Regulation of fibronectin expression in rat regenerating liver

M. Caputi, C. A. Melo and F. E. Baralle*

International Centre for Genetic Engineering and Biotechnology–UNIDO, Padriciano 99, Area Science Park, 34012 Trieste, Italy

Received October 21, 1994; Revised and Accepted December 14, 1994

ABSTRACT

Fibronectin (FN) expression displays a complex regulation that results in precisely defined isoform patterns during different developmental stages, aging and injury. The qualitative and quantitative changes that are observed derive from modulation of the rate of transcription of the single FN pre-mRNA and its specific differential processing in the EIIIA, EIIIB and V regions of rat FN. The liver is the major source of plasma FN which is characterised by the absence of the EIIIA and EIIIB exons. Here we show that in the rat regenerating liver there is a significant reprogramming of the splicing machinery that results in the synthesis by the liver of up to 17% of EIIIA+ FN linked with all the three V forms. On the other hand the EIIIB+ form is totally absent both in normal and regenerating liver. Furthermore there is a variation of the V pattern observed in the regenerating tissue, the V120 form (linked to both EIIIA+ and EIIIA− messengers) increases from 11 to 32%. The quantitative RT-PCR method was used to estimate the FN transcription rate, before and after partial hepatectomy. We have shown a 3-fold increase in FN mRNA in liver that is specifically linked to the regeneration process and not to the surgical stress.

INTRODUCTION

Fibronectins (FNs) are high molecular weight, multi-domain/multi-functional glycoproteins present in plasma and extracellular matrices. FN subunits are composed of a series of structural domains displaying three types of sequence homologies, named type I, type II and type III (Fig. 1). The domain structure and the genomic organisation are well conserved among human, rat, bovine and chicken. They are mostly dimers of similar but not identical subunits of about 250 000 Da. Through the specific binding to other macromolecules such as collagen, fibrin, heparin and integrins, FNs play key roles in cell adhesion, spreading and migration, embryonic development, blood clotting, wound healing, oncogenic transformation and metastasis (1–3).

The expression of the FN gene has been analysed in detail and has been shown to be regulated at both the transcriptional and pre-mRNA processing level (4–11). The FN gene promoter structure shows sequence homologies that indicate possible targets of corticosteroid and acute phase effectors (10). The FN gene transcript is subjected to alternative splicing that accounts for exon skipping in two type III segments (EIIIA and EIIIB) and for a more complex mechanism in the V region that generates three alternative forms in rat, five in human and two in chicken (Fig. 1). The alternative splicing pattern observed in the different embryo and adult cell types (12,13) undergoes dramatic changes in pathological conditions such as healing (14) and myocardial infarction (15).

The hepatic tissue is the major source of circulating plasma FN. Although similar in the overall structure to the extracellular matrix form, plasma FN is characterised by the absence of the EIIIA and EIIIB regions. Previous studies (16–18) have shown that the adult liver presents almost undetectable levels of both the EIIIA and EIIIB. Nevertheless, in vitro studies have evidenced the ability of the hepatic cell types to produce the cellular FN form (19,20). Using partial hepatectomy (21) we have evaluated in an in vivo experimental model the ability of this tissue to undergo reprogramming in case of massive cell proliferation and tissue reorganisation. The rat regenerating liver is an excellent model to study the regulatory mechanisms involved. New methodologies such as RT-PCR and the quantitative-PCR allowed us to address the study of the changes in the rat regenerating liver model both at the transcriptional and at the pre-mRNA processing level.

MATERIALS AND METHODS

Regenerating liver tissue preparation and RNA isolation

Six month old rats were subject to 70% hepatectomy (median and left lateral lobe) as described by Waynfort and Flecknell (21) under anaesthesia with ketamina-clorohidrate (Park Davies). All the hepatic tissue taken was immediately frozen in liquid nitrogen. Control rats were sham operated. Total RNA was prepared from 0.5–0.7 g of frozen tissue using the methods of Chomczynski and Sacchi (22). In the first experiment three hepatectomized rats were sacrificed at 8, 16 and 24 h and the regenerating hepatic tissue was recovered. Northern blot analysis

* To whom correspondence should be addressed
was then performed on the mRNA extracted from these hepatic tissues (23).

After the results of the Northern blot analysis, described later, a further four rats were partially hepatectomized along with two other control rats. All the rats were then sacrificed after 24 h and the hepatic tissue recovered. All the regenerating liver tissues were carefully taken avoiding the scar tissue. Histological examination of the regenerating liver tissue showed several mitotic figures but no evidence of inflammatory cell infiltration.

cDNA synthesis and polymerase chain reaction

The cDNAs were obtained using the ‘First Strand cDNA Synthesis Kit’ supplied from Pharmacia. For each reverse transcription reaction 5 μg of total RNA and specific primers (cDNAFN or GAPDH cDNA) at a final concentration of 2 μM were used. The amplifications of the target sequences were carried out in the following conditions: 3 μl of the first strand cDNA synthesis mixture were added to the amplification reaction mix, which included, in a final volume of 100 μl, 0.2 mM each dNTP, 0.185 MBq of [α-32P]-dCTP, 3% of dimethyl-sulfoxide, 5 U of Tag DNA polymerase (Promega) and each set of primers to a final concentration of 1 μM. The amplifications were performed on a Perkin-Elmer Cetus DNA thermo Cycler. Each cycle was carried out as follows: denaturation step (1 min at 93 °C), annealing step (1 min at 56 °C), extension step (1 min, 30 s at 72 °C). The number of amplification cycles ranged from 21 to 24. The following primers were used: GAPDH cDNA (5'-TGTAAAAAGGTAGGCTGTG-3'), cDNAFN (5'-CTCAGAATCTCCTTGGGAT-3'), 5'OUT (5'-TATGCTTCAAGGACACA-3'), 3'OUT (5'-CTGCTTCTTTTCCTTCCAA-3'), 5'GAPDH (5'-ACATGGTTCGATGAC-TCT-3'), 3'GAPDH (5'-ACGGAAGGCGCAAGGTA-3'), EIIIBS' (CTATCTCTGTATAACGAT), EIIIB3' (ACACTGAC- TAGGTACTCAGT), EIIIA5' (CCTGTTAGACTCAGTG- GA), EIIIA3' (ACTGCTAGGCTGAGGTTT), PREIIA+ (TCAGACTGCACTGACAA), PREIIA- (TCAGACTGCACTGACCC), V3' (GTCTGAGGACCACTTCTT).

Location of the FN primers on the corresponding mRNA molecule is shown in Figure 1.

Analysis of the PCR products and Northern blots

Five μl of each reaction product were analysed by vertical electrophoresis on a 1.2% agarose (SeaKem) gel, stained with ethidium bromide. Verification of the fragment size was carried out by visualisation of their migration relative to molecular weight standard (1 kb DNA Ladder, Bio-England Research Laboratories), whereas verification of the sequence was accomplished by sequencing of the clones obtained from the purified PCR products. The gel was then dried under vacuum and exposed on Kodak X-Omat film. Exposure intensities of the bands, produced by the radio labelled products on the film, were submitted to densitometric analysis. The value assigned to each band was then integrated taking into account the cytosine content and the amount of [α-32P]-dCTP incorporated in each fragment. The Northern blot analysis was performed using 10 μg of total RNA from each sample following standard laboratory protocols (23). The pFH111 clone (4) was used as specific probe for the FN transcript.

Constructs and quantitative PCR assay

The enzymes for DNA work were obtained from Boehringer Mannheim and New England Biolabs. Total mRNA extracted from rat liver tissues was reverse transcribed using the primers FNcDNA and GAPDHcDNA. FN and GAPDH cDNAs were then amplified using the primers 5'OUT, 3'OUT and 5'GAPDH,
Figure 2. Quantitative PCR analysis. (A) Locations of the primers 5'OUT–3'OUT on the FN cDNA and pRFout260 construct and 5'GAPDH–3'GAPDH on the GAPDH cDNA and pGAPDH260 construct. The size of the amplified products are also indicated. (B) Row A shows the PCR set of reactions in which an equal amount of cDNA, synthesized from the mRNA of the liver taken at the time of the operation (liver h0), is titrated, in a series of reactions, with a decreasing amount of the competitor plasmid pRFout260. In row C the same set of reactions is represented for the regenerating liver taken 24 h after the operation (Liver h24). Row E shows the reactions carried out for the control liver analysed 24 h after the sham operation. In rows B, D and F there are the results of the competitive PCR carried out to quantify the GAPDH messenger in the liver taken during the operation, the regenerating and the control one, respectively. (C) The graph shows the ratio between the O.D. of the liver FN PCR product and the competitor (pRFout260) PCR product plotted against the competitor quantity in each reaction. From the intersection of each line with the value 1 of the axis (equivalence point between the two template species) a more accurate quantification is reached. In the sample of liver taken during the operation the equivalence point is at 10 pg, the control sample shows an increase to 12 pg, whereas the regenerating liver shows an increase of up to 30 pg.

RESULTS

Fibronectin mRNA quantification

Preliminary experiments aimed to evidence changes in the Fn mRNA levels were performed using Northern blot analysis on total liver mRNA extracted from partially hepatecomised rats 8, 16 and 24 h after the operation. Although no proper quantification was carried out, there was a noticeable increase in the FN messenger in the regenerating liver particularly at 16 and 24 h (data not shown). The latter time point was chosen for proper quantitative studies using PCR analysis (25). The technique, which has been successfully applied to viral RNA quantification (26), consists in coamplifying two templates that slightly differ in length but are recognised by the same set of primers. This ensures the same amplification efficiency for both template species. If the quantity of one template is known the amount of the other can be extrapolated from the ratio between the two products of the amplification reaction.
This method allows a reliable quantification of small amounts of DNA and RNA. We have therefore developed a competitive RT-PCR assay to detect variations in FN expression levels between the normal and the regenerating liver tissue. The efficiency of the RT step and the equal amount of the total mRNA used in the reactions were controlled by comparing the results with the competitive PCR analysis of the glyceraldehyde-phosphate-dehydrogenase (GAPDH) mRNA levels. GAPDH is a housekeeping enzyme whose mRNA was seen to remain constant regardless of the replication stimuli received by the cell.

The quantification of the FN mRNA levels in the normal, control and hepatectomized liver was carried out by coamplification of the cDNA synthesized from the total RNA extracted from the tissues and the plasmid pRFout260 with the primers 5'OUT and 3'OUT. Quantification of the GAPDH mRNA levels in the same samples was achieved by coamplifying the cDNAs and the plasmid pGAPDH260 with the primers 5'GAPDH and 3'GAPDH. Five independent RNA extractions and cDNA synthesis were carried out for each sample. Figure 2B (rows B, D and F) shows that the equivalence point between the GAPDH mRNA template and its competitor (plasmid pGAPDH260) remains constant (2-4 pg) both in the liver taken during the hepatectomy and 24 h after the partial hepatectomy. Moreover in the sham operated control there is no detectable variation in the overall transcriptional activity of GAPDH during the regenerating process. On the other hand, for the FN messenger (rows A, C and E, and Fig. 2C), its equivalence point with the competitor (plasmid pRFout260) is reached at 10 pg in the sample taken at the time of the hepatectomy (row A). There is an increase up to 30 pg in the FN transcript expression levels measured in the regenerating liver after 24 h (row C). Conversely, the control sample taken 24 h after the sham operation shows only a slight increase up to 12 pg (row E).

**Fibronectin pre-mRNA processing**

Since the highest FN levels were detected 24 h after the operation, the regenerating hepatic tissue of those rats was analysed to characterise any change in the FN's splicing pattern when compared with the tissue taken at the time of the operation. The tissues were amplified by PCR to analyse the three different splice sites (EIIIB, EIIIA and V), their level of expression and the linkage of the different forms.

The amplification of the fragment including the EIIIB site was performed with the primers EIIIB+ and EIIIB3'. This reaction gives two products, one of 543 bp which includes the EIIIB exon (EIIIB+), and the other of 270 bp lacking the EIIIB exon (EIIIB−). The tissues taken at time 0 and after 24 h in both hepatectomized and control rats show only the shorter product. Therefore only the EIIIB− FN mRNA forms are present in the liver even during the regenerative process (Fig. 3A).

The amplification of the region that includes the EIIIA site was performed with the primers EIIIA5' and EIIIA3'. This amplification gave two different products one of 200 bp in which the EIIIA region is included (EIIIA+) and one of 470 bp in which the region EIIIA is excluded (EIIIA−). In the hepatic tissue taken at the time of operation the EIIIA+ form is below 1% of the total (Fig. 3B, lane 1), but the tissue taken at 24 h shows a remarkable increase of that form, up to 17% of the total (lane 2). In contrast no changes were observed in the sham operated control tissue (lane 3). This result shows a strong correlation between the involvement of the EIIIA region, normally absent in the FN synthesized by the liver, and the regenerating event. This phenomena is not due to the surgical stress as proved by the barely detectable level of EIIIA+ in the control tissue.

The changes in the synthesis of the V variants (V120, V95 and V0) and their linkage to the appearance of the EIIIA region was also studied by two different PCR reactions for each sample. The primers PREIIIA− and V3' were used to detect the V pattern linked to the EIIIA− form while the primers PREIIIA+ and V3' were used to detect the V pattern linked to the EIIIA+ form (Fig. 1). As expected for the tissue extracted at the time of operation and for the surgical stress control there were no mRNAs carrying the EIIIA region, the EIIIA− mRNA shows a relative abundance of the V0 form (57%) (Fig. 4, lane 1). In the analysis of the regenerating tissue there was a significant variation in the splicing pattern of the V region that was equal in both the EIIIA+ and the EIIIA− forms with a relative increase of the V120 form from 11%
of the total in the tissue taken at the time of the operation to 32% of the total in the regenerating tissue (Fig. 4, lanes 1 and 3). It is clear therefore, that the new types of messengers carrying the EIIIA region arising with the regeneration process are not associated to a particular V form.

**DISCUSSION**

The rat regenerating liver provides an excellent model to study the changes that occur to FN transcription and pre mRNA processing during the *in vivo* physiological response to environmental changes. It has been previously shown that during development, wound healing and other pathological situations (14-18,27,28) consistent changes occur on the FN isoforms and total FN production.

The V120 FN form has been shown to be abundant in liver fetal tissue in human, chick and rat (16,18,29). We have now shown that V120 also increase in the regenerating liver. The functional significance of this variance probably involves the integrin α4β1, the cellular receptor for the CS1 cell binding site included in the V120 form. Indeed, FN can provide a stimulus for cellular spreading and growth via its integrin receptors (30,31). Our results however do not establish definitively if the observed increase in expression is due to a reprogramming of the RNA processing in the hepatocyte, the endothelial cells or any other cell type that is actively contributing to liver regeneration. However, since we know from previous work (unpublished results) that the V pattern is specific for different tissues, the invariance of the V0/V95 ratio suggests that the hepatocyte reprogramming hypothesis is the more likely. The same is valid for the appearance of the EIIIA+ exon, where the EIIA+ forms are linked to the V0, V95 and V120 forms in the same proportion as to the EIIA- forms. Thus, EIIA+ and EIIA- messengers are associated with the same V pattern, reinforcing the hypothesis of the same cellular origin for both messengers types. This is consistent with the observations of Odenthal et al. (19) that, after 3 days in culture hepatocytes start producing EIIA+ FN. Our results suggest a role in cell proliferation and tissue modelling for the EIIA+ and V120. The two regions seem to be subjected to independent regulatory mechanisms and we have shown here that they are not exclusively linked in the same mRNA species. There is not a full reprogramming to an embryo-like splicing pattern in the regenerating liver. In fact the EIIIB+ messenger, the fetal isoform by excellence, is absent. This confirms previous results on the independent regulation of the alternative splicing of the EIIA and EIIIB regions (12,32,33). This result makes unlikely the hypothesis for a common function, at least in this model, of the two sites and differentiates the liver regenerating process from others, such as wound healing in which there is an increase of both EIIA and EIIIB (14).

It was of interest to analyse the absolute amount of FN mRNA during regeneration and to establish if the increases in plasma FN observed in many pathological conditions involving stress and acute phase reactions (15,28,34) were due to an increase in transcription. The quantitative PCR method used allowed, for the first time, this problem to be addressed in a precise and effective way. We demonstrate that there is a specific increase of FN transcripts in the regenerating liver up to 3-fold 24 h after the operation while the sham operated control shows only a slight increase. It is clear that the hepatocyte proliferative stimuli induced by the hepatocyte in early regeneration has resulted in a reprogramming of the FN transcription and RNA processing pathways. These variations are specifically due to the hepatectomy as the changes due to surgical stress are minimal. On the other hand further experiments should be carried out to investigate on the translational control of the messenger.

We have shown that regenerating liver undergoes specific quantitative and qualitative changes in the absolute level of FN mRNA and in its different transcript isoform. To understnd the precise localisation and functional role of these changes we are currently performing *in situ* hybridisation studies of the regenerating tissue.

**ACKNOWLEDGEMENTS**

We thank Helen Mardon and Franco Pagani for their preliminary work on the rat regenerating liver system. This work supported in part by the grant of the Progetto Finalizzato BTBS Biotecnologie e Biostromattura of the Consiglio Nazionale delle Ricerche.

**REFERENCES**