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# Expression of a human α-globin/fibronectin gene hybrid generates two mRNAs by alternative splicing

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**We have isolated genomic clones for human fibronectin (FN), by screening a human gene library with previously isolated FN cDNA clones. We have recently reported two different FN mRNAs, one of them containing an additional 270 nucleotide insert coding for a structural domain ED. Restriction mapping and DNA sequencing of the genomic clones show that the ED type III unit corresponds to exactly one exon in the gene, whilst the two flanking type III units are split in two exons at variable positions. When an α-globin/FN gene hybrid construct, containing the ED exon, flanking introns and neighbouring FN exons, is transfected into HeLa cells, two hybrid mRNAs differing by the ED exon are synthesized. These experiments confirmed that the two FN mRNAs observed *in vivo* arise from the same gene by alternative splicing.**

*Key words:* fibronectin gene/alternative splicing/transient expression in HeLa cells

## Introduction

Fibronectin (FN) is a large, adhesive glycoprotein, important in cell adhesion, cell migration, maintenance of normal cell morphology, opsonization, differentiation and wound healing. FN is found in blood plasma as a heterodimer of two very similar chains of mol. wt. 230 000 and 250 000, and in extracellular matrices as dimers or multimers of chains which are slightly different from those of the plasma form. It has discrete domains (listed from the N terminus) responsible for binding to fibrin (and *Staphylococcus aureus*), collagen, DNA, cell surfaces, heparin, fibrin and itself [for reviews, see Mosher (1980) and Yamada (1983)]. The amino acid sequence of ~60% of bovine plasma FN (Skorstengaard *et al.*, 1982; Petersen *et al.*, 1983) and cDNA sequence of parts of bovine (Kornblihtt *et al.*, 1983), rat (Schwarzbauer *et al.*, 1983) and human (Kornblihtt *et al.*, 1983; Oldberg *et al.*, 1983) FN mRNAs reveal a structure with three types of homologous repeats called homology type I, II and III (Petersen *et al.*, 1983).

We have previously reported (Kornblihtt *et al.*, 1984a) that in a human cell line (and in cultures of normal human fibroblasts), there are two different FN mRNAs (mRNAI, mRNAII). One of them (mRNAI) has an extra internal 270 nucleotide piece, coding for exactly one type III homology domain (the extra domain, ED), which is flanked by other type III homologies. Human liver contains only mRNAII, without the ED. Recently, Schwarzbauer *et al.* (1983) have reported that rat liver has three different FN mRNAs, differing in another region, nearer the 3′ end of the mRNA.

Here we report the isolation and characterization of genomic clones for part of the human FN gene. The extra type III domain ED, encoded by mRNAI but not mRNAII, corresponds to exactly one exon. More interestingly, we have studied the transient expression of a hybrid gene construct in which the segment of the human FN gene is transcribed under the control of the human α-globin promoter. Both structural and functional studies indicated that mRNAs I and II arise from the same primary transcript by alternative splicing.

## Results

We have previously isolated overlapping cDNA clones corresponding to the C-terminal 60% of human FN (Kornblihtt *et al.*, 1983,1984a,1984b). The inserts of these clones were used as probes to screen a human gene library in bacteriophage λ. Six positive plaques were identified. The plaques represent at least two independent clones (see Materials and methods) named λFN4 (15.8 kb) and λFN5 (15.3 kb). Both contain the extra domain (ED) (Figure 1A).

Restriction enzyme mapping by Southern blotting showed that the ED and the adjacent regions of the cDNA were contained in a 4.3-kb *Eco*RI fragment of λFN5. This fragment was sub-cloned and the resulting recombinant plasmid (pFN5RI 4.3) was mapped in detail (Figure 1C). The nucleotide sequence of its exons and of the exon/intron boundaries was determined as outlined in Figure 1C. The extra domain ED corresponds to precisely one exon (270 bp), which encodes a full type III homology, whereas the exon just 5′ to the ED (exon −1) and just 3′ to it (exon +1) are shorter (114 and 188 bp, respectively) and encode only part of a type III unit. The sequences of the exon/intron boundaries are shown in Figure 2. The sequence of the exons (not shown) and that of the corresponding area in the cDNA clones (Kornblihtt *et al.*, 1984a) are identical, except for one base exchange. This variation does not change any amino acids, but creates a restriction enzyme polymorphism since an *Xho*I site (CTCGAG) is present in the middle of the ED in the cDNA clones, but absent (CTCTAG) in the ED exon of the isolated genomic clones.

Southern blot analysis of restriction enzyme digests (results not shown) of both genomic clones using several cDNA probes allowed us to draw the map of part of the human FN gene shown in Figure 1B. λFN4 and λFN5 overlap by 2.7 kb and together span a central 28-kb portion of the FN gene. Although the size of the human gene has not been determined, it is likely to be similar to that of the chicken FN gene (~50 kb) (Hirano *et al.*, 1983). The approximate position of the exons −3, −2 and +2, +3 on the gene map (Figure 1B) was also determined by blot analysis.

A 3.5 kb long *Bgl*II/*Eco*RI fragment of pFN5RI which contains exons ED−1, ED and ED+1 and flanking introns was sub-cloned in an expression vector containing the gene for the human α1 globin and SV40 origin of replication and enhancer sequences (pSVEDα1W) (Higgs *et al.*, 1983). The FN gene fragment was inserted in the third exon of the α1 globin gene into a unique *Bst*EII site. This construct (pSVED-

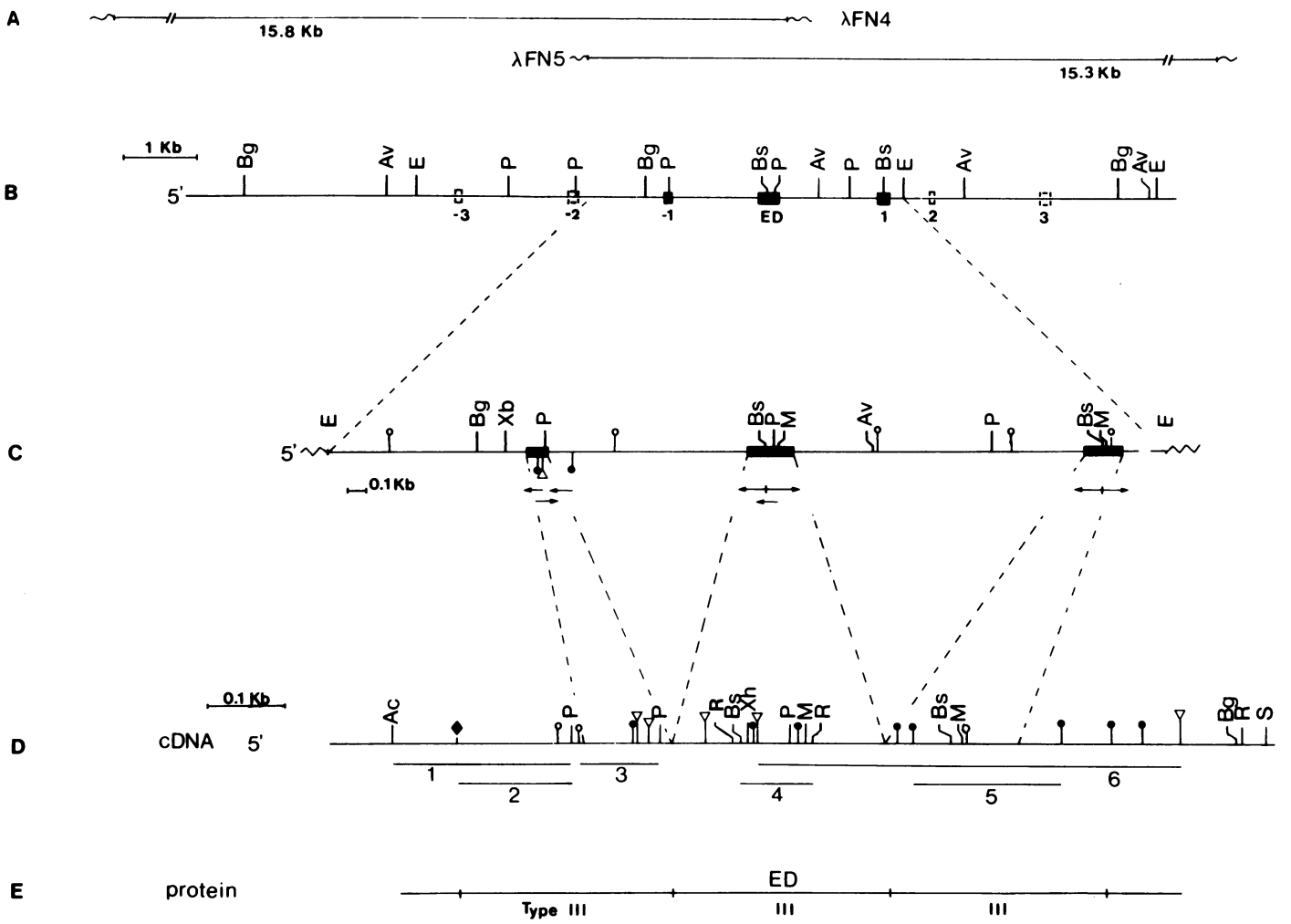


**Fig. 1. (A)** The two overlapping FN-λ clones. Wavy lines represent λ DNA. **(B)** Map of part of the human FN gene. Restriction sites: Av = *Aval*, Bg = *Bgl*II, Bs = *Bst*EII, E = *Eco*RI, P = *Pst*I, R = *Rsa*I. The exons named ED +1 and −1 are marked with solid boxes. The next exons in both directions are marked with dotted boxes. Their exact position and length are not known yet (see text). **(C)** Expanded map of the part of the gene that corresponds to the subclone pFN5RI 4.3. The strategy for Maxam and Gilbert (1977) sequencing is shown with arrows. The sequence from the *Pst*I site in the ED exon and 150 bases downstream has been determined by the chain terminator method. Restriction sites as in **(B)** and M = *Msp*I, Xb = *Xba*I, ⚲ = *Dde*I, ⚲ = *Hinf*I, ⚲ = *Sau*961. Not all *Msp*I, *Dde*I and *Hinf*I sites are on the map. Zig-zag lines represent vector DNA. **(D)** Map of part of the cDNA clones for human FN (Kornblihtt *et al.*, 1984a). Restriction sites as above, plus Ac = *Acc*I, Xh = *Xho*I. pFH111 covers this area completely. pFH23 spans from the diamond to the 3′ end. Probe 1 is an *Acc*I-*Pst*I fragment from the insert of pFH111, probe 2 is a fragment from pFH23 with its 5′-end at the *Hind*III site in the vector and its 3′ end at a *Pst*I site in the insert. Probes 3−6 have been obtained from the insert of pFH23. **(E)** Outline of the FN type III homologies in this area.

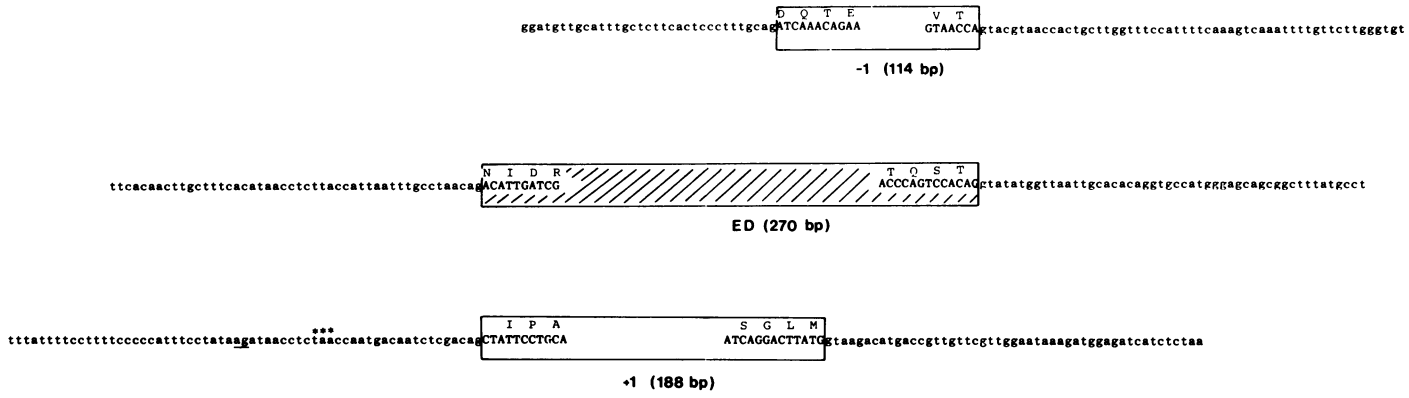


**Fig. 2.** Sequence of the exon/intron boundaries of the ED exon (hatched) and the two exons flanking it (−1 and +1). Intron sequences are in lower case letters. Underlined: putative alternative acceptor junction followed by a termination codon in frame (***).

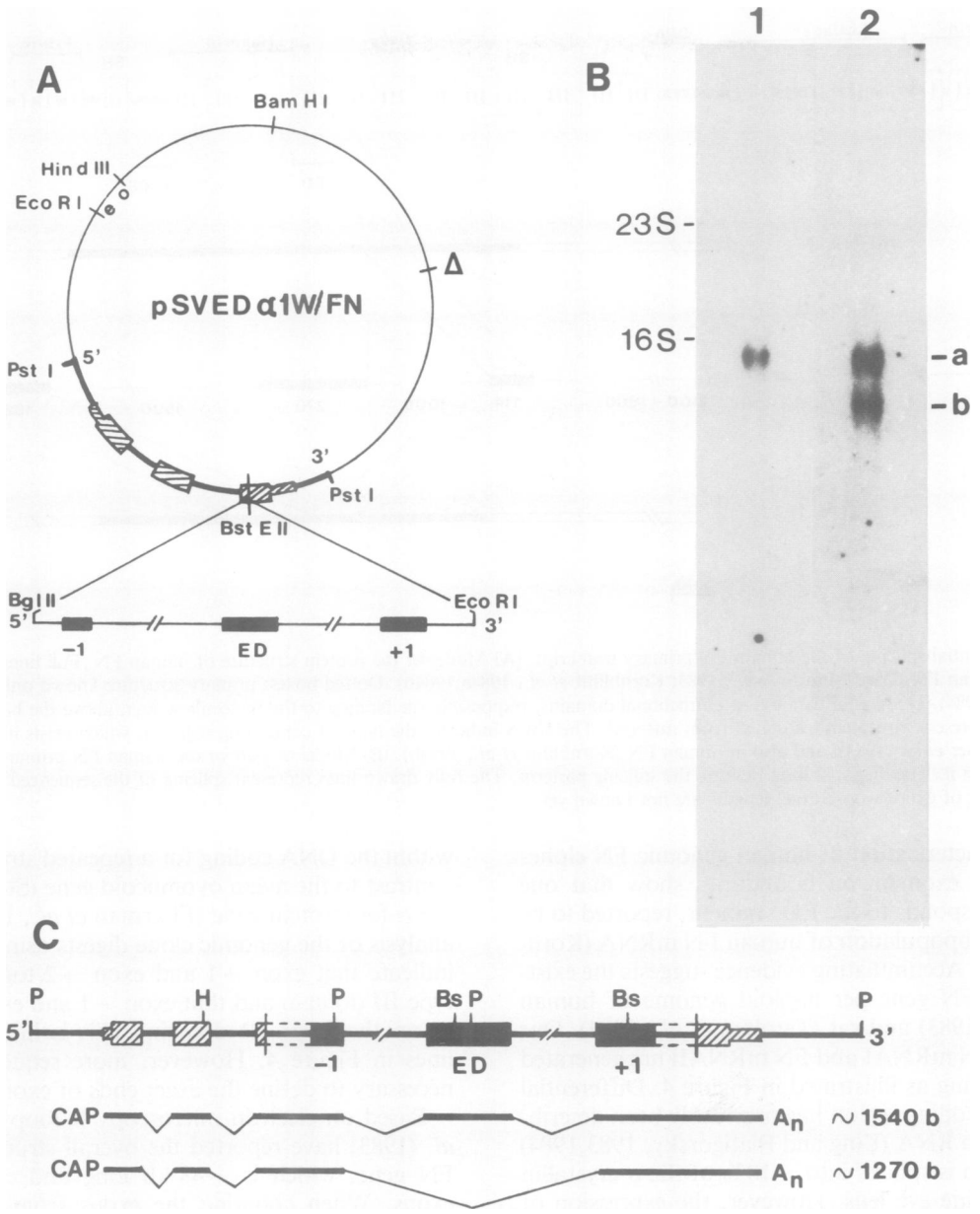


**Fig. 3. (A)** A physical map of the human α-globin/fibronectin gene construct used for transient expression, pSVEDα1W/FN. Striped boxes, human α-globin exons. 5′ and 3′ non-coding regions are denoted by narrower boxes. Black boxes, human fibronectin exons. △, deletion of nucleotides 1426–2490 of pBR322. e and o, SV40 enhancer and origin of replication sequences. **(B)** Northern blot of cytoplasmic RNA from HeLa cells transfected with pSVEDα1W/FN and pβ5′SV *Bgl*II 9.7 (Higgs *et al.*, 1983). **Lane 1**, hybridized to an ED-specific cDNA probe, M13ED1. **Lane 2**, hybridized to a cDNA probe containing exons −1, ED and +1 (the insert of clone pFH23). The position of bacterial ribosomal RNA bands is indicated (23S, ~3.7 kb; 16S, ~1.7 kb). **(C)** Detailed map of the α-globin/FN hybrid insert. Striped boxes, α-globin exons. Black boxes, fibronectin exons. Dotted boxes, sequences originally belonging to FN introns but that form part of α-globin/FN hybrid exons in the construct. The lines under the map indicate the two ways of splicing.

αlW/FN) (Figure 3A) was used for transient expression in HeLa cells, using a second construct [pβ5′DV *Bgl*II (9.7 kb)] (Grosveld *et al.*, 1982) as a co-transfection control. Total cytoplasmic RNA was extracted from the transfected cells and analysed by Northern blotting using either an ED-specific cDNA probe (M13ED1) or a FN cDNA probe covering all three exons of the construct (insert of clone pFH23). The ED-specific probe hybridized only to a 1500 nucleotide long RNA, band a (Figure 3B, lane 1), whereas the pFH23 probe hybridized to both the 1500 bases long band a and to a lower band b, corresponding to a RNA species ~270 bases smaller (Figure 3B, lane 2). The size of band a is consistent with the predicted size for a processed transcript arising from the α-globin/FN hybrid construct (Figure 3C) and carrying the ED exon. The size of band b is consistent with the one predicted for a similar transcript, but lacking the ED exon. Furthermore, the fact that hybridization with an ED-specific cDNA probe only shows up band a and that hybridization with a cDNA probe carrying sequences flanking the ED shows up both bands a and b, indicates that the two RNA transcripts observed differ by the presence of ED sequences.

## Discussion

These results provide the first structural and functional evidence that the primary transcript of FN undergoes alternative splicing in the generation of two mRNAs differing by the ED

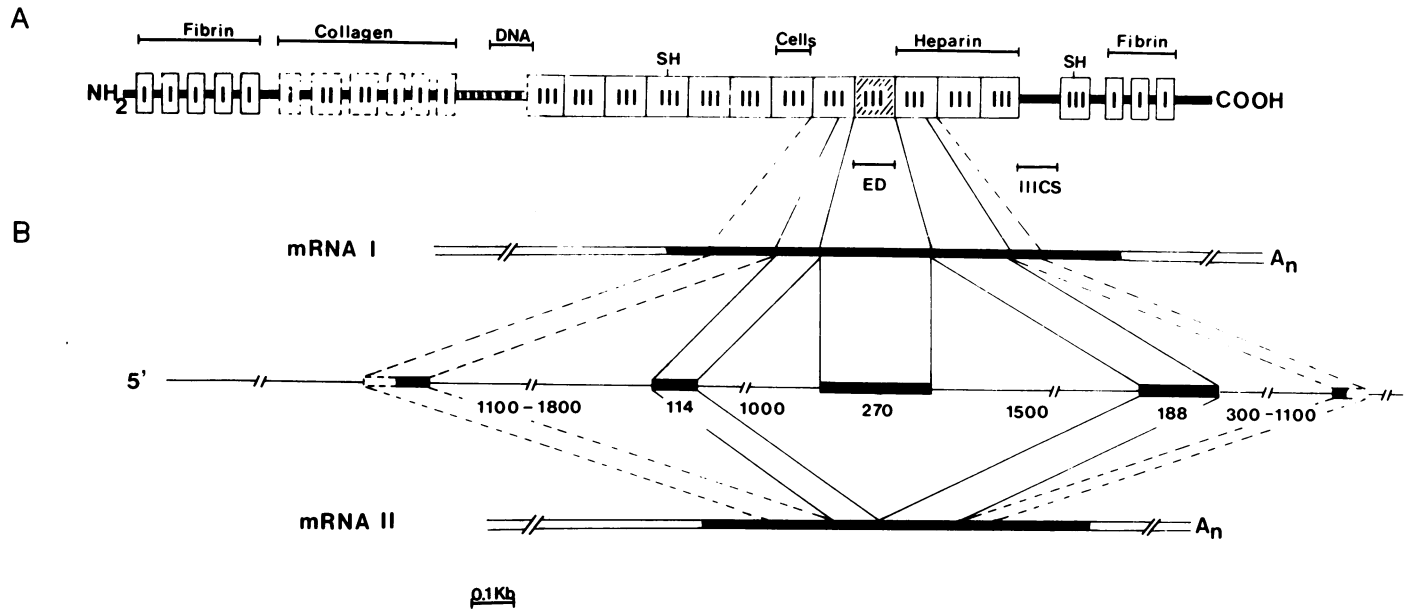


**Fig. 4.** Model of differential splicing of the human FN primary transcript. **(A)** Model of the protein structure of human FN. Full lines and boxes: primary structure known in human FN (Garcia-Pardo *et al.*, 1983; Kornblihtt *et al.*, 1984a,1984b). Dotted boxes: primary structure known only in bovine FN (Skorstengaard *et al.*, 1984). Horizontal bars indicate functional domains, responsible for binding to the molecule written above the bar. The extra domain (ED) (hatched) is only present in protein translated from mRNAI. The IIICS indicates the type III connecting segment, which exists in different forms in rat plasma FN (Schwarzbauer *et al.*, 1983), and also in human FN (Kornblihtt *et al.*, 1984b). **(B)** Model of part of the human FN primary transcript, and the two mRNAs arisen from it. The diagonal lines indicate the splicing patterns. The fully drawn lines represent splicing of the sequenced exons. The dotted lines are the expected splicing of exons whose exact lengths are not known yet.

segment. The characterization of human genomic FN clones and sequencing of exon/intron boundaries show that one separate exon corresponds to the ED segment, reported to be present only in a subpopulation of human FN mRNA (Kornblihtt *et al.*, 1984a). Accumulating evidence suggests the existence of only one FN gene per haploid genome of human (Kornblihtt *et al.*, 1983) and rat (Tamkun *et al.*, 1984). Our results show that FN mRNAI and FN mRNAII are generated by differential splicing as illustrated in Figure 4. Differential splicing within the coding region has previously been described in the α-crystallin RNA (King and Piatigorsky, 1983,1984) where an extra exon is spliced into ~10% of the α-crystallin mRNA in the murine eye lens. However, the expression of FN mRNA with the ED is tissue-specific, hepatoma cells, cultured fibroblasts and transformed epithelial cells all produce both forms of FN mRNA whereas liver expresses only mRNA without the ED (Kornblihtt *et al.*, 1984a,1984b). Since liver has been reported to make plasma FN (Tamkun and Hynes, 1983), it is possible that ED is a particular feature of cellular FN.

The peculiar primary structure of FN with 12 homology type I repeats (Skorstengaard *et al.*, 1982; Petersen *et al.*, 1983), two type II repeats (Skorstengaard *et al.*, 1984) and at least 13 type III repeats (Kornblihtt *et al.*, 1984b) (Figure 4A) has probably evolved through a series of gene duplications. An interesting question is whether each homology unit corresponds to one exon. In fact, the ED exon corresponds exactly to one type III domain, whereas the exon −1 and exon +1 are both shorter (Figure 4). Exon −1 codes for the last 38 of the 90 amino acid long type III domain before the ED, whereas exon +1 codes for the first two-thirds (62 amino acids) of the 91 amino acid long type III domain after the ED (Figure 2). The introns separating exon −1 from −2 and exon +1 from +2 are therefore not in similar positions in the protein domains. This non-conserved position of the introns within the DNA coding for a repeated structural domain is in contrast to the avian ovomucoid gene (Stein *et al.*, 1980) and the α-fetoprotein gene (Eiferman *et al.*, 1981). Southern blot analysis of the genomic clone digests using FN cDNA probes indicate that exon +1 and exon +2 together code for one type III domain and that exon −1 and exon −2 also add up to another type III domain. This is illustrated with dotted lines in Figure 4. However, more sequence information is necessary to define the exact ends of exon −2 and exon +2.

Based on electron microscopy R loop analysis, Hirano *et al.* (1983) have reported the overall structure of the chicken FN gene, which is ~48 kb long and contains at least 48 exons. When counting the exons from the 3′ end of the chicken gene, assuming exon 48 to be the 3′ non-coding region and allowing one exon for type I homology, two exons per type III homology and one exon for the IIICS region (see Figure 4A), exon 35 is in an analogous position to the human ED exon. Most interestingly, exon 35 is considerably longer (247 ± 97 bp) than most of the other chicken exons (147 ± 35 bp on average). Both in the human and chicken gene, the ED exon might therefore be a fused exon.

The intron/exon junctions around the exons ED−1, ED and ED+1 all obey the AG/GT rule, and have reasonable resemblance to the consensus acceptor [(Y)11-15NYAG/G] and donor [AG/GTRAGT] sequences described by Mount (1982). This is in contrast to the α-crystallin case, where an unusual AG/GC sequence flanks the exon, which is only occasionally spliced in, and the differential splicing could therefore be merely a chance event (King and Piatigorsky, 1984).

When a α-globin/FN gene hybrid containing exons −1, ED and +1 (Figure 3A) is transfected into HeLa cells, two hybrid mRNAs differing by the ED exon are produced. This experiment not only confirms the structural evidence that only one FN gene is sufficient to generate both mRNAs I and

II, but it also shows, for the first time, that alternative splicing can be reproduced in a transient expression system in cultured cells. The differential splicing of the ED exon seems to be independent from transcription of the rest of the FN gene, not represented in pSVEDα1W/FN. This rules out any participation in the ED splicing event of FN-specific promoter and regulation sequences as well as any conformation requirement due to the secondary structure of the whole primary transcript of FN. Furthermore, the fact that two mRNAs differing by the ED exon were produced in a cell line in which the endogenous FN gene is not expressed (Northern blot analysis, not shown) suggests that the alternative splicing event is either completely controlled by information within the nucleotide sequence of the transcript itself or determined by factors or enzymes, if any, that are of general character and not specific for FN sequences. In any case, the system described in this paper provides a basis for the investigation of the DNA sequences and/or factors responsible for alternative splicing.

Schwarzbauer *et al.* (1983) reported the occurrence of three rat liver FN mRNAs differing in an area coding for the connecting strand between the last two type III homologies (IIICS) in Figure 4A. In the human cDNA clone pFH1, the IIICS sequence is highly homologous to the rat sequence, but with a length different from any of the three in rat. Tamkun *et al.* (1984) recently demonstrated that the three mRNAs differing in the IIICS area are generated by a process of alternative splicing in which one donor site can be paired with any of three acceptor sites, one at the beginning of, and two within, a single exon. This pattern of alternative splicing in the IIICS area is different from the one described in this communication for the ED area. This means that two different mechanisms of alternative splicing might be working in the same molecule. It is likely that the differences in the IIICS area account for the differences between the chains of plasma FN, while the presence of the ED seems to be a particular feature of cellular FN.

Further investigation of the sequence of the FN gene and of the splicing of its primary transcript under different circumstances, will not only provide insight into the structural basis for functionally different FN molecules, but also contribute to solving the question of the control of the splicing mechanism in general.

## Materials and methods

*cDNA probes*

We used the overlapping cDNA clones already described (Kornblihtt *et al.*, 1984a). pFH1 has its 3′ end at the polyA tail and it is 2.2 kb long. pFH23 (1.1 kb), pFH37 (0.8 kb), pFH111 (1.9 kb) and pFH154 (2.3 kb) all have their 3′ end 200 bp downstream of the 5′ end of pFH1. Only pFH23 and pFH111 contain the ED. The probes were restriction fragments either 3′ end labelled by filling in with the Klenow fragment of DNA polymerase I (Maxam and Gilbert, 1977) and eluted from acrylamide gels, or eluted from agarose gels (Girwitz *et al.*, 1980) and nick translated (Rigby *et al.*, 1977).

*Isolation of λ clones*

Approximately 350 000 recombinants from the *Hae*III/*Alu*I human gene library in λ Charon 4A (Lawn *et al.*, 1978) were screened with a mixture of nick-translated inserts of pFH1 and pFH111. Six positive plaques were found (λ FN1-6), representing three independent genomic clones, all of them hybridizing to an ED probe. Two of them (λFN4 and λFN5) were analysed more extensively. Only λFN4 hybridized to the 5′ end of clone pFH154, whilst only λFN5 hybridized to the 5′ end of clone pFH1.

*Sub-cloning*

A total *Eco*RI digest of λFN5 was cloned in the pAT153/*Pvu*II/8 vector (Anson *et al.*, 1984), and a clone (pFN5RI 4.3), which hybridized to the ED probe (probe 4, Figure 1D) was chosen for further analysis.

*Transient expression*

A *Bgl*II/*Eco*RI digest of pFN5RI 4.3 was filled-in with the Klenow fragment of DNA polymerase I and cloned into the unique, filled-in *Bst*EII site of pSVEDα1W (Higgs *et al.*, 1983) (a kind gift from Dr N.J.Proudfoot). A clone (pSVEDα1W/FN) hybridizing to an ED-specific cDNA probe contained the 3.5 kb-long *Bgl*II-*Eco*RI fragment from the insert of clone pFN5RI 4.3. The orientation of the FN insert was such that the α-globin and FN coding sequences were in the same strand of DNA. pSVEDα1W/FN was used in transient expression experiments: 20 μg of each pSVEDαW/FN and pβ5′SV *Bgl*II (Grosveld *et al.*, 1983) were co-precipitated with calcium phosphate (Chu and Sharp, 1981) and added to sub-confluent HeLa cells as described in Grosveld *et al.* (1983). Cytoplasmic RNA was extracted by the Nonidet P40 lysis method (Maniatis *et al.*, 1982), run on formaldehyde containing agarose gels (Lehrach *et al.*, 1977) and blotted to Gene Screen membranes (New England Nuclear). Hybridization probes were either the nick-translated insert of clone pFH23 (Kornblihtt *et al.*, 1984a) or a $^{32}$P-labelled, primer-extended (Shoulders and Baralle, 1982) insert of clone M13ED1. M13ED1 is a M13mp8 (Messing and Vieira, 1982) vector carrying a 160-base fibronectin insert, corresponding to a region within the ED (positions 2004 – 2164 in Figure 2 of Kornblihtt *et al.*, 1984b). Its construction involved digestion of the insert of pFH111 with *Pst*I and *Sau*3AI and ligation to a *Pst*I/*Bam*HI restricted M13mp8 vector. After ligation, the mixture was digested with *Bam*HI, in order to prevent transformation originated from self-ligated vector, and used to transform JM101 competent cells (Messing *et al.*, 1981). The orientation of the ED insert is such that extension of a 'universal' sequencing primer (Duckworth *et al.*, 1981) with Klenow polymerase using M13ED1 DNA as template results in a single-stranded probe complementary to ED mRNA sequences.

*DNA sequencing*

3′ End-labelled fragments were sequenced according to Maxam and Gilbert (1977). A *Pst*I digest of pFN5RI 4.3 was subcloned in the *Pst*I site of M13mp9 (Messing and Vieira, 1982) and recombinants were sequenced by the chain terminator method (Sanger *et al.*, 1977).

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