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The α1(IX) collagen gene gives rise to two different transcripts in both mouse embryonic and human fetal RNA

(collagen gene transcription/*COL9A1*/*Col9a-1*)

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ABSTRACT **We have isolated and characterized portions of the α1(IX) collagen gene from mouse and human DNA. Nucleotide sequence analysis and comparison with the chicken gene suggest that the mammalian genes contain an alternative exon that is located within the intron between exons 6 and 7. Using oligonucleotide primers specific for exons 4, 8, and the alternative exon (exon 1*), we demonstrated by the polymerase chain reaction that embryonic mouse and fetal human RNAs contain two types of α1(IX) collagen transcripts. One type of transcript does not contain the sequence encoded by exon 1*; the second type of transcript contains this exon. Both mouse and human α1(IX) collagen genes give rise, therefore, to (at least) two mRNA transcripts.**

Collagen fibrils provide structural elements of high tensile strength in extracellular matrices. They are composed of staggered arrays of long, rod-like triple-helical molecules. The polypeptide subunits of such fibrillar collagen molecules are products of multiexon genes that form a distinct homology group (for review, see ref. 1). The group consists of (at least) nine genes in vertebrates, and the polypeptides encoded by its various members form distinct homo- or heterotrimeric molecules called fibrillar collagens (types I, II, III, V, and XI) (1). The fibrillar collagen types may be coexpressed in various tissues and even copolymerized within the same fibrils (2–4). It is likely that such heterotypic interactions between different types of molecules play an essential part of the mechanism by which fibril diameters are regulated in tissues (5).

Interactions between collagen fibrils and other matrix components and cells are likely to provide the basis for the precise three-dimensional patterns of fibril arrangement in tissues. Such interactions may range from entanglement with other polymer systems (elastic fibers, anchoring fibrils of type VII collagen, and networks containing type VI collagen) to short-range interactions with nonfibrillar components at the fibril surface. The recently discovered type IX collagen-proteoglycan (collagen IX) provides an example of a matrix component that interacts in a specific manner with fibrillar surfaces (6–8). Collagen IX molecules are composed of three genetically distinct chains that form a long and a short triple-helical arm connected by a flexible non-triple-helical hinge (7, 9). The long arm is located along the surface of type II-containing collagen fibrils in cartilage (8) and other type II collagen-containing tissues, such as the vitreous body of the eye (10). The short arm projects into the perifibrillar matrix and is thought to interact with other, yet unidentified, matrix components.

The gene encoding one of the three collagen IX subunits, α1(IX), has been shown to give rise to transcripts of different size in two different tissues of the developing chick embryo (11). In chondrocytes, the majority of the transcripts encode α1(IX) chains with a large amino-terminal globular domain (12), whereas in the cornea, at the time of synthesis of the primary corneal stroma by the epithelial cells, the majority of the α1(IX) transcripts are generated from a transcription start site that is located ≈20 kilobases (kb) downstream of that used in chondrocytes (11, 13). This shorter corneal transcript encodes α1(IX) chains that are identical with those of cartilage in their triple-helical sequences but are very different in their amino-terminal non-triple-helical domains. The synthesis of type II collagen (and of type IX) during cornea morphogenesis (14) may be unique to birds because type II collagen has not been found in developing mammalian corneas. Therefore, although a small amount of the short transcript has been detected in chicken cartilage by the polymerase chain reaction (PCR) (11), it is not clear to what extent the observations on the alternative transcription of the α1(IX) collagen gene in the chicken is of general significance and can be extended to mammals. This raises the question of whether the α1(IX) collagen gene has the same exon structure in mammals as in chickens. To address this question, we have isolated and characterized portions of *Col9a-1* and *COL9A1* from both mouse and human[‖], and we report here that the α1(IX) collagen gene contains two translation start codons located within two alternative exons and gives rise to two alternative transcripts in mammals. We therefore suggest that this represents a general mechanism used by cells in different locations to modulate the surface properties of type II-containing collagen fibrils and their interactions with other matrix components.

MATERIALS AND METHODS

Preparation of RNA and Genomic DNA. RNA was extracted from whole 19-day mouse (BALB/c) embryos with guanidine hydrochloride (15) and from cultured human chondrocytes with guanidine thiocyanate (16, 17). Poly(A)$^+$ RNA was separated from rRNA by oligo(dT)-cellulose chromatography (18). Genomic DNA was isolated from liver and spleen of adult BALB/c mice and from human peripheral blood leukocytes by using a standard protocol (19). Human fetal RNA was obtained from a 14-week fetus after therapeutic termination and with approval of the local committee gov-

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Abbreviation: PCR, polymerase chain reaction.
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[‖]The sequences reported in this paper have been deposited in the GenBank data base (accession nos. M32132, M32133, M32134, M32135, M32136, and M32137).

erning ethical experimentation. Tissue was snap-frozen in liquid nitrogen immediately after removal, ground under liquid nitrogen, and then suspended in ice-cold 4.5 M guanidine thiocyanate/50 mM EDTA/25 mM sodium citrate, pH 7/0.1 M 2-mercaptoethanol/2% Sarcosyl. The suspension was homogenized with a Polytron for 5 min on ice at medium setting. Gross debris was removed by centrifuging at 4000 rpm (2500 × g) for 20 min at 4°C, and cesium chloride was added to the supernatant to a final concentration of 0.2 g/ml. This supernatant was then layered on a cushion of 5.7 M cesium chloride/50 mM EDTA, pH 8, and spun at 48,000 rpm for 12 hr at 4°C. RNA pellets were retrieved, pooled, and washed in 70% (vol/vol) ethanol. The RNA was then dissolved and extracted three times with phenol/chloroform, twice with chloroform, and then precipitated from 0.3 M lithium chloride with 2.5 vol of absolute ethanol.

Genomic Libraries and Screening Procedures. A mouse genomic library, consisting of 8- to 22-kb pieces of adult BALB/c mouse DNA cloned in the vector EMBL3 was purchased from Clontech. Partial mouse and human genomic libraries were prepared by digesting genomic DNA with *Xba* I (mouse) or *Eco*RI (human), fractionating the digested DNA by agarose gel electrophoresis, and ligating specific-sized fractions into λZAP (mouse) or λgt10 (human). Library filters were screened with hybridization probes in the presence of Sarcosyl (20). Phage purification and recombinant DNA isolations were performed as described elsewhere (21).

PCR. Double-stranded cDNAs were synthesized with mouse embryonic or human fetal poly(A)$^+$ RNA as template using a primer-extension method (11). For mouse RNA, the cDNA primer was a synthetic oligonucleotide 5'-ATCAATGCCATCTATGC-3' with a reverse and complementary sequence of part of exon 8 within the α1(IX) collagen gene, whereas for human RNA, the cDNA primer was 5'-GTCGATGCCATCGATGC-3'. The double-stranded cDNA was then used as template for the PCR. A combination of 5' and 3' primers was used for the reaction with *Thermus aquaticus* (Taq) polymerase (22). Thirty PCR cycles were performed with Programmable Dri-Block (Techne, Inc., Princeton Junction, NJ) at 95°C for 0.5 min, at 65°C for 10 min, and an additional 7 min at 65°C at the end of cycles.

PCR was also used to amplify a portion of the human α1(IX) gene. Human genomic DNA (1 μg) was combined with 5' and 3' oligonucleotide primers, and PCR was carried out for 30 cycles. The conditions were 1 min at 95°C, 2 min at 55°C, 3 min at 72°C, and an additional 7 min at 72°C at the end of cycles.

Nucleotide Sequence Analyses. Nucleotide sequence analyses were performed with the dideoxy nucleotide chain-termination technique (23). Computer analysis of sequence data was done at the National Institutes of Health-supported Molecular Biology Computer Research Resource of the Dana-Farber Cancer Institute/Harvard School of Public Health.

RESULTS AND DISCUSSION

Isolation and Partial Characterization of the Mouse α1(IX) Gene. In preliminary experiments, we determined that fragments of the human cartilage α1(IX) collagen cDNA, YMh509 (Y.M., T. Kimura, Y.N., and B.R.O., unpublished work) (Fig. 1), hybridized to transfer-blots of restriction endonuclease-digested mouse genomic DNA. Therefore, we used a 340-base-pair (bp)-long *Eco*RI–*Xba* I fragment (Fig. 1, probe 1) from the 5' portion of YMh509 as probe to screen a mouse genomic library. This procedure isolated the genomic clone YMm513, containing an ≈15-kb insert (Fig. 2). Because mapping and Southern blotting showed that the probe recognized sequences in the 3' portion of the insert of YMm513, this region (≈2.4 kb) was sequenced. Comparison with the chicken and human α1(IX) cDNA sequences allowed unambiguous identification of exons 1–4. The transcription start site was tentatively defined by comparison with the chicken genomic α1(IX) sequence, and the translation start within exon 1 could be identified based on a high degree of sequence similarity among the three species within the 3' region of exon 1. Four and two-thirds codons of the signal peptide are encoded by exon 1 in all three species (Fig. 3).

The transcription start site in the α1(IX) collagen-encoding gene used by embryonic chicken cornea cells is located ≈20 kb downstream of the start site used by chondrocytes. This portion of the gene encodes sequences at the carboxyl end of the amino-terminal globular domain (Fig. 1) and is within the region covered by probe 2 of the human cDNA YMh509 (Fig. 1).

To isolate the corresponding portion of the mouse gene, we first determined that a 330-bp-long *Hin*cII–*Pst* I fragment (probe 2) of YMh509 (Fig. 1) hybridized to a band of 1.6 kb in transfer-blots of *Xba* I-digested mouse genomic DNA. Mouse genomic DNA was digested with *Xba* I and electrophoresed through a 0.8% agarose gel. DNA was eluted from the 1.6-kb region, purified, and ligated to arms of λZAP, predigested with *Xba* I. This λZAP library was screened with probe 2, and the clone YMm341 was isolated (Fig. 2).

Sequence analysis of ≈800 bp of the 1.6-kb insert of clone YMm341 demonstrated that it contained exon 6 of the mouse α1(IX) collagen gene. Clone YMm341 was therefore used as a probe to screen a complete mouse genomic library. This screening isolated the clones YMm911 and YMm413 (Fig. 2). Mapping of the inserts of these two clones showed that they represented overlapping fragments of the same gene. Sequence analysis of ≈3 kb of the common portion of the overlapping clones revealed the presence of exons 6, 7, and 8 (Fig. 2).

Isolation and Partial Characterization of the Human α1(IX) Collagen Gene. Transfer-blot analysis of *Eco*RI-digested human genomic DNA showed that a 520-bp-long *Pst* I–*Hin*cII fragment of clone YMh509 (Fig. 1, probe 3) hybridized to a 5-kb band. DNA was, therefore, eluted from the 5-kb region of a preparative gel and ligated to the arms of λgt10. The library thus generated was screened with probe 3, and clone YMh304 was isolated. Sequence analysis of ≈2.5 kb of its insert showed that clone YMh304 contained exons 1–4 of the human α1(IX) gene (Fig. 2).

Isolation of a fragment of human *COL9A1*, containing exons 6 and 7, was accomplished by amplifying a portion of the gene by PCR. For the reaction we used two synthetic oligonucleotide primers and leukocyte DNA as template. The 5' sense primer, 5'-CAATGGATGCTGATCCA-3', corresponded to a portion of the exon 6 sequence within clone YMh509 cDNA (based on comparison with chicken sequence). The 3' anti-sense primer, 5'-CTCGTCGGTGGTC-

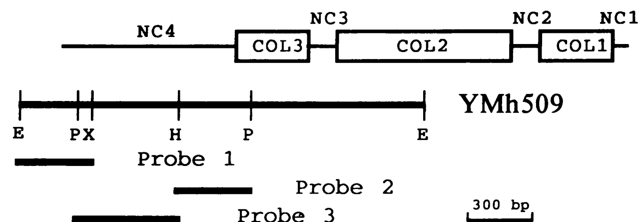

FIG. 1. Diagram showing the domain structure of cartilage α1(IX) collagen chains. Triple-helical sequence domains (COL1, COL2, and COL3) are indicated by boxed-in regions; non-triple-helical sequences (NC1, NC2, NC3, and NC4) are indicated by lines. Below the diagram is a simplified restriction map of the human cartilage α1(IX) cDNA YMh509 and the relative sizes and locations of the three restriction endonuclease fragments used as probes (probe 1, probe 2, and probe 3). E, *Eco*RI; P, *Pst* I; X, *Xba* I; and H, *Hin*dIII.

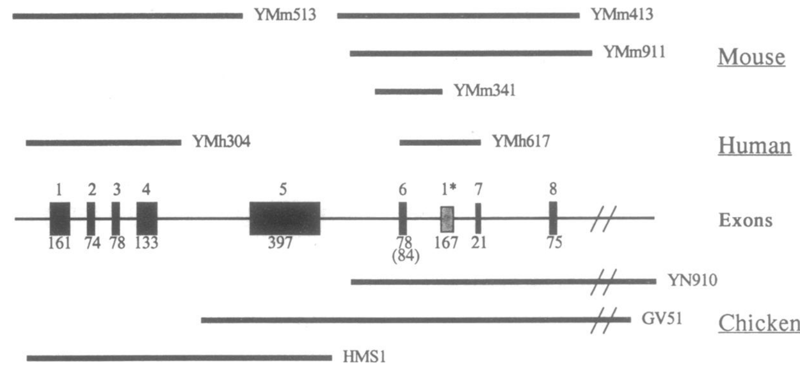

FIG. 2. Diagram showing the exon structure of the 5′ region of the chicken, mouse, and human α1(IX) collagen gene. Exons are numbered from the 5′ end, as indicated by numbers above exons. Sizes of the exons (in bp) in chicken gene (11, 12) are given below exons. Exon 6 is 6 nucleotides longer in the mouse and human genes (84 bp) than in the chicken gene (78 bp). The alternative exon 1 (exon 1*) is 167 bp in the chicken gene. The precise size in mouse and human genes is still unknown. Also, because we have not determined the precise transcription start sites in the mouse and human genes, it is not yet known whether the alternative exon is the 5′-most exon of the alternative transcript, as in the chicken. For simplicity we will nevertheless refer to it as exon 1* in mouse and human genes. Above the diagram are the relative sizes and location of mouse and human genomic clones. For comparison, the chicken genomic clones described previously (12) are shown.

TGGCTG-3′, corresponded to a sequence within exon 7 (see Fig. 2). Agarose gel electrophoresis of the PCR product after 30 cycles of amplification showed a 900-bp band, hybridizing to an α1(IX) cDNA probe. DNA in this band was isolated and cloned into *Sma* I-digested pBluescript, generating clone YMh617.

A comparison between exons 1, 2, 3, 4, 6, 7, and 8 in the mouse, human, and chicken α1(IX) genes demonstrates a

A

Exon 1

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Mouse    --------AGA GAA CCC ACT GGG AAA ATG AAG AAC TTC TG  gt
                                          M   K   N   F
Human    --------AGA AAA TCA ACT GGG AAA ATG AAG ACC TGC TG  gt
                                          M   K   T   C
Chicken  --------AGA AAA CCA GCA GAG AAG ATG AAA AGC AAC TG  gt
                                          M   K   S   N
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B

Exon 6

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Mouse    ag TTT GAA CTC CAG TGG ATG CTG ATT CAT TGT GAC CCC CTG AGA
             F   E   L   Q   W   M   L   I   H   C   D   P   L   R
Human    ag TTT GAA CTT CAA TGG ATG CTG ATC CAT TGT GAC CCC CTG CGG
             F   E   L   Q   W   M   L   I   H   C   D   P   L   R
Chicken  ag TTT GAA GTC CAG TGG ATG CCG ATT CAC TGC GAT CCC CTG CGG
             F   E   V   Q   W   M   P   I   H   C   D   P   L   R

Mouse       CCC AGG AGA GAA ACC TGT CAT GAG CTG CCA ATC AGA ATC ACA gt
             P   R   R   E   T   C   H   E   L   P   I   R   I   T
Human       CCC AGG AGA GAA ACT TGC CAT GAG CTG CCA GCC AGA ATA ACG gt
             P   R   R   E   T   C   H   E   L   P   A   R   I   T
Chicken     CCC CAG AGA GAA GGT TGT GGT GAG CTC CCA GCC CGG gt
             P   Q   R   E   G   C   G   E   L   P   A   R
```

Exon 1*

```
Mouse    -------ATG GCC TGG GCT GCC TGG GGA CGA GGA GTG CTT GGG
                 M   A   W   A   A   W   G   R   G   V   L   G
Human    -------ATG GCC TGG ACT GCG CGG GAC CGC GGG GCC CTG GGG
                 M   A   W   T   A   R   D   R   G   A   L   G
Chicken  -------ATG GCC TGG GCT GCA TGG GGC CCT CTG CTT CTC GGG
                 M   A   W   A   A   W   G   P   L   L   L   G

Mouse       CTG TCA CTG ATG CTG TCT GGG CTC CGC TTG TGT GCT GCT CAA gt
             L   S   L   M   L   S   G   L   R   L   C   A   A   Q
Human       CTG     CTG     CTG TTG GGG CTC TGC TTG TGC GCG GCT CAA gt
             L       L       L   L   G   L   C   L   C   A   A   Q
Chicken     CTT     TTC     TTG CAG ATT TTT TGC CTC TGC CTT GCT CAA gt
             L       F       L   Q   I   F   C   L   C   L   A   Q
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Exon 7

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Mouse    ag ACC AGC CAG ACC ACT GAT GAG gt
             T   S   Q   T   T   D   E
Human    ag CCC AGC CAG ACC ACC GAC GAG gt
             P   S   Q   T   T   D   E
Chicken  ag ATA AGC CAG ACA GTG ATT GAG gt
             I   S   Q   T   V   I   E
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FIG. 3. (*A*) Nucleotide and derived amino acid (in one-letter code) sequence of the 3′ region of exon 1 in mouse and human α1(IX) collagen genes. For comparison, the chicken sequence (11, 12) is also shown. Nucleotides within introns are shown in lowercase letters. Exon 1 encodes 4⅔ of the codons within the signal peptide of long α1(IX) collagen translation products. (*B*) Nucleotide and derived amino acid (in one-letter code) sequences of exons 6 and 7 and the alternative exon 1 (exon 1*) within human and mouse α1(IX) collagen genes. For comparison, the chicken sequences (11) are also shown. Nucleotides within introns are shown in lowercase letters. For exon 1* only the 3′ region of the exons, starting with the translation start codon, is shown. To maximize the sequence similarities during alignment of exon 1* sequences, two gaps of one codon each were introduced in the human and chicken sequences. Exon 6 is 6 nucleotides longer in mouse and human than in chicken.

very high degree of conservation in exon sizes (Fig. 2). Except for an insertion of two codons in exon 6 of the mouse and human genes (Fig. 3B), some uncertainty concerning the precise transcription start sites and the precise size of exon 5 in mouse and human, exon sizes are the same in all three genes. Intron sizes are also quite similar in this part of the α1(IX) gene among the three species.

The intron between exons 6 and 7 in the chicken gene contains a 167 bp-long alternative exon 1 (exon 1*) that is used for synthesis of a short α1(IX) transcript in chicken embryonic cornea (11). Inspection of the nucleotide sequence of the intron between exons 6 and 7 in the mouse and human genes revealed the presence of a similar exon in mammals (Fig. 3B). The degree of similarity between this exon in mouse and human DNA and that of the chicken gene was as high as that between the cartilage exons 1, 2, 3, 4, 6, and 7. In contrast, the remaining intron sequences were different between the three species. We conclude, therefore, that the α1(IX) collagen gene contains an alternative exon in the intron between exons 6 and 7 in both mouse and human.

Two Alternative Transcripts are Generated from the α1(IX) Collagen Gene. For chicken α1(IX) gene, Northern blot analysis shows that the sequences of exons 1 and 1* are found within alternative α1(IX) collagen transcripts in cartilage and cornea (11, 13). To find out whether the synthesis of such alternative transcripts is a general property of the α1(IX) collagen gene, the PCR was used to demonstrate two different α1(IX) collagen transcripts in mouse embryonic and human fetal poly(A)$^+$ RNA. Poly(A)$^+$ RNA was used to synthesize double-stranded cDNA, and the cDNA was used as template for PCR. For mouse, two 5' sense primers were used. One primer (primer 1), 5'-GTAGACTTCAGGATTC-CA-3', encoded part of exon 4 and was specific for the long form of the α1(IX) transcript. The second primer (primer 2), 5'-ATGGCCTGGGCTGCCTGG-3', encoded part of exon 1* and was specific for the short form of the α1(IX) transcript. The 3' anti-sense primer (primer 3), 5'-CCGGAACTCCAG-GAGGC-3', encoded part of exon 8 and was common to both forms of the transcript. Use of primers 1 and 3 for PCR should generate a product of 583 bp in the presence of α1(IX) transcripts containing the sequences of exons 4–8. Use of primers 2 and 3 should generate a PCR product of 136 bp in the presence of transcripts containing the sequence of exons 1* and 8. The presence of transcripts generated by alternative splice patterns involving exon 1*, such as exons 4, 5, 1*, 7, and 8 or exons 4, 5, 6, 1*, and 8, should generate additional bands with primers 2 and 3 or primers 1 and 3.

As indicated in Fig. 4, agarose gel electrophoresis of the PCR products showed that use of primer 1 gave rise to a single band of the predicted size, 583 bp. In contrast, use of primer 2 gave rise to a 136-bp PCR product; this is the size predicted for a transcript in which the sequence of exon 1* is spliced to the sequence of exon 8.

For human DNA, two 5' sense primers were also used for PCR. One primer (primer 1) encoding part of exon 4 was identical to the corresponding mouse primer. The second primer (primer 2), 5'-ATGGCCTGGACTGCGCGG-3', encoded part of exon 1* and was specific for the short form of the transcript. The 3' anti-sense primer (primer 3), 5'-CTG-GAACTCCAGGGGGGC-3', encoded part of exon 8 and was common to both forms of the transcript. Fig. 4 shows that the use of primer 1 gave rise to the predicted PCR product of 583 bp (as for mouse) and that the use of primer 2 gave rise to a 130-bp product. The difference between mouse and human in the size of the PCR products generated with primer 2 is due to the fact that exon 1* is 6 nucleotides longer in mouse than in human (Fig. 3). Identification of the two PCR products was based on cloning and nucleotide sequence analysis.

We conclude from these data that mammalian embryos contain two different α1(IX) collagen transcripts. One of the transcripts contains sequences of exon 1*, whereas the second transcript does not. Both transcripts contain triple-helical exon 8 sequences. Because exon 1* contains a translation start codon and the coding sequence of a hydrophobic signal peptide, transcripts containing exon 1* would encode short α1(IX) chains with this signal peptide at the amino terminus of the triple-helical domain COL3 (see Fig. 1). In contrast, the nucleotide sequence of the human cDNA YMh509 (Y.M., T. Kimura, Y.N., and B.R.O., unpublished work) shows that the transcript which contains exon 4 encodes a long α1(IX) collagen chain with a signal peptide derived from exons 1 and 2 and a globular sequence derived from exons 2–7 at the amino terminus of the triple-helical domain COL3.

The PCR experiments were done with RNA from whole embryos. Therefore, we do not know whether the short and long α1(IX) collagen chains are expressed by different cells in a tissue-specific manner or whether they are coexpressed in the same tissue. *In situ* hybridization studies with probes specific for the two types of transcripts should be useful in addressing this question. The mouse and human DNA sequences described here should make such studies possible.

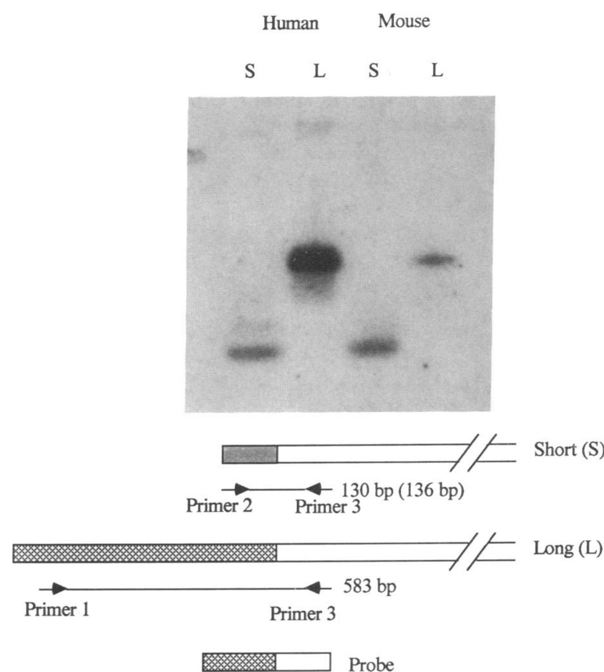

FIG. 4. Use of a common 3' primer (primer 3) from within exon 8 and 5' primers from within exon 1* (primer 2) or exon 4 (primer 1) generates a PCR product of 130 bp (human) or 136 bp (mouse) when the transcript contains exon 1* spliced to exon 8, or a 583-bp product when the transcript contains sequences of exons 4–8. Triple-helical regions of the long (L) and short (S) α1(IX) chains are indicated by open boxes; the non-triple-helical regions encoded by exon 1* and exons 4–8 are indicated by shaded and cross-hatched areas, respectively. The PCR products with human fetal RNA and primer 2 (human, lane S) or primer 1 (human, lane L) and the PCR products with embryonic mouse RNA and primer 2 (mouse, lane S) or primer 1 (mouse, lane L) were analyzed by 2% agarose gel electrophoresis. The PCR bands were identified by Southern blotting by using a mouse probe containing sequences of exons 6–8. In addition, the human PCR products were cloned in pBluescript and sequenced. To synthesize mouse cDNA containing sequences of exons 6–8, double-stranded mouse cDNA was used as template for PCR. The 5' sense primer was the oligonucleotide 5'-TGAACTCCAGTGGATGG-3', and the 3' antisense primer was the oligonucleotide 5'-CCGGAAC-TCCAGGAGGC-3'. The PCR product was phosphorylated with T4 polynucleotide kinase, purified by agarose gel electrophoresis, and ligated into the *Sma* I site of pBluescript. Identity of the cloned cDNA was confirmed by sequence analysis.

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