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Cis-acting elements regulate alternative splicing of exons 6A, 6B and 8 of the α1(XI) collagen gene and contribute to the regional diversification of collagen XI matrices

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Abstract

Consecutive exons 6A, 6B, 7 and 8 that encode the variable region of the amino-terminal domain (NTD) of the col11a1 gene product undergo a complex pattern of alternative splicing that is both tissue-dependent and developmentally regulated. Expression of col11a1 is predominantly associated with cartilage where it plays a critical role in skeletal development. At least five splice-forms (6B-7-8, 6A-7-8, 7-8, 6B-7 and 7) are found in cartilage. Splice-forms containing exon 6B or 8 have distinct distributions in the long bone during development, while in non-cartilage tissues, splice-form 6A-7-8 is typically expressed. In order to study this complex and tissue-specific alternative splicing, a mini-gene that contains mouse genomic sequence from exon 5 to 11, flanking the variable region of α1(XI)-NTD, was constructed. The minigene was transfected into chondrocytic (RCS) and non-chondrocytic (A204) cell lines that endogenously express α1(XI), as well as 293 cells which do not express α1(XI). Alternative splicing in RCS and A204 cells reflected the appropriate cartilage and non-cartilage patterns while 293 cells produced only 6A-7-8. This suggests that 6A-7-8 is the default splicing pathway and that cell or tissue-specific *trans*-acting factors are required to obtain pattern of the alternative splicing of α1(XI) pre-mRNA observed in chondrocytes. Deletional analysis was used to identify *cis*-acting regions important for regulating splicing. The presence of the intact exon 7 was required to generate the full complex chondrocytic pattern of splicing. Furthermore, deletional mapping of exon 6B identified sequences required for expression of exon 6B in RCS cells and these may correspond to purine-rich (ESE) and AC-rich (ACE) exonic splicing enhancers.

Keywords: Collagen, α1(XI); Alternative splicing; Exon enhancer; Regulation

1. Introduction

Type XI collagen is a heterotrimeric member of the fibrillar collagens with a structurally complex N-terminal domain (NTD) (Bernard et al., 1988; Morris et al., 2000; Kimura et al., 1989; Yoshioka and Ramirez, 1990; Zhidkova et al., 1993). The NTDs of the α1 and α2 chains are composed of a minor triple helix, a variable region and a propeptide (Npp domain) (Fig. 1). The structure of the N-terminal domain of type XI collagen is modulated by alternative splicing of the exons comprising the variable region in a developmentally regulated and cell and tissue-specific manner (Oxford et al., 1995; Morris et al., 2000; Iyama et al., 2001; Davies et al., 1998). Alternative splicing in the variable region involves exons 6A, 6B and 8 of α1(XI) (Oxford et al., 1995; Yoshioka et al., 1995; Zhidkova et al., 1995) and exons 6, 7 and 8 of α2(XI) (Tsumaki

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and Kimura, 1995; Zhidkova et al., 1995). Because it is derived from the type II collagen gene, the structure of the NTD of the α3 chain is modulated by the inclusion or exclusion of exon 2, corresponding to collagen type IIA and IIB respectively (Wu and Eyre, 1995; Ryan and Sandell, 1990). Upon differentiation from mesenchymal cell to chondrocyte, exon 2 of α3 (XI) is excluded (Davies et al., 1998) and exons 6, 7 and 8 of α2(XI) are largely excluded (Sugimoto et al., 1998; Tsumaki and Kimura, 1995). This parallels the well-characterized switch from collagen type IIA to IIB during chondrocyte differentiation (Sandell et al., 1994, 1991; Ryan and Sandell, 1990). On the other hand, the splicing pattern of the α1(XI) chain shifts from 6A-7-8 in prechondrocytic cells to the expression of a complex pattern of splice-forms including 6A-7-8, 6B-7-8, 7-8, 6B-7 and 7 in chondrocytes (Davies et al., 1998). 6A-7-8 appears to be the predominant splice-form when α1(XI) is expressed in non-cartilaginous tissues (Yoshioka et al., 1995; Oxford et al., 1995). Exon 6B-7 and exon 7 splice-forms are the most abundant within cartilage. Quantitative analysis indicates that exons 6A and 6B are mutually exclusive and exon 6B is predominantly expressed without exon 8 while exon 6A is predominantly expressed with exon 8 (Davies et al., 1998). This indicates that splice site selection on either side of exon 7 is co-ordinated. At the protein level, isoforms containing the basic p6B peptide (encoded by exon 6B) are restricted to the cartilage in the diaphysis adjacent to perichondrium, a pattern that arises de novo, and are absent from epiphyseal cartilage and the cartilage of the developing cuboidal bone (Morris et al., 2000). Isoforms containing the acidic peptide p8 (encoded by exon 8) are more uniformly distributed at the beginning of chondrogenesis but then recede first from the diaphysis and then the epiphysis as development proceeds, and are eventually restricted to the forming articular cartilage overlying the epiphysis (Fig. 1) (Morris et al., 2000).

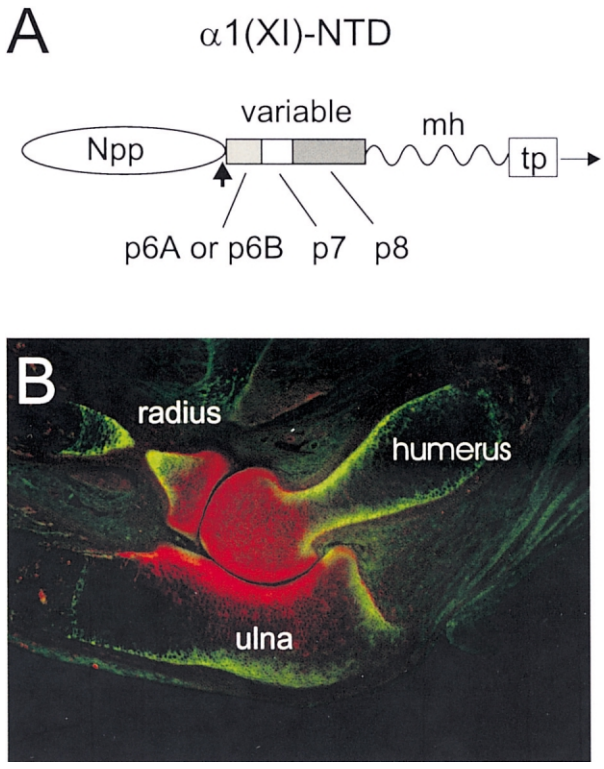

Fig. 1. Structure of N-terminal domain and distribution of α1(XI) collagen isoforms. (A) The amino terminal domain of the α1(XI) collagen chain contains an amino propeptide (Npp), a variable region modulated by alternative splicing, and the minor collagen triple helix (mh) and telopeptide (tp) characteristic of fibrillar collagens. The variable region is composed of peptides p6a, p6b, p7, and p8 encoded by the corresponding exon. The p7 peptide is constitutively expressed while the others are included in different combinations. Because p6b is very basic and p6a and p8 are very acidic, the different combinations result in very diverse compositions of the variable domain. Proteolytic processing occurs between the Npp and the variable region rather than after the telopeptide, so the variable region is retained in the mature type XI collagen molecule. (B) Antibodies to p6B (green) and to p8 (red) were used to stain the elbow of an 18 day rat fetus by indirect immunofluorescence. Isoforms containing p6b were found primarily in the diaphysis adjacent to the perichondrium while p8-containing isoforms were found in the less mature epiphyseal cartilage at this stage.

As a minor component of fetal cartilage collagen fibrils (Mendler et al., 1989; Wu et al., 1992; Wu and Eyre, 1995), type XI collagen plays a critical role in regulating fibrillogenesis of type II collagen that is required for normal skeletal development (Li et al., 1995; Seegmiller et al., 1971). Proteolytic processing of the type XI procollagen removes the *N*-propeptide, albeit slowly (Moradi-Ameli et al., 1998; Thom and Morris, 1991), but the variable region with its extended structure (Gregory et al., 2000) is retained in the mature molecule and can be localized to the fibril surface (Morris et al., 2000; Keene et al., 1995). Because the N-terminal domain cannot be accommodated within the fibril, the propeptide and the variable region of type XI collagen sterically hinder further addition of type II collagen molecules and thus limits fibril diameter (Keene et al., 1995; Gregory et al., 2000; Blaschke et al., 2000).

The α1(XI) chain forms a heterotrimer with α2(XI) and α1(II) in cartilage and associates with chains of the closely related type V collagen in non-cartilage tissues (Niyibizi and Eyre, 1989; Kypreos et al., 2000; Mayne et al., 1993; Kleman et al., 1992). This regional diversification of collagen XI matrices is amplified by the differential distribution of variable region isoforms of α1(XI). The tissue-specific and developmental regulation of alternative splicing suggest the potential for additional functions as has been proposed for exon 2 of type II collagen (Zhu et al., 1999) and provide the impetus for this initial attempt to identify

cis-acting elements that are involved in regulating this complex system of alternative splicing.

Typically exon-skipping results from splice site sequences that deviate significantly from the consensus and therefore interact poorly with the normal exon splicing machinery (Black, 1995). This defect can be overcome by the participation of additional sequences, both enhancers and silencers, either in the intron or in the exon (Cooper and Mattox, 1997; Coulter et al., 1997; Schaal and Maniatis, 1999; Muro et al., 1998). Exon enhancers interact with transacting factors, SR proteins, to promote utilization of weak splice sites. Purine-rich exon splice enhancers (ESE) and AC-rich exon enhancers (ACE) have been identified (Cooper and Mattox, 1997). Utilization of an exon in one cell but not another is mediated by the expression of specific splice factors or specific combinations of splice factors (Zahler et al., 1993). We have evaluated the conventional splice site sequences of the α1(XI) gene from exon 5 to exon 9 encompassing the variable region and using a minigene construct identified putative purine-rich and AC-rich enhancer elements required for the inclusion of cartilage-specific exon 6B. We have also examined the potential for constitutively included exon 7 to regulate the co-ordinated expression of exons 6A, 6B and 8.

2. Results

2.1. Validation of the splicing assay

In order to examine *cis*-acting elements involved in the regulation of alternative splicing in the variable region of the collagen α1(XI) chain, a minigene construct encompassing most of exon 5 through the 3′end of exon 11 was cloned into pCDNA-3 (Fig. 2). Minigene transcripts were amplified by RT-PCR using primers anchored in the vector sequence. Endogenous transcripts were amplified using genomic-based primers (Fig. 2). In some cases, the cDNA was resolved by denaturing gel electrophoresis after end labeling with ^{32}P. In others experiments, the general pattern was visualized by probing a Southernblot of the RT-PCR products with a probe to exon 5, which is common to all splice forms. In all cases, identification of each of the products was based first on comparison to the pattern established previously (Oxford et al., 1995). In addition, the identity of each band was confirmed by Southern analysis using probes specific to exons 6A, 6B, 7, and 8.

To validate the splicing assay using the minigene, mRNA transcripts derived from the minigene were compared to endogenous transcripts in the following cell lines: Rat chondrosarcoma (RCS) as a model for chondrocytes (Oxford et al., 1995); A204 rhabdomyosarcoma cells, a non-chondrocyte cell line expressing α1(Xl) (Kleman et al., 1992); and 293 cells which are not known to make α1(XI) (Fig. 3). The endogenous transcript showed the expected patterns of splicing. Splice-forms 6A-7-8, 7-8 and abundant 6B-7 and 7 (null) were detected in RCS cells, while A204 cells showed only 6A-7-8, the splice-form more typical of non-chondrocytes. No α1(XI) transcripts were detected in 293 cells. In the human cell lines, A204 and 293, different primers (nested with respect to the Rat primers, Fig. 2) were used for PCR so the 6A-7-8 product was smaller in size. The minigene generated splicing patterns that were qualitatively similar to the endogenous transcripts. The four isoforms of RCS were present but 6B-7 was somewhat under represented. A204 and 293 both yielded 6A-7-8 but both also produced traces of 7-8 as well. Never-

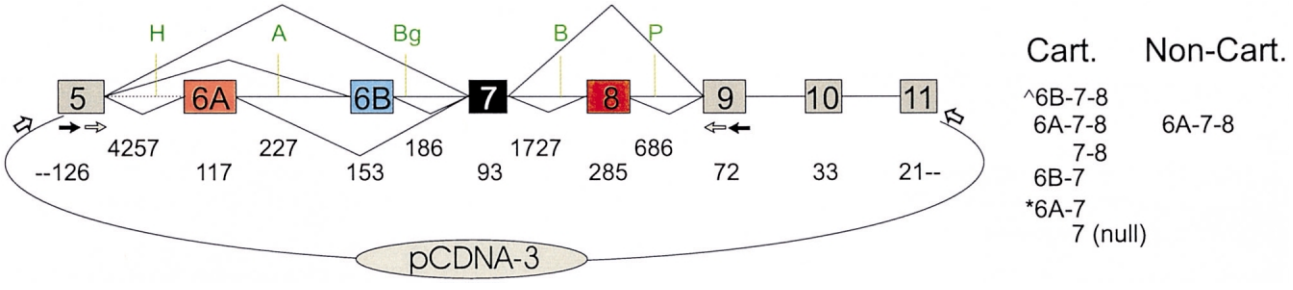

Fig. 2. Minigene construct for the analysis of alternative splicing. A minigene was constructed from a genomic fragment of α1(XI) containing the 3′ end of exon 5 through the 5′ end of exon 11 cloned into pCDNA-3. The mini gene was approximately 12 kb and the sizes of the exons and most of the introns are shown. The options for splicing of each exon are indicated. The open arrows indicate the location of the vector-based primers used to analyze the minigene transcripts by RT-PCR. Primers in exons 5 and 9 were used to characterize endogenous transcripts in the cell lines used (rat primers: black arrows; human primers: gray arrows). Because the human primers are nested relative to the rat primers, the human splice-form products are smaller than their corresponding rat splice-forms. The restriction sites used for exon deletion are Hind III (*H*), AluI (*A*), Bg1II (*Bg*), BarnHI (*B*), and PstI (*P*). At the right are shown the splice-forms typically associated with non-cartilaginous tissues (NC) and those found in cartilage (C). ^6B-7-8 is low but variable in amount and *6A-7 is almost undetectable. The splice-form, -7-, without any of the alternatively spliced exons is also referred to as null.

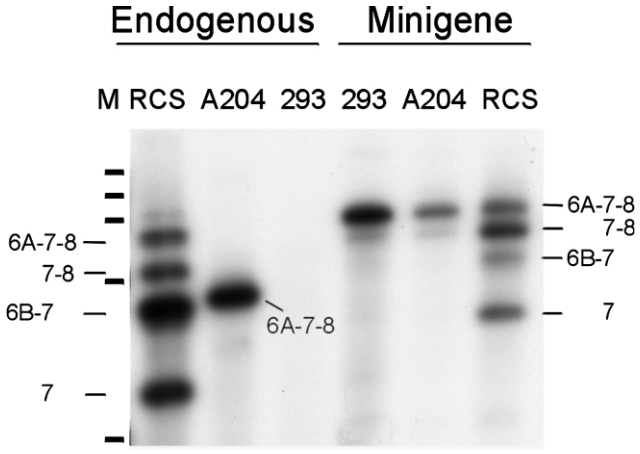

Fig. 3. Comparison of minigene with endogenous transcripts. Rat chondrosarcoma (RCS) and human cell lines A204 and 293 were examined for the expression of α1(XI) isoforms by RT-PCR before (endogenous) or after transfection with the minigene. PCR products were end-labeled with ^{32}P and examined by electrophoresis on an alkaline denaturing agarose gel. The identity of each band is indicated and was verified by Southern analysis using exon-specific probes. Note that 6A-7-8 from A204 is smaller than 6A-7-8 from RCS due to the nested position of the human primers relative to the rat primers. The markers are 1353, 1078, 872, 603, 310 bp.

theless, splicing was sufficiently authentic to permit further analysis.

2.2. Operation of conventional splice signals

A survey of splice acceptor and donor sequences surrounding exons 6A, 6B, 7 and 8 from mouse, chick, rat, and human revealed weaker splice signals flanking the alternatively spliced exons (Fig. 4). Particularly with regard to the splice acceptor, only exon 7, which is constitutively expressed, met the criteria of the consensus sequence. All of the exons except 6B have a branch point within 40 nucleotides. To test whether splice signals account for the failure of non-chondrocytes to express exon 6B, a new potential branch point within 40 bp of exon 6B was introduced by conversion of TTCT**C**AC to TTCT**A**AC (YNYTRAY). Alternatively, the 3′ acceptor site, cagA, was changed to cagG (yagG), but neither of these changes induced the expression-of exon 6B in A204 cells (data not shown).

2.3. Co-ordination of alternative splicing

To examine the effect of exon sequences on the splicing of the other exons, each of the exons with some flanking intron was deleted in turn. Exon 6A was deleted at the intron-exon boundaries in order to preserve any intronic sequence involved in the splicing of exon 6B. Splicing of the resultant minigene constructs was analyzed in RCS and A204 cells to see if exon deletion altered the pattern of chondrocytic and non-chondrocytic splicing, other than changes due to the absence of the exon itself (Fig. 5). The general pattern was obtained by Southern analysis with an exon 5-specific probe (Fig. 5A) while bands containing exon 6A or 6B were identified by probing with exon 6A and 6B specific sequences (Fig. 5B). In the A204 cells, deletion of exon 6A converts the 6A-7-8 band to 7-8, deletion of exon 8 converts 6A-7-8 to 6A-7 and deletion of exon 7 converts 6A-7-8 to 6A-8. Deletion of exon 6B has no effect and deletion of all four exons of the variable region, ΔVR, gives the expected splicing product, exon 5 linked to exon 9. These results suggested that the generation of the non-chondrogenic splicing pattern did not require the participation of any silencer or enhancer sequences located within these four exons.

In RCS cells a more complex response to exon deletion was observed. Deletion of exons 6A, 6B or 8 had no effect on the pattern of splicing other than the absence of the splice forms containing those exons, suggesting no negative *cis*-acting elements. There was also no significant alteration in the relative proportion of the remaining splice-forms. However, deletion of exon 7 had a pronounced effect on the co-ordination of exon splicing. The 6B-7 splice form was absent and instead exon 6B was associated with exon 8 (6B-8). This was unusual since endogenous exon 6B is mostly expressed in the absence of exon 8 (Fig. 3, RCS endogenous). Also there was a relative loss of intensity of the null band (exon 5-linked to exon 9 in the absence of exon 7). These two observations suggested that deletion of exon 7 promoted inclusion of exon 8.

The origin of this effect of exon 7 was explored by constructing a deletion series (Fig. 6A and 6B). The original construct, Δ7 deleted all of exon 7 as well as most of the upstream and part of the downstream intron. A7-1 contained a deletion of exon 7 at the intron-exon boundaries. Δ7-2 contained a deletion of the central 69 bp, leaving the flanking 12 bp on either side of the deletion. Δ7-3 contained a deletion of the central 27 bp, leaving the flanking 33 bp on either side. All of the deletions were in-frame to exclude potential effects on mRNA stability due to introduction of stop codons (Frischmeyer et al., 1999). Δ7-1 and Δ7-2 yielded splice patterns similar to the original Δ7-deletion indicating the requirement of a portion of exon 7 for normal splicing. With Δ7-3, involving a central 27 bp deletion of exon 7 (exon $7^{\Delta 27}$), a more normal pattern of splicing was obtained in that splice-form $7^{\Delta 27}$-8 was restored. However, 6B-$7^{\Delta 27}$ is very low or absent and instead exon 6B is associated in an unresolved band as 6B-$7^{\Delta 27}$-8 with 6A-$7^{\Delta 27}$-8. Thus normal splicing appears to require an intact exon 7 including the central 27 bp.

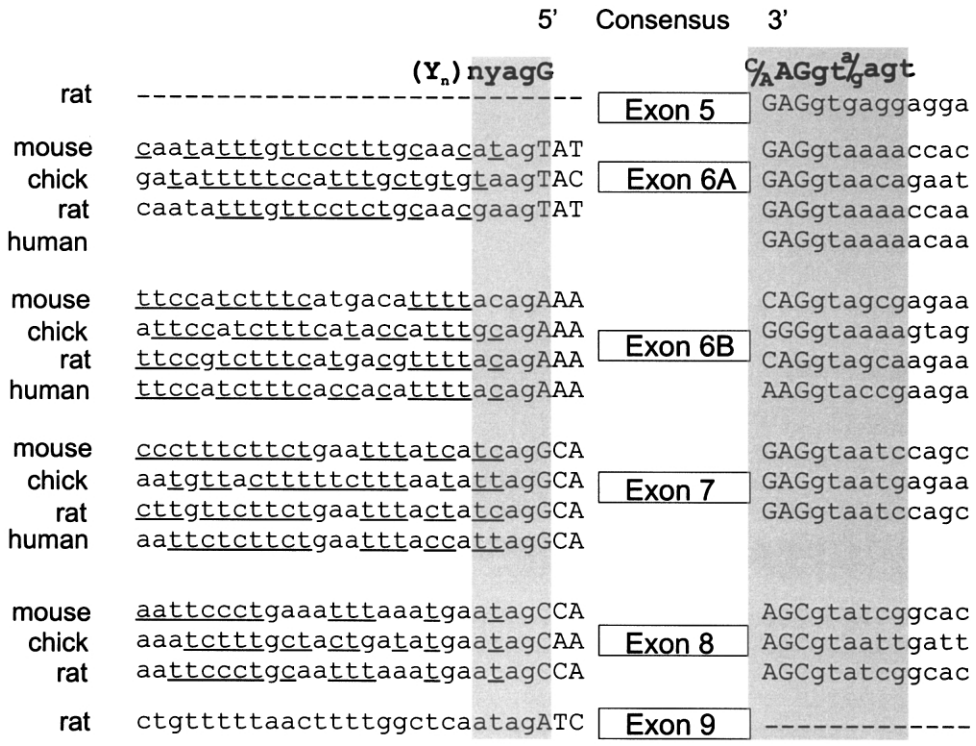

Fig. 4. Splice signals in variable region introns of α1(XI). The intron sequences flanking exons 5 though 9 from human and chick (Zhidkova et al., 1995) (R. Mayne, personal communication), mouse (Iyama et al., 2001) and rat (Oxford et al., 1995) are shown. The 5′(acceptor) and 3′ (donor) sequences are in shaded boxes and the consensus acceptor and donor sequences are shown for comparison. Pyrimidine tracts are underlined.

2.4. Regulation of exon 6B splicing

Since none of the deletions examined altered the level of expression of exon 6B in RCS or A204 cells, it seemed likely that sequences within exon 6B might act as exon enhancer elements (EXE) required to promote the inclusion of exon 6B in chondrocytes. To test this possibility, exon 6B was modified by deletion or replacement (Fig. 7). Replacement of all of exon 6B except the trinucleotides at the intron-exon boundaries with an in-frame 117 bp fragment of exon 5 (6Br5) abolished the expression of exon 6B (Fig. 7B). A series of in-frame 21 bp deletions within exon 6B (Fig. 7A) was also examined (Fig. 7B). Deletions 6B-3 and 6B-4 had no effect on splicing. However the overlapping pair, Δ6B-1 and Δ6B-2, as well as deletions 6B-5 and 6B-6 resulted in the loss of exon 6B. These results indicate that specific sequences within exon 6B are required for the inclusion of exon 6B during the splicing of α1(XI) mRNA transcripts in RCS cells. Comparison of these sequences between species indicated that they are highly conserved, as is the entire exon (Fig. 8). The four deletions that block exon 6B expression encompass or overlap putative purine rich (ESE) or AC-rich (ACE) exonic splicing enhancer elements identified in the figure.

3. Discussion

It is clear from studies of the Cho mutant mouse, harboring a functional deletion of the col11a1 gene, that type XI collagen plays a critical role in the organization of fibril architecture and in the structural integrity of developing cartilage (Li et al., 1995; Seegmiller et al., 1971). It seems reasonable to conclude that the α1(XI) chain has a similar or related function in the elaboration of non-cartilaginous tissues that contain type XI collagen in order to meet the specific requirements of those tissues (Schwarze et al., 2000; Andrikopoulos et al., 1995; Spranger, 1998). The ability to modulate fibrillogenesis (Blaschke et al., 2000) is mediated in part by the structure of the N-terminal domains (Gregory et al., 2000), their slow and incomplete processing (Thom and Morris, 1991; Moradi-Ameli et al., 1998) and their potential to mediate interactions between other matrix-resident molecules and the fibril surface. Alternative splicing of exons 6A, 6B and 8 in the variable region further contributes to the specialization of collagen XI matrices in a process that is both tissue and cell-specific and developmentally regulated. The expression of the 6A-7-8 splice-form (encoding acidic peptides, p6A and p8) in non-cartilaginous tissues and the temporal and spatial segregation of exon 8 and exon 6B-containing

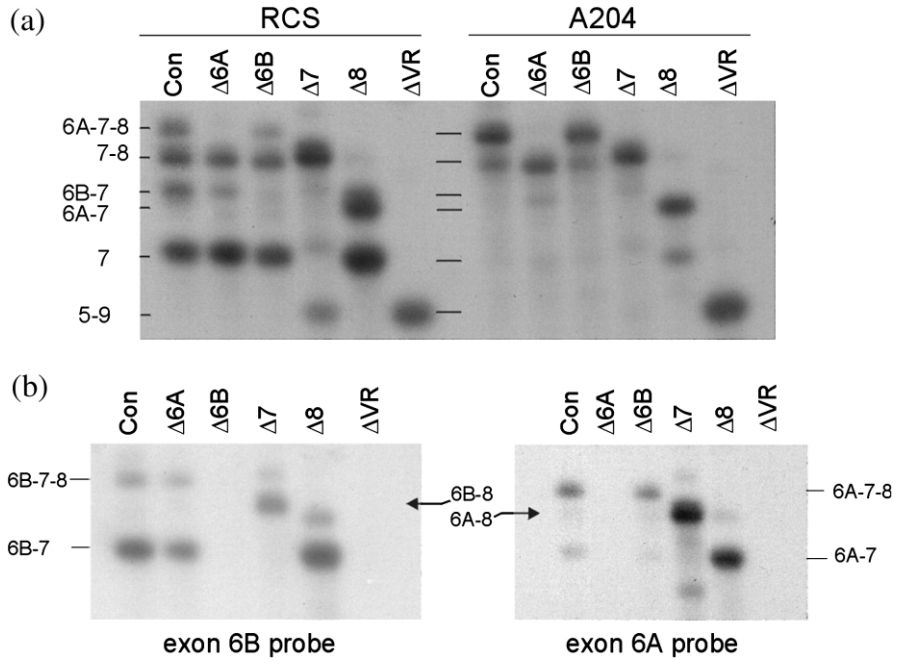

Fig. 5. Analysis of the effect of deletions on the splicing of the minigene in RCS and A204 cells. (A) Either the original minigene (con) or the minigene harboring a deletion of one of the exons (Δ6A, Δ6B, Δ7, Δ8) were transfected into either RCS or A204 cells and examined by RT-PCR. ΔVR represents a deletion of all four variable region exons, 6A, 6B, 7, 8. All of the deletions except Δ6A included deletion of some flanking intron sequence. In this experiment, Δ6A was deleted at the intron exon boundaries. The RT-PCR products were analyzed by alkaline denaturing gel electrophoresis and the band visualized by Southern blot with a probe to exon 5, common to all splice forms. The identity of the bands is indicated. (B) Because the deletion of exon 7 altered the pattern of splicing, the identity of the bands was confirmed by Southern analysis with specific probes to exon 6A and exon 6B. Probes to exon 7 and 8 were also utilized to complete the analysis (not shown). The indication of 6B-8 and 6A-8 between the gels (arrows) corresponds to the prominent band in the Δ7 lane in each get. In the Δ8 lane, the weaker band above either 6B-7 or 6A-7 is 6A-6B-7, since it is labeled with both probes. However, this band is barely detectable with the exon 5 probe and so is not likely a legitimate product of splicing.

splice forms (encoding highly basic peptide, p6B) within cartilage suggest that this structural diversity has functional consequences in cartilage as well as other tissues (Morris et al., 2000; Davies et al., 1998). To begin to understand the basis of the regulation of alternative splicing in the col11a1 gene, we examined the involvement of *cis*-acting elements. Within the limits of the minigene construct and experimental model employed, inclusion of exons 6A and 8 together (6A-7-8) is the default splicing pathway and likely accounts for the predominant expression of this splice-form in non-cartilage. Furthermore, inclusion of exon 6B in a defined subset of chondrocytes in developing cartilage appears to depend on sequences within exon 6B, which may be defined as splice enhancers.

The regulatory mechanism underlying the alternative splicing of the col11a1 gene is complex since three exons are involved and there appears to be some co-ordination between the splicing on either side of exon 7; exon 6B tends to be expressed without exon 8 and exon 6A with 8. Alternative splicing generally requires weak splice sites to allow regulated usage (Black, 1995). Not surprisingly, only the constitutively expressed exon 7 has the perfectly conserved sequence elements within the intron and at the intron-exon boundaries.

Expression of the minigene construct in RCS and A204 cells qualitatively replicated the endogenous pattern and was sufficiently authentic to permit further analysis (Oxford et al., 1995; Davies et al., 1998). Expression of the minigene in 293 cells was revealing. This cell line is not known to express α1(XI) and no endogenous transcripts were detected. When forced to express α1(XI) by transfection with the minigene, the typical non-chondrocytic pattern was observed. This suggests that the splice-form, 6A-7-8, represents the default splicing pathway and that no specific splicing factors are required to produce this isoform in A204 cells, placenta (Yoshioka and Ramirez, 1990), brain, skin or other tissues (Iyama et al., 2001; Oxford et al., 1995). Interestingly, inclusion of 6A and 8 occurs despite sub optimal splice signals so that 6A-7-8 rather than null is the default pathway.

The deletion of exon 7 significantly altered the splice pattern. Linkage of 6A with 8 was not unexpected. However, linkage of 6B with 8 and the suppression of the null form were unexpected. It is possible that in the absence of exon 7, splicing of exon 6B to the nearer exon 8 is preferred competitively over

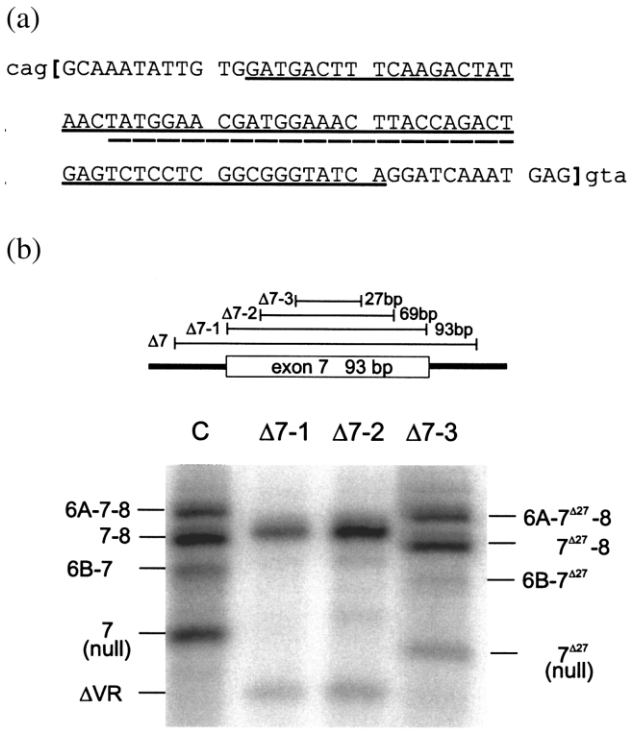

Fig. 6. Effects of exon 7 deletion on the pattern of alternative splicing. In order to identify the origin of the Δ7 sequences whose deletion altered the pattern of splicing, an additional set of exon 7 deletions were prepared. (A) Δ7-1, exon 7 was deleted at the intron exon boundaries (brackets); Δ7-2, the central 69 of 93 bps of exon 7 was deleted (underline); and Δ7-3, the central 27 bp of exon 7 was deleted (dashed underline). (B) Analysis of the transcripts: The deleted constructs are indicated schematically above the gel. The intact minigene is included as a control (C) The RT-PCR products were end-labeled with ^{32}P and resolved by alkaline denaturing gel electrophoresis. The identities of the bands in the control and in Δ7-3 are indicated. In Δ7-1, the lowest band is ΔVR, which corresponding to exon 5 linked directly to exon 9 and is equivalent to the null form without exon 7. The most prominent band in Δ7-1, located between 6A-7-8 and 7-8, is a mixture of 6A-8 and 6B-8 as determined by southern analysis. In Δ7-2, the 24 bp exon$7^{\Delta 69}$ is not utilized and the pattern and composition is nearly identical to Δ7-1. In Δ7-3, the -7-band lacks the central 27 bp.

the more distant exon 9. However, deletion of the central 69 of 93 bp of exon 7 sequence yielded the same result and reducing the deletion to the central 27 bp (Δ7-3) still did not fully restore the pattern. Splice-forrn $7^{\Delta 27}$-8 became apparent indicating restoration of the ability to exclude exon 6A. However, the central 27 bp appear to be required to suppress exon 8 and to promote exon 6B as evidenced by the absence or under-representation of 6B-$7^{\Delta 27}$ or $7^{\Delta 27}$ (null). It would appear that an interaction between this central region of exon 7 and chondrocyte specific splicing factors and/or neighboring sequences involved in splice signals derived from RNA secondary structure (Coleman and Roesser, 1998; Muro et al., 1999) are necessary for the expression of the pattern of α1(XI) splice-forms found in cartilage.

Since none of the manipulation of the genomic sequences outside of exon 6B affected the inclusion of 6B in RCS, it is likely that sequences within exon 6B in the form of splice enhancers are involved in its expression. Weak splice acceptor sites require splicing enhancers to ensure that the weak splice signals are not ignored. These enhancer sequences can be located within exons and typically fall into two classes, purine-rich exonic splicing enhancer (ESE) (Muro et al., 1998; Manley and Tacke, 1996; Cooper and Mattox, 1997; Lavigueur et al., 1993; Reed and Maniatis, 1986) and AC-rich enhancers (ACE) (Coulter et al., 1997; Cooper and Mattox, 1997). Consistent with this model, replacement of the internal 147 of the 153 bps of exon 6B with sequences from exon 5 abolished expression of the hybrid exon even though the intron-exon boundaries and 75% of the length were conserved.

Deletional analysis pointed to specific sets of sequences being important for the expression of exon 6B. Two of these, both GAAGAAGA, correspond to purine-rich ESE motifs defined as GAR or GRA (where R = G or A) (Zheng et al., 1997), although quite unrelated sequences can serve as ESEs (Cooper and Mattox, 1997). The putative ESE was found in the overlap of the regions deleted in Δ6B-1 and

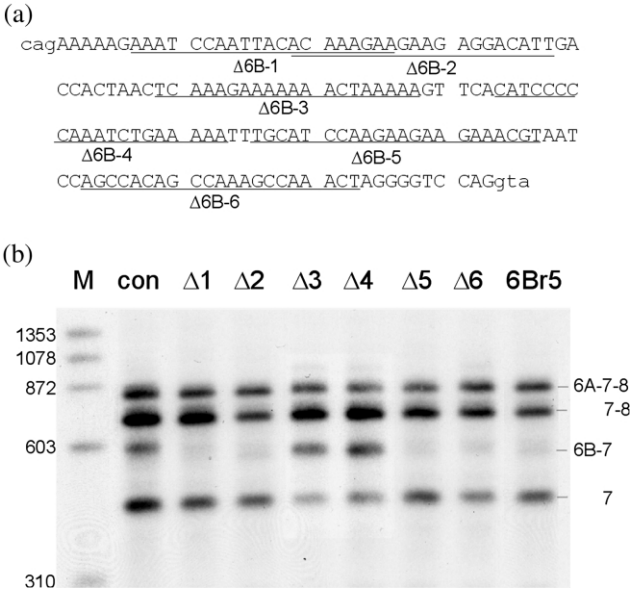

Fig. 7. Deletional analysis of exon 6B. In order to test whether sequences within exon 6B were necessary for its inclusion during splicing, a series of deletion or replacement constructs were prepared. (**A**) 21 bp sequences were deleted by site-directed mutagenesis, Δ6B-1 through Δ6B-6, as underlined in the sequence. Finally, all 6B except the first and last condons (shaded) was replaced by an in-frame sequence from exon 5 of about the same length. (**B**) The constructs were transfected into RCS cells and the transcripts analyzed by RT-PCR, end-labeling and alkaline gel electrophoresis. The markers are indicated by size in bp of the left and the splice-form is identified on the right.

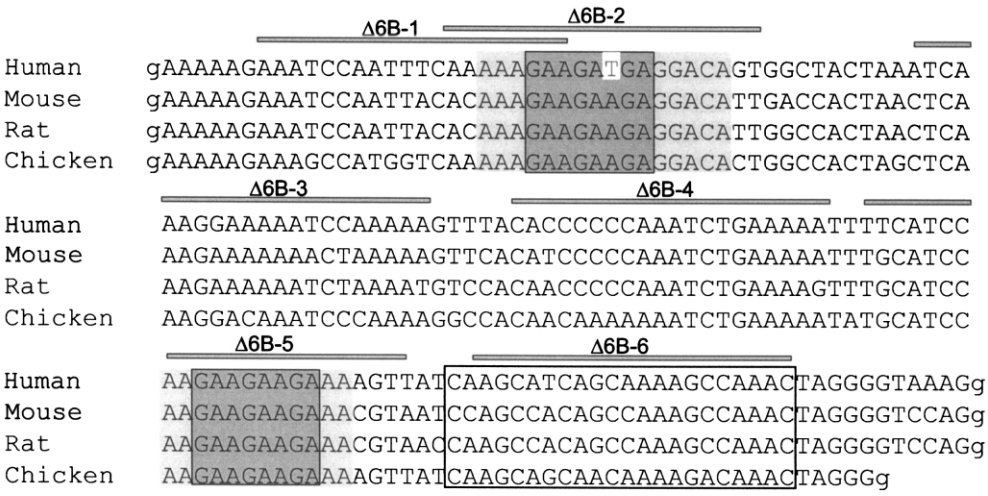

Fig. 8. Comparison of exon 6B sequences and identification of putative exonic enhancer elements. The sequences of exon 6B from human (Zhidkova et al., 1995), mouse, rat (Oxford et al., 1995), and chick (Zhidkova et al., 1995) are shown. Purine rich regions are shown in light shaded boxes and putative ESEs are shown in dark shaded boxes. The putative ACE is shown in the unshaded box. The positions of the different deletions of exon 6B from Fig. 7 are shown above the sequences.

Δ6B-2 and appears again in Δ6B-5 deletion (Fig. 8). This ESE sequence in both positions is perfectly conserved among chick, rat, mouse and human except that the 5′ ESE in human has an A to T substitution. This putative ESE is very similar to the purine-rich ESE in exon 5 of CD44 and is identical to the one found to be involved in regulating the expression of the EIIIA exon of fibronectin during chondrogenesis (Caputi et al., 1994; Lavigueur et al., 1993).

The sequence deleted in Δ6B-6 is A/C-rich and could correspond to an ACE element. The A/C rich region is present in all four species examined (Fig. 8) and while extensive (18 or 19 of 22 bp) the sequence itself is not highly conserved. However, identified ACE sequences lack consensus (Cooper and Mattox, 1997), so it is possible that the A/C-rich sequences encompassed by Δ6B-6 in all four species shown in Fig. 8 are similar enough to be functional. Thus three separate ESE and ACE enhancer elements may be required for the inclusion of exon 6B in chondrocytes. Their effect seems to be specific since Δ6B-3 (purine-rich, 17/21 bp) and Δ6B-4 (A/C-rich, 17/121bp) had no effect on the expression of exon 6B.

The function of exonic splicing enhancer sequences is mediated by their binding of SR proteins, a conserved family of constitutive and essential splicing factors (Graveley, 2000; Fu, 1995; Manley and Tacke, 1996; Staknis and Reed, 1994; Zuo and Maniatis, 1996). Regulation of the function of these enhancer elements in cell or tissue-dependent alternative splicing is achieved by differential expression of members of the SR protein family (Hanamura et al., 1998; Kamma et al., 1995; Zahler et al., 1993) or the expression of specific cell or tissue-dependent splicing factors (Stoss et al., 2001). Presumably, distinct patterns SR protein expression, expression of specific splicing factors or both (Hanamura et al., 1998; Kamma et al., 1995; Stoss et al., 2001; Zahler et al., 1993) account for the expression of exon 6B in a subpopulation of chondrocytes during development (Davies et al., 1998; Morris et al., 2000; Oxford et al., 1995). As previously mentioned, the central 27 bp of exon 7 are also required for expression of exon 6B suggesting interaction of factors binding to both exons. We cannot rule out that the effects of these deletions on exon 6B expression derive all or in part from changes in pre-mRNA secondary structure (Muro et al., 1999; Coleman and Roesser, 1998) since this was not evaluated.

The structural versatility of cartilage matrix macromolecules derived by alternative exon splicing appears to have functional significance for cartilage development. For example, exon EIIIA of fibronectin may be required for mesenchymal condensation while its absence is essential for further differentiation into chondrocytes (Gehris et al., 1996, 1997) Exon 2 of type II collagen encodes an amino-terminal cysteine-rich propeptide domain with structural and functional homology to the BMP inhibitor chordin (Larrain et al., 2000; Oganesian et al., 1997; Sandell et al., 1991, 1994; Zhu et al., 1999). Differentiated chondrocytes do not utilize this exon in the synthesis of type II collagen (type IIB), but prechondrogenic cells, which can become either chondrocytes or osteoblasts, do utilize this exon (type IIA) (Sandell et al., 1994; Nah et al., 2000). Type IIA is also expressed at mesenchymal epithelial boundaries during skin development. Alternative splicing of exon 2 of type II collagen may thus modulate a function for this molecule in BMP signaling separate from the structural role of type II

collagen in the formation of cartilage collagen fibrils and also serves as a model for the functional possibilities of alternative splicing in type XI collagen.

4. Experimental procedures

4.1. Minigene constructs

A minigene construct containing the mouse genomic DNA from exon 5 to 11 of COL11A1 was cloned into Kpn I/Xho I sites of pCDNA3 vector (Invitrogen) by PCR using as template a combination of rat genomic clones gHY101 and gHY114 (Iyama et al., 2001). From this control construct several mutated minigenes were prepared. Restriction and other modifying enzymes were obtained from Promega). Exon-deletion constructs Δ6B (Alu I and Bgl II), Δ7 (Bg1 II and BamH 1), Δ8 (BamH I and Pst I) and ΔVR (deletion of all four variable region exons) (Hind III and PstI) were generated by utilizing convenient restriction sites in the flanking introns. Exon deletions at the intron/exon boundaries were created for exon 6A (Δ6A), exon 7 (A7-1) and within exon 7 (Δ7-2, leaving the distal 12 nucleotides at each end, and Δ7-3 deleting the central 27 nucleotides) by linking upstream and down stream segments obtained by PCR. Using control construct as a template, the weak branch point (bp) and the splice acceptor site (as) of exon 6B were converted to the consensus sequence by site-directed mutagenesis (Clonetech) resulting in constructs 6Bbp and 6Bas, respectively. A series of 21 nucleotides deletions within exon 6B (Δ6B1, Δ6B2, Δ6B3, Δ6B4, Δ6B5 and Δ6B6, respectively) were created also by site-direct mutagenesis. To replace exon 6B with the fragment of exon5 (6Br5), exon 6B was deleted from the control minigene leaving the distal 3 nucleotides of exon 6B left at both ends and a new Pvu I site in the middle. A fragment of exon5 from 836 to 949 (accession # NM_007729) (Yoshioka et al., 1995) with compatible Pvu I ends was obtained by oligonucleotide synthesis and cloned into the Pvu I site.

4.2. Cell transfection and RT-PCR assays

RCS cells and A204 cells were cultured in DMEM medium with 10% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin while 293 cells were cultured with DMEM/F12 medium with the same supplements (GibcoBRL). 8×10^5 cells/60-mm dish of each cell line were plated the day before transfection. Cells were transfected with 10 μl Superfect Transfection Reagent (Qiagen) plus 5 μg DNA per well. The cells were collected 36 h after transfection. Total RNA was purified using Trizol Reagent (Gibcol/BRL) following manufacturer's directions. cDNAs were synthesized using random primers (Promega). For endogenous transcripts, RT-PCR was performed using rat or human primers in exons 5 and 9, flanking the variable region. For the products of the minigene constructs, primers were derived from the flanking vector sequence. After 30 cycles of amplification the PCR products from constructs of Δ6A, Δ6B, Δ7, Δ7-1, Δ7-2, Δ7-3, Δ8 and AVR were resolved on 3% agarose, alkaline denaturing gels, transferred to a nylon membrane and blotted successively with ^{32}P-labeled probes corresponding to the full lengths of exons 5, 6A and 6B. In some cases RT-PCR products were end-labeled with [γ-^{32}P]-ATP and then analyzed by electrophoresis on alkaline denaturing gels. The sequence of primers and oligonucleotides is available upon request.

4.3. Immunohistochemistry

Acetone-fixed and paraffin embedded E18 fetal rat forelimb was analyzed by indirect immunofluorescence staining as previously described (Morris et al., 2000) using a monoclonal antibody to p6b and a polyclonal rabbit antiserum to p8 (Davies et al., 1998; Morris et al., 2000). FITC and CY3-conjugated secondary antibodies were obtained from Sigma.

4.4. COL11A1 genomic sequences

Mouse: GenBank, AB066244; Rat: GenBank, U20118, Human and Chicken: Zhidkova et al., 1995.

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