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Alternative Splicing of Fibronectin mRNAs in Chondrosarcoma Cells: Role of far Upstream Intron Sequences

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Abstract The fibronectin (FN) gene encodes multiple mRNAs through the process of alternative splicing, and production of certain isoforms is characteristic of a given cell type. Chondrocytes produce FNs that completely lack alternative exon EIIIA, and loss of inclusion of the exon is tightly linked to chondrogenic condensation of mesenchymal cells. The inclusion of a second exon, EIIIB, is high in embryonic cartilage, but declines with age. Multiple exons are omitted to produce the (V + C)-form that is highly specific for cartilage and chondrocytes. A rat chondrosarcoma cell line, RCS, was identified that preserves key features of the cartilage-specific splicing phenotype. RCS cells, which exclude exon EIIIA, and HeLa cells, which include exon EIIIA similar to mesenchymal cells, were used to assess the contribution of intron sequences flanking exon EIIIA to splicing regulation. Deletion of most of the intron downstream of the exon had little effect on splicing in either cell type. However, deletions within upstream intron 32-A reduced inclusion of the alternative exon in both cell types. The sequences involved lie more than 200 nucleotides away from the exon, but could not be localized to a single region by deletion mapping. These intronic sequences contribute to the efficiency of exon EIIIA recognition, but not to cell-type specific regulation. The normally inhibitory factor polypyrimidine tract binding protein promotes exon EIIIA inclusion in a manner that is partially dependent on the regulatory sequences within intron 32-A. J. Cell. Biochem. 90: 709–718, 2003.

Key words: extracellular matrix; mRNA splicing; intronic splicing enhancer; PTB

Most vertebrate genes are split into two or more exons separated by introns; the introns must be removed from the primary transcript and the exons spliced together. Genes may have dozens of exons that must be spliced precisely to yield a correct protein product. Although terminal exons tend to be a few hundred nucleotides in length, internal exons typically are short, with most less than 300 base pairs. In contrast, intron length is more variable, with lengths of more than 1 kb common in human genes [Consortium, 2001]. The cellular splicing machinery must be able to locate exons accurately, but the sequence signals present at intron–exon junctions are quite short, suggesting that other factors contribute to exon recognition. A complex contribution of sequence context to splice site recognition is emphasized by the discovery that genes may produce two or more transcripts due to alternative mRNA splicing. Estimates of the number of genes that undergo alternative splicing range as high as 40–60% [Modrek and Lee, 2002]. Although there are no rules that apply to all alternative splicing events, alternative exons tend to be flanked by weak splicing signals, which might favor regulation [Chabot, 1996; Black, 2000]. Many alternative exons contain exonic splicing enhancer elements (ESEs), and ESEs may be paired with nearby silencer elements [Smith and Valcárcel, 2000]. Intronic sequences that lie outside the consensus splice site signals and branch site have also been implicated in regulating splicing events [Modafferi and Black,

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1997; Goldstrohm et al., 2001; Hastings et al., 2001].

One of the first cellular genes shown to produce multiple mRNAs via alternative splicing is that encoding the extracellular matrix (ECM) protein fibronectin (FN). Patterns of FN alternative splicing vary in a cell and tissue-specific manner [ffrench-Constant, 1995], presumably as a means to modulate the structural and functional properties of the local ECM. One well-characterized example of regulated FN splicing has been the shift in splicing of alternative exon EIIIA that accompanies the differentiation of mesenchyme into cartilage. FN mRNAs from undifferentiated chick limb mesenchyme include exon EIIIA (A+ mRNA), whereas the condensation of mesenchymal cells associated with chondrogenic differentiation is accompanied by loss of EIIIA (A− mRNA) [Bennett et al., 1991; Gehris et al., 1996]. A similar cartilage-associated loss of exon EIIIA inclusion is seen in mammalian species [Zhang et al., 1995; Peters et al., 1996]. The functional importance of this change is suggested by studies using antibodies to the EIIIA segment to perturb chondrogenesis in vitro and limb formation in ovo [Gehris et al., 1997]. More recently, a novel FN isoform has been described that is apparently restricted to chondrocytes and cartilage [MacLeod et al., 1996; Burton-Wurster et al., 1997]. Production of the isoform, (V + C)-, involves the omission of three adjacent exons, resulting in a protein deleted internally for the corresponding protein coding segments.

In previous studies, we described splicing reporter RSV-chA and identified exon sequences and cellular factors involved in the cell type-specific regulation of FN alternative exon EIIIA splicing [Uporova et al., 1999; Kuo et al., 2002]. Analysis of reporter-derived mRNAs demonstrated that exon EIIIA was included in chick limb mesenchymal cells and mostly excluded in vertebral chondrocytes, consistent with the behavior of the endogenous gene. However, the transfection efficiency of primary cell types was low and variable, motivating us to investigate whether established cell lines could provide similar splicing environments. A+ mRNA was produced efficiently in HeLa cells transfected with RSV-chA, suggesting that these cells possess a splicing environment similar to the mesenchymal cells [Kuo et al., 2002]. In the present study, we have characterized the FN mRNA isoform content of a chondrosarcoma cell line, RCS, and demonstrated the presence of isoforms typical of chondrocytes and cartilage. RCS cells also splice the RSV-chA reporter in a manner resembling chondrocytes. This pair of cell lines was used to evaluate the influence of intron sequences on alternative splicing of exon EIIIA and to investigate the effect of overexpression of the splicing factor polypyrimidine tract binding protein (PTB).

MATERIALS AND METHODS

Cell Culture and Transfection

RCS swarm rat chondrosarcoma cells [Oegama et al., 1975; Smith et al., 1975] were obtained from J. Oxford (Boise State University), and cultured in DMEM supplemented with 10% fetal calf serum. RCS cells were transfected with 3.0 μg of each splicing reporter construct (or as indicated) using Superfect (Qiagen). HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% horse serum. HeLa cells were transfected with 4 μg of each splicing reporter using Geneporter2 (Gene Therapy Systems, San Diego, CA).

Plasmids Used

The construction of splicing reporter construct RSV-chA was described previously [Uporova et al., 1999]. Briefly, a 3.5 kb fragment of the chicken FN gene containing alternative exon EIIIA along with flanking introns and exons have been placed under the control of the RSV LTR; the SV40 late poly A site provides the signals for 3′ end maturation. Deletion mutants were generated using available restriction sites, as indicated in Figures 2 and 3. To generate RSV-chAXN+, complementary oligodeoxynucleotides XNS and XNAS (Table I) were annealed and ligated between the XmaI and NheI sites. An expression construct for PTB-1 was prepared by subcloning the insert from pET-PTB ([Pérez et al., 1997] kind gift of J. Patton, Vanderbilt Univ.) into pCMV-Sport6 (Invitrogen, Carlsbad, CA), generating pCMV-PTB

RNA Isolation and RT-PCR Analysis of Endogenous FN mRNAs

Procedures for RNA isolation and RT-PCR analysis of exon EIIIB inclusion were performed as described previously [Górski et al., 1996], using primers AF, AR, BF, and BR

TABLE I. Oligodeoxynucleotides Used in This Study

Primer	Sequence (5′ to 3′)	Orientation	Location
VF	GGAGCCAGGAACCGAGTACACC	Sense	86 nt 5′ of exon V
VR	ATCTTGTAGTTGACACCGTT	Antisense	445 nt 3′ of exon V
BF	CATGCTGATCAGAGTTCCTG	Sense	121 nt 5′ of exon EIIIB
BR	GGTGAGTAGCGCACCAAGAG	Antisense	115 nt 3′ of exon EIIIB
AF	GAAATGACCATTGAAGGTTTG	Sense	106 nt 5′ of exon EIIIA
AR	TTCTTTCATTGGTCCTGTCTT	Antisense	140 nt 3′ of exon EIIIA
LPA3	CATCAATGTATCTTATCATGTC	Antisense	Vector 3′ untranslated
11F	ATGGTCAGCGTCTATGCTCAGAA	Sense	Exon 32
12R	CCCGGTCTTCTCTTTGGGGTTCAC	Antisense	Exon 33
XNS	CCGGGCTGAGACTGCCATCCTGCTGTCTGGCG	Sense	Intron 32-A
XNAS	CTAGCGCCAGACAGCAGGATGGCAGTCTCAGC	Antisense	Intron 32-A

(Table I). Amplification of the (V + C) region was performed using the Elongase kit (Invitrogen) as described [Górski et al., 1996], using primers VF and VR (Table I). PCR was performed for 30 cycles of 0.5 min at 94°C, 0.5 min at 55°C, and 1.5 min at 68°C.

RT-PCR Assay for Exon EIIIA Splicing

Procedures were essentially as described previously [Kuo et al., 2002]. Total cellular RNA was isolated from cells 48 h post-transfection using TRIzol (Invitrogen), DNaseI treated, and resuspended in 10 μl of water. Reverse transcription (RT) was performed with Superscript II MoMLV reverse transcriptase (Invitrogen) and primer LPA3 (Table I), which is specific for minigene-derived mRNAs. Amplification was performed with Taq DNA polymerase (Stratagene, La Jolla, CA) for 25 (HeLa) or 35 (RCS) cycles of 1 min at 94°C, 1 min at 52°C (HeLa) or 50°C (RCS), and 1 min at 72°C. Forward primer 11F was used for PCR along with primers 12R for HeLa RNA or LPA3 for RCS RNA. PCR products were separated on 5% polyacrylamide gels and visualized by ethidium bromide staining. Images were captured digitally using an AlphaInnotech imaging system with AlphaEase software (Alpha Innotech Corp., San Leando, CA).

RNA Sequence Analysis

Prediction of RNA secondary structure was performed using mfold [Mathews et al., 1999]. Analysis of pentameric repeats found to be enriched in small human introns [Lim and Burge, 2001] was performed using Findpatterns in the Wisconsin Package Version 10.3 software suite (Accelrys, Inc., San Diego, CA).

RESULTS

Characterization of FN mRNA Isoforms in RCS Cells

The FN gene can produce multiple mRNAs due to alternative splicing events that occur at three positions (Fig. 1A). As previously reported [Kuo et al., 2002], and shown in Figure 1B, RCS cells derived from the rat Swarm chondrosarcoma produced FN mRNAs that lack alternative exon EIIIA, as is characteristic of primary chondrocytes derived from embryonic cartilage [Bennett et al., 1991]. Other tested chondrocytic cell lines produced both A+ and A− FN mRNAs (data not shown). To fully catalog the FN mRNA isoform content of RCS cells, RT-PCR was performed to assess whether these cells omit or include exon EIIIB, as well as their ability to produce the (V + C)-isoform that is highly chondrocyte-specific. This analysis demonstrated

TABLE II. Ratio of A+/A− mRNA Produced by Each Construct

	HeLa[a]	Weighted[b]	RCS[a]	Weighted[b]
RSV-chA	7.2+/−1.8	3.0	0.98+/−0.21	0.44
RSV-AXP	4.0+/−0.4	1.7	0.47+/−0.04	0.22
RSV-AXN	2.7+/−0.4	1.1	0.42+/−0.02	0.19
RSV-AXS	7.5+/−1.5	3.1	0.82+/−0.13	0.37
RSV-ASN	5.7+/−1.5	2.4	0.73+/−0.04	0.33
RSV-AXA	5.6+/−0.4	2.3	0.97+/−0.09	0.44
RSV-AAN	2.5+/−0.6	1.0	0.59+/−0.10	0.27

[a]The relative ratio of A+ to A− mRNA was quantitated for each sample, and then mean and standard error were calculated. Each mean was derived from at least three transfection experiments.
[b]Weighted; ratios calculated from integrated densities have been adjusted for the relative length of the PCR products so as to more accurately reflect the relative abundance of the two RNA isoforms.

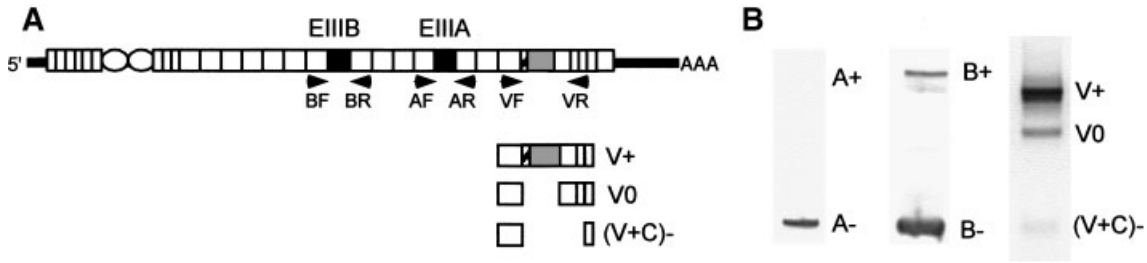

Fig. 1. Characterization of FN mRNA isoforms produced by RCS cells. **A**: Schematic diagram of mature FN mRNA, reflecting the modular structure of the encoded protein, with the open rectangles, ovals, and squares denoting repeating protein domains of structures FN types I, II, and III, respectively. Alternative segments EIIIB and EIIIA are indicated as filled squares. Gray shaded and striped segments represent the V (for variable) region. The bars represent the 5′ and 3′ untranslated regions. The variant amplification products derived from alternative splicing in the vicinity of the V region are diagrammed below. Arrowheads indicate primers used for amplification. **B**: RT-PCR detection of exon EIIIA (**left**) and exon EIIIB (**center**) alternative splicing, and V+C region splicing (**right**). Identity of PCR products is indicated along with the predicted position of band derived from the A+ form. Product structure was confirmed by DNA sequencing. Images of ethidium bromide stained gels are contrast reversed to enhance detection of faint bands. Fragment lengths are A+, 515 bp; A−, 245; B+, 505 bp; B−, 232 bp; V+, 891 bp; V0, 531 bp; (V + C)-, 122 bp.

that approximately 25% of mRNAs included the exon EIIIB (Fig. 1B). Amplification across the region including the V segment revealed mostly V+ FN, but the highly cartilage specific (V + C)-isoform was detected, along with the V0 isoform that lacks the entire V region, but retains the C segments (Fig. 1B). Adjusting for the different lengths of the PCR products, which affects staining intensity, the ratio of V+:V0: (V + C)- was 57:24:19. Thus, the RCS cells preserve a splicing phenotype highly characteristic of differentiated chondrocytes.

Behavior of a Splicing Reporter in a Chondrocytic Cell Line

To evaluate whether RCS cells would be useful for transient transfection analysis of exon EIIIA splicing, the splicing reporter RSV-chA was introduced into RCS cells (a chondrocytic splicing environment) or into HeLa cells (a non-chondrocytic environment). Two days post-transfection, RNA was isolated and RT-PCR was performed to measure the relative ratio of A+ and A− mRNA. As expected from previous results [Kuo et al., 2002], transfection of HeLa cells with RSV-chA yielded predominantly A+ mRNA (Fig. 2B, lane 1). In contrast, transfection of RCS cells resulted in a mixture of A+ and A− forms derived from the splicing reporter (Fig. 2B, lane 3). This pattern resembled the pattern observed with the RSV-chA reporter in primary chondrocytes [Uporova et al., 1999; Kuo et al., 2002], in that splicing of reporter-derived mRNAs is not as tightly regulated as endogenous mRNAs. The production of both A+ and A− mRNAs in RCS cells offers a cellular context in which to assay both positive and negative roles of *cis*-acting sequences and *trans*-acting factors on exon EIIIA splicing.

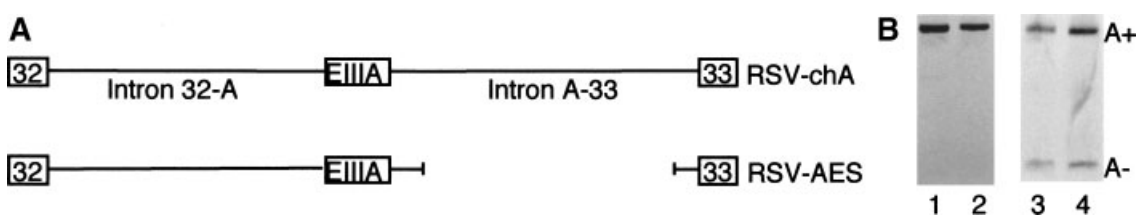

Fig. 2. A large deletion within downstream intron A-33 does not affect exon EIIIA splicing. **A**: Schematic diagram of splicing reporter RSV-chA. Boxes, exons; lines, introns. Each intron is approximately 1.5 kb in length. Below, diagram of intron A-33 deletion construct RSV-AES. The EcoNI site forms the left deletion boundary; the StuI site forms the **right**. **B**: RT-PCR analysis of exon EIIIA splicing. HeLa cells (**lanes 1** and **2**) or RCS cells (**lanes 3** and **4**) were transfected with RSV-chA (lanes 1 and 3) or RSV-AES (lanes 2 and 4). RT-PCR was used to determine the structure of mRNAs derived from the splicing reporter. The positions of bands corresponding to A+ and A− mRNA isoforms are indicated at the **right**. Images are contrast reversed to enhance detection of faint bands.

Effect of a Large Deletion Within Intron A-33 on Exon EIIIA Inclusion

Several deletions were introduced into splicing reporter RSV-chA to evaluate the potential contribution of intron sequences to exon EIIIA splicing. As many intronic regulatory elements lie downstream of the target exon, a 1.25 kb EcoNI–StuI fragment was deleted from the central region of the 1.54 kb intron A-33 that lies downstream of alternative exon EIIIA. The resulting plasmid, RSV-AES (Fig. 2A), lacked all but 191 nucleotides adjacent to exon EIIIA and 93 nucleotides upstream of exon 33. HeLa cells and RCS chondrosarcoma cells were transfected with RSV-AES and splicing was analyzed by RT-PCR assay as above. No obvious difference in the splicing of exon EIIIA in either cell type was observed as a consequence of the deletion within intron A-33 (Fig. 2B, compare lanes 1 vs. 2, and 3 vs. 4). Densitometric analysis of two independent transfections established A+:A− mRNA ratios of 2.2 for RSV-chA and 1.8 for RSV-AES, suggesting that the deleted intron A-33 sequences have little effect on exon EIIIA splicing.

Effects of Deletions Within Intron 32-A on Exon EIIIA Inclusion

A similar approach was taken to address the possible role of upstream intron 32-A sequences in exon EIIIA splicing. First, a deletion of 1.34 kb was introduced into 1.49 kb intron 32-A, generating plasmid RSV-XP (Fig. 3A). The deletion left 82 nucleotides adjacent to exon EIIIA and 74 nucleotides adjacent to exon 32. HeLa and RCS cells were transfected with RSV-XP, and the resulting mRNA analyzed as above. A decrease in the ratio of A+/A− mRNA relative to RSV-chA was observed in the transfected HeLa cells (Fig. 3B, compare lanes 1 and 2). A comparable change in splicing was observed in the RCS cells as a consequence of the deletion (Fig. 3C, lanes 1 and 2). Thus, sequences within intron 32-A positively influenced the inclusion of exon EIIIA.

To localize the sequences within intron 32-A that modulate exon EIIIA splicing, additional deletion mutants were analyzed (Fig. 3A). The deletion in RSV-XP interrupted a conserved region that lies ca. 90 nucleotides upstream of exon EIIIA [Norton et al., 1998]. These sequences, as well as additional 100 nucleotides, were re-inserted in plasmid RSV-XN, and splicing pattern determined in HeLa and RCS cells. Restoration of these sequences did not revert splicing to wild type in either cell type (Fig. 3B,C, lanes 3). Next, the two halves of the intron were deleted separately in plasmids RSV-XS and RSV-SN. Both of these plasmids behaved similarly to RSV-chA in both cell types (Fig. 3B,C, lanes 4 and 5), suggesting that

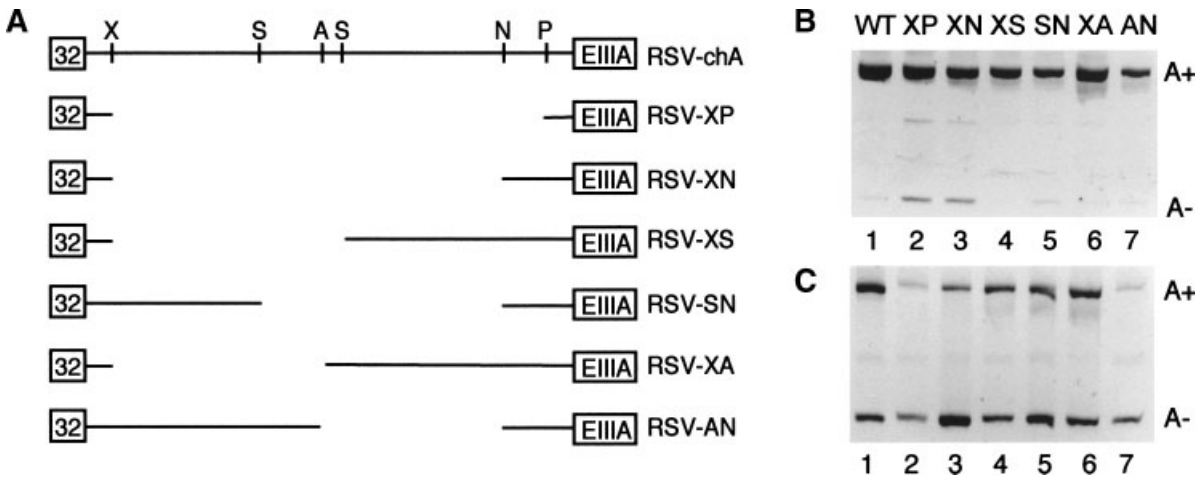

Fig. 3. Certain deletions within upstream intron 32-A reduce exon EIIIA splicing. **A**: Top, diagram of intron 32-A in RSV-chA. Restriction sites used in the construction of various deletion mutants, below, are indicated. X, XmaI; S, SacI; A, AccI; N, NheI; P, NspI. **B**: RT-PCR analysis of exon EIIIA splicing. HeLa cells were transfected with RSV-chA (**lane 1**, WT) or the indicated deletion mutant construct (**lanes 2–7**; only the last two letters of each construct are given). **C**: RT-PCR analysis of exon EIIIA splicing. RCS cells were transfected with RSV-chA (**lane 1**) or the indicated deletion construct (**lanes 2–7**). In panels B and C, the positions of bands corresponding to A+ and A− mRNA isoforms are indicated at the **right**. Images are contrast reversed to enhance detection of faint bands.

either half of the intron could restore higher levels of exon EIIIA inclusion. This finding could be explained by functional redundancy, with equivalent regulatory elements residing in both halves of the intron. To explore this possibility further, plasmids RSV-XA and RSV-AN were generated and analyzed as above. RSV-XA produced the same ratio of mRNA isoforms as the parent construct (lanes 6), but RSV-AN behaved the same as the AXP and AXN deletions (lanes 7). The relative ratio of A+/A− mRNAs produced by all the constructs is summarized in Table II.

Inspection of intron 32-A revealed a short stretch of 30 nucleotides containing five copies of the tri-nucleotide 5′-CUG immediately upstream of the NheI site used to generate deletion RSV-XN (Fig. 4A). As CUG repeats have been implicated in alternative splicing (see Discussion), the possible involvement of these sequences in exon EIIIA splicing was tested by reinsertion of the CUG-rich stretch into RSV-XN, creating RSV-XN+. RCS cells were transfected with RSV-XN+ and the ratio of spliced products determined. Replacement of the CUG-rich region did not revert splicing of exon EIIIA to wild type levels in RCS cells (Fig. 4B) or in HeLa cells (data not shown). These results suggest that the CUG-rich stretch is not sufficient to restore efficient splicing of intron 32-A.

PTB Increases Exon EIIIA Inclusion in a Manner Partially Dependent on Intron 32-A

Splicing factor PTB has been shown to repress the inclusion of several alternative exons, including FN exon EIIIB [Wagner and Garcia-Blanco, 2002]. To evaluate whether this factor plays a similar role with respect to exon EIIIA, RCS cells were co-transfected with RSV-chA and an expression construct for PTB, pCMV-PTB. PTB over-expression resulted in an increased level of exon EIIIA inclusion (Fig. 4C). Densitometric analysis of three experiments indicated a 1.9 fold increase in exon EIIIA inclusion versus co-transfection of the splicing reporter with vector alone. To evaluate whether this PTB-dependent increase required sequences within intron 32-A, a similar co-transfection experiment was performed using splicing reporter RSV-XN (Fig. 4C). As expected, the ratio of A+:A− was lower compared with RSV-chA, but a modest increase in A+ mRNA was observed with PTB co-transfection. Densitometric analysis of three independent transfections revealed that PTB over-expression increased exon EIIIA inclusion by an average of 1.4 fold, suggesting that the deleted intron sequences play a role in PTB-dependent exon EIIIA inclusion.

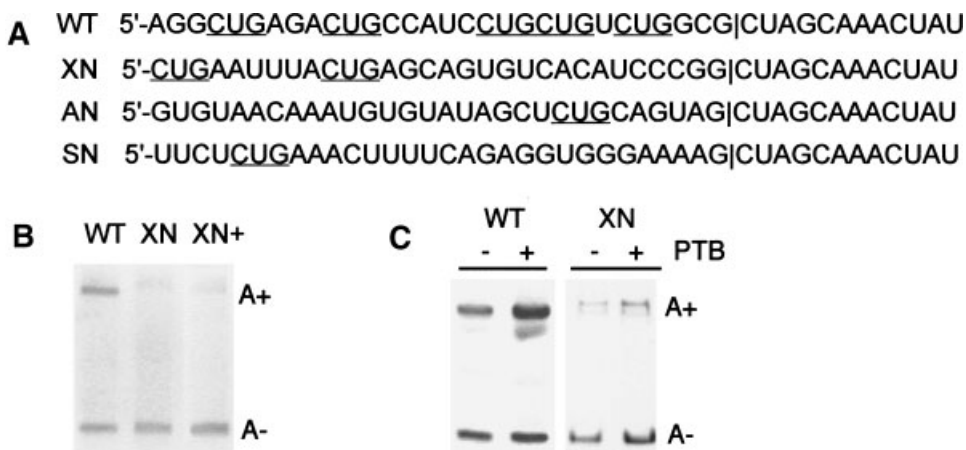

Fig. 4. Role of specific intron 32-A sequences and splicing factor PTB in exon EIIIA splicing. **A**: Comparison of the sequences juxtaposed near exon EIIIA. The sequences to the **right** of the vertical line are those found in intron 32-A beginning at the NheI site approximately 200 nucleotides upstream of the exon. The sequences to the left of the line are those found immediately upstream of the NheI site in RSV-chA (WT) or various deletion constructs, as indicated. The positions of bands corresponding to A+ and A− mRNA isoforms are indicated at the **right**. Images are contrast reversed. **B**: RT-PCR analysis to compare splicing of mRNAs derived from RSV-XN+ with RSV-XN and wild-type (WT) RSV-chA. RCS cells were transfected with the indicated construct and RT-PCR was performed as above. **C**: RT-PCR analysis to assess the ability of PTB to affect exon EIIIA splicing. RCS cells were transfected with 1 μg of RSV-chA (WT) or RSV-XN (XN), and 2 μg of either pCMV-Sport6 (−) or 2 μg of pCMV-PTB (+) and mRNAs were detected by RT-PCR. A+ and A− mRNAs are indicated at the **right**.

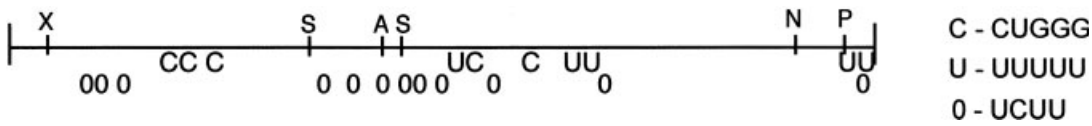

Fig. 5. Analyses of sequences within intron 32-A. The intron is diagrammed with the relevant restriction sites indicated as in the legend to Figure 2. The symbols indicating the location of the pentameric repeats (see text) and predicted PTB binding sites are defined at the **right**.

DISCUSSION

An initial goal of the present study was to complete the characterization of the FN isoforms produced in RCS chondrosarcoma cells and establish whether these cells preserve a chondrocytic splicing environment. Previous work had demonstrated that exon EIIIA was omitted in these cells [Kuo et al., 2002], typical of chondrocytes and cartilage of both embryonic and adult origin [Bennett et al., 1991; Rencic et al., 1995; Zhang et al., 1995; Peters and Hynes, 1996; Peters et al., 1996]. Approximately 25% of FN mRNAs in RCS cells included the alternative EIIIB exon, which corresponds well to estimates for most adult cartilages derived from humans or other mammals [Rencic et al., 1995; Zhang et al., 1995]. The RCS cells produced the highly cartilage-specific (V+C)-isoform as well as the longer forms that include all or part of the V region, a pattern that resembles that seen in freshly isolated chondrocytes, although the (V+C)-isoform is lost with prolonged culture [MacLeod et al., 1996]. The (V+C)-form is apparently absent from non-cartilage tissues [MacLeod et al., 1996; Matsui et al., 2001; Parker et al., 2002]. Thus, the presence of this diagnostic isoform in the RCS cells, the low level of EIIIB+ mRNA, and the production of all A− FN mRNAs together indicate that this cell line preserves a chondrocyte-specific splicing environment. Although the exact roles of the various chondrocyte and cartilage-enriched FN isoforms remain to be established, the protein segment encoded by exon EIIIA appears to be important for correct chondrogenic condensation in vitro and in vivo [Gehris et al., 1997]. As discussed in a recent review, FN isoforms may possess distinct cell signaling properties via differential interactions with the cell surface, and alter ECM architecture via differential interactions with other ECM components [Burton-Wurster et al., 1997].

The above results suggested that RCS cells might be useful for studies of FN alternative splicing, as they were for identifying *cis*-acting sequences involved in chondrocyte-associated splicing of procollagen α1(XI) mRNAs [Chen et al., 2001]. Thus, RCS cells were employed to dissect potential intronic *cis*-acting sequences that influence exon EIIIA splicing. The results of the present study indicate that sequences within upstream intron 32-A that lie 200 or more nucleotides away from alternative exon EIIIA play a positive role in the inclusion of the exon. These intronic sequences promote exon EIIIA inclusion in a non-cell type specific manner, as their deletion had similar effects in both HeLa and RCS cells. The fairly high level of exon inclusion observed with RSV-XP suggested that all sequences needed for use of the 3′ splice site, including branch site and polypyrimidine tract, must be present within the 84 nucleotides that remain adjacent to exon EIIIA. We had previously predicted that the most likely branch site sequence was at −27 relative to exon EIIIA [Norton et al., 1998]; current results are consistent with use of that site.

Several reports have identified sequences downstream of alternative exons that play a role in the selection of that exon by the splicing machinery (for instance, see [Modafferi and Black, 1997] and references therein). Most of the sequences within downstream intron A-33 appear to have no effect on exon EIIIA inclusion. The absence of a requirement for sequences within this intron contrasts with the situation within another alternative exon of the FN gene, exon EIIIB. Inclusion of exon EIIIB requires multiple copies of the hexameric sequence UGCAUG; there are eight exact copies distributed throughout the ca. 1 kb intron [Huh and Hynes, 1993, 1994; Lim and Sharp, 1998]. This hexamer has been implicated in the splicing of other alternative exons [Kawamoto, 1996; Del Gatto et al., 1997; Hedjran et al., 1997; Modafferi and Black, 1997] and computer predictions suggest an enrichment of this motif downstream of several brain-specific exons [Brudno et al., 2001]. Examination of the sequence of intron A-33 revealed no exact copies of

this motif. There are eight occurrences that match five bases out of six, but the RSV-AES deletion retains only a single copy, suggesting that seven copies are non-essential for exon EIIIA splicing. Thus, the molecular mechanisms that govern inclusion of the two alternative FN exons appear to be distinct, as predicted from their independent regulation in various cells and tissues [ffrench-Constant, 1995].

Using other characterized genes as precedents, we set out to define the intron 32-A sequences involved in enhancing the splicing of exon EIIIA. From the deletion analysis, we were unable to identify a discrete intronic enhancer element. Specifically, reduction in exon EIIIA inclusion for RSV-AN cannot be explained by deletion of a single regulatory sequence, as this construct retains all sequences present in RSV-SN, which includes the exon at a higher level. A restriction on intron length also does not seem to account for the different behavior of RSV-AN (914 nucleotides) and RSV-XA (841 nucleotides). Repositioning different sequences near exon EIIIA might alter the ability of the splicing apparatus to recognize the exon. In this case, we might expect that the sequences brought into the proximity of exon EIIIA in RSV-SN should provide an environment that resembles that of wild type. Simple inspection of the sequence junctions failed to reveal any obvious similarities in primary sequence (Fig. 4A). However, an unusual cluster of CUG trinucleotides, including one tandem pair is present immediately upstream of the NheI site. Tandem CUG repeats function as intronic enhancers in the cardiac troponin T gene, and these serve as binding sites for the members of the CELF family of splicing regulators [Ladd et al., 2001]. Similarly, CUG repeats have been implicated in alternative splicing of the smooth muscle α-actinin and α-tropomyosin mRNAs [Gromak and Smith, 2002; Gromak et al., 2003]. Reinsertion of the CUG-rich sequence in RSV-XN+ had no effect on the level of exon EIIIA inclusion, suggesting that the CUG repeats are not sufficient to restore intron 32-A splicing to wild type levels.

Other possible scenarios are that deletions either disrupt higher order structure within the intron, or remove some subset of multiple redundant elements. Computer analysis of the sequence of intron 32-A predicts an extended stem-loop structure that overlaps the NspI site (data not shown); the RSV-XP deletion would disrupt this structure. As RSV-XP behaves identically to RSV-XN, which retains the entire predicted stem-loop region, it seems unlikely that this structure plays a key role in exon EIIIA splicing. Finally, the intron was examined for the presence of unusual features of primary sequence. A set of nine pentamers have been found to be represented at high frequency in short introns [Lim and Burge, 2001]. The sequence of intron 32-A was analyzed for the presence of these sequence motifs; both 5′-CUGGG and 5′-UUUUU were over represented, with five copies of each. The distribution of these repeats, relative to the boundaries of the various deletions, is shown in Figure 5. All of the deletions that depressed exon EIIIA inclusion lack the cluster of repeats located between the SacI and NheI sites. However, in the case of deletion RSV-SN, the more distal cluster is now brought into closer proximity to exon EIIIA, which might compensate.

Since splicing factor PTB has been associated with the exclusion of FN alternative exon, EIIIB [Norton, 1994; Wagner and Garcia-Blanco, 2002], we investigated whether PTB could affect the inclusion of exon EIIIA. PTB overexpression resulted in a modest increase in exon EIIIA inclusion (Fig. 4C). This result contrasts with that observed for exon EIIIB; indeed, PTB binding generally tends to repress the recognition of nearby exons by the splicing apparatus (see [Wagner and Garcia-Blanco, 2002], and references therein). The site(s) of action of PTB has not been defined in the current study, but the lower level of PTB-dependent exon EIIIA inclusion seen for RSV-XN suggests that the missing intron 32-A sequences contribute. Intron 32-A contains 12 copies of the core PTB binding site UCUU [Pérez et al., 1997]; 11 lie scattered within the deleted XN fragment (Fig. 5), and several fall within more extended pyrimidine-rich regions (data not shown). The one exception is an occurrence very near the 3′ splice site; however, we were unable to detect PTB binding in this region in an earlier study employing UV crosslinking [Norton et al., 1998]. As PTB can bind to a variety of pyrimidine-rich sequence motifs, including poly-U [Garcia-Blanco et al., 1989], other sequence motifs including the UUUUU repeats might also recruit PTB. Thus, the number of potential PTB binding sites is quite large. The significance of individual binding sites and of their spatial organization will require detailed analysis.

Although quite a few examples have been reported of intron sequences upstream of a regulated exon affecting splicing efficiency, most of these involve the branch site and/or the polypyrimidine tract. Other sequences a short distance (<50 nucleotides) upstream of the branch site also can negatively affect splicing of a downstream exon [Kanopka et al., 1996; Zheng et al., 1996]. A few examples of regulatory elements that lie >100 nucleotides upstream from an alternative exon have been described. An intronic element within the hnRNP A1 gene modulates use of alternative exon 7B more than 100 nucleotides downstream, apparently by promoting use of the competing 5′ splice site of exon 7 [Chabot et al., 1997]. A bi-functional element that lies between two mutually exclusive exons of the FGF-R2 gene activates the upstream exon and inhibits the downstream exon, with both activities occurring at a distance [Carstens et al., 1998]. In a situation resembling that described here, a region >100 nucleotides upstream of alternative exon 6 of the tau gene affects splicing of the exon [Wei et al., 2000]. Moreover, a short deletion affected splicing when a longer deletion encompassing the same region had no effect, similar to the situation with mutants RSV-AN versus RSV-SN. However, the shorter deletion was associated with a substantial decline in the amount of spliced product present, which is not the case for plasmid RSV-AN (Fig. 3 and data not shown). In summary, our results suggest that intron 32-A contains multiple distributed sequence elements that promote the inclusion of alternative exon EIIIA in a manner that is not dependent upon cellular context. Splicing factor PTB appears to play a positive role toward exon EIIIA inclusion, in part acting through intron 32-A sequences.

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