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Quantitative In Situ Localization Of Tenascin-C Alternatively Spliced Transcripts In The Avian Optic Tectum

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Purpose: Tenascin-C is an extracellular matrix glycoprotein found at sites of embryonic cell motility, including the developing visual system. Numerous alternatively spliced variants of tenascin-C have been identified, and these variants have distinctive properties in vitro. The purpose of this study was to use quantitative in situ hybridization to determine the relative abundance of transcripts encoding the alternatively spliced fibronectin type III repeats of tenascin-C in the embryonic avian optic tectum.

Methods: Similarly sized DNA probes specific for sequences encoding the alternatively spliced repeats of tenascin-C were labeled with 35-S and applied to frozen sections of the E10 optic tectum. After determining the linear period of exposure, silver grain densities in the ventricular zone were calculated.

Results: Densitometric analysis revealed that more than half of the total tenascin-C mRNAs expressed in the ventricular zone of the E10 optic tectum encode the variable fibronectin type III repeats "A," "AD1" and "D." Transcripts encoding other variable repeats were detectable, but at considerably lower levels.

Conclusions: The most abundant form of tenascin-C in the developing optic tectum has a molecular weight of 230 kDa. Most of this form contains the variable repeats "A," "AD1," and "D," a combination of fibronectin type III repeats that has previously been identified only in a tumor-derived cell line.

Tenascin-C is an extracellular matrix glycoprotein found in the embryo at sites of epithelial-mesenchymal interactions, cell motility and tissue modeling [1,2]. It is particularly abundant in the developing visual system [3-5], where it is made by radial glia, glial precursors, and by displaced amacrine cells as they migrate into the inner plexiform layer [5]. The function of tenascin-C in the nervous system is probably related to promoting cell motility and establishing selective barriers to neurite outgrowth. Antibodies to tenascin-C inhibit the migration of cranial neural crest cells in vivo [6] and granule cells in cerebellar slice cultures [7], and the severe behavioral deficits observed in a tenascin-C knockout mouse may be related to the abnormal migration or differentiation of specific populations of neuroblasts [8-10].

Tenascin-C, like many other large glycoproteins, is composed of repeated amino acid motifs. In birds, there are 12.5 epidermal growth factor (EGF)-like repeats in the amino half of the molecule, followed by 8 or more fibronectin type III repeats and a terminal fibrinogen-like domain (Figure 1). Western blotting of avian tenascin-C reveals several bands with apparent molecular weights ranging from 190 kDa to 230 kDa. These bands are the result of alternative mRNA splicing of one or more "variable" fibronectin type III repeats between the fifth and sixth "constant" fibronectin type III repeats [11-13]. The variable repeats give different properties to tenascin-C. Different splice variants have different affinities for other extracellular matrix molecules [14] and for putative tenascin-C receptors [15,16]. Larger forms of tenascin-C are usually associated with motile cells during development and disease, whereas tenascin-C without variable repeats is found around cells not typically considered invasive [17]. Complicating the analysis of tenascin-C function has been the identification of previously unidentified variable repeats, AD2 and AD1 [13]. These novel repeats can be expressed in tandem in different combinations with the previously identified repeats [17], meaning that not all "Tn230," the tenascin-C form with 3 variable repeats, is the same (Figure 1). This has necessitated the development of recombinant "designer tenascins" that have known combinations of variable repeats for in vitro studies. The recombinant variants studied so far have distinctive effects on cell shape and adhesion in vitro [18].

Here, the polymerase chain reaction (PCR) was used to generate probes that are specific for the sequences encoding each of the 6 variable fibronectin type III repeats of avian tenascin-C. The similarly sized probes were labeled with 35-S and used for quantitative in situ hybridization analysis of tenascin-C expression in one of its most abundant sites of synthesis: the embryonic optic tectum.

METHODS

Variable Domain-Specific Probes— PCR products corresponding to the sequences encoding each of the avian variable fibronectin type III repeats were amplified from poly (A) RNA (Micro Fast Track, Invitrogen, San Diego, CA) isolated from embryonic day 10 (E10) chicken brain (UC Davis Department of Avian Sciences) using the Perkin Elmer-Cetus (Emoryville, CA) RT-PCR kit. Primers corresponded to the first and last 20 nucleotides of chicken tenascin-C repeats A, B, AD2, AD1, C and D. In addition, a 260 bp PCR product corresponding to the fifth constant fibronectin type III repeat

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(cFN5) was amplified from chicken genomic DNA (Promega, Madison, WI) using Taq and nucleotides from Gibco BRL (Gaithersburg, MD). Each PCR product (cFN5, cA, cB, cAD2, cAD1, cC, and cD) was cloned using the TA-cloning system (Invitrogen) and sequenced to confirm its identity.

In Situ Hybridization— Three E10 quail embryos (UC Davis Department of Avian Sciences) were fixed overnight in 4% paraformaldehyde in PBS, cryoprotected in sucrose, frozen and sectioned at 14 µm. A total of 24 slides with 3 sections from each embryo were collected. The sections were subjected to in situ hybridization with 100,000 cpm of the probes cFN5, cA, cB, cAD2, cAD1, cC, cD, or a pUC19-derived control probe using previously described methods and reagents [17]. After washing and air drying, the slides were exposed to Hyperfilm-beta (Amersham, Arlington Heights, IL) for 1 day, 3 days, 7 days and 9 days. The films from the sections hybridized with cFN5 and the pUC control probe were digitized and analyzed with NIH Image to determine the linear period of silver grain exposure, as previously described [17]. Sections exposed for 3 days were chosen for densitometric analysis. Final values for each probe represent the mean percentage of the cFN5 signal (after subtracting the pUC value) from the ventricular zones of the central optic tectum from 3 different E10 quail. The values for each embryo were calculated by averaging the densitometric values of 3 sections to account for minor variations in section thickness.

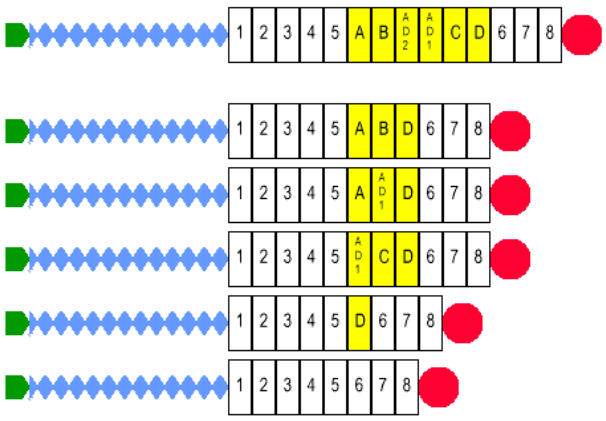

Figure 1. Tenascin-C Variants. Stick diagrams illustrating the domain organization of avian tenascin-C and a few of the known alternatively spliced variants. The EGF-like repeats are indicated with blue diamonds, the constant fibronectin type III repeats are numbered white boxes, the variable fibronectin type III repeats are lettered yellow boxes, and the fibrinogen-like terminal domain is a red circle. The tenascin-C shown at the top is hypothetical; it shows the relationships of the 6 variable repeats within the variable domain. At the bottom are 5 known splice variants (see [13] and [17] for other variants). Three contain 3 variable repeats and would have apparent molecular weights of approximately 230 kDa (Tn230). One has a single repeat and would have an apparent molecular weight of 200 kDa (Tn200). The tenascin-C at the bottom does not have any variable repeats, and would have a molecular weight of 190 kDa (Tn190).

RESULTS

A representative collection of cross sections through the E10 quail optic tectum hybridized with the probes to tenascin-C variable repeats is illustrated in Figure 2. As shown previously [3-5,19], the most prominent signal is in the ventricular zone,

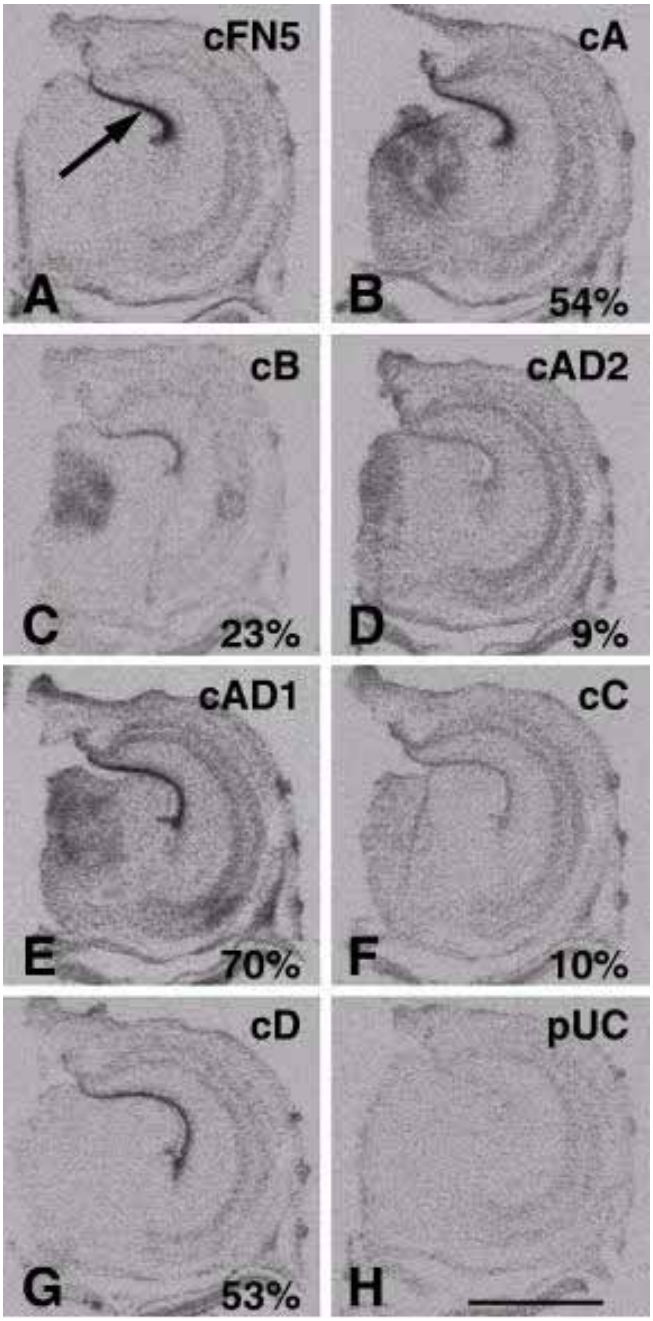

Figure 2. Tenascin-C Variants in the Optic Tectum. High-resolution X-ray film overlays of cross sections through the E10 quail optic tectum hybridized with a probe that recognizes all tenascin-C transcripts (A) or only transcripts encoding a specific variable repeat (B-G). A pUC control for spurious labeling and probe trapping is also shown (H). The strongest signal in the E10 optic tectum is within the ventricular zone (arrow). The probe used for each image is shown in the upper right-hand corner. The result of densitometric analysis of the films (shown as a percentage of the cFN5 signal; n = 3) is shown in the lower right-hand corner. The bar corresponds to 1 mm.

which is consistent with tenascin-C being made by radial glia and glial precursors. The mean percentage of the total tenascin-C transcript (i.e. cFN5) in the ventricular zone calculated for each probe is summarized in Figure 2 and here (probe, mean percent of Fn5 [±standard deviation]): cA, 54% (±25%); cB, 23% (±16%); cAD2, 9% (±8%); cAD1, 70% (±5%); cC, 10% (±6%); cD, 53% (±12%). Although each probe generated a hybridization signal over background, three probes, cA, cAD1 and cD, generated signals that corresponded to more than half of the total tenascin-C signal.

DISCUSSION

Tenascin-C is a prominent part of the extracellular matrix of the developing avian visual system [3-5]. Since tenascin-C inhibits retinal neurite outgrowth in vitro [4,5], the optic tectum's tenascin-C may act as an inhibitory guidance molecule, slowing neurite outgrowth within the stratum opticum. Another possible role is suggested by Chuong et al. [7], who found that antibodies to tenascin-C inhibited the migration of cerebellar granule cells along radial glial fibers. The radial glia of the optic tectum similarly support the migration of neuronal precursors from the ventricular zone to their appropriate layer from E4 till E12 [20], and radial glia-derived tenascin-C may provide an environment that is conducive to motility. Note that these two models seem to be contradictory: on the one hand, tenascin-C may inhibit motility, and on the other hand it may promote motility. In fact, tenascin-C could do both, since different splice variants of tenascin-C have different adhesive properties [18]. This paper represents an effort to determine the variants of tenascin-C that are expressed in the optic tectum to clarify its role in the development of the visual system.

The first 3 avian tenascin-C variable repeats to be identified corresponded to human tenascin-C's variable repeats A, B and D; no C repeat was found [11,12]. Later, PCR techniques resulted in the identification of the avian homolog of the C repeat as well as 2 additional repeats between repeats B and C [13]. These repeats are named AD1 [21] and AD2. The human homolog of the AD2 repeat has recently been found [22]. Genomic sequencing has confirmed that repeats A, B, AD2, AD1, C and D represent all of the variable repeats of chicken tenascin-C [13]. Our previous studies showed that tenascin-C with repeat C is concentrated in connective tissue and at sites of epithelial-mesenchymal interactions, but not in the avian nervous system [13]. In contrast, others [23] studying the mouse have found transcripts encoding the repeat C in the developing CNS. Here we show that tenascin-C mRNAs encoding the variable repeat C are, in fact, present in the avian central nervous system, albeit at relatively low levels (10% of the total transcripts).

Immunoblotting of homogenates of the E10 optic tectum showed that Tn190, Tn200 and Tn230 are all present, and that Tn230 is the most abundant form [3]. The results of the current study demonstrate that the optic tectum's Tn230 is mostly A/AD1/D tenascin-C (see Figure 1), and not the A/B/D form of Tn230 sequenced from fibroblasts and whole brain [11,12]. The A/AD1/D variant of tenascin-C has been identified as the most common variant made by an invasive quail tumor-derived cell line [17], but until this study there was no evidence that A/AD1/D was made anywhere but in tumors. Future studies should be directed toward in vitro analysis of recombinant tenascin-C with these 3 particular variable repeats to determine if it promotes or inhibits cell attachment and migration, or promotes or inhibits retinal neurite outgrowth in vitro.

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