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A novel tenascin type III repeat is part of a complex of tenascin mRNA alternative splices

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ABSTRACT

Sequence analysis of two human tenascin encoding cDNA clones from a cDNA library of the U251 glioblastoma cell line revealed the presence of a novel 276 bp tenascin type III fibronectin like repeat. This alternatively spliced type III repeat designated AD1 is located between the previously identified repeats 10 and 11 and has sequence homology with human, chicken and mouse tenascin type III repeats. These results show that tenascin has at least 16 consecutive fibronectin like type III repeats. PCR amplification of random primed mRNA with specific type III repeat primers revealed a pattern of multiple alternative splices of AD1 and flanking type III repeats. The alternative splice variants were confirmed by direct sequencing. Differences were observed in the expression of the various alternative splices of tenascin mRNA between tumor and normal cells and may thus indicate differences in tenascin isoform expression and function in normal and tumor cells. PCR and Southern analysis of genomic DNA indicate that AD1 is coded by a single exon present in both human and mouse genome.

INTRODUCTION

Tenascin is a multifunctional extracellular matrix glycoprotein which is characterized by its expression in fetal tissues and association with the neovasculature and stroma of tumors (1–5). It is involved in numerous cellular functions including adhesion, migration and differentiation, angiogenesis, wound healing, embryonic development and tumor cell metastasis (6, and reviewed in 3,4). Structurally, tenascin is composed of repeating EGF-like repeats and fibronectin-type III-like domains (7). At least one tenascin type III repeat, repeat 3, contains a specific cell binding site. This cell binding site serve as a ligand for RGD dependent integrin class receptors (8,9). In addition there may be a heparin binding site in repeat 12 (10). Other type III repeats as well as EGF repeats may mediate other cellular interactions involved in cell proliferation, neural cell adhesion, and immune modulation (11–15). It is likely therefore that multiple type III repeats may mediate specific matrix or receptor interactions. It is also apparent that multiple tenascin isoforms are detectable at the mRNA and protein level. Alternative splicing is one mechanism by which tenascin isoforms are generated. Such isoforms and their relative abundance could mediate specific cell type or matrix interactions.

This mechanism of functional regulation by alternative splicing has been demonstrated for collagen II, collagen VIII, collagen IX, elastin and fibronectin (16-24). Multiple forms of fibronectin have been shown to arise as a consequence of alternative splicing of the mRNA at three sites (25,26). Fibronectin alternative splice forms in the V region result in the alternative expression of an $\alpha_4\beta_1$ integrin binding site in cellular fibronectin and its deletion in plasma fibronectin (27). Similarly, several alternative splice forms have been observed in tenascin which appear to be regulated during development (28,29). The most obvious splice forms involve splicing out repeats 6 to 11, coding for nearly 100 kd of the molecular weight of human tenascin (30–34). However, in an examination of human tenascin cDNA and type III alternative splicing we have found an additional type III domain encoded between the previously identified type III repeats 10 and 11, bringing the total number of type III repeats to 16. This novel type III domain appears to be spliced as part of a complex pattern of alternative splices among its flanking type III repeats, thus indicating that multiple tenascin isoforms may be present in tissues and cells of normal and neoplastic origin. These alternative splices have been identified at the mRNA level through cDNA sequencing, PCR analysis and PCR product sequencing. The alternative splicing of tenascin mRNA may well result in the expression of at least 8 tenascin isoforms. We speculate that such isoforms may well result in varied functional populations of tenascin. At least one alternative splice involving AD1 may be tumor associated.

MATERIALS AND METHODS

Isolation of tenascin cDNA clones from U251 MG glioma cDNA library and their sequencing

A random primed human glioblastoma U251 MG cDNA library in λZAP II was custom made by Stratagene (La Jolla, CA) from poly-A mRNA. Multiple cDNA clones were identified by screening the U251 library with a bacterial lysate absorbed polyclonal antiserum against human tenascin. The positive cDNA clones were plaque purified and the Bluescript phagemid (Stratagene) rescued. Sequencing was performed on plasmid DNA by the method of Sanger (35). The Sequenase Version 2.0

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(United States Biochemical) and ^{35}S-dATP (Amersham) were used to sequence cDNA clones. Overlapping regions of the DNA were sequenced by using specific oligonucleotides as sequencing primers.

RNA extraction and first strand cDNA synthesis

Total RNA from human glioblastoma lines: U251MG and U87MG; neuroblastoma: SK-N-SH; osteosarcoma: MG-63; human fibroblast cell line IMR 90, and human umbilical vein endothelial cells (HUVEC) was isolated by using the guanidinium isothiocynate/cesium chloride method (36). Poly-A RNA was prepared using a mRNA purification kit (Stratagene La Jolla, CA) and random primed with poly hexanucleotides (Pharmacia) for the preparation of cDNA using M-MLV reverse transcriptase (BRL) (100 units/μl).

Analysis of the alternate splicing pattern of poly-A RNA by RT-PCR and Southern blot

Reverse transcribed mRNA from the different cell lines was PCR amplified using flanking oligonucleotides. These include 3'AD15: TGTAATGACAAAGGCAGTGA; 5'AD13:ACCAAAGCCA-CAGTTGGGCA; 3'8E2:GCTGTCAAAGGCATTCTCCGAT-GCC; 5'3200: GCTACCCCCTAGTACTGATTTTATTGTC-TA; 3'5316:CTGGTCTGAGTCTTG-GTTCCGTCC. PCR was performed for 30 cycles (94°C for 1 min, 55°C for 30 sec and 72°C for 2 min) with final soak at 72°C for 10 min using the GeneAmp kit (Perkin Elmer Cetus). The PCR amplified DNA samples (15%) were run on an agarose gel and the different fragments purified using the PCR Prep kit (Promega). Each of the PCR products was sequenced either directly with specific oligonucleotides as sequencing primers using ^{32}P-dATP or subcloned into pCR-1000 vector (Invitrogen) and the plasmid DNA containing the different inserts from each of the PCR analysis were sequenced using M13 forward and reverse primers or specific primers as described above. All sequences were analyzed using the PC gene software package (Intelligenetics). Southern analysis was performed with 30 μg of human genomic DNA on nylon membrane as described (36). AD13 and AD15 oligonucleotide probes were end labelled with γ^{32}P-ATP and hybridized at 55°C overnight. The blot was washed twice with 2×SSC/0.1%SDS at room temp and once with 0.1×SSC/0.1%SDS at 55°C at high stringency and exposed at −70°C.

RESULTS

Sequencing of two tenascin cDNA clones (TN3.1 and H1) selected from a U251 MG human glioma cDNA library revealed a new 276 bp sequence designated AD1 (additional domain 1) between previously identified type III repeats 10 and 11. The sequence codes for 92 amino acids inframe with the known tenascin coding sequence (Fig. 1). The AD1 protein sequence is clearly a type III repeat based on its overall homology with the other type III repeats of human tenascin. Alignment of the protein sequence of AD1 with human tenascin type III repeats is shown in Fig. 2. The alignments are ordered by their degree of homology.

Searches of the Genbank, EMBL, and PIR data bases with the AD1 nucleotide and protein sequences did not find an identical match. However, the searches did find related sequences. The highest levels of protein sequence homology were with human tenascin type III repeat 11, mouse tenascin type III repeat 9 and chicken tenascin type III repeat 7 with 36%, 35% and 37%

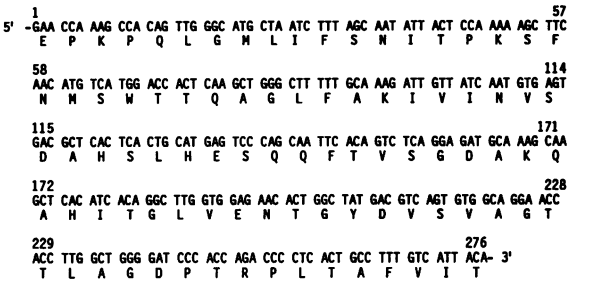

Figure 1. Nucleotide and amino acid sequence of AD1 as determined from clones TN3.1 and H1. A U251 cDNA library was screened with bacterial lysate absorbed polyclonal antiserum against human tenascin. Tenascin coding clones were obtained, subcloned into bluescript and sequenced using either T3 and T7 primers or used internal primers derived from cDNA sequencing.

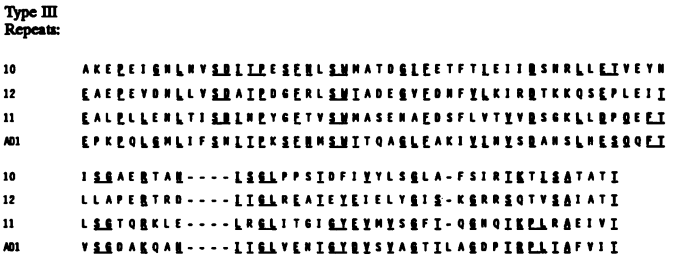

Figure 2. Alignment of the protein sequence of AD1 with other human type III repeats. The amino acid alignments as determined by computer analysis (PC gene software) are ordered by their degree of homology. Amino acids identical or lightly conserved with AD1 are underlined in type III repeat.

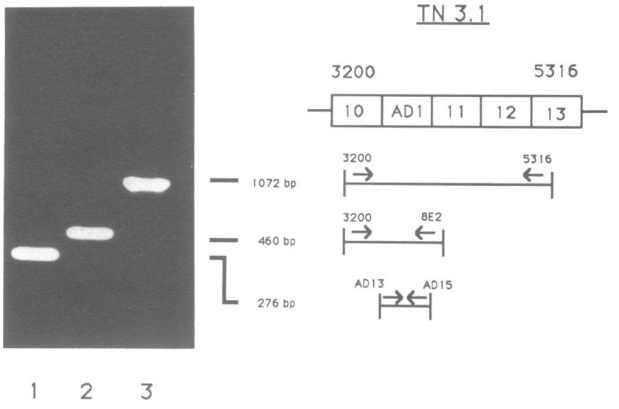

Figure 3. PCR analysis of clone TN3.1. cDNA from clone TN3.1 was subjected to PCR analysis with primer pairs (1) AD13/AD15; internal primer pair for AD1, (2) 3200/8E2; corresponding to repeats 10 and 11, and (3) 3200/5316; corresponding to repeats 10 and 13. The PCR was performed for 30 cycles and the samples were analyzed on 1.2% agarose gel.

identity respectively. In addition, the AD1 protein sequence was found to have 23 to 27% homology with type III repeats from fibronectin of various species. Comparing the AD1 protein sequence with that of its flanking type III repeats 10 and 11 revealed that AD1 had 34% and 36% identity respectively, with these repeats. Many of these conserved amino acid residues are at positions characteristic for type III repeats (Fig. 2).

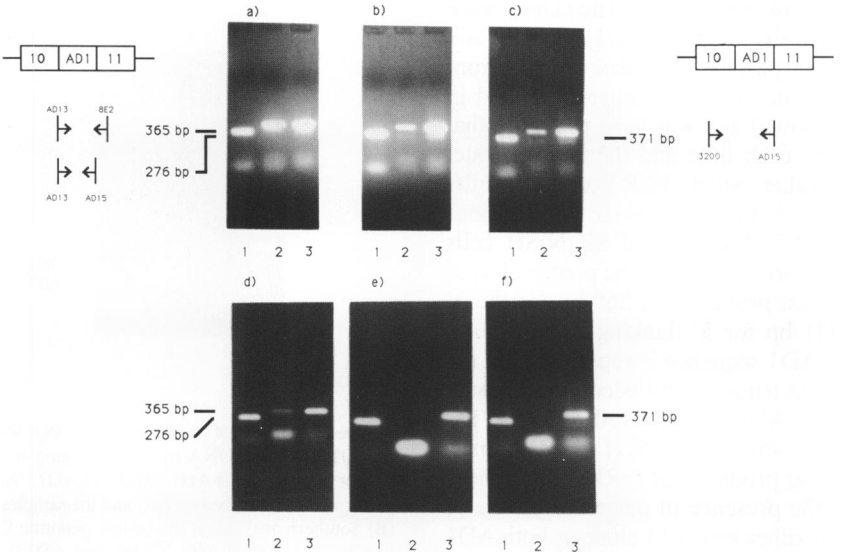

Figure 4. RT-PCR of mRNA from (**a**) U251, (**b**) U87, (**c**) MG 63, (**d**) SK-N-SH, (**e**) IMR-90 and (**f**) HUVEC. One μg of mRNA was reverse transcribed using M-MLV RT and subjected to PCR using the following internal primer pairs: (1) AD13/AD15, (2) AD13/8E2 (3) AD15/3200. The PCR amplified samples were analyzed on a 1.2% agarose gel.

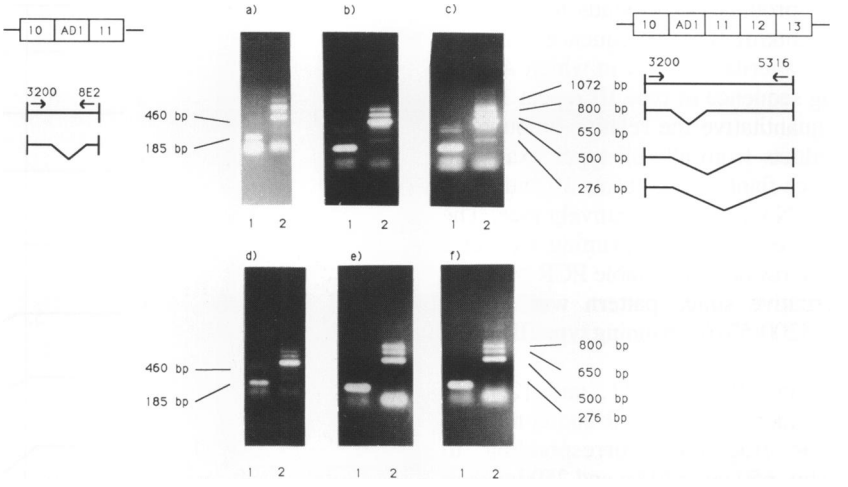

Figure 5. Alternate splicing of tenascin mRNA in different cell lines by PCR. mRNA from (**a**) U251, (**b**) U87, (**c**) MG 63, (**d**) SK-N-SH, (**e**) IMR-90 and (**f**) HUVEC was evaluated by RT-PCR as described above. The cDNA was PCR amplified with the following internal primer pairs: (1) 3200/8E2 corresponding to primers from repeats 10 and 11, and (2) 3200/5316 corresponding to primers from repeats 10 and 13. The samples analyzed on a 1.2% agarose gel, the fragments purified and sequenced.

The AD1 domain appears to be spliced as part of a pattern of multiple alternative splices. Sequence analysis of the two cDNA clones TN 3.1 and H1 revealed that clone H1 had type III repeats 11 and 12 spliced out and the AD1 domain was contiguous with repeats 10 and 13 while TN3.1 was not spliced and the AD1 domain was contiguous with repeats 10 and 11. Additional splice variants were identified in mRNA from different cell types by PCR. Primers were prepared from the AD1 sequence at its 5′ (#AD13) and 3′ (#AD15) ends, and at positions 86 bp 5′ of AD1 in the type III repeat 10 (#3200), and 95 bp 3′ of AD1 in type III repeat 11 (#8E2). These primer pairs allowed for PCR not only of the AD1 sequence but across its junction with either flanking type III repeat or across the junction of both 5′ and 3′ flanking repeats. In addition, a 3′ primer located 140 bp from the 5′ end of type III repeat 13 (#5316) was made to allow for PCR flanking type III repeats AD1, 11 and 12 (Fig. 3).

PCR analysis of the TN3.1 cDNA with internal primer pairs from AD1 (ie., AD13/AD15), repeats 10 and 11 (ie., 8E2/3200), and repeats 10 and 13 (3200/5316) resulted in the generation of the expected full length products of 276, 460 and 1072bp confirming the sequencing data from cDNA (Fig. 3). The mRNA from 4 tumor cell lines including the glioblastomas: U251, U87, neuroblastoma: SK-N-SH, osteosarcoma: MG 63; and two normal cell types: normal lung fibroblast, IMR-90 and HUVEC's expressing tenascin (as determined by ELISA, data not shown)

were primed for cDNA using random primers. The cDNA were then subjected to PCR with the internal AD1 primers and appropriate AD1 and flanking primers. Primers derived from human β actin were used as internal PCR controls resulted in a 623 bp product (data not shown) and was used to ensure that the PCR worked consistently each time and the actin product acted as an additional size marker. Single PCR products for the AD1 internal, 5′ flanking, and 3′ flanking primer combinations were observed for U251, U87, MG 63 and SK-N-SH cells (Fig. 4a–d). These products corresponded to the predicted sizes of 276 bp for the AD1 internal primer pair, 365 bp for the 3′ flanking primer pair and 371 bp for 5′ flanking primer pairs. The results confirm that the AD1 sequence is represented in the mRNA of cell lines expressing tenascin and is contiguous with the known tenascin sequence. Although, IMR90 and HUVEC did yield the predicted 276 bp product for the AD1 internal primer pair and the 371 bp 5′ flanking product, the mRNA from these normal cells did not reveal the presence of detectable levels of the 3′ product suggesting that either repeat 11 alone or both AD1 and type III repeat 11 were spliced in normal cell lines (Fig. 4).

We next examined the larger pattern of PCR products resulting from PCR priming across type III repeats 10, AD1, 11 and repeats 10, AD1, 11, 12 and 13. The primer pair immediately flanking AD1 on both the 5′ and 3′ ends produced two products; a 460 bp fragment which included AD1 and a second 185 bp fragment in which AD1 is spliced (Fig. 5). As determined by sequencing, the larger PCR product corresponds to the AD1 sequence plus the 5′ and 3′ flanking type III sequence while the 185 bp product represents an alternative form in which AD1 is spliced and only the flanking sequence of type III repeat 10 and 11 are present. While not quantitative the relative amounts of the 461 bp and 185 bp products from all cell types examined indicate that the AD1 sequence flanked by repeats 10 and 11 as represented by cDNA clone TN3.1 may be relatively rare. The results are similar to those in Fig. 4 in that priming with 8E2 from type III repeat 11 did not result in detectable PCR products in normal cells. The alternative splice pattern was further examined with primer pairs (3200/5316) spanning type III repeat 10 to 13 (Fig. 5).

For the four related repeats 10, AD1, 11, and 12, four alternative splice forms were identified by PCR and confirmed by sequencing. Five PCR fragments corresponding to approximately 1072 bp, 800 bp, 650 bp, 520 bp and 250 bp were detected. Sequencing of the PCR fragments confirmed that the largest PCR fragment observed in the U251 mRNA included type III repeats AD1, 11, 12 and flanking type III repeats 10 and 13 sequences. The predicted size of this fragment was 1076 bp, however, the full length fragment was not discernable in the other cell lines tested suggesting that it's occurrence is rare. Alternatively the low levels detected could be the result of disproportionate priming of the shorter PCR products. The 800 bp fragment which was observed in all the cell types analyzed represents mRNA in which AD1 was spliced out. The 520 bp fragment is the major PCR product observed and sequencing of this fragment obtained from the different cell types indicated that repeats AD1 and 11 are spliced together. The smallest PCR product includes only the 5′ flanking repeat 10 sequence and 3′ flanking repeat 13 sequence. In each case sequencing confirmed a single sequence and an in frame translation product with the expected type III repeat junctions. The 800, 520 and 250 bp fragments indicate that the type III repeat AD1 is spliced alone or in combination with repeats 11 or 11 and 12, respectively.

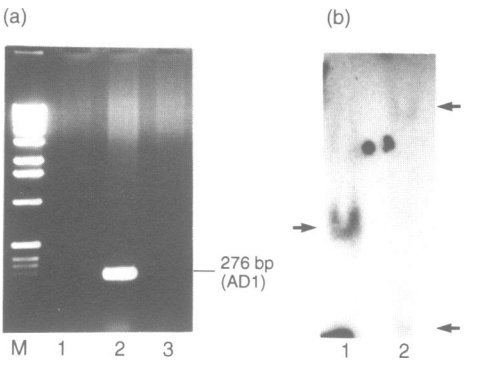

Figure 6. (**A**) PCR of human genomic DNA for the analysis of exon organization of AD1. Genomic DNA from human lung fibroblasts was PCR amplified with primer pairs from (1) AD13/8E2, (2) AD13/AD15 and (3) 3200/8E2. PCR was performed as described earlier, and the samples analyzed on a 1.2% agarose gel. (**B**) Southern analysis of the human genomic DNA digested with (1) EcoR1 (2) BamH1 and probed with ^{32}P-labelled AD13/AD15 primers.

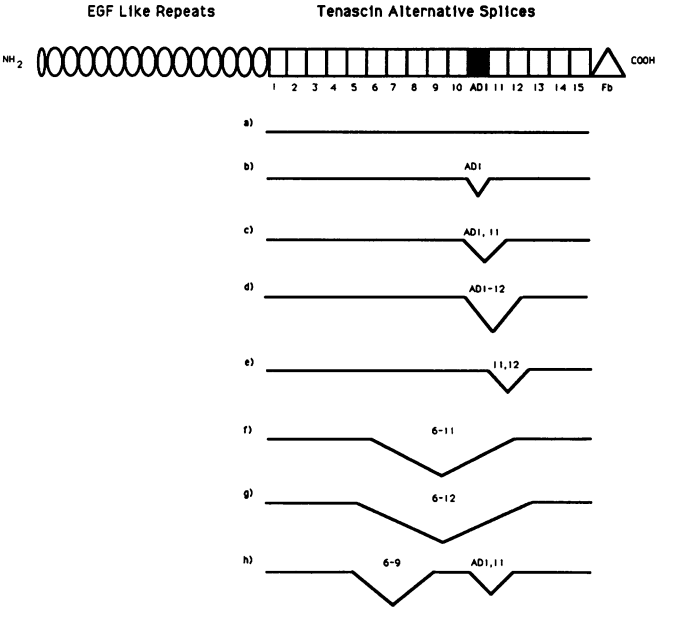

Figure 7. Diagrammatic representation of AD1 within the 15 type III repeats of human tenascin and possible alternative variants as identified by cDNA cloning and PCR analysis. **a**) clone TN3.1 (present investigation), **b**) clone P29 (29), **c**) clone HT-33 (32), **d**) PCR analysis, **e**) clone H1 and **f**) clone H4 (present investigation), **g**) clone P31 (29), and **h**) clone HT-5 and PCR analysis (32). The EGF repeats are shown in circles. The squares represent the tenascin type III repeats. The carboxy terminal fibrinogen like domain is shown as a triangle.

In each of these splice variants entire type III repeats are spliced out. However, an additional 650 bp PCR fragment was observed in the different cell lines tested. Although this fragment size could conceivably represent an internal exon splice indicating the presence of one and a half type III repeats being spliced, sequence analysis of the cloned 650bp fragment from the different cell types did not correspond to any of the known tenascin type III repeats.

We have inferred from the pattern of type III repeat alternative splices (34) that AD1 corresponded to a single exon. To examine this question, human genomic DNA was analyzed by PCR and

Southern analysis. The 5′ and 3′ AD1 internal primers amplified a single 276 bp product, corresponding to the complete AD1 type III repeat (Fig. 6a). The exon/intron junctions are inferred from PCR with primers flanking the type three repeats junctions between 10 and AD1 and AD1 and 11. No PCR product was observed with these primer pairs indicating there are large intervening sequences between the putative exons (Fig 6a). Similarly, Southern analysis suggests that AD1 is encoded by a single gene (Fig. 6b). Human genomic DNA was digested with either EcoR1 or BamH1 and probed with the internal AD1 primers. Hybridization of the EcoR1 digested genomic DNA with γ^{32}P-ATP labelled AD13/AD15 primer pair from the 5′ and 3′ ends of AD1 resulted in the presence of a single restriction fragment. Digestion of genomic DNA with BamH1 which has one restriction site within the AD1 domain resulted in the detection of 2 fragments (Fig. 6b). These results together confirm the presence of the AD1 sequence in the genome and indicate that this type III repeat is encoded by a single gene. In addition the presence of a 276 bp PCR product was also observed in mouse genomic DNA when primed with the AD1 internal primers. Sequence analysis of the subcloned mouse 276 bp PCR product revealed it to be a homologue of the human AD1 sequence. The results demonstrate the presence of AD1 in human and mouse genomic DNA.

DISCUSSION

The results presented here describe the identification from tenascin coding cDNA of a new alternatively spliced exon sequence encoding a single 92 amino acid type III repeat between the previously identified tenth and eleventh type III repeats. Tenascin, therefore, has at least 16 consecutive type III repeats. Tenascin mRNA from human, mouse, and chicken are highly homologous, leading us to speculate that human AD1 related repeats may be present in mouse and chicken tenascin genes. This has been subsequently confirmed for mouse.

Structurally the AD1 repeat is closely related to the three flanking repeats 10, 11 and 12 based on both its nucleotide sequence and the deduced amino acid sequence (Fig. 2). These four type III repeats form one of several type III homology groups. Based on their level of homology the type III repeats 6−9 form a group as do repeats 3, 5, 14 and 15. It would appear that duplications of type III repeats have given rise to the present group of type III repeats. What functions the AD1 and flanking repeats may have is unknown but it is not unlikely that one or another of these repeats represent functional domains. At least two type III repeats of tenascin contain functional sites. The repeat 3 includes an RGD cell binding site which interacts with integrin receptors (8,9) and repeat 12 likely includes a heparin binding site (10).

Within the group of repeats 10, AD1, 11 and 12, we have identified at least five alternative splices (Fig. 4 and 5). Two tenascin cDNA clones that we identified to have the AD1 sequence indicate the presence of two possible splice forms ie., a full length transcript (clone TN3.1), and one in which repeat 11 and 12 are spliced (clone H1). PCR analysis of a random primed mRNA from several cell lines revealed additional tenascin splice variants. The PCR of mRNA with primers from type III repeat 10 and type III repeat 13 revealed multiple PCR products (Fig. 5). The largest product corresponds to predicted full length product spanning the 3′ end of repeat 10, AD1, 11, 12 and the 5′ end of repeat 13. In descending order of size the PCR products corresponded to an 800 bp product in which AD1 is spliced; a 500 bp product in which AD1 and repeat 11 are spliced; and finally a 250 bp product which included only the repeat 10 and repeat 13 sequences. For each of the PCR products we have confirmed by sequencing the splices described. In each case the splices result in appropriate splice junctions and a single open reading frame.

In examining the alternative splice patterns of mRNA from normal and neoplastic cells differences are seen. When PCR was performed with internal primers AD13 and 8E2 flanking AD1 and repeat 11, the mRNA of all the tumor cell types studied (U251, U87, MG-63 and SK-N-SH) revealed the presence of a 365bp product. However, the two normal cell type (fibroblasts and endothelial cells) mRNA lacked this band and this product was not observed when PCR was performed for up to 35 cycles, thus suggesting that repeats AD1 and 11 either singly or together were spliced out in these normal cells (Fig. 4). This was further substantiated by PCR using primers flanking AD1 in repeats 10 and 11. Although the 460 bp full length product was observed in all tumor cell lines, this fragment was not present in both normal cell types analyzed, consistent with the finding that AD1 and repeat 11 singly or together were spliced out in normal cells (Fig. 5). Additionally the full length product spanning repeats 10−13 was observed only in tumor cell lines, particularly in U251 mRNA although its occurrence appears to be rare in the different cell lines tested. The absence of certain splice variants involving the AD1 in normal cells even after performing PCR at saturating levels, suggest that there may be a tumor associated pattern of splicing involving AD1 and flanking repeats. Such tumor associated splice variants have been suggested for the splicing of type III repeats 6−11 and 6−12 (37).

In addition to the splice variants involving AD1 and its flanking type III repeats other splice variants have been described (Fig.7). These alternative splices include deletion of type III repeats 6−9 in combination with deletion of repeat 11 or deletion of 10−12 (32−34). We now know these splice variants must also include deletion of AD1. Sequencing of yet another cDNA clone H4 that we have recently identified (Fig. 7), revealed that type III repeats 6−11 including the AD1 could be spliced out. This splice and the splices represented in clone P31 (30) in which repeats 6−12 were spliced represent splice variants equivalent to previously identified splices in chicken tenascin mRNA (11). Based on results from the present investigation and those described by others (11,29−32) it appears that alternative splicing of tenascin mRNA may result in the expression of at least 8 variants of tenascin (Fig. 7). In addition we have observed the presence of a highly conserved AD1 repeat homologue in the mouse genomic DNA.

Alternative splicing appears to be a means by which multiple tenascin isoforms could be generated. We would speculate that like fibronectin, tenascin isoforms could represent functionally discrete forms of tenascin. Alternate splicing of fibronectin mRNA is an important process that helps in tissue specific and developmentally regulated gene expression in eukaryotes (25,26,38,39). Analysis of the alternative splicing in the fibronectin primary transcript, reveals that multiple forms arise because of splicing at three sites; EIIIA, EIIIB and the variable (V) region (IIICS) (22−26). EIIIA and EIIIB are single type III repeats encoded by a single exon that are either skipped or included during splicing. These variants are found in a subset of cellular fibronectin subunits. Such a splicing within the coding sequences gives rise to 5 variants in humans (25). In addition,

12 different combinations of the three alternatively spliced segments of fibronectin have been observed in rats (40). The requirement of different combinations of alternative spliced fibronectin variants for the secretion of fibronectin dimers has also been demonstrated (41). Further, the expression of a $\alpha 4\beta 1$ binding site in the cellular form and its deletion in plasma fibronectin and the involvement of fibronectin in blood clotting as well as secretion and synthesis of fibronectin have all been reported to be differentially affected by alternative splicing (27,41). These results lend support to the idea that tenascin alternative splice forms may also have functional significance at the protein level.

Further insights into the differential expression of alternatively spliced variants of tenascin will help us to understand differences between normal and tumor cell expression and function.

ACKNOWLEDGMENTS

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