Novel tenascin variants with a distinctive pattern of expression in the avian embryo

R. P. Tucker¹, J. Spring², S. Baumgartner², D. Martin², C. Hagios², P. M. Poss¹ and R. Chiquet-Ehrismann²,*

¹Department of Neurobiology and Anatomy, Neuroscience Program, Bowman Gray School of Medicine, Wake Forest University, Winston-Salem, NC 27157-1010, USA
²Friedrich-Miescher Institut, Postfach 2543, CH-4002 Basel, Switzerland

*Author for correspondence

SUMMARY

Previous studies have shown that several forms of the glycoprotein tenascin are present in the embryonic extracellular matrix. These forms are the result of alternative splicing, which generates tenascin variants with different numbers of fibronectin type III repeats. We have used degenerate primers and PCR to isolate a novel tenasin exon from an avian genomic library. Genomic clones contained a sequence encoding a fibronectin type III repeat that corresponds to repeat ‘C’ from the variable domain of human tenascin. To demonstrate that tenascin containing repeat ‘C’ is actually synthesized by avian cells, a monospecific antiserum was raised against a repeat ‘C’ fusion protein. This antiserum recognized a novel high-molecular-weight variant on immunoblots of tenasin isolated from chicken embryo fibroblast-conditioned medium, and stained tendons on frozen sections of chicken embryos. A cDNA probe specific for mRNA encoding repeat ‘C’ was used for in situ hybridization. This probe hybridized in a subset of the embryonic tissues labelled with a universal tenascin probe, including tendons, ligaments and mesenchyme at sites of epithelial-mesenchymal interactions. Finally, we provide evidence that additional fibronectin type III repeats, one corresponding to a recently discovered human repeat as well as one entirely novel sequence, also exists in chicken tenasin mRNA. These data indicate that tenascin is present in the embryonic matrix in a multitude of forms and that these forms have distinctive distributions that may reflect more than one function for tenascin in development.

Key words: cytotactin, splice variants, in situ hybridization, development, extracellular matrix

INTRODUCTION

Tenascin is a multimeric glycoprotein found in the extracellular matrix of embryos and tumors (Chiquet and Fambrough, 1984; Chiquet-Ehrismann et al., 1986; Grumet et al., 1985). Tenascin is especially prominent in the developing central nervous system, in the matrix lining the pathways of migratory cells, in mesenchyme at sites of mesenchymal-epithelial interactions and in developing connective tissues (for reviews see Erickson and Bourdon, 1989; Chiquet et al., 1991b; Chiquet-Ehrismann, 1993). Unlike fibronectin, which has a more ubiquitous distribution in embryonic mesenchyme, tenascin acts as an anti-adhesive substratum for cells in vitro. Cells will attach and migrate on tenascin-coated tissue culture plastic, but they fail to spread and form strong adhesions (Spring et al., 1989; Halfter et al., 1989; Lotz et al., 1989). Moreover, when cells are first allowed to spread on fibronectin or native basal laminae and tenascin is then added to the culture medium, these cells will lose their strong attachments and become rounded (Chiquet-Ehrismann et al., 1988; Halfter et al., 1989; Lotz et al., 1989). If the cells used are from wing bud cultures, the cell rounding apparently promotes chondrogenesis (Mackie et al., 1987). For these reasons, tenascin is believed to play a role in regulating cell-matrix interactions in a way that may promote cell motility and/or differentiation.

Several forms of tenascin have been found in the extracellular matrix that are the result of differential mRNA splicing. Three splice variants have been identified in the chicken (Jones et al., 1989; Spring et al., 1989) with apparent relative molecular masses of 230×10^3 (Tn230), 200×10^3 (Tn200) and 190×10^3 (Tn190). These forms differ from one another in their number of fibronectin type III repeats. Tn230 has eleven fibronectin type III repeats; two of these repeats are not found in Tn200 and exons encoding three of these repeats are spliced from transcripts encoding Tn190. This diversity may reflect different functional forms of tenascin, as splice variants have different affinities for other matrix molecules (Chiquet-Ehrismann et al., 1991) and cell surface glycoproteins (Zisch et al., 1992). Both immunohistochemistry with splice variant-specific antibodies (Matsuoka et al., 1990; Chiquet-Ehrismann et al., 1991; Kaplony et al., 1991) and in situ hybridization with splice variant-specific probes (Prieto et al., 1990; Mackie and...
Tucker, 1992; Tucker, 1993) have revealed distinct tissue distributions and cellular origins for the variants as well. Comparison of avian tenasin with tenascins from other species shows that the spliced, variable domain of the molecule has been conserved through evolution (Gulcher et al., 1989; Saga et al., 1991; Weller et al., 1991).

It was noted earlier that the primary sequences of fibronectin type III repeats from chicken and human tenasin correspond with one another colinearly. That is, the first repeat from human tenasin is more similar to the first repeat in chicken than to other human fibronectin type III repeats, and so on. Within the variable domain, sequence analysis revealed that one of the human fibronectin type III repeats, repeat 'C', did not have a counterpart in the chicken. For this reason, we have undertaken a study of genomic sequence and have found a homologous avian exon. Monospeciﬁc antibodies raised against avian repeat 'C' fusion proteins have identiﬁed this previously unknown splicing variant both in tenasin from chicken embryo ﬁbroblast-conditioned medium and in tissue sections. Finally, the tissue distribution of transcripts encoding tenasin with repeat 'C' was examined by reverse transcriptase PCR and in situ hybridization. By investigating the ﬁbronectin type III repeats surrounding repeat 'C'-containing mRNA, two further novel ﬁbronectin type III repeats were discovered. Whereas one of these repeats represents an entirely new sequence, the other one is the homologue of the recently published human tenasin 'AD1' repeat (Sriramarao and Bourdon, 1993). Thus our results provide further evidence that the structure of tenasin is phylogenetically conserved, and that tenasin may have multiple functions in development that are regulated via the expression of different forms of the molecule.

MATERIALS AND METHODS

Cloning and sequencing of tenasin variants and production of fusion proteins

Genomic clones of the tenasin gene were isolated from a library in EMBL3 (Clontech CL1004D) using a probe encompassing tenasin repeats 'A' and 'B'. One of the clones isolated ended in repeat 'D'. This DNA was used as a template for the PCR reaction using degenerate primers developed after the sequence of human tenasin repeat 'C'. The following oligonucleotides were used: 5'-GATGAAAATC-GAYATHAAYCCTAYGG-3' and 5'-CTAGTCGACSNAGCATNACYTCTRA-3' (corresponding to the regions marked in Fig. 1). The resulting PCR product was subcloned and sequenced. Oligonucleotides corresponding to the obtained internal sequence were used to sequence the entire chicken repeat 'C' plus the adjacent introns of the original genomic clone. Oligonucleotides were then synthesized using the terminal sequences of this repeat to amplify the entire repeat for the subcloning into the bacterial expression vectors and to screen a chicken embryo cDNA library (Clontech) to isolate a cDNA clone containing repeat 'C'. PCR products and cDNAs were subcloned into the Bluescript vector (Strategen). Both strands were sequenced using the Sequenase Kit (U.S. Biochemicals).

For the production of fusion proteins containing selected fibronectin type III repeats of tenasin, the commercially available plasmid pEX-1 (Genofit) was used. Since it was shown that after the deletion of the C-terminal half of the β-galactosidase the stability and efficiency of the production of fusion proteins increased, the plasmid was modiﬁed according to Wingender et al. (1989). The plasmid pEX-1 was cleaved at the unique EcoRV and XbaI sites to delete part of the β-galactosidase coding region and the two partially complementary oligonucleotides 5'-GTTAATGTTGACCGGGTGTTAATTAT- TAAT-3' and 5'-CTAGATTTAATATTACCACCAT- TACC-3' were inserted at this site. With this insertion, the modiﬁed plasmid pEX-1 contains 3861 bp. After the truncated β-galactosidase-coding region, the aminooacids NGDPC precede the restriction enzyme recognition site Smal. Stop codons in all three reading frames follow after the Smal site. Blunt end cloning into the Smal site links the insert to the third position of the reading frame of the β-galactosidase part. Thus the fusion proteins produced contain the N-terminal part of the β-galactosidase migrating at 54×10^3 M^−1s^−1 on SDS-PAGE plus the added molecular weight of the attached tenasin fragments. They were puriﬁed from inclusion bodies according to the procedure of Wingender et al. (1989). Alternatively tenasin fragments were expressed as fusion proteins with glutathione-S-transferase using the pGEX series of vectors (Pharmacia). Since also these fusion proteins were recovered in inclusion bodies, they were puriﬁed using the same procedure as for the β-galactosidase fusion proteins.

Antibodies, immunoblotting and immunohistochemistry

The monoclonal antibodies against tenasin and the location of their epitopes have been described previously (cf. Chiquet-Ehrismann et al., 1991). The antiserum against the glutathione-S-transferase repeat 'C' fusion protein has been raised in a rabbit using standard procedures. The antiserum was aﬃnity puriﬁed using β-galactosidase repeat 'C' fusion protein coupled to CNBr-activated Sepharose (Pharmacia). The monospeciﬁc antibodies were eluted using 0.1 M glycine/HCl (pH 2.6) and the eluate was immediately neutralized. Their speciﬁcity was tested using an ELISA procedure as described in Chiquet-Ehrismann et al. (1988).

The tenasin for the immunoblotting experiment was puriﬁed from chicken embryo ﬁbroblast conditioned medium according to Chiquet et al. (1991a). SDS-PAGE and immunoblotting was performed as described earlier (Hofer et al., 1990), with the exception that the chemiluminescence detection kit (ECL, Amersham) was used to develop the ﬁlter. Immunofluorescence of cryosections was performed as described by Mackie et al. (1987).

In situ localization of splice variants

Tenasin variant transcripts were identiﬁed in frozen sections of chicken embryos at a variety of developmental stages using in situ hybridization. To identify the mRNA encoding repeat 'C', an upstream anti-sense primer corresponding to the ﬁrst 22 nucleic acids of repeat 'C' (5'-AGGCCATGCCCCCACCTGAAAG-3') and a downstream sense primer corresponding to the ﬁnal 21 nucleic acids of repeat 'C' (5'-CTGTAACAGCCACATGCTTA-3') were used to generate a 272 bp reverse transcriptase PCR product (TNC). TNC was identiﬁed as a probe to the sequence encoded in the 'C' repeat by a restriction digest with HindIII. The in situ hybridization pattern with TNC was compared to the pattern obtained with two other probes: TN230 (Tucker, 1993), a 545 bp PCR product generated from primers corresponding to the beginning of repeat 'A' and the end of the repeat 'B'; and cTn8, an 1899 bp chicken tenasin cDNA that corresponds to the portion of the tenasin mRNA encoding the heptad repeats, the EGF-like repeats and the ﬁrst one and one-half ﬁbronectin type III repeats (Pearson et al., 1988). Thus, TN230 hybridizes to tenasin transcripts encoding variants with repeats 'A' and 'B', and cTn8 hybridizes to all known variant mRNAs. All probes were labelled with 35S-dCTP using random primers (Promega), and unincorporated nucleotides were removed with a Sephadex G-50 mini-spin column (Worthington). To control for spurious hybridization, a 955 bp TaqI/Sau3AI pUC19 restriction fragment was similarly puriﬁed and labelled.

Chicken embryos (Hubbard Farms) at embryonic day 7 (E7), E10 and E14 were ﬁxed by immersion overnight in ice-cold 4% paraformaldehyde in phosphate-buﬀered saline (PBS), cryoprotected overnight in 25% sucrose in PBS and embedded in OCT compound.
Serial 12-14 μm sections were cut in a cryostat and collected on poly-L-lysine-subbed slides. Details of the in situ hybridization protocol have been published previously (Tucker and McKay, 1991). In brief, air-dried sections were prehybridized for 1 hour at room temperature in 5× SSC and 5× Denhardt’s solution, with 100 μg/ml salmon sperm DNA and 20 mM β-mercaptoethanol. Slides were then dehydrated in ethanol, and 5×10^6 cts/minute of the appropriate probe in hybridization buffer (prehybridization buffer with 50% formamide, 20 mM Tris, 0.1% lauryl sulfate and 1% dextran sulfate; all reagents from Sigma) was added and allowed to hybridize overnight at 42°C. The next day the slides were rinsed with 1× SSC at room temperature and at 42°C. Sections were exposed to 11-day embryos and RNA was prepared from skin of 11-day embryos and RNA was prepared after the procedure of Chomczynski and Sacchi (1987). Poly(A) RNA was isolated using the mRNA kit from Pharmacia. For the RT-PCR (reverse transcriptase and Taq-polymerase, Boehringer-Mannheim) of chicken embryo fibroblast mRNA, the following primers were used:

repeat ‘C’ forward (5′-TGAATTCCTAGCCAT-GCCCCAAGGTA-3′),
repeat ‘C’ reverse (5′-TGAATTCCTAGTTAAGCCACAGGTGC-3′),
repeat ‘D’ reverse (5′-ATGTCGACGGTCCAC-CTGGTTCTGCG-3′),
repeat ‘F’ forward (5′-ATGTCGACCAACCAT-CAAGGGTTCGA-3′),
repeat ‘A’ forward (5′-ATGTCGACGCTGGT-GATTCCGGCTAC-3′),
repeat ‘6’ reverse (5′-TGAATTCCTAGT-TTTCGAAATTTGC-3′),
repeat ‘B’ forward (5′-ATGTCGACCTAAGT-GCTGGGCTTCA-3′),
repeat ‘A’ reverse (5′-ATGTCGACCTAGAT-GTTGAGCGCCTGC-3′).

All products of the reactions shown in Fig. 9.II were sequenced to confirm its identity. Lanes e-g show immunoblots of tenascin isolated from chicken embryo fibroblast conditioned medium, reacted with a general anti-tenascin antiserum (lanes e-f), anti-tenascin repeat ‘C’ (lane g) and stained for protein (lane h). Anti-tenascin repeat ‘C’ recognizes a high-molecular-weight tenascin band, migrating above the three major tenascin variants seen after staining the tenascin (lane h). From chicken embryo fibroblast mRNA using primers from the beginning of repeat ‘1’ to the end of repeat ‘2’ (lane a), the beginning of repeat ‘C’ to the end of repeat ‘D’ (lane b) and from the beginning of repeat ‘C’ to the beginning of repeat ‘D’ (lane c).

**RESULTS**

**Does a fibronectin type III repeat ‘C’ exist in chicken tenasin?**

When the fibronectin type III repeats of chicken and human tenasin are aligned with each other, it is evident that the order of the fibronectin III repeats is conserved. Thus, we assigned numbers to identify the homologous constant repeats and letters to identify the corresponding repeats known to be subject to alternative splicing (cf. Vrčinič-Filippi and Chiquet-Ehrismann, 1993; Aukhil et al., 1993; Fig. 9). Repeat ‘A’ occurs in 4 versions in human tenasin, but only once in the chicken. Human repeat ‘C’ shares 35% identity with repeat ‘B’ of chicken tenasin, but this did not seem to be enough similarity to qualify as an authentic homologue, since all other homologous repeats have a counterpart in the human repeat ‘C’ exon.

**RT-PCR with chicken embryo fibroblast mRNA**

Chicken embryo fibroblast cultures were prepared from skin of 11-day embryos and RNA was prepared after the procedure of Chomczynski and Sacchi (1987). Poly(A) RNA was isolated using the mRNA kit from Pharmacia. For the RT-PCR (reverse transcriptase and Taq-polymerase, Boehringer-Mannheim) of chicken embryo fibroblast mRNA, the following primers were used:

repeat ‘C’ forward (5′-TGAATTCCTAGCCAT-GCCCCAAGGTA-3′),
repeat ‘C’ reverse (5′-TGAATTCCTAGTTAAGCCACAGGTGC-3′),
repeat ‘D’ reverse (5′-ATGTCGACGGTCCAC-CTGGTTCTGCG-3′),
repeat ‘F’ forward (5′-ATGTCGACCAACCAT-CAAGGGTTCGA-3′),
repeat ‘A’ forward (5′-ATGTCGACGCTGGT-GATTCCGGCTAC-3′),
repeat ‘6’ reverse (5′-TGAATTCCTAGT-TTTCGAAATTTGC-3′),
repeat ‘B’ forward (5′-ATGTCGACCTAAGT-GCTGGGCTTCA-3′),
repeat ‘A’ reverse (5′-ATGTCGACCTAGAT-GTTGAGCGCCTGC-3′).

All products of the reactions shown in Fig. 9.II were subcloned and sequenced.
Fig. 3. X-ray film overlay images of cross sections through E7 (A-H), E10 (I,K,M,O) and E14 (J,L,N,P) chicken embryos following incubation with $^{35}$S-labelled cTN8 (8), cTN230 (230), cTNC (C) or a 955 bp pUC19 TaqI/SauAI restriction fragment (pUC). At E7 cTN8 hybridizes in areas of connective tissue morphogenesis, the dorsal aorta (a), the tips of lung (l) buds (arrows in A), and in kidney (k) mesenchyme (arrows in E). There is also a ‘spot’ of hybridization in the spinal cord (sc). cTN230 hybridizes in the same regions, but the signal is greatly reduced in areas of chondrogenesis. The signal with cTNC is similar to that seen with cTN230, except that no hybridization could be detected in the spinal cord. At E10 cTN8 (I) hybridizes in the spinal cord, in areas of chondrogenesis, at the base of developing feathers (small arrows) and in tendon (large arrow). The cTN230 (K) signal is similar, but as at E7 there is a very weak signal in areas of chondrogenesis. The only significant signal with cTNC (M) at E10 is in tendons (large arrow) and at the base of feathers (small arrows). At E14 both cTN8 (J) and cTN230 (L) hybridize in ventral white matter, but the signal with cTNC (N) is indistinguishable from the control (P). f, feathers; g, gut; t, tendon; vb, vertebra; w, wing.
Novel tenascin variants

chicken with over 50% amino acid identity. Therefore, we postulated that in the chicken a previously unidentified ‘C’ repeat could exist between the known repeats ‘B’ and ‘D’.

In our earlier studies (Spring et al., 1989), none of the cDNA clones analyzed revealed a potential repeat ‘C’. Therefore, we decided to determine if the chicken tenascin gene contains an exon encoding repeat ‘C’. Genomic clones were isolated that hybridized with repeats ‘A’ and ‘B’. One of these clones ended within repeat ‘D’ and should, if repeat ‘C’ exists in chicken, contain the putative exon. Using repeat ‘C’-specific degenerate primers (derived from the human sequence corresponding to the two regions marked by arrows in Fig. 1), we were able to amplify by PCR a product of the predicted length using the genomic clone as a template. The sequence of the amplified fragment and of the corresponding exon in the genomic clone (including flanking intron sequences) confirmed that it was indeed derived from an exon encoding a chicken repeat ‘C’ (Fig. 2). This repeat has an identity of 80% with human repeat ‘C’.

Fig. 4. Cross sections through brachial-level chicken spinal cord at E7 (A-D) and E10 (E-G) stained with anti-tenascin (TN) or [35S]-labelled cTN8 (8), cTN230 (230) or cTNC (C). The sections treated for in situ hybridization are shown in dark field. Anti-tenascin staining first appears in the E7 spinal cord as a discrete portion of the ependymal layer just dorsal to the ventral floor plate (arrow in A). Both cTN8 and cTN230 hybridize in this same region (arrows in B and C). The signal with cTNC (D), however, is indistinguishable from controls (not shown). At E10 there is an intense hybridization signal with both cTN8 (E) and cTN230(F) in the spinal cord ependymal layer in cells found in the ventral horn and ventral white matter. In contrast, the cTNC signal (G) is identical to controls (not shown). Thus, ‘C’ repeat-containing mRNA can not be detected in the developing spinal cord by in situ hybridization, indicating that cTN230 is the most abundant high-molecular-weight form of tenascin.
Fig. 5. Cross sections through E7 (A-F) and E10 (G-I) chicken embryos incubated with 35S-labelled cTN8 (8), cTN230 (230), cTNC (C) or stained with anti-tenascin (TN). At E7, all three tenascin cDNA probes (A-C) hybridize in the connective tissue linking adjacent vertebral laminae (vl). There is no signal in the dorsal root ganglion (drg). Similarly, cTN8 (D), cTN230 (E) and cTNC (F) all hybridize in the endoderm-derived epithelium at the growing tips of the lung bronchioles (b, arrows). (G) At E10, the only tissues where cTNC hybridizes are tendons (t) and in the mesenchyme at the base of feather buds (fb, arrow). Anti-tenascin also stains the base of feather buds (H, arrow) and tendons (I, t) in nearby sections. Thus, repeat ‘C’-containing mRNA is relatively abundant in some embryonic connective tissues, as well as at sites of epithelial-mesenchymal interactions.
Do chicken fibroblasts express tenascin containing the ‘C’ repeat?

An antiserum was raised against a recombinant glutathione-S-transferase repeat ‘C’ fusion protein (GST-C, Fig. 2A). In order to purify the antibodies from this serum specific for the ‘C’ repeat, a second fusion protein between β-galactosidase and repeat ‘C’ was prepared (β-gal-C). β-gal-C was coupled to CnBr-activated Sepharose 4B and the antiserum was applied to the column. Bound antibodies were eluted by low pH and the eluate was immediately neutralized. The specificity of the antiserum and the purified monospecific antibodies were then tested by ELISA (Fig. 2A). The antiserum loaded onto the column (L) reacted both with GST-C and β-gal-C, but not with a different β-galactosidase fusion protein (β-gal-D). The flow through (FT) still reacted with the GST fusion protein, but not with β-gal-C. The eluate (E), as expected, reacts with both types of repeat ‘C’ fusion proteins.

To determine whether chicken embryo fibroblasts produce mRNA and tenascin protein containing repeat ‘C’, mRNA was isolated from chicken embryo fibroblast cultures and used for RT-PCR. Primers to amplify the constant repeats ‘1’ and ‘2’ were used in a control reaction (Fig. 2B, lane a). Primers of sequences from either end of repeat ‘C’ (Fig. 2B, lane b) or a sense primer from the beginning of repeat ‘C’ and an anti-sense primer from the beginning of repeat ‘D’ (Fig. 2B, lane c) were used to test for the presence of repeat ‘C’. In all cases, we were able to amplify a PCR product of the appropriate size. The products also contained the predicted internal restriction sites (not shown). Thus, repeat ‘C’ is present in fibroblast tenascin mRNA adjacent to repeat ‘D’. Furthermore, tenascin isolated from the conditioned medium of chicken embryo fibroblasts was analyzed by immunoblotting using the ‘C’ repeat monospecific antibody. The ‘C’ repeat antibody recognized exclusively a high-molecular-weight tenascin variant that migrated just above the larger of the three major tenascin bands recognized by an antibody that recognizes all tenascin forms (Fig. 2B, lanes e-h). Thus, both the mRNA encoding the ‘C’ repeat, and a tenascin variant containing the ‘C’ repeat, are made in primary cultures of chicken embryo fibroblasts. However, the repeat ‘C’-containing tenascin is only a minor fraction of the tenascin produced in these cells, since it is not visible in the lane containing the purified tenascin stained by Coomassie Blue and it could only be detected with the general anti-tenascin antiserum in a lane loaded with a ten-fold higher tenascin concentration than was used to reveal the three major variants (Fig. 2B).

Tissue distribution of tenascin containing repeat ‘C’

The distribution of tenascin mRNA encoding repeat ‘C’ was determined by in situ hybridization in sections of chicken embryos at several stages of development. The distribution of repeat ‘C’ transcripts was compared with the distribution of
mRNAs containing the variable ‘AB’ repeats as well as the distribution of total tenascin transcript.

Fig. 3A-H shows low magnification overviews (X-ray film overlays) of cross sections through two axial levels of E7 chicken embryos hybridized with tenasin probes and a pUC control probe. cTn8, which hybridizes to the mRNAs of all known tenasin forms (Spring et al., 1989), hybridizes in the perichondrium, in endothelial cells of the aorta, in the connective tissue associated with forming vertebrae, as well as in lung buds and kidney mesenchyme (Fig. 3A). Some of these regions are shown at higher magnification in Figs 4 and 5, which show dark-field images of silver grains following autoradiography. The small, punctate signal seen near the center of the E7 spinal cord with cTn8 at low magnification is clearly present in a restricted portion of the ependymal layer just dorsal to the ventral floor plate (Fig. 4B; Tucker and McKay, 1991). This ‘spot’ corresponds to the strongest concentration of tenasin itself as seen with anti-tenasin staining of a nearby section (Fig. 4A). The strong cTn8 hybridization signal seen associated with the vertebrae at low magnification is actually present in connective tissue linking adjacent vertebral laminae, i.e. the developing ligamentum flavum (Fig. 5A). In lung, the cTn8 signal is present within the epithelial cells at the very tip of the bronchiole buds, as described previously (Koch et al., 1991). The signals seen with TN230, which recognizes mRNA sequences encoding variable repeats ‘A’ and ‘B’, are a subset of the regions where cTn8 hybridizes: kidney mesenchyme, bronchiole tips, ligamentum flavum, aorta endothelium and spinal cord ependyma are all ‘AB’ positive (Figs 3B,F, 5B,E, 4C). In turn, the hybridization pattern with TNC is a subset of the TN230 pattern. The TNC hybridization signal is strong in the ligamentum flavum and at the tips of bronchioles, and is also seen (albeit more weakly) in the kidney mesenchyme and aorta endothelium (Figs 3C,D, 5G), in the same regions where anti-tenasin stains the extracellular matrix (Fig. 5H,I). Thus, tenasin mRNA encoding repeat ‘C’ is concentrated in a subset of the cells that synthesize tenasin with repeats ‘A’ and ‘B’. Repeat ‘C’ transcripts are not detectable by in situ hybridization in perichondrium or in the spinal cord, though other tenasin mRNAs are found in these regions.

The restricted distribution of repeat ‘C’-containing mRNA and the prominent expression in embryonic tendon was confirmed by immunostaining. We stained sections of chicken
embryos with the ‘C’ repeat monospecific antibody. The only prominent staining was found in the flight tendon of a chicken wing in a 10-day embryo (Fig. 6). Fig. 6A shows an overview over the section stained with anti-TnM1, which stains all tenascin variants and therefore shows the most widespread distribution. This staining was compared to the staining pattern obtained by other monoclonal tenascin antibodies and the repeat ‘C’ antibody as shown Fig. 6B-E. Anti-Tn32 does not recognize the smallest tenasin variant without extra repeats and shows a subset of the anti-TnM1 staining pattern, reflecting the presence of tenascin variants containing extra repeats ‘A’ and ‘B’ (Fig. 6C). Even more restricted is the staining using anti-Tn26, which recognizes a large tenasin variant containing extra repeat ‘D’ (Fig. 6D). The ‘C’ repeat antibody consistently stained the tendon exclusively (Fig. 6E). There was no staining of tendons with control antisera (not shown). Whereas the perichondrium was equally stained by the three monoclonal anti-tenasin antibodies (only shown for M1, Fig. 6A), another connective tissue structure (marked by a star) was brightly stained by anti-TnM1, moderately stained by anti-Tn32 and not stained at all by anti-Tn26 or repeat ‘C’ antibody.

### Are there more tenasin variants?

The next set of experiments was undertaken to investigate the nature of the tenasin variants containing repeat ‘C’. First we screened a chicken embryo cDNA library with a probe specific for repeat ‘C’. One of the clones isolated contained repeat ‘D’, preceded by repeat ‘C’, which surprisingly was not joined to a known repeat, but was preceded by a novel fibronectin type III sequence (Fig. 7). This new repeat revealed an identity of 59% to a recently discovered additional repeat in human tenasin and termed ‘AD1’ by Srimarao and Bourdon (1993). We thus decided to adopt this nomenclature and refer to this repeat as the chicken ‘AD1’ (cf Fig. 7; chAD1). We then performed three sets of RT-PCR reactions using chicken embryo fibroblast mRNA. In the first set using different combinations of primers from the published chicken tenasin repeats, bands corresponding to the previously described three tenasin variants could be amplified exclusively (Figs 8e-g, 9I). To our surprise, when we performed RT-PCR with a primer from repeat ‘AD1’ together with primers from repeats ‘S’, ‘A’ and ‘B’, we obtained, in the latter case, a product of about 270 bp larger size than expected (Figs 8d, 9II). The cloning and sequencing of this product revealed a further entirely novel fibronectin type III sequence, which we decided to term ‘AD2’ (Fig. 8; chAD2). A third set of RT-PCR reactions using primers of repeat ‘S’ together with repeat ‘C’ primers resulted in a number of bands spaced by about 270 bp (marked by white dots in Fig. 8h), which by restriction digests could be identified as the splicing variants shown in Fig. 9III. Thus, chicken tenasin mRNA can contain up to six fibronectin type III repeats subject to alternative splicing as shown in the model of Fig. 9, and repeat ‘C’ occurs in multiple tenasin splice variants.

### DISCUSSION

The importance of tenasin for development has recently been questioned by Saga et al. (1992), since they discovered that mice lacking tenasin developed apparently normally. This result led Erickson (1993a) to propose that tenasin could be a superfluous protein and that the sites of prominent expression of tenasin are not necessarily the sites where tenasin has a function. Tenasin is the product of a member of a multigene family consisting of three or four members (Erickson, 1993b; Chiquet-Ehrismann et al., 1993). Clearly the knocking out of genes belonging to multigene families often has no or only minor effects on the deficient mice and frequently the defects are a surprise and unpredictable. However, since in most of these cases the loss of a gene product is compensated for by other genes, the normal function of the deleted gene can not be found by this approach. Certainly redundant proteins are not superfluous, and if a protein has any function (which presumably every protein has cf. Brookfield, 1992), this function has to be
performed where the protein is present. We therefore believe that
it is still important to investigate the structure, function and
expression pattern of tenascin in normal embryos as well as in
tissue culture models to find possible functions of tenascin.

Evidence from both biochemical and morphological studies
indicate that different splice variants of tenascin may have
different functions. For example, low-molecular-weight
 tenascin has a higher affinity for both fibronectin (Chiquet-
Ehrismann et al., 1991) and contactin/F11 (Zisch et al., 1992),
than tenascin containing the variable domain. Moreover, when
bovine endothelial cells are exposed to a fusion protein corre-
sponding to the variable domain of human tenascin, they lose
their focal adhesions (Murphy-Ullrich et al., 1991). In the
embryo, individual motile cells like osteoblasts and glial cells
make high-molecular-weight tenascin, whereas non-motile
chondroblasts are a primary source of low-molecular-weight
tenascin (Mackie and Tucker, 1992; Tucker, 1993). A possible
role for high-molecular-weight tenascin in promoting invasive
behavior is also provided by studies of tumors and in vitro
transformation. For example, Borsi et al. (1992) have recently
found that high-molecular-weight tenascin is preferentially
expressed by invasive human breast carcinomas, whereas low-
molecular-weight tenascin is expressed in normal breast tissue
and by benign breast lesions. Moreover, when chicken embryo
fibroblasts are transformed by Rous sarcoma virus middle T
(Matsuoka et al., 1990), or when 3T3 cells are treated with
bFGF (Tucker et al., 1993), there is a selective induction of
tenascin containing the variable fibronectin type III repeats.
Taken together, these data indicate that the key to understand-
ing tenascin function may lie in our understanding the differ-
ences between alternatively spliced variants of the molecule.

Comparison of the amino acid sequences of the fibronectin
type III repeats of human (Gulcher et al., 1989) and chicken
(Spring et al., 1989; Jones et al., 1989) tenascin indicated that
the C repeat from the variable domain of human tenascin had
yet to be identified in the chicken. Through a combination of
genomic cloning with probes flanking the putative C repeat
and PCR amplification with degenerate oligonucleotides, we
have found a C repeat in chicken tenascin. Here we provide
evidence that this previously unknown fibronectin type III
repeat is actually present in avian tenascin: (1) monospecific
antibodies to an avian repeat C fusion protein recognize a
band corresponding to a high-molecular-weight splice variant
of tenascin from medium conditioned by chicken embryo
fibroblasts, (2) these antibodies stain a subset of the embryonic
extracellular matrix stained by antibodies that recognize all
tenascin forms, (3) appropriately sized PCR products with
restriction sites characteristic for repeat C can be amplified
from chicken embryo fibroblast mRNA using primers designed
from avian C repeat sequence, and (4) a DNA probe specific for
mRNA encoding repeat C hybridizes within a subset of the
embryonic tissues where other tenasin probes hybridize.
Previously, we have shown that tenasin containing repeats A
and B is made by migrating cells (osteoblasts and glia cells),
at the sites of epithelial-mesenchymal interactions (feather
buds, kidney and at the tips of growing lung bronchioles), and
is found in embryonic tendons (Tucker, 1993). We now know
that the high-molecular-weight tenasin found in these diverse
regions is not all the same type. Within the CNS, most of the
high-molecular-weight tenasin apparently lacks the C repeat.
This is evident from the absence of a significant hybridization
signal in developing spinal cord with TNC. In contrast, the
strong signal with TNC in tendon, lung and kidney and at the
base of feather buds indicates that tenasin with repeat C is a
major component of high-molecular-weight tenasin concen-
trated at sites of epithelial-mesenchymal interactions as well as
in embryonic tendon. These different sites of expression
implicate repeat C in functions that may be associated with
inductive events, cell proliferation, or the binding of non-
neuronal matrix components.

With the aim of determining in which type(s) of splicing
variants the fibronectin type III repeat C occurs, we dis-
covered two novel repeats preceding repeat C. The homologue
of one of them has also been identified recently in human
tenascin and has been termed repeat AD1 for alternative
domain 1 by Sriramarao and Bourdon (1993). We thus decided
to adopt this nomenclature for the chicken tenasin repeats
and termed our novel repeats AD1 preceded by ADA2. In the
case of the human ‘AD1’ repeat, it was suggested that its expres-
sion may be tumor associated (Sriramarao and Bourdon, 1993).
It will be the subject of future studies to investigate the
expression pattern of these novel repeats in the developing
chicken. Furthermore, it remains to be seen whether
mammalian tenasin also contains a homologue of the chicken
‘AD2’ repeat. Repeat C does not always occur together with
‘AD1’, but appears to be present in a multitude of splicing
variants in mRNA isolated from chicken embryo fibroblast
cultures. These mRNAs are, however, a minority of the
tenascin mRNA in these cultures, since they can only be
detected using primers specific for either of the three novel
repeats. In future studies, it will be most interesting to find out
whether the novel repeats confer new activities to tenascin.

The sequences reported in this article can be obtained from the
EMBL data base under the accession number X73833.

This work was supported in part by a grant from the National
Science Foundation (BNS-9021124) to R. P. T.

REFERENCES

Aukhil, I., Joshi, P., Yan, Y., and Erickson, H. P. (1993). Cell- and heparin-
binding domains of the hexabrachion arm identified by tenasin expression
Borsi, L., Carnemolla, B., Nicolò, G., Spina, B., Tanara, G. and Zardi, L.
(1992). Expression of different tenasin isoforms in normal and neoplastic
A monoclonal antibody as a marker for tendon and muscle morphogenesis. J.
Cell Biol. 98, 1926-1936.
Chiquet, M., Vrucinic-Filipi, N., Schenk, S., Beck, K. and Chiquet-
(cytoactin): an extracellular matrix protein involved in morphogenesis of the
(1986). Tenasin: An extracellular matrix protein involved in tissue
interactions during fetal development and oncogenesis. Cell 47, 131-139.
Chiquet-Ehrismann, R., Kalla, P., Pearson, C. A., Beck, K. and Chiquet,
Chiquet-Ehrismann, R., Matsuoka, Y., Hofer, U., Spring, J., Bernasconi,
fibronectin and distinct distribution in cell cultures and tissues. Cell Regul. 2, 927-938.


(Submitted 9 December 1993)

Note added in proof

We have recently sequenced the entire genomic region of about 9 kb of the chicken tenasin gene encompassing the exon encoding repeat “A” to the exon encoding repeat “C”. We confirmed the presence of the exons of all extra repeats described in the present manuscript, but did not find any other unknown exons encoding novel repeats. Therefore, we can conclude that the tenasin model presented in this paper most likely includes a complete set of all possible fibronectin type III repeats of chicken tenasin.