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Alternative Splicing During Chondrogenesis: Modulation of Fibronectin Exon EIIIA Splicing by SR Proteins

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Abstract The alternative exon EIIIA of the fibronectin gene is included in mRNAs produced in undifferentiated mesenchymal cells but excluded from differentiated chondrocytes. As members of the SR protein family of splicing factors have been demonstrated to be involved in the alternative splicing of other mRNAs, the role of SR proteins in chondrogenesis-associated EIIIA splicing was investigated. SR proteins interacted with chick exon EIIIA sequences that are required for exon inclusion in a gel mobility shift assay. Addition of SR proteins to in vitro splicing reactions increased the rate and extent of exon EIIIA inclusion. Co-transfection studies employing cDNAs encoding individual SR proteins revealed that SRp20 decreased mRNA accumulation in HeLa cells, which make A+ mRNA, apparently by interfering with pre-mRNA splicing. Co-transfection studies also demonstrated that SRp40 increased exon EIIIA inclusion in chondrocytes, but not in HeLa cells, suggesting the importance of cellular context for SR protein activity. Immunoblot analysis did not reveal a relative depletion of SRp40 in chondrocytic cells. Possible mechanisms for regulation of EIIIA splicing in particular, and chondrogenesis associated splicing in general, are discussed. J. Cell. Biochem. 86: 45–55, 2002.

Key words: fibronectin; chondrogenesis; alternative splicing; SR proteins

Chondrogenesis, the process of cartilage differentiation from mesenchyme, is accompanied by striking changes in the composition of the extracellular matrix. The matrix produced by mesenchymal cells is rich in collagen type I. In contrast, the matrix produced by chondrocytes is enriched in collagen type II and the proteoglycan aggrecan [Kuettner, 1992; Mayne, 1989]. It is thought that these differences in matrix structure are key to the distinct functions of these different cell types. Fibronectin (FN[1]) is a dimeric extracellular matrix glycoprotein found in both undifferentiated limb mesenchymal tissue and differentiated cartilage. However, careful analyses revealed that changes in the FN isoforms produced in the developing embryonic limb result from a change in alternative mRNA splicing [Bennett et al., 1991; Peters and Hynes, 1996]. Specifically, undifferentiated mesenchymal cells produce FN mRNAs that contain alternative exon EIIIA, whereas chondrocytes produce FN mRNA that lacks the exon EIIIA [Bennett et al., 1991]. Thus, although FNs are present throughout chondrogenesis, their functions might change due to the alteration in protein structure resulting from the splicing change. In support of this hypothesis, treatment with antibodies directed to the protein segment encoded by exon EIIIA results in reduced mesenchymal cell condensation in vitro and structural anomalies in limbs in vivo [Gehris et al., 1997]. The mechanisms that regulate the alternative splicing of exon

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EIIIA are thus of relevance to understanding the process of chondrogenesis.

Pre-mRNA splicing is a major function of metazoan nuclei, as most genes are interrupted by one or more introns that must be removed from each pre-mRNA to yield mature mRNA. Correct cleavage at each exon border is directed by short conserved sequence motifs [Moore et al., 1993]. However, many genes produce alternative mRNAs as a means to expand protein diversity. Alternative splicing events are frequently modulated in a cell and tissue specific manner, but the regulatory mechanisms are incompletely understood. Multiple sequences at and near splice sites likely cooperate to regulate a particular splicing event [Norton, 1994; Black, 1995; Chabot, 1996], and the requirement for specific sequence elements may vary from one cell type to another. For instance, splicing of minigene RSV-chA, containing chick exon EIIIA and flanking introns and exons under transcriptional control of the RSV LTR, was regulated differentially in transfected limb mesenchymal cells and vertebral chondrocytes [Uporova et al., 1999]. Two sequence elements within exon EIIIA appear to be required for inclusion of the exon in mesenchymal cells. One of these, termed the ESE for exon splicing enhancer, appears to be important for inclusion in several cell types [Mardon et al., 1987; Lavigueur et al., 1993; Caputi et al., 1994]. In contrast, the ESS, for exon splicing silencer, may have a negative effect on splicing in other cell types [Caputi et al., 1994]. The sequence at the 5′ splice site of the exon also may influence exon recognition [Muro et al., 1998]. Finally, another silencer, based on an extended secondary structure, has been identified within the 5′ end of the exon itself [Staffa et al., 1997; Muro et al., 1999]. Establishing the cell-type-specific requirements for these various sequence elements remains an important goal for understanding the basis for regulated alternative mRNA splicing.

In addition to cis signals, trans-acting factors also have been implicated in the control of alternative splicing. SR proteins are a family of highly conserved splicing factors that participate in both constitutive and alternative splicing events [Fu, 1995; Manley and Tacke, 1996; Blencowe et al., 1999]. SR proteins as a group can complement a splicing deficient S100 extract, and generally tend to enhance splicing in the presence of nuclear extract. The ability of individual SR proteins to influence splicing varies for different pre-mRNAs [Fu, 1993; Zahler et al., 1993]. It has been reported that SR proteins interact with the purine-rich ESE within human FN exon EIIIA/EDA [Lavigueur et al., 1993]. In the present study, we have evaluated the influence of SR proteins on alternative exon EIIIA splicing, and assessed whether SR proteins might play a role in splicing changes associated with chondrogenesis.

MATERIALS AND METHODS

Cell Culture and Transfections

HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% horse serum. HeLa cells were transfected with 20 μl Geneporter (Gene Therapy Systems) plus 4 μg total DNA in 1.0 ml of OptiMEM (Gibco-BRL) for 3 h followed by the addition of 1.0 ml of DMEM supplemented with serum to a concentration of 20%. Following overnight incubation, the medium was replaced and cells incubated for an additional 24 h. Limb mesenchymal cells, tendon fibroblasts and primary chick chondrocytes were isolated from chick embryos as described previously [Bennett and Adams, 1987]. The chondrocytes were cultured in suspension in DMEM containing 10% fetal bovine serum for one week, then treated with hyaluronidase as described [Uporova et al., 1999] prior to seeding at 10^6 cells per 35 mm well for transfection. Cells were transfected with splicing reporter genes (2 μg DNA) and 8 μl Superfect (Qiagen). RCS Swarm rat chondrosarcoma cells were obtained from J. Oxford (Oregon Health Sciences University), and cultured in DMEM supplemented as above. Transfections were performed as described above. Hybridoma cells producing MAb104 were obtained from the American Type Culture Collection.

Plasmids Used

The chicken fibronectin exon A-containing mini-genes RSV-chA and RSV-chAdE (deletion of exon splicing enhancer, ESE) were described previously [Uporova et al., 1999]. The same mini-gene inserts also were cloned into pBluescript, as follows. Short oligonucleotides encoding a 5′ splice site (GDF, 5′-GATCAACAG/GTGAGTTCG-3′ and GDR, 5′-GATCCGAACTCACCTGTT-3′) were annealed and ligated into the BamHI site downstream of exon 33, to form

pFNA1 and pFNA1dE respectively. These constructs served as templates to generate substrates for in vitro splicing experiments. Human SRp55 cDNA was generated from total HeLa RNA by reverse transcription with random hexanucleotides primers and Superscript (Gibco-BRL) as suggested by the manufacturer. SRp55 cDNA was amplified by PCR with primers B3 and B4 (B3: 5′- GCGAATTCCATGGCGCGCGTCTACATAGGACGC -3′; B4: 5′- GCGAATTCTTAATCTCTGGAACTCGACCTGGAC -3′) and ligated into the EcoRI site of mammalian expression vector pCI-neo (Promega). In vitro transcription/translation confirmed production of an appropriately sized protein. SRp20, SRp40, and SRp30a/ASF/SF2 expression vectors were obtained from G. Screaton [Screaton et al., 1995] and R. Lafyatis [Sarkissian et al., 1996].

Nuclear Extract and SR Protein Isolation and RNA-Protein Binding Experiments

HeLa nuclear extracts were prepared from 4×10^9 HeLa cells, obtained from the Cell Culture Center, Minneapolis, MN [Dignam et al., 1983]. Total SR proteins were isolated from 10^{10} HeLa cells [Zahler et al., 1992]. SR proteins were reconstituted at 10 mg/ml in buffer D. Preparation of radiolabeled fragments of chick exon EIIIA was as described previously [Uporova et al., 1999]. Fragment chA consists of the entire exon, chA2 is the central 66 nucleotides of the exon and chA2dE is identical to chA2 but for the deletion of the nine nucleotide ESE sequence. Gel retardation was performed as described [Uporova et al., 1999] using 0.5–1.0 μg of total HeLa SR proteins.

In Vitro Splicing Reactions

Pre-mRNAs used for in vitro splicing assays were synthesized with T7 RNA polymerase (Stratagene), DNase I treated, purified over a Sephadex G-50 spin-column, and quantitated by measuring absorbance at 260 nm. A standard 25 μl splicing reaction contained 50 fmoles of pre-mRNA, 10 μl of HeLa nuclear extract, 10 mM HEPES (pH 7.6), 5 mM creatine phosphate, 1.6 mM ATP, 3 mM $MgCl_2$, and 1 U of RNaseBlock (Stratagene). Incubation was at 30°C for 2 h unless otherwise noted. Where indicated, various amounts of total SR proteins were added to the reaction mix after addition of nuclear extract and prior to addition of pre-mRNA. Spliced products were recovered as described [Norton and Hynes, 1990] and resuspended in 20 μl of water.

RNA Isolation and RT-PCR Assay

Total cellular RNA was isolated from cells 48 h post-transfection using TRIzol (LifeTechnologies), DNaseI treated, and resuspended in 10 μl of water. Reverse transcription (RT) of RSV-chA and FN1A spliced products was performed with Superscript II MoMLV reverse transcriptase (LifeTechnologies). Reverse transcription reactions included 1 μl of total RNA and return primers GDR for in vitro splicing products, 12R (5′-CCCGGTCTTCTCTTTGGGGTTCAC-3′) for HeLa cell RNA, and LPA3 (5′-CATCAATGTATCTTATCATGTC-3′) for chondrocyte RNA. Amplification was performed with Taq DNA polymerase (Stratagene) for 25 cycles of one minute at 94°C, 1 min at 58°C, and 1 min at 72°C, unless otherwise specified. Forward primer 11F (5′-ATGGTCAGCGTCTATGCTCAGAA-3′) was used for PCR along with primers 12R for in vitro spliced mRNA, 201 new (5′- AGACTGGTAGGAGTTACCTGAGTGAACTTCAG -3′) for HeLa RNA, and LPA3 for chondrocyte RNA. The structure of RCS fibronectin mRNA was determined by RT-PCR as described previously [Górski et al., 1996]. PCR products were separated on 5% polyacrylamide gels and visualized by ethidium bromide staining. Gels were photographed; scanned images were contrast inverted and densitometric analysis was performed using NIH Image (zippy.nimh.nih.gov).

Immunoblot Assay

For chick cells, proteins were extracted in 50 mM Tris, pH 6.8, 20 mM EDTA, 5% SDS, 1 mM aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 5 mM β-glycerophosphate [Zahler et al., 1993]. Following protein determination (BCA, Pierce), 100 μg total protein was loaded per lane onto a 10 % polyacrylamide gel. For HeLa or RCS cell lysates, 2.5×10^5 cells were lysed directly in electrophoresis sample buffer and heated to 100°C. Proteins were separated on 12% SDS–polyacrylamide gels. Following electrophoresis, proteins were transferred to nitrocellulose, and blots were incubated with supernatant from hybridoma cells producing the anti-SR monoclonal MAb104 [Roth et al., 1991], followed by horseradish peroxidase-conjugated goat anti-mouse IgG (BioRad). Blots were incubated with SuperSignal reagents (Pierce) and exposed to X-ray film.

RESULTS

Binding of SR Proteins to Exon EIIIA

In a previous study, a gel mobility shift assay was used to detect a complex that formed on exon EIIIA in nuclear extract prepared from chick limb mesenchymal cells [Uporova et al., 1999]. This factor or factors interacted with chick exon EIIIA in a manner that was dependent on the presence of an exonic splicing enhancer, ESE, within the exon. Attempts to fractionate the mesenchymal extract to further characterize the binding activity were unsuccessful, due in part to the limited amounts of material obtained from the tissue. Thus, we pursued a candidate factor approach, and used the gel mobility shift assay to determine whether SR proteins interact with exon EIIIA in a sequence-dependent fashion. SR proteins isolated from HeLa cells were incubated with radiolabeled exon EIIIA RNA (Fig. 1A). The resulting complexes were subjected to native gel electrophoresis, and heparin-resistant complexes of reduced mobility relative to free probe were observed (Fig. 1B, lanes 1–3). Deletion of the ESE eliminated these SR protein-RNA complexes (lanes 4–6), suggesting sequence specificity. SR proteins also form a specific, heparin-resistant complex on the A2 fragment (lanes 7–9), which contains the ESE. Removal of the ESE eliminated the complex (lanes 10–12). These results indicate that SR proteins interact with exon EIIIA in a manner dependent upon the ESE, as expected for the mesenchymal factor.

In Vivo and In Vitro Splicing Assays for Exon EIIIA

The RNA-protein interactions observed in the experiments described above are consistent with a role for SR proteins in the splicing of exon EIIIA. Using a transfection based splicing assay, it was demonstrated that inclusion of exon EIIIA in chick limb mesenchymal cells required the presence of the ESE [Uporova et al., 1999]. To investigate the relationship between exon inclusion and SR protein binding, we wished to use an in vitro splicing assay based on HeLa nuclear extract [Kuo and Norton, 1999]. However, first it was necessary to establish the behavior of the chick-derived RSV-chA splicing reporter in human HeLa cells. Cells were transfected with expression construct RSV-chA (Fig. 2A) or derivative RSV-chAdE, which

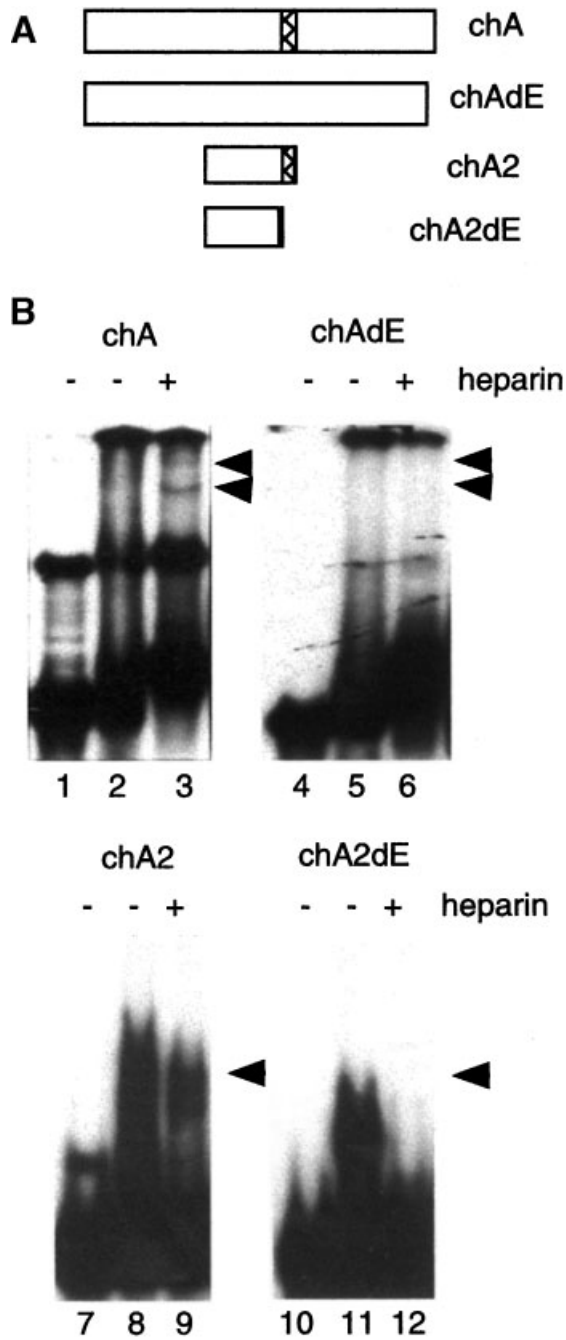

Fig. 1. Binding of SR proteins to exon EIIIA. Top, diagram of exon EIIIA fragments used in gel mobility shift assays. Below, purified HeLa SR proteins were incubated with radiolabeled fragments consisting of the entire exon EIIIA (chA, **lanes 1–30**), the central third of the exon containing the ESE (chA2, lanes 4–6) or a version of chA2 lacking the nine nucleotide ESE sequence (chAdE, lanes 7–9). RNA protein complexes were either loaded directly onto a native 5% acrylamide gel, or pretreated with heparin, as indicated. Heparin resistant complexes formed between SR proteins and RNA probes are indicated by arrowheads; the arrowhead in brackets indicates the absence of such a complex in the absence of the ESE.

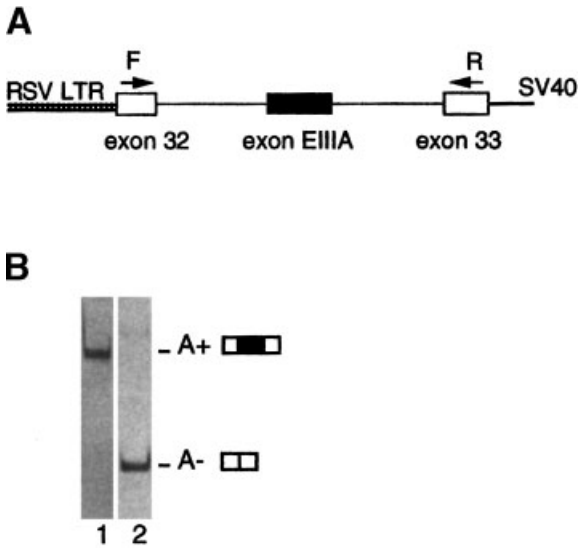

Fig. 2. Inclusion of chick exon EIIIA in HeLa cells requires the ESE. **A**: Diagram of expression construct RSV-chA. Boxes: exons; lines: introns and other non-coding sequences. Positions of forward and reverse primers used in the RT-PCR assay are indicated. **B**: RT-PCR analysis of spliced mRNAs produced by HeLa cells transfected with either RSV-chA or RSV-chAdE. Products were separated by electrophoresis through 5% polyacrylamide gels and detected by ethidium bromide staining. The contrast of the image has been reversed. Structure of the corresponding A+ and A− mRNAs are diagrammed to the right.

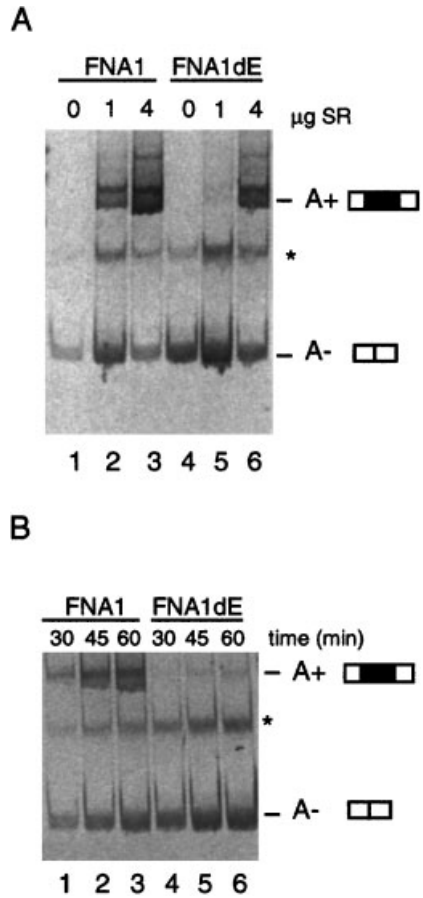

Fig. 3. In vitro splicing of pre-mRNAs FNA1 and FNA1dE. **A**: Pre-mRNAs were prepared by in vitro transcription and incubated in the presence of HeLa extract plus the indicated amount of SR proteins. Splicing products were detected by RT-PCR assay and are identified to the right. Asterisk: PCR artifact arising from primer 12R alone. **B**: Kinetics of accumulation of spliced products. Splicing reactions supplemented with 1 µg of SR proteins were incubated at 30°C for the indicated times. Detection of PCR products was as in Figure 2.

lacks the 9 base pair ESE. RNA was isolated two days later and used for RT-PCR analysis using primers based on flanking exons 32 and 33. These results demonstrated that the chick exon EIIIA is included in the HeLa cells (Fig. 2B, lane 1), as reported for human EIIIA-containing minigene constructs [Caputi et al., 1994]. In contrast, only EIIIA−mRNA is produced from the ESE-deleted construct (Fig. 2B, lane 2). Thus, the chick sequences are spliced appropriately in cells of mammalian origin, and the ESE is required for exon inclusion in the HeLa cells as it is in the mesenchymal cells.

A chick minigene fragment containing exon EIIIA and flanking exons 32 and 33 was subcloned into pBluescript, generating pFNA1, and used as a template to synthesize a pre-mRNA substrate for in vitro splicing (Fig. 2A). A consensus 5′ splice site was included immediately downstream of exon 33 to improve splicing of upstream intron IIIA-33. Pre-mRNA FNA1 was prepared from pFNA1 by in vitro transcription, and incubated with HeLa nuclear extract under conditions that permit splicing. RNA was isolated from splicing reactions and exon EIIIA splicing was analyzed by RT-PCR using primers based on exons 32 and 33. Considerable accumulation of A− mRNA was observed (Fig. 3A, lane 1), but the three exon A+ form was not detected, in contrast to exon EIIIA splicing observed in HeLa cells.

SR Proteins Promote Exon EIIIA Inclusion in the In Vitro Splicing Assay

To assess whether SR proteins could drive exon inclusion, increasing amounts of SR proteins were added to splicing reactions, and the resulting mRNAs analyzed as above. Addition of SR proteins resulted in the dose-dependent inclusion of exon EIIIA, with detectable levels of EIIIA+ mRNA observed at the lowest level of

added protein (Fig. 3A, lanes 1–3). Thus, the interactions between SR proteins and exon EIIIA shown in Figure 1 may have functional consequences for exon recognition.

If SR proteins participate in exon EIIIA inclusion by binding directly to exon sequences, the production of A+ mRNA should be dependent upon the presence of the ESE. To evaluate whether SR protein-mediated exon EIIIA inclusion required the ESE element, pre-mRNA FNA1dE, lacking the nine nucleotide ESE, was prepared. In vitro splicing of FNA1dE produced no A+ mRNA without added SR proteins (Fig. 3A, lanes 4–6). Although exon EIIIA inclusion increased with SR protein addition, the extent of inclusion of the mutant exon was considerably less than for wild type at all protein concentrations tested (Fig. 3A data not shown). The relative extent of SR protein-mediated exon EIIIA inclusion is evident when splicing kinetics are monitored. Accumulation of A+ mRNA is present at early time points in reactions containing the wild type (Fig. 3B, lanes 1–3) but not the mutant substrate (lanes 4–6). Thus, the ESE sequence represents a functionally important SR binding site within exon EIIIA.

Effects of SR Proteins on Exon EIIIA Splicing In Vivo

The in vitro splicing experiments utilized SR protein preparations that include multiple members of this protein family (26). To evaluate the contributions of individual SR proteins, we performed co-transfection of expression constructs for individual SR proteins along with a splicing reporter. First, HeLa cells were co-transfected with splicing reporter plasmid RSV-chA and expression constructs for SRp55, SRp20, SRp40, or SRp30a (ASF/SF2). SRp55 overexpression had little effect on exon EIIIA splicing (Fig. 4A, compare lanes 1 and 2), whereas SRp30a, SRp40, and most notably SRp20 reduced the production of A+ mRNA to an increasing extent, without a concomitant increase in A− mRNA (lanes 3–5). An overall reduction of splicing also was observed when SRp20 was co-transfected with RSV-chAdE. This reporter normally produces mostly A− mRNA, but in the presence of SRp20, this species is diminished, without the appearance of A+ mRNA (Fig. 4B, compare lanes 1 and 2). The apparent negative consequences of overexpression of SRp20 were investigated further by co-transfecting decreasing amounts of the SRp20 construct along with a constant amount of RSV-chA. Even at concentrations that do not eliminate A+ mRNA, no A− mRNA is observed (Fig. 4C). A similar trend was observed when the same experiment was performed with decreasing amounts of SRp40 (data not shown). Thus, SRp20, and to a lesser extent, SRp40 and SRp30a negatively affect production of mRNAs from RSV-chA pre-mRNAs in HeLa cells, which normally include exon EIIIA.

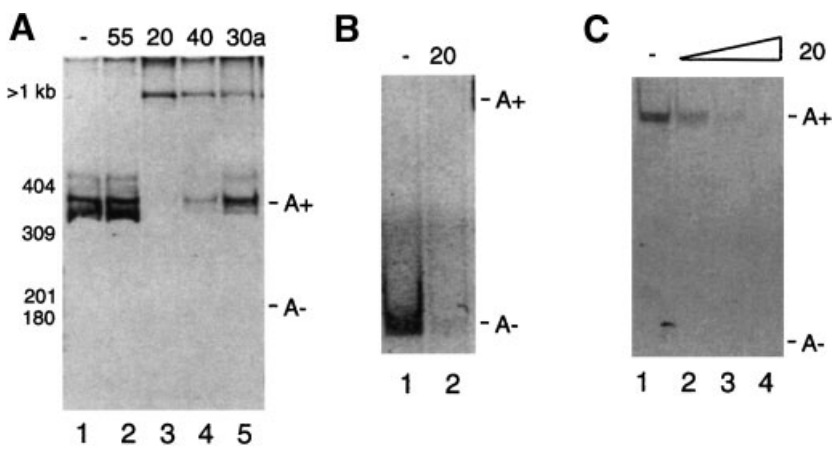

Fig. 4. Transfection of HeLa cells with RSV-chA and RSV-chAdE in the presence of individual SR protein cDNAs. **A**: HeLa cells were transfected with 1.0 μg of RSV-chA alone (**lane 1**) or with 1.0 μg of the indicated SR protein expression constructs (**lanes 2–5**). Carrier plasmid DNA was added to obtain 4.0 μg total DNA per sample. Spliced products were analyzed and detected as in Figure 2. The expected positions of products arising from A+ and A− mRNAs are indicated at right. At left, positions of molecular size markers are indicated. **B**: HeLa cells were transfected with RSV-chAdE with (**lane 2**) or without (**lane 1**) the construct encoding SRp20. **C**: HeLa cells were transfected with RSV-ChA (**lane 1**) and increasing amounts of the SRp20 expression construct (**lane 2**, 0.005 μg; **lane 3**, 0.17 μg; **lane 4**, 0.5 μg). Expected positions of products representing A+ and A− mRNAs are indicated.

Based on the interaction between the exon EIIIA ESE and SR proteins detected in the gel mobility shift assay and their role in enhancement of EIIIA inclusion in vitro, we postulated that one or more family members may play a positive role in the splicing of the exon. However, the high level of exon inclusion observed in the transfected HeLa cells precluded detection of a further increase in the co-transfection experiments. Thus, the same co-transfection experiments were performed using chondrocytes, which produce mostly A− mRNA. In the absence of added SR proteins, most of the mRNA detected from either RSV-chA or RSV-chAde was of the A−form (Fig. 5A and B, lanes 1), as expected from previous S1 nuclease analyses (10). Co-transfection of with either SRp55 or SRp30a had little effect on exon EIIIA splicing (lanes 2 and 5). In contrast, SRp40 overexpression stimulated exon EIIIA inclusion (Fig. 5A, lane 3). The increase in exon inclusion was dependent upon the presence of the ESE (Fig. 5B, lanes 1 and 4). SRp20 reduced exon EIIIA inclusion to a level that was nearly undetectable, but had little effect on production of A− mRNA; the presence or absence of the ESE made no difference (Fig. 5A, lane 4 and 5B, lane 3). Thus, overexpression of SRp40 can stimulate recognition of exon EIIIA in chondrocytes, a cellular environment normally nonpermissive for inclusion of this exon.

SR Protein Content of Chondrocytic Cells

One possible explanation for the ability of SRp40 to stimulate EIIIA inclusion in chondrocytes is that this factor is relatively deficient in these cells. To investigate whether changes in the levels SR proteins correlated with difference in exon EIIIA splicing, the SR protein content of isolated chick limb mesenchymal cells and tendon fibroblasts was compared to that of chondrocytes by immunoblotting using the monoclonal antibody MAb104, which recognizes an epitope common to a number of SR proteins [Roth et al., 1991]. There were a number of differences in the protein profile of the chondrocytes (A-) versus the mesenchymal cells and fibroblasts (A+), but a species comigrating with SRp40 was detected in both chondrocyte preparations as well as in mesenchymal cells (compare lanes 2 and 3 with lane 4), but not tendon fibroblasts (lane 5). However, as not all of the proteins co-migrated with known HeLa SR proteins (Fig. 6A, lane 1), the identities of the chick proteins could not be determined unambiguously.

The rat chondrosarcoma-derived cell line RCS retains several characteristics of differentiated chondrocytes [Oegama et al., 1975; Smith et al., 1975] Available sequence information indicates that SRp40 is highly conserved between human and rat [Diamond et al., 1993], suggesting that identification of individual SR proteins should be feasible in the rat cells. RT-PCR established that exon EIIIA was omitted in RCS cells, so as to produce A−FN mRNA, consistent with a chondrocytic phenotype (Fig. 6B). Total cell lysates were prepared from these cells and compared with lysates prepared from an equal number of HeLa cells (A+ mRNA) by

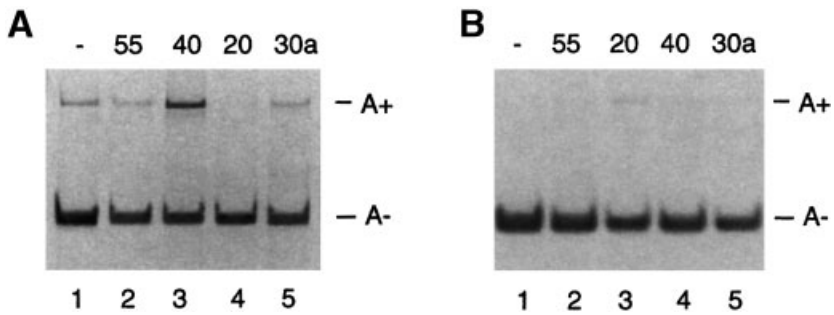

Fig. 5. Transfection of chondrocytes with RSV-chA and RSV-chAdE in the presence of individual SR protein cDNAs. **A**: Primary vertebral chondrocytes were transfected with 0.5 μg of RSV-chA alone (**lane 1**) or with 1.0 μg of the indicated SR protein expression constructs (**lanes 2–5**). Carrier plasmid DNA was added for a total of 2.0 μg DNA. Spliced products were analyzed and detected as in Figure 2. The expected positions of products arising from A+ and A− mRNAs are indicated at right. The results shown are representative of three independent experiments. **B**: Primary vertebral chondrocytes were transfected with 0.5 μg of RSV-chAdE alone (**lane 1**) or with 1.0 μg of the indicated SR protein expression constructs (**lanes 2–5**). Analysis was performed as described for panel A, but note that the order of SR protein addition is not the same in the two panels.

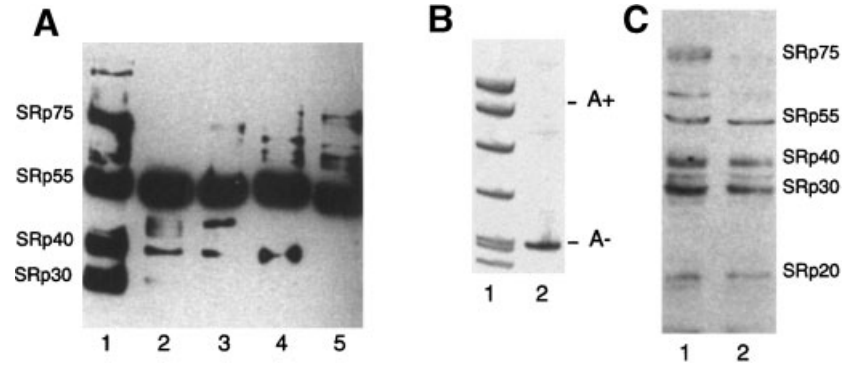

Fig. 6. Analysis of EIIIA splicing and SRp40 expression in chondrocytic cells. **A**: Proteins were extracted from the cells and subjected to immunoblot analysis with MAb104. **Lane 1**, HeLa cells; **lane 2**, vertebral chondrocytes; **lane 3**, sternal chondrocytes; **lane 4**, mesenchymal cells; **lane 5**, tendon fibroblasts. SR proteins are identified at left; a non-specific band between SRp55 and SRp75 is observed [Zahler et al., 1993]. Note that SRp20 was not detected in this experiment and has been detected variably in these tissues (data not shown). **B**: Total RNA was isolated from RCS cells and exon EIIIA inclusion was determined by RT-PCR (**lane 2**) as described previously [Górski et al., 1996]. A+ mRNA produces a band of 515 bp; A−, 245. **Lane 1**, pBR322 DNA cut with MspI; fragments are 622, 527, 404, 309, 242/238, and 217 bp in length. **C**: Total cell lysates were prepared from HeLa or RCS cells and fractionated by SDS–PAGE through a 12% polyacrylamide gel. Following transfer, SR proteins were detected using monoclonal antibody MAb104. SR proteins in the HeLa (**lane 1**) and RCS (**lane 2**) lysates are identified based on co-migration with purified HeLa SR proteins, indicated to the right.

immunoblotting using MAb104 to reveal relative SR protein content. Overall, the pattern of SR proteins in the RCS cells resembles that of the HeLa cells; similar results were obtained when lysates were normalized for total protein content (data not shown). The most obvious difference was that SRp75 was enriched in the HeLa cells. The relative level of SRp40 was similar in the two cell types (Fig. 6C), although subtle differences in protein mobility were observed in some experiments. These results suggest that the abundance of SRp40 is not the single factor limiting exon EIIIA inclusion in cells of chondrocyte origin.

DISCUSSION

We have investigated the role of SR proteins in mediating the change in fibronectin exon EIIIA splicing that accompanies the differentiation of limb mesenchymal cells into chondrocytes. One or more of the proteins can interact directly with the alternative exon in a gel mobility shift assay, and a mixture of SR proteins was able to enhance EIIIA inclusion in an in vitro splicing assay. Co-transfection studies revealed that SRp40 overexpression alone resulted in a significant increase in the level of EIIIA inclusion in chondrocytes, which normally produce only A− mRNA. However, immunoblotting results indicated that SRp40 levels were not reduced in cells such as chondrocytes that exclude exon EIIIA compared with those that include the exon, suggesting the involvement of additional factors.

The inability of the HeLa nuclear extract to produce A+ mRNA without SR protein supplementation suggests that the balance of factors present in the extract does not favor recognition of alternative exon EIIIA. Previous reports suggested that SR proteins are involved in exon EIIIA recognition. SRp30a has been implicated in the inclusion of the corresponding exon (termed ED-1) of the human fibronectin gene in HeLa cells, and the purine rich ESE element was a key binding site [Lavigueur et al., 1993]. In another study, human exon ED-1 inclusion was increased in an ESE-dependent fashion by SRp30a or 9G8 co-transfection in Hep3B cells [Cramer et al., 1999]. SRp40 overexpression increased exon ED-1 inclusion to a lesser extent in these cells, in contrast to our results in transfected chondrocytes. These discrepancies could be due to either species-specific (chick versus human) or cell type-specific differences. We prefer the latter explanation as the pattern of EIIIA splicing in chondrocytes is conserved between species [Zhang et al., 1995], and the chick splicing reporter appears to be spliced correctly in mammalian cells (Fig. 2, and unpublished results). The conserved purine-rich ESE sequence is important for SR protein-stimulated exon inclusion in many cell types [Cramer et al., 1999; Muro et al., 1999], but the

factors that bind to the ESE may vary from one cell type to another [Uporova et al., 1999]. Although the relative distribution of SR proteins varies in different tissues and cell types [Zahler et al., 1993; Hanamura et al., 1998], the effect of an individual SR protein on a transcript in the context of different cell types has not been explored extensively.

Overexpression of SRp20 inhibited production of A+ mRNA in HeLa cells, without inducing a switch to A− mRNA (Fig. 4). It has been reported that overexpression of individual SR proteins, including SRp20, can result in export of unspliced RNAs from the nucleus [Wang and Manley, 1995; Huang and Steitz, 2001]. In some of our experiments, larger species consistent with intron-containing RNAs were observed (for example, see Fig. 4), but PCR conditions were not optimized to permit the amplification of large (>1 kb) products. However, a general inhibition of pre-mRNA RSV-chA splicing by SRp20 is supported by both the dose-dependence of the effect and by the reduction in A− mRNA produced from the ESE deleted construct. Other factors must determine the relative ratio of A+ to A− mRNA. It is interesting that the cellular environment appears to dictate the response of the reporter to SRp20 overexpression, as SRp20 did not affect the level of A− mRNA produced by either RSV-chA or RSV-chAdE (Fig. 5). Similarly, SRp30a and SRp40 decreased exon EIIIA inclusion in HeLa cells, but not chondrocytes, with the latter protein having the opposite effect. These observations all suggest the importance of cellular context in modulating the activity of individual SR proteins.

Our previous gel mobility shift analyses revealed that one or more factors interact with EIIIA in chondrocyte nuclear extract, and these factors appear distinct from those enriched in the undifferentiated limb mesenchymal cells [Uporova et al., 1999]. The results presented in Figure 5 suggested that SRp40 is an excellent candidate for being a mesenchymal cell-enriched splicing factor that enhances exon EIIIA inclusion in an ESE-dependent fashion. SRp40 has been implicated in the alternative splicing of several exons to date [Zahler et al., 1993; Ramchatesingh et al., 1995; Screaton et al., 1995], as well as in the recognition of another alternative exon of the fibronectin gene, EIIIB [Du et al., 1997; Kuo and Norton, 1999]. However, the failure to include EIIIA in chondrocytes does not appear to be due simply to insufficient SRp40, as this protein clearly was present in the RCS cells, which produce A− mRNA, and has been tentatively identified in the chick chondrocytes (Fig. 6). One alternative possibility is that a factor enriched in chondrocytes antagonizes the action of SRp40. Candidate antagonistic factors include the hnRNP proteins A1 and H, which have been implicated in other alternative splicing reactions [Mayeda and Krainer, 1992 ;Chen et al., 1999]. Another possibility is that SRp40-dependent inclusion of exon EIIIA requires a second factor that is limiting in chondrocytes. Stable association of SRp40 with a purine rich repeat sequence was found to require at least one additional protein of unknown identity [Yeakley et al., 1996]. Also, the phosphorylation state of individual SR proteins can influence their activity [Tacke et al., 1997; Prasad et al., 1999; Sanford and Bruzik, 1999], and alternative splicing of SR protein transcripts may add further complexity [Jumaa and Nielsen, 1997]. Finally, there may be functional redundancy between SRp40 and one or more factors, such as SRp75. We are in the process of testing these various models for regulation of EIIIA splicing in chondrocytic cells.

Although the presence of the EIIIA segment in FN has functional consequences for mesenchymal cell condensation and limb development [Gehris et al., 1997], the precise function of this protein domain is unknown. However, it may be relevant that the inclusion of exon EIIIA has been shown to influence the cell adhesive activity of FN, as well as cell cycle progression [Manabe et al., 1997; Manabe et al., 1999]. Moreover, the change in fibronectin exon EIIIA alternative splicing is not the only splicing change that accompanies chondrogenesis. For example, alternative splicing also affects the structures of cartilage-enriched type II procollagen, as well as α1(XI) and α2(XI) procollagens (Table I). In addition, a novel cartilage-specific isoform of fibronectin that lacks three consecutive exons has been reported in canine, rabbit, and equine articular cartilage [MacLeod et al., 1996]. These parallel changes in splicing suggest that a common mode of regulation may exist, and there are certain features that link these alternative exons. First, chondrocyte maturation correlates with decreased inclusion of alternative exons, resulting in shorter protein isoforms. Second, the protein segments encoded

TABLE I. Potential ESEs in Alternative Exons Excluded in Chondrocytes

Gene product (exon)[a]	Species[a]	Potential ESE[b]	References
Procollagen a1 (11) (exon 2)	Human	GGGCAGAGG	[Ryan and Sandell, 1990]
Procollagen a1 (XI) (exon V1a/6a)	Rat/chick	GGGGANRCAGA	[Oxford et al., 1995; Zhidkova et al., 1995]
Procollagen a1 (XI) (exon V2/8)	Rat/chick	GGAGAGGA	ibid.
Procollagen a2 (XI) (exon 7)	Human/mouse	AAGAGGAAG	[Lui et al., 1996; Tsumaki and Kimura, 1995]
Fibronectin (exon 1-10)	Canine	GGAGAGGAG	[MacLeod et al., 1996]
Fibronectin (exon EIIIA/EDA)	Human/chick	GAAGAAGAC	[Caputi et al., 1994; Lavigueur et al., 1993; Uporova et al., 1999]

[a]Only selected examples of exons undergoing alternative splicing and species of origin are listed.
[b]The longest purine-rich tract in each exon is listed, along with the EIIIA ESE analyzed in the present work for comparison.

by the excluded exons are fairly acidic. Third, these exons all contain purine-rich tracts similar to the EIIIA ESE (Table I). Purine-rich codons GAU or GAC (aspartic acid) and GAG or GAA (glutamic acid) specify acidic amino acids, raising the possibility that a mechanism has developed to skip exons based on coding potential. A similar compositional bias is not observed for constitutively included exons or for a set of exons that undergo alternative splicing in neurons [Stamm et al., 1994]. Whether the same factors that play a role in EIIIA splicing regulation are also involved in other chondrogenesis-associated splicing changes remains to be determined.

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