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A role for exon sequences in alternative splicing of the human fibronectin gene

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ABSTRACT

Exon EDIIIA of the fibronectin (Fn) gene is alternatively spliced via pathways which either skip or include the whole exon in the messenger RNA (mRNA). We have investigated the role of EDIIIA exon sequences in the human Fn gene in determining alternative splicing of this exon during transient expression of α globin/Fn minigene hybrids in HeLa cells. We demonstrate that a DNA sequence of 81bp within the central region of exon EDIIIA is required for alternative splicing during processing of the primary transcript to generate both EDIIIA$^+$ and EDIIIA$^-$ mRNA's. Furthermore, alternative splicing of EDIIIA only occurs when this sequence is present in the correct orientation since when it is in antisense orientation splicing always occurs via exon-skipping generating EDIIIA$^-$ mRNA.

INTRODUCTION

Fibronectin (Fn) is involved in processes of cell adhesion. It is widely distributed in the extra cellular matrix (cellular Fn) and is secreted into the plasma (plasma Fn) by hepatocytes (for reviews see 1-3). The Fn protein is composed of three types of amino acid sequence homologies, types I, II and III (Figure 1A). Type I and type II homologies are encoded by one exon each. Most of the type III homologies are encoded by two exons of variable size separated by an intron. However at least two type III homologies are encoded by a single contiguous exon. Differential RNA processing of a single primary Fn transcript can potentially generate twenty Fn messenger RNA (mRNA) isoforms. Fn protein variants corresponding to differentially processed mRNA's have been identified in the functionally distinct plasma and cellular Fn's (3).

It is now evident that alternative splicing of a single primary gene transcript is of widespread importance in the regulation of gene expression. Differential RNA processing can generate variant proteins from a single gene which may have different functions (4-6) and may be expressed in a developmental stage and/or tissue-specific fashion (7-11). Although the

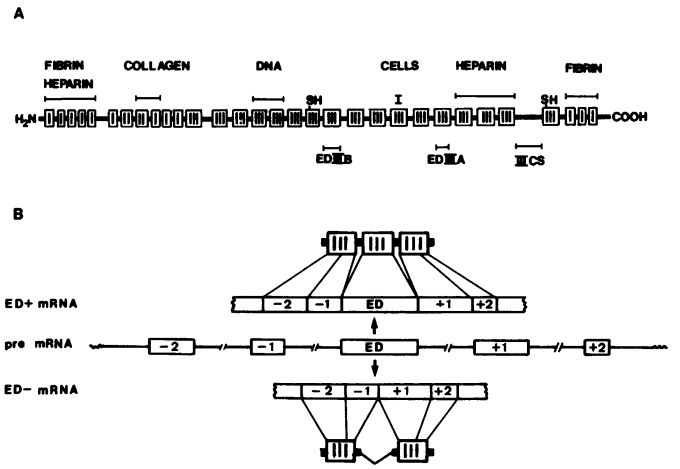

Fig. 1.A. Schematic representation of Fn amino acid sequence homologies types I, II and III. The location of various binding domains are shown above and the three regions which undergo alternative splicing (EDIIIA, EDIIIB and IIICS) are shown below. B. Open boxes in the pre-mRNA correspond to exons (numbered -2, -1, +1, +2 with respect to EDIIIA) and lines between exons corresponds to introns. Alternative splicing of the pre-mRNA generates either ED^+ mRNA (above) or ED^- mRNA (below). EDIIIA encodes one complete type III homology whereas those adjacent to it are encoded by two exons (-2,-1 and +1,+2) of different sizes. The intron is inserted at variable positions between two exons coding for a type III repeating unit. ED^+ mRNA gives rise to a Fn peptide which thus contains an extra type III repeating unit corresponding to EDIIIA.

phenomenon of alternative splicing of mRNA precursors occurs in a number of gene systems, the mechanism(s) remain unclear.

Alternative RNA processing of human (12-14) and rat (15) Fn pre-mRNA occurs in three regions of the primary transcript (Figure 1A). In two regions, each encoding a complete type III homology and corresponding to exons EDIIIA and EDIIIB, two alternative pathways are available whereby the whole exon is either included or omitted from the mRNA (Figure 1B). The third region of alternative RNA processing, IIICS, is a type III homology connecting segment. The splicing pathways in IIICS are more complex and processing occurs via the selective use of alternative 5′ and 3′ splice junctions within the coding region (16).

Differential tissue specific expression of exon EDIIIA is well defined, and the expression of EDIIIA splice variants is modulated during development and by oncogenic transformation. The 100% omission of EDIIIA during RNA

processing, generating exclusively EDIIIA$^-$ mRNA and protein, has only been observed so far in plasma Fn. There is a switch in the splicing pathways which results in the expression of a proportion of EDIIIA$^+$ (cellular) Fn isoforms in transformed liver cells, in liver cells from early developmental stages and in cells from other tissues (our unpublished observations). A simplified pre-mRNA that faithfully reproduces natural splicing patterns can be obtained by transient expression in HeLa cells of an α globin/Fn mini gene hybrid containing exon EDIIIA. In this system, all of the exon/intron borders are spliced accurately as shown by SI mapping of the transcripts in previous experiments (17). The EDIIIA region of human Fn has provided us with a naturally-occurring model system of alternative splicing. We have exploited these features to investigate the possible role of sequences within the exon in the mechanism of alternative splicing.

MATERIALS and METHODS

Construction of α globin/Fn hybrid genes

1) FNED:- A 1.3kb PstI/BstEII genomic Fn fragment consisting of the 3′ 12 bp of exon -1, intron -1, and the 5′ 100bp of EDIIIA was cloned into the HincII site of pSP64 (24);a 2 kb BstEII/BstEII genomic Fn fragment consisting of the 3′ 170 bp of EDIIIA, intron +1 and the 5′ 105 bp of exon +1 was cloned into the BamHI site of the same pSP64/Fn clone. The pSP64 polylinker sequence containing both halves of the Fn minigene was excised with HindIII and EcoRI and cloned into the BstEII site in the third exon of the human α globin gene in the expression construct pSVED α 1W which also carries the SV40 origin of replication and early promoter region (25). Polylinker restriction sites were thus available which were used to manipulate the Fn gene in pSP64. The mutant Fn minigenes were then excised and cloned into psVEDαIW as for FNED producing the following constructs. 2) FNEDΔ81:-the XbaI/StuI 81 bp fragment was excised from EDIIIA, the isolated plasmid blunt-ended using the Klenow fragment of DNA polymerase I and the ends religated using T4 DNA ligase. 3) FNEDREC81S:- the XbaI/StuI fragment from FNEDΔ81 was blunt-ended and cloned into the FNEDΔ81 at XbaI/StuI in sense orientation, thus reconstructing FNED with minor sequence changes at the XbaI site. 4) FNEDREC81aS:- The XbaI/StuI blunt-ended fragment was cloned back into the FNEDΔ81 at XbaI/StuI in antisense orientation. The clones were analysed at each stage of subcloning by PAGE of [α-^{32}P] dATP end-labelled restriction fragments.

DNA transfections and analysis of RNA

HeLa cells were maintained in 150mm Petri dishes in Dulbecco's MEM containing 10% foetal calf serum. Subconfluent cultures were transfected with 25μg of a plasmid DNA construct by Ca PO_4 precipitation (26). Cells were cotransfected with an equal amount of p βS'SVBglII (27) which expresses the large T antigen necessary for SV40 replication. The culture medium was changed 8h after transfection.

Total cell RNA was prepared from transfected cells after 48 h by lysis in guanidine thiocyanate followed by centrifugation through high density CsCl gradients (28). 15μg of each RNA sample was run through 1.3% agarose denaturing gels, and RNA species transferred onto nitrocellulose filters (Schleicher and Schuell) by blotting with 20 x SSC (29). Filters were baked for 2h at 80°C, and pre-hybridised in 5xSSC; 50% deionised formamide; .04% Denhardts solution; 1% sodium dodecyl sulphate; 250μg/ml denatured herring sperm DNA for 4h at 42°C. 2-3 x 10^6 cpm (sp.act. 10^8 cpm/μg) of denatured DNA probe was added and hybridised for 16h at 42°C.

Blot A was hybridised with a fragment from a human Fn cDNA clone pFH23 (12) which was nick translated in the presence of [α-^{32}P] dATP and hybridises to both ED^+ and ED^- mRNA. Blot B was hybridised with a fragment specific to EDIIIA cloned into M13mp9 (17) which was primer-extended in the presence of [α-^{32}P] dATP.

RESULTS and DISCUSSION

All four splice junctions involved in alternative splicing of EDIIIA contain sequences which conform to the consensus donor and acceptor sequences (17,18). We investigated the possible participation of intra-exonic sequences (which may be unique to Fn EDIIIA exon) in alternative splicing by testing the qualitative splicing capacity of EDIIIA mutant constructs carrying an 81 bp deletion within the 270 bp EDIIIA exon. This 81bp sequence is located within the region corresponding to least amino acid sequence homology with other type III repeating units, and was chosen because it represents a contiguity found only in the alternatively spliced exons (EDIIIA and EDIIIB) coding for complete type III amino acid sequence homologies.

Construction of an artificial α-globin/Fn minigene hybrid provides an excellent tool for studying specific structural features in a small region of the Fn gene. To facilitate the manipulation of EDIIIA sequences, a construct which incorporates polylinker sequences within the EDIIIA exon and

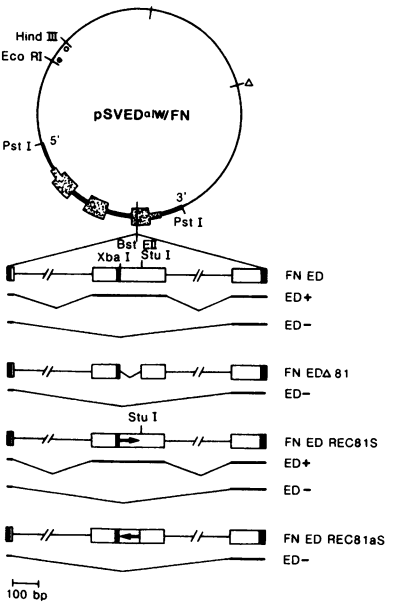

Fig. 2. Construction of α globin/Fn minigene hybrids in expression vector pSVED. △ is a deletion in pBR32; o and e are the origin of replication and early promoter of SV40. Stippled boxes and connecting lines represent human α globin exon sequences and introns. Fn genomic fragments cloned into the BstEII site of α globin are shown underneath. Empty boxes represent Fn exon sequence; connecting lines correspond to introns; solid boxes are pSP64 polylinker sequence. The middle Fn exon in each construct is EDIIIA and exons -1 and +1 are 5′ and 3′ with respect to ED. The 81bp deletion is represented by ⌄ and the orientation of the 81bp fragment in FNEDREC81S and FNEDREC81aS is indicated by the direction of the arrow. The pathways of splicing during processing of the pre-mRNA according to the data shown in Figure 3, are shown under each construct.

at the 5′ and 3′ ends of the Fn minigene in the α globin/Fn expression construct (construct FNED in Figure 2) was designed. The mutants FNEDΔ81, FNEDREC81S and FNEDREC81aS shown in Figure 2 were constructed from FNED. These minigenes retain all the relevant Fn splice junction sequences.

Northern blot analysis of total cell RNA from HeLa cells transfected with the α globin/Fn expression constructs is shown in Figure 3. Blot A was hybridised with a cDNA probe containing EDIIIA plus flanking sequences and therefore both ED^+ and ED^- RNA species can be detected. Confirmation that the larger mRNA bands in blot A contain EDIIIA was given by hybridisation of duplicate RNA samples on blot B to a probe specific to EDIIIA. The splicing pathways used in processing of the RNA during transient expression of the

constructs is summarised underneath each construct in Figure 2. The alternative splicing of EDIIIA was retained by construct FNED from which both ED^+ and ED^- transcripts were generated, thus establishing that the additional polylinker sequences did not affect alternative processing pathways.

The mutant construct FNEDΔ81 has the 81bp deletion in central region of EDIIIA. This construct was not permissive for alternative splicing since one mRNA species (blot A in Figure 3) was processed via a pathway which skipped the EDIIIA exon generating ED^- mRNA. This was confirmed by the absence of hybridisation of the mRNA to the EDIIIA probe in blot B, Figure 3. Reinsertion of the 81bp fragment into FNEDΔ81 in the correct orientation at the site of excision produced the construct FNEDREC81S which, compared to FNED, has minor base changes at the sites of reinsertion. In transient expression of this construct alternative splicing activity was reconstituted via pathways which generated both ED^+ and ED^- mRNA (two bands in blot A; the upper band corresponds to Ed^+ as confirmed in blot B, Figure 3). The fourth construct, FNEDREC81aS in which the 81bp fragment deleted in FNEDΔ81 was cloned back into the deletion in antisense orientation, did not reconstitute alternative splicing activity and, as in the case of deletion mutant FNEDΔ81, only ED^- mRNA was generated.

In those mRNA's in which EDIIIA was spliced out (i.e. from FNEDΔ81 and FNEDREC81aS) the exon was present in the pre-mRNA, as shown in the Northern blot in Figure 3, blot B. It is interesting to note that the mRNA precursors from constructs FNED and FNEDREC81S appear to be more abundant than those from FNEDΔ81 and FNEDREC81aS in Figure 3. The fact that those precursors which undergo alternative splicing are more abundant than those which generate only ED^- mRNA may reflect a difference in the kinetics of RNA processing when different splicing options are available.

These data demonstrate that exon EDIIIA in the human Fn gene contains an 81bp sequence located in the central region of the exon which is required for alternative splicing. This requirement is directional, which further suggests that the sequence is of functional rather than simply spatial importance in alternative splicing. Two further constructs separately reconstructing the 26 bp 5' region and the 55 bp 3' region of the deleted 81 bp fragment, did not reconstitute alternative splicing activity (data not shown).

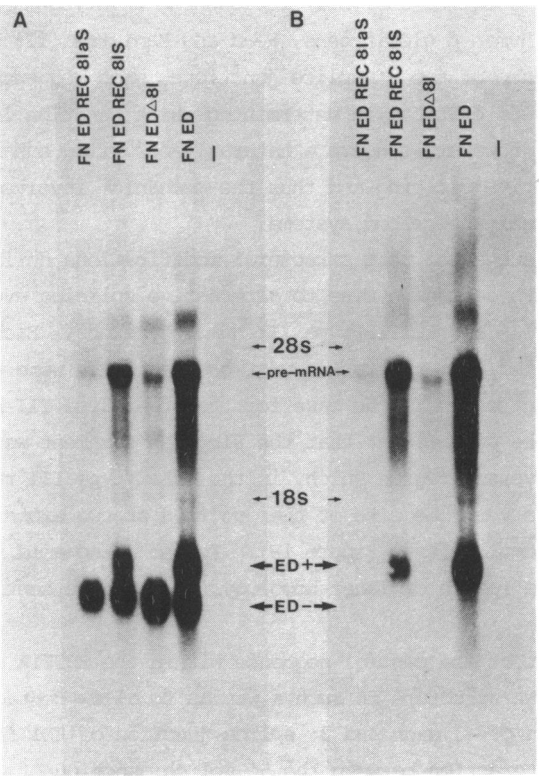

Fig. 3. Northern blot analysis of total RNA recovered from HeLa cells transfected with α globin/Fn minigene hybrid constructs. Blot A was hybridised with a fragment from a Fn cDNA, pFH23, which hybridises to mRNA's which contain EDIIIA (ED^+, upper band of 930bp) and those which do not (ED^- lower band, 660bp). Blot B was hybridised with a DNA fragment specific to EDIIIA in M13, confirming that the larger transcripts from in blot A are ED^+ and the smaller transcripts are ED^-. Neither probe hybridised to RNA from mock-transfected HeLa cells (track -). Higher molecular weight Fn mRNA precursors from all the constructs (pre-mRNA) hybridised to both pFH23 and the EDIIIA probes. The positions of the 18S and 28S ribosomal RNA bands are marked by arrows.

Recently, various structural features which modify splice site selection and alternative splicing patterns in artificially created gene systems have been described These features include RNA secondary structure (19), and the integrity of splice site sequences within a hierarchy of splice sites (20). Mutation of exon sequences has been found to quantitatively modulate alternative splicing of SV40 large T and small t

exons (21). In a detailed analysis of sequences involved in splice site selection in the human β globin gene, Read and Maniatis (22) demonstrated that in the presence of two 3′ splice junctions, exon sequences adjacent to the 3′ junction were critical in determining which junction is selected. Our observations, however, concern a naturally-occurring eukaryote gene system of alternative splicing and thus the mechanism involved may not be related to previously described systems.

It has been suggested that structural modifications during the evolution of the Fn gene gave rise to alternative splicing events (23) which are unique to EDIIIA and EDIIIB type III homology exons. Each of these two exons may be derived from the fusion of two exons which were at some stage separated by an intron as is the case for the other type III homology coding regions. It may be significant that the 81bp DNA fragment we have described in exon EDIIIA covers a region which, in the other type III repeating units, corresponds to the variable site of the junction of two exons coding for a single type III homology (see Figure 1B). In the amino acid sequence this corresponds to the region of least homology found in the centre of type III repeating units.

It is clear that the central sequence within the EDIIIA exon must modify the pre-mRNA structure in such a way as to allow contact between the 5′ splice junction of -1 exon and 3′ splice junction of EDIIIA without preventing the interaction between the 5′ splice junction of -1 exon and the 3′ splice junction of +1 exon. Furthermore, it should be noted that there is no obvious structural feature which would prevent the 100% inclusion of EDIIIA since all the splice junctions appear to be normal. Other features, as yet unknown, repress the splicing pathway which includes EDIIIA in the mRNA. This is counteracted by the functionality of a sequence or sequences within the central 81 bp in EDIIIA, which in turn may involve regulation by tissue-specific cellular factors.

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