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Regulation of expression of alternatively spliced human fibronectin IIICS mRNA variants

Hershberger, Richard Paul, Ph.D.

Case Western Reserve University (Health Sciences), 1991
REGULATION OF EXPRESSION OF ALTERNATIVELY-SPliced
HUMAN FIBRONECTIN IIICS mRNA VARIANTS

by
Richard P. Hershberger

Submitted in partial fulfillment of the requirements
for the Degree of Doctor of Philosophy

Thesis Advisor: Lloyd A. Culp, Ph.D.

Department of Molecular Biology and Microbiology
Case Western Reserve University

August 1991
CASE WESTERN RESERVE UNIVERSITY

GRADUATE STUDIES

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(Chairman)

John H. Litt

S. R. Rottenmark

M. H. Rottenmark

Date May 23, 1991

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REGULATION OF EXPRESSION OF ALTERNATIVELY-SPliced
HUMAN FIBRONECTIn IIICS mRNA VARIANTS

Abstract
by
Richard P. Hershberger

Fibronectin polypeptide diversity can be generated by alternative splicing of fibronectin primary transcripts at three sites, including a unique region termed the type-III connecting segment (IIICS). A novel double primer extension assay was developed to measure each of the five human IIICS mRNA splicing variants. Expression of IIICS mRNAs was analyzed in a variety of human normal and tumor cells, and in liver tissue. Differences in IIICS expression patterns were observed among different cell types, among fibroblasts of different tissue origins, and between normal and transformed cells. The most predominant differences were in the abundance of the one IIICS\textsuperscript{\texttt{-}} mRNA variant relative to the four IIICS\textsuperscript{\texttt{+}} variants. The expression of the IIICS\textsuperscript{\texttt{-}} variant was elevated in liver tissue relative to cultured cells, indicative of differences in subunit composition between cellular and plasma fibronectins. Additional changes in expression levels of the four IIICS\textsuperscript{\texttt{+}} variants are consistent with a model in which regulation of IIICS mRNA expression occurs predominantly at the level of alternative 3' splice site selection.

The degree to which the IIICS alternative splicing process is subject to ongoing regulation within individual cell types was experimentally...
examined. Possible alterations in IIICS mRNA expression patterns during \textit{in vitro} or \textit{in vivo} aging of human dermal fibroblasts were tested. The possible effects on IIICS expression of factors that regulate matrix synthesis (TGF-β, glucocorticoids) were studied.

As a prelude to studies on the sequences and factors required to mediate regulated IIICS alternative splicing, several plasmids were constructed directing synthesis of a human IIICS pre-mRNA splicing substrate.

This work was designed to provide insights into the regulation of alternative splicing of human fibronectin pre-mRNAs in both generating and responding to adhesive preferences, and also addresses the possible regulation of the process of pre-mRNA splicing.
This thesis is dedicated to the memory
    of my late father,
    Paul George Hershberger
    Staff Sergeant, United States Army
    (b. July 1915 - d. December 1978)

He may not have bought me every toy
    I wanted while I was growing up,
    but he never said no
    to buying me any book.

Thanks, Dad.
Acknowledgements

My sincerest appreciation goes to:

Dr. Robert Hampson for suggesting the double primer extension approach for IIICS study, and Mike Hall and Kelly Flickinger for their technical contributions in dermal fibroblast culture and RNA isolations.

My thesis advisory committee, Drs. David Setzer, Fritz Rottman, John Nilson, and Urs Rutishauser, for their ongoing interest, stimulating discussions, helpful input, and challenging critique of my work.

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My laboratory colleagues for their immeasurable technical input and support; Wen-chang Lin, Kelly Flickinger, Mike Hall, Katey Houmiel, Dr. Gabriele Mugnai, and Kristine Lewandowska.

My dear friends and colleagues who stimulated me scientifically, challenged me athletically, and accompanied me socially; Sandy “Squid” Siepka, Dr. Bruce (“uff-da”) Horazdovsky (keg-master and porch-sitter extraordinaire), Ed Goodwin, Steve Slater, Tom Flickinger, Dr. Elizabeth Walter (a metaphor for Cleveland), and “Chief” Paresh Shrimankar (our very own Cleveland Indian).

The Yeastie Boys Brewing Company for keeping up my spirits (or is that in spirits); Rich Schneeberger, Dave Singleton, Joel Kreps, and Bruce Campell, and the “Yeastie Girls”, Joann, Joan, and Lori.
The Friends of Harvey Softball team for making Fridays worth looking forward to: Jack Ryan, John Gidley, Wessel Dirksen, Mike "Spike" Schneider, Dr. Ed Lancy, Kris O'Dee, Howie, Han, John, and Margaret, and our founder and mutual friend and colleague, the late Harvey Werber.

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Everyone with whom I camped, backpacked, drank, talked science, worked, watched sports, talked politics, golfed, campaigned, studied, governed, and generally hung-out, for making my time in the Land of Cleve an enjoyable, enlightening and forever memorable experience.

The Martello family, for raising such a bright and charming daughter, and making me a part of such a close and supportive family.

My mother, Blandine (Schwarzmeier) Hershberger, for supporting my many years of education and for giving me my German heritage, and my brothers and their families, for tolerating my many years of under-paid existence as a student.

Twilight, for his patience while waiting to become a doctor's cat.

Most of all, my wife Pam. My indebtedness to her, and my appreciation for all the things that she is and all the things that she has taught and given me, could fill the rest of these pages. My highest ambition in life is to justify her love for me.

Introducing the team of Dr. and Dr. Hershberger...
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<th>Definition</th>
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</thead>
<tbody>
<tr>
<td>AMP</td>
<td>adenosine monophosphate</td>
</tr>
<tr>
<td>AMV</td>
<td>avian myeloblastosis virus</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>cFN</td>
<td>cellular fibronectin</td>
</tr>
<tr>
<td>Dex</td>
<td>dexamethasone</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified Eagle medium</td>
</tr>
<tr>
<td>dNTP</td>
<td>deoxyribonucleoside triphosphate</td>
</tr>
<tr>
<td>DPE</td>
<td>double primer extension</td>
</tr>
<tr>
<td>ED</td>
<td>extra domain (type-III homology repeat)</td>
</tr>
<tr>
<td>FN</td>
<td>fibronectin</td>
</tr>
<tr>
<td>NCS</td>
<td>neonatal calf serum</td>
</tr>
<tr>
<td>nt</td>
<td>nucleotides</td>
</tr>
<tr>
<td>NTP</td>
<td>ribonucleoside triphosphate</td>
</tr>
<tr>
<td>Pap</td>
<td>papillary</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>pFN</td>
<td>plasma fibronectin</td>
</tr>
<tr>
<td>phFN-IIICS</td>
<td>plasmid containing human IIICS-region cDNA sequence</td>
</tr>
<tr>
<td>phIIICSg, p(X)-hIIICSg</td>
<td>plasmids containing human IIICS-region genomic sequence expressed from promoter (X)</td>
</tr>
<tr>
<td>placZ, p(X)-lacZ</td>
<td>plasmids containing the E. coli β-galactosidase (lacZ) gene expressed from promoter (X)</td>
</tr>
<tr>
<td>Ret</td>
<td>reticular</td>
</tr>
<tr>
<td>rIIICS-ABC</td>
<td>IIICS RNA probe transcribed from phFN-IIICS</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>RGD</td>
<td>Arg-Gly-Asp cell binding sequence of fibronectin</td>
</tr>
<tr>
<td>RNase</td>
<td>ribonuclease</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris-Borate-EDTA buffer</td>
</tr>
<tr>
<td>TGF-β</td>
<td>transforming growth factor-β</td>
</tr>
<tr>
<td>UTP</td>
<td>uridine triphosphate</td>
</tr>
<tr>
<td>III CS</td>
<td>type-III connecting segment</td>
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</table>
Chapter I. Introduction

Extracellular Matrix: Regulating Cellular Fate and Function

It is an inherent characteristic of metazoan species that multiple different cell types must arise in or move to precisely defined locations within the organism and then differentiate to assume a specific identity and function at each site while forming organs and tissues of significant cellular and physiologic complexity. The mechanisms by which such precise positional information is encoded within a complex tissue environment and the means by which individual cell types recognize and respond to this information are issues that are fundamental to our understanding of the cell biology, developmental biology and physiology in multicellular organisms.

The complex interplay of factors that define and maintain the differentiated phenotype of different cell types are as yet poorly defined. It is clear that cells respond to many such factors, and that the overall biosynthetic activity of a cell is a result of its processing of multiple stimuli. Many of these inputs are in the form of soluble factors such as growth factors and hormones. These soluble factors may be synthesized by the responding cell type itself (autocrine regulation), by neighboring cell types within the same tissue with a localized range of action, or by different tissues or organs at distant sites of the body providing synchronization of metabolic functions throughout the organism. The specific sites of synthesis of such factors, their range of action, and their mode of transport throughout the body probably provide significant positional information for different cell types during the morphogenesis
of organs and tissues and during the determination and differentiation of their unique cell types.

Another mechanism for the creation of positional information is through the composition of each cell’s immediate extracellular environment. The components of the extracellular matrix provide the adhesive scaffold for cell attachment and migration. The specificity of interactions between different cell types and extracellular matrices throughout the body play a considerable role in defining the paths of migration during development, the formation of complex tissues and the differentiation of multiple cell types (Ekblom et al., 1986; Reichardt and Tomaselli, 1991; Duband et al., 1990; Liesi, 1990; Springer, 1990). Examples include the migration of neural crest cells over long distances in the developing vertebrate embryo (Thiery et al., 1985; Duband, 1990), gastrulation and mesoderm induction in amphibians (Smith et al., 1990), and differentiation of lymphocytes in the bone marrow (Abraham et al., 1989; Luikart et al., 1987). Thus a further understanding of the mechanisms by which cells interact with, recognize, and respond to the components of their extracellular matrix environment, and how the composition of the extracellular matrix is regulated will provide valuable insights into these complex developmental processes.

Mechanisms for Specificity in Cell-Matrix Interactions

Components of the Extracellular Matrix

The extracellular matrix contains a large variety of constituent macromolecules. Among the protein components are collagens, laminin, elastin, vitronectin, thrombospondin, and fibronectin, all of which exhibit
adhesive activities for cells and other matrix molecules (Yamada, 1983; Martin and Timpl, 1987; Hakomori et al., 1984). In addition, extracellular matrices contain oligosaccharide components called glycosaminoglycans and protein-linked oligosaccharides termed proteoglycans (Ruoslhti, 1989). Glycosaminoglycans and proteoglycans consist of multiple classes (heparan sulfate, chondroitin sulfate, dermatan sulfate, hyaluronic acid) based on their oligomeric carbohydrate structures. Molecular heterogeneity is also observed among collagens, which comprise over fifteen identified types with subunits derived from multiple genes. Many of these collagen types exhibit restricted tissue distributions. Given the number and heterogeneity of different matrix components and their tissue specific expression patterns, the different adhesive properties of extracellular matrices throughout the body may arise, at least in part, from the unique mixture of matrix components present. The composition of any given matrix can be regulated at a number of levels, including the rate of synthesis of each component (transcription, translation, protein processing and transport), the incorporation and cross-linking of each component into the complex matrix, and the rate of degradation of each component through the action of matrix-specific proteases.

**Cell Surface Matrix Receptors: Integrins**

Specificity in the adhesive interactions between cells and extracellular matrices of different composition requires the existence of multiple cell surface receptors for these multiple matrix molecules. The most actively studied class of these are the integrin family of matrix receptors (Hynes, 1987; Buck and Horwitz, 1987; Ruoslhti, 1991; Humphries, 1990; Albelda
Integrins are transmembrane glycoproteins comprised of one α and one β subunit encoded by separate families of genes. The matrix ligand specificity of each integrin is defined by the unique pairing of subunits from among the multiple α and β subunit species (Table 1).

<table>
<thead>
<tr>
<th></th>
<th>FN</th>
<th>LN</th>
<th>COL</th>
<th>FG</th>
<th>VN</th>
<th>VWF</th>
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<tr>
<td>αIIbβ3</td>
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<td>C3bi</td>
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Table 1. Ligand Specificities of Integrin Heterodimers. The known ligand specificities of identified integrin α/β heterodimers are represented by black dots for the matrix molecules fibronectin (FN), laminin (LN), collagen (COL), fibrinogen (FG), vitronectin (VN), thrombospondin (TSP), and von Willebrand factor (VWF). The site on fibronectin with which a particular integrin interacts is listed, where known. Other adhesive ligands for integrins include intercellular adhesion molecules (ICAMs), vascular cell adhesion molecule (VCAM), peyer's patch addressin (*) and complement component (C3bi).
The mechanisms by which different integrins bind their ligands have some shared and some unique features. Several integrins bind at an Arg-Gly-Asp (RGD) sequence present in several matrix molecules (including fibronectin) whereas several other integrins bind in an RGD-independent manner. It is interesting to note that for certain integrins substitution of either the α or β subunit can add to or change the group of ligands to which it can bind (α4β1 vs. αVβ1 vs. αVβ5 for example). Some integrins are specific for a single matrix molecule (α5β1) and some bind to unique sets of ligands (α4β1, α3β1). Similarly some ligands are bound by a single integrin (ICAMs) while others are bound by several different integrins (laminin, fibronectin, etc.). In the case of fibronectin, multiple integrins can bind the same matrix molecule at different sites through presumably different mechanisms (α4β1 at the CS-1 site and α5β1 at an Arg-Gly-Asp or RGD sequence; more on this point later in this chapter). Thus the multiplicity of integrin subunit combinations and their resulting ligand specificities illustrate an interesting mechanism for generating adhesive specificities.

*Molecular Heterogeneity in Matrix Components*

In addition to the variety of distinct gene products that constitute the extracellular matrix, the molecular complexity of each matrix can arise through heterogeneity within each class of macromolecules. Among glycosaminoglycans and proteoglycans, heterogeneity is observed in the length and composition of the oligosaccharide chains and their linkage to different core proteins (Ruoslhaki, 1989; Höök et al., 1984; Lark and Culp, 1984). Alternative N-linked and O-linked glycosylation of matrix
glycoproteins also exhibit tissue-specific or developmentally regulated patterns of expression (Matsuura et al., 1988; Nichols et al., 1986; Feinberg et al., 1991).

A considerable degree of heterogeneity among many classes of matrix proteins arises as a result of alternative splicing of pre-messenger RNAs. Synthesis of functional protein isoforms through alternative splicing has been demonstrated for fibronectins (Paul et al., 1986; Tamkun et al., 1984; Kornblihtt et al., 1984a; Carnemolla et al., 1987; Gutman and Kornblihtt, 1987), elastins (Indik et al., 1989; Heim et al., 1991; Parks and Deak, 1990; Pollock et al., 1990), tenascin (also called cytotactin or hexabrachion) (Spring et al., 1989; Jones et al., 1989; Prieto et al., 1990; Gulcher et al., 1989), proteoglycan core protein (Baldwin et al., 1989), and several types of collagen (Ryan and Sandell, 1990; Saitta et al., 1990; Pihlajaniemi and Tamminen, 1990; Tikka et al., 1988). For both fibronectin and tenascin, alternative splicing results in the synthesis of polypeptide isoforms with altered adhesive function (Humphries et al., 1986; Humphries et al., 1987; Spring et al., 1989).

In addition to the role of alternative splicing in regulating the adhesive activity of certain matrix components, in several instances inherited mutations that perturb normal splicing patterns are associated with pathological conditions affecting the extracellular matrix. Such genetic disorders are probably a consequence of the synthesis of protein isoforms that are disruptive to the architecture or adhesive function of certain matrices. Inherited splice site mutations and resulting abnormal protein isoforms have been identified in several type I and type III
collagen subunits expressed in patients with Ehlers-Danlos syndrome type VII (Weil et al., 1990; Weil et al., 1989; Weil et al., 1989), Ehlers-Danlos syndrome type IV (Kuivaniemi et al., 1990; Kontusaari et al., 1990; Cole et al., 1990), and osteogenesis imperfecta (Bonadio et al., 1990; Tromp and Prockop, 1988). These examples illustrate how the process of pre-mRNA splicing serves both as a potential mechanism for generating adhesive specificities and, when disrupted through mutation, as a cause of matrix pathologies and disease.

Fibronectin: A Multivalent Ligand Linking Cells and Matrices

The matrix glycoprotein fibronectin (FN) serves as a model for studying many of the aforementioned mechanisms by which cells differentially bind, recognize, and respond to extracellular matrices of distinct compositions (Hynes, 1990; Ruoslahti, 1988; Akiyama and Yamada, 1986; Hynes, 1986). Fibronectins exhibit developmentally regulated and tissue-specific patterns of expression and localization within matrices throughout the body, binding to multiple other matrix macromolecules. Many cell types differentially adhere to fibronectin-containing matrices by way of several distinct classes of cell surface fibronectin receptors, producing variable morphologic, differentiative, and biosynthetic responses. In addition, alternative splicing of the fibronectin pre-mRNA results in the synthesis of multiple subunit isoforms with distinct adhesive properties. One such site of alternative splicing encodes for a unique adhesive domain which is recognized by a distinct member of the integrin family of matrix receptors. Thus the study of fibronectin structure, expression, and activity provides a paradigm for understanding
the multiple mechanisms for the regulation of matrix function in general.

Subunit Structure and Genomic Organization

Fibronectin has traditionally been categorized into two classes. Plasma fibronectin (pFN) is synthesized by hepatocytes and secreted into the blood plasma (Tamkun and Hynes, 1983). Cellular fibronectin (cFN) is synthesized by many different cell types and is a significant component of the interstitial matrix in a wide variety of tissues.

Fibronectins are composed of two nearly identical polypeptide subunits, each approximately 200-240kD molecular weight and joined near their carboxy-termini by a pair of disulfide bonds (Figure 1). Approximately 90% of the amino acid sequence of each subunit is contained in long series of structural domains displaying three patterns of amino acid sequence homology, termed type I, type II, and type III homology repeats (Garcia-Pardo et al., 1987; Kornblihtt et al., 1985; Petersen et al., 1983) of 41-52, 60, and 90 amino acids respectively. These repeat structures are comparatively protease-resistant; proteolytic digestion typically cleaves within the short stretches of non-homologous sequence, releasing individual repeats or clusters of repeats.

The exon structure of the single FN gene reflects this pattern of repeated structural units (Hynes, 1985; Odermatt et al., 1985; Patel et al., 1987), with each homology repeat encoded either by 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 fibronectin subunits isolated from different sources (Hayashi and Yamada, 1981; Paul and Hynes, 1984; Sekiguchi and Titani, 1989) arises in part from alternative splicing of the primary fibronectin transcript at three distinct locations (Schwarzbauer et al., 1983; Kornblihit et al., 1984a; Gutman and Kornblihit, 1987; Carnemolla et al., 1989; Paul et al., 1986). Alternative splicing at the two extra domain exons (EDa and EDb; Figure 1) involves the inclusion or removal of the coding region for an individual type III homology repeat (Gutman and Kornblihit, 1987; Kornblihit et al., 1984b; Norton and Hynes, 1987). A complex pattern of alternative splicing in the type III connecting segment (IIICS; Figure 1) results in the inclusion of varying amounts of sequence.

Figure 1. FN Subunit Structure and Adhesion Domains. Type I, II, and III homology repeats are shown as boxes containing one, two, or three dashes, respectively. Non-homologous regions are shown as thin lines. Adhesion domains (black bars: Hep, heparan sulfate proteoglycans; Fib, fibrin) and the RGDS cell binding site (black triangles) are present in all subunits. Alternatively spliced regions are shown stippled, as is the alternatively expressed CS-1 cell adhesion site.
that encodes polypeptide segments with no homology to other sites of the fibronectin subunits (Schwarzauer et al., 1983; Kornblihtt et al., 1985; Bernard et al., 1985). In each of these instances, alternative splicing maintains the same reading frame, resulting only in the inclusion of additional polypeptide sequences. The patterns of alternative splicing, their regulation, and the functional consequences of the altered sequences in the fibronectin subunits will be discussed in detail in a later section.

Binding Activities and Cell Biological Functions

Each fibronectin subunit contains multiple binding domains for both cell surface and extracellular ligands, thus serving as a molecular bridge between cells and their extracellular environment (Yamada, 1983; Akiyama and Yamada, 1986; Ruoslahti, 1988; Hynes, 1986). Specific binding domains have been mapped on the fibronectin molecule through the isolation of individual biologically active fragments released through limited proteolytic digestion. Extracellular matrix ligands for fibronectin include collagen, fibrin, the glycosaminoglycan heparin, and heparan sulfate proteoglycan (Figure 1).

Cells bind to fibronectin using a variety of cell surface ligands, including proteoglycans and gangliosides. In addition, several members of the integrin family of matrix receptors bind fibronectin at distinct sites and through separate mechanisms (Table 1). The prototype of the integrin family is the α5β1 140kD complex originally identified as the human fibronectin receptor (Pytela et al., 1985). This receptor is specific for the central cell binding domain of fibronectin (Cell1 in Figure 1), and its binding can be inhibited by synthetic peptides containing the Arg-Gly-Asp-
Ser sequence (RGDS in Figure 1) from the cell binding domain (Pierschbacher et al., 1981; Pierschbacher and Ruoslahti., 1984). Several other matrix receptors of the integrin family share this RGD sequence specificity, and some can recognize more than one type of matrix molecule. The α4β1 integrin binds at a distinct site on fibronectin subunits containing the alternatively spliced IIICS (Cell3 in Figure 1) (Humphries et al., 1987; Wayner et al., 1989; Mould et al., 1990; Guan and Hynes, 1990). This receptor for the IIICS will be discussed in detail in a later section.

Different cell types cultured on substrata coated with intact plasma fibronectin show varying degrees of sensitivity to inhibition of adhesion by added soluble RGD-containing peptides (Yamada and Kennedy, 1987; Humphries et al., 1986; Mugnai and Culp, 1987; Waite et al., 1987), indicating that multiple cell surface ligands contribute to the overall fibronectin binding activity, some in an RGD-independent manner. Such studies have established that cell surface heparan-sulfate proteoglycan contributes to the overall cell adhesion response on fibronectin (Culp et al., 1986), either by independently binding to the heparin-binding domains or by influencing the interaction of other cell surface receptors with their recognition sites. Thus individual cell types can establish different morphological and biochemical effects and adhesive preferences by binding fibronectins through specific combinations of cell surface ligands of different classes.

Through its multiplicity of adhesive activities, fibronectin can fulfill key roles in a variety of cell adhesion phenomena (Mosher, 1984; Hynes,
Fibronectin serves as a substrate for cell migration during gastrulation, morphogenesis, and wound healing (Duband et al., 1990; Dufour et al., 1988b; Donaldson et al., 1985; Thiery et al., 1985; Smith et al., 1990). It is cross-linked into newly-synthesized matrices during thrombosis, and adheres to bacterial surfaces during opsonization. Cellular interaction with fibronectin is a contributing factor in regulating the proliferation, morphology, and/or biosynthetic phenotype of various cell types. This has been demonstrated with cultured adipocytes (Spiegelman and Ginty, 1983), cholinergic neurons (Kamagata and Donahoe, 1985), astroglia (Goetschy et al., 1987), arterial smooth-muscle cells (Hedin and Thyberg, 1987; Hedin et al., 1988) neural crest cells (Sieber-Blum et al., 1981) and chondroblasts (West et al., 1984). It is hoped that through a detailed understanding of the regulation of fibronectin's multiple binding activities and their cell surface ligands will come insights into these important matrix-mediated physiological processes.

**Regulation of Fibronectin Synthesis and Adhesive Activity**

Various growth factors and hormones are known to regulate the expression of fibronectin and influence cellular adhesion processes. Several of these agents modulate the steady state levels of fibronectin mRNA either transcriptionally or post-transcriptionally. Retinoids can either up- or down-regulate fibronectin protein synthesis in a variety of cell types (Kenney et al., 1986; Kim and Wolf, 1987; Varani et al., 1989). Fibronectins synthesized by retinoid-treated chondrocytes exhibit a slight alteration in electrophoretic mobility and enhanced binding to the chondrocyte cell surface, inducing a more flattened morphology (Hassel et
al., 1979). Whether these differences in mobility and binding are due to alterations in fibronectin pre-mRNA splicing or post-translational modifications is not known.

Treatment of cells with dibutyryl cyclic AMP or forskolin, an activator of adenylate cyclase, regulates transcription from the fibronectin gene, acting through cAMP response elements in the fibronectin promoter in either a stimulatory (Dean et al., 1988; Dean et al., 1989; Bowlius et al., 1991) or inhibitory manner (Bernath et al., 1990). Transforming growth factor-β (TGF-β) (Massague, 1990) also regulates the steady state levels of mRNAs for fibronectin and other matrix proteins and matrix receptors (Varga et al., 1987; Ignoz et al., 1987; Ignoz and Massague, 1987; Ignoz and Massague, 1986) through a TGF-β response element in the promoters of these genes (Thompson et al., 1988; Dean et al., 1988; Dean et al., 1989; Rossi et al., 1988). The stimulation of integrin matrix receptor expression by TGF-β appears to modulate the binding of exogenous fibronectin to cell surfaces (Allen-Hoffmann et al., 1988). In addition, alternative splicing of both the EDa and EDb exons are sensitive to TGF-β treatment (Balza et al., 1988; Borsi et al., 1987; Borsi et al., 1990). The possible role of TGF-β in regulating fibronectin adhesive activity through modulation of IIICS alternative splicing is as yet undemonstrated.

The synthetic glucocorticoid dexamethasone regulates steady state fibronectin mRNA levels in a variety of cell types (Oliver et al., 1983; Nimmer et al., 1987; Raghow et al., 1986). Unlike TGF-β and cAMP stimulation of fibronectin mRNA synthesis at the transcriptional level, dexamethasone appears to regulate fibronectin mRNA levels by stabilizing
the mRNA against degradation (Dean et al., 1988). Dexamethasone stimulates the synthesis of fibronectin subunits of reduced adhesive activity in a hepatoma cell line (Baumann and Eldredge, 1982), however the mechanism underlying this alteration of adhesive activity, perhaps through regulation of alternative splicing, remains to be elucidated.

It is clear that a variety of growth factors and hormones can influence fibronectin mRNA steady state levels in many different cell types through several mechanisms. However the possible effects of these agents on the regulation of alternative splicing of fibronectin pre-mRNAs, and the functional consequences of such alterations of fibronectin subunit structure on its adhesive properties have not been fully examined. Fibronectins have been described that exhibit altered subunit structure or biological activity. Dexamethasone treatment of a rat hepatoma cell line induces synthesis of a fibronectin of altered electrophoretic mobility and reduced binding affinity for normal fibroblasts as compared to pFN or fibroblast-derived cFN (Baumann and Eldredge, 1982). Another demonstration of a fibronectin diminished in activity is that synthesized by senescent human skin fibroblasts. Fibronectin from the conditioned media of late passage fibroblasts was shown to have a reduced activity in cell adhesion assays compared to that of early passage fibroblasts (Chandrasekhar and Millis, 1980; Chandrasekhar et al., 1983a) and exhibited reduced collagen binding activity (Chandrasekhar et al., 1983b). Alterations in subunit structure were also demonstrated in fibronectins isolated from aging cells, either antigenically or electrophoretically (Porter et al., 1990; Sorrentino and Millis, 1984).
Whether these examples of structural alteration and diminished binding activity result from alterations in primary sequence (via alternative splicing) or post-translational modifications remains unknown. However, nonglycosylated cFN, isolated from tunicamycin-treated fibroblasts, shows no loss in activity in a variety of adhesion assays compared to the glycosylated form (Olden et al., 1979). These studies raise the possibility that alterations in primary sequence arising from alternative splicing may play a role in modulating the biological activity of fibronectins.

Alternative Splicing and the Regulation of Fibronectin Activity

Fibronectin subunit variants isolated from different sources can exhibit diversity in amino acid sequence at multiple sites. This subunit diversity arises from alternative splicing of the fibronectin pre-messenger RNA transcribed from a single gene (Hynes, 1985; Hynes, 1990). Alternative splicing occurs at two extra domain exons (EDA and EDB) and one type III connecting segment (IIICS) exon.

Extra Domain Alternative Splicing

The two extra domain exons each encode a single type III homology repeat at two sites in the central portion of the fibronectin polypeptide subunit (Figure 1). Through a process described as "exon skipping", each 270 nucleotide exon can be independently included or excised from the mature fibronectin mRNA through alternative splicing (Figure 2), resulting in ED+ and ED- mRNA variants and subunit isoforms at each site (Paolella et al., 1988; Kornblihtt et al., 1984b; Kornblihtt et al., 1984a; Gutman and Kornblihtt, 1987; Schwarzbauer et al., 1987; Norton and
Hynes, 1987). This process is similar for human, rat, and chicken fibronectins. The single exons that encode these two ED repeats are unique in that all other type III repeats are encoded by a pair of exons (Odermatt et al., 1985; Patel et al., 1987). Exon sequences appear to be involved in the recognition of the human EDa as an alternatively spliced exon (Mardon et al., 1987).

Cell type or tissue specificity of expression of both ED regions has been demonstrated at both the protein (Borsi et al., 1987; Carnemolla et al., 1989; Paul et al., 1986) and mRNA levels (Gutman and Kornblith, 1987;  

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**Figure 2. Alternative Splicing of ED Exons.** (A) *Genomic structure.* Dark boxes represent exon sequences constitutively included in mature mRNAs. Open boxes denote alternatively spliced sequences. Thin dashed lines represent introns. Consensus 5' and 3' splice sites and the distances separating them are labeled. (B) *ED Alternative Splicing.* V-shaped lines above each splice variant reflect the splicing events that give rise to each mRNA variant. (C) *ED mRNA Variants.* Mature mRNA variants are designated ED⁺ or ED⁻.
Kornblihtt et al., 1984a; Kornblihtt et al., 1984b; Norton and Hynes, 1987) Both ED exons are absent in all mature pFN transcripts (those synthesized by hepatocytes), and present in approximately 25-30% (EDa) or 10% (EDb) of mature cFN transcripts in the cell lines examined (Kornblihtt et al., 1984a; Kornblihtt et al., 1984b; Gutman and Kornblihtt, 1987). Regulation of ED isoform expression has been observed during wound healing (ffrench-Constant et al., 1989), development (ffrench-Constant and Hynes, 1989; ffrench-Constant and Hynes, 1988; Oyama et al., 1990; Oyama et al., 1989; Vartio et al., 1987), and oncogenesis (Oyama et al., 1990; Oyama et al., 1989; Carnemolla et al., 1989; Zardi et al., 1987; Colombi et al., 1986; Castellani et al., 1986). In addition, TGF-β treatment modulates the expression of both EDa and EDb protein isoforms and mRNA variants (Balza et al., 1988; Borsi et al., 1987; Borsi et al., 1990).

Despite the tissue specificity and developmental regulation of its expression, the effect of ED region expression on the biological function of fibronectin subunits is not yet fully understood. Studies on tumor cell adhesion and neuroblastoma neurite extension have suggested a role for ED sequences in modulating adhesive responses (Lewandowska et al., 1990; Radinsky et al., 1990; Mugnai et al., 1988).

**IIICS Alternative Splicing**

The third location of alternative splicing of the primary fibronectin transcript is termed the type III connecting segment, or 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 fibronectin 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 (Figure 3).

Through "exon subdivision", portions of the IIICS can be alternatively expressed in mature fibronectin transcripts. Sequencing of cDNA (Schwarzbauer et al., 1983; Kornbluh et al., 1984a; Bernard et al., 1985) and

![Diagram](image)

Figure 3. Alternative Splicing of the Human IIICS Exon. (A) Genomic structure. Exons, introns, splice sites, and distances are as depicted in Figure 2. Alternatively spliced subsegments of the IIICS are designated by abbreviations A, B, and C. The CS-1 encoding A-subsegment is stippled. (B) IIICS Alternative Splicing Patterns. (C) IIICS mRNA Variants. Each mature mRNA variant is named using an abbreviation (center) listing the IIICS subsegments contained in that variant.
genomic clones (Tamkun et al., 1984; Vibe-Pedersen et al., 1986) of the IIICS revealed three consensus 3' splice acceptor sites within this complex exon (Figure 3a). These 3' acceptor sites are present and functional in both rat and human fibronectin mRNAs (Schwarzbauer et al., 1983; Tamkun et al., 1984; Vibe-Pedersen et al., 1986; Kornblihtt et al., 1984a; Bernard et al., 1985) and give rise to three IIICS mRNA variants in rats (ABC, BC, and O variants; Figure 3b).

In addition to the three alternative 3' splice sites, a single nucleotide difference in sequence creates a functional alternative 5' splice donor site in the human IIICS (Vibe-Pedersen et al., 1986) which is absent in the rat sequence (Tamkun et al., 1984). This complexity of splice site choices allows for the hypothetical existence of five human IIICS mRNA variants (Figures 3b and 3c), four of which (all except the BC variant) have been demonstrated to exist through cDNA cloning (Bernard et al., 1985; Kornblihtt et al., 1984a; Sekiguchi et al., 1986) and nuclease protection experiments (Oyama et al., 1989; Sekiguchi et al., 1986).

In efforts to characterize cell type-specificity in IIICS expression at the protein level, several investigators have used various proteolysis schemes and domain-specific monoclonal antibodies to identify differences in fibronectin subunits from different sources (Matsuura and Hakomori., 1985; Paul et al., 1986; Sekiguchi and Titani, 1989). These studies indicate that the pattern of IIICS expression differs among fibronectin subunits from different cell types. Such analyses of expression of IIICS isoforms are limited by the assay involved, because only the relative abundance of α subunits (those containing at least a portion of the proteolysis-sensitive
IIICS region) versus β subunits (those lacking all IIICS sequence) could be quantified (Castellani et al., 1986). Even without resolving the various IIICS-containing isoforms, cell type-specific differences in subunit composition are observed between pFN, cFN secreted by normal fibroblasts, and cFN from tumor and transformed cells. Lacking an assay for the expression of each possible IIICS polypeptide variant, further studies on cell type-specific patterns or transformation-associated changes in qualitative fibronectin expression await the development of an accurate assay for mRNA splicing variants.

How the alternative expression of the peptide sequences of the IIICS influences the biological activities of fibronectin subunit isoforms is an issue currently under vigorous investigation. Initial studies relied on the use of substrata coated with purified proteolytic fragments isolated from different subunit variants. Sets of fragments have been isolated that differ only in their expression of the IIICS sequence. Using such overlapping sets of proteolytic fragments, in conjunction with synthetic peptides derived from IIICS sequences, the functional significance of the IIICS peptide segment has been firmly established.

Two distinct IIICS sequences have been identified that are capable of promoting the attachment and spreading of B16-F10 murine melanoma cells but not baby hamster kidney fibroblasts. Synthetic peptides CS-1, comprised of the A-subsegment of the human IIICS (Figures 1 and 3), and CS-5, containing an REDV peptide sequence from the C-subsegment, both exhibited adhesion-promoting activity specifically for melanoma cells (Humphries et al., 1986; Humphries et al., 1987), 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 (Humphries et al., 1988), spreading and motility of avian neural crest cells (Dufour et al., 1988a) and lymphocyte adhesion to high endothelial cells (Ager and Humphries, 1990).

The receptor on lymphocytes and melanoma cells for the CS-1 adhesion signal has been identified as the α4β1 member of the integrin matrix receptor family (Wayner et al., 1989; Mould et al., 1990; Guan and Hynes, 1990). This is structurally and functionally distinct from the α5β1 fibronectin receptor integrin, sometimes present on the same cell types, that mediates adhesion to the constitutively-expressed RGDS-dependent cell adhesion domain of fibronectin (Wayner et al., 1989). The α4β1 and α5β1 integrins are also known as VLA-4 and VLA-5 respectively, members of a set of VLA proteins (very late antigens; all integrins) induced on lymphocytes upon activation (Hemler, 1990; Hemler et al., 1987; Springer, 1990). The importance of the differential expression of multiple sets of these VLA-family integrins on different subsets of lymphocytes upon activation (Ferreira et al., 1991; Gismondi et al., 1991; Picker et al., 1990; Rosemblatt et al., 1991; Kohn et al., 1991) and their role in mediating selective adhesive interactions and cellular responses in the immune system are well documented (Holzmann et al., 1989; Miyake et al., 1991; Yamada et al., 1991; Ferreira et al., 1990).

Recent experiments demonstrate the complex nature of α4β1 integrin binding to the IIICS. The α4β1 integrin on cell surfaces can bind to
substratum-bound CS-1-IgG conjugates via a Leu-Asp-Val (LDV) sequence present in the IIICS A-subsegment, and to CS-5-IgG conjugates via an Arg-Glu-Asp-Val (REDV) sequence present in the human IIICS C-subsegment. Either peptide, when provided as a soluble inhibitor, inhibited α4β1 binding to either CS-1-IgG or CS-5-IgG conjugates (Mould et al., 1991). Thus the binding site for α4β1 is composed of sequences from two different alternatively spliced subsegments of the IIICS. This observation has important implications for the possible role of regulated alternative splicing at multiple distinct sites within the IIICS in modulating the adhesive function of fibronectin subunits.

It is important to note that the expression of the CS-5 REDV sequence in two of the five human IIICS splicing variants may be affected during the splicing process by either of two mechanisms. Regulated alternative 3' splice site selection (any use of the 3'-most acceptor site removes the sequence) or alternative 5' splice site selection (use of the internal 5' donor site removes the sequence) both could independently or cooperatively modulate CS-5 expression. Interestingly, a similar Arg-Gly-Asp-Val (RGDV) sequence is present in the rat and bovine IIICS due to a single nucleotide change. Whether the rat and bovine RGDV is also active in modulating α4β1 recognition is unknown.

The adhesive activity of the IIICS for lymphoid, melanoma and neuronal cells, but not fibroblasts, demonstrates the considerable cell type specificity in the multiple mechanisms with which differentiated cell types adhere, spread, or migrate on FN substrates. This results from the preferential expression and 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.

The Mechanics of Alternative RNA Splicing

The splicing of messenger RNA precursors to form mature polypeptide-encoding transcripts is a general feature of the pathway of eukaryotic gene expression (Green, 1986; Padgett et al., 1986; Sharp, 1987). The reaction that removes intervening sequences is catalyzed by a multicomponent complex termed a spliceosome (Grabowski et al., 1985; Brody and Abelson, 1985; Frendewey and Keller, 1985) and comprised of several small nuclear ribonucleoproteins (snRNPs) (Maniatis and Reed, 1987; Guthrie and Patterson, 1988; Lührmann et al., 1990; Dahlberg and Lund, 1988; Choi et al., 1986). The spliceosome complex forms through the recognition of consensus splice site sequences located at or near the end of each intron (Mount, 1982; Ruskin and Green, 1985; Mattaj, 1989; Eperon et al., 1986; Mount et al., 1983; Chabot et al., 1985; Tazi et al., 1986; Gerke and Steitz, 1986).

Although much is known about the mechanism of pre-mRNA splicing, the components involved, and their assembly into a splicing complex, little is known of how these or other components are involved in the regulation of cell type-specific alternative splicing (Smith et al., 1989; Leff et al., 1986; Andreadis et al., 1987; Breitbart et al., 1987; Maniatis, 1991). Cell type-specific trans-acting factors appear to regulate alternative splice site choices in fibronectin ED region splicing (Barone et al., 1989) and in
other alternatively spliced mRNAs (Breitbart and Nadal-Ginard, 1987; Leff et al., 1987). In addition, some of these factors appear to be developmentally regulated (Breitbart and Nadal-Ginard, 1987), as are several snRNP species (Forbes et al., 1984; Mattaj and Hamm, 1989).

Alternative splicing patterns may be regulated by the affinity of RNA-RNA interactions based on 5' splice site sequence complementarity with U1 snRNA (the RNA component of U1 snRNP) (Kuo et al., 1991). In select instances, protein factors have been identified that modulate alternative splicing. Such factors include multiple Drosophila gene products (Sex-lethal, transformer, and transformer-2) identified genetically based on their regulatory effects on other mRNAs that determine sexual identity (Bingham et al., 1988; Bell et al., 1988; Boggs et al., 1987). Purified protein factors that regulate alternative splicing patterns in vitro include splicing factor 2 (SF2) (Krainer et al., 1990a; Krainer et al., 1990b) and alternative splicing factor (ASF) (Noble et al., 1989; Ge and Manley, 1990). These two factors were identified independently in different laboratories but share structural and functional characteristics indicating that they may be the same protein.

Despite these recent insights into possible mechanisms for regulating alternative splicing, many questions remain unanswered. Of particular interest is whether alternative splicing factors are specific for single pre-mRNAs or act globally, altering the splicing apparatus and changing the efficiency of multiple splicing events on many different pre-mRNAs. In addition, the regulation of splicing factor expression or activity and the functional consequences on the target pre-mRNAs remain to be described.
Rationale and Research Goals

Despite our broadening understanding of the functional role multiple IIICS sequences play in fibronectin adhesive function, little is known about the regulation of expression of alternatively spliced IIICS mRNA variants encoding fibronectin subunits with different subsets of IIICS sequence. The overall hypothesis to be addressed through this program of research is that different cell types can exhibit distinct patterns of IIICS mRNA variant expression, and that these patterns are regulated during development, cellular aging, and/or in response to the extracellular environment. In examining the regulation of alternative fibronectin IIICS splicing, the following experimental goals have been pursued:

1. Two different assays for quantifying all human IIICS region mRNA splicing variants have been established, providing increased accuracy of quantification through comparison of data derived from two distinct experimental protocols. These assay systems are (a) an RNase protection assay using uniformly-labelled SP6-generated probes from cloned fibronectin cDNA templates, and (b) a protocol involving the synthesis of double-stranded cDNA copies of the fibronectin mRNA population defined at both ends by fibronectin-specific oligonucleotide primers.

2. The pattern of alternative splicing of fibronectin IIICS pre-mRNA have been assayed in a variety of established human cell lines and tissues. Comparisons were made between several normal, virally-transformed, and tumor cell lines. Through these studies, the cell type specificity of IIICS expression patterns have been demonstrated, and insights were gained into the possible mechanisms by which IIICS splicing is regulated.
3. Possible changes in IIICS mRNA variant expression patterns associated with *in vivo* and *in vitro* aging have been examined using various cultured human dermal fibroblast cell strains.

4. Regulation of IIICS expression patterns in response to extracellular influences have been examined using growth factors and hormones known to regulate extracellular matrix and fibronectin biosynthesis.

5. The development of an exogenous IIICS pre-mRNA splicing substrate was pursued. Through the use of plasmids transcribing fibronectin IIICS genomic sequences in transient transfection experiments, the sequences regulating IIICS alternative splicing and the existence of cell type specific trans-acting splicing factors could be experimentally addressed.
Chapter II. Development of an Assay for IIICS mRNA Variant Expression

Introduction

Any experimental analysis of fibronectin IIICS alternative splicing must have as a prerequisite the development of an assay by which the abundance of each variant mRNA in a sample can be measured and compared. Moreover a complete appraisal of the mRNA variant expression pattern in any sample, and hence an understanding of its underlying regulation, depends on an assay system that is accurate, quantitative, reproducible, and capable of measuring the abundance of each variant species simultaneously within a given experimental RNA sample. This last criterion presents no significant obstacle when examining rat or chicken IIICS variants. The lack of an internal alternative 5' splice donor site in these species results in the synthesis of multiple mRNA variants (ABC, BC, and O variants in rat (Schwarzbauer et al., 1983); ABC and BC in chicken (Norton and Hynes, 1987)) due only to alternative 3' splice site selection (Figure 3). This relatively simple pattern of alternative splicing permits simultaneous measurement of all variants within a sample using a standard S1 nuclease or RNase protection assay protocol. Hybridization of rat or chicken IIICS mRNA variants to either a 5' end-labeled DNA probe or a uniformly-labeled RNA probe (spanning all alternative 3' splice sites) results in the protection of one diagnostic probe fragment for each individual IIICS mRNA variant.

The task of simultaneously measuring each IIICS mRNA variant within a sample of human RNA is complicated by the multiple overlapping alternative splicing patterns available. As a consequence of
both alternative 5’ splice site selection (removal of the C-subsegment) and alternative 3’ splice site selection (removal of the A-subsegment or the entire IIICS), sequence heterogeneity occurs at both ends of the IIICS region. This precludes utilization of a nuclease protection protocol based on either a 5’ end-labeled or 3’ end-labeled probe as different IIICS variants would protect identical end-labeled probe fragments.

To overcome this experimental limitation, two assays based on different technologies were developed in an effort to simultaneously quantify all human IIICS mRNA variants within an experimental sample. Initially, an RNase protection protocol (Zinn et al., 1983) was tested using a uniformly-labeled RNA probe spanning the entire IIICS sequence. Subsequently, a novel double primer extension, or DPE, assay was created based on the synthesis of double-stranded cDNAs from target fibronectin mRNA populations primed using oligonucleotides flanking the alternatively spliced sequences. The DPE assay proved to be the optimal assay method for the identification and accurate quantification of all five human IIICS variants, including the postulated but previously unidentified BC variant.

Experimental Procedures

*Materials and Methods*

Unless otherwise noted, reagent-grade chemicals were obtained from Sigma (St. Louis, MO) and Fisher (Pittsburgh, PA), and enzymes were obtained from United States Biochemicals (Cleveland, OH), Boehringer Mannheim (Indianapolis, IN), and New England Biolabs (Beverly, MA). Cell culture reagents were from Gibco (Grand Island, NY). Radiolabeled
nucleotides were from Amersham (Arlington Heights, IL) and NEN/DuPont (Boston, MA).

Where not specifically detailed, all molecular biological procedures were performed using standard established techniques (Maniatis et al., 1982; Sambrook et al., 1989; Ausubel et al., 1987).

**Cell Culture**

The HT-1080 fibrosarcoma human tumor cell line was obtained from the American Type Culture Collection (Rockville, MD). Cells were cultured *Mycoplasma*-free in DMEM supplemented with 10% neonatal calf serum (Biologos, Naperville, IL), 250 U/ml penicillin and 250 µg/ml streptomycin sulfate at 37°C in a 10% CO₂-humidified air mixture.

**RNA Isolation**

Total cellular RNA was purified from confluent cell cultures by the guanidinium isothiocyanate/hot phenol method (Chirgwin et al., 1979; Maniatis et al., 1982), followed by treatment with proteinase K. Cells were lysed directly in the tissue culture flask with the hot guanidinium solution to avoid possible detachment-associated alterations in RNA metabolism. RNA solution concentrations and purity were determined spectrophotometrically at 260nm and 280nm, respectively.

**RNase Protection Assay**

**Construction of phFN-IIICS In Vitro Transcription Plasmid**

Plasmid phFN-IIICS was prepared by isolating a 1036bp XbaI - HindIII fragment from the human fibronectin cDNA plasmid FN421 (provided by Dr. M.-L. Chu, Jefferson Medical College, Philadelphia, PA) (Bernard et al., 1985) and subcloning it into the dual promoter plasmid pSP6/T7-19
linearized within the multiple cloning site by cleaving with XbaI and HindIII. The 1036bp insert fragment contains pBR322 sequence at its HindIII end (HindIII to EcoRI) followed by a poly-C:poly-C stretch, both derived from the cloning vector used in the construction of FN421 (Bernard et al., 1985). The identity of the FN421 plasmid and the structure and insert orientation of phFN-IIICS after subcloning were confirmed by restriction mapping.

**In Vitro Synthesis of RNA Probes**

A gel-purified 1451bp HaeII restriction fragment of phFN-IIICS was used as a template for *in vitro* transcription using the bacteriophage SP6 RNA polymerase and standard protocols (Melton et al., 1984). SP6 transcription from the phFN-IIICS HaeII fragment in the presence of [α-32P]UTP yields a uniformly-labeled 1165 nucleotide RNA probe (rIIICS-ABC) complementary to the entire 360 nucleotide IIICS region (ABC variant) and neighboring exon sequences of human fibronectin mRNAs (Figure 4). SP6 transcription reaction conditions were: 40mM Tris-Cl pH 7.5, 6mM MgCl₂, 2mM spermidine, 500mM each NTP, 10mM dithiothreitol, 1U/μl RNasin ribonuclease inhibitor, 100μg/ml bovine serum albumin, 50-100μg/ml linear DNA, 6mM [α-32P]UTP (800Ci/mmol, 20mCi/ml), 0.1U/μl SP6 RNA polymerase. Reactions were performed at 40°C for one hour, then treated with RNase-free DNase to degrade template DNA. Ethanol-precipitated probe RNA was dissolved and denatured at 90°C in denaturing loading buffer (90% deionized formamide, 1x TBE, 0.2% bromophenol blue, 0.2% xylene cyanol), and size fractionated on a 5% polyacrylamide / 7M urea denaturing gel. Labeled
reaction products were visualized by autoradiography and the full-length probe RNA was excised from the gel and eluted.

**RNase T2 Protection Assay**

HT-1080 fibrosarcoma cell total RNA (40μg) and ~400,000 dpm radiolabeled rIIICS-ABC probe RNA were co-precipitated, dissolved in 10μl of RNase T2 hybridization buffer (80% deionized formamide, 40mM PIPES pH 6.5, 400mM NaCl, 1mM EDTA pH 8.0), denatured briefly at 90°C, and hybridized overnight at 50°C. RNase T2 digestion buffer (90μl with concentrations: 300mM NaCl, 20mM NaOAc pH 4.5) and 2-20 units of RNase T2 (Calbiochem, San Diego, CA) were added and incubated at 37°C for 45 minutes. RNase T2 reaction mixtures were treated with proteinase K, extracted with phenol-chloroform, and ethanol-precipitated.

**Double Primer Extension Assay**

**Primer Synthesis and 5' End-Labeling**

Oligorucleotide primers IIICS-5' (5'-ACTGGCCTGGAACCGGG-3'), IIICS-C (5'-CCCTGGGAAATGCTGACCAATTTTG-3'), and IIICS-3' (5'-GTGTTCCTGGAATGGGGCCC-3') were synthesized using an Applied Biosystems (Foster City, CA) model 381A DNA synthesizer in accordance with the manufacturers instructions, followed by purification by isolation from a 20% denaturing polyacrylamide gel. These primers are complementary to human FN mRNA sequences that lie 5' of the IIICS region (IIICS-5'), within the IIICS C-subsegment (IIICS-C), or 3' of the IIICS region (IIICS-3'; see Appendix A). Primer IIICS-5' was end-labeled (Maniatis et al., 1982) using [γ-32P]ATP (3000-5000Ci/mmol) and T4 polynucleotide kinase (United States Biochemicals, Cleveland, OH).
1st Strand cDNA Synthesis - Reverse Transcription

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 AMV reverse transcriptase (United States Biochemicals, Cleveland, OH). Total cellular RNA (50μg) was precipitated with ethanol, resuspended in 40 μl of 100mM KCl, 50mM Tris-Cl pH 8.3 with 0.1 picomole primer IIICS-3' (or IIICS-C), denatured at 95°C for 5 minutes, and incubated at 50°C for 45 minutes for primer annealing. Reverse transcriptase reaction mix (60 μl with concentrations: 50mM Tris-Cl pH 8.3, 10mM MgCl2, 3mM dithiothreitol, 500μM each dNTP, 3 units AMV reverse transcriptase) was added for extending the annealed primer at 50°C for 45 minutes. Test studies using purified template IIICS-ABC RNA transcribed in vitro from phFN-IIICS using T7 RNA polymerase demonstrated that these experimental conditions reliably synthesize first-strand cDNAs extending well past the IIICS-5' primer binding site and that the template RNA is degraded during reverse transcription by the RNase H activity of AMV reverse transcriptase (data not shown).

2nd Strand cDNA Synthesis - Sequenase Reaction

Second-strand cDNA run-off extension products were synthesized from first-strand cDNA templates by extension of an annealed 32P-IIICS-5' primer using Sequenase (modified T7 DNA polymerase: United States Biochemicals, Cleveland, OH). Ethanol-precipitated reverse transcriptase reaction products were resuspended in 40μl of 40mM Tris-Cl pH 7.5, 50mM NaCl, 20mM MgCl2 with 0.1 picomole 32P-IIICS-5' primer (0.05-0.1 μCi), denatured at 95°C for 5 minutes, and incubated at 50°C for 45
minutes for primer annealing. Sequenase reaction mix (20 μl with concentrations: 10mM Tris-Cl pH 7.5, 12.5mM NaCl, 5mM MgCl₂, 20mM dithiothreitol, 1.25mM each dNTP, 3 units Sequenase) was added and incubated at 37°C for 15 minutes. Test studies using reverse transcription reaction products derived from *in vitro*-synthesized ABC variant RNA (T7-transcribed from phFN-IIIICS) resulted in the reliable synthesis of full length (506 nucleotide) ABC variant second-strand extension products (data not shown).

**Restriction Analysis of DPE Products**

For restriction analysis of FN-specific DPE products, double-stranded DNA products of DPE reactions were precipitated with ethanol and digested with the restriction enzymes listed in the figure legends in accordance with manufacturers specifications.

**Denaturing Gel Electrophoresis and Densitometry**

In order to visualize the radiolabeled cDNA products of the DPE assay or the RNase-protected RNA probe fragments from the RNase protection assay, reaction products were precipitated with ethanol, resuspended in denaturing loading buffer (90% deionized formamide, 1x TBE, 0.2% bromophenol blue, 0.2% xylene cyanol) and size-fractionated on a 5% polyacrylamide 7M urea denaturing gel (Maniatis et al., 1982). ³²P-labeled products were visualized by exposing dried gels to x-ray film (Kodak X-Omat AR).

The relative abundance of products corresponding to IIIICS mRNA variants were quantified by densitometrically scanning autoradiographs on an LKB Bromma 2222-010 UltraScan XL laser densitometer using LKB
Bromma Gel-Scan XL data analysis software. For the DPE assay autoradiograms, the upper limit of the linear range of the film was determined empirically to be the area value for the AB band (the most abundant band) above which the AB band exposure saturates, resulting in the numerical reduction in the AB percentage of the total and a compensatory increase in the percentages of the other bands. The lower limit of the linear range was an exposure below which the least abundant variant bands could not be accurately discerned from background.

For quantification of the relative molar abundance of RNase-protected RNA probe fragments, the peak areas were divided by the number of $^{32}$P-uridine residues present in each fragment. This compensates for the differences in probe fragment length and uridine content.

Results

**RNase Protection Assay**

Initial efforts at developing an assay for IIICS mRNA variant expression were based on a modification of the RNase protection protocol (Zinn et al., 1983). This assay protocol utilizes a uniformly-labeled RNA probe (IIICS-ABC) transcribed *in vitro* using SP6 RNA polymerase (Melton et al., 1984) from a cloned FN IIICS cDNA (Figure 4). Hybridization of the RNA probe to a human FN mRNA sample results in the formation of double-stranded heteroduplex regions between the FN mRNA and the RNA probe which are protected from hydrolysis upon treatment with the single-strand specific RNase T2. Among the resulting protected probe fragments several are diagnostic for individual IIICS splice variants whereas other fragments arise from multiple variant mRNAs.
Plasmid phFN-IIICS was prepared as a template for RNA probe synthesis. A fibronectin cDNA sequence (from the FN cDNA clone FN421) containing the entire IIICS region (ABC variant) and neighboring exon sequences was cloned into a site between the bacteriophage T7 and SP6 promoters on pSP6/T7-19 (Figure 4). SP6 transcription from the 1451bp HaeII fragment of phFN-IIICS in the presence of [α-32P]UTP yields a uniformly-labeled 1165 nucleotide IIICS-ABC probe RNA.

HT-1080 human fibrosarcoma cell RNA was used as a test target for the RNase T2 protection assay at a range of RNase concentrations (Figure 5).

**Figure 4. RNase Protection Assay.** A uniformly 32P-labeled RNA probe (IIICS-ABC) is transcribed from the SP6 promoter on a HaeII fragment of phFN-IIICS. The solid and stippled boxes indicate common FN sequences and the IIICS region respectively. Open boxes indicate vector sequences. Numbers indicate the lengths of the full-length RNA probe and the probe fragments protected by IIICS variant mRNAs after hybridization and RNase treatment.
Four of the five possible IIICS variants (those containing some or all of the IIICS) produced characteristic protected fragments allowing direct measurement of their abundance. However, the O variant yields no characteristic probe fragment; its protected fragments are common to other splice variants. To measure O variant expression, the intensities of the B band plus that of the AB or BC bands must be subtracted from that of the bands common to more than one splicing variant (AB+B+O and BC+B+O). Thus quantification of the O variant relies on accurate measurement of two pairs of other bands. This was not possible due to the high background even at high RNase concentrations.

Quantification of the unique probe fragments showed that the AB variant is the predominant FN mRNA species in HT-1080 cells, comprising approximately 65-80% of the detectable FN mRNA pool. Estimates for the other variants are: ABC, 10-13%; B, 5-20%; and BC, less than 1%. The consistent absence of a BC+B+O band (in a number of repetitions of this assay) precluded any estimate as to O variant abundance.

**Figure 5. RNase Protection Assay on HT-1080 RNA.** *(next page) (A)* Overview of the rIIICS-ABC probe and RNase-protected fragments, as depicted in Figure 4. *(B)* 40µg of HT-1080 total cellular RNA was hybridized with the rIIICS-ABC probe, treated with RNase T2, and fractionated on a denaturing 5% polyacrylamide gel. Arrows indicate protected probe fragments derived from IIICS mRNAs. Above each lane is listed the amount of RNase T2 used in the digestion. Lane M contains RNA markers. Undigested probe is shown in the left lane.
Double Primer Extension Assay

The inability to accurately measure the abundance of all IIICS mRNA variants in a sample using the RNase protection protocol necessitated the development of an alternative assay. A double primer extension (DPE) assay was developed in which a family of double-stranded cDNAs are created from the FN mRNA pool through successive primer extension reactions (Figure 6).

An oligonucleotide primer complementary to a site 3’ of the alternatively spliced human IIICS is annealed to the sample RNA and first strand cDNA synthesis is performed with AMV reverse transcriptase, with the RNA strand being digested during DNA polymerization by the RNase H activity of reverse transcriptase. The sample is denatured and a second 5’ end-labeled oligonucleotide primer complementary to a region of the first-strand cDNA 5’ of the IIICS is annealed and extended using Sequenase (modified T7 DNA polymerase). This results in the synthesis of a partial double-stranded copy of the IIICS region of each fibronectin mRNA (Figure 6A). Each radiolabeled second-strand cDNA is defined at its ends by the sites of the primers 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 (Figure 6B). Because the regions to which both oligonucleotides hybridize are sequences common to all fibronectin transcripts, the primers should show no bias toward any of the mRNA variants, as is conceivable with long uniformly-labeled RNase-protection probes containing alternatively expressed sequences.
DPE analysis of HT-1080 fibrosarcoma cell RNA identified all five IIICS variants (Figure 7), including the postulated but previously undemonstrated BC variant (Figure 7C). 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.

Figure 6. Double Primer Extension Assay. (A) The steps in the DPE assay are diagrammed showing the locations of the oligonucleotide primers (thick lines), the first-strand cDNA products (dashed lines), second-strand cDNA products (solid lines), alternatively spliced regions of variable length (white boxes) and the $^{32}$P label on the 5' primer (asterisks). (B) The structure of each IIICS mRNA is depicted (as in Figure 3) with its DPE products. Numbers indicate the size of the radiolabeled run-off DPE product expected for each mRNA variant.
The observed molar distribution of variant cDNAs was not altered by increasing the amount of primers used, increasing the duration of the reactions, or altering the primer annealing temperatures (data not shown). Changes in the amount of RNA assayed (mimicking differences in the FN mRNA abundance among different cell lines) resulted in changes in the overall amount of 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 on individual variant mRNAs under these experimental conditions.

Figure 7. Double Primer Extension Assay and Restriction Analysis of HT-1080 RNA. (next page) (A) Numbers indicate the size in nucleotides of full-length radiolabeled DPE products (using primers IIICS-5' and IIICS-3') and the products after digestion with the restriction enzymes listed. (B) Autoradiogram of denatured DPE products from HT-1080 RNA assayed with primers IIICS-5' and IIICS-3'. Numbers indicate the sizes of marker DNA fragments. Run-off DPE products (first lane) of IIICS mRNAs are indicated by their abbreviations. Additional lanes show DPE products treated with restriction enzymes prior to electrophoresis. (C) Extended electrophoresis of (B) to resolve the weak BC band from the AB band. (D) Products of C-specific DPE assay on HT-1080 RNA using primers IIICS-5' and IIICS-C. Variants containing the C-subsegment are labeled.
No DPE products of a longer size corresponding to unprocessed or partially processed FN primary transcripts (those retaining the preceding ~1kb intron) were observed. This suggests that these unspliced precursors comprise a very low percentage of the total FN RNA pool.

To verify the identity of each product on 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 (Figure 7A). In all cases, treatment with the appropriate restriction enzymes resulted in the predicted shifts in electrophoretic mobility (Figure 7B 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 (Figure 7C). 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 IIIICS-C, complementary to the IIIICS C-subsegment, for IIIICS-3' as the primer for first-strand cDNA synthesis. This creates DPE products corresponding to only those mRNA variants containing the C-subsegment (ABC and BC). The relative amounts of ABC and BC products observed in this C-specific assay (Figure 7D) were identical to those derived from the comprehensive IIIICS assay (Figure 7C).

Densitometric quantification of the products of DPE analysis of HT-1080 RNA provided the following values for the relative molar abundance of
each IIICS variant: ABC, 5%; BC, <0.5%; AB, 78%; B, 9%; O, 8%. These values are comparable with the approximate abundance values derived from the RNase protection assay for those variants detected in that assay, thus confirming the accuracy of the DPE assay. The reproducibility of the quantitative measurements derived from the DPE assay will be addressed in the following chapter.

These results taken together indicate that the DPE assay provides a highly suitable 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.

Discussion

The intended study of the expression of IIICS splicing variants and their regulation required an assay capable of identifying and quantifying the relative abundance of each variant RNA within a experimental sample. To accomplish this initial objective, two experimental protocols were tested. Initially an RNase T2 protection protocol using a uniformly-labeled RNA probe was employed. A cDNA fragment containing the entire IIICS region was subcloned into an SP6/T7 transcription vector to serve as a template for in vitro synthesis of the rIIICS-ABC RNA probe. An RNase T2 protection assay using this probe and human total cellular RNA resulted in the protection of four unique probe fragments, diagnostic for the IIICS-containing variants (ABC, AB, BC, and B), plus two fragments derived from multiple variants (Figure 4).
This assay protocol proved unsatisfactory due to its high background and the lack of a diagnostic fragment for the O variant (Figure 5). As will be discussed in the following chapter, the accurate quantification of the O variant abundance within a sample proved to be an indispensable requirement of a IIICS assay because it is the O variant that exhibits the greatest cell type-specific regulation. The RNase protection assay failed to demonstrate any cell type-specific changes in IIICS expression patterns among several human cell types tested because of this limitation (data not shown). However, the relative abundances of the four IIICS-containing variants measured in the RNase assay (Figure 5) were in good agreement with the values derived from the novel double primer extension assay developed subsequently, thus the RNase protection assay served as a confirmation of the accuracy of the 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 double primer extension assay was developed. Through the synthesis of cDNA copies of each FN RNA (Figure 6), this assay was able to simultaneously demonstrate all five IIICS variants and allow for their accurate quantification. The DPE protocol is applicable to other instances of mRNA variation, adapting the oligonucleotide primers to specific mRNA templates or sites of variation, as demonstrated with the C-specific DPE assay for those IIICS variants that contain the C-subsegment (Figure 7D). Heterogeneity among mRNA transcripts can be identified and quantitated on the basis of DPE run-off product length, and the
sequence content of the DPE products (and hence the specificity of the primer extension reactions) can be confirmed through restriction mapping (Figure 7C). The identification of the weakly expressed BC variant (which had not been previously identified either through cDNA cloning or nuclease protection experiments) demonstrates the sensitivity of the DPE assay, particularly when using the C-specific primer IIICS-C in order 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.

The DPE assay proved completely satisfactory for the purpose of accurately measuring IIICS variant abundances within experimental samples. This assay protocol would thus provide the means for conducting the analyses of IIICS RNA variant regulation presented in the following work.
Chapter III. Cell Type-Specific Expression of IIICS mRNA Variants

Introduction

Given the functional consequences of alternative splicing in the human IIICS on the adhesive activity of fibronectin subunits, and the multiple possible mechanisms regulating the preferential utilization of multiple 5' and 3' splice sites within the human IIICS, it is important to identify the range of expression patterns of IIICS mRNA variants exhibited by different human cell types and tissues. Such studies have been performed on rat and chicken cells in which the splicing patterns are dictated exclusively by alternative 3' splice site use (three 3' sites in rats, two in chickens) (Schwarzbauer et al., 1983; Norton and Hynes, 1987; ffrench-Constant and Hynes, 1989). Studies in these species demonstrated the cell type-specificity of IIICS mRNA variant expression, particularly with regard to the increased expression of the O variant (encoding the IIICS- subunit) in liver tissue (pFN mRNAs) versus cultured fibroblasts and other tissues (cFN mRNAs).

Several studies of the expression of fibronectin polypeptide subunit isoforms in human cells have suggested that alternative splicing in the IIICS is regulated in a cell type-specific manner. These results once again demonstrated the increased expression of the IIICS- subunit isoform (encoded by the O mRNA variant) in pFN as compared to fibroblast cFN (Castellani et al., 1986). In addition the synthesis of IIICS- subunits appeared to be reduced in tumor cells and SV-40-transformed fibroblasts relative to normal fibroblasts. Other researchers have demonstrated a reduction in the expression of C-subsegment-containing subunit isoforms.
in pFN relative to fibroblast cFN, suggesting cell type-specific regulation of the use of the human alternative 5' splice donor site (Sekiguchi and Titani, 1989).

In this study, the cell type-specificity in the qualitative expression of the human IIICS mRNA variants and the underlying regulatory mechanisms of IIICS alternative splicing were examined. The DPE assay was used to examine IIICS mRNA expression patterns in a variety of human normal and tumor cell lines and in human liver tissue. Cell type-specific differences in IIICS mRNA variant expression patterns were observed, and the unique composition of human liver pFN mRNA was demonstrated. In addition, transformed cells appeared to down-regulate their O variant expression relative to normal cells. Based on 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 5' splice donor site selection in establishing specific patterns of IIICS variant expression.

Experimental Procedures

Except were specifically noted, cell culture manipulations, RNA isolations, DPE assays, and densitometry were performed as described in Chapter 2.

Human Cell Lines and Tissues

8 Pap and 13 Pap human dermal papillary fibroblasts and human keratinocytes "K2" (Schafer et al., 1989; Beyth and Culp, 1985) were isolated by Drs. 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 (Mene et al., 1989) were isolated by Dr. 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, NJ, fetal skin fibroblasts AG-4449 from the National Institute of Aging cell repository, Camden, NJ, and the remaining human cell lines were from the American Type Culture Collection, Rockville, MD. Human transformed and tumor cell lines are: HepG2, hepatocellular carcinoma; VA-13, SV40-transformed WI-38 fibroblasts; RD, rhabdomyosarcoma; HT-1080, fibrosarcoma; A172, glioblastoma; A549, adenocarcinoma; Hs294T, melanoma. Normal cell strains are: CCD-18Co, colon fibroblasts; WI-38, MRC-9 and MRC-5, fetal lung fibroblasts; WI-1003, adult lung fibroblasts. Normal human liver tissue samples were obtained from the Tissue Collection Facility, University Hospitals of Cleveland.

Densitometric Quantification

Densitometric scans were performed on multiple linear range exposures of DPE autoradiograms, and individual values for the relative abundance of each DPE product were calculated for each lane as a percentage of the total amount of FN-specific DPE products (area of individual peak + sum of all peak areas = relative percentage of individual variant). Individual values derived from identical lanes on multiple autoradiogram exposures were averaged to produce relative percentage values for each assayed RNA sample. Multiple RNA samples were assayed for each cell strain or tissue examined, and the relative percentage values and error bars presented on the graphs in this chapter for each cell
type are the averages and standard deviations for the values derived from these multiple RNA samples. Thus each column represents assay measurements of multiple RNA samples from the same cell strain. The variability in the values derived among multiple exposures of the same assayed RNA sample were consistently smaller that the variability observed among multiple RNA samples from the same cell or tissue source, which are the error values presented.

Results

IIICS mRNA Expression Patterns in Human Cells

A wide variety of human normal and tumor cell lines, as well as liver tissue, were tested using the DPE assay to examine how the patterns of expression of IIICS variants differ among various cell types (Figure 8). 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 comprise anywhere from 3% to 57% of the total fibronectin mRNA pool when comparing many cell types, based on densitometric quantification of the molar abundance of each variant (Figure 9). 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 fibronectin mRNA pool.

The observed changes in IIICS expression patterns do not exhibit any simple correlation with the significant changes in the quantitative abundance of fibronectin mRNA among these cell lines. For instance, HepG2 cells (3% O variant) contained steady state levels of fibronectin
mRNA comparable to skin fibroblasts 8 Pap and 13 Pap (42% O variant), and liver RNA (57% O) contained much less fibronectin mRNA than skin fibroblasts.

Tumor and transformed cells consistently exhibited low levels of O variant expression (HepG2 through Hs294T; Figures 8A and 9) compared with the high levels of O variant expression in some normal cells (AG4449 through HMC; Figures 8B and 9). In direct comparisons, the SV-40 transformed VA-13 cell line (Figure 8A; 4% O) exhibited reduced O variant expression compared to its parental WI-38 normal fibroblast cell strain (Figure 8B; 22% O). Similarly, HepG2 hepatoma cells (Figure 8A) exhibited a drastic reduction in O variant expression compared to liver tissue (Figure 8B; 3% versus 57%).

Figure 8. DPE Assay of RNAs from Various Human Cell Types. (next page) The DPE assay was performed on RNAs from tumor (A) and normal cells and liver tissue (B) using the IIICS-5' and IIICS-3' primers. Varying amounts of DPE reaction mixtures were loaded to provide roughly equivalent AB band signals, compensating for differences in FN mRNA abundance among RNA samples. The four major IIICS variants are labeled on the left by their abbreviations. (C) The C-specific DPE assay using primer IIICS-C was performed on selected samples and the amounts of DPE reaction products loaded were standardized for a constant ABC band signal. The two C+ variants are labeled by their abbreviations.
These observations would suggest that transformation significantly alters fibronectin 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. The demonstration of transformation-sensitive alterations in IIICS expression would be best performed using syngeneic cell strains in which the transformed phenotype is the direct result of the introduction of an individual oncogene, either as an activated oncogene or in a temperature-sensitive or regulatable form.

Figure 9. Quantification of IIICS mRNA Variant Expression in Various Human Cell Types. (next page) Varying exposures of DPE autoradiograms (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). Error bars represent the standard deviations among percentage values obtained from assays of multiple RNA samples from each cell strain or tissue type. Results are grouped among tumor and transformed cell lines (left), normal cell strains (center), and liver tissue (right).
Among normal cell strains tested, O variant expression displayed significant tissue and cell type-specificity (Figures 8B and 9). 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). If the IIICS mRNA expression patterns of these cells in vitro accurately reflect their fibronectin 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 fibronectin 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 splice site pairing versus the four remaining splicing patterns. If the O variant is the single mRNA species subject to either of these mechanisms of regulation, one would expect 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 (Figure 10) 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 (Figure 10). Unlike all cultured cell lines tested, liver tissue RNA exhibited a level of B variant expression comparable to that of the AB variant (Figures 8B and 9), 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.

Figure 10. AB and B Variant Levels Relative to O Variant Expression in Various Human Cell Types. The percentages of AB variants (•) and B variants (x) are plotted against O variant levels for each cell type. Percentage values and errors are as in Figure 9.
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' splice site to the same constitutive 5' splice site. The expression of these mRNAs vary independently (Figure 10) presumably through regulation of 3' site choice. The AB and B variants lack the C-subsegment due to an additional splicing event involving the internal alternative 5' splice site. It is not known whether these two separate splicing events occur in a particular order, or if the result of one splicing event may affect the splicing reaction at the other location. If these two splicing events are independent, one may predict that differences in 3' splice site use 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 assaying the ABC and BC variants using the C-specific DPE protocol (demonstrated in Figure 7C). The BC variant was present at low levels in all cells tested (<2% of the total FN mRNA pool; Figure 8C). Figure 11 presents densitometric scans of DPE autoradiograms for normal cell samples selected on the basis of their significant differences in O or B variant expression. 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; Figure 11, left column) and the C+ variants (ABC/BC ratios; Figure 11, right column). These 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).
Figure 11. Densitometric Scans Comparing Changes in BC and B Variant Expression. Densitometric scans of linear range autoradiograms from normal (left) and C-specific DPE assays (right) of selected cell types were normalized to display equivalent AB or ABC peak areas, respectively. Peak identities are labeled at the bottom.
The co-regulation of the B and BC variants relative to the AB and ABC variants suggests that IIICS⁺ variants can be grouped into A⁺ (ABC+AB) and A⁻ (BC+B) “families” (Figure 12). These families, in addition to the O variant, result from 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 (Figures 10 and 11). The most dramatic differences in IIICS mRNA expression patterns among different cell types can be attributed within this model to modulation of the three variant families through regulation of alternative 3’ splice site choice (Figure 12B).

**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 (Figures 11 and 12C and 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 Figures 9, 11, and 12C). In liver RNA less than 5% of the A⁺ transcripts fail to use the internal 5’ site (Figure 11). Although the degree of utilization of the internal alternative 5’ splice site does vary somewhat among different cell types (Figure 12C), this regulation at the level of 5’ splice site choice accounts for much less variation in overall IIICS expression patterns than does the regulation at the level of 3’ splice site choice (Figure 12B).
Discussion

To address the issue of cell type-specific alternative splicing regulation in the complex IICS exon, the DPE assay was used to identify cell type-specific changes in IICS expression patterns among different human cell types and to suggest the possible regulatory mechanisms responsible for such changes.

Figure 12. Model for Cell Type-Specific Regulation of IICS Alternative Splicing. (A) IICS mRNAs are grouped by family (A⁺, A⁻, and O) by the use of alternative 3' splice sites joined with the common upstream 5' splice site (sites highlighted). (B) The average abundance of each variant family is expressed as a percentage of the total FN RNA pool. (C) The average percentage of mRNAs within each family that are C⁺ (ABC or BC) or C⁻ (AB or B) reflect alternative use of the internal 5' splice site.
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 (Figures 8 and 9). 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 coworkers (Castellani et al., 1986) demonstrated the variability in IIICS- subunit expression. Plasma fibronectin subunits (synthesized in the liver) contained the highest percentages of IIICS- subunits, cFN from normal fibroblasts an intermediate amount, and tumor and transformed cell cFN the lowest levels. Both the patterns of these cell type-specific changes in IIICS- expression and the percentage values obtained at the protein level (Castellani et al., 1986) are quite similar to the changes in O mRNA percentages observed in this study (Figure 9). 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 cFN purified from WI-38 fibroblasts (Sekiguchi and Titani, 1989). This is consistent with the underexpression of C+ mRNAs in liver tissue RNA compared to fibroblasts as observed in this study.

The similarity of the results presented here on mRNA variant levels with 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 fibronectins at both the mRNA and protein levels are comparable to those observed among rat fibronectins. Rat liver RNA exhibits higher O mRNA levels than do rat fibroblasts (Schwarzbauer et al., 1983). At the protein level, studies on rat fibronectins using a novel chemical cleavage-immunoblot technique (Paul et al., 1986) demonstrated the elevated expression of both the O and BC subunits (compared to ABC levels) in pFN compared to 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, where the C-subsegment is removed from a vast majority of transcripts through 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 fibronectin transcripts.

In recent experiments examining the intracellular transport and secretion of cDNA-encoded partial FN-polypeptides ("deminctins"), it has been demonstrated that homodimers of rat IIICS- deminctins form intracellularly but are not secreted, whereas heterodimers of IIICS- IIICS+
(ABC variant) demineccins are properly secreted (Schwarzbauer et al., 1989). This is particularly interesting in light of the observation in this study that O variants never comprise a significant majority of human IIICS transcripts (Figure 9; maximum O variant expression=57% in liver, significantly less in all other cell types). If those IIICS- fibronectin subunits that are in excess of the available IIICS+ subunits are not secreted, then little is gained for the cell by synthesizing more than approximately 50% of its fibronectin transcripts in the O variant form.

**Transformation Effects on IIICS Expression**

It was noted that tumor and transformed cells appear to underexpress the O mRNA variant compared to several normal fibroblasts, mesangial cells, and liver tissue, as demonstrated previously for IIICS- subunit expression (Castellani et al., 1986). 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 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 comparison of appropriate isogenic cell models for transformation and tumorigenesis, as well as direct experimental manipulation of the transformed phenotype.

**Implications for the Mechanism of Regulated IIICS Alternative Splicing**

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+AB, BC+B, and O variant levels among various
cell lines suggests the existence of independently regulated sets or "families" of mRNA variants. As outlined in Figure 12, each family differs with regard to which alternative 3' splice site is joined to the common 5' splice site. 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 (Figure 12B). 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 (Figure 12C). The prevalence of 3' splice site regulation over 5' splice site regulation 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 normal fibroblasts) in both human pFN (Figures 9 and 12) (Castellani et al., 1986) and rat pFN (Paul et al., 1986; Schwarzbauer et al., 1983) which lacks an alternative 5' splice site.

A model of 3' splice site regulation is proposed (Figure 12) based on 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 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 co-regulation 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. Although differential fibronectin mRNA stability among various cell lines appears to affect the steady state abundance of the total fibronectin mRNA pool (Dean et al., 1988), any role it plays in establishing cell type-specific patterns of qualitative IIICS variant expression remains to be demonstrated.

Recognition of the 3' splice site and the branchpoint region by splicing components are required initial events in the formation of an active spliceosome (Chabot et al., 1985; Frendewey and Keller, 1985). The branchpoint is recognized by U2-snRNP in concert with a required auxiliary factor, U2AF, which also recognized 3' splice sites (Ruskin et al., 1988). 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 IIICS 3' splice site use could occur. The observed alterations in the efficiency of alternative 5' splice site utilization may be
influenced by the action of several recently described splicing factors (SF2 and ASF) (Ge and Manley, 1990; Krainer et al., 1990a; Krainer et al., 1990b) or U1-snRNP (Kuo et al., 1991) for which a role in alternative 5' splice site selection have been described.

**Cell Biological Implications - Expression of CS-1-Encoding mRNAs**

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 (Humphries et al., 1986; Humphries et al., 1987; Mould et al., 1991) 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 (Figure 12B). The A+ transcripts constitute the majority of fibronectin mRNAs in all of the cultured cell types examined, although their percentage abundance varies among cell types (Figure 12). This contrasts with liver fibronectin mRNA, believed to encode pFNs (Tamkun and Hynes, 1983), in which the A+ transcripts are in the minority (Figure 12). It is not known if 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 fibronectins. 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.
Chapter IV. IIICS Expression in Aging Dermal Fibroblasts

Introduction

Human skin is an architecturally and cytologically complex organ made up of three compartments, the epidermis, dermis, and subcutis, each of which are comprised of multiple cell types with different specialized functions. Extracellular matrix components play an important role in many of skin's functions by regulating the adhesive interactions of different cell types within the complex tissue structure, such as during the repair of damaged skin (Grinnell, 1984).

Fibronectin and Skin Pathologies

Proper structure and function of skin relies on the accurate synthesis and assembly of its extracellular matrices, as demonstrated by various skin pathologies associated with altered expression, structure, or processing of extracellular matrix components (Holbrook and Byers, 1987; Bauer et al., 1987). These include aberrant responses to skin wounding (keloids), systemic abnormalities with associated dermal manifestations (scleroderma), and changes in dermal properties during biological aging or genetically-determined premature aging (Down's and Werner's syndromes). A thorough description of the mechanisms involved in altered matrix synthesis and assembly will contribute significantly to the understanding of the biochemical and cellular basis of these skin pathologies.

Multiple physiologic and biochemical changes occur in skin during the aging process resulting in varying deficits in its mechanical properties and biological functions (Balin and Kligman, 1989). Such physicochemical
changes in skin are associated with, and may be the direct consequence of, alterations in matrix biosynthesis (Kohn and Schnider, 1989; Perlish et al., 1989). These aging-related changes may reflect alterations in cellular proliferation or gene expression either in response to long-term environmental influences or in accord with a developmental program of gene regulation (Smith and Pereira-Smith, 1989). Cultured fibroblast populations isolated from dermis serve as an effective in vitro model for the study of adhesion processes and matrix gene expression in skin (Norwood and Smith, 1985; Schneider, 1989).

**Alterations in Fibronectin Structure and Activity During Aging**

During aging in vitro, fibroblasts exhibit changes in a variety of properties, including loss of proliferative capacity (Hayflick, 1965) and changes in cell surface properties (Maciera-Coelho, 1983). These changes may be either the cause or effect of altered cell-cell or cell-matrix adhesion processes (Hayflick, 1977). Late in vitro passage human fibroblasts exhibit reduced amounts of cell-associated fibronectin and an altered organization of fibronectin fibrils on their cell surfaces (Edick and Millis, 1984; Vogel et al., 1981; Aizawa et al., 1980) while retaining their ability to synthesize and secrete fibrinectin into the culture medium (Chandrasekhar and Millis, 1980; Vogel et al., 1981). This could be the result of altered extracellular processing and matrix assembly by aged fibroblasts, or of synthesis of qualitatively distinct fibronectin subunits, perhaps through regulated alternative splicing.

Fibronectin from the conditioned media of late passage fibroblasts was shown to have a reduced activity in cell adhesion assays compared to that
of early passage fibroblasts (Chandrakekhar and Millis, 1980; Chandrasekhar et al., 1983a). Furthermore, both early- and late-passage fibroblasts exhibited reduced adhesion and altered morphology on purified aged-cell fibronectin, while young-cell fibronectin mediated normal adhesion and morphology of both early- and late-passage fibroblasts (Chandrakekhar and Millis, 1980). The cellular fibronectin produced by late-passage fibroblasts is deficient in collagen-binding activity (Chandrakekhar et al., 1983b) and is electrophoretically and antigenically distinct from early-passage fibronectin (Porter et al., 1990; Sorrentino and Millis, 1984).

Whether these examples of structural alteration, changes in extracellular processing, and diminished biological activity in aged-cell fibronectin result from alterations in primary sequence (via alternative splicing) or post-translational modifications remains unknown. However, nonglycosylated cellular fibronectin, isolated from tunicamycin-treated fibroblasts, shows no loss of activity in adhesion assays compared to the glycosylated form (Olden et al., 1979). These studies raise the possibility that alterations in primary sequence arising from alternative splicing may be responsible for aging-related deficiencies in fibronectin function.

Reticular and papillary subpopulations of human dermal fibroblasts, aged in vitro (i.e. senescing) or in vivo (isolated from young or very old patients), have been used in this laboratory and others to examine aging-related changes in cell adhesion processes (Hall et al., 1988; Kent et al., 1986; Flickinger and Culp, 1990a; Schafer et al., 1989; Beyth and Culp, 1985)
and to extend these observations to a human genetic model of premature aging, Down's syndrome (Flickinger and Culp, 1990b). Our laboratory has demonstrated in vivo and in vitro aging-related changes in the cell biology and biochemistry of adhesion of human dermal fibroblast populations to coated substrata of fibronectin or fibronectin proteolytic fragments containing defined adhesion domains. An aging-related shift was observed in the relative importance of multiple cell surface fibronectin receptor classes responsible for fibroblast adhesion and cytoskeletal reorganization (Hall et al., 1988). These studies demonstrate the utility of these cultured cell systems in studying alterations in cell adhesion functions associated with both normal chronologic aging and premature genetic aging.

In light of previous findings of altered structure and activity of fibronectin subunits synthesized by in vitro aged fibroblasts, the effects of in vivo and in vitro aging on qualitative fibronectin expression in normal dermal fibroblasts will be explored. Use of such an in vitro skin cell system should allow for the identification of those gene regulatory mechanisms that result in altered matrix gene expression and activity.

**Experimental Procedures**

Except were specifically noted, cell culture manipulations, RNA isolations, DPE assays, and densitometry were performed as described in Chapter 2.

*Dermal Fibroblast Cell Strains and Cell Culture*

Reticular (Ret) and papillary (Pap) subpopulations of human dermal fibroblasts were isolated by Drs. Bryan Davis and Irwin Schafer, Cleveland
Metropolitan General Hospital, from several patients of ages 1 day (patient 5) and 78 years (patient 8) (Schafer et al., 1985; Schafer et al., 1989; Beyth and Culp, 1985). Primary cultures of these cell types were propagated in vitro for varying lengths of time up to senescence to serve as a model for comparing in vitro aging to in vivo aging. To examine cells exhibiting a genetic predisposition for premature aging, AG07096 and AG04823 fibroblasts from Down's syndrome patients were obtained from the National Institute of Aging Cell Repository, Camden NJ.

For the isolation of RNA from fibroblasts of increasing in vitro passage level, a single frozen stock of each fibroblast strain was used to establish the initial culture which was continuously maintained. At increasing passage levels, portions of this stock culture were used for RNA isolations. Subculturing was performed at 1:4 dilutions of cells, thus the passage number equals approximately one-half the number of population doublings. By passage numbers 18-19 (patient 8 cells) or 23-24 (patient 5 cells) (Kent et al., 1986), these fibroblast cell strains begin to exhibit the slower cell doubling times typically associated with in vitro aging and cellular senescence of normal fibroblast cell strains (Hayflick, 1965). Total cellular RNA was isolated from confluent (contact-inhibited) cell cultures at the passage levels listed. Propagation of fibroblast cell cultures and RNA isolations were performed by K. Flickinger of this laboratory.

**Densitometric Quantification**

The percentage values and error bars on the graphs in this chapter are the averages and standard deviations derived from densitometric scans of multiple autoradiographic exposures of lanes showing DPE products for
an RNA sample isolated from a single cell culture. Thus each column represents measurements on a single RNA sample.

Results

Human dermal fibroblast strains isolated from patients of different chronological age (patient 5, 1 day; patient 8, 78 years) were cultured to various in vitro passage levels, upon which time total cellular RNA was isolated from confluent cultures and assayed using the DPE protocol. Although papillary fibroblast cultures from both young and old donors exhibited sizeable differences in their IIICS mRNA variant expression patterns at various in vitro passage levels, these changes did not show any correlation with the in vitro age of the cell cultures (Figure 13). Against this background variability in IIICS expression among individual RNA samples from different cultures of the same cell strain, no differences between in vivo aged fibroblast strains from donors of different ages could be discerned (Figure 13, patient 5 versus patient 8). In addition, fibroblasts from Down’s syndrome patients and reticular fibroblast strains from patients 5 and 8 at different in vitro passage levels all exhibited IIICS expression patterns well within the ranges observed among the larger number of papillary fibroblast cultures assayed (data not shown).

Figure 13. Quantification of IIICS mRNA Variant Expression in Aging Dermal Fibroblasts. (next page) Densitometric quantification of DPE variant abundances are plotted for two human dermal papillary fibroblast cell strains (5 Pap and 8 Pap) at increasing in vitro passage number (indicated by the number after 5 Pap or 8 Pap in each column).
The IIICS expression pattern within any individual RNA sample was consistent in multiple DPE assays of the same RNA sample. Thus the observed variability in IIICS expression among individual cultures derived from the same continuously maintained stock may reflect subtle uncontrolled differences in the way in which the cells were subcultured and grown to confluence. These normal fibroblast strains exhibit longer cell doubling times and lower cell densities at confluence with increasing \textit{in vitro} age (Schafer et al., 1985) as is typical for normal human diploid fibroblasts (Hayflick, 1965). Cell cultures at later passage levels would thus require longer amounts of time after subculturing to achieve a cell density that was deemed fully confluent, and would contain a higher percentage of cells that had stopped dividing. The period required to reach confluence, the frequency of media changes (3-5 days), the time between the final media change and RNA isolation, the cell density at confluence and RNA isolation, and the amount of time the cells spent at confluence are all possible sources of the variability observed among individual cell cultures.

In an attempt to identify the possible differences in cell culture conditions responsible for the observed variability in IIICS expression among different cultures of the same fibroblast strain, one fibroblast stock was subcultured at dilutions of 1:1, 1:2, 1:4, and 1:8. These cultures were grown under identical conditions for 4 days, at which time the 1:1 and 1:2 cultures were deemed fully confluent, the 1:4 culture was approximately 70-80\% confluent, and the 1:8 culture was approximately 40\% confluent. DPE assays of the RNAs isolated from these cultures demonstrated
uniform IIICS expression patterns in the cells grown under these conditions (data not shown). Thus it appears that the cell density (subconfluent versus confluent) is not the source of the variability observed in the aging cell cultures. This does not rule out the possibility that IIICS expression patterns may be altered over longer time periods at confluence as non-dividing (contact-inhibited or quiescent) cells continue to synthesize and restructure their extracellular matrix. In addition, IIICS expression may be sensitive to changes in the growth medium as cells reach confluence during the different amounts of time between the final media change and RNA isolation.

Whatever the influence(s) may be that result in the regulation of IIICS expression within these individual fibroblast strains, the patterns observed in those changes show a striking similarity to the cell type-specific differences demonstrated in Chapter 3 (Figure 10). When comparing the relative abundances of the three predominant IIICS variants, increased O variant abundance in any given culture is linked to a compensatory reduction in AB variant abundance. In contrast the B variant levels are relatively uniform over the wide range of O and AB variant expression levels observed (Figure 14).

This demonstrates the independent regulation of the AB, B, and O variants within several individual cell strains, consistent with the model of regulated 3' splice site choice advanced in Chapter 2 (Figure 12). These results indicate that the same mechanisms by which IIICS mRNA variant expression is regulated in different cell types may also act within an individual cell type to modulate its IIICS expression, probably in response
to changes in its culture environment. These changes may either be a result of subtle inconsistencies in culture technique, or may reflect changes in fibroblast biosynthetic activity during prolonged maintenance in a confluent state.

Discussion

Fibronectins isolated from late in vitro passage human fibroblasts have been previously shown to exhibit structural and functional alterations relative to early passage cells (Porter et al., 1990; Chandrasekhar et al., 1983a; Chandrasekhar et al., 1983b; Chandrasekhar and Millis, 1980;

Figure 14. AB and B Variant Levels Relative to O Variant Expression in Aging Dermal Fibroblasts. The percentages of AB variants (●) and B variants (x) are plotted against O variant levels for each aging fibroblast cell culture shown in Figure 13. Percentage values and errors are as in Figure 13.
Sorrentino and Millis, 1984). In this study the possible role of IIICS splicing in these aging-related alterations was addressed. The IIICS expression patterns were measured in human reticular and papillary dermal fibroblasts from donors 1 day and 78 years old (in vivo aging) at different in vitro passage levels. In addition, fibroblasts from Down syndrome patients (a genetic predisposition for premature aging) were analyzed.

Individual cultures grown from the same fibroblast stock to different passage levels exhibited a sizeable heterogeneity with respect to their IIICS expression patterns (Figure 13). This was observed for both reticular and papillary fibroblast populations derived from two different donors. However these alterations in IIICS expression did not correlate either with the chronological age of the donor or the in vitro passage level of the fibroblast cultures. Reticular and papillary fibroblast cultures exhibited similar ranges of IIICS expression heterogeneity among individual cultures. In addition, Down’s syndrome fibroblasts had expression patterns within the ranges observed in normal fibroblasts. Given the significant culture-to-culture variability within these fibroblast strains, no aging-related alterations in IIICS expression could be convincingly demonstrated.

The cause of the variability in IIICS expression among individual fibroblast cultures remains unclear. Multiple DPE assays on the same RNA sample yielded identical results, thus the variability must be a result of subtle inconsistencies in culture conditions to which IIICS expression
patterns are sensitive. Attempts to identify the cause of this variability have been unsuccessful.

Whatever the cause or mechanism, dermal fibroblasts do appear to regulate their IIICS expression as evidenced by the wide range of IIICS patterns observed among individual cultures. The A+ family of mRNA variants comprised anywhere from 13% to 56% of the total fibronectin mRNA pool (Figure 13). These changes could give rise to the synthesis of fibronectins with significant differences in their expression of the CS-1 adhesion signal.

As was observed among different cell types (Figure 10), the predominant AB, B, and O variants are regulated independently within the individual fibroblast cell strains examined (Figure 14). Thus it appears that the same mechanisms that regulate IIICS expression in a cell type-specific manner also act within dermal fibroblasts to regulate IIICS expression, presumably in response to culture conditions. This regulation predominantly modulates the expression of mRNA variants that differ in their use of one of the three available alternative 3' splice sites in conjunction with the common 5' splice site.
Chapter V. Growth Factor and Hormone Regulation of IIICS Expression

Introduction

It has been demonstrated in Chapter 3 that human IIICS alternative splicing is subject to cell type- and tissue-specific control. Furthermore, results presented in Chapter 4 suggest that individual cell types may regulate their IIICS expression in response to changes in culture conditions. It is not known if IIICS splicing is subject to ongoing regulation within a single cell type in response to defined environmental influences such as growth factors and hormones.

A variety of molecules have been characterized that regulate the quantitative synthesis of fibronectin and its incorporation into the extracellular matrix. These include retinoids, glucocorticoids, transforming growth factor-β (TGF-β), and agents which modulate intracellular cyclic AMP (cAMP) levels. Several of these agents also influence cellular morphology and adhesion, and in some select cases result in the synthesis of fibronectins with altered structure or activity.

Retinoic acid (vitamin A) reduces steady-state levels of fibronectin mRNA in hepatocytes from vitamin A-deficient rats (Kim and Wolf, 1987), stimulates fibronectin protein synthesis in rabbit corneal keratocytes (Kenney et al., 1986), and reduces fibronectin protein synthesis in human keratinocytes (Varani et al., 1989). Fibronectins synthesized by vitamin A-treated chick sternal chondrocytes exhibit a slight alteration in electrophoretic mobility, but adhered to cells with the same affinity as fibronectin from untreated chondrocytes (Hassel et al., 1979). Whether
this difference in mobility is due to alterations in splicing or post-translational modifications is unknown.

The pathway of fibronectin biosynthesis appears to be affected by glucocorticoids at several levels. Treatment of rat hepatoma cells with dexamethasone (a synthetic glucocorticoid) increases the synthesis of a partially active fibronectin with altered electrophoretic mobility (Baumann and Eldredge, 1982). Additionally, it stimulates the incorporation of exogenously added fibronectin into matrix by HT-1080 fibrosarcoma cells (Furcht et al., 1979), and of endogenous fibronectin into matrix by hepatocytes (Marceau et al., 1980) in vitro, perhaps by inducing a "matrix assembly receptor" (McKeown-Longo and Etzler., 1987).

Dexamethasone treatment stimulates the synthesis of fibronectin in both tumor cells (10-fold in HT-1080 fibrosarcoma cells) and normal fibroblasts (2-fold) through an increase in the steady state levels of fibronectin mRNA (Oliver et al., 1983; Dean et al., 1988; Raghow et al., 1986). The mechanisms underlying this regulation appear to be different in individual cell types. Increases in fibronectin transcript steady state levels upon dexamethasone treatment of HT-1080 cells are accounted for by an increase in fibronectin mRNA half-life (Dean et al., 1988). In contrast, dexamethasone treatment of normal human fibroblast strains increases the transcription rate of the fibronectin gene (Dean et al., 1988). Thus multiple steps in the pathway of fibronectin mRNA biosynthesis appear to be responsive to the action of glucocorticoids in different cell types.
Blood platelets store relatively large amounts of transforming growth factor-β, suggesting that the release of this growth factor may play a role in wound healing, perhaps by stimulating the early event of matrix deposition (Massague, 1987; Massague, 1990). TGF-β stimulates synthesis of several matrix receptors of the integrin family (Ignotz and Massague, 1987; Heino et al., 1989; Ignotz et al., 1989). It also stimulates the synthesis of both fibronectin and collagen in a variety of normal and transformed cell lines (Ignotz and Massague, 1986; Ignotz et al., 1987; Dean et al., 1988) and increases the incorporation of fibronectin into matrix (Allen-Hoffmann et al., 1988). These increases in the rates of fibronectin, collagen, and integrin protein synthesis are matched by similar increases in the steady-state levels of their mRNAs (Ignotz et al., 1987; Keski-Oja et al., 1988; Varga et al., 1987; Roberts et al., 1988; Ignotz and Massague, 1987). TGF-β acts to stimulate transcription from the α2(I) collagen gene through the action of the transcription factor NF1 (Rossi et al., 1988). TGF-β also acts on the fibronectin promoter to stimulate transcription in HT-1080 cells and various fibroblast strains (Dean et al., 1988; Thompson et al., 1988).

Transcription from the fibronectin promoter is also modulated in response to intracellular levels of cAMP. Forskolin, an activator of adenylate cyclase, increases fibronectin gene transcription in HT-1080 tumor cells and normal fibroblasts (Dean et al., 1988). This transcriptional stimulation is mediated by several cAMP-response elements in the human fibronectin promoter (Dean et al., 1990; Dean et al., 1989; Bowlius et al., 1991; Dean et al., 1987). In one identified cell line, fibronectin
transcription can be repressed via the cAMP-response elements (Bernath et al., 1990).

It appears from these previous studies that steady state levels of fibronectin mRNA are subject to regulation both transcriptionally and through regulated mRNA stability. Indeed both mechanisms function within HT-1080 cells where fibronectin transcription is regulated by TGF-β and forskolin, and mRNA stability is regulated by dexamethasone (Dean et al., 1988). The influence these regulatory mechanisms may have on the differential steady state levels of multiple IIICS mRNA variants remains unknown.

In several select instances, these same agents which regulate quantitative fibronectin expression have been shown to alter the qualitative abundance of certain fibronectin mRNA variants and subunit isoforms. TGF-β treatment of normal human fibroblasts results in increased synthesis of the EDa+ subunit isoform and EDa+ and EDb+ mRNA variants (Balza et al., 1988; Borsi et al., 1990). EDa+ subunit expression is reduced in canine chondrocytes upon treatment with dibutyryl-cAMP (Burton-Wurster et al., 1988). These studies indicate that the possible regulatory influence of hormones, growth factors, and cAMP levels on the qualitative expression of fibronectin mRNA variants is worth further examination. In this study, the expression patterns of IIICS mRNA variants were measured in human cells treated with TGF-β, dexamethasone, retinoic acid, and forskolin.
Experimental Procedures

Except were specifically noted, cell culture manipulations, RNA isolations, DPE assays, and densitometry were performed as described in Chapter 2. The presentation of densitometric quantification data is as described in Chapter 4. For each graph, each column represents measurements of a single RNA sample isolated from an individual cell culture plate.

Materials and Stock Solutions

Transforming growth factor-β isolated from human platelets was obtained from R&D Systems (Minneapolis, MN). One microgram of lyophilized TGF-β was dissolved in 800μl of 4mM HCl + 1mg/ml heat-denatured bovine serum albumin for a stock concentration of 50nM. Dexamethasone, forskolin, and all-trans retinoic acid were obtained from Sigma (St. Louis, MO) and were dissolved in 100% ethanol for stock concentrations of 10^-3M, 10^-2M, and 10^-2M, respectively.

Cell Culture Treatments

Cells were plated in 100mm-diameter tissue culture dishes and allowed to grow for several days. Media was removed, cells were washed with phosphate buffered saline, and fresh media was added containing the reagents at the final concentrations listed in the figure legends. Ethanol-only controls (EtOH) or mock TGF-treated controls were treated with identical volumes of the stock solution solvent present in treated cultures (ethanol for dexamethasone, retinoic acid, and forskolin; 4mM HCl + 1mg/ml albumin for TGF-β ). At various time points, RNA was isolated by lysing cells directly in the dish with the hot-guanidinium solution.
Results

Initial Survey of Growth Factors and Hormones

Dexamethasone, forskolin, retinoic acid and TGF-β were tested for their possible effects on IIICS mRNA variant expression in HT-1080 cells (Figure 15). RNA samples from 48 hour, but not 96 hour, dexamethasone-treated cultures produced a greater amount of DPE products than control, forskolin-, and retinoid-treated cultures, reflecting the increased steady state level of fibronectin mRNA in HT-1080 cells at this time point of dexamethasone treatment (Dean et al., 1988). No quantitative effect of forskolin or TGF-β treatment was observed at either the 48 or 96 hour time points. The increase in fibronectin mRNA levels in these cells under these treatments is known to be transient, peaking at about 24 hours (Dean et al., 1988), thus the quantitative effect of these treatments may not be reflected at later time points.

Figure 15. DPE Assay of IIICS mRNA Variant Expression in HT-1080 Cells upon Various Treatments. (next page) DPE assays were performed on RNA samples from HT-1080 cells treated for 48 or 96 hours with $10^{-7}$M dexamethasone, $10^{-5}$M forskolin, $10^{-6}$M retinoic acid, or $2 \times 10^{-10}$M TGF-β. Controls for dexamethasone, forskolin, and retinoic acid were treated with ethanol (EtOH). Controls for TGF-β (mock TGFb) were treated with the solvent used in the TGF-β stock solution. Untreated cultures (NCS) received no solvent treatment. DPE products synthesized from identical amounts of sample RNA were loaded in each lane.
Expression of the O mRNA variant exhibits the greatest variation between different cell types (Figure 9) and upon different treatments (Figure 16). At 48 hours after treatment, forskolin- and retinoid-treated cultures exhibited expression levels for all IIICS variants indistinguishable from those observed in untreated and ethanol-treated control cultures (O variant levels of 13%-16% with one exception among controls). In contrast, dexamethasone treatment resulted in O variant levels of 20%-23%, an average increase of seven percentage points over control, forskolin-, and retinoid-treated cultures. TGF-β-treated cultures exhibited a similar increase in O variant levels relative to mock TGF-β controls at the 48 hour time point (20%-22% versus 12%-13%).

At the 96 hour time point, dexamethasone-treated cultures exhibited a more variable increase in O variant expression over control, forskolin-, and retinoid-treated cultures (22%-29% versus 14%-23%). TGF-β-treated cultures retained increased O variant levels at this time point relative to mock-TGF controls (22%-24% versus 13%-17%). The reason for the increased variability at 96 hours among replicate cultures is unknown.

Figure 16. Quantification of IIICS O-Variant Expression in HT-1080 Cells upon Various Treatments. (next page) Densitometric quantification of DPE O variant abundances are plotted for samples described in Figure 15. Error bars represent the standard deviations among percentage values obtained from scanning identical lanes on multiple autoradiographic exposures. (Dexamethasone, Dex; forskolin, Forsk.; retinoic acid, Ret. acid; ethanol, EtOH; untreated, NCS)
The data in Figure 16 gave indications of dexamethasone- and TGF-β-mediated alterations in IIICS mRNA variant expression. The effects of these two factors were studied more closely in a series of time course and dose-response experiments.

**Dexamethasone**

Treatment of HT-1080 cells with 10^{-7}M dexamethasone resulted in a ~4-fold increase in the steady state amount of fibronectin mRNA at 24 hours, as measured by the amount of DPE products synthesized from equal amounts of total RNA (Figure 17). This diminished to a ~2-fold effect by 48 and 72 hours. No quantitative effect was observed at lower concentrations of dexamethasone (≤10^{-9}M, approximately the K_d of the glucocorticoid receptor) at any time point. These results were consistent for duplicate or triplicate cultures treated identically. The time course, dose response, and overall magnitude of the effect are in general agreement with previous descriptions of dexamethasone effects on fibronectin mRNA levels in HT-1080 or other cells (Dean et al., 1988).

**Figure 17. Time Course and Dose Response of Dexamethasone Effects in HT-1080 Cells.** (next page) DPE assays were performed on RNA samples from HT-1080 cells treated for 24 (A), 48 (B), or 72 hours (C) with the indicated concentrations of dexamethasone. Controls were treated with a volume of ethanol (EtOH) equal to that present in dexamethasone treated cultures. DPE products synthesized from identical amounts of sample RNA were loaded in each lane.
At 24 hours, cultures treated with $10^{-7}$M dexamethasone exhibited a very slight increase in O variant abundance relative to controls (Figure 18; 10%-13% versus 7%-8%), although variability was observed among replicate cultures. This effect continued through 48 hours, and became indiscernible by 72 hours. Cultures treated with lower concentrations of dexamethasone at all time points exhibited O mRNA percentages in the range observed in control cultures, with one exception. No alterations in the expression of other IIICS mRNA variants were observed.

The basal level of O variant expression among untreated cells in this experiment was approximately one-half that observed in the experiment presented in Figure 16 (7%-8% versus 13%-16%). Thus the ability to convincingly demonstrate a dexamethasone effect may be facilitated in cell types or under conditions exhibiting a higher basal level of O variant expression. Under such conditions, differences in O variant levels between treated and control cultures may become significantly larger than the variance observed in these experiments among replicate cultures.

**Figure 18. Quantification of Dexamethasone Time Course and Dose Response Data.** (next page) Densitometric quantification was performed on DPE assay autoradiograms (Figure 17) for HT-1080 cells treated with the indicated concentrations of dexamethasone (no Dex: ethanol-treated control). O variant percentages are plotted for RNA samples from each individual culture. Error bars reflect the variance among data derived from scans of multiple exposures of each sample lane. Where no error bar is shown, data from the scan of a single exposure is presented.
Transforming Growth Factor-β

Treatment of HT-1080 cells with 200pM TGF-β resulted in a ~5-fold increase in steady state fibronectin mRNA levels at 24 hours (data not shown). No increase in DPE product amounts were observed between control and TGF-β-treated cultures at 48 and 72 hours, consistent with previous results for HT-1080 and other cells (Dean et al., 1988). Expression levels for the O variant (and other IIICS mRNA variants) were comparable for all concentrations of TGF-β and mock-treated controls (Figure 20). Thus the small increase in O variant expression upon TGF-β treatment described in Figure 16 could not be confirmed.

Figure 19. Quantification of TGF-β Time Course and Dose Response Data. (next page) Densitometric quantification was performed on DPE assay autoradiograms for HT-1080 cells treated with the indicated concentrations of TGF-β for 24, 48, or 72 hours. Data are presented as described in Figure 18.
Discussion

Steady state fibronectin mRNA levels in HT-1080 cells are greatly increased by dexamethasone, forskolin, and TGF-β through multiple mechanisms. The magnitude of these responses is significantly larger in HT-1080 cells than in normal fibroblasts where the basal fibronectin mRNA levels are much greater. In this study, the possible effect of these reagents on IIICS expression was examined.

HT-1080 cells were chosen for this study because much is known about the mechanisms by which steady state fibronectin mRNA levels are regulated in these cells (Dean et al., 1988). Transcriptional activity at the fibronectin promoter is enhanced by treatment with TGF-β or forskolin. Alternatively a two-fold increase in fibronectin mRNA stability is observed in dexamethasone-treated cells. The analysis of the possible gene regulatory mechanisms involved in regulating IIICS mRNA variant expression levels could be facilitated by using a cell line in which either the rate of fibronectin mRNA synthesis or decay can be experimentally manipulated.

Treatment of HT-1080 cells with forskolin or retinoic acid did not produce observable alterations in IIICS mRNA variant expression patterns (Figures 15 and 16). A modest increase in O mRNA variant expression was observed in TGF-β-treated cells in one experiment (Figure 16), although this observation could not be confirmed upon repeating the experiment (Figure 19). It is possible that the modest TGF-β-induced alterations in IIICS expression patterns in HT-1080 cells may be more dramatic in other cell types in which the basal O variant expression is
greater and subject to more sizeable modulations, such as in human dermal fibroblasts (Chapter 4, Figure 13).

Dexamethasone treatment of HT-1080 cells produced a small, reproducible increase in the relative expression of the O mRNA variant (Figures 16 and 18). This effect was observed at $10^{-7}$M dexamethasone, but not at $10^{-9}$M dexamethasone. The magnitude of this increase decayed with time, as did the increase in the total amount of fibronectin mRNA. It remains unclear whether the increase in O variant levels is linked mechanistically to the dexamethasone-induced stabilization of fibronectin mRNA previously described in these cells (Dean et al., 1988). The increase in O variant expression was greatest in those cultures which exhibited the highest steady state fibronectin mRNA levels. This correlation between the maximal time points for both the quantitative effect on steady state levels and the qualitative effect on O variant expression may suggest that the IIICS mRNA variants may have differential mRNA stabilities. This hypothesis would require further testing in an experimental system in which the decay rates of different IIICS mRNAs could be independently measured.

At best, dexamethasone increases the relative expression of the O mRNA variant by HT-1080 cells in culture by about 10% or less. Given the overwhelming predominance of AB and ABC variants encoding the CS-1 adhesion signal among both control and dexamethasone-treated cells, it is difficult to envision a significant alteration in fibronectin activity resulting from such an increase in IIICS- subunit synthesis. However, constitutive stimulation of different cell types in vivo may effect subunit expression
patterns over long periods of time, thus synthesizing matrices of altered subunit composition and adhesive activity.

In order for factor-induced changes in IIICS splicing to be evident under experimental conditions involving treatment over several hours or a few days, the changes in IIICS splicing patterns would have to be dramatic enough to produce observable shifts in the steady state IIICS variant levels. Thus newly-synthesized and spliced transcripts would have to accumulate in large enough amounts to be observed in the DPE assay against a background of transcripts formed before the experimental treatment. This is complicated by the fact that some cells (particularly dermal fibroblasts) can possess large steady state pools of mature fibronectin mRNAs that are highly stable (half-life on the order of several days in fibroblasts). Further studies on factor-induced changes in IIICS alternative splicing would benefit from the use of a plasmid-encoded IIICS pre-mRNA splicing substrate. This would allow one to monitor the splicing of newly-synthesized fibronectin transcripts under different experimental conditions.
Chapter VI. Expression of a IIICS Mini-Gene Splicing Substrate

Introduction

From the observed patterns of cell type-specific changes in IIICS mRNA variant levels it was hypothesized that regulated selection among the three alternative 3’ splice acceptor sites within the IIICS plays a fundamental role in establishing IIICS expression patterns (Figure 12 in Chapter 3). This hypothesis requires further testing in an experimental system allowing controlled disruption of regulatory sequences in the pre-mRNA. This will allow for elucidation of the sequences responsible for this regulation, and the mechanisms by which IIICS splicing processes are altered in a cell type- or tissue-specific manner or in response to environmental influences.

Several plasmid-borne fibronectin IIICS mini-gene constructs were assembled that will permit the examination of newly-synthesized and processed fibronectin IIICS transcripts after transfection into human cells exhibiting distinct patterns of IIICS steady state expression. If the introduced mini-gene transcripts exhibit cell type-specific changes in splicing patterns analogous to those seen among endogenous fibronectin mRNAs, it would indicate that cell-specific changes in the expression, abundance, or activity of trans-acting factors acting on the splicing apparatus regulate IIICS alternative splicing.

Alterations in the IIICS splicing patterns for newly-synthesized IIICS pre-mRNAs containing defined introduced mutations should provide valuable insights into the molecular mechanisms and components involved in regulated alternative splicing. Furthermore, transfection of
wild-type constructs into cells treated with dexamethasone, TGF-β, or other regulatory factors will permit the analysis of mini-gene transcripts synthesized in the presence of these factors separate from those transcripts transcribed from the endogenous fibronectin gene both before and after factor addition.

**Experimental Procedures**

Except were specifically noted, all standard molecular biology techniques were performed using established protocols (Maniatis et al., 1982; Sambrook et al., 1989; Ausubel et al., 1987). Synthesis of primers, cell culture manipulations, RNA isolations, and DPE assays were performed as described in Chapter 2.

**PCR Primer Design**

Polymerase chain reaction (PCR) oligonucleotide primer IIICS-5’PBEH (5’-CTGCAGGATCCGAATTCAAGCTTGGAAACGGGACCGAATATAC-3’) contains fibronectin sense sequence from the 5’ end of the exon preceding the IIICS exon, preceded by sequences encoding PstI, BamHI, EcoRI, and HindIII restriction sites (Figure A1). Primer IIICS-3’XXN (5’-GCGGCCGCTCTAGACTGAGATGTTTGTCTGAGAGAGACCTTC-3’) contains sequence complimentary to fibronectin sense sequence at a site in the constitutive portion of the IIICS exon internal to the IIICS-3’ primer site, and contains XhoI, XbaI, and NotI restriction sites.

**PCR Amplification of Human IIICS Genomic Sequences**

Human genomic DNA was isolated from a subclone of A549 adenocarcinoma cells (TE238; DNA provided by the laboratory of Dr. David Goldthwaite, CWRU Department of Biochemistry). Bulk PCR
reactions were performed at the following component concentrations: 2µg/ml genomic DNA, 50nM each of primers IIICS-5'PBEH and IIICS-3'XXN, 500µM each dNTP, 10mM Tris-HCl (pH 8.3), 50mM KCl, 50µg/ml bovine serum albumin, 2.5mM MgCl₂, and 20 units/ml Taq DNA polymerase (Perkin Elmer Cetus). The bulk PCR reaction mix (4ml) was aliquoted at 500µl per amplification reaction tube, overlaided with mineral oil, and amplified 50 cycles (95°C for 1 minute, 50°C for 2 minutes, and 72°C for 3 minutes with no cycle extensions) in a Perkins Elmer Cetus Thermal Cycler. Samples were pooled, extracted once with phenol-chloroform, precipitated from 70% ethanol, and redissolved in water. The identity of the single ~1500bp PCR product was confirmed to be a fibronectin-specific amplification product by restriction analysis with BgIII and BstEII (sites within the IIICS exon), and its size was identical to the same PCR product synthesized from HT-1080 cell DNA.

**Cloning: Creation of phIIICSg Expression Plasmids**

The PCR product was cleaved within the primer sequences by treatment with restriction enzymes HindIII (5' end) and XbaI (3' end). The fragment DNA was resolved and eluted from a 1% agarose gel using the NaI-glass bead protocol (USBioClean kit, United States Biochemicals). The correct structure of the restricted ends of the PCR fragment was confirmed with a self-ligation reaction, generating a ladder of fragment concatamers.

The phIIICSg family of plasmids were generated by ligating the PCR insert separately into HindIII+XbaI-cleaved vectors pRc-RSV, pRc-CMV (Invitrogen), and pECE, creating plasmids pRSV-hIIICSg, pCMV-hIIICSg, and pSV-hIIICSg, respectively. After overnight ligation, products
resulting from the self-ligation of vector DNA (re-insertion of the multiple cloning site) were rendered non-transforming by treatment with restriction enzymes SpeI (pRc-RSV) or NotI (pRc-CMV) which cleave within the vectors multiple cloning site between the HindIII and XbaI sites. These enzymes do not cleave the PCR product.

Treated ligation products were transformed into competent *E. coli* DH5α cells (Bethesda Research Laboratories), and plasmid DNA was isolated from cultures grown from resulting ampicillin-resistant colonies. The desired recombinant clones were identified by restriction analysis based on known vector and human fibronectin cDNA sequence.

Simultaneous with the generation of the IIICS-containing expression plasmids, a HindIII-XbaI fragment bearing the *E. coli* β-galactosidase gene (*lacZ*; provided by Wen-chang Lin of this laboratory) was cloned into each vector, creating plasmids pRSV-lacZ, pCMV-lacZ, and pSV-lacZ. Staining of human cells co-transfected with these *lacZ*-expressing plasmids with the chromogenic substrate X-gal generates a blue color in the cytoplasm (Lin et al., 1990), serving as a marker for transfection efficiencies and proper plasmid-based expression from these vectors.

**Transfection of Cultured Cells**

Transfections of plasmids into human cells (100mm plates) were performed using the CaPO₄-precipitation method (ProFection Kit from Promega) or the Lipofectin reagent (Bethesda Research Laboratories) in accordance with the manufacturers instructions. The CaPO₄-precipitant or the Lipofection solution (containing 20μg each of pHIIICSg plasmid and placZ plasmid) was left on the cells for 6 or 18 hours before the media was
changed. Cells were stained 48 or 72 hours after transfection and RNA was isolated from cultures 72 hours after transfection.

**DPE Assays of Endogenous and Mini-Gene Transcripts**

Oligonucleotide pRc-3' (5'-GGCACAGTCGAGGCTGATCAGCG-3') complementary to transcribed vector sequences 3' of the fibronectin-vector cloning junction was synthesized for use in DPE assays for selective reverse-transcription of mini-gene transcripts. Primer IIICS-3' (which lies outside the PCR amplified mini-gene sequence) was used for reverse-transcription of endogenous fibronectin transcripts. Oligonucleotide IIICS-5' (5'-GCTTGGAAACCGGAACCGAATATAC-3') (Figure A1) was used as the radiolabeled second-strand primer for DPE assays of both endogenous and mini-gene transcripts.

**Results**

Using the polymerase chain reaction (PCR), the IIICS region of the human fibronectin gene was amplified from human genomic DNA with oligonucleotide primers specific for exon sequences flanking the alternatively spliced IIICS region. A ~1.5 kilobase amplification product was observed in PCR reactions from A549 adenocarcinoma cell and HT-1080 fibrosarcoma cell genomic DNAs, suggesting no gross alterations in fibronectin gene structure in these cells.

Blunt-end cloning of PCR products proved problematic, as Taq DNA polymerase adds non-templated nucleotide extensions onto the ends of synthesized strands, resulting in 3' overhangs. This difficulty was circumvented through the addition of restriction site sequences (those found in the vector multiple cloning sites) onto the 5' ends of the PCR
primers, allowing directional ligation of restricted PCR fragments into vectors. Using this approach, the IIICS genomic PCR product was cloned into several vectors which drive the transcription of insert sequences from different eukaryotic promoters. In mammalian cells, the pRSV-hIIICSg, pCMV-hIIICSg, and pSV-hIIICSg plasmids drive transcription of IIICS insert sequences from the vector-encoded Rous sarcoma virus LTR promoter, the human cytomegalovirus immediate-early promoter, or the simian virus-40 early promoter, respectively (Figure 20).

Plasmid pRSV-hIIICSg or pCMV-hIIICSg were introduced into human cells, co-transfected with pRSV-lacZ or pCMV-lacZ, respectively. Transfection of either plasmid pair into HT-1080 cells using the Lipofectin reagent under a variety of transfection conditions yielded very few blue cells after X-gal staining (∼1 in 10^4 cells). Improved transfection efficiencies were obtained for HT-1080 cells using the CaPO4-precipitation protocol (∼1 in 10^2-10^3). Neither protocol yielded any blue-stained transfected cells among human dermal fibroblasts.

**Figure 20. Construction of pIIICSg Expression Plasmids. (next page)** (A) *Plasmid construction.* Fibronectin sequences are represented by black (constitutive) and stippled boxes (alternative spliced) with splice sites, subsegments, and primer restriction sites noted. Vector sequences transcribed in mammalian cells are represented by white boxes. (B) *Structure of exogenous and endogenous transcripts.* The locations of primers used in PCR amplification and selective DPE assays are shown by thin and thick short black lines, respectively.
RNAs isolated from co-transfected HT-1080 cells or dermal fibroblasts were assayed using the DPE protocol. IIICS mRNA variants arising from endogenous fibronectin gene transcripts were identified using the oligonucleotide IIICS-3' as the primer for first-strand synthesis. Because the primer for PCR amplification of the phIIICSg plasmid (IIICS-3'XXN) insert lies 5' of IIICS-3', the binding site for IIICS-3' is not present on plasmid-encoded transcripts (Figure 20). IIICS expression patterns among endogenous fibronectin transcripts in HT-1080 cells and fibroblasts were identical in transfected and non-transfected cultures and were those expected for these cell types (data not shown).

The DPE assay was performed on the same sample RNAs using oligonucleotide pRc-3' as a primer for first-strand synthesis. This primer is complementary to downstream vector sequences expressed on the same transcript as either IIICS or lacZ insert sequences. Second-strand synthesis using the fibronectin-specific primer IIICS-5'* ensures the exclusive synthesis of DPE products from phIIICSg transcripts. No plasmid-encoded IIICS transcripts were identified in RNA samples from multiple transfected cultures (data not shown).

Discussion

The inability to detect phIIICSg-encoded transcripts in transfected cells may have several possible causes. Transfection efficiencies were generally rather low (1.0-0.1%) as measured by X-gal staining for placZ expression. Although transcripts encoded by the placZ plasmids are expressed in these cells, as evidenced by positive staining for β-galactosidase activity, the mRNA abundance of placZ and phIIICSg transcripts is unknown. In
addition, the stability of the short IIICS transcript in these cells is a concern, particularly since it lacks a translation initiation codon, hence it is not protected from degradation by translating ribosomes covering the transcript. Lastly, the sensitivity of the DPE assay, relying on an end-labeled primer for visualization of products, may need to be improved (extension reactions in the presence of $^{32}$P-labeled dNTPs) so that the transcripts synthesized in the small fraction of successfully transfected cells can be detected.

Several avenues of investigation will become available upon the successful expression of a IIICS pre-mRNA splicing substrate in human cells. Individual consensus 3' splice acceptor sites and branchpoint sequences in the IIICS sequence can be altered using site-directed mutagenesis, creating a series of mutant IIICS mini-gene constructs that can be used to test the model of regulated 3' splice site choice advanced in Chapter 3. In addition, transfection of IIICS pre-mRNA-expressing plasmids simultaneous with treatment with growth factors or hormones would permit the examination of the splicing of newly-synthesized pre-mRNAs, removing the contribution of the stable pre-spliced fibronectin mRNAs present in cells prior to experimental manipulation.
Chapter VII. Summary

This work involved the examination of the regulation of expression of alternatively-spliced human fibronectin IIICS mRNA variants. At the time these studies were initiated, our understanding of the IIICS region of the fibronectin molecule was limited to knowledge of the locations of multiple splice sites within rat and human IIICS pre-mRNAs that potentiate alternative splicing of fibronectin transcripts. Since that time, many investigators have dramatically advanced our understanding of the functional significance of the IIICS polypeptide sequence as a distinct cell adhesion domain, and our appreciation of the possible importance of IIICS alternative splicing as a modulator of fibronectin adhesive activity. Through these discoveries a convergence of interest in matrix-mediated adhesion processes has drawn together researchers in the fields of cell biology, molecular biology, immunology, dermatology, and tumor biology. Current work in many of these fields centers on the roles of multiple members of the integrin family of matrix receptors, including the \( \alpha 4 \beta 1 \) IIICS-receptor, in regulating a host of physiological processes.

Despite our greater understanding of the functional importance of the IIICS region, relatively little is known in any detail about how the process of IIICS alternative splicing is regulated, in response to what influences this regulation occurs, and in what contexts regulated alternative splicing results in modulation of cell-cell or cell-matrix adhesion events with physiologically important consequences. It was with an interest in addressing these concerns that this work was undertaken.
Development of DPE Assay

Two assay systems were developed for the accurate measurement of human IIICS mRNA variant expression within experimental RNA samples (Chapter 2). Using a novel double primer extension assay, all five human IIICS mRNAs could be simultaneously identified and their the relative abundances quantified. Using a more sensitive modification of this assay, the low-level expression of the hypothesized but previously-unidentified BC variant was established.

Cell Type-Specific Regulation of IIICS Variant Expression

In a survey of a variety of human normal, tumor, and transformed cell types, IIICS mRNA variants were shown to exhibit a wide range of steady state expression patterns (Chapter 3). Expression of the O variant, encoding IIICS-polypeptide subunits, exhibited the greatest variability among different cell types. However, differences in the relative expression of several other IIICS variants indicate that IIICS alternative splicing may be regulated through multiple mechanisms. Coordinate changes in the levels of certain pairs of IIICS mRNA variants in different cells suggest that regulated utilization of the three alternative 3' splice acceptor sites may account for much of the cell type-specific regulation observed.

These studies demonstrated that the A+ mRNA variants encoding the CS-1 adhesion site are in the majority in all cultured cells tested, but constitute a minority of transcripts in human liver tissue. This may have important implications for the specialized roles of cellular fibronectins versus plasma fibronectin.
IIICS Variant Expression During In Vitro and In Vivo Aging Processes

The possible alteration of IIICS expression patterns during cellular aging processes was examined in populations of human dermal fibroblasts. These cells exhibited a wide range of IIICS expression patterns, however these changes did not correlate with either the in vivo or in vitro age of the cell cultures. These results suggest that dermal fibroblasts regulate their IIICS expression in response to environmental cues or physiological changes during culture. The cause or mechanism underlying this regulation is yet to be identified.

Regulation of IIICS Variant Expression by Extracellular Factors

Several growth factors, hormones, and other reagents are known to influence quantitative fibronectin expression through changes in the synthesis or degradation of fibronectin mRNAs. The effects of these agents on qualitative fibronectin expression was tested. Sizeable increases in the steady state amounts of fibronectin were observed in HT-1080 cells upon treatment with dexamethasone or TGF-β. These changes were matched by very modest increases in the relative abundance of the O variant upon dexamethasone treatment only.

A Substrate for IIICS Alternative Splicing

A family of IIICS mini-gene constructs were assembled for use in studies requiring the introduction of a IIICS pre-mRNA substrate into mammalian cells. The IIICS-encoding region of the human fibronectin gene was amplified using the polymerase chain reaction. This DNA fragment was cloned into several plasmid vectors such that transcription
of IIICS pre-mRNA sequences (IIICS exon and preceding intron and exon) is directed from various plasmid-borne eukaryotic promoters.

Conclusion

Although the cell biology of adhesion to IIICS polypeptide sequences remains a hotly investigated topic, and studies on the alternative splicing of extra domain (ED) exons are well represented in the literature, little progress has been made in understanding the molecular biology of IIICS alternative splicing and its regulation. This may be a consequence of the splicing complexity in the human IIICS exon and hence the difficulties encountered in its analysis. It is hoped that through the studies described in this work, I have contributed some insights into the molecular biology of this interesting exon, and have provided future investigators the tools necessary for its further study.

Portions of this work were previously published in the journal *Molecular and Cellular Biology* (Hershberger and Culp, 1990)
Figure A1. IIICS Sequence. (next page) The DNA sequences of the
human IIICS exon (B) and its preceding exon (A) are shown with their
encoded amino acids and (where known) neighboring intron sequences
(italicized letters). Sequence is listed 5' to 3' for the sense strand. Squares
indicate 5' splice donor sites and diamonds denote 3' splice acceptor sites
(black if an alternative site). Ns designate undetermined lengths of
unsequenced nucleotides. Underlined nucleotides match consensus
splice site sequences (Mount, 1982). Selected restriction enzyme
recognition sites are labeled. Black arrows denote the locations and
orientations (arrows pointing 5' to 3') of the fibronectin-specific sequence
contained in various DPE and PCR oligonucleotide primers.
A. 

1. TACCTGGN........NGCCCTGGAACCGGAAACCGATAGCTATTGTACATTGCC

   \[\text{LeuGluProGlyThrGluTyrThrIleTyrValIleAla}\]

   \(\text{oligo-5'}\) & \(\text{oligo-5'}^{*}\) & \(\text{oligo-5'}^{\text{PBEH}}\)

64. CTGAGAACATATAACGAGGACGGGACCCCTTGAGGGAAGAGACACAGNNNNNNNNNN

14. \[\text{LeuLysAsnAsnGlnLysSerGluProLeuIleGlyArgLysLysThr}\]

B. 

1. NNCTCTCTTGCTGAGACTCAGCTTCCCTCCAACACTGTAACCCCTCCAACACCTCATGGA

   \[\text{AspGluLeuProGlnLeuValThrLeuProHisProAsnLeuHisGly}\]

   \(\text{BglII}\)

64. CCAGAGACATTTGAGGTCTCTCAGGTCTAGCAACACCAGTTGTGGGCAACAA

17. \[\text{ProGluIleLeuAspValProSerThrValGlnLysThrProPheValThrHisProGlyTyr}\]

127. GACACTGGAAAAGTTAGTTTACACTCTGCTAGCAACACCCGACTTGTGGGCAACAA

38. \[\text{AspThrGlyAsnGlyIleGlnLeuProGlyThrSerGlyGlnGlnProSerValGlyGlnGln}\]

190. ATGATCTTTGGAGAACATGGTTTCTGACCGGACCACAGGCGGGACACCCAGGGCCACCATAGGG

59. \[\text{MetIlePheGluGluHisGlyPheArgArgThrThrProProThrThrAlaThrProIleArg}\]

253. CATAGGGCAAGCCATACCCGCCAGTATGATGAGGGAATCCAAATTTGCTCAGTTCCAGGG

80. \[\text{HisArgProArgProTyrProProAsnValGlyGluIleGlnIleGlyHisIleProArg}\]

   \(\text{Accl}\)

316. GAAGATGTTAAGACTATACCTGTACCCACACGGGCTCGGCTCAATCCAAATGCTCTTGAGGA

101. \[\text{GluAspValAspTyrHisLeuTyrProHisGlyProGlyLeuAsnProAsnAlaSerThrGly}\]

   \(\text{DdeI}\)

379. CAAGAAGCTCCTCTCATGAGACACCATCTCATGGCCATCCAGGACACTCTGTGAGTACAC

122. \[\text{GlnGluAlaLeuSerGlnThrIleSerTrpAlaProPheGlnAspThrSerTyrIle}\]

   \(\text{oligo-3'XXN}\)

442. ATTTGCATGTCATCGTGGGACTGTGAAGAACACCTCTACAGGTAATTAATTTGCTCTTCAC

143. \[\text{IleSerCysHisProValGlyThrAspGluProLeuGln}\]

505. TTCTCATGGGG
Literature Cited


