Evidence for Combinatorial Variability of Tenascin-C Isoforms and Developmental Regulation in the Mouse Central Nervous System*

Angret Joester‡ and Andreas Faissner‡§¶

From the ‡Department of Neurobiology, University of Heidelberg, Im Neuenheimer Feld 364, D-69120 Heidelberg, Germany and §Laboratoire de Neurobiologie du Développement et de la Régénération, UPR 1352, Centre de Neurochimie du CNRS et Université Louis Pasteur, F-67084 Strasbourg, France

The extracellular matrix glycoprotein tenascin-C (TN-C) displays a restricted and developmentally regulated distribution in the mouse central nervous system. Defined modules of the molecule have been shown to mediate specific functions, such as neuron migration, neurite outgrowth, cell adhesion, and cell proliferation. The smallest TN-C form contains a stretch of eight fibronectin type III (FNIII) domains, which are common to all TN-C isoforms. Unrestricted and independent alternative splicing of six consecutive FNIII cassettes between the fifth and sixth constitutive FNIII domain bears the potential to generate 64 different combinations that might code for TN-C proteins with subtly different functions. To explore TN-C isoform variability in mouse brain, the alternatively spliced region of TN-C mRNAs was examined by the reverse transcription-polymerase chain reaction technique. Polymerase chain reaction products of uniform size were subcloned and analyzed using domain-specific probes to reveal the expression of particular combinations of alternatively spliced FNIII domains. 27 TN-C isoforms were identified to be expressed in mouse central nervous system, of which 22 are novel. Furthermore, during development, specific TN-C isoforms were found to occur in distinct relative frequencies, as demonstrated for isoforms containing two alternatively spliced FNIII domains. We conclude that TN-C is expressed in a complex and regulated pattern in mouse central nervous system. These findings highlight the potential role of TN-C in mediating specific neuron glia interactions.

Tenascin-C (TN-C) is a glycoprotein of the extracellular matrix. Similar to many other extracellular matrix molecules, it is composed of serially arranged structural units called protein domains (1). The amino-terminal sequence contributes to the central knob, a cysteine-rich structure that assembles six TN-C monomers via disulfide bridges to form the native hexameric protein (the hexabrachion; Ref. 2). This region is followed by 14.5 epidermal growth factor-type repeats and eight FNIII domains in the shortest TN-C variant. The sequence is terminated by a domain homologous to the β and γ chains of fibrinogen (3, 4). Larger isoforms of mouse TN-C are generated by the insertion of up to six additionally spliced FNIII domains between the fifth and sixth FNIII domain of the smallest TN-C variant. These alternatively spliced domains have been named A1, A2, A4, B, C, and D, according to their counterparts in human TN-C (5, 6).

TN-C is expressed in a variety of tissues and is thought to play a role in mesenchymal-epidermal interactions during organ development but also under conditions of tissue remodeling in the adult organism such as wound healing and tumor growth (7–9). In the central nervous system, TN-C is predominantly expressed by astrocytes and radial glia at early stages of development, whereas its expression is down-regulated during tissue differentiation (10–12). In the adult animal, the protein is restricted to a few brain regions, e.g. the molecular layer of the cerebellum, the olfactory bulb, the retina, and the optic nerve head (10, 13–15). The expression of TN-C in the central nervous system (CNS) has attracted special interest because of the inhibitory (16) and stimulatory actions the molecule can exert in different in vitro assays (summarized in Ref. 9). The diverse functions of the molecule could be localized to distinct functional domains within the protein using proteolytic fragments or fusion proteins encompassing defined structural units of TN-C (17–20). In this context, the alternatively spliced FNIII domains have attracted particular interest. Fusion proteins comprising all alternatively spliced domains have been found to down-regulate focal adhesion integrity of aortic endothelial cells (21) and to support short-term adhesion of embryonic and early postnatal neurons, but not long-term adhesion (19). Fusion proteins consisting of the FNIII domains A1, A2, and A4 (TNfnA124) exhibited repulsive properties, whereas the fusion proteins TNfnBD and, to a lesser extent, TNfnD6 showed a pronounced neurite outgrowth-promoting effect on hippocampal neurons (19).

The functional role of TN-C in vivo might be modified either by alternative splicing of the primary transcript or by post-translational modifications. Interestingly, most of the potential glycosylation sites are found within the FNIII domains that are subject to alternative splicing (22). With six alternatively spliced FNIII domains, each of which can potentially be absent or present, up to 64 isoforms can theoretically be generated. Of these, only seven have been identified thus far in mouse tissue or cell lines (3, 4, 6).

Previous studies have primarily focused on combinations of adjacent domains. These investigations do not take into account that nonadjacent FNIII domains also might be combined by alternative splicing, thereby possibly generating novel rec-
Tenascin-C Isoforms in Mouse Brain

TABLE I

| Name  | Restriction site | Sequence (5′ → 3′) |
|-------|------------------|--------------------|
| 5-s   | BamHI            | cggagtccgaaggttctctctccggg |
| A1-s  | Xhol             | cgctcggagagctgcctctcctgg |
| A1-as | HindIII          | cgcaagcttattggagacctccgagag |
| A2-s  | Xhol             | cgctcggagagctgcctctcctgg |
| A2-as | HindIII          | cgcaagctttattggagacctccgagag |
| A4-s  | Xhol             | cgctcggagagctgcctctcctgg |
| B-as  | HindIII          | cgcaagctttattggagacctccgagag |
| B-s   | Xhol             | cgctcggagagctgcctctcctgg |
| C-as  | HindIII          | cgcaagcttattggagacctccgagag |
| C-a   | HindIII          | cgcaagctttattggagacctccgagag |
| D-s   | BamHI            | cggagtccgaaggttctctctccggg |
| D-as  | HindIII          | cgcaagctttattggagacctccgagag |
| 6-s   | Xhol             | cgctcggagagctgcctctcctgg |
| 6-as  | EcoRI            | cgctcggagagctgcctctcctgg |
| 5fowr | Xhol             | cgctcggagagctgcctctcctgg |
| 5rev  | HindIII          | cgcaagctttattggagacctccgagag |

Sequences that correspond to the coding sequence of TN-C are shown in italics. "-s" and "-as" designate sense and antisense primers, respectively. Sense primers hybridize to the exact 5′ end and the antisense primers to the 9′ end of the respective FNIHII domains. Primers 5forw and 6rev hybridize to the 5′ end of the fifth and the 5′ end of the sixth FNIHII domain, respectively. Restriction sites (bold) were added to the 5′ ends of the primers to facilitate cloning of the resulting PCR products. A stop codon (underlined) was introduced 5′ to the coding sequence in antisense primers to ensure correct termination of fusion proteins, in case that TN-C fragments obtained with these primers would later be cloned into expression vectors.

Materials and Methods

Animals—NMRI mice were used for the preparation of RNA. The day a vaginal plug was observed was designated embryonic day 0 (E0), and the day of birth was designated postnatal day 0 (P0). All animals were raised in the animal facility of the University of Heidelberg (INF 345, Heidelberg, Germany).

Amplification of Tenascin-C Fragments by Reverse Transcription-Polymerase Chain Reaction (RT-PCR)—For the amplification of two FNIHII domain fragments of TN-C, RNA from mouse brain tissues of different ages was reverse-transcribed using hexameric random primers (Amersham Pharmacia Biotech) 4 μg of total RNA and 200 ng of random primer in a total volume of 13 μl were incubated at 70 °C for 10 min and then cooled slowly to room temperature. After the addition of 4 μl of 5X First Strand Buffer (Life Technologies, Inc.), 2 μl of 0.1 M dithiothreitol, and 1 μl of 10 mM deoxynucleotide triphosphates, the reactions were heated to 37 °C, and 1 μl of SuperScript™ reverse transcriptase (Life Technologies, Inc.) was added. The reactions were incubated for 15 min at 37 °C, 15 min at 40 °C, 15 min at 42 °C, 15 min at 45 °C, and 15 min at 50 °C. Finally, the reverse polymerase was heat denatured during a 5-min incubation at 95 °C. The reactions were diluted with 30 μl of TE buffer (10 mM Tris/HCl, pH 7.2, and 1 mM EDTA) 0.5 μl of the reverse transcription reaction was used per 25 μl of PCR reaction with 10 pmol of the appropriate sense and antisense primers (see Table I). The primers were designed to hybridize to the 5′ and 3′ ends of the respective domains. The reaction conditions were as follows: 60 mM Tris/HCl, 15 mM (NH₄)₂SO₄, 2 mM MgCl₂, 1 mM deoxynucleotide triphosphates, 5 mM dithiothreitol, and 0.25 μl of rRNas RNAse inhibitor (Promega) in a 20 μl reaction at 42 °C for 1 h. After denaturation of 15 min, the PCR was performed in 25 μl reactions using 5 μl of the reverse transcription product with the addition of oligonucleotides and buffer components to achieve a final concentration of 60 mM Tris/HCl, 15 mM (NH₄)₂SO₄, 2 mM MgCl₂, 0.2 mM deoxynucleotide triphosphates, 1 mM dithiothreitol, 0.4 μM 5forw, 0.4 μM 6rev, and 1 unit of Taq polymerase (AGS) per 50 μl reaction. Cycling conditions were as detailed above; the only modification was that 20 cycles were run. To obtain a sufficient amount of amplification product for cloning, reverse transcription was upscaled to 100 μl, and PCR was performed in 20 parallel 5 μl reactions.

Generation of FNIHII Domain-specific Probes—The TN-C FNIHII domain fragments A1, A2, A4, B, C, D, and 6 were amplified by RT-PCR using mouse brain RNA and the primers specified in Table I. The primers were designed to hybridize to the 5′ and 3′ ends of the respective domains and contained restriction sites for directional cloning. RT-PCR was performed as outlined above. The resulting fragments were cloned into pBluescript II KS+ (Stratagene) yielding the plasmids pA1, pA2, pA4, pB, pC, pD, and p6. The plasmids were sequenced to confirm that they contained the respective FNIHII domains (Sequenase Version 2.0, Amersham Pharmacia Biotech). Fluorescein-labeled probes were generated by PCR using 10 pg of the respective plasmid, 0.2 μl the domain-specific sense and antisense primer, 20 μl dATP, 20 μl dCTP, 20 μl dGTP, 17.5 μl dTTP, 2.5 μl fluorescente-11-dUTP (Amersham Pharmacia Biotech), 2 mM MgCl₂, 60 mM Tris/HCl, 15 mM (NH₄)₂SO₄, and 1 unit of Taq polymerase (AGS) per reaction. Cycling conditions were as follows: 60 s at 94 °C and 30 cycles of 30 s at 94 °C, 30 s at 57 °C, 15 min at 57 °C, 90 s at 72 °C, and 8 min at 72 °C.

The amount of the different probes used in the hybridization reaction was adjusted to obtain comparable signals for identical amounts of target sequence using dot blots with defined concentrations of target plasmid. Dot blots were prepared using a 96-well dot blot device (Schleicher & Schuell). In brief, Hybrid N°+ membrane (Amersham Pharmacia Biotech) was prewetted in 10× SSC buffer (1.5 M NaCl and 0.15 M sodium citrate, pH 7.0) and mounted in the dot blot machine according to the manufacturer’s instructions. 400 μl of 10× SSC buffer were applied to each well and filtered through the membrane with very low suction. Dilution series of the plasmids were prepared in 10× SSC buffer, the samples were heat denatured, and 100 μl/dilution step were loaded per well. After disassembly of the device, the DNA loaded on the membrane was blocked by incubating the filter in 0.001% blocking solution of native casein and 0.5% (v/v) liquid block (Amersham Pharmacia Biotech) for 30 min at 72 °C, and the labeled probes were denatured at 95 °C for 5 min, quick chilled on ice, and added to the prehybridization solution. After the first round of adjustment experiments, the amount of probes used in 3 ml of hybridization solution was 1.5 μl for A1, 2.5 μl for A2, 2 μl for A4, 0.22 μl for B, 7 μl for C, 0.7 μl for D, and 10 μl for 6. The labeled probes were stored at −20 °C and showed a slight decrease in target recognition sensitivity over a period of 18 months. This was most probably due to degradation of the fluorescein group. For this reason, the amount of probe used for hybridization was regularly readjusted. To ensure specificity of target recognition, high stringency hybridization and washing conditions were developed. In detail, hybridization was performed at 72 °C for 2.5→16 h, depending on the amount of target on the blot, followed by two washes at 72 °C with 0.5% (v/v) SDS and 0.1× SSC for 15 min each. After stringency washes, the hybridized probes were visualized by binding an alkaline phosphatase-coupled anti-fluorescein antibody that was developed with a dioxetane-based substrate (Amersham Pharmacia Biotech). The resulting emission of light was detected on autoradiographic films.

Screening Tenascin-C Isoform-specific RT-PCR Fragments—RT-PCR bands of defined size representing multiples of FNIHII domains were ligated into the plasmid pBluescript II KS+ (Stratagene) using restriction sites that had been included at the 5′ ends of the oligonucleotide primers used for amplification and transformed into the bacterial strain XL1-blue (Stratagene). The resulting transformants were checked by PCR for TN-C fragments of the expected size. The PCR products were
were carried out as described for dot blots. ciein-labeled FNIII domain-specific probes. Hybridization and detection seven analogous blots. These were hybridized separately to the fluores- 
mented in mouse brain (∙; Ref. 6), in primary cultures of mouse fibroblasts (●; Ref. 3), and in a mouse mammary tumor cell line (○; Ref. 4).

RESULTS

Alternatively Spliced FNIII Domains of Tenascin-C in the Murine CNS—A restricted number of FNIII isoforms had been described for mouse TN-C (summarized in Fig. 1). Five of these were found to be expressed in mouse brain (Fig. 1; Ref. 6). All known isoforms of the mouse are generated by the insertion of up to six alternatively spliced FNIII domains, namely, A1, A2, A4, B, C, and D, between the fifth and sixth FNIII domain of the shortest TN-C variant. The derived amino acid sequence of FNIII domain C has been published previously (6), but its nucleotide sequence was not available from databases. The sequence, as demonstrated here (Fig. 2), displays a 95% identity with human FNIII domain C (23) and therefore represents the mouse homologue of this domain.

A search for additional FNIII domains corresponding either to the A5 domain found in human TN-C or to the newly identified domains AD1 and AD2, which have been shown to be present in both human and chick TN-C (24–26), was performed using RT-PCR techniques (Fig. 3). Experiments designed to amplify sets of two adjacent FNIII domains from mouse brain of different developmental stages did not yield products larger than two domains. The amplification of the domain pairs A2A4 and BD did not reveal larger products that might hint at the expression of mouse homologues of the human domains A3, AD1, or AD2. Therefore, we conclude that additional domains, if present at all in the mouse TN-C gene, are not expressed in mouse brain.

Tenascin-C Isoforms of All Possible Sizes Are Expressed in Mouse Brain—Differential splicing of mouse TN-C in the CNS was studied using brain tissue of postnatal day 6 (P6) mice, comparing cerebellar tissue with brain tissue without cerebellum. The cerebellum has been previously shown to contain high amounts and different isoforms of TN-C mRNA and protein at this developmental stage (10). RT-PCR performed with oligonucleotide primers flanking the site of alternative splicing

yielded six products that corresponded in size to one to six FNIII domains (Fig. 4). This result indicates that TN-C isoforms of the sizes expected for multiples of maximally six FNIII cassettes are expressed in mouse brain. Interestingly, variants that contain two or three alternatively spliced FNIII domains that had not previously been described in mouse brain were also detected.

When equivalent amounts of cerebellar RNA and of RNA from brain tissue devoid of cerebellum were subjected to RT-PCR, less product was obtained for the latter (Fig. 4). This is in agreement with recent observations suggesting that at P6 the cerebellum is the main source of TN-C mRNA (2). In both tissues, the most intense amplification pertained to the inserts comprising one and six cassettes, whereas the three-FNIII domain PCR product proved to be the weakest. This result is indicative of differential expression of TN-C isoforms of distinct sizes.

Design and Adjustment of FNIII Domain-specific Probes—Following the assumption that the six alternatively spliced FNIII domains of TN-C are independently combined to form different TN-C isoforms, only the largest product assembling six FNIII domains is of unambiguous composition because it

2 A. Joester and A. Faissner, unpublished observations.
Tenascin-C Isoforms in Mouse Brain

must include all available domains: A1, A2, A4, B, C, and D. In contrast, the amplified inserts of smaller size might represent various combinations of these domains. For example, RT-PCR products consisting of three FNIII domains could potentially encompass up to 20 possible combinations (Fig. 5). To further examine the composition of these inserts, specific fluorescein-labeled probes for the FNIII domains A1, A2, A4, B, C, and D and the constitutively expressed FNIII domain 6 were produced using PCR. The concentrations of the probes used for hybridization were adjusted to ensure comparable target detection sensitivity (Fig. 6A). Furthermore, the conditions for probe hybridization and the subsequent washing steps were modified to obtain a high specificity of target recognition (Fig. 6B). This is particularly important because of the considerable sequence similarities that prevail between the alternatively spliced FNIII domains.

Identification of Tenascin-C Isoforms—To unravel the exact domain compositions of the TN-C isofrom-specific products (Fig. 4), the six RT-PCR amplified bands were separately cloned into the plasmid pBluescript II KS+. The resulting bacterial colonies were screened using the fluorescein-tagged FNIII domain-specific probes. A representative example of original screening data is shown in Fig. 7.

Starting with RT-PCR products of RNA from P6 cerebellum, screening of more than 200 transformant colonies for the products containing one FNIII domain, screening of more than 100 colonies for each of the products containing two to five FNIII domains, and screening of 22 colonies for the six FNIII domain product resulted in the detection of 25 combinations of alternatively spliced FNIII domains expressed in mouse CNS (Fig. 8). Of these, 19 are entirely novel, 2 have as yet only been described in tumor cell lines (4), and 4 combinations have been previously detected in mouse brain (6).

TN-C isoforms in P6 cerebellum possessing one alternatively spliced FNIII domain predominantly carried the FNIII domain D (98%), whereas only 2% contained the domain A1. None of the four other alternatively spliced domains was found to be exclusively inserted between the fifth and sixth constitutive FNIII domain.

Of the two-FNIII domain RT-PCR products, about two-thirds consisted of the domains C and D, and one-third consisted of the domains A1 and D. The two other combinations found (A1A2 and A4B) represented only 10% of this RT-PCR product.

The combination of three FNIII domains implies the highest potential for variation, i.e. 20 different combinations are possible, as shown in Fig. 5. The detection of eight combinations for cerebellar tissue at a specific time point during development indicates that this potential is used to a high extent in vivo. However, taking into account the very low intensity of the three-FNIII domain RT-PCR product (Fig. 4), these forms probably represent only a minor portion of brain TN-C isoforms. Nevertheless, the expression of these isoforms seems to be regulated, as demonstrated by their distinct frequencies.

Seven combinations of four alternatively spliced FNIII domains were identified. However, only three of these made up 87% of the four-domain RT-PCR product. This again points to a regulated expression of TN-C isoforms. Interestingly, the two most frequent combinations A1A2A4B (36%) and A4BCD (31%) consisted of a series of FNIII domains following the order of the respective exons on the TN-C gene.

The major combination with five domains consisted of A1A2A4BD (90%). This TN-C splice variant had previously been documented in fibroblasts, tumor cell lines and brain tissue (3, 4, 6). The two other five-domain splice variants detected in this screen, one missing domain A1 and the other missing domain A4, represented only about 10% of the colonies subcloned from this RT-PCR product.

Screening of 22 transformant colonies with six FNIII domains led to unambiguous hybridization signals with all six probes, consistent with the assumption that no alternatively spliced FNIII domains other than A1, A2, A4, B, C, and D are expressed in mouse brain.

Within the subset of TN-C isoforms that was found to be expressed in P6 cerebellum, none of the alternatively spliced FNIII domains was restricted to only one specific isoform. For example, FNIII domain C that had previously been attributed to the largest TN-C isoform only (Fig. 1; Ref. 6) was also found in shorter TN-C variants, preferentially in those containing two alternatively spliced domains (Figs. 4 and 9). Interestingly, FNIII domain C was never found to be spliced directly to the sixth FNIII domain, thereby omitting domain D. In addition, no isoforms were identified where the FNIII domain A4 was directly linked to the C domain. In contrast, all other possible links between pairs of alternatively spliced FNIII domains were found to be realized within the 25 TN-C isoforms identified in the P6 cerebellum.

When using an independent RNA preparation, the RT-PCR products of distinct sizes were found to consist of the same combinations of FNIII domains that, by and large, were present in the same relative proportions as in the experiment shown in Fig. 7. One additional combination (A2BD) was detected but was found to represent a minor portion of the three-domain amplification products.

Tenascin-C Isoform Expression Changes during Development—To assess variations of TN-C isoform expression during brain development, RNA of embryonic day 13 to adult mouse CNS was prepared and subjected to RT-PCR, using the same primers and cycling conditions described above. Southern blots of the resulting amplification products were hybridized with the probes for the alternatively spliced domains (Fig. 9). Overall expression of differentially sized TN-C isoforms clearly

Fig. 3. No evidence for A3, AD1, and AD2 in mouse brain. RT-PCR was performed on RNA of E16 (a), P0 (b), and P7 (c) mouse brain using primer pairs 1–7, which are indicated with pairs of arrows (see Table I for details). Sense primers were chosen to hybridize to the 5’ end, and antisense primers were chosen to hybridize to the 3’ end of the respective FNIII domains. RT-PCR with primer pair 8 was performed to ensure that the experimental conditions allowed the amplification of products larger than two FNIII repeats. The positions of amplification products representing one to four FNIII domains (273, 546, 819, and 1092 base pairs, respectively) are depicted to the right of the figure (100-base pair ladder). Note that the RT-PCR products obtained with primer pairs 1–7 had the expected size of two FNIII repeats, indicating that no supplementary FNIII repeats corresponding to the human domains A3, AD2, and AD1 are expressed in mouse brain at the developmental stages studied.
peaked around birth. Isoform expression decreased substantially at the end of the second postnatal week, and only splice variants with a single D domain were detected in the adult mouse brain. Considering the isoforms that contain one alternatively spliced FNIII domain, the Southern blot results further substantiated the previous observation that domains A1 and D are present in single-domain isoforms, whereas only extremely weak signals were obtained with probes for A2 and A4, and no signal was detected with probes for B and C.

When the signal intensities obtained with the different probes were compared for specific RT-PCR products, a similar developmental pattern was observed for five of the six bands, i.e., low signal intensity in early and late development, but high intensity at P0. A notable exception was detected for the two-FNIII domain product that developed the strongest signal for A1 and A2 at P0, whereas the signals obtained using probes C and D peaked at E16, with probe C showing a more prominent decrease toward P0 than probe D. This observation suggested a shift of expression for the TN-C isoforms comprising two alternatively spliced FNIII domains from CD at E16 toward an A1- and/or A2-containing combination at P0.

This interpretation was explored using the screening method described above for brain tissue of E16, P0, and P6 mice (Fig. 10). The predominant domain composition of the two-FNIII domain TN-C isoforms detected at the late embryonic state E16 was CD (91%), but its expression was found to be decreased toward P0 (38%). The combinations A1A2 and A1D represented only a minor portion of the two-FNIII domain isoforms at E16,
but their expression levels increased to 38% and 20%, respectively, at P0. At P6, the combination A1A2 only represented 7% of the isoforms of this size, whereas the expression of A1D was slightly increased (32%), and the expression of CD was clearly increased (59%).

**DISCUSSION**

The study of TN-C isoforms on the mRNA level in mouse brain revealed the expression of six alternatively spliced FNIII domains. In contrast, a total of nine alternatively spliced domains has been identified in human TN-C (23, 24, 26, 27). These are, following the 5' to 3' arrangement on the gene, the domains A1, A2, A3, A4, B, AD2, AD1, C, and D. TN-C sequence motifs in different species display a high degree of homology, which reaches 95% for analogous FNIII domains. On this basis, the six alternatively spliced FNIII domains found in mouse TN-C were identified as the mouse homologues of human A1, A2, A4, B, C, and D (5, 6). Studies on the expression of the FNIII domains AD1 and AD2 in E10 chick spinal cord revealed low expression of AD1 in a restricted subpopulation of TN-C-expressing cells, e.g. a subpopulation of cells within the ependymal layer, but no expression by radial glia. In contrast, expression of AD2 was not detected (28). In E14 brain, only cells located in the ventricular zone of the optic tectum showed expression of AD1, which was not included in TN-C mRNA in other brain areas (28). It is unclear whether these findings can be transferred to the mouse nervous system, because the territories of TN-C expression vary in different species (29). Along these lines, FNIII domain C was concluded to be CNS-specific according to in situ hybridization studies carried out on E15 mouse (6), but it was found to be completely absent from spinal cord in chicken E10 embryos (28). It has been proposed that in the human the expression of particular splice variants comprising AD1 is tumor-associated (24), and domain AD2 was documented in two of four specimens of squamous cell carcinomas studied (26). In the present study using a RT-PCR-based strategy, no evidence was obtained for the expression of the mouse homologues of FNIII domains A3, AD2, or AD1 in brain tissue at any of the developmental stages studied. Furthermore, no hints at the expression of alternatively spliced FNIII domains other than A1, A2, A4, B, C, and D were obtained by screening more than 1,000 RT-PCR products spanning the splice site of mouse TN-C. Nevertheless, the expression of FNIII domains equivalent to human TN-C domains A3, AD1, and AD2 in other mouse tissues or in the CNS at developmental stages different from those investigated in this study cannot be excluded. From an evolutionary point of view, the existence of mouse TN-C domains equivalent to the recently discovered AD1 and AD2 is highly probable because both domains were identified in avian and human TN-C. In contrast to these, at present, domain A3 has only been detected in human TN-C. In the well-studied chicken TN-C molecule, only one A domain has been described (25). These findings may suggest that the domains AD1 and AD2 arose from duplication events that occurred before the avian and mammalian species diverged and should thus be inherited by rodents as well. In contrast, the different A domains found in mammals presumably evolved later; therefore, the A3 domain might be unique for primate TN-C. A careful analysis of the genomic sequence is required to ultimately identify the subset of alternatively spliced FNIII domains present in the mouse TN-C gene.

The RT-PCR strategy using primers upstream and downstream of the splice site located between the fifth and sixth FNIII cassette of TN-C yielded products that represented integral multiples of one to six FNIII domains. This result supports the conclusion that TN-C isoforms of all possible sizes that can
be generated with six domains are expressed in mouse brain. Although RT-PCR is primarily a qualitative technique, it can provide some insight into the relative abundance of alternatively spliced transcripts of given sizes. Interestingly, the amount of product obtained by RT-PCR for the different size classes differed in a way that was not directly correlated with the lengths of the inserts. This finding opposes the general experience that smaller products are amplified more readily than larger ones in PCR. In the case of TN-C, the largest alternatively spliced RT-PCR product exhibited an intensity second only to that of the shortest product, whereas the intermediated sized products of three and four FNIII domains were amplified to a lower extent. Thus, the RT-PCR results support the notion that expression of differentially sized TN-C isoforms is regulated and is not merely a reflection of a random production of variants due to the failure of the splicing apparatus to provide some insight into the relative abundance of alternatively spliced transcripts of given sizes. Interestingly, the amount of product obtained by RT-PCR for the different size classes differed in a way that was not directly correlated with the lengths of the inserts. This finding opposes the general experience that smaller products are amplified more readily than larger ones in PCR. In the case of TN-C, the largest alternatively spliced RT-PCR product exhibited an intensity second only to that of the shortest product, whereas the intermediated sized products of three and four FNIII domains were amplified to a lower extent. Thus, the RT-PCR results support the notion that expression of differentially sized TN-C isoforms is regulated and is not merely a reflection of a random production of variants due to the failure of the splicing apparatus to include some or all of the potentially available domains.

The FNIII domain-specific probes that were generated for the purpose of this study proved to be efficient tools for decoding TN-C isoforms. Due to the fluorescein label, the probes could reliably be used over a prolonged time period without a substantial loss of activity. Adjustment of the concentrations applied in hybridization procedures was required due to the differential labeling efficiency of the various probes. No clear relationship between sequence composition, e.g. AT content, and labeling efficiency could be established. After the adjustment of concentrations, the seven different probes recognized the respective target sequences with comparable sensitivities. High stringency conditions were chosen for hybridization to avoid cross-reactions of the probes with nonspecific target sequences. On average, the alternatively spliced FNIII domains share 52% of their nucleotide sequence. The lowest degree of similarity is recorded between A2 and D (41%), and the highest degree of similarity was recorded between A1 and A4 (80%). Interestingly, the constitutively expressed FNIII domains only display an average degree of sequence identity of 44%, whereas the sequence homology between the constitutive and the alternatively spliced FNIII domains is even lower, with an average value of 40%. This points to close evolutionary relationships within these two groups of FNIII domains.

Screening of RT-PCR products of the alternatively spliced transcripts revealed the expression of a complex but restricted repertoire of different TN-C isoforms in the developing mouse brain. 27 TN-C isoforms were identified, of which 22 had not been previously reported. The 27 TN-C isoforms identified in mouse brain represent 42% of the 64 possible combinatorial TN-C variants that can be achieved with six independently arranged cassettes. The present study is the first that combines the amplification of the complete alternatively spliced region with a systematic analysis of representative numbers of single RT-PCR products. Other investigations dealing with the isoform variability of TN-C have used RT-PCR to amplify shorter stretches within the region of alternative splicing, thereby identifying combinations of up to four domains, but not entire isoforms (25, 26). Others have not taken into account that TN-C isoforms of identical size might not be homogeneous with regard to domain composition (6). The screening strategy developed in this study allowed the resolution of six populations of RT-PCR products. Within each size class, substantial heterogeneity of FNIII domain composition was revealed. Due to the screening procedure, the relative frequencies of given isoforms could only be compared within a given size category. For example, the CD variant made up two-thirds of the two-domain group of TN-C isoforms, whereas CD was the only one of the three-domain isoforms that was detected. This indicates that the expression of differentially sized TN-C isoforms is developmentally regulated. Two-FNIII domain RT-PCR products obtained with the primer pairs described in Fig. 4 from RNA of E16, P0, and P6 mouse brain were cloned, and the resulting transformant bacterial clones were screened as outlined in Fig. 7. The prevalence of the different two-domain TN-C isoforms is indicated as a percentage of the total number of bacterial clones tested for each developmental stage. Note that at E16, the combination CD accounts for nearly all of the isoforms of this size. In contrast, at P0, the prevalence of CD equals that of the combination A1A2, whereas the FNIII domain pair A1D represents one-fifth of these isoforms. At P6, the combination CD is dominant again (59%), whereas A1D accounts for one-third of the two-domain isoforms.

**Fig. 10.** The expression of tenascin-C isoforms with two FNIII domains is developmentally regulated. Two-FNIII domain RT-PCR products obtained with the primer pairs described in Fig. 4 from RNA of E16, P0, and P6 mouse brain were cloned, and the resulting transformant bacterial clones were screened as outlined in Fig. 7. The prevalence of the different two-domain TN-C isoforms is indicated as a percentage of the total number of bacterial clones tested for each developmental stage. Note that at E16, the combination CD accounts for nearly all of the isoforms of this size. In contrast, at P0, the prevalence of CD equals that of the combination A1A2, whereas the FNIII domain pair A1D represents one-fifth of these isoforms. At P6, the combination CD is dominant again (59%), whereas A1D accounts for one-third of the two-domain isoforms.
isofoms in P6 cerebellum, whereas the combination A1D represented the other third of these isofoms. Two other variants, namely, A1A2 and A4B, were found to be relatively rare.

FNIIIdomain D was detected in most of the splice variants expressed in mouse brain. Within each size class of isofoms, the D domain containing splice variants made up more than 88% of the inserts. The only exception was noted within the four-domain forms, where D was present in only 65% of all the RT products analyzed, whereas domain A4 was found in 95% of these isofoms in P6 cerebellum. Considering the general organizational features of TN-C isofoms, it is striking that FNIIIdomain C was found to occur in conjunction with domain D in all cases studied thus far, thereby excluding the direct transition between domain C and 6. This observation is in agreement with analogous reports for normal, malignant, and reactive oral mucosae (26). Interestingly, of the 28 possible transitions between pairs of FNIIIdomains, only 2 were not realized in TN-C isofoms in the developing mouse brain: (a) the transition between domains C and 6, and (b) that between domains A4 and C. Total exclusion of these junctions for any size class would decrease the number of potential TN-C isofoms by 20. Additional studies have to show whether these junctions are generally prohibited. If this proves to be the case, these sequences, in comparison to those found frequently, might be interesting candidates for the study of specific splicing factors.

None of the six alternatively spliced FNIIIdomains was confined exclusively to one specific TN-C isofom. In particular, domain C, which had been assigned earlier to the largest TN-C isofom only (6), was also detected in smaller forms. The occurrence of these isofoms in domains with two additional FNIII domains proved to be of special interest in the context of developmental regulation. The expression of the CD-containing isoform dominated during late embryonic development (E16), representing more than 90% of the isofoms of this size. The prevalence of this variant decreased toward birth and increased again at the end of the first postnatal week. This might indicate the involvement of this isoform in specific developmental processes. In late embryonic development, neuron generation reaches a maximum, and the neurons migrate toward their destination within the forming cortical layers and finally differentiate. Neuronal migration and neurite outgrowth are processes that have been found to be influenced by TN-C in vitro (summarized in Ref. 30). During the first two postnatal weeks, the cerebellum is generating its structure, being one of the last parts of the brain to differentiate (31). In situ hybridization has shown the cerebellum to be the major source of TN-C at P6. Therefore, the CD isoform might be of particular importance for the process of differentiation at E16 as well as at P6.

Until now, studies on TN-C isoform expression, regulation, and function have focused primarily on the comparison of the smallest TN-C isoform with the largest TN-C isoform. Generally, high molecular weight TN-C transcripts or proteins were found to be associated with motile cells (32) and enriched in tissues undergoing dynamic remodeling (33). Different factors have been described to influence the expression of TN-C isoforms, for example, extracellular pH, TGF-β1, and bFGF (34–38). The present study shows that a large variety of TN-C isoforms are expressed in the developing brain and that their expression is developmentally regulated. With its many isoforms, TN-C has the potential of encoding positional specificities of astrocytes and, consequently, specific microenvironments for different neuronal populations. Whether particular isofoms display topologically restricted expression patterns will be the focus of additional studies.

Acknowledgments—We thank Drs. M. J. Hannah and J. Garwood for comments on the manuscript, D. Schörr for technical assistance, and Prof. Dr. W. B. Huttner for ongoing support.

REFERENCES

1. Engel, J. (1991) Curr. Opin. Cell Biol. 3, 779–785
2. Erickson, H. P., and Inglesias, J. L. (1984) Nature 311, 267–269
3. Weller, A., Beck, S., and Ekkblom, P. (1991) J. Cell Biol. 112, 355–362
4. Saga, Y., Tsukamoto, T., Jing, N., Kusakabe, M., and Sakakura, T. (1991) Gene (Amst.) 104, 177–185
5. Vuvinic-Filipi, N., and Chiquet-Ehrißman, R. (1993) Symp. Soc. Exp. Biol. 47, 155–162
6. Dörries, U., and Schachner, M. (1994) J. Neurosci. Res. 37, 336–347
7. Erickson, H. P., and Bourdon, M. A. (1989) Annu. Rev. Cell Biol. 5, 71–92
8. Chiquet-Ehrißman, R., Hagios, C., and Schenk, S. (1995) Bioessays 17, 873–878
9. Faisened, A. (1997) Cell Tissue Res. 290, 331–341
10. Bartsch, S., Bartsch, U., Dörries, U., Faisened, A., Weller, A., Ekkblom, P., and Schachner, M. (1992) J. Neurosci. 12, 736–749
11. Mittrovic, N., Dörries, U., and Schachner, M. (1994) J. Neurocytol. 23, 364–378
12. Götz, M., Bolz, J., Joester, A., and Faisened, A. (1997) Eur. J. Neurosci. 9, 496–506
13. Mirzall, P., and Dermitzel, R. (1992) Morroc. Res. Tech. 23, 157–172
14. Bartsch, S., Bartsch, U., Dörries, U., and Schachner, M. (1992) Eur. J. Neurosci. 4, 338–352
15. Bartsch, U., Faisened, A., Trotter, J., Dörries, U., Bartsch, S., Mohajeri, H., and Schachner, M. (1994) J. Neurosci. 14, 4756–4768
16. Faisened, A., and Kruse, J. (1990) Neurov 5, 627–637
17. Spring, J., Beck, K., and Chiquet-Ehrißman, R. (1998) Cell 59, 325–334
18. Prieto, A. L., Andersson-Fison, C., and Crossin, K. L. (1992) J. Cell Biol. 119, 663–678
19. Götz, M., Scholze, A., Clement, A., Joester, A., Schuette, K., Wigger, F., Frank, R., Spiess, E., Ekkblom, P., and Faisened, A. (1996) J. Cell Biol. 132, 681–699
20. Dörries, U., Taylor, J., Xio, Z., Lockter, A., Montag, D., and Schachner, M. (1996) J. Neurosci. Res. 43, 420–438
21. Murphy-Ullrich, J. E., Lightner, V. A., Aukhil, I., Yan, Y. Z., Erickson, H. P., and Hooik, M. (1991) J. Cell Biol. 115, 1127–1136
22. Gulcher, J. R., Nies, D. E., Marton, L. S., and Steinfosnn, K. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 1588–1592
23. Sari, A., Cermollia, B., Saginati, M., Casari, G., Baralle, F., and Zardi, L. (1991) Nucleic Acids Res. 19, 525–531
24. Sriramarao, P., and Bourdon, M. A. (1993) Nucleic Acids Res. 21, 163–168
25. Tucker, R. P., Spring, J., Baumgartner, S., Martin, D., Hagios, C., Poss, P. M., and Chiquet-Ehrißman, R. (1994) Development 120, 637–647
26. Mittel, A. J., Thompson, J., Hame, W. J., Markham, A. F., and Robinson, P. A. (1997) Int. J. Cancer 72, 236–240
27. Nies, D. E., Hemesath, T. J., Kim, J. H., Gulcher, J. R., and Steinfosnn, K. (1991) J. Biol. Chem. 266, 2818–2823
28. Derr, L. B., Chiquet-Ehrißman, R., Gandour-Edwards, R., Spence, J., and Tucker, R. P. (1997) Differentiation 62, 71–82
29. Retting, W. J., Hoffman, S., Su, S. L., and Garin-Chesa, P. (1992) Brain Res. 590, 219–228
30. Faisened, A. (1996) Tenascin and Other Counteradhesive Molecules of the Extracellular Matrix (Crossin, K. L., ed), pp. 47–87, Harwood Academic, Amsterdam, The Netherlands
31. Altmann, J. (1982) in The Cerebellum: New Vistas. (Palay, S., and Chun-Palay, V., eds), pp. 8–49, Springer, Berlin, Germany
32. Kaplon, A., Zimmermann, D. R., Fischer, R. W., Imhof, B. A., Odermann, B. F., Winterhalter, K. H., and Vaughan, L. (1991) Development 112, 605–614
33. Tucker, R. P. (1993) Development 117, 347–358
34. Bors, L., Allemann, G., Gagerno, B., and Zardi, L. (1996) Int. J. Cancer 66, 632–635
35. Zhao, Y., and Young, S. L. (1995) Am. J. Physiol. 268, L173–L180
36. Pearson, C. A., Pearson, D., Shibahara, S., Hofteene, J., and Chiquet-Ehrißman, R. (1988) EMBO J. 7, 2977–2982
37. Tucker, R. P., Hammelback, J. A., Jenath, D. A., Mackie, E. J., and Xu, Y. (1993) J. Cell Sci. 104, 69–76
38. Meiners, S., Marone, M., Rittenhouse, J. L., and Geller, H. M. (1993) Dev. Biol. 160, 480–493