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# Cell-Type-Specific Expression of Alternatively Spliced Human Fibronectin IIICS mRNAs

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**Fibronectin polypeptide diversity is generated to a large extent by alternative splicing of the fibronectin primary transcript at three sites: two extra domain exons encoding extra structural repeats and a region of nonhomologous sequence termed the type-III connecting segment (IIICS). A novel double primer extension assay was developed to identify and quantify simultaneously each of the five human IIICS mRNA splicing variants. Expression of the five IIICS variants was analyzed in a variety of human normal and tumor cell types as well as in human liver. Differences in IIICS expression patterns were observed among different cell types, among fibroblasts of different tissue origins, and between comparable normal and transformed cells. The most predominant cell-type-specific differences were in the abundance of the one IIICS$^-$ mRNA variant relative to the four IIICS$^+$ variants. The percentage of O variant (IIICS$^-$) mRNAs within the total fibronectin mRNA pool varied between 3 and 17% among tumor cells and between 7 and 46% among normal cells. The O variant composed 57% of the fibronectin mRNA in liver tissue, correlating with the previously described increased abundance of IIICS$^-$ polypeptide subunits in plasma fibronectin, compared with those in cellular fibronectins. Additional cell-type-specific changes among the expression levels of the four IIICS$^+$ mRNA variants are consistent with a proposed model in which regulation of an alternative selection of a 3′ splice site predominates over regulation of the selection of a 5′ splice site in generating specific patterns of IIICS mRNA expression.**

Fibronectin (FN) is a large adhesive glycoprotein of the extracellular matrix composed of two nearly identical polypeptide subunits (1, 36). Each subunit contains binding domains for a variety of cell surface and extracellular ligands, including collagen, proteoglycans, fibrin, and the integrin family of matrix receptors (23). Through this multiplicity of adhesive activities, FN can fulfill key roles in a broad spectrum of physiological processes, such as morphogenesis, wound healing, opsonization, differentiation, and cell attachment and migration.

FN polypeptide subunits are composed of a long series of structural domains displaying three patterns of amino acid sequence homology, termed type I, type II, and type III homology repeats (15, 24, 35). The exon structure of the single FN gene reflects this pattern of repeated structural units (22), with each homology repeat encoded by either a single exon or a discrete pair of exons. The repeated domain structure of the polypeptide and its underlying genomic organization are well conserved among the human, rat, bovine, and chicken species, where analyzed.

Heterogeneity among FN subunits isolated from different sources (18, 33, 40) arises in part from alternative splicing of the primary FN transcript at three distinct locations (8, 34). At two of these regions, termed extra domain exons (17, 26, 30), single type III homology repeats, each encoded by a single exon, are alternatively expressed in the mature polypeptide. Cell type or tissue specificity of expression of both extra domain regions has been demonstrated at both the protein (6, 8, 34) and mRNA (17, 25, 26, 30) levels. The third location of alternative splicing of the primary FN transcript is termed the type III connecting segment (IIICS). The alternatively spliced 5′ portion of the IIICS exon encodes a stretch of 120 amino acids which bears no homology to any other region in the FN mRNA sequence. The 3′ region of the IIICS exon encodes the first portion of the most C-terminal type III homology repeat and is constitutively included in all mature transcripts.

The sequencing of cDNA (4, 25, 38) and genomic clones (43, 44) of the IIICS region revealed three consensus 3′ splice acceptor sites within this complex exon (Fig. 1A). These 3′ acceptor sites are present and functional in both rat and human FN (38, 43, 44). In addition, a functional alternative 5′ splice donor site is present within the human IIICS alternatively spliced sequence (44) but is absent in the rat sequence (43). This complexity of choices for splice sites allows for the hypothetical existence of five human IIICS mRNA variants, four of which (all except the BC variant) (Fig. 1A) have been demonstrated to exist through cDNA cloning (4, 25, 39) and nuclease protection experiments (31, 39).

The functional significance of the IIICS peptide segment has been demonstrated by the studies of Humphries et al., who identified two distinct IIICS sequences capable of promoting the attachment and spreading of B16-F10 murine melanoma cells but not BHK fibroblasts (19, 21). Synthetic peptides CS-1, composed of the A subsegment of the IIICS (Fig. 1), and CS-5, containing an REDV peptide sequence from the C subsegment, both exhibited adhesion-promoting activity specifically for melanoma cells (19, 21), with the CS-1 peptide exhibiting significantly greater activity. The adhesive activity of the CS-1 sequence was further demonstrated for neurite extension from chick peripheral nervous system neurons (20) and the spreading and motility of avian neural crest cells (13). The receptor on T lymphocytes for the CS-1 adhesion signal has been identified as a member of the integrin matrix receptor family distinct from the integrin on these same cells that mediate adhesion to the constitutively expressed RGDS-dependent cell adhesion domain of FN (45). The adhesive activity of the IIICS for melanoma and neuronal cells but not fibroblasts demonstrates the

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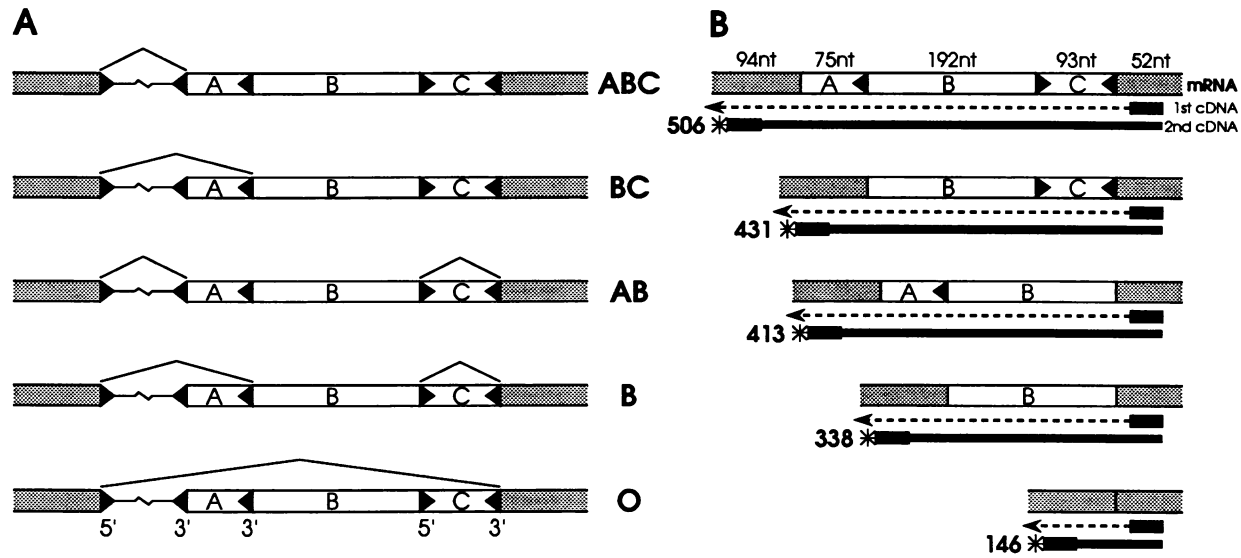


FIG. 1. Model of the DPE assay for evaluating products of alternative splicing in the human FN IIICS region. (A) The genomic structure of the IIICS region of the human FN gene. Exon sequences constitutively included in mature mRNA transcripts are represented by stippled boxes. Alternatively spliced sequences are represented by open boxes, with uppercase letters (A, B, and C) denoting subsegments of the IIICS region. Intron sequences are represented by jagged lines. Splice site sequences are denoted by rightward-pointing (5′ splice donor) and leftward-pointing (3′ splice acceptor) solid triangles (labeled 5′ and 3′ at the bottom of the diagram). V-shaped lines above each splice variant reflect the splicing events that give rise to each of the five mRNA variants. Each variant mRNA is named, using uppercase letters (to the right of each variant) to reflect the IIICS subsegments contained in that variant. (B) Derivation of full-length DPE products, using primers IIICS-5′ and IIICS-3′. The structure of each mature mRNA is shown with stippled (constitutive sequence) or open (IIICS; alternatively spliced sequence) boxes. Numbers above the diagram indicate the distances (in nucleotides [nt]) between neighboring splice junctions or between a splice junction and the distal end of a primer binding site. Wide solid lines indicate oligonucleotide primers, and asterisks indicate the radioactive label on the IIICS-5′ primer. Dashed lines represent the first-strand cDNA reverse transcription products, and solid lines represent the second-strand Sequenase primer extension products. Numbers to the left of each variant indicate the expected size (in nucleotides) of the radiolabeled second-strand runoff DPE product corresponding to that mRNA variant.

considerable cell type specificity in the multiple mechanisms with which differentiated cell types adhere, spread, or migrate on FN substrates, suggesting the utilization of different sets of FN receptor components for these physiological processes. These findings also point out how regulation of the qualitative distribution of different FN subunits via alternative splicing may serve to modulate cell-type-specific adhesion to FN-containing matrices of different subunit composition.

In this study, we examined cell type specificity in the qualitative expression of the human IIICS mRNA variants and the underlying regulatory mechanisms of IIICS alternative splicing. To pursue these goals, a novel double primer extension (DPE) assay that simultaneously identifies all five IIICS mRNA variants in human cells was developed. This technique has been used to examine IIICS mRNA expression patterns in a variety of human normal and tumor cell lines and in human liver tissue. On the basis of the differences in IIICS expression among different cell lines, a model is proposed in which cell-type-specific preferences in the utilization of alternative 3′ splice acceptor sites is more important than the selection of 5′ splice donor sites in establishing specific patterns of IIICS variant expression.

## MATERIALS AND METHODS

**Human cell lines.** 8 Pap and 13 Pap human dermal papillary fibroblasts and human K2 keratinocytes (5, 37) were isolated by Bryan Davis and Irwin Schafer, Cleveland Metropolitan General Hospital, from patients 78 years old (8 Pap) or 17 years old (13 Pap). HMC human kidney mesangial cells (P. Mene, M. S. Simonson, and M. J. Dunn, Physiol. Rev., in press) were isolated by Michael J. Dunn, Division of Nephrology, University Hospitals of Cleveland. Fetal lung fibroblasts GM-5387 were obtained from the National Institute of General Medical Sciences cell repository, Camden, N.J.; fetal skin fibroblasts AG-4449 were obtained from the National Institute on Aging cell repository, Camden, N.J.; and the remaining human cell lines were obtained from the American Type Culture Collection, Rockville, Md. Human transformed and tumor cell lines were HepG2, hepatocellular carcinoma; VA-13, simian virus 40-transformed WI-38 fibroblasts; RD, rhabdomyosarcoma; HT-1080, fibrosarcoma; A172, glioblastoma; A549, adenocarcinoma; and Hs294T, melanoma. Normal cell strains were CCD-18Co, colon fibroblasts; WI-38, MRC-9, and MRC-5, fetal lung fibroblasts; and WI-1003, adult lung fibroblasts. Normal human liver tissue samples were obtained from the Tissue Collection Facility, University Hospitals of Cleveland.

**Cell culture and RNA purification.** Cells were cultured *Mycoplasma*-free in Dulbecco modified Eagle medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 10% neonatal calf serum (Biologos, Naperville, Ill.), 250 U of penicillin per ml, and 250 μg of streptomycin sulfate per ml at 37°C in a 10% $CO_2$-humidified air mixture.

Total cellular RNA was purified from confluent cell cultures by the guanidinium isothiocyanate-hot phenol method (11, 28) and then treated with proteinase K (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). Cells were lysed directly in the tissue culture flask with the hot guanidinium solution to avoid possible detachment-associated alterations in RNA metabolism.

**DPE assay.** Oligonucleotide primers IIICS-5′ (5′-ACT

GGCCTGGAACCGGG-3'), IIICS-C (5'-CCCTGGGAAT GTGACCAATTTGG-3'), and IIICS-3' (5'-GTGTCCTGGA-ATGGGGCCC-3') were synthesized by using an Applied Biosystems (Foster City, Calif.) model 381A DNA synthesizer in accordance with the instructions of the manufacturer and then purified by isolation from a 20% denaturing polyacrylamide gel. These primers are complementary to human FN mRNA sequences that lie within the IIICS C subsegment (IIICS-C) or 3' of the IIICS region (IIICS-3'). Primer IIICS-5' is identical to FN mRNA sequences that lie 5' of the IIICS region (Fig. 1B). Primer IIICS-5' was $^{32}P$ end labeled (28), using [$\gamma$-$^{32}P$]ATP (>5,000 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) and T4 polynucleotide kinase (United States Biochemicals, Cleveland, Ohio).

A first-strand cDNA copy of the target FN mRNA pool was synthesized by extension of primer IIICS-3' (or in select cases, primer IIICS-C as noted), using avian myeloblastosis virus reverse transcriptase (United States Biochemicals). Total cellular RNA (50 μg) was precipitated with ethanol, suspended in 40 μl of 100 mM KCl–50 mM Tris hydrochloride (pH 8.3) with 0.1 pmol of primer IIICS-3' (or IIICS-C), denatured at 95°C for 5 min, and incubated at 50°C for 45 min for primer annealing. Reverse transcriptase reaction mix (60 μl with 50 mM Tris hydrochloride [pH 8.3]–10 mM $MgCl_2$–3 mM dithiothreitol–500 μM each deoxynucleoside triphosphate–3 U of avian myeloblastosis virus reverse transcriptase) was added for extending the annealed primer at 50°C for 45 min. Test studies with purified RNA (transcribed in vitro from cloned FN IIICS ABC variant cDNA sequences) (kindly provided by M.-L. Chu, Thomas Jefferson University, Philadelphia, Pa.) (4) demonstrated that these experimental conditions reliably synthesize first-strand cDNAs extending well past the IIICS-5' primer binding site (data not shown).

Second-strand cDNA runoff extension products were synthesized from first-strand cDNA templates by extension of an annealed $^{32}P$-IIICS-5' primer, using Sequenase (modified T7 DNA polymerase; United States Biochemicals). Ethanol-precipitated reverse transcriptase reaction products were suspended in 40 μl of 40 mM Tris hydrochloride (pH 7.5)–50 mM NaCl–20 mM $MgCl_2$ with 0.1 pmol of $^{32}P$-IIICS-5' primer (0.05 to 0.1 μCi), denatured at 95°C for 5 min, and incubated at 50°C for 45 min for primer annealing. Sequenase reaction mix (20 μl with 10 mM Tris hydrochloride [pH 7.5]–12.5 mM NaCl–5 mM $MgCl_2$–20 mM dithiothreitol–1.25 mM each deoxynucleoside triphosphate–3 U of Sequenase) was added and incubated at 37°C for 15 min. Test studies with reverse transcription reaction products derived from in vitro-transcribed ABC variant RNA resulted in the reliable synthesis of full-length (506-nucleotide) ABC variant second-strand extension products (data not shown).

For restriction analysis of FN-specific DPE products, double-stranded DNA products of DPE reactions were precipitated with ethanol and digested with restriction enzymes (shown in Fig. 2A) in accordance with the specifications of the manufacturer (Boehringer Mannheim).

In order to visualize the radiolabeled cDNAs, DPE reaction products were precipitated with ethanol, suspended in denaturing loading buffer (90% deionized formamide–1× TBE [Tris-borate-EDTA buffer]–0.2% bromophenol blue–0.2% xylene cyanol) and size fractionated on a 5% polyacrylamide 7 M urea denaturing gel (28). $^{32}P$-labeled DPE products were visualized by exposing dried gels to X-ray film (Kodak X-Omat AR). The relative abundance of products corresponding to IIICS mRNA variants were quantified by densitometrically scanning autoradiograms (exposures within the linear range of the film) on an LKB Bromma 2222-010 UltraScan XL laser densitometer, using LKB Bromma Gel-Scan XL data analysis software.

## RESULTS

**Identification and quantitation of the five human IIICS mRNA variants.** A DPE assay to allow simultaneous identification of all human IIICS mRNA variants was developed. In this assay, a family of double-stranded cDNAs are created through primer extension from the FN mRNA pool (Fig. 1B). Each radiolabeled second-strand cDNA is defined at its ends by the sites of oligonucleotides used to initiate each primer extension reaction. The second-strand cDNAs differ in length corresponding to the amount of alternatively spliced IIICS sequence present in the mRNA template between the flanking primer sites.

DPE analysis of HT-1080 fibrosarcoma cell RNA identified all five IIICS variants (Fig. 2B and C), including the postulated but previously undemonstrated BC variant (Fig. 2C). Because primer IIICS-5' is end labeled, the intensity of the radioactive signal in each band should reflect the molar abundance of each mRNA variant in the template cellular RNA sample. The observed molar distribution of variant cDNAs was not altered by increasing the amount of oligonucleotide primers used, increasing the duration of the reaction, or altering the primer annealing temperature (data not shown). Changes in the amount of total cellular RNA assayed (mimicking differences in the FN mRNA abundance among different cell lines) resulted in changes in the overall amount of resulting DPE products but not in the molar distribution of each variant as a percentage of the total amount of DPE products (data not shown). Thus, the observed patterns cannot be attributed to selective primer annealing or extension of individual variant mRNAs under suboptimal experimental conditions. This strongly indicates that this assay provides an accurate and reproducible quantitation of the relative abundance of each FN variant mRNA within the total RNA sample. The absence of longer DPE products corresponding to unprocessed or partially processed FN primary transcripts (those retaining the preceding ~0.6-kilobase intron) suggests that these precursors compose a very low percentage of the total FN RNA pool.

To verify the identity of each product by a criterion other than length or electrophoretic mobility, restriction analysis was used to map the presence of predicted restriction sites within the double-stranded DPE products (Fig. 2A). In all cases, treatment with the appropriate restriction enzymes resulted in the predicted shifts in electrophoretic mobility (Fig. 2B and C). This proves that each of the observed DPE products arises specifically from primer extension of mature FN transcripts.

In order to demonstrate unambiguously the weakly expressed BC variant, a combination of restriction enzyme treatment and extended electrophoresis was initially employed (Fig. 2C). This clearly resolved the small amount of BC product from the large amount of AB product. The assay for BC expression was subsequently simplified by substituting the oligonucleotide IIICS-C, complementary to the IIICS C subsegment, for IIICS-3', as the primer for first-strand cDNA synthesis. This creates DPE products corresponding only to those mRNA variants containing the C subsegment (ABC and BC). The relative amounts of ABC and BC products observed in this C-specific assay (Fig. 2D) were identical to those derived from the comprehensive IIICS assay (Fig. 2C).

These results taken together indicate that the DPE assay provides an accurate, reproducible experimental approach for examining alternative splicing patterns in the FN IIICS region. Its advantages include simultaneous identification of all potential splicing variants, verification of template specificity through restriction analysis, and adaptability through the choice of variant-specific primers.

**Cell type specificity of IIICS mRNA variant expression.** A wide variety of human normal and tumor cell lines as well as liver tissue were tested with the DPE assay to examine how the patterns of expression of IIICS variants differ among different cell types (Fig. 3). The most striking observation from this comparison is that the molar abundance of the O variant, which lacks all IIICS sequence, displayed the greatest variation in amounts among different cell lines. The O variant can compose anywhere from 3 to 57% of the total FN mRNA pool when comparing many different cell types, on the basis of densitometric quantitation of the molar abundance of each variant (Fig. 4A). Thus, cell type specificity in IIICS variant expression appears for the most part to affect the ratio of IIICS$^-$ mRNAs (O variants) to IIICS$^+$ mRNAs (all others) in the steady-state FN mRNA pool. The observed changes in IIICS expression patterns do not exhibit any simple correlation with the significant changes in the quantitative abundance of FN mRNA among these cell lines. For instance, HepG2 cells (3% O variant) contained steady-state levels of FN mRNA comparable to those of skin fibroblasts 8 Pap and 13 Pap (42% O variant), and liver RNA (57% O) contained much less FN mRNA than did skin fibroblasts.

Tumor and transformed cells consistently exhibited low levels of O variant expression (HepG2 through Hs294T) (Fig. 3A and 4A) compared with the high levels of O variant expression in some normal cells (AG4449 through HMC) (Fig. 3B and 4A). In direct comparisons, the simian virus-40-transformed VA-13 cell line (Fig. 3A) (4% O) exhibited reduced O variant expression compared with its parental WI-38 normal fibroblast cell strain (Fig. 3B) (22% O). Similarly, HepG2 hepatoma cells (Fig. 3A) exhibited a drastic reduction in O variant expression compared with liver tissue (Fig. 3B) (3% versus 57%). These observations would suggest that transformation significantly alters FN IIICS expression patterns. However, this correlation between the transformed phenotype and reduced O variant expression is tenuous in that several tumor cell lines (A172 glioblastoma, A549 adenocarcinoma, and Hs294T melanoma) synthesize as much or more O mRNA than do several normal cell strains (CCD-18Co colon fibroblasts, K2 keratinocytes, and several fetal and adult lung fibroblasts). Unfortunately, normal cell progenitors of these tumor cells are not available for direct comparisons.

Among normal cell strains tested, O variant expression displayed significant tissue and cell type specificity (Fig. 3B and 4A). Similar cultured cell types isolated from different tissues exhibited different IIICS patterns, as observed for fibroblasts from skin (fetal AG4449 and adult 9 Pap and 13 Pap) versus fibroblasts from colon (CCD-18Co) or lung (fetal MRC-5, MRC-9, GM5387, WI-38, and adult WI-1003). Different cell types displayed different patterns (K2 keratinocytes versus HMC mesangial cells) even when originating from the same tissue (keratinocytes versus skin fibroblasts).

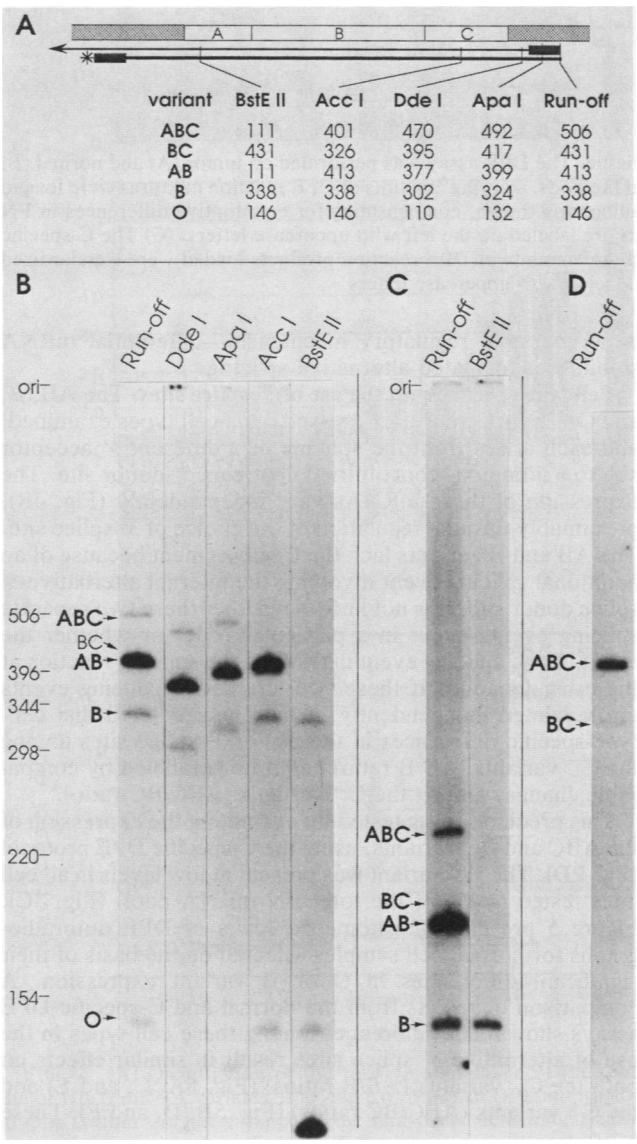


FIG. 2. DPE and restriction analysis of HT-1080 human fibrosarcoma cell RNA. (A) Thin lines indicate the locations of informative restriction sites along the DPE products. Numbers in the table indicate the expected size (in nucleotides) of each radiolabeled DPE product (using primers IIICS-5′ and IIICS-3′) after digestion with the restriction enzymes listed. (B) Autoradiogram of denatured DPE products from HT-1080 fibrosarcoma cell total RNA assayed as described in Materials and Methods. Numbers to the left of the autoradiogram indicate the sizes (in nucleotides) of radiolabeled marker DNA fragments. Runoff DPE products corresponding to mature mRNA variants are indicated by uppercase letters to the left of each variant. Additional lanes reflect the results of digestion of double-stranded DPE products with restriction enzymes whose cleavage sites are present either in all variants (*Dde*I and *Apa*I) or only in those variants that retain the C subsegment (*Acc*I; ABC and BC variants) or the A subsegment (*Bst*EII; ABC and AB variants) of the IIICS region. (C) Autoradiograph of DPE products as described in panel B after extended electrophoresis to better resolve the closely spaced BC and AB variants. The AB band in the *Bst*EII lane is the result of incomplete restriction enzyme digestion, the amount of which varied between repetitions of the restriction treatment depending on enzyme digestion conditions. (D) Autoradiograph of HT-1080 DPE products substituting oligonucleotide IIICS-C (complementary to a site within the C subsegment) for IIICS-3′ as the primer for first-strand cDNA synthesis. Products corresponding to mRNA variants containing the C subsegment are labeled with uppercase letters.

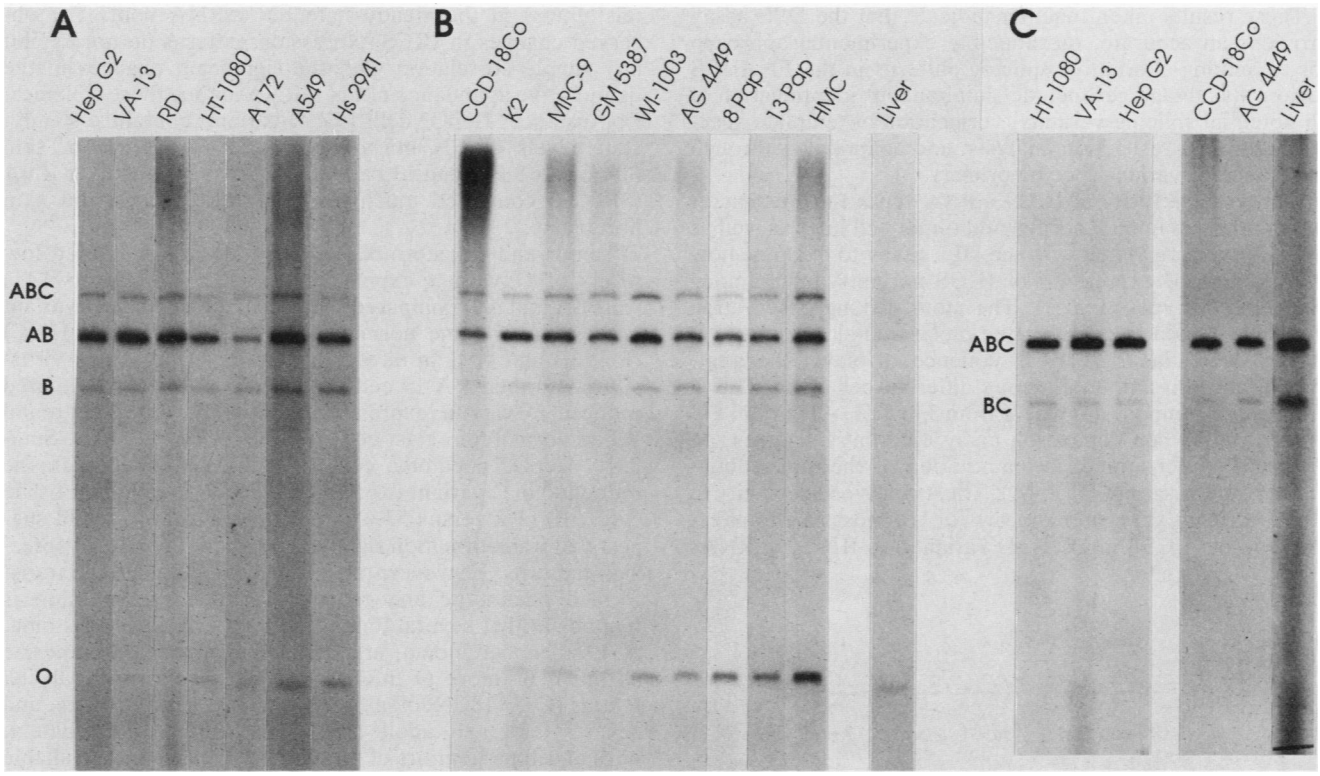


FIG. 3. DPE analysis of human normal and tumor cell lines and liver tissue. The DPE assay was performed on tumor (A) and normal (B) cells, using the IIICS-3′ and IIICS-5′ primers as described in Materials and Methods. Varying amounts of DPE reaction mixtures were loaded on the gels to provide roughly equivalent AB band signals on the autoradiograms shown, compensating for quantitative differences in FN expression among cell lines. The locations of the four major variant bands are labeled on the left with uppercase letters. (C) The C-specific DPE assay with primer IIICS-C was performed on selected samples, and the amounts of DPE reaction products loaded were standardized for a constant ABC band signal. Locations for the two $C^+$ products are labeled with uppercase letters.

If the IIICS mRNA expression patterns of these cells in vitro accurately reflect their FN synthesis patterns in vivo, the significant differences in the ratio of $IIICS^-$ to $IIICS^+$ transcripts suggest that complex tissues may contain matrix microenvironments whose unique FN subunit composition may be regulated by the IIICS expression patterns of the multiple neighboring cell types.

Cell-type-specific regulation of O variant expression could occur through two possible mechanisms. The mature O mRNA may be selectively prone to (or protected against) mRNA degradation in different cell types. Alternatively, changes in the RNA splicing machinery may regulate the use of the O-specific pair of splice sites versus the four remaining splicing patterns. If the O variant is the single mRNA species subject to either of these mechanisms of regulation, it would be expected that increasing O variant expression would result in roughly proportional decreases in the relative abundance of each of the other four variants. However, a comparison of AB variant and B variant levels relative to O variant levels (Fig. 4B) suggests that this is not the case. B variant levels increased modestly with increased percentages of O, in contrast to the significant decrease in AB levels with increasing O expression (Fig. 4B). Unlike all cultured cell lines tested, liver tissue RNA exhibited a level of B variant expression comparable to that of the AB variant (Fig. 3B and 4A), providing the most striking example of this phenomenon. These results strongly suggest that the relative expression of the AB, B, and O variants can each be adjusted independently in a cell-type-specific manner, indicating that the O mRNA variant is not the sole target of either possible regulatory mechanism—differential mRNA stability or regulated alternative splicing.

**Cell type specificity in the use of 3′ splice sites.** The AB, B, and O variants are well expressed in all cell types examined, and each arises from the splicing of a different 3′ acceptor site to a common (constitutive) upstream 5′ donor site. The expression of these mRNAs vary independently (Fig. 4B), presumably through regulation of the choice of 3′ splice site. The AB and B variants lack the C subsegment because of an additional splicing event involving the internal alternative 5′ splice donor site. It is not known whether these two separate splicing events occur in a particular order or whether the result of one splicing event may affect the splicing reaction at the other location. If these two alternative splicing events are regulated independently, it may be predicted that cell-type-specific differences in the use of 3′ splice sites among the $C^-$ variants (AB/B ratio) might be paralleled by comparable changes among the $C^+$ variants (ABC/BC ratio).

This prediction was tested by examining the expression of the ABC and BC variants, using the C-specific DPE protocol (Fig. 2D). The BC variant was present at low levels in all cell lines tested (<2% of the total FN mRNA pool) (Fig. 3C). Figure 5 presents densitometric scans of DPE autoradiographs for normal cell samples selected on the basis of their significant differences in O or B variant expression. A comparison of results from the normal and C-specific DPE assays shows that differences among these cell types in the use of alternative 3′ splice sites result in similar effects on both the $C^-$ variants (AB/B ratios) (Fig. 5A, C, and E) and the $C^+$ variants (ABC/BC ratios) (Fig. 5B, D, and F). These

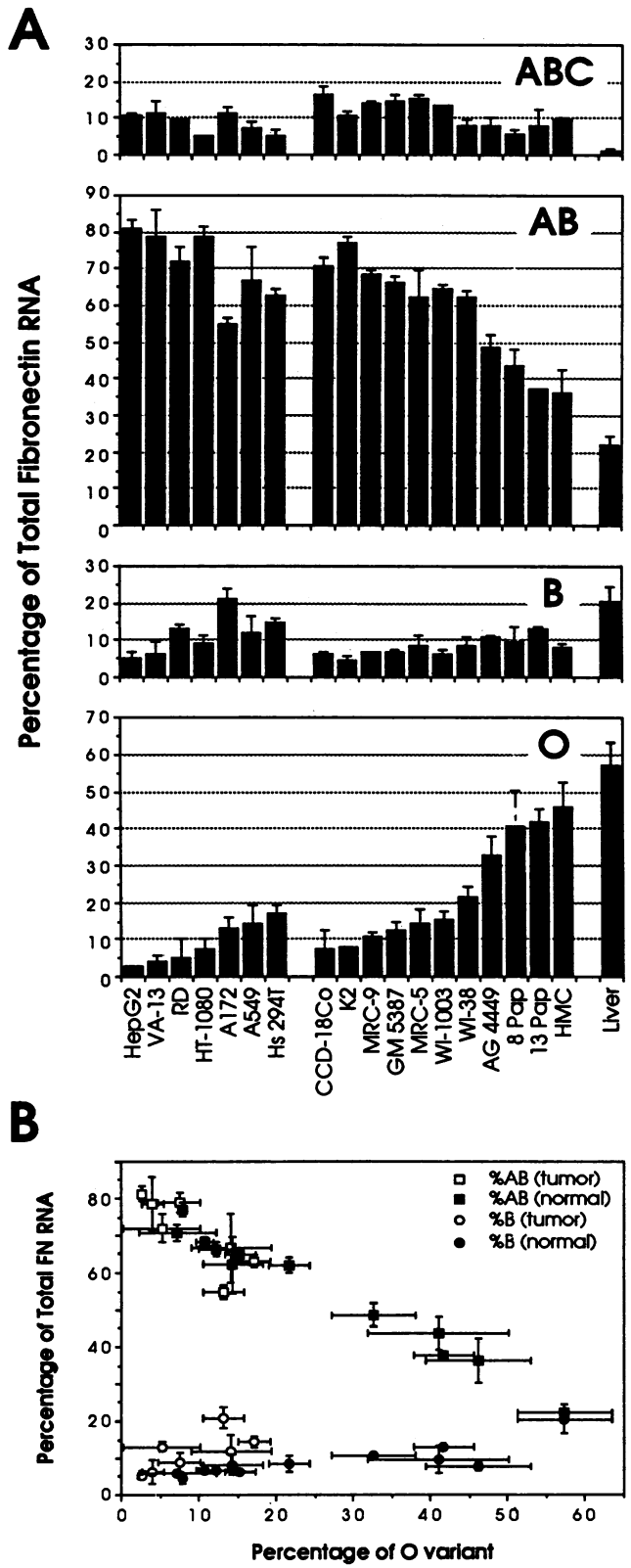


FIG. 4. (A) Quantitation of the relative abundance of the four major IIICS mRNA variants. Varying exposures of DPE autoradiographs (within the linear range of the film) were densitometrically scanned to derive values for the relative abundance of each IIICS variant as a percentage of the total amount of FN mRNA (total of DPE products). Bars represent the standard errors among percentage values obtained from multiple assays of each cell line. Results are grouped among tumor and transformed cell lines (left), normal cell strains (center), and liver tissue (right). (B) AB and B variant levels in relation to O variant expression. The percentages of AB variants (squares) and B variants (circles) are plotted against O variant levels for each tumor (open symbols) and normal (filled symbols) cell type. Percentage values and errors are as described for panel A.

proportional changes in the AB/B and ABC/BC ratios were also observed in tumor cells HepG2, VA-13, and HT-1080 (data not shown).

The coregulation of the B and BC variants relative to the AB and ABC variants suggests that IIICS$^+$ variants can be grouped into A$^+$ (ABC plus AB) and A$^-$ (BC plus B) families (Fig. 6A). These families, in addition to the O variant, result from the use of different alternative 3′ splice acceptor sites in conjunction with a common 5′ splice donor site. Each variant family exhibits cell-type-specific levels of expression that vary independently (Fig. 4B). The most dramatic differences in IIICS mRNA expression patterns among different cell types (Fig. 4B) can be attributed within this model to modulation of the three variant families through the regulation of the choice of alternative 3′ splice sites (Fig. 6B).

**Cell type specificity in the use of 5′ splice sites.** A comparable minority of transcripts within both the A$^+$ and A$^-$ families retain the C subsegment because the human-specific internal 5′ splice donor site is not utilized (Fig. 5 and 6C) (additional tumor cell lines not shown). Among normal cells, from 10 to 20% of the A$^+$ transcripts are not spliced at the internal 5′ splice site (ABC and AB levels in Fig. 4A, 5A, and 5C). In liver RNA, less than 5% of the A$^+$ transcripts fail to use the internal 5′ site (Fig. 5E). Although the degree of utilization of the internal alternative 5′ splice site varies somewhat among different cell types (Fig. 6C), this regulation of the choice of 5′ splice site accounts for much less variation in overall IIICS expression patterns than does the regulation of the choice of 3′ splice site (Fig. 6B).

## DISCUSSION

The alternatively expressed IIICS region of the FN molecule has become the focus of much experimental attention. A thorough understanding of the significance of this region will arise from knowledge of the mechanisms at the molecular level by which subunit heterogeneity is regulated as well as the functional consequences of this regulation on FN activity. To address the issue of alternative splicing regulation in this complex exon, a novel assay was developed to quantify expression of all human IIICS mRNA variants. This assay was used to identify cell-type-specific changes in IIICS expression patterns among different human cell types and to suggest the possible regulatory mechanisms responsible for such changes.

**DPE assay.** The presence of several alternatively spliced subsegments with the human IIICS region prevents nuclease protection protocols from assaying all of the possible mRNA subpopulations simultaneously. To overcome this limitation, the DPE assay was developed. In examining FN IIICS expression, this assay could simultaneously demonstrate all five IIICS variants and allow for their accurate quantitation. The DPE protocol is applicable to other instances of mRNA variation, adapting the oligonucleotide primers to specific mRNA templates or sites of variation. Heterogeneity among mRNA transcripts can be identified and quantitated on the basis of DPE runoff product length, sequence content with

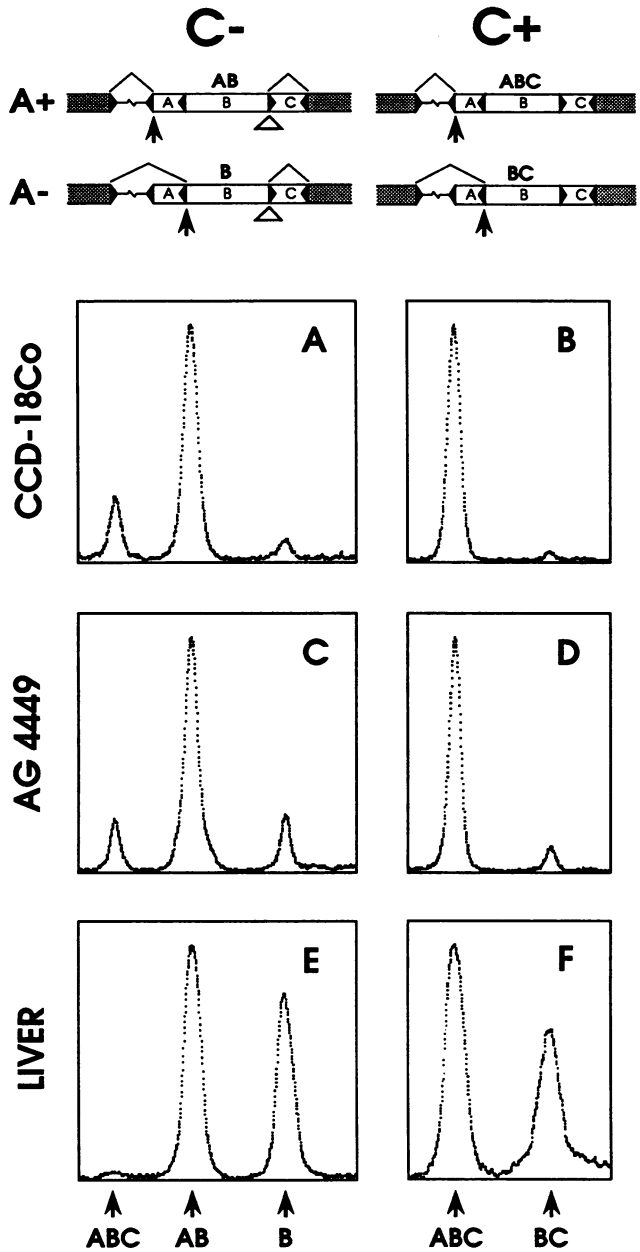


FIG. 5. Comparison of changes in BC and B variant expression. Densitometric scans of linear range autoradiograms from normal (A, C, and E) and C-specific (B, D, and F) DPE assays were normalized to display equivalent AB or ABC peak areas, respectively. Peak identities are labeled at the bottom of the scans with their variant designations. Above the scans for each DPE assay are representations of the splicing events that give rise to the variants within the $A^+$ (top) and $A^-$ (bottom) families that are $C^-$ (left) or $C^+$ (right), diagrammed as in Fig. 1. Arrows indicate the 3′ splice sites used in synthesizing the $A^+$ or $A^-$ family mRNAs. Open triangles indicate the internal 5′ splice site used in splicing out the C subsegment, generating the $C^-$ transcripts.

restriction mapping, or primer specificity. The identification of the weakly expressed BC variant (which had not been previously identified through either cDNA cloning or nuclease protection experiments) demonstrates the sensitivity of the DPE assay, particularly when the C-specific primer IIICS-C is used to assay this minor variant against a background of more strongly expressed variants, and suggests a strategy by which such a minor mRNA species could be cloned.

**Cell type specificity in IIICS mRNA variant expression.** Examining IIICS expression patterns among different normal and tumor cell types revealed that expression of the O variant, which lacks all IIICS sequence, exhibits considerable variation among different cell types (Fig. 3 and 4A). This observation concerning O mRNA expression complements the results of previous studies of $IIICS^-$ expression at the protein level. Assaying for the presence of a protease-sensitive site within $IIICS^+$ subunits, Zardi and co-workers (9) demonstrated the variability in $IIICS^-$ subunit expression. Plasma FN (pFN) subunits (synthesized in the liver) contained the highest percentages of $IIICS^-$ subunits, cellular FN (cFN) from normal fibroblasts contained an intermediate amount, and tumor and transformed cell cFN contained the lowest levels (9). Both the patterns of these cell-type-specific changes in $IIICS^-$ expression and the percentage values obtained at the protein level (9) are quite similar to the changes in O mRNA percentages observed in this study (Fig. 4A). Studies using antibodies specific for the C subsegment of the human IIICS peptide sequence demonstrated that pFN contains one-fifth as many $C^+$ subunits as does cFN purified from WI-38 fibroblasts (41). This is consistent with the underexpression of $C^+$ mRNAs in liver tissue RNA compared with that of fibroblasts as observed in this study.

The similarity of the results presented here on mRNA variant levels with those of comparable studies on protein subunit expression strongly suggests that cell-type-specific patterns of subunit diversity are regulated for the most part by alterations in IIICS mRNA metabolism, either through regulated alternative splicing or differential mRNA stability among IIICS mRNAs. Furthermore, these results indicate that the largest cell-type-specific changes in IIICS subunit expression among cultured cells are those involving the relative abundance of the $IIICS^-$ O mRNA variant versus the four $IIICS^+$ variants.

Interestingly, the liver-specific patterns of IIICS expression observed in human FNs at both the mRNA and protein levels are comparable to those observed among rat FNs. Rat liver RNA exhibits higher O mRNA levels than do rat fibroblasts (38). At the protein level, studies on rat FNs using a novel chemical cleavage-immunoblot technique (34) demonstrated the elevated expression of both the O and BC subunits (compared with ABC levels) in pFN compared with those in fibroblast cFN. The absence of an alternative 5′ splice site within the rat IIICS limits any cell-type-specific regulation to a process of selecting among alternative 3′ splice sites. Therefore, these liver-specific rat IIICS patterns probably correspond to similar patterns observed in this study in the predominant AB, B, and O mRNA levels in human liver mRNA, in which the C subsegment is removed from a vast majority of transcripts through the use of the human-specific internal 5′ splice site. This indicates that the liver-specific regulation of IIICS expression is quite similar between rats and humans, despite the evolution of an alternative 5′ splice site in human FN transcripts.

**Transformation effects.** It was noted that tumor and transformed cells appear to underexpress the O mRNA variant, unlike several normal fibroblasts, mesangial cells, and liver tissue, as demonstrated previously for $IIICS^-$ subunit expression (9). In those select instances in which direct comparisons could be made, viral transformation (VA-13 versus WI-38) or in vivo tumorigenesis (HepG2 versus liver

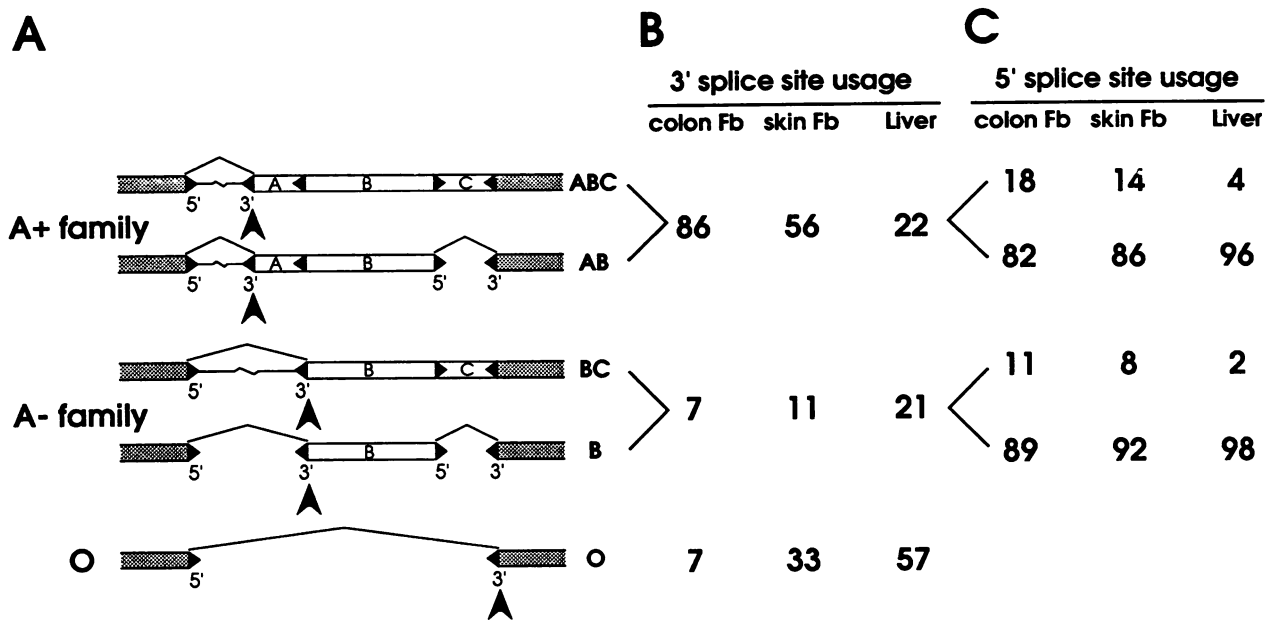


FIG. 6. Proposed model of cell-type-specific regulation of IIICS alternative splicing. (A) The five IIICS mRNA variants are diagrammed as in Fig. 1 and grouped by family ($A^+$, $A^-$, and O) according to changes in the relative use of each alternative 3′ splice site (indicated with arrowheads) in conjunction with the common upstream 5′ splice site (see Results). (B) The values for the average relative abundance of each variant family are presented (expressed as percentages of the total FN RNA pool) for the samples analyzed in Fig. 5 (average values and error bars for individual variants are as described in the legend to Fig. 4A). (C) Values represent the average percentage of transcripts within each variant family that are $C^+$ (ABC or BC) or $C^-$ (AB or B) due to alternative use of the internal 5′ splice site. Fb, Fibroblasts.

tissue) correlated with a reduction in the expression of the O mRNA variant. Firm experimental evidence for any such transformation-related effects on IIICS expression will require a comparison of appropriate isogenic cell models for transformation and tumorigenesis as well as direct experimental manipulation of the transformed phenotype.

**Predominant regulation of the choice of 3′ splice acceptor site.** Although the levels of the O variant varied most dramatically, it appears not to be the only regulated variant. Examination of the cell-type-specific changes in ABC plus AB, BC plus B, and O variant levels among various cell lines suggests the existence of independently regulated sets or families of mRNA variants. Each family differs with regard to which alternative 3′ splice site is joined to the common 5′ splice site (Fig. 6A). It appears that differential utilization of each of the 3′ splice sites plays a fundamental role in establishing cell-type-specific IIICS expression patterns, as indicated by the sizable differences in the relative abundances of each family, even among fibroblasts isolated from different tissues (Fig. 6B). A more modest degree of cell type specificity in IIICS expression arises as a result of modest differences among various cell types in the relative utilization within each family of the alternative 5′ splice site within the IIICS (Fig. 6C). The prevalence of the regulation of the 3′ splice site over the regulation of the 5′ splice site in human cells is further supported by the striking similarity in the liver-specific IIICS pattern (increased relative amounts of O and $A^-$ families compared with those of normal fibroblasts) in both human pFN (Fig. 4A and 6) (9) and rat pFN (34), which lacks an alternative 5′ splice site.

A model of 3′ splice site regulation is proposed (Fig. 6) on the basis of cell-type-specific differences in IIICS expression. The cell lines used in this study were cultured under similar experimental conditions; therefore, the observed changes in IIICS patterns are probably a consequence of the differentiated phenotype of each cell type. It will be important to test this model by examining the regulation of IIICS expression within individual cell types in response to growth conditions or external stimuli as well as in situ versus in vitro environments. It is possible that similar or alternative mechanisms of IIICS regulation exist within each cell for regulating IIICS expression in response to its extracellular environment.

Because the DPE assay quantifies steady-state levels of each mRNA variant, the role of differential mRNA stability among the IIICS variants in regulating these steady-state levels could not be addressed in this study. A regulatory model based on mRNA stability would need to account for the three independently regulated mRNA families observed, the coregulation of $C^+$ variants within each family, and the significant differences in expression levels among the mRNA families. These observed results are most clearly reconciled within a regulatory model based on cell-type-specific alternative splicing. Preliminary experiments using dactinomycin to inhibit FN transcription failed to detect any differences in mRNA stability among the IIICS variants (data not shown). Although differential FN mRNA stability among different cell lines appears to affect the steady-state abundance of the total FN mRNA pool (12), any role it plays in establishing cell-type-specific patterns of qualitative IIICS variant expression remains to be demonstrated.

Although much is known about the mechanism of pre-mRNA splicing, the components involved, and their assembly into a splicing complex (16, 29, 32), little is known of how these or other components are involved in the regulation of cell-type-specific alternative splicing (2). Cell-type-specific *trans*-acting factors appear to regulate choices of alternative splice sites in FN extra domain region splicing (3) and in other alternatively spliced genes (7, 27). In addition, some of these factors appear to be developmentally regulated (7).

Recognition of the 3′ splice site and the branchpoint region by splicing components are required initial events in the formation of an active spliceosome (10, 14). Differences in the efficiency with which splicing complex precursors form at the alternative IIICS 3′ splice sites and the involvement of cell-type-specific auxiliary factors are two possible mechanisms by which the observed differences in the use of the IIICS 3′ splice sites could occur.

**Cell biological implications.** One goal of this study was to examine the variability among different cell types in the expression of transcripts encoding the CS-1 cell-binding domain (13, 19, 20, 21) within the A subsegment. The $A^+$ family of IIICS transcripts is an independently regulated family of transcripts based on cell-type-specific utilization of the upstream 3′ splice site (Fig. 6B). The $A^+$ transcripts constitute the majority of FN mRNAs in all of the cultured cell types examined, although their percentage of abundance varies among cell types (Fig. 6). This contrasts with liver FN mRNA, believed to encode pFNs (42), in which the $A^+$ transcripts are in the minority (Fig. 6). It is not known whether this difference in the relative expression of the CS-1 adhesion site between soluble pFN and matrix-associated cFNs has any effect on the biological activities of these two classes of FNs. It is possible that the abundance of the CS-1 adhesion site in FN-containing matrices in vivo may exhibit temporal or spatial regulation not discernible in studies of cultured cells. Furthermore, such in vivo regulation of IIICS splicing patterns may be responsive to cell-cell or cell-matrix interactions, resulting in CS-1 expression levels unique to individual tissue environments.

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