Tissue-specific Splicing Pattern of Fibronectin Messenger RNA Precursor during Development and Aging in Rat

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Abstract. Fibronectin isoforms are generated by the alternative splicing of a primary transcript derived from a single gene. In rat at least three regions of the molecule are involved: EIIIA, EIIIB, and V. This study investigated the splicing patterns of these regions during development and aging, by means of ribonuclease protection analysis. Between fetal and adult rat, the extent of inclusion of the EIIIA and/or EIIIB region in fibronectin mRNA varied according to the type of tissue analyzed; but the inclusion of the V region, and in particular the V25 alternative variant, was significantly higher in all fetal than in adult tissues. These data suggest a crucial role of the V25 variant, possibly related to its interaction with the α4β1 integrin receptor during development. On the other hand, during aging, the only significant change observed in the splicing pattern was a decrease in the EIIIA variant in brain. The high inclusion levels of the EIIIA and EIIIB regions in young adult brain suggest that these segments may play an important role in differentiated brain tissue. The decreasing levels of inclusion of the EIIIA segment in brain fibronectin mRNA during aging may be an age-related marker with functional consequences.

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1. Abbreviation used in this paper: FN, fibronectin.
associated with the synthetic or modulated phenotype of smooth muscle cells (SMCs) (16). This phenotype is observed with aging (when SMCs migrate to the progressively thickening intima, where they proliferate and secrete extracellular matrix components), as well as in atherosclerotic lesions (16). Recently, the cell type-specific fashion of the alternative splicing of rat and human FN gene transcript has been shown to be a subject for modulation during transformation both in cultured cells and in tumors (1, 2, 5, 6). To understand the expression and regulation of FN variants in vivo, the pattern of FN pre-mRNA splicing of the EIIIA, EIIIB, and V regions in different rat tissues was evaluated during both development (from fetus to 5 mo of age) and aging (from 5 to 24 mo of age).

**Materials and Methods**

**Animals and RNA Preparation**

All of the animals were housed in specific pathogen-free facilities and fed ad lib. on a standard rat chow diet at the Fidia Research Laboratory (Abano Terme, Italy). Sprague-Dawley male rats of the following ages were used: 17 d (fetuses; El7), and 5, 12, 17 and 24 mo. Three of each age animals were used, with the exception of El7 and 17-mo-old rats, for which only two animals were studied. Rats were decapitated and their organs were immediately frozen in liquid nitrogen. Brain, liver, kidney, lung, and heart were obtained from rats and brain, liver, kidney, and lung from fetus. Total RNA was prepared from 0.5-2 g of frozen tissues homogenized in guanidinium thiocyanate and ultracentrifuged in cesium-chloride gradient (7).

**Probe Preparation**

Three sets of oligonucleotide DNA primers complementary to the published sequence of rat fibronectin (29, 40, 45) were synthesized using a DNA synthesizer (model 3080A; Applied Biosystems; Foster City, CA). The sequences appear below.

EIIIA1 5'-TCA GAA CCG GAA CCG AGA AA-3'
EIIIA2 5'-ACA AGC TTC ACG GAG GTG CTG TCT GGA GAA A-3'
EIIIB1 5'-TAT CTA GAG TCA TCC TAC CAG TGC CCC A-3'
EIIIB2 5'-TAC TGC AGT CCA GAC GTG TGT TTT GAC G-3'
VAR1 5'-ATA CTG CAG ACC ATC TAT GTC ATC GCA CTG AA-3'
VAR2 5'-ACA AGC TTA GCC TTC TCC TAC GTC GCC AA-3'

These primers spanned the EIIIA, EIIIB, and V regions of FN mRNA plus a 3' 171 bp and 5' 8 bp for EIIIA, 3' 285 bp and 5' 12 bp for EIIIB, and 3' 102 bp and 5' 67 bp for V. EIIIA1, EIIIA2, EIIIB1, EIIIB2, VAR1, and VAR2 primers were modified by the addition of noncomplementary bases at the 3' end to create artificial recognition sites for restriction enzymes. A specific first-strand cDNA copy of the three rat FN regions was made by using one of the two oligonucleotide primers in each set that was complementary to the RNA strand and that served as a primer for reverse transcriptase. The synthesis of cDNA was carried out in a 50-μl reaction volume using Murine Leukemia Virus reverse transcriptase (Bethesda Research Laboratories, Gaithersburg, MD), used under the conditions suggested by the supplier with the omission of dactinomycin. Amplification of these first-strand cDNA was performed by means of TAE polymerase (Perkin-Elmer Cetus Corp., Norwalk, CT) according to Saiki et al. (38). 10 μl of the first-strand cDNA mixture was included in a 100-μl volume with 50 pmol of each corresponding set of primers and 5 U of TAE polymerase. 40 cycles of polymerase chain reaction (PCR) amplification were carried out, each consisting of 1 min at 93°C, 30 s at 48°C, and 3 min at 70°C. After DNA amplification, the PCR mixture was digested with 5 μl of ribonuclease A at 37°C for 30 min and then analyzed by ethidium bromide on agarose gels. The amplified fragments corresponding to the EIIIA, EIIIB, and V inclusions (497, 572, and 529 bases for V) and the 3' bases from pTZ19R vector. 10 1000 copies of single-strand RNA probe were hybridized overnight at 51°C with 20–40 μg of total RNA in 40 μl of 80% formamide, 20 mm Tris pH 7.4, 400 mM NaCl, 1 mM EDTA and 0.1% SDS. The RNA/RNA hybrids were then digested in 350 μl of RNase solution (300 μm NaCl, 5 mM EDTA, 10 mM Tris pH 7.4, 40 μg/ml RNase A, 3 μg/ml RNase T1) and incubated at 37°C for 75 min.

After the addition of 30 μg of proteinase K and 20 μl of SDS 10%, the RNAs were incubated at 37°C for 15 min, extracted with phenol and then EtOH precipitated. The protected fragments were analyzed on 4% denaturing polyacrylamide gels and by subsequent autoradiography with preflashed Fuji x-ray film. In some experiments, an artifact band that was slightly smaller than the intact probe was observed. This band also appeared in the absence of cellular RNA and must derive from probe sequences that are resistant to the ribonucleases under our experimental conditions. For densitometric analysis, only signals in the linear range of film sensitivity were used.

The relative radioactivity of the ribonuclease-protected fragments was determined by means of a Zeiss Kontron Image Analyzer IBAS 2000 (Oberkochen, Germany) and expressed as a ratio between the radioactivities of the upper and lower bands. Because different amounts of radiolabeled nucleotides are incorporated according to the length of the protected fragments, this ratio is not canonical, but it is an indicator of the fluctuations of various mRNA concentrations.

**Results**

**Expression of the EIIIA Region during Development and Aging**

Alternative splicing at the EIIIA region generates two types of FN mRNA that differ in terms of the presence of the EIIIA sequence. Fig. 1 schematically shows the two FN mRNA variants, EIIIA+ and EIIIA−, and the sequences covered by the probe used to study the splicing pattern of EIIIA region by means of ribonuclease protection analysis.

Total RNAs prepared from various rat tissues at El7 and at the different months of age were analyzed by ribonuclease protection assay using the EIIIA probe. Hybridization of the RNA probe with FN mRNAs (with and without the EIIIA sequence), followed by ribonuclease digestion, yielded a 456-base and a 171-base fragment, respectively (Figs. 1, 2, and 3). Samples of the experimental results are shown in Figs. 2 and 3. Table I summarizes the EIIIA+/EIIIA−-protected fragment ratio obtained by means of densitometric analysis of the autoradiograms representing the average of three independent experiments.

During development, the highest EIIIA+/EIIIA− ratio was found in kidney with a marked decrease from 19.80 to 0.57 between El7 and 5 mo of age (Fig. 2, Table I). In the same tissue, between 5 and 24 mo of age, the EIIIA+/EIIIA− ratio remained constant at ~0.5 (Table I).

In adult liver, the EIIIA inclusion levels were very low but still detectable; in fetal liver it was clearly evident (Fig. 2). The EIIIA+/EIIIA− ratio changed from 0.14 to 0.01 between El7 and 5 mo of age (Table I). During aging, the EIIIA+/EIIIA− ratio in liver tissue remained low (Table I).

On the contrary, lung and brain tissues appeared to be relatively stable during ontogeny (Fig. 2). The EIIIA+/EIIIA−...
Figure 1. (A) Schematic map of the rat fibronectin molecule. Type I, II, and III repeats are shown as small rectangles, ovals, and large rectangles, respectively. Binding sites are indicated. Alternative spliced exons are shown in black. (B) Schematic representation of the three RNA probes used in RNase protection experiments and the putative fragments protected with the FN mRNA variants. The RNA probes and their protected putative fragments are shown as shaded boxes and their length is indicated. The RNA probes contain extra nucleotides derived from the cloning vector at its 3' end (indicated by a wavy line).

Expression of the EIIIB Region during Development and Aging

Alternative splicing at the EIIIB region generates two types of FN mRNA that differ in term of the presence of the EIIIB sequence (Fig. 1). Total RNAs prepared from various rat tissues at E17 and at the different months of age were analyzed by ribonuclease protection assay using the EIIIB probe. Two protected fragments were detected: a 572- and a 285-base fragment, respectively corresponding to EIIIB+ and EIIIB−. Samples of the experimental results are shown in Fig. 4, and Table I summarizes the EIIIB+/EIIIB− protected fragment ratio obtained by means of densitometric analysis of the autoradiograms representing the average of two independent experiments.

During development, the EIIIB+/EIIIB− ratio in liver, kidney and lung tissues showed a marked decrease from fetus to 5 mo of age. At 5 mo of age, EIIIB inclusion was undetectable in liver and lung (even when the autoradiographic exposure time was increased) and was present at low levels in kidney. On the other hand, the EIIIB region in brain tissue was less regulated during development. In fact, in this tissue, the EIIIB+/EIIIB− ratio was 1.80 at E17 and 0.47 at 5 mo of age.

During aging, no significant changes in the EIIIB+/EIIIB− ratio were evident in any of the tissues analyzed.

Expression of the V Region during Development and Aging

The V region shows a more complex pattern of alternative splicing than either EIIIA or EIIIB and three different forms can be found in rat. Fig. 1 schematically shows the three FN mRNA variants (V1, V2, and V3) and the sequences covered by the probe used to study the splicing pattern of the V region by ribonuclease protection analysis.

Hybridization of the RNA probe with the FN mRNAs in the three different forms, followed by ribonuclease digestion, yielded a 529-, a 387-, and a 102-base fragment, respectively corresponding to V1, V2, and V3 (Fig. 1). The low intensity of the V3 protected band in most tissues required longer exposures before it could be visualized. This led to the linear
range of film sensitivity of the higher V1 and V2 bands being lost. Hence, in this study only the V1 and V2 bands were considered for densitometric analysis.

Total RNAs, prepared from the various rat tissues at El7 and at the different months of age, were analyzed by ribonuclease protection assay using the V probe. Fig. 5 shows the different splicing patterns of this variable region in the different tissues between El7 and 5 mo of age. Table I summarizes the V1/V2 mRNA ratio revealed by means of densitometric analysis of the autoradiograms obtained from ribonuclease protection assays, representing the average of three independent experiments.

All of the tissues examined showed a marked decrease in the presence of the V1 form between El7 and 5 mo of age (Fig. 5, Table I). On the contrary, despite increased exposure time, no difference in band intensity was apparent for the V3 band (data not shown).

In adult tissues, the highest V1/V2 mRNA ratio was observed in kidney (2.71) and the lowest in liver (0.7). During aging, neither the V1/V2 mRNA ratio nor the intensity of the V3 band changed significantly in any of the analyzed tissues.

**Discussion**

The alternative splicings of FN pre-mRNA and hence their possible FN isoform translations seem to be regulated by nuclear trans-acting factors that in turn may be modulated by the environment in which the cell is present. There are two levels of regulation: the stable program that can be seen in tissue culture lines and adult organs, and the reprogramming induced by external agents (growth factors, transforming agents, physical stimuli, position in the embryo, et cetera).

The present study considered the changes occurring in FN pre-mRNA processing in various rat tissues during the lifespan of the animal: after 17 d of gestation (fetal) and at 5 mo (the developmental period), and at 12, 17, and 24 mo (aging).

During development (from fetus to adult age), the three alternatively spliced regions of FN have a tissue-specific regulation in which three different patterns of expression can be observed. The first is in kidney and liver tissues, where all of the three variants (EIIIA, EIIIB, and V) are developmentally regulated (Figs. 2, 4, and 5; Table I). The second pattern is observed in lung, where only the EIIIB and V variants
Table I. Fibronectin mRNA Ratios for EIIIA, EIIIB, and V Alternately Spliced Regions in Rat Tissues as a Function of Age Expressed in Months

<table>
<thead>
<tr>
<th>mRNA ratio</th>
<th>Tissue</th>
<th>Age (mo)</th>
<th>E17</th>
<th>5</th>
<th>12</th>
<th>17</th>
<th>24</th>
</tr>
</thead>
<tbody>
<tr>
<td>EIIIA+/EIIIA-</td>
<td>Liver</td>
<td>0.14</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Kidney</td>
<td>19.80</td>
<td>0.57</td>
<td>0.57</td>
<td>0.48</td>
<td>0.55</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Brain</td>
<td>3.90</td>
<td>3.44</td>
<td>3.31</td>
<td>2.60</td>
<td>1.23</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lung</td>
<td>1.40</td>
<td>0.66</td>
<td>0.51</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Heart</td>
<td>ND</td>
<td>0.89</td>
<td>0.85</td>
<td>0.90</td>
<td>0.70</td>
<td></td>
</tr>
<tr>
<td>EIIIB+/EIIIB-</td>
<td>Liver</td>
<td>0.10</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Kidney</td>
<td>15.00</td>
<td>0.03</td>
<td>0.03</td>
<td>0.05</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Brain</td>
<td>1.80</td>
<td>0.47</td>
<td>0.55</td>
<td>0.60</td>
<td>0.42</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lung</td>
<td>1.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>V1/V2</td>
<td>Liver</td>
<td>10.00</td>
<td>0.70</td>
<td>0.87</td>
<td>0.91</td>
<td>0.95</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Kidney</td>
<td>14.40</td>
<td>2.71</td>
<td>3.35</td>
<td>ND</td>
<td>2.50</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Brain</td>
<td>11.20</td>
<td>2.13</td>
<td>2.22</td>
<td>2.00</td>
<td>1.80</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lung</td>
<td>15.00</td>
<td>1.16</td>
<td>1.18</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Heart</td>
<td>ND</td>
<td>1.52</td>
<td>1.30</td>
<td>0.90</td>
<td>1.41</td>
<td></td>
</tr>
</tbody>
</table>

Data obtained by densitometric analysis of autoradiograms deriving from ribonuclease protection experiments represent the average of three independent experiments for the EIIIA+/EIIIA- and V1/V2 mRNA ratios and the average of two independent experiments for the EIIIB+/EIIIB- mRNA ratio. $E_{17} = \text{fetal d } 17; <0.01 \text{ means that the high molecular weight protected band was undetectable even when the exposure time of autoradiographic films was increased.}$

show changes, and where the EIIIA appears to be less closely regulated, as shown by the EIIIA+/EIIIA- mRNA ratio between E17 and 5 mo of age (1.40 vs. 0.66). The third pattern can be seen in brain, where only the V25 variant is developmentally regulated. In this tissue, between E17 and 5 mo of age, the EIIIB+/EIIIB- mRNA ratio slightly decreased (1.80 vs. 0.47) and the EIIIA+/EIIIA- mRNA ratio did not change (3.90 vs. 3.44).

As far as the EIIIA and EIIIB regions are concerned, developmental changes in the relative inclusion of these segments in FN mature transcripts have been previously reported in some human and chicken tissues (10, 11, 31). Oyama et al. (37) have shown that EDA in humans is developmentally regulated in liver but not in lung; we observed the same thing in the corresponding rat tissues. During chicken development, ffrench-Constant et al. (10, 11) have reported the presence of all of the three alternatively spliced forms in early embryo. However, at later embryonic stages (16 d), the EIIIA and EIIIB regions were virtually absent in liver and in the other tissues, the amount of EIIIB was less than that of EIIIA (which was still present in large amounts at this time). In fetal rat liver, both EIIIA and EIIIB were clearly detectable in FN mRNA at E17 but had disappeared from the livers of 5-mo-old rats. Both of these variants were also the predominant forms in fetal rat kidney (Table I). In rat brain, there was only a slight decrease in the inclusion of the EIIIB region between the ages of E17 and 5 mo, whereas there was no change in the EIIIA region. Some of these differences may be due to the species-specific timing of alternative splicing during fetal development.

Changes in EIIIA and/or EIIIB inclusion have been previously observed not only during development but also in experimental conditions of induced FN reprogramming, such as wound healing (12) and the malignant transformation of cells (2, 5). These changes have been interpreted as indicating that these segments (in particular the EIIIB segment) play
a major role in the migration and/or proliferation of cells. However, under these experimental conditions, no different expression in the V region has been clearly demonstrated. The vast majority of previous studies did not test for a selective specific exclusion of the V25 peptide (21, 40). The only study that specifically analyzes the V region in detail did not detect a differential expression during embryonic development in chicken (11), although it must be said that the alternative splicing of FN in embryos was not compared with that in adult chicken tissues. Furthermore, unlike in rat, chicken FN at the V region showed only two alternatively spliced forms (26). The V region of the chicken is only 50% homologous with the amino acid residues of the corresponding region in mammals, which suggests that in avian species the V region may not possess a similar cell binding function.

In our study, the VI/V2 ratio represents the presence of the V25 peptide in mature protein, if we assume that the mRNA forms correspond to the FN isoforms previously described (3, 35, 41, 44, 45). This peptide can be selectively spliced out independently of the rest of the V region (11, 40) and is identical in human, rat, and cow fibronectins (34). This region (also called CS-1 in human FN) interacts with a specific integrin receptor localized on the surface of some transformed cells (20, 24). In neural avian crest cells in vitro, this is the only segment which promotes attachment and, in cooperation with the RGDS cell binding site and the synergic site domain of FN, it is the segment responsible for migration (9). The high level of inclusion of the V25 variant in all of the fetal tissues analyzed suggests that it plays a major role during fetal development, which is possibly related to an interaction with the α4β1 integrin receptor (20, 34).

The alternative splicing of FN pre-mRNA was also investigated in aging animals. Perhaps the most striking result observed was the change in FN mRNA in the EIIIA region of the aging brain (Fig. 3, Table I). This is the first report of tissue-specific alterations in FN pre-mRNA processing in vivo during aging. The observed change in FN pre-mRNA alternative splicing in brain may be due to the occurrence of a cell specific alteration of the genetic program during aging (17, 42), age-related modifications in brain cell populations (8, 46), or even a combination of both.

The cell type localization of FN synthesis in the brain is not known. Neural avian crest cells and their derivatives do not synthesize FN, as detected by in situ hybridization (10, 25), but they appear to be very responsive to surrounding FN and in vitro migrate on layers of the molecule (47). On the contrary, rat astrocytes have been shown to synthesize FN in vitro (35) and this FN includes all of the three alternative spliced segments (41). The high level of inclusion of EIIIA and EIIIB, as expressed by the corresponding mRNA ratios observed in adult rat brain (Table I) indicates the important role of these segments in differentiated brain tissue. Previous studies using specific mAbs have failed to identify the EDA and EDB segments in adult human brain tissue (which respectively correspond to rat EIIIA and EIIIB) at protein level (2, 5). It should be noted that overall FN expression in lower adult rat brain tissue and that immunohistochemical assays are probably less sensitive in detecting low quantities of FN isoforms than RNase protection techniques. However, it cannot be excluded that rat and human brain tissue may behave differently in this respect and that the alternatively spliced forms of FN pre-mRNA might be differently translated in this tissue. To understand the functional role of selective fibronectin changes in aging brain, we are currently performing further studies to examine cell localization and regulation of pre-mRNA alternative splicing in this tissue.

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