn6-8/fbg were tested for interference with substrate-bound TN.L and TN.S. All of these antibodies cross-react with full-length tenascin on Western blots. The fn1-5 antibody also cross-reacts with a bacterial expression protein corresponding to domains fn1-5 but not with expression proteins corresponding to fnA-D or fn6-8; the fnA-D antibody cross-reacts with an expression protein corresponding to domains fnA-D but not with expression proteins corresponding to fn1-5 or fn6-8; and the fn6-8/fbg antibody cross-reacts with an expression protein corresponding to domain fn6-8 but not with expression proteins corresponding to fn1-5 or fnA-D (data not shown).

E17 cerebral cortical neurons were cultured for 24 h on BHK cells in the presence of bound tenascin or bound tenascin and antibodies. Data (Fig. 5) are plotted as the percentage increase or decrease over the control median length (38 µm for neurons cultured on BHK cells without added tenascin or antibodies). Cumulative frequency distribution plots showed that for any change in median values, neurite lengths for the entire distribution were evenly shifted (data not shown). Neurite outgrowth on bound TN.S was not altered by either the fn1-5 or the fnA-D antibody but was reduced to control

levels in the presence of the fn6-8/fbg antibody; hence bound TN.S does not appear to have any promoting sites outside of the fn6-8/fbg sequence. Neurite outgrowth on bound TN.L also was not altered by the fn1-5 antibody, whereas both fnA-D and fn6-8/fbg antibodies partially reduced its permissive properties. These results suggest that at least two sites, one within fnA-D and one within fn6-8/fbg contribute to the effect of TN.L. Blocking bound TN.L with both antibodies simultaneously abolished the effects of TN.L (data not shown), suggesting that there are not likely to be other sites involved in the promotion of neurite outgrowth. Note that the fn6-8/fbg antibody was a more effective blocking reagent, suggesting either that the fn6-8/fbg epitope is a better promoter than the fnA-D epitope or that the fn6-8/fbg antibody has a greater affinity for its epitope than does the fnA-D antibody. None of the antibodies modified neurite extension on BHK cells in the absence of bound tenascin (data not shown). To ensure that the antibodies were not cross-reacting with neighboring domains on bound tenascin, we also cross-adsorbed the fn1-5 antibody with fnA-D and fn6-8 expression proteins, the fnA-D antibody with fn1-5 and fn6-8 expression proteins, and the fn6-8/fbg antibody with fn1-5 and fnA-D expression proteins. Identical results were achieved in neurite outgrowth assays using the cross-adsorbed antibodies (data not shown).

In some conditions, neurons were cultured on bound tenascin in the presence of excess soluble tenascin or on



**FIG. 5.** Neurite outgrowth in the presence of FN-III antibodies. TN.L and TN.S were bound to BHK cells. Cerebral cortical neurons were cultured on the cells for 24 h in the presence of fn1-5, fnA-D, or fn6-8/fbg antibodies. In some experiments, soluble tenascin was added to the cells along with antibodies. The median total neurite length was determined for a sample of 100 neurons for each condition. One representative experiment of three is shown. Columns represent the percentage increase or decrease over the control value (38- $\mu$ m median length for neurons grown on BHK cells in the absence of tenascin or antibodies). The fn1-5 antibody did not alter regulation of neurite outgrowth by either splice variant, bound or soluble. The fnA-D antibody partially reduced the permissive properties of bound TN.L and caused soluble TN.L to become an inhibitory molecule; fnA-D antibody did not affect the neurite outgrowth properties of TN.S. The fn6-8/fbg antibody also partially reduced the permissive properties of bound TN.L; on the other hand, it caused soluble TN.L to become a permissive molecule. Fn6-8/fbg antibody totally reduced the permissive properties of bound TN.S and neutralized the inhibitory properties of soluble TN.S. As described in the legend to Fig. 3, neurites were significantly longer on bound TN.L as opposed to bound TN.S (asterisks) ( $P < 0.05$ ; Kolmogorov-Smirnov test).

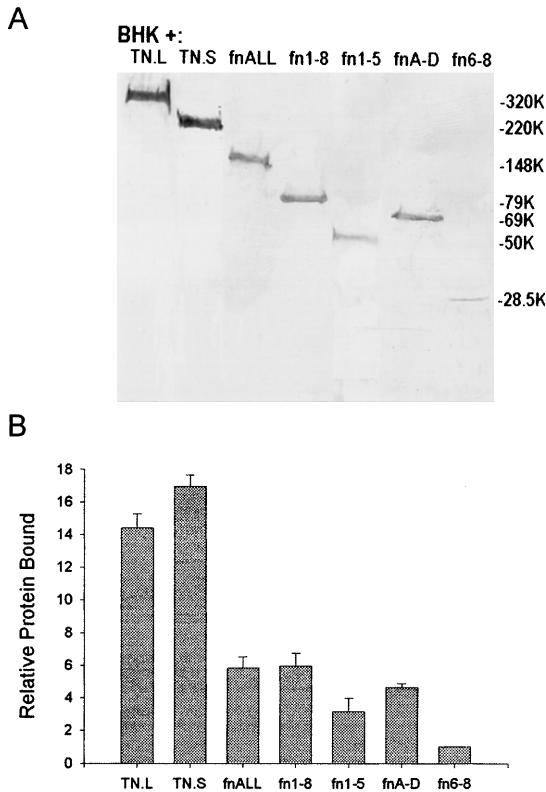
bound tenascin in the presence of excess soluble tenascin and antibodies. As with the bound splice variants, the fn1-5 antibody had no effect on neurite outgrowth. The inhibitory properties of soluble TN.S were unaltered by the fnA-D antibody and neutralized by the fn6-8/fbg antibody. Thus the fn6-8/fbg domains are responsible for the inhibitory actions of soluble TN.S. Since the fn6-8/fbg region in the bound molecule promoted outgrowth, this supports the hypothesis (Lochter *et al.*, 1991) that bound and soluble tenascin, and even bound and soluble tenascin fragments, adopt different conformations such that different functional domains are exposed. Alternatively, the inhibitory site within fn6-8/fbg might be exposed in soluble TN.S and obliterated, or masked, in bound TN.S by interaction with the BHK cell surface.

Soluble TN.L became inhibitory rather than inactive when blocked with the fnA-D antibody, and permissive when blocked with the fn6-8/fbg antibody. These data raise the possibility that a permissive site exposed

within fnA-D and an inhibitory site exposed within fn6-8/fbg counteract each other's activities in soluble TN.L. When bound + soluble TN.L were blocked with both antibodies simultaneously, outgrowth was the same as on control BHK cells (data not shown), suggesting that all regulatory domains were obstructed.

### **Bacterial Expression Proteins Confirm Roles for FN-III Domains**

Bacterial expression proteins corresponding to defined FN-III domains of TN.L and TN.S were utilized to confirm roles for these sequences suggested by the antibody blocking experiments. Proteins included universal domains fn1-5 and fn6-8, which are found in both TN.L and TN.S; fnA-D, the alternatively spliced domains of TN.L; fn1-8, the FN-III domains of TN.S; and fnALL, the FN-III domains of TN.L (see Fig. 1). BHK cells were incubated with expression proteins (10  $\mu$ g/ml) for 6 h at 4°C for binding assays or 37°C for neurite



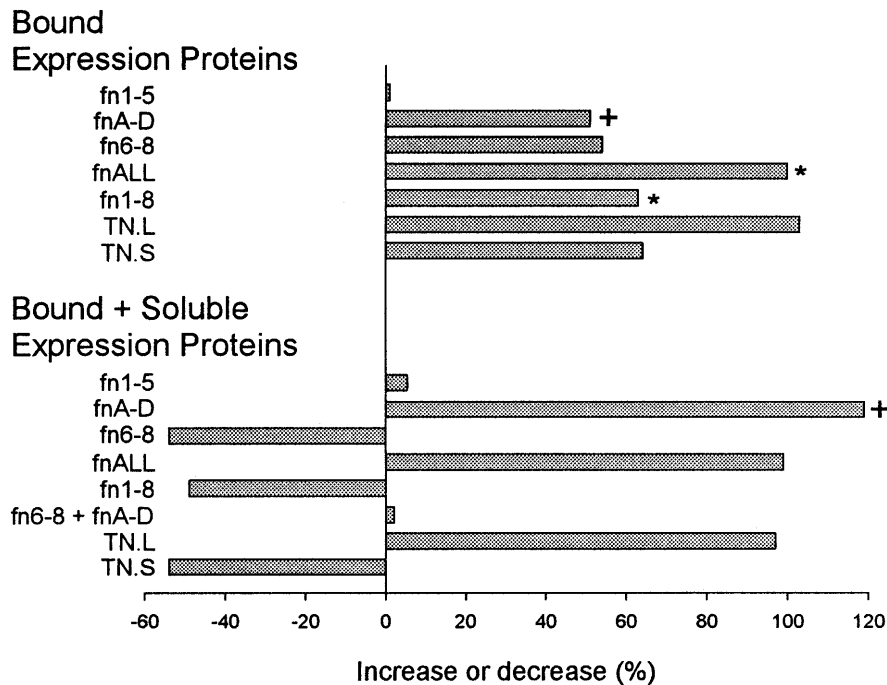
**FIG. 6.** Binding of FN-III bacterial expression proteins to BHK cells. (A) Cell extracts (25  $\mu$ g) were subjected to Western blot analysis with full-length tenascin antibody as described in the legend to Fig. 4. FnALL, fn1-8, fn1-5, fnA-D, and fn6-8 all bound to BHK cells, but to different degrees. TN.L and TN.S are also shown for comparison. (B) Blots were scanned and the level of bound protein in each lane was quantified and normalized to the level of bound fn6-8, which was assigned a value of 1. Bars represent mean  $\pm$  SEM ( $n = 3$ ).

outgrowth assays as described above for tenascin splice variants. Cells bound similar amounts of protein at the two temperatures, but internalization of protein did not occur at 4°C (data not shown). Western blot analysis with full-length tenascin antibody revealed that the expression proteins all bound to BHK cells (Fig. 6A), but to different degrees; bound TN.L and TN.S are also shown for comparison. Labeling for the  $M_r$  28,500 fn6-8 polypeptide was always very faint, indicating a low affinity for BHK cells. Western blots were analyzed by quantitative scanning densitometry as reported previously (Meiners *et al.*, 1993). Within each blot, the levels of bound protein were normalized to the level of bound fn6-8, which was assigned a value of 1 (Fig. 6B). Levels of fn1-5, fnA-D, fnALL, and fn1-8 were all in the same range, approximately 4- to 6-fold higher. TN.L and TN.S had the greatest affinity for BHK cells; levels were about 14- and 17-fold higher, respectively, than the level of

fn6-8. However, caution must be exercised in the interpretation of these results, given that the polyclonal tenascin antibody might have different affinities for the different FN-III domains and that the transfer efficiency and binding efficiency to the filter might be different for different FN-III domain proteins.

We also verified the binding of the expression proteins by immunocytochemical analysis with full-length tenascin antibody. Staining for bound fn1-8 was fibrillar, whereas staining for bound fnALL was punctate (data not shown; see also Chung *et al.*, 1995), which is similar to the staining patterns observed for intact tenascin splice variants (Fig. 3A). Staining for fn1-5 was also fibrillar, in keeping with its binding to fibronectin (Chung *et al.*, 1995), whereas immunoreactivity for fn6-8 was barely detectable. On the other hand, staining for fnA-D was punctate (see Fig. 8A) and resembled the pattern observed for fnALL and TN.L.

To assess roles for specific FN-III sequences in the regulation of neurite outgrowth by tenascin, E17 cerebral cortical neurons were cultured on BHK cells in the presence of bound expression proteins. Process extension was measured at 24 h. Proteins remained bound to the BHK cells throughout the course of the experiment (data not shown). Data are plotted as the percentage increase or decrease over the control median length (37  $\mu$ m for neurons cultured on BHK cells without added expression proteins). Outgrowth values on bound TN.L and bound TN.S are shown for comparison. Outgrowth on bound fn1-5 was the same as outgrowth on control BHK cells (Fig. 7). Bound fnA-D, fn6-8, fnALL, and fn1-8, on the other hand, all increased neurite outgrowth. This suggests, albeit indirectly, that the neurite outgrowth promoting site blocked by the fn6-8/fbg antibody (above) may reside within fn6-8 rather than the fbg sequence. Other experiments utilizing the purified fbg domain have indicated more clearly that this region does not appear to promote neurite outgrowth (Phillips *et al.*, 1995; Dorries *et al.*, 1996; Gotz *et al.*, 1996). FnA-D and fn6-8 promoted outgrowth to a similar extent on BHK cells, even though the cells bound about 5-fold less fn6-8 than fnA-D (Fig. 6). This is consistent with the hypothesis that the fn6-8 epitope is a more potent promoter than the fnA-D epitope. Outgrowth values on bound fnALL and fn1-8 were similar to outgrowth values on bound TN.L and bound TN.S; in agreement with results from the antibody blocking experiments, this suggests that the FN-III domains are necessary and sufficient for the permissive properties of bound tenascin. This also implies that the higher levels of TN.L and TN.S bound to BHK cells (Fig. 6) are saturating to process extension.



**FIG. 7.** Differential effects of FN-III expression proteins on neurite outgrowth. Cerebral cortical neurons were cultured on BHK cells for 24 h in the presence of bound or bound + soluble fn1-5, fn6-8, fn1-8, fnA-D, or fnALL expression proteins. TN.L and TN.S are shown for comparison. The total neurite length was determined for a sample of 100 neurons for each condition. One representative experiment of three is shown. Columns represent the percentage increase or decrease over the control value (37  $\mu$ m for neurons grown on BHK cells in the absence of expression proteins). Fn1-5 did not affect outgrowth in either bound or soluble form, whereas bound fn6-8, fn1-8, fnA-D, and fnALL all increased outgrowth. Outgrowth on fnALL was better than on fn1-8. Neurite lengths on bound fnALL were significantly different from those on bound fn1-8 (asterisks) ( $P < 0.05$ ; Kolmogorov-Smirnov test). Outgrowth was impaired in the presence of soluble fn6-8 and fn1-8. Outgrowth was enhanced in the presence of soluble fnA-D; outgrowth values were significantly higher (crosses) in comparison to values on bound fnA-D alone ( $P < 0.01$ ; Kolmogorov-Smirnov test). Neurite outgrowth was also enhanced in the presence of soluble fnALL; however, outgrowth values were no different from those in the presence of bound ligand only. Process extension from neurons cultured in the presence of a mixture of soluble fn6-8 and fnA-D was unaltered in comparison to outgrowth on control cells.

Outgrowth values were higher on bound fnALL as compared to bound fn1-8; they were also higher on bound fnALL than on bound fn6-8 or bound fnA-D. This is consistent with the hypothesis that neurite outgrowth promoting sites within both fn6-8 and fnA-D contribute to the permissive properties of fnALL, but that only one site within fn6-8 contributes to the permissive properties of fn1-8. Outgrowth values on fn1-8 and fn6-8 were similar; this is somewhat surprising, given the approximately 6-fold lower levels of fn6-8 protein bound to BHK cells. The most parsimonious explanation is that the neurite outgrowth promoting site within fn6-8 is so strong that low levels of the protein are saturating to process extension. Alternatively, fn6-8 and fn1-8 may adopt different conformations upon binding to BHK cells such that the promoting site is more accessible to neurons in the shorter fragment.

Cerebral cortical neurons were also cultured for 24 h on bound expression proteins in the presence of excess

soluble expression proteins (10  $\mu$ g/ml). Figure 7 shows that the addition of soluble fn1-5 had no effect on neurite outgrowth, whereas soluble fn6-8 and fn1-8 both impaired neurite outgrowth. Neurite outgrowth in the presence of either soluble expression protein was similar to growth in the presence of soluble TN.S, suggesting that an inhibitory site within the fn6-8 sequence is wholly responsible for the inhibitory properties of the molecule. In contrast to fn6-8 and fn1-8, the addition of soluble fnA-D significantly enhanced outgrowth in comparison to values on bound fnA-D alone. Therefore, in agreement with the antibody blocking experiments, one or more permissive sites seems to be exposed in both bound and soluble forms of the fnA-D sequence.

Similarly to TN.L, the addition of soluble fnALL did not alter neurite outgrowth beyond that stimulated by bound fnALL. As proposed above, a neurite outgrowth promoting site within fnA-D and a neurite outgrowth

inhibiting site within fn6–8 may neutralize each other's effects in soluble fnALL and TN.L. To investigate whether fnA–D and fn6–8 could counteract each other's effects in solution, neurons were cultured on BHK cells in the presence of a mixture of FN-III expression proteins (fnA–D + fn6–8; 10 µg/ml of each). Indeed, outgrowth values were indistinguishable from those on control cells (Fig. 7).

### **Monoclonal Antibodies Reveal at Least Two Neurite Outgrowth Promoting Sites within the Alternatively Spliced Region of TN.L**

To further map the neurite outgrowth promoting site(s) within the alternatively spliced domains, the fnA–D expression protein was first bound to BHK cells (Fig. 8). In some cases excess soluble fnA–D was added to the cultures. Blocking experiments were conducted with mAbs J1/tn1, J1/tn2, and BC-2, all of which recognize epitopes on bound fnA–D (Fig. 8A). E17 cerebral cortical neurons were cultured for 24 h on BHK cells in the presence of bound or soluble fnA–D or bound or soluble fnA–D and mAbs. The extent of neurite outgrowth was then measured. Data (Fig. 8B) are plotted as the percentage increase or decrease over the control median length (36 µm for neurons cultured on BHK cells without added expression protein or mAbs). As shown above, bound and soluble fnA–D both promoted neurite outgrowth. The mAb J1/tn2, which reacts in fnD, partially reduced the permissive properties of both bound and soluble fnA–D, as did mAbs J1/tn1 and BC-2. The blocking activity of J1/tn1 and BC-2 was very similar, which is not surprising given that they react within the same region of the molecule (fnA1,A2,A4 for J1/tn1; fnA1,A4 for BC-2). Therefore, at least two distinct neurite outgrowth promoting sites seem to be exposed within the alternatively spliced segment, one within fnD and one within the N terminal region of the protein. The mAb α-A2, which reacts in fnA2, had no effect on neurite outgrowth even though it does recognize epitopes on bound fnA–D in immunocytochemical assays (data not shown). This suggests that the neurite outgrowth promoting site obstructed by mAbs J1/tn1 and BC-2 was in fnA1 and/or fnA4 and not in fnA2. Simultaneous blocking of bound or soluble fnA–D with mAbs J1/tn1 and J1/tn2 or BC-2 and J1/tn2 (data not shown) reduced outgrowth to control values; hence no additional permissive domains are exposed outside of fnD and fnA1/A4. The mAbs J1/tn1, J1/tn2, and BC-2 also partially reduced the permissive properties of bound fnALL and TN.L and caused soluble fnALL and TN.L to become inhibitory rather than

inactive, but were without effect on fn1–5, fn1–8, or TN.S (data not shown).

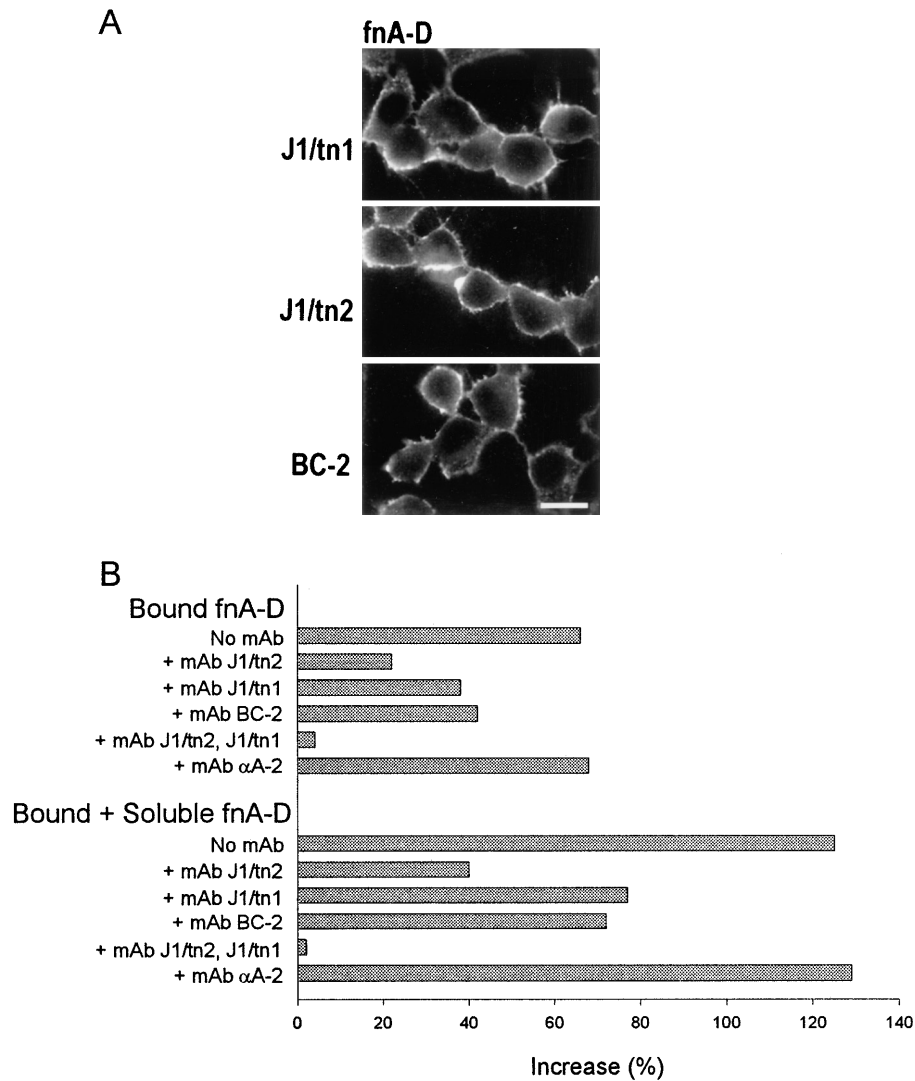
Figure 9 presents a model of the FN-III domains of tenascin indicating the regulatory sites we have described herein. The permissive properties of both bound TN.L and bound TN.S are attributed to a common neurite outgrowth promoting site within fn6–8 (probably within fn6). Additional promoting sites recognized by mAbs J1/tn1, BC-2, and J1/tn2 are depicted within the alternatively spliced region (fnA–D) of TN.L. Bound TN.L is more permissive than bound TN.S due to the presence of these sites. Our data also suggest that the permissive sites within fnA–D and an impairing site within fn6–8 neutralize each other's actions in soluble TN.L. TN.S lacks the permissive site within fnA–D; thus soluble TN.S is inhibitory to neurite outgrowth.

## **DISCUSSION**

We investigated the hypothesis that the varied and sometimes conflicting activities ascribed to tenascin may be in fact due to different functional roles of naturally occurring splice variants. Our studies demonstrate that the largest and smallest variants of human tenascin, TN.L and TN.S, respectively, both promote neurite outgrowth *in vitro* when bound to the surface of BHK cells. This is in agreement with the work of Chiquet and Wehrle-Haller (1994), who isolated three tenascin variants from chick and found that all were permissive when bound to glass. Our work extends this observation, in that addition of excess TN.S in solution to neuron-BHK cocultures impaired process extension, whereas addition of excess soluble TN.L had no inhibitory effect. Thus the net effect of tenascin is a function of the type of splice variants present, as well as the form or conformation of those variants.

### **FN-III Domains and Neurite Outgrowth Sites**

While tenascin also promoted outgrowth from CNS neurons when adsorbed to a plastic substrate, addition of the soluble molecule to the cultures inhibited neurite outgrowth on substrate-bound tenascin, laminin, or fibronectin (Lochter *et al.*, 1991). This work utilized a mixture of splice variants isolated from mouse brain. MAb J1/tn2 largely abolished the permissive effects of bound tenascin but not the inhibitory effects of soluble tenascin. The J1/tn2 epitope has subsequently been mapped to alternatively spliced FN-III domain D (fnD), which is present in mouse tenascin only in large isoforms. However, the antibody occasionally cross reacts

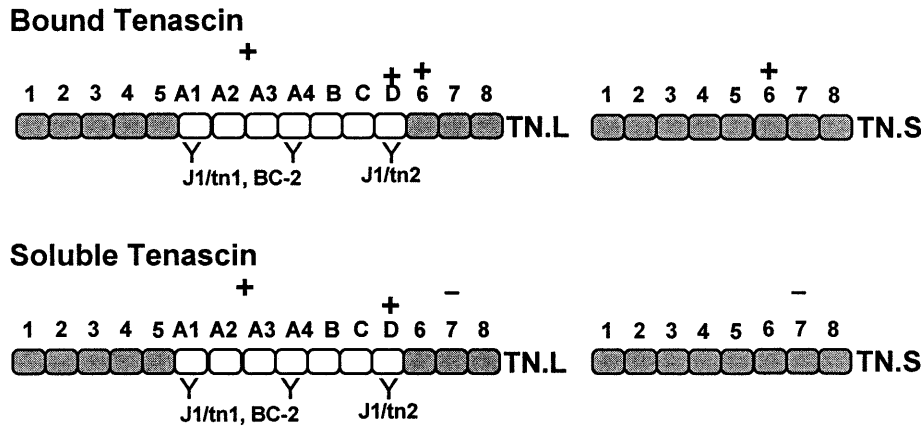


**FIG. 8.** (A) Cultures were immunostained with mAbs J1/tn1, J1/tn2, or BC-2 followed by fluorescein-conjugated secondary antibodies. BHK cells incubated with fnA-D revealed punctate labeling with all of the mAbs. (B) Monoclonal antibodies reveal at least two neurite outgrowth promoting domains within the alternatively spliced region of TN.L. The fnA-D expression protein was bound to BHK cells. Cerebral cortical neurons were cultured on the cells for 24 h in the presence of mAbs J1/tn2, J1/tn1, BC-2, or  $\alpha$ -A2. In some experiments, soluble fnA-D was added to the cells along with mAbs. The median total neurite length was determined for a sample of 100 neurons for each condition. One representative experiment of three is shown. Columns represent the percentage increase over the control value (36- $\mu$ m median length for neurons grown on BHK cells in the absence of expression protein or mAbs). All three mAbs partially reduced the permissive properties of both bound and soluble fnA-D. J1/tn1 and BC-2, which react within fnA1,A2,A4, and fnA1,A4, respectively, had similar activities, while J1/tn2, which reacts within fnD, was a somewhat more effective blocking reagent. The mAb  $\alpha$ -A2, which reacts in fnA2, did not affect neurite outgrowth. Simultaneous blocking of bound or soluble fnA-D with mAbs J1/tn2 and J1/tn1 reduced outgrowth to control levels.

with universal FN-III domain 6 (fn6), which is present in all splice variants, and may also sterically obstruct fn6 (Gotz *et al.*, 1996). Hence, the neurite outgrowth promoting properties of bound tenascin could potentially be ascribed to either alternatively spliced or universal FN-III domains. To this point, recent investigations (Phillips *et al.*, 1995; Gotz *et al.*, 1996) have demonstrated

an active site for neurite promotion in fn6, and our results indicate that a common site within the fn6-8 sequence promotes neurite outgrowth on bound TN.L and TN.S.

Bound TN.L was a more permissive substrate than bound TN.S in our studies, supporting the presence of at least one active site within the alternatively spliced



**FIG. 9.** Model of the FN-III domains of human tenascin indicating promoting and inhibiting sites of bound and soluble forms. A promoting site within fn6 (+) is depicted for substrate-bound human TN.L and TN.S. At least two promoting sites recognized by mAbs J1/tn1, BC-2, and J1/tn2 are shown within the alternatively spliced domains fnA–D of TN.L; the J1/tn2 site is assigned to fnD and the J1/tn1, BC-2 site is assigned to fnA1 and/or fnA4. An inhibiting site (rather than a promoting site) is exposed somewhere within the fn6–8 sequence (–) of soluble tenascin. This may be a result of conformational rearrangements, or the inhibiting site may be obliterated in bound tenascin by interaction with the cell surface and only exposed in soluble tenascin. The promoting sites within fnA–D are also shown as exposed in soluble TN.L, where they can potentially counteract the inhibiting site within fn6–8. Thus, soluble TN.L does not effect neurite outgrowth in this model, whereas soluble TN.S, which lacks the second permissive site within fnA–D, is inhibitory to neurite outgrowth.

domains of TN.L. In addition, antibodies against the alternatively spliced domains reduced the permissive properties of bound TN.L, and a bacterial expression protein corresponding to the same region promoted neurite outgrowth when bound to BHK cells. Gotz *et al.* (1996) have also demonstrated that bacterial expression proteins corresponding to fnB–D and fnD-6 of mouse tenascin enhance neurite outgrowth on a plastic substrate, suggesting that a neurite promoting site resides in either fnB or fnD. Our blocking experiments with mAb J1/tn2 indicate that this site resides in fnD for the human protein. Our work also demonstrates an additional promoting site within fnA1,A4 of human tenascin, which is obstructed by mAbs J1/tn1 and BC-2. Therefore, we now suggest that the permissive properties of the alternatively spliced segment can be ascribed to at least two distinct regions.

Lochter *et al.* (1991) suggest that the dichotomy in the effects observed for bound vs soluble tenascin may be related to the presentation of the molecule as a substrate-bound vs soluble ligand. Hence, tenascin may adopt different conformations such that distinct neurite outgrowth promoting sites are exposed in the bound form, and a distinct inhibitory site is exposed in the soluble form. It is also possible that the inhibitory site is involved in the binding of tenascin to the cell surface, and thus would only be exposed to neurons in the soluble form. In either case, our results with tenascin FN-III bacterial expression proteins and antibodies sug-

gest that the inhibitory site resides within the fn6–8 sequence. Our results also support the hypothesis that at least one additional outgrowth promoting site within the alternatively spliced domains is exposed in soluble TN.L, where it is able to counteract the inhibitory site within the fn6–8 sequence; thus, soluble TN.L does not affect neurite outgrowth. TN.S lacks the additional permissive site(s); thus soluble TN.S is inhibitory to neurite outgrowth.

The current study indicates that the FN-III domains are necessary and sufficient for the regulation of process outgrowth by human tenascin in the context of a cell. This extends the work of Lochter *et al.* (1991), who demonstrated that mAb J1/tn2 neutralized promotion of neurite outgrowth by mouse tenascin bound to plastic, suggesting that all active sites resided within the FN-III domains. On the other hand, Gotz *et al.* (1996) and Dorries *et al.* (1996) reported that bacterial expression proteins corresponding to both the EGF domains and the FN-III domains of mouse tenascin enhanced process extension on plastic. This suggests that the EGF protein fragment may have different activities within the context of the intact molecule, and on a living cell, as opposed to on a plastic substrate. Significantly, mAb J1/tn1 did not have any effect on neurite outgrowth in the Lochter *et al.* (1991) study, whereas J1/tn2 and J1/tn1 both partially reduced promotion of neurite outgrowth in our work. The possibility also exists that human tenascin has an additional permissive site not

present on the mouse homolog, or alternatively, that the J1/tn1 site is strictly dependent upon conformation and is revealed only when tenascin is bound to a cell surface receptor.

### **Tenascin in the Context of Other Matrix Molecules and Cell Substrates**

In these experiments, tenascin consistently promoted neurite outgrowth when bound to BHK cells. However, the maximal neurite lengths achieved were less than those observed on more natural substrata, such as neonatal astrocytes. Flat neonatal astrocytes (as opposed to tenascin/chondroitin sulfate proteoglycan-rich "rocky" neonatal astrocytes) produce negligible amounts of tenascin (Meiners *et al.*, 1995), so other molecules are clearly responsible for the neurite outgrowth promoting properties of astrocytes. Candidate molecules include fibronectin and laminin, both of which are more potent stimulators of neurite outgrowth than substrate-bound tenascin in *in vitro* assays (Faissner and Kruse, 1990). However, BHK cells also produce high levels of fibronectin (Chung *et al.*, 1995) and laminin (data not shown), yet neurites grow longer on astrocytes, implying that still other astrocyte-derived molecules promote process extension, or alternatively, that BHK-derived molecules inhibit it. Hence the neurite outgrowth regulatory properties of tenascin or any other matrix protein can at best be discussed in a relative sense. The fact that tenascin antibodies increased neurite growth on living tissue explants (Gates *et al.*, 1996) suggests either that the explants secrete inhibitory tenascin isoforms, or that bound tenascin is inhibitory in the context of a CNS wound and permissive in other cellular contexts. The permissive and inhibitory effects of tenascin splice variants documented in this study extend beyond the BHK cell substrate; for example, TN.L and TN.S continue to stimulate neurite outgrowth when bound to the surface of neonatal astrocytes or immortalized astrocyte cell lines (the percentage increase in neurite length is nearly the same as when tenascin is bound to BHK cells), and soluble TN.S continues to impair outgrowth (the percentage decrease is even greater) (data not shown). The fact that neurons respond to tenascin in either of these cellular contexts demonstrates that tenascin can regulate neurite outgrowth in a complex cellular environment as would be found *in vivo*, rather than on an artificial substrate.

### **Soluble Matrix Molecules**

The question arises as to the potential role of soluble tenascin variants in the neuronal injury response *in vivo*,

a point which has not been directly addressed in the literature. However, a close examination of published figures that examine tenascin in relation to wounds reveals that not all tenascin immunoreactivity is associated with identified cells (e.g., Fig. 5B in McKeon *et al.*, 1991). This same figure clearly demonstrates the reactive astrocyte as a major source of tenascin. Since the initial response to a traumatic insult is the formation of a wound cavity, secreted tenascin may well accumulate in this area just at the beginning of the repair process. In fact, our early work has demonstrated just such a localization following a stab wound injury to the adult rat brain, and we have also established that both TN.L and TN.S are upregulated in this type of CNS injury (unpublished data). Thus, our results on the effects of soluble vs bound tenascin are directly applicable to the *in vivo* situation. The idea that a matrix molecule can function as a soluble component *in vivo* is certainly not unique; consider the example of the netrins, secreted laminin-related proteins which function as long-range chemoattractants to guide cells and axons during development (Colamarino and Tessier-Lavigne, 1995; Wadsworth *et al.*, 1996).

In conclusion, we have demonstrated an isoform-specific and context-specific regulation of neurite outgrowth by human tenascin in a cellular environment, and our results support a functional role for the alternative splicing of tenascin glycoproteins. Our results are consistent with an involvement of both large and small splice variants during phases of enhanced neurite growth and regeneration, provided the variants are bound to cell surfaces or extracellular matrices and with an involvement of the soluble short variant in the inhibition of these processes. Nonetheless, the true function of tenascin splice variants *in vivo* remains to be determined.

## **EXPERIMENTAL METHODS**

### **Cells, Proteins, and Antibodies**

Transfected BHK cells, bacterial expression proteins, and rabbit polyclonal tenascin antibodies were the gift of Dr. Harold Erickson (Department of Cell Biology, Duke University Medical Center, Durham, NC). Splice variants of human tenascin were produced in the transfected cells (Aukhil *et al.*, 1993). Native TN.L and TN.S were purified from culture supernatants of these cells by gelatin-Sepharose and hydroxyapatite chromatography (Aukhil *et al.*, 1990; Erickson and Briscoe, 1995) followed by electroelution from nondenaturing gels (S.-Y. Ho, S. Palnitkar, and S. Meiners, unpublished data). Bacterial

expression proteins (Aukhil *et al.*, 1993) corresponded to universal FN-III domains 1–5 and 6–8 (fn1–5 and fn6–8); fn1–8, the FN-III domains of TN.S; fnA–D, the alternatively spliced domains of TN.L; and fnALL, the FN-III domains of TN.L. The full-length tenascin antibody was prepared against highly purified tenascin from U251-MG glioma cells, which is almost entirely TN.L (Erickson and Bourdon, 1989). Fn1–5 and fnA–D antibodies were prepared against the corresponding expression protein, while the fn6–8/fbg antibody was prepared against a mixture of fn6–8 and fbg expression proteins. All reagents cited correspond to the human protein.

Monoclonal antibodies (mAbs) against mouse tenascin, J1/tn1 and J1/tn2, were the gift of Dr. Andreas Faissner (Department of Neurobiology, University of Heidelberg, D-69120 Heidelberg, Germany), whereas mAbs against human tenascin, BC-2 and  $\alpha$ -A2, were the gift of Dr. Luciano Zardi (Laboratory of Cell Biology, Istituto Nazionale per la Ricerca sul Cancro, Viale Benedetto XV 10, 16132 Genoa, Italy). The epitope for mAb J1/tn1 is contained on fnA1,A2,A4 of mouse tenascin but has not been mapped to a specific domain, whereas the epitope for mAb J1/tn2 is contained on fnD (Gotz *et al.*, 1996). The mAb BC-2 reacts in both fnA1 and fnA4 of human tenascin, which can be explained by the 83% similarity between the two domains (Balza *et al.*, 1993), and the mAb  $\alpha$ -A2 reacts in fnA2.

### Neuronal Cell Culture

Cerebral cortical and hypothalamic neuronal cultures were prepared as described by Ventimiglia and Geller (1987). Embryonic rats were isolated from timed-pregnant females by Caesarian section. The appearance of a sperm plug was defined as embryonic day 1 (E1); neuronal cultures were cultivated on E17. Embryonic brains were removed into a Petri dish containing 5 ml of BME with 2 M HEPES buffer (BME-HEPES). Meninges were peeled off, and selected brain regions (cerebral cortex; hypothalamus) were removed and minced into fine pieces (<0.5 mm) with dissecting knives. Any remaining meninges and blood vessels were removed at this time to ensure minimal contamination from endothelial cells. The minced tissue was then incubated in BME-HEPES containing 0.025% trypsin and 0.1% collagenase for 30 min at 37°C. Following incubation, the trypsinization was halted by adding 1 ml of BME containing 0.025% soy bean trypsin inhibitor and 0.05% DNase I. The tissue was then gently triturated through a fire-polished pasteur pipette until it was dispersed into a homogeneous suspension. The suspension was centrifuged for 5 min at 1000 rpm. The pellet of embry-

onic cells was resuspended in DMEM/10% heat-inactivated FCS, filtered through a fine nylon mesh (40  $\mu$ m; Tetko), and used for neurite outgrowth assays as described below.

### Neurite Outgrowth Assays

BHK cells transfected with TN.L or TN.S (BHK-TN.L and BHK-TN.S cells) were grown to confluence in DMEM, high glucose, supplemented with 10% FCS. Media were removed from confluent cultures on poly-L-lysine-coated 12-mm glass coverslips and replaced with fresh DMEM + 2% FCS 24 h prior to the start of the experiment; this created a defined time period in which the cells secreted tenascin into the culture environment. Dissociated E17 cerebral cortical cells were plated onto the monolayers and allowed to extend processes for 24 h, at which time the extent of neurite outgrowth was determined via carboxyfluorescein diacetate (CFDA) labeling (Petroski and Geller, 1994). CFDA (Sigma Chemical Co., St. Louis, MO) intensely stains the soma and all processes of cultured, living neurons. Images of the cultures were captured using a Macintosh Quadra 700 with a Scion LG-3 frame grabber board and analyzed with the NIH Image Software (available at <http://rsw.info.nih.gov/nih.image>). A sample of 100 neurons with lengths of processes equal to or greater than one cell soma diameter was considered for each condition. The length of each primary process and its branches was measured for each neuron, and the total neurite length was calculated as the sum of the lengths of individual neurites.

### Growth of Neurons in the Presence of Bound and Soluble Tenascin Isoforms

To investigate the neurite outgrowth promoting properties of bound vs soluble tenascin, purified splice variants were added and bound to untransfected cultures. Cells were incubated with the appropriate variant (10  $\mu$ g/ml in DMEM + 2% FCS) for 6 h at 37°C; the tenascin solution was then removed and replaced with DMEM + 2% FCS. To verify binding, living cultures were immunostained with full-length tenascin or fn1–5, fnA–D, or fn6–8/fbg antibodies. BHK cells on glass coverslips were labeled with the appropriate primary antibody diluted 1:100 in PBS (0.14 M NaCl, 2.7 mM KCl, 1.5 mM KH<sub>2</sub>PO<sub>4</sub>, 4.3 mM NaHPO<sub>4</sub>) containing 0.1% sodium azide (PBS-azide) and 10% FCS (PBS-azide-serum) for 30 min at 4°C. After rinsing in PBS-azide, the coverslips were incubated with fluorescein-

conjugated goat anti-rabbit secondary antibodies (Organon-Technika Cappel, Durham, NC) diluted 1:100 in PBS azide-serum for 30 min at 4°C. The cells were again rinsed in PBS-azide and fixed with acetic acid/ethanol (5%/95%) for 5 min at -20°C. Following fixation, coverslips were rinsed in PBS-azide and mounted in Fluoromount-G (Southern Biotechnology Associates, Birmingham, AL) on microscope slides. Nonspecific binding of secondary antibodies was controlled for by omitting the appropriate primary antibody in parallel cultures. Cultures were examined using a Zeiss Axioptan microscope equipped with an epifluorescence illuminator with a fluorescein filter set. Images were captured using a Macintosh Quadra 700.

In other experiments, BHK cells were incubated with TN.L or TN.S for 6 h at 4°C instead of at 37°C to control for internalization of protein. Surface tenascin was then examined using Western blotting techniques. Cultures were removed from 12-well chambers by aspirating off the tenascin solution, rinsing with 0.5 M EDTA, and scraping the cells into 150 µl of Laemmli sample buffer (Laemmli, 1970) using a rubber policeman. Protein concentrations were determined using the BioRad-DC protein assay. Cell extracts (20 µg) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) and then transferred to nitrocellulose paper (400 mA, 1.5 h) (Towbin *et al.*, 1979). The blots were incubated in blocking solution containing 5% dry milk (Carnation brand) in TBS (20 mM Tris-HCl, pH 7.5, 0.5 M NaCl) for 1 to 2 h. They were then incubated for 3 h in a solution of full-length tenascin antibody diluted 1:1000 in 3% dry milk/TBS and washed. The washing procedure consisted of four 10-min washes in TBS containing 0.05% Tween 20. The blot was then incubated with alkaline phosphatase-conjugated goat anti-rabbit IgG (Sigma Chemical Co., St. Louis, MO) at a 1:000 dilution and washed as above. Bound tenascin antibody was detected colorimetrically using 5-bromo-4-chloro-3-indoyl-phosphate and nitroblue tetrazolium. All antibody incubations and washes were done at room temperature.

Cerebral cortical or hypothalamic neurons were then plated onto BHK cells in the presence of bound splice variants and allowed to extend neurites for 24 h. To study the effects of soluble tenascin, TN.L and TN.S were first bound to the cell surface as described above. Excess tenascin in solution (10 µg/ml of the appropriate splice variant in DMEM + 2% FCS) was added, and neurons were then plated onto the cells and cultured in the presence of bound + soluble tenascin.

### **Blocking Experiments with Polyclonal Antibodies**

To investigate the role of the universal and alternatively spliced FN-III domains in the regulation of neurite outgrowth, blocking experiments were conducted using polyclonal antibodies to universal domains fn1-5 and fn6-8/fbg, and to alternatively spliced domains fnA-D. TN.L and TN.S were bound to the surface of BHK cells as described above. Cells were incubated with 100 µg/ml of antibody in DMEM + 2% FCS for 1 h at 37°C. In some experiments, soluble TN.L or TN.S (10 µg/ml) were added to the cultures along with antibodies. One hour after the addition of antibodies, cerebral cortical neurons were plated onto the cells and cultured for 24 h in the presence of antibodies. The extent of neurite outgrowth was then determined.

### **Growth of Neurons in the Presence of FN-III Expression Proteins**

BHK monolayers were incubated with expression proteins corresponding to FN-III domains fn1-5, fnA-D, fn6-8, fn1-8, or fnALL (10 µg/ml in DMEM + 2% FCS) for 6 h at 37°C for neurite outgrowth assays or for 6 h at 4°C for binding studies, as described above for TN.L and TN.S. Relative levels of protein bound were compared and quantified by scanning densitometry as we have described previously (Meiners *et al.*, 1993, 1995). Briefly, Western blots of cell extracts were scanned at 600 dots per inch and 256-grey-scale resolution on a Microtek 600G scanner. Images were analyzed using the NIH Image Program with the Gel Analysis Macro routine. A profile of intensities of immunoreactivity within each lane was first created and used as an indicator of the level of tenascin binding. Western blots containing serial dilutions of cell extracts were also scanned and the intensity values were found to increase linearly with increasing volumes of cell extract. Within each gel, the levels of bound protein were normalized to the level of bound fn6-8, which was assigned a value of 1. The data from three independent experiments were then pooled and analyzed using the Instat Program (GraphPad Software, San Diego, CA).

Cerebral cortical neurons were plated onto the cultures in the presence of bound expression proteins, and the amount of neurite outgrowth was assessed at 24 h. To study the effects of soluble FN-III expression proteins, proteins were first bound to the surface of BHK cells. Excess protein in solution (10 µg/ml in DMEM + 2% FCS) was added immediately prior to the plating of neurons.

### Blocking Experiments with Monoclonal Antibodies

The role of the alternatively spliced FN-III domains in the regulation of neurite outgrowth was further evaluated as follows. The fnA-D expression protein was bound to BHK cells as described above. Blocking experiments were conducted using mAbs J1/tn1, J1/tn2, BC-2, and  $\alpha$ -A2, which are specific for this region. Cells were incubated with 75  $\mu$ g/ml of mAb in DMEM + 2% FCS for 1 h at 37°C. Soluble fnA-D (10  $\mu$ g/ml) was added to some cultures along with mAbs. One hour later, cerebral cortical neurons were plated onto the cells and allowed to extend neurites for 24 h in the presence of mAbs.

### ACKNOWLEDGMENTS

This work was supported by Research Fellowship Grant P2MF6C from the New Jersey Governor's Council on the Prevention of Mental Retardation and Developmental Disabilities to S.M. and by NIH R01 NS24168 to H.G. We thank Dr. Harold Erickson for helpful discussions and the generous gift of BHK cells, antibodies, and bacterial expression proteins; Elizabeth M. Powell for helpful discussions and critical reading of the manuscript; and Shu-Yin Ho for excellent technical assistance. We thank Drs. Andreas Faissner and Luciano Zardi for monoclonal antibodies and helpful discussions, and Dr. Faissner for insightful suggestions regarding the J1/tn1 and J1/tn2 epitopes on tenascin bound to plastic vs tenascin bound to cells. We also note that after the conclusion of this study, a paper appeared (Gotz *et al.* (1997) *Eur. J. Neurosci.* **9**: 496–506) which proposed similar mechanisms of the actions of the alternatively spliced region of TN.L. We thank the reviewers of this manuscript for their helpful comments, in particular the insightful suggestions regarding the mechanism of action of substrate-bound vs soluble tenascin.

### REFERENCES

- Aukhil, I., Slemp, C. C., Lightner, V. A., Nishimura, K., Briscoe, G., and Erickson, H. P. (1990). Purification of hexabrachion (tenascin) from cell culture conditioned medium, and separation from a cell adhesion factor. *Matrix* **10**: 98–111.
- Aukhil, I., Joshi, P., Yan, Y., and Erickson, H. P. (1993). Cell- and heparin-binding domains of the hexabrachion arm identified by tenascin expression proteins. *J. Biol. Chem.* **268**: 2542–2553.
- Balza, E., Siri, A., Ponassi, M., Caocci, F., Linnala, A., Virtanen, I., and Zardi, L. (1993). Production and characterization of monoclonal antibodies specific for different epitopes of human tenascin. *FEBS Lett.* **332**: 39–43.
- Bartsch, S., Bartsch, U., Dorries, U., Faissner, A., Weller, A., Ekblom, P., and Schachner, M. (1992). Expression of tenascin in the developing and adult cerebellar cortex. *J. Neurosci.* **12**: 736–749.
- Chiquet, M., and Wehrle-Haller, B. (1994). Tenascin-C in peripheral nerve morphogenesis. *Perspec. Dev. Neurobiol.* **2**: 67–74.
- Chung, C.-Y., Zardi, L., and Erickson, H. P. (1995). Binding of tenascin-C to soluble fibronectin and matrix fibrils. *J. Biol. Chem.* **270**: 29012–29017.
- Colamarino, S. A., and Tessier-Lavigne, M. (1995). The role of the floor plate in axon guidance. *Annu. Rev. Neurosci.* **18**: 497–529.
- Crossin, K. L., Hoffman, S., Tan, S. S., and Edelman, G. M. (1989). Cytotactin and its proteoglycan ligand mark structural and functional boundaries in somatosensory cortex of the early postnatal mouse. *Dev. Biol.* **136**: 381–392.
- Crossin, K. L. (1996). Tenascin-a multifunctional extracellular matrix protein with a restricted distribution in development and disease. *J. Cell. Biochem.* **61**: 592–598.
- Dorries, U., Taylor, J., Xioa, Z., Lochter, A., Montag, D., and Schachner, M. (1996). Distinct effects of recombinant tenascin domains on neuronal cell adhesion, growth cone guidance, and neuronal polarity. *J. Neurosci. Res.* **43**: 420–438.
- Erickson, H. P., and Bourdon, M. A. (1989). Tenascin: An extracellular matrix protein prominent in specialized embryonic tissues and tumors. *Annu. Rev. Cell Biol.* **5**: 71–92.
- Erickson, H. P., and Briscoe, G. (1995). Tenascin, laminin, and fibronectin produced by cultured cells. In *Extracellular Matrix: A Practical Approach* (M. A. Haralson and J. R. Hassell, Eds.), pp. 187–198. Oxford Univ. Press, Oxford, UK.
- Faissner, A., and Kruse, J. (1990). J1/tenascin is a repulsive substrate for central nervous system neurons. *Neuron* **5**: 627–637.
- Forsberg, E., Hirsch, E., Frolich, L., Meyer, M., Ekblom, P., Aszodi, A., Werner, S., and Fassler, R. (1996). Skin wounds and severed nerves heal normally in mice lacking tenascin-C. *Proc. Natl. Acad. Sci. USA* **93**: 6594–6599.
- Fukamaki, F., Mataga, N., Wang, Y. J., Sato, S., Yoshiki, A., and Kusakabe, M. (1996). Abnormal behavior and neurotransmissions of tenascin gene knockout mice. *Biochem. Biophys. Res. Commun.* **221**: 151–156.
- Gates, M. A., Fillmore, H., and Steindler, D. A. (1996). Chondroitin sulfate proteoglycan and tenascin in the wounded adult mouse neostriatum in vitro: Dopamine neuron attachment and process outgrowth. *J. Neurosci.* **16**: 8005–8018.
- Gotz, B., Scholze, A., Clement, A., Joester, A., Schutte, K., Wigger, F., Frank, R., Spiess, E., Ekblom, P., and Faissner, A. (1996). Tenascin-C contains distinct adhesive, anti-adhesive, and neurite outgrowth promoting sites for neurons. *J. Cell Biol.* **132**: 681–699.
- Husmann, K., Faissner, A., and Schachner, M. (1992). Tenascin promotes cerebellar granule cell migration and neurite outgrowth by different domains in the fibronectin type III repeats. *J. Cell Biol.* **116**: 1475–1486.
- Jones, F. S., Hoffman, S., Cunningham, B. A., and Edelman, G. M. (1989). A detailed structural model of cytotactin: Protein homologies, alternative RNA splicing, and binding regions. *Proc. Natl. Acad. Sci. USA* **86**: 1905–1909.
- Joshi, P., Chung, C. Y., Aukhil, I., and Erickson, H. P. (1993). Endothelial cells adhere to the RGD domain and the fibrinogen-like terminal knob of tenascin. *J. Cell Sci.* **106**: 389–400.
- Kaplony, A., Zimmermann, D. R., Fischer, R. W., Imhof, B. A., Odermatt, B. F., Winterhalter, K. H., and Vaughan, L. (1991). Tenascin Mr 220 000 isoform expression correlates with corneal cell migration. *Development* **112**: 605–614.
- Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680–685.
- Laywell, E. D., Dorries, U., Bartsch, U., Faissner, A., Schachner, M., and Steindler, D. A. (1992). Enhanced expression of the developmentally regulated extracellular matrix molecule tenascin following adult brain injury. *Proc. Natl. Acad. Sci. USA* **89**: 2634–2638.
- Letourneau, P. C., Condic, M. L., and Snow, D. M. (1994). Interactions of developing neurons with the extracellular matrix. *J. Neurosci.* **14**: 915–928.
- Lochter, A., Vaughan, L., Kaplony, A., Prochiantz, A., Schachner, M., and Faissner, A. (1991). J1/tenascin in substrate-bound and soluble

- form displays contrary effects on neurite outgrowth. *J. Cell Biol.* **113**: 1159–1171.
- McKeon, R. J., Schreiber, R. C., Rudge, J. S., and Silver, J. (1991). Reduction of neurite outgrowth in a model of glial scarring following CNS injury is correlated with the expression of inhibitory molecules on reactive astrocytes. *J. Neurosci.* **11**: 3398–3411.
- Meiners, S., Marone, M., Rittenhouse, J. L., and Geller, H. M. (1993). Regulation of astrocytic tenascin by basic fibroblast growth factor. *Dev. Biol.* **160**: 480–493.
- Meiners, S., Powell, E. M., and Geller, H. M. (1995). A distinct subset of tenascin/CSPG-rich astrocytes restricts neuronal growth *in vitro*. *J. Neurosci.* **15**: 8096–8108.
- Mitashov, V. I., Arsanto, J. P., Markitantova, Y. V., and Thouveny, Y. (1995). Remodeling processes during neural retinal regeneration in adult urodeles—an immunohistochemical survey. *Int. J. Dev. Biol.* **39**: 993–1003.
- O'Brien, T. F., Faissner, A., Schachner, M., and Steindler, D. A. (1992). Afferent-boundary interactions in the developing neostriatal mosaic. *Dev. Brain Res.* **65**: 259–267.
- Petroski, R. E., and Geller, H. M. (1994). Selective labeling of embryonic neurons cultured on astrocyte monolayers with 5(6)-carboxy-fluorescein diacetate (CFDA). *J. Neurosci. Meth.* **52**: 23–32.
- Phillips, G. R., Edelman, G. M., and Crossin, K. L. (1995). Separate cell binding sites within cytotactin/tenascin differentially promote neurite outgrowth. *Cell Adh. Commun.* **3**: 257–271.
- Pindzola, R. R., Doller, C., and Silver, J. (1993). Putative inhibitory extracellular matrix molecules at the dorsal root entry zone of the spinal cord during development and after root and sciatic nerve lesions. *Dev. Biol.* **156**: 34–48.
- 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.
- Prieto, A. L., Andersson-Fisone, C., and Crossin, K. L. (1992). Characterization of the multiple adhesive and counteradhesive domains in the extracellular matrix protein cytotactin. *J. Cell Biol.* **119**: 663–678.
- Prieto, A. L., Edelman, G. M., and Crossin, K. L. (1993). Multiple integrins mediate cell attachment to cytotactin/tenascin. *Proc. Natl. Acad. Sci. USA* **90**: 10154–10158.
- Saga, Y., Yagi, T., Ikawa, Y., Sakakura, T., and Aizawa, S. (1992). Mice develop normally without tenascin. *Genes Dev.* **6**: 1821–1831.
- Spring, J., Beck, K., and Chiquet-Ehrismann, R. (1989). Two contrary functions of tenascin: Dissection of the active site by recombinant tenascin fragments. *Cell* **59**: 325–334.
- Steindler, D. A., Cooper, N. G., Faissner, A., and Schachner, M. (1989). Boundaries defined by adhesion molecules during development of the cerebral cortex: The J1/tenascin glycoprotein in the mouse somatosensory cortical barrel field. *Dev. Biol.* **131**: 243–260.
- Steindler, D. A., Settles, D., Erickson, H. P., Laywell, E. D., Yoshiki, A., Faissner, A., and Kuskabe, M. (1995). Tenascin knockout mice: Barrels, boundary molecules, and glial scars. *J. Neurosci.* **15**: 1971–1983.
- Towbin, H., Staehelin, T., and Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedures and some applications. *Proc. Natl. Acad. Sci. USA* **76**: 4350–4354.
- Tucker, R. P. (1993). The *in situ* localization of tenascin splice variants and thrombospondin 2 mRNA in the avian embryo. *Development* **117**: 347–358.
- Ventimiglia, R., and Geller, H. M. (1987). Cell types and cell-substrate interactions in serum-free dissociated cultures of rat hypothalamus. *Brain Res.* **436**: 339–351.
- Wadsworth, W. G., Bhatt, H., and Hedgecock, E. M. (1996). Neuroglia and pioneer neurons express UNC-6 to provide global and local netrin cues for guiding migrations in *C. elegans*. *Neuron* **16**: 35–46.
- Wehrle, B., and Chiquet, M. (1990). Tenascin is accumulated along developing peripheral nerves and allows neurite outgrowth *in vitro*. *Development* **110**: 401–415.
- Wehrle-Haller, B., and Chiquet, M. (1993). Dual function of tenascin: simultaneous promotion of neurite growth and inhibition of glial migration. *J. Cell Sci.* **106**: 597–610.
- Zhang, Y., Anderson, P. N., Campbell, G., Mohajeri, H., Schachner, M., and Lieberman, A. R. (1995a). Tenascin-C expression by neurons and glial cells in the rat spinal cord: Changes during development and after dorsal root or sciatic nerve injury. *J. Neurocytol.* **24**: 585–601.
- Zhang, Y., Campbell, G., Anderson, P. N., Martini, R., Schachner, M., and Lieberman, A. R. (1995b). Molecular basis of interactions between regenerating adult rat thalamic axons and Schwann cells in peripheral nerve grafts. 2. Tenascin-C. *J. Comp. Neurol.* **1995**: 210–224.

Received June 18, 1997