Journal of Cellular Biochemistry 76:341–351 (1999)

Alternative Splicing During Chondrogenesis: cis and trans Factors Involved in Splicing of Fibronectin Exon EIIIA

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Abstract Primary chicken mesenchymal cells from limb buds and vertebral chondrocytes have been used to study the changes that occur in alternative mRNA splicing of fibronectin exon EIIIA during chondrogenesis. The mesenchymal cell phenotype (exon EIIIA included) and chondrocyte phenotype (exon EIIIA excluded) were preserved in culture. Both primary cell types were transfected with an EIIIA minigene and alternative splicing was monitored by S1 protection assay. Differential cell-specific splicing of the reporter was observed. The roles of two regulatory elements, an exon splicing enhancer (ESE) and an exon splicing silencer (ESS) were examined. Both elements were required for EIIIA inclusion into mRNA in mesenchymal cells. Gel mobility shift assays revealed that both chondrocyte- and mesenchymal cell-derived nuclear extracts contained exon EIIIA binding factors, but the RNA binding factors present in the two cell types appeared to be distinct. The ESE and ESS appeared to cooperate in the formation of both cell type-specific complexes. These results suggest a model in which inhibitory factors enriched in chondrocytes compete with positive factors enriched in mesenchymal cells for binding to exon EIIIA, determining whether the exon is included. J. Cell. Biochem. 76:341–351, 1999. © 1999 Wiley-Liss, Inc.

Key words: fibronectins; alternative mRNA splicing; regulatory elements; exon enhancer element; cartilage; limb development

Differentiation of mesenchymal tissue to cartilage is accompanied by striking changes in the composition of the extracellular matrix. The mesenchymal matrix is rich in collagen type I, whereas the nascent cartilage matrix contains collagen types II, IX, and XI and the proteoglycan aggrecan [Kuettner, 1992; Mayne, 1989]. The extracellular matrix protein fibronectin is present in both mesenchymal tissue and in cartilage, although expression peaks during the condensation event that precedes overt chondrogenic differentiation [Kulyk et al., 1989]. Fibronectins (FNs) are large dimeric glycoproteins present in plasma and in the extracellular matrices of many tissues [Hynes, 1990; Mosher, 1989]. Each FN monomer is composed of a series of modular repeating units, referred to as FN-I, FN-II, or FN-III-type repeats. Distinct regions of each chain possess binding activities for the extracellular molecules collagen, fibrin and heparin, as well as for members of the integrin family of cell surface receptors. These diverse binding activities suggest that FN functions as a connecting molecule linking the matrix with the cell surface.

The functional versatility of the FN molecule is expanded further by alternative splicing of a single primary transcript, which results in the production of multiple protein isoforms. The FN mRNAs and proteins may differ at three positions, and the patterns of splicing at each of these three positions are regulated independently both temporally and spatially [ffrench-Constant, 1995; Kornblihtt et al., 1996]. Studies of mammalian FN genes have indicated that

Grant sponsor: National Institutes of Health; Grant number: AR42887.

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Received 15 March 1999; Accepted 26 July 1999

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This article published online in Wiley InterScience, December 1999.

alternative protein segments EIIIA and EIIIB, termed EDA and EDB in humans, are each derived from the inclusion of single optional exons. By contrast, variation at the V (or IIICS in human) region stems from complex subdivision of a large exon. Alternative splicing also occurs at all three positions in birds and amphibians, and the patterns of inclusion or exclusion of individual segments are conserved, although the precise subdivision that occurs at the V region varies from one species to another [DeSimone et al., 1992; Norton and Hynes, 1987]. Alternative splicing of exons EIIIA and EIIIB occurs independently of each other, suggesting the involvement of distinct sets of regulatory factors [Paolella et al., 1988]. The conservation of both sequence and splicing pattern have led to the belief that the alternative segments confer distinct activities onto the FN isoforms.

In addition to the condensation-associated increase in FN, the pattern of FN mRNA splicing changes during chondrogenesis. Specifically, FN mRNAs from chick limb mesenchyme before overt chondrogenesis or myogenesis contain exons EIIIA and EIIIB (B^+A^+), whereas FN mRNAs in cartilage contain exon EIIIB but not exon EIIIA (B^+A-) [Bennett et al., 1991; Gehris et al., 1996]. The protein segment encoded by exon EIIIA has been implicated in cell adhesion and activation of hepatic stellate cells and myofibroblasts [Hashimoto-Uoshima et al., 1997; Jarnagin et al., 1994; Serini et al., 1998; Xia and Culp, 1995]; the findings are consistent with an active role for this segment in modulating cell growth and differentiation. Recent results from our laboratory indicate that the protein sequences encoded by exon EIIIA are involved in the critical step of mesenchymal condensation, suggesting that the splicing change may be necessary for the chondrogenic transition [Gehris et al., 1997]. The modulation of the splicing pattern of FN exon EIIIA during cartilage differentiation and chondrogenesis provides an ideal system for examination of the mechanism(s) regulating tissue-specific alternative splicing by exon skipping.

Alternative exons frequently are flanked by weak splicing signals [Black, 1995; Chabot, 1996; Norton, 1994]; presumably, it is more difficult for the splicing apparatus to modulate the use of strong splice sites. In addition, sequences within a number of alternative exons have been implicated in regulating the recognition of the exon. So-called exon splicing enhancer elements (ESEs) have been discovered in a variety of regulated exons [Berget, 1995; Chabot, 1996]; a key characteristic of these elements is that deletion will result in skipping of the exon. A number of enhancers identified to date are purine rich and serve as binding sites for members of the SR protein family of splicing regulators [Manley and Tacke, 1996]. Thus, numerous cis elements and trans factors likely collaborate to ensure accurate regulation of splicing.

A few studies have begun to address the cis and trans factors involved in the alternative splicing of human FN exon EDA/EIIIA (we use the term EIIIA to refer to the exon in all species). It was demonstrated that sequences within the regulated exon are required for its inclusion in HeLa cells [Mardon et al., 1987]. Subsequently, the sequences that constitute this positive regulatory element have been defined more narrowly. Exon recognition by the HeLa splicing apparatus requires a short purine-rich tract that lies near the center of the 270-bp-long exon [Caputi et al., 1994]; this purine-rich sequence behaves as an ESE element. The exon EIIIA ESE can stimulate the splicing of the upstream intron [Lavigueur et al., 1993; Staffa and Cochrane, 1995]. In addition, the exon EIIIA ESE interacts with the SR protein ASF/SF2 [Lavigueur et al., 1993], suggesting that this family of splicing regulators might play a role in exon EIIIA alternative splicing. A negatively acting element, termed an exon splicing silencer (ESS), has been identified a short distance downstream of the ESE [Caputi et al., 1994]. Another ESS element that lies between the enhancer and the upstream 3′ splice site tends to suppress splicing due to the formation of secondary structure [Staffa et al., 1997]. Finally, we have identified a protein enriched in limb mesenchymal cell nuclear extract versus chondrocyte extract that binds to the highly conserved polypyrimidine tract region upstream of exon EIIIA [Norton et al., 1998], suggesting that this region also may play a role in regulating exon EIIIA inclusion.

In this report, we have investigated the cis elements and trans-acting factors involved in the regulation of chicken FN exon EIIIA splicing during chondrogenesis, with emphasis on establishing the roles of the ESE and ESS elements. Splicing reporter constructs containing exon EIIIA and its flanking exons and introns were introduced into either primary cultures of

embryonic chick limb mesenchymal cells or chick vertebral chondrocytes. It was established that both the ESE and ESS elements are essential for inclusion in mesenchymal cells. The results of the transfection studies suggest the existence of distinct trans-acting factors in the two cell types, and gel mobility shift data are presented in support of this hypothesis.

MATERIALS AND METHODS

DNA Constructs

Chicken fibronectin minigenes. Various exon EIIIA-containing chicken FN constructs were derived from the 3.5-kb chicken FN exon EIIIA minigene described previously [Norton et al., 1998]. A genomic fragment containing exon EIIIA and its flanking introns and exons 32 and 33 was subcloned into pBluescript using *Bam*H1 and *Xho*1 sites. The structural organization of the EIIIA minigene is shown in Figure 2A. Sequence coordinates refer to GenBank accession number AF003198. Eukaryotic expression constructs are based on the modified version of the RSV-promoter vector RSV0ntLPA (a gift of Dr. S. Adams), which contains the SV40 late polyadenylation signal; this construct is expressed efficiently in primary chicken chondrocytes (S. Adams, personal communication). For purposes of subcloning, the unique *Hin*dIII site was converted to an *Spe*I site. The *Xho*I site preceding the 5′ end of the exon EIIIA minigene also was converted to a *Spe*I site (see Fig. 2). Another *Spe*I site is present in pBluescript. A fragment was released by *Spe*I hydrolysis and subcloned into RSV0ntLPA to generate the expression construct RSV-chA. The RSV/EIIIA constructs were modified to produce deletions of either the ESE or the ESS, or both. In RSV-chA, the ESE is located at 1740–1749 bp and the ESS lies at 1761–1766 bp (Fig. 2). To delete the ESE, the region between *Nhe*I and *Cel*II restriction sites (1392–1752 bp) was amplified using reverse primer *Cel*II#1 lacking the 9-base ESE (5′-TGCAGCTCAGCAGT//GCCACCTGG) and forward primer *Nhe*I (5′-TCGAGCTAG-CAAACTATCAGC). Subcloning of the PCR product into RSV-chA at the *Nhe*I and *Cel*II sites produced RSV-chAdE. To delete the ESS element, a *Cel*II-*Avr*II fragment of exon EIIIA sequence was amplified (1752–2041 bp) using forward primer *Cel*II#2 lacking the 5-base ESS (5′-TGACGCTGAGCTG//CCTCAGGCCAG) and reverse primer *Avr*II (5′-TGCACCTAGGGCA-CACACGGGCT). The PCR product was subcloned into the *Cel*II and *Avr*II sites of exon EIIIA, producing RSV-chAdS. A construct lacking both regulatory elements was generated by combining two PCR products with the deletions described above in one plasmid, RSV-chAdES.

Substrates for RNA-protein binding experiments. Exon EIIIA and variants were subcloned into pGEM-2 under the control of the SP6 RNA polymerase promoter. Subcloning of the native exon EIIIA was described previously (chA)[Gehris et al., 1996]. Mutant versions of exon EIIIA that lacked either the ESE or ESS, or both, were produced by polymerase chain reaction (PCR) amplification, using the RSV constructs as templates. Forward primers incorporated *Eco*RI sites; reverse primers incorporated *Bam*HI sites. All the PCR fragments were subcloned into the *Eco*RI and *Bam*HI sites of pGEM2 to produce plasmids chAdE, chAdS, and chAdES.

Plasmid pchA was manipulated so as to divide the exon into three fragments (see Figs. 2, 5). The 5′ portion of the exon was isolated by deletion of a *Bst*EII-*Bam*HI fragment to generate pchA1. The central portion of the exon was obtained in two steps. First, an *Eco*RI-*Bst*EII fragment was deleted from pchA, resulting in plasmid pchA23. Next, pchA23 was digested with *Bbs*I and *Bam*HI, treated with Klenow and religated to generate pchA2 (the *Bbs*I site cuts 2 base pairs (bp) upstream of the *Cel*II site). The 3′ portion of the exon was obtained by deletion of a *Bbs*I-*Eco*RI fragment to generate pchA3.

Tissue Culture and Transfection Studies

Limb buds from Hamburger-Hamilton stage 23/24 chick embryos were used to obtain mesenchymal cells [Ahrens et al., 1977; San Antonio and Tuan, 1986] for transfection experiments and for preparation of nuclear extracts. The cells were plated in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) on 6-well plates at 3×10^6 cells/well. After the cells formed a homogeneous monolayer of approximately 80–90% confluency without signs of chondrogenesis (usually on the next day), they were transfected with the Rous sarcoma virus (RSV) constructs in the presence of 10 µl of LipofectAMINE (Gibco/BRL) plus 2.0 mg DNA, or 9.0 ml Superfect Reagent (Qiagen) plus 1.5 µg DNA per well. Both methods proved equally effective. The cells were collected 48–72 h after transfection, before the appearance

of foci of chondrogenesis. Total RNA was purified using Trizol Reagent (Gibco/BRL) following manufacturer's directions, and treated with DNase I.

Primary chondrocytes were obtained from the vertebral cartilage of 12- to 13-day-old chick embryos as described previously [Bennett and Adams, 1987]. The cells were plated onto Falcon dishes at 2.0×10^6 cells/100-mm dish in DMEM with 10% FBS. After 1 week of incubation in suspension at 37°C, the cells were trypsinized for 30 min and resuspended in medium containing 4 U/ml of hyaluronidase (H′ase) to eliminate extracellular matrix and improve DNA uptake (S. Adams, personal communication). The cells were replated on bacterial dishes at a concentration of ca. 1.0×10^7 cells in 10 ml of medium. Chondrocytes cultured in this fashion retain their differentiated properties after H′ase treatment [Adams et al., 1977]. After overnight incubation the cells were collected and washed with Hank's balanced salt solution (HBSS). For every plate, 30 µg DNA was combined with 60 µl of Superfect solution in 300 ml DMEM without serum or antibiotics. After 10 min, the complex was added dropwise to the cell cultures. The cells were harvested after 48-h incubation, pellets were resuspended in Trizol Reagent and RNA isolated as above.

RNA Analysis

S1 nuclease protection assay. A probe for S1 protection assay was prepared by PCR amplification of the FN cDNA region spanning exon EIIIA and the portion of exon 33 present in the EIIIA minigene, and subcloning into *Eco*RI/*Bam*HI-cleaved pBluescript. The forward primer contained an *Eco*RI site and exon EIIIA sequence. The reverse primer spanned both the exon 33 region and the *Bam*HI site within pBluescript; this polylinker-derived site was included in the RSV constructs in order to distinguish plasmid-derived mRNAs from endogenous FN mRNAs (see Fig. 3A).

To prepare the labeled probe (see Fig. 3), the plasmid was linearized with *Bam*HI, dephosphorylated, digested with *Kpn*I, and labeled with ^{32}P-γ-ATP using T4 polynucleotide kinase (Boehringer-Mannheim). The DNA was denatured by boiling for 5 min, and the strands were separated on a 6% denaturing gel. After autoradiography, the appropriate band was excised and DNA was eluted, ethanol-precipitated, and resuspended in S1 hybridization buffer (80% formamide, 40 mM Pipes, pH 6.4, 400 mM NaCl, 1 mM EDTA). Total cellular RNA samples (5—20 µg) were dried, then resuspended in 30 µl of S1 hybridization buffer; 1-µl of probe (5–20×10^3 dpm) was added to each RNA sample. Samples were denatured for 10 min at 80°C and hybridized overnight at 40°C. A total of 300 µl of cold S1 nuclease buffer (30 mM sodium acetate, pH 5.0, 250 mM NaCl, 1 mM $ZnCl_2$, 5% glycerol) containing 500 U of S1 nuclease (Promega, Madison, WI) was added to each reaction. After a 30- to 60-min incubation at room temperature, S1 digestions were terminated by phenol extraction, and DNA was precipitated with ethanol. The protected RNA-DNA hybrids were dissolved in formamide loading buffer and resolved on a 6% polyacrylamide gel with 8 M urea. The sizes of the bands were determined by comparison with pGEM marker (Promega).

RT-PCR

A Perkin-Elmer GeneAmp RNA PCR Kit containing MuLV reverse transcriptase (RT) was used for synthesis of the first-strand cDNA. The RT reaction included 1 µg of total cellular RNA and reverse primer 12chA, which is complementary to the region at 3445–3414 bp of exon 33 (5′-AGACTGGTAGGAGTTACCTGAGTGAACTTCAG). The reaction was carried out in a total volume 20 µl at 42°C for 15 min. After denaturing for 5 min at 99°C, the reaction mixture was diluted to 200 µl; 1 µl of the diluted RT reaction was amplified in a volume of 100 µl. For DNA amplification with AmpliTaq DNA polymerase (Perkin-Elmer Cetus), the same reverse primer was used as for the initial RT. Forward primer 11chB corresponds region at 23–52 bp in the exon 32 (5′-ATGGTACAGCGTCTATGCTCAGAATCAGAA). Thirty cycles of amplification were performed; each cycle included 1 min at 94°C, 2 min at 55°C, and 3 min 72°C; 40 µl of each reaction mixture was analyzed by electrophoresis through a 5% polyacrylamide gel. Gels were stained in ethidium bromide and photographed, and the contrast of the image reversed. The sizes of the expected RT-PCR products for this pair of primers are 388 bp (A^+ form) and 118 bp (A− form).

RNA-Protein Binding Experiments

Nuclear extract preparation. A modification of the method described by Dignam et al. [1983] was used to prepare the nuclear extracts (NE) from primary chondrocytes, mesenchymal

cells, or dissected limb buds of stage 23–24 chicken embryos. Pellets of cells or limb buds were resuspended in 4–5 vol of 10 mM Hepes, pH 7.5, 10 mM KCl, 1 mM EGTA plus protease inhibitors (phenylmethylsulfonylfluoride [PMSF], dithiothreitol, pepstatin, L-(tosylamido-2-phenyl)ethyl chloromethyl ketone, leupeptin, trypsin inhibitor, spermidine, and spermine). After 30 min on ice, cell pellets were lysed with a Dounce homogenizer. Nuclei were recovered by centrifugation at 12,000 rpm for 20 min at 4°C and resuspended in 20 mM Hepes pH 7.5, 25% glycerol, 0.42 M KCI, 1 mM EGTA, 0.2 mM EDTA, and protease inhibitors. The nuclei were shaken gently at 4°C for 30 min, followed by centrifugation at 12,000 rpm for 30 min at 4°C. The clear supernatant, NE, was aliquoted, frozen in liquid nitrogen, and stored at −80°C. Protein concentration was determined using Coomassie Plus Protein Reagent (Pierce).

Gel mobility shift assay. Radiolabeled transcripts were prepared from subcloned exon EIIIA variants that had been linearized by digestion with *Bam*HI (pchA, pchAdE, pchAdS, and pchAdES) or *Hin*dIII (pchA1, pchA2, pchA3, and pchA23). In vitro transcription was performed with SP6 RNA polymerase in the presence of α-^{32}P-CTP [Norton et al., 1998]. Labeled RNAs were purified by electrophoresis through denaturing polyacrylamide gels. RNA-protein binding was carried out for 20–30 min at room temperature in a final volume of 15–20 µl that included 10 mM Hepes, pH 7.9, 10% glycerol, 5 mM $MgCl_2$, 20 mM KCl, 1 mM DTT, 5–7.5 µg of protein and 5×10^4-1.5×10^5 cpm of α-^{32}P-CTP-labeled probe. Heparin was added at the end of the incubation to a final concentration of 6 µg/ml. For gel mobility shift assay, samples were loaded directly on 4.5% gels (acrylamide-to-bisacrylamide ratio of 60:1) in Tris-borate-EDTA buffer, and complexes were detected by autoradiography of dried gels.

RESULTS

Establishment of Transfection Conditions That Maintain Primary Limb Mesenchymal Cells and Vertebral Chondrocytes in Their Respective States of Differentiation

For transfection studies, primary cells were isolated from either stage 23/24 chick limb bud mesenchyme or day 12–13 vertebral cartilage and placed into culture. Cells were cultured for either 1 day (mesenchymal cells) or 1 week (chondrocytes) before transfection with splicing reporter RSV-βgal to assess transfection efficiency. Several methods of introducing DNA into these two cell types were tested. It has been reported that calcium phosphate precipitation can be used to introduce DNA into high density cultured limb mesenchymal cells [Szabó et al., 1995], but this procedure resulted in significant cytotoxicity in our hands. However, both LipofectAMINE and Superfect reagents were effective (transfection efficiency of ca. 5–10%, not shown), with reduced toxicity. LipofectAMINE also has been used for transfection of chondrocytes [Pallante et al., 1996]. Therefore, these two reagents were used to test the ability of the cells to produce A^+ or A^- mRNAs from EIIIA-containing splicing reporter constructs (see Materials and Methods for additional details).

It was important to verify that cell- and differentiation-specific splicing of FN mRNAs was preserved after the cell culture and transfection manipulations. Mesenchymal cells and chondrocytes were transfected with the eukaryotic expression construct pRSV0ntLPA with no insert. RNA was isolated 2–3 days post-transfection, and FN alternative splicing was analyzed by RT-PCR using primers that flank exon EIIIA. This analysis demonstrated that mesenchymal cells produce largely A^+ FN mRNA and chondrocytes produce largely A− FN mRNA (Fig. 1). Thus, both cell types maintained their differentiated phenotype with respect to FN alternative splicing throughout the culture and transfection manipulations.

Transfection of Mesenchymal Cells and Chondrocytes Demonstrates Differential Splicing of Exon EIIIA From a Minigene Reporter Construct

The chicken genomic region containing alternative FN exon EIIIA and flanking introns and exons was subcloned into RSV promoter vector RSV0ntLPA to produce splicing reporter construct RSV-chA (Fig. 2). Primary cells were transfected with RSV-chA as described above, and total RNA was isolated 2–3 days later. An S1 nuclease protection assay was devised to monitor splicing of the plasmid-derived RNAs independent of the endogenous chick FN mRNA. The structure of the probe and the protected fragments expected to arise from each mRNA are diagrammically represented in Figure 3. Note that the 5′ end label is added at a *Bam*HI

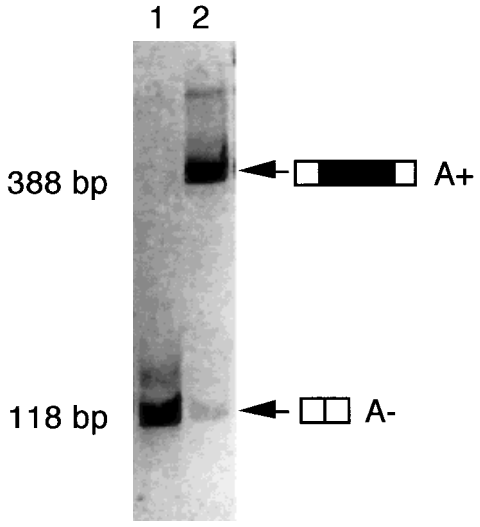

Fig. 1. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of the splicing pattern of exon EIIIA was carried out on total endogenous RNA purified from transfected primary cells. PCR products were fractionated on a 5% polyacrylamide gel and detected by ethidium bromide staining. A^+ and A^- mRNAs are indicated by the diagrams to the right; open boxes, flanking exons 32 and 33; black box, exon EIIIA. **Lane 1**, cultured and transfected chondrocytes; **lane 2**, cultured and transfected mesenchymal cells. Sizes of the products shown at left were determined on the basis of molecular size standards.

site that lies outside the FN sequences in the reporter construct, so that endogenous FN mRNAs will not yield a protected product.

The pattern of RSV-chA splicing in the two cell types is compared in Figure 3. The predominant RSV-chA-derived mRNA detected in mesenchymal cells is the exon EIIIA+ form, whereas approximately two-thirds of the reporter-derived mRNA detected in chondrocytes is the A^- form (cf. lanes 1 and 5). These results generally are consistent with the pattern of splicing of the endogenous fibronectin transcripts in these cell types, although possible reasons for the incomplete exclusion of exon EIIIA in chondrocytes are considered in the Discussion. Thus, the RSV-chA construct can be exploited to investigate the cis sequences and trans factors that mediate exon EIIIA inclusion in mesenchymal cells and exclusion in chondrocytes.

Deletion of Exon Sequences Reduces Exon IIIA Inclusion in Mesenchymal Cells but Does Not Activate Inclusion in Chondrocytes

It was previously reported that both positive and negative cis-regulatory elements reside within exon EIIIA of the human FN gene [Caputi et al., 1994]. The equivalent sequences in the chicken gene are indicated in Figure 2 as ESE or ESS, after the nomenclature of Caputi et al. [1994]. We investigated the possible roles of these elements in cell type-specific alternative splicing of chick exon EIIIA by deleting each element individually (RSV-chAdE and RSV-chAdS) or in combination (RSV-chAdES) from construct RSV-chA. These constructs were introduced into mesenchymal cells and chondrocytes and the pattern of exon EIIIA inclusion was monitored by S1 nuclease protection assay (Fig. 3B). Note that exon EIIIA-included products protect slightly smaller fragments due to mismatches between the wild-type probe and the target RNAs resulting from the deletion constructs (diagrammed in Fig. 3A). These analyses revealed that deletion of the ESE in RSV-chAdE reduced the level of exon EIIIA inclusion in mesenchymal cells (lane 2), as would be expected if this element played a positive role in exon inclusion. Deletion of the ESS also reduced exon EIIIA inclusion in mesenchymal cells (lane 3), as did deletion of both sites in RSV-chAdES (lane 4). These results indicate that both the ESE and ESS are required for efficient exon EIIIA inclusion in mesenchymal cells, and raise the possibility that the ESE and ESS act in concert.

In chondrocytes, deletion of the ESE had no effect on splicing pattern (cf. lanes 5 and 6). By contrast, deletion of the ESS eliminated the atypical exon EIIIA inclusion (lane 7). The double mutant RSV-chAdES also produced A^- mRNA exclusively (lane 8). These results suggest that the partial exon EIIIA inclusion observed in the chondrocytes occurs via a mechanistically distinct pathway from exon EIIIA inclusion in mesenchymal cells. We also note that neither deletion activated exon EIIIA inclusion in chondrocytes. The distinct effects of the ESE and ESS mutations in the two cells types suggest the presence of different cell-specific trans-acting factors.

Cell-Type Specificity of Nuclear Factors That Bind to Exon EIIIA

To explore the possible existence of cell type specific factors that interact with exon EIIIA sequences, nuclear extracts were prepared from chick limb mesenchyme or from vertebral chondrocytes. Each extract was incubated either with labeled chA RNA, representing intact exon EIIIA, or with versions of the exon that were

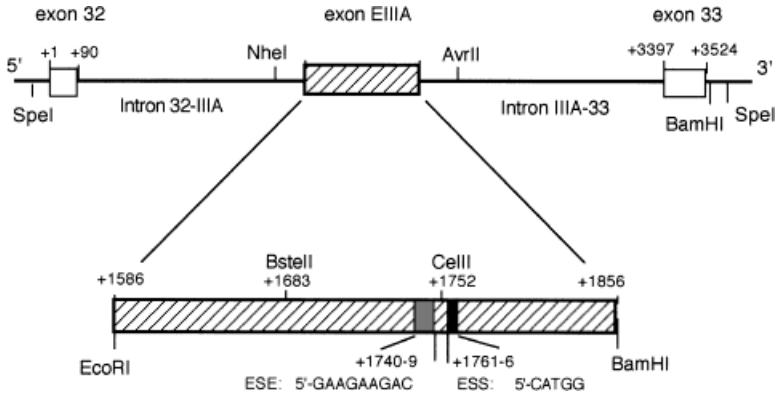

Fig. 2. Structural organization of the chicken FN exon EIIIA minigene, RSV-chA. The coordinates of all structural elements are based on GenBank accession number AF003198 and are given in base pairs. Above, diagram of the RSV-chA minigene subcloned in pBluescript; the T7 promoter is upstream of the insert. The *Spe*I fragment was subcloned into pRSV0ntLPA such that the insert is under the control of the RSV LTR. Lines, introns; boxes, exons; alternative exon EIIIA is shaded. Below, exon EIIIA is expanded to show sequence elements that were mutated to generate the RSV-chAdE, RSV-chAdS, and RSV-chAdES versions.

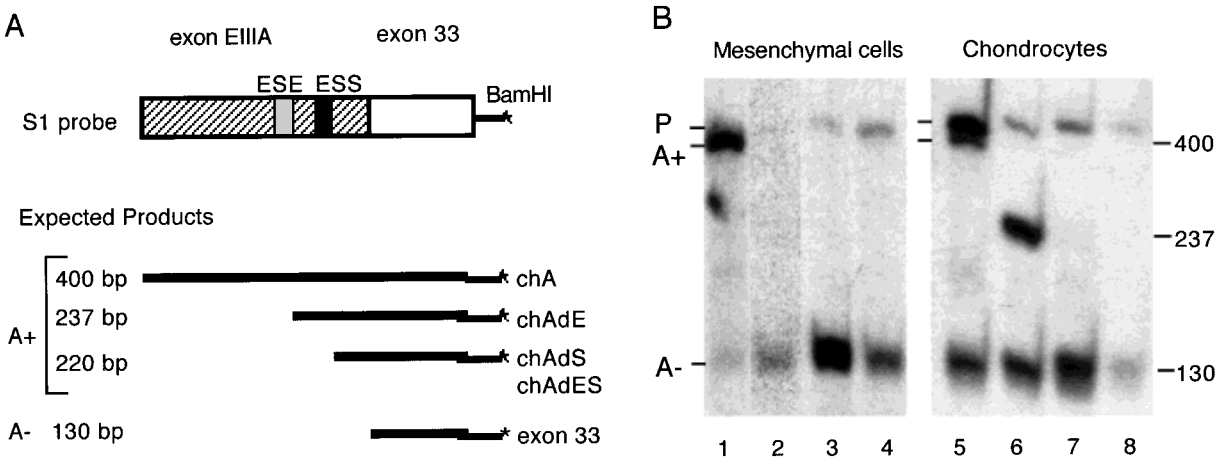

Fig. 3. S1 nuclease protection analysis of reporter-derived mRNA splicing. **A:** Schematic illustration of the probe used for S1 analysis and the protected products expected to result from the various reporter constructs. Exon EIIIA sequence is indicated by the hatched region, exon 33 by the open rectangle and the exon splicing element (ESE) and exon splicing silencer (ESS) sequences are depicted with light and dark shaded regions, respectively. Note that A^+ mRNAs from deletion mutants are expected to protect shorter fragments due to mismatch with the wild-type probe. **B:** Fragments protected from digestion by S1 nuclease are resolved on denaturing polyacrylamide gels. Fragment sizes were determined by molecular size markers run in parallel. Either mesenchymal cells or chondrocytes, as indicated, were transfected with RSV-chA (**lanes 1, 5**), RSV-chAdE (**lanes 2, 6**), RSV-chAdS (**lanes 3, 7**), or RSV-chAdES (**lanes 4, 8**). P, undigested probe, which is slightly larger than the A^+ protected fragment die to the presence of polylinker sequence on the 3′ end of the probe.

deleted for either the ESE (chAdE) or ESS (chAdS), or both (chAdES). RNA binding activity was assessed in presence of heparin to reduce nonspecific interactions, and RNA-protein complexes were detected by native gel electrophoresis. These studies revealed that both chondrocyte and mesenchymal extracts were capable of forming complexes on the exon EIIIA RNA, with distinct, heparin-resistant complexes observed for the two extracts (Fig. 4, arrow labeled M and bracket labeled C indicate complexes in lanes 2 and 3). Deletion of either the ESE or ESS appeared to eliminate both complexes (lanes 4–12), suggesting a role for these sequences in the observed RNA-protein interactions.

To better define the sequence requirements for RNA-protein complex formation, exon EIIIA was subdivided, and the corresponding transcripts were analyzed in the gel mobility shift assay. Fragment chA1 did not form any heparin-resistant cell-type specific complexes (data not

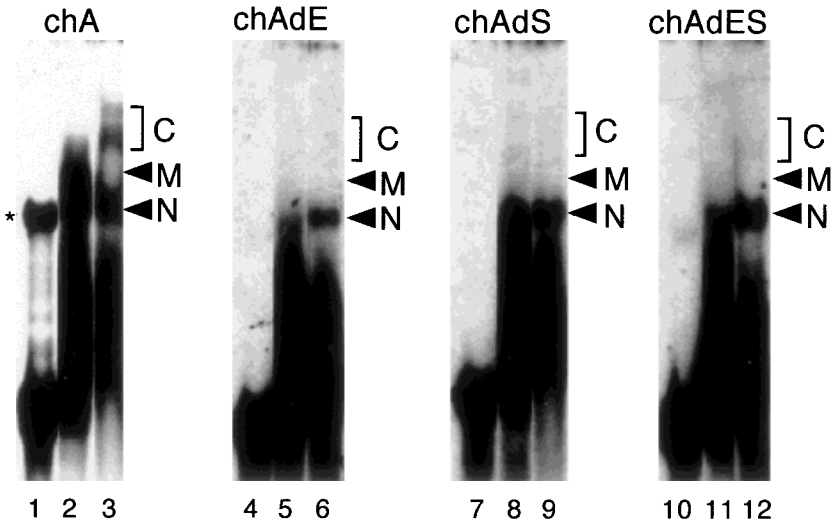

Fig. 4. Gel mobility shift analysis of exon EIIIA binding proteins. Labeled RNAs chA (**lanes 1–3**), chAdE (**lanes 4–6**), chAdS (**lanes 7–9**), and chAdES (**lanes 10–12**) were incubated with nuclear extract prepared from mesenchymal cells (**lanes 2, 5, 8, 11**) or chondrocytes (**lanes 3, 6, 9, 12**), as indicated. **Lanes 1, 4, 7, 10**, probe alone. Arrows, the positions of heparin-resistant complexes that form in mesenchymal cell extract (**lane 2, M**) or chondrocyte extract (**lane 3, C**) on chA RNA. Note that these complexes fail to assemble on any of the mutant RNAs. Arrow N, a complex that forms in a non-cell-specific fashion. The upper band in lane 1 (asterisk) apparently is due to secondary structure within the chA RNA, as the same preparations run as a single band under denaturing conditions.

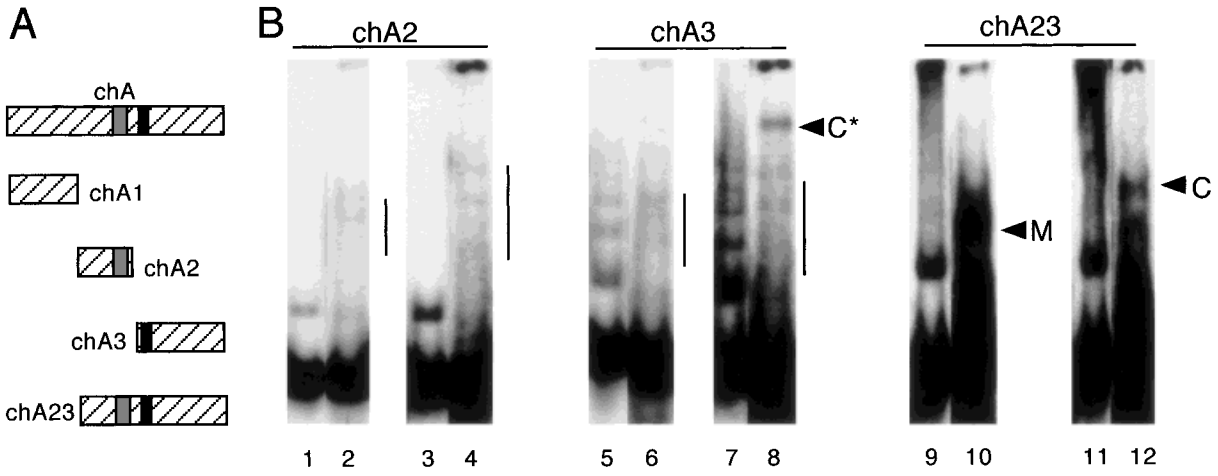

Fig. 5. Gel mobility shift analysis with subfragments of exon EIIIA. **A:** Diagrammatic representation of the various labeled chA-derived RNA fragments, with the exon splicing element (ESE) indicated in gray and the exon splicing silencer (ESS) in black. **B:** Gel mobility shift analysis of complex formation on RNAs chA2 (**lanes 1–4**), chA3 (**lanes 5–8**), and chA23 (**lanes 9–12**). Faint heparin-resistant complexes formed in both extracts on chA2 and chA3 RNAs are indicated with a line. Arrows labeled M or C, complexes that form in mesenchymal cell or chondrocyte extract on chA23 RNA. Arrow labeled C*, a complex that forms on chA3 in chondrocyte extract.

shown). Fragment chA2, containing the ESE, formed diffuse heparin-resistant complexes with both mesenchymal cell and chondrocyte extracts (Fig. 5, lanes 2 and 4). Fragment chA3 formed a heparin-resistant complex with chondrocyte extract (lane 8, C*), but the amount of this complex was variable. In contrast, both chondrocyte and mesenchymal cell extract assembled distinct heparin-resistant complexes on RNA fragment chA23 (lanes 10, M, and 12, C). The M and C complexes resemble those that form on intact chA RNA (Fig 4, lanes 2 and 3), based on mobility relative to unbound probe. Similarly, the C and C* complexes do not appear to be equivalent. The results suggest that the factors that bind to exon EIIIA differ in the

two cell types. Moreover, formation of the mesenchymal cell complex requires both the ESE and ESS sites, consistent with the positive role observed for both sites in the transfection experiments.

DISCUSSION

The pattern of alternative splicing of the FN exon EIIIA undergoes a striking shift during chondrogenesis, from complete inclusion in the undifferentiated mesenchymal cells to complete exclusion in the differentiated chondrocytes. Recent findings from our laboratory suggest that the A^+ isoforms of FN play a functionally important role in the condensation event that precedes overt chondrogenesis, and we hypothesize that subsequent loss of this segment is needed for terminal chondrocyte differentiation [Gehris et al., 1997]. In the present study, we have investigated both the cis elements and trans-acting factors that are involved in the splicing change.

We first established transfection procedures that permitted both cultured primary mesenchymal cells and chondrocytes to retain their differentiation state-specific splicing of the FN exon EIIIA (Fig. 1). Specifically, mesenchymal cells produced A+ FN mRNA and chondrocytes produced A^- FN mRNA. Transfection of minigene splicing reporter construct RSV-chA also resulted in differential splicing in the two cell types, but some minigene-derived A^+ mRNA was observed in the chondrocytes, whereas the endogenous FN mRNA was almost exclusively A^- (cf. Figs. 1 and 3). Other workers have noted discrepancies between the splicing pattern of transcripts arising from endogenous or stably transfected genes versus those produced from transiently transfected plasmids [Carstens et al., 1998; Huh and Hynes, 1993; Libri et al., 1989]. The reasons for these differences are not well understood but they might relate to the abnormally high level of a single pre-mRNA in the transfected cells, as appears to be the case for the fibroblast growth factor receptor 2 gene [Carstens et al., 1998]. The discrepancy between the transfected and endogenous transcripts suggests the interesting possibility that exon EIIIA exclusion in chondrocytes requires factors that are titrated out by high pre-mRNA levels in transfected cells. An alternative explanation is that a less differentiated subpopulation of the chondrocytes is transfected preferentially, skewing the splicing pattern of the reporter.

Transfection of the two primary cell types was used to establish the roles of two exon sequence motifs in exon EIIIA splicing. A motif referred to as the ESE had been reported to be a positively acting element, promoting splicing of an upstream intron in vitro and inclusion of exon EIIIA (termed EDA or ED-1) in HeLa cells [Caputi et al., 1994; Lavigueur et al., 1993; Staffa and Cochrane, 1995]. A motif referred to as the ESS was thought to play a negative role recognition of the exon in HeLa cells [Caputi et al., 1994]. By contrast, our results indicate that both motifs are required for exon EIIIA inclusion in mesenchymal cells, as mutation of either sequence resulted in exon skipping (Fig. 3). Neither mutation reduced exon EIIIA skipping in chondrocytes, although we note that the inappropriate exon inclusion observed in the chondrocytes is eliminated by deletion of the ESS. The discrepancy between our data and those of Caputi et al. [1994] could be attributable to either cell type-specific or species-specific differences in ESS function. Inspection of the chicken and human exon EIIIA sequences shows that the ESS sequence, CAAGG in human FN, is CAUGG in the chick gene. A recent report suggests that the mouse ESE element (CAGGG) is functionally equivalent to the human version [Muro et al., 1998]. Further work is needed to assess the consequences of these base substitutions.

The different pattern of splicing of the RSV-chA reporter RNA in the two cell types leads us to hypothesize that different splicing factors are present in the two cell types. We have reported previously that a protein enriched in limb mesenchymal cell nuclear extract versus chondrocyte extract crosslinks to the highly conserved polypyrimidine tract region upstream of exon EIIIA [Norton et al., 1998]; this factor thus represents a candidate positive regulatory factor for exon EIIIA inclusion. We wished to investigate whether other, cell-specific differences in RNA binding proteins might exist between the two cell types. Specifically, the ability of the exon EIIIA ESE and ESS mutations to alter splicing suggested that there might be differences in proteins that bind to the exon itself. Gel mobility shift studies revealed that distinct, heparin-resistant complexes were formed on a labeled exon EIIIA fragment in nuclear extracts derived from the two cell types

(Fig. 4). The mesenchymal cell-specific protein-RNA interactions were dependent on the presence of both ESE and ESS sites (Figs. 4, 5). The requirement for both sites is consistent with the transfection results, which demonstrated that exon EIIIA inclusion requires both elements. We hypothesize that the ESE and ESS cooperate in mesenchymal cells to recruit factors that drive exon inclusion. The 12 nucleotide distance between the two sequence elements is compatible with either two factors, one recognizing each site, or a single factor interacting with both sequence elements.

The pattern of protein binding to exon EIIIA in chondrocyte extract suggests a more complex situation. The dependence of chondrocyte-specific protein-RNA interactions on either the ESE and ESS could not be predicted from the transfection experiments; nevertheless, deletion of either the ESE or the ESS from full-length chA RNA prevented complex formation (Fig. 4). However, it appeared that the ESS-containing fragment chA3 was capable of interacting with chondrocyte factors despite the absence of the ESE (Fig. 5), although the relationship between this complex and the complexes that form on larger fragments chA23 and chA is unclear. Examination of the vector-derived sequence present immediately upstream of the exon EIIIA sequences in the chA3 transcript provides a possible explanation. Normally, the ESE (5′-GAAGAAGAC) lies 12 nt upstream of the ESS. In the chA3 transcript, a purine-rich sequence (5′-GAATACACGGAA) lies 11 nt upstream of the ESS. It is possible that this sequence provides partial ESE function. The simplest interpretation appears to be that in chondrocyte extract a complex forms on chA and chA23 RNAs in a manner that is dependent on both the ESE and ESS, but that this complex is distinct from the one that forms on the same sequences in mesenchymal cell extract.

Taken together, these results suggest a model in which exon EIIIA splicing is regulated by a balance between positive and negative factors competing for binding to a common region that includes both the ESE and the ESS. In mesenchymal cells, positive factors require both elements to facilitate exon recognition by the splicing apparatus. As deletion of the same elements does not activate exon inclusion in chondrocytes, we postulate that the function of chondrocyte-enriched factors must be to antagonize the activity of positive factors by competing for RNA binding sites. It has been reported previously that the ESE can interact with one member of the SR protein family of splicing factors [Lavigueur et al., 1993]. These proteins tend to promote exon inclusion by virtue of sequence-specific binding [Fu, 1995; Manley and Tacke, 1996]. We are currently evaluating whether SR proteins may play a role in exon EIIIA inclusion in mesenchymal cells.

ACKNOWLEDGMENTS

This work is dedicated to the memory of Dr. Vickie D. Bennett, deceased January 4, 1998. The authors thank J.W. Hall and D.G. White for expert technical assistance and B.A. Kuo for many helpful suggestions during the course of the project. This work was supported by NIH grant AR42887 (to V.D.B.).

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