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Structural analysis of the regulatory elements of the type-II procollagen gene

Conservation of promoter and first intron sequences between human and mouse

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Transcription of the type-II procollagen gene (COL2A1) is very specifically restricted to a limited number of tissues, particularly cartilages. In order to identify transcription-control motifs we have sequenced the promoter region and the first intron of the human and mouse COL2A1 genes. With the assumption that these motifs should be well conserved during evolution, we have searched for potential elements important for the tissue-specific transcription of the COL2A1 gene by aligning the two sequences with each other and with the available rat type-II procollagen sequence for the promoter. With this approach we could identify specific evolutionarily well-conserved motifs in the promoter area. On the other hand, several suggested regulatory elements in the promoter region did not show evolutionary conservation. In the middle of the first intron we found a cluster of well-conserved transcription-control elements and we conclude that these conserved motifs most probably possess a significant function in the control of the tissue-specific transcription of the COL2A1 gene. We also describe locations of additional, highly conserved nucleotide stretches, which are good candidate regions in the search for binding sites of yet-uncharacterized cartilage-specific transcription regulators of the COL2A1 gene.

INTRODUCTION

Collagens, the major structural components in many different human tissues, form a large family of proteins with a wide variety of functions. Over 25 genes coding for collagen chains have already been characterized and localized in several chromosomes. Types I, II, III, V and XI, which form the subgroup of fibrillar collagens, exhibit remarkable conservation of gene structure but very diverse expression patterns [1,2]. Type-I and type-III procollagen genes are expressed in several tissues but the ratio of these proteins varies considerably from tissue to tissue [3]. The regulatory mechanisms mediating these complex expression patterns are only partially understood, although a considerable amount of information on the regulatory elements in the promoters and first introns of these genes has accumulated [1,2]. The α1(II) procollagen (COL2A1) gene demonstrates a highly specific tissue expression. The gene product, type-II collagen, is only found in cartilages, nucleus pulposus and vitreous humour [4]. Difficulties in analysing these tissues probably explain why much less is known about the regulatory elements of this gene. The current information comes from sequence analyses [5–7] and transfection experiments with constructs containing short segments of the promoter and/or the first intron of the gene [8–10].

Since type-II collagen is the major structural component of vertebrate hyaline cartilage, it is not surprising that the corresponding gene (COL2A1) has been linked to different cartilage disorders. These include both hereditary chondrodysplasias [11,12] and an inherited predisposition to an acquired cartilage disease, early-onset osteoarthrosis [13,14]. The mutations characterized so far in these diseases include point mutations, deletion of an exon and a partial duplication of an exon in the triple-helical domain [15–18]. In one case a point mutation creating an effective null allele (nonsense codon in the triple helix) has been associated with the Stickler syndrome suggesting that underexpression of the COL2A1 gene alone can result in a disease phenotype [19]. Therefore mutations in the regulatory areas of the COL2A1 gene, resulting in deficient expression of an allele could predispose to late-onset cartilage diseases. A prerequisite for a search for such mutations is detailed knowledge of the regulatory regions. Here we report the complete sequence and structural analysis of 1.3 kb of the upstream promoter and the entire first intron of the human (4.1 kb) and mouse (3.8 kb) COL2A1 genes. Our strategy to identify significant regulatory regions is based on the assumption that highly conserved regions between mouse, rat and man can guide us to functionally important sequences.

MATERIALS AND METHODS

Cloning strategy

Human. A cosmid library was constructed of partially *Sph*I-digested DNA from an individual heterozygous for two intragenic polymorphic restriction-enzyme-cleavage sites (*Hin*dIII and *Pvu*II) in the COL2A1 gene [20]. These polymorphic sites facilitated the recognition of the two alleles. Fragments of 20–30 kb in size were ligated to a cosmid vector derived from pJB8 [21]. The library containing approx. 700000 clones was screened with a radiolabelled 4.5 kb *Sal*I–*Bam*HI fragment from the 5′ end of the COL2A1 gene [22]. Forty positive clones were identified. The two alleles were identified by amplifying

Abbreviation used: COL2A1, α1(II) procollagen gene.

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The sequence of the human COL2A1 gene has been submitted to the EMBL Nucleotide Sequence Database under the accession number X58709, and the sequence of the mouse COL2A1 gene has been submitted to the GenBank Nucleotide Sequence Database under the accession number M65161.

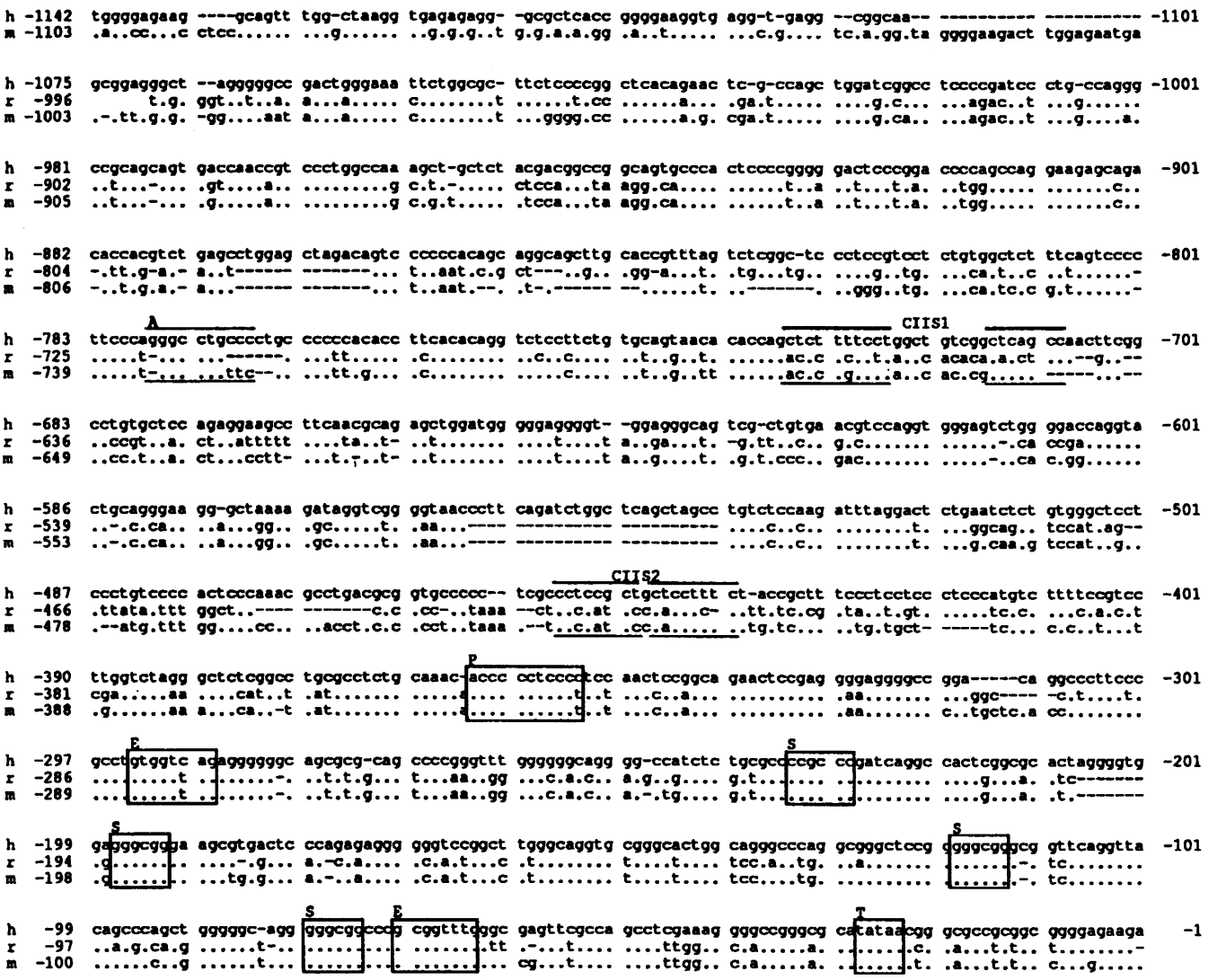


Fig. 1. Sequence comparison of the COL2A1 gene promoters

Line h, human gene sequence; line r, rat gene sequence (from 5); line m, mouse gene sequence. The nucleotides of each promoter are numbered on the left, the consensus nucleotides on the right. Only nucleotides differing from human are shown in the rat and mouse sequences; identical nucleotides are marked with a point (.), and deletions with a hyphen (-). The sequences were aligned with the PCGENE program. The potential Sp1-binding sites (S) are boxed. Also marked are the TATAA box (T), two enhancer core elements (E) and a conserved pyrimidine-rich sequence (P). Marked with horizontal lines are the partially conserved AP-2 site (A) and the locations of the two putative silencer elements (CIIS1 and CIIS2) described in the rat sequence. Over 500 bp of human promoter sequence upstream from consensus position −710 represents previously unpublished data. Only 217 bp of the mouse promoter have been published [24] and from consensus location −227 upstream the sequence represents new data.

the DNA spanning the polymorphic *Pvu*II site directly from lysed bacteria of the 40 clones by PCR [23]. The primers used were 5′-GGGAAGTGGAGTCCTTGTGG and 5′-TTGCCGGGAGCACCCTAAGG and the program for the amplification was 95 °C for 1 min, 56 °C for 1 min and 72 °C for 1 min for 30 cycles. The PCR products were digested with *Pvu*II and analysed on 1 % agarose gels. Both alleles were isolated and these cosmid clones were subcloned as *Eco*RI and *Pvu*II restriction fragments into the pGem3Blue and pSP72 vectors (Promega, Madison, WI, U.S.A.) respectively to obtain overlapping subclones.

Mouse. The genomic sequence of the mouse COL2A1 gene was obtained from several overlapping cosmid clones [24]. The entire gene and approximately 1.3 kb of upstream promoter was characterized by sequencing. The sequence reported here was obtained by using the original cosmid clone and 20 subclones in the Bluescript SK− vector (Stratagene, La Jolla, CA, U.S.A.).

Sequence analyses

The subclones of the human COL2A1 gene were sequenced using the SP6 and T_7 sequencing primers and 12 additional oligonucleotides, and the subclones of the mouse gene were sequenced using the T_3 and T_7 sequencing primers and six synthetic oligonucleotides. The sequencing was performed using the Sequenase reagent kit (United States Biochemical, Cleveland, OH, U.S.A.).

The structural analyses of the human and mouse sequences and the previously published rat promoter sequence were performed using the SEQIN, NALIGN and NMATPUS options in

Table 1. Location of recognition sequences for transcription factors in the promoters of the human, rat and mouse COL2A1 gene

The locations given indicate the 5′-nucleotide of recognition sequence in the consensus sequence shown in Fig. 1. c, Coding strand; n, non-coding strand; *, one base mismatch in the recognition sequence.

Sequence	Description of factor	Location of 5′-nucleotide		
		Human	Rat	Mouse
TATAA	RNA polymerase II	−28 c	−28 c	−28 c
GGGCGG	Sp1	−80 c	−80 c	−80 c
		−115 c		
		−119 c	−119 c	−119 c
				−132 c
		−198 c	−198 c	−198 c
		−234 n	−234 n	−234 n
			−473 n	
CCAAT	CAT-binding protein		−210 n	
			−869 c	−869 c
TGAGTCAG	AP-1	−555 n		
G(CG)(CG)(TA)G(GC)CC	AP-2	−128 n	−132 n	
			−168 c	−168 c
			−241 c	−241 c
			−253 c	
			−321 c	
		−794 n	−794 n	−794 n*
		−792 c	−798 c	
			−949 n	−949 n
				−995 c*
		−1024 n		
CAGCTGTGG	AP-4		−156 c*	−156 c*
GTGG(AT)(AT)(AT)G	Viral enhancer core	−71 c*	−71 c*	−71 c*
		−296 c*	−296 c	−296 c
		−444 n*	−411 n*	
				−726 n*
				−868 n*
		−902 n*		
GNNTTGGTGA	Transforming growth factor-β inhibitory element		−182 n*	−182 n*
				−1114 c
TGGGAATT	E2A (EIIaE-Cβ)			−462 n*
			−621 c*	−748 c*
		−1077 c*		

the PCGENE program (Version 6.25, IntelliGenetics, Geel, Belgium).

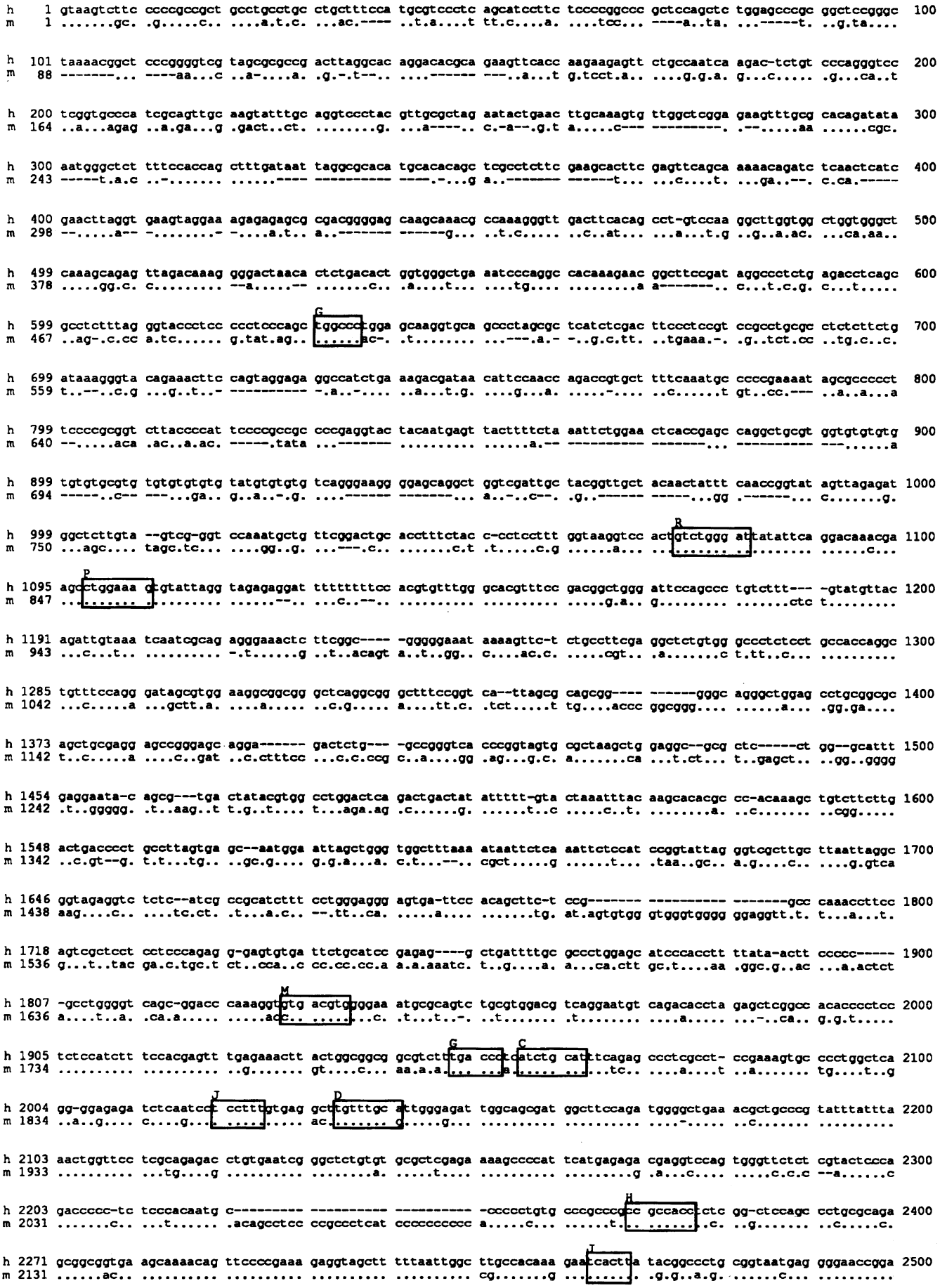
RESULTS AND DISCUSSION

The promoter region

For the analysis of the promoter region we sequenced 1542 bp of the human gene and 1103 bp of the mouse gene upstream of the transcription initiation site. These two sequences were aligned with each other and with the published 995 bp rat COL2A1 promoter sequence (Fig. 1) [5]. Some of the putative regulatory elements in the human promoter between nucleotides −694 and −1 have been reported previously [6,7] but no evidence for their functional significance is available. Within this 694 bp sequence, nine differences were observed between the new sequence and the previously published data [7].

Since functionally important regulatory elements are considered to be well conserved during evolution, we performed a detailed comparison of the regulatory motifs of the promoter regions of COL2A1 genes between mouse, rat and man (Fig. 1). Experimental evidence supporting this hypothesis comes from the work on chimeric mice which express a human COL2A1 transgene in a tissue-specific manner [25] and from studies on the expression of rat COL2A1 promoter/enhancer constructs in chick chondrocytes [9] and in transgenic mice [10].

All three genes were found to contain the same canonical TATAA box, important for binding of the RNA polymerase II, in an identical location between nucleotides −28 and −24 (Fig. 1). No CCAAT box, typical for most eukaryotic genes, was found in the immediate upstream region in any of these genes. The mouse and rat genes have a CCAAT sequence between nucleotides −869 and −865. The functional importance of this motif may be minimal since in the human promoter it is replaced by CCCCA (Fig. 1) and all of the flanking sequences are greatly divergent from the consensus sequence [26]. Another CCAAT motif is present in the non-coding strand in the rat gene between nucleotides −210 and −199 in a region of marked divergence between the three species analysed (Fig. 1). Three GGGCGG sequences were seen at identical locations in the three promoters at positions −80 to −75, −119 to −114, and −198 to −193 (Fig. 1). An inverted Sp1-recognition sequence (CCCGCC) was also observed in all three genes between nucleotides −234 and −229 (Fig. 1). The fact that these sequences are conserved between species and especially in the absence of the CCAAT box means that the four Sp1-binding sites (GGGCGG) probably play an important role in determining the basal promoter activity. The 'reverse CAT box' (GGTTA) reported in the human promoter between nucleotides −105 and −101 [7] is found also in the mouse and rat gene. However, since no factor capable of binding to this sequence has been described, its importance remains unclear.



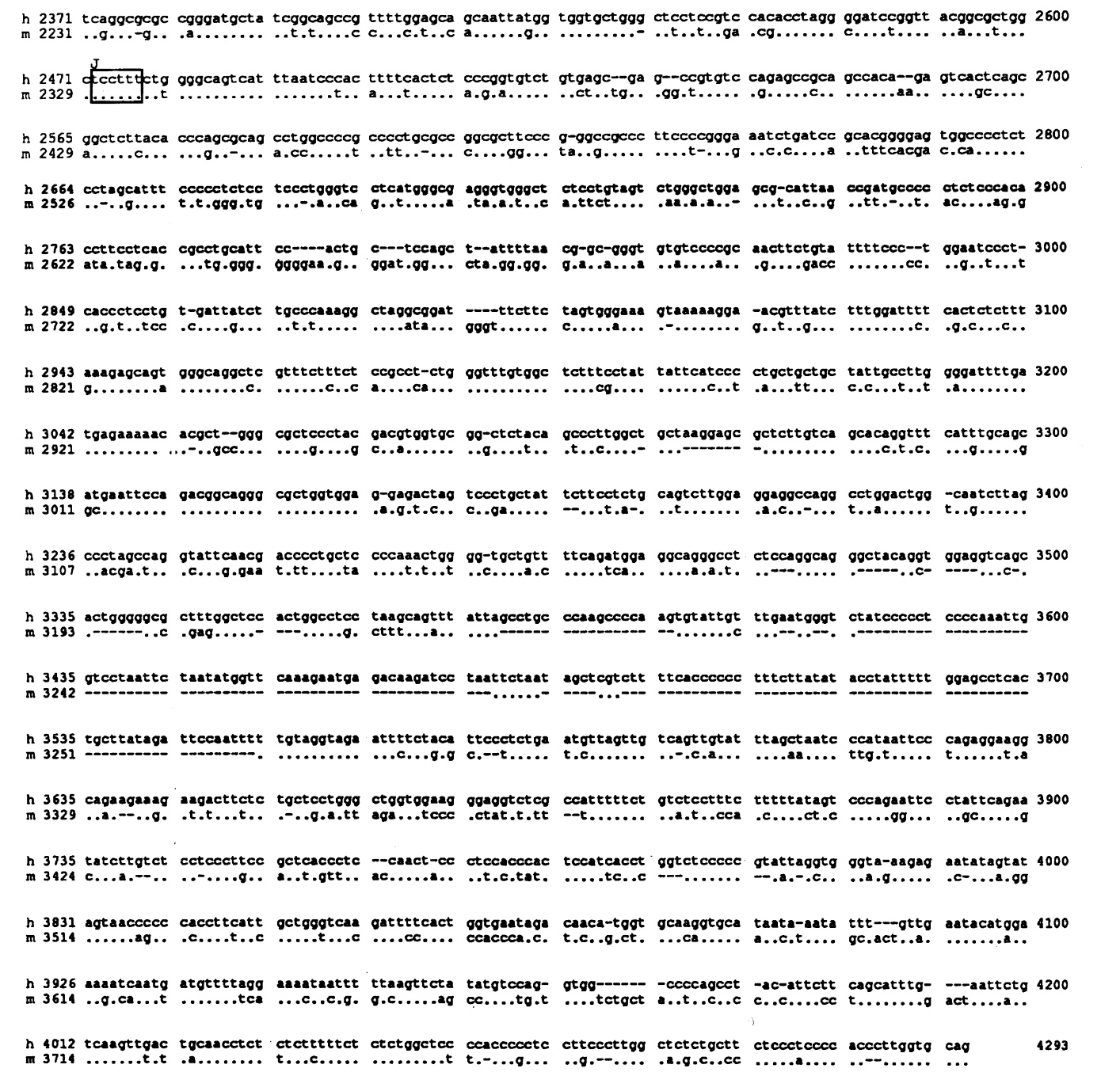


Fig. 2. Sequence comparison of the first intron of the human and mouse COL2A1 genes

Line h, human gene sequence; line m, mouse gene sequence. The nucleotides numbered on the left are for the individual sequences; the consensus nucleotides are numbered on the right. In the mouse sequence, only nucleotides differing from human are shown; identical nucleotides are marked with a point (.), and deletions with a hyphen (-). The sequences were aligned with the PCGENE program. The potential recognition sequences conserved in man and mouse are boxed, the letter above referring to Table 2.

All promoter sequences were screened on both strands with a panel of recognition sequences for known regulatory elements [26]. Several elements were found, but most of them are in locations not conserved between human, rat and mouse promoters (Table 1). This indicates that these sites do not represent key regulatory domains. For example, the human promoter has a nearly consensus AP-1/jun binding-site sequence between nucleotides −555 and −548 in a domain which is lacking in the rat and mouse genes. Several putative AP-2-binding sites were also found, but none were located in conserved domains of the promoters (Table 1).

A novel CTC factor capable of stimulating transcription of type-IV collagen genes has been recently described (E. Pöschl & K. Kühn, unpublished work). The binding-site consensus sequence (CCCTYCC) of this factor can also be identified twice in the promoter area of human COL2A1 gene (Fig. 1). Neither of these, however, was found fully conserved in the interspecies comparison.

Since all three promoters analysed share regions of considerable homology (Fig. 1), we paid particular attention to these in our search for potential recognition sites for transcription factors. Two conserved viral enhancer core sequences were found

in all three promoters at nucleotides −71 to −64, and at nucleotides −296 to −289. One of the two pyrimidine-rich sequences (ACCCCCTCCCCT) reported earlier by Ryan *et al.* [7] at −364 to −353 is located in a conserved region in all three genes whereas the other one (TTCCCTCCTCCCTCCC) at −431 to −416 is not conserved.

Analysis of the promoters for stem loops revealed a large loop of 40 bp with a stem of 11 bp and free energy value of −137.3 kJ between nucleotides −323 and −362 in the human gene. The corresponding loop cannot be expected to be formed in the mouse or in the rat gene owing to several mismatches in the stem structure. These types of loop have been proposed to act as enhancers for transcription [27]. Although the loop structure is obviously not well conserved, its location adjacent to other regulatory elements suggests that it might be significant for transcription of the human gene.

Using constructs with fragments of promoter region linked to a (chloramphenicol acetyltransferase) marker gene, two putative silencer motifs have been identified in rat COL2A1 promoter [9]. In our comparisons, the putative silencer regions between nucleotides −734 and −708 and between nucleotides −456 and −439 (Fig. 1) show, however, considerable variation between all three species, except for the CIIS2 motif (−456 to −448) which is present in both rat and mouse genes. Thus the significance of these silencer elements is doubtful. Since reasonably good tissue-specific control of the human and rat COL2A1 promoters has been experimentally shown in transgenic mice [10,25], other important silencer elements in the promoters should be searched for from the evolutionarily conserved regions. All the conserved regulatory sequences identified in man, rat and mouse are shown in Fig. 1.

We conclude that in addition to the TATA box and the four Sp1-recognition sequences, the completely conserved motifs in the promoter include only two enhancer core elements and one pyrimidine-rich region. Since so many other conserved regions were found in the promoter, it is likely that novel regulatory motifs will eventually be identified.

The first intron

Evidence has been accumulating for the presence of tissue-specific enhancer-type elements in the first introns of some collagen genes like COL1A2 coding for type-1 procollagen and COL4A1 coding for type-IV procollagen [28–31]. Horton *et al.* have used marker-gene (chloramphenicol acetyltransferase) constructs to demonstrate that a 550 bp region of the first intron in rat COL2A1 gene contains a tissue-specific enhancer [8]. The first intron in COL2A1 gene is remarkably long, 4105 bp in man and 3802 bp in mouse, when compared with the other introns of this gene which average 410 bp in man [32]. Consequently, it is highly possible that the first intron of the COL2A1 gene contains regulatory elements for transcription.

Our original motive to sequence the first intron of the human COL2A1 gene was a suspicion of a non-functional allele in a patient [13] (M. Vikkula, A. Palotie, J. Ott, L. Ala-Kokko, U. Sievers, K. Aho & L. Peltonen, unpublished work). When comparing the sequence obtained with the published sequence [7], we found both alleles of the patient to exhibit 117 single nucleotide differences, one additional stretch of 30 nucleotides and a reverse orientation of a 291 bp *Eco*RI fragment. Consequently, any search for an intronic mutation became impossible. We confirmed that the additional 30 bp did not represent a polymorphism of this gene by sequencing of both strands of both alleles and by PCR analyses of several unrelated individuals (results not shown). Since all the sequence given here is obtained by sequencing two alleles, the discrepancies cannot represent exceptionally high individual variation in the intronic sequence. When comparing our 4105 bp sequence of two alleles we found only two polymorphisms: one in position 1807 (G or A) and the second in position 3192 (C or A), altering the recognition site of the restriction enzyme *Mnl*I.

Table 2. Transcription-control motifs (from [26]) identified in the human and mouse COL2A1 sequences

	Transcription-control motif	Factor
A	G(CG)(CG)(TA)G(GC)CC	AP-2
B	CAGCTGTGG	AP-4
C	ATTTGCAT	Octamer (NF-3 binding)
D	TGTTTGCT	LTF-E-Site
E	TGACGTCA	Cyclic AMP-response element
F	GTG(GC)GGTG	Sterol-dependent repressor
G	TG(GA)(AC)CC	LF-A1
H	GCGCCACC	fos BLE-1
I	TGAGTCAG	AP-1
J	TC(AC)(CT)TT	β-Interferon silencer B
K	TGGGAATT	E2A, E4F2
L	TTTCGCGC	E2F
M	(GA)TGACGT(GA)	ATF
N	GCCACACCC	CACCC-box
O	TCNTACTC	C/EBP
P	GTGG(AT)(AT)(AT)G	Enhancer core
Q	ANCCTCTC(TC)	Lysozyme silencer
R	(AT)T(CG)TGGGA(AT)	APRE-2
S	AANCCAAA	Keratinocyte enhancer
T	GNNTTGGTGA	Transforming growth factor-β inhibitory element
U	GAAANNGAAA(CG)(CT)	VRE

The overall homology between the sequences of the first intron of COL2A1 gene of man and mouse was as high as 71.6%. Additionally, the 4105 bp intron contains several clusters of 300–400 bp where the homology is 80–90% (Fig. 2). We tried to identify the putative transcription-control sequences in the first intron by searching first for the homologies of 69 known motifs listed by Locker & Buzard [26]. The motifs identified on either strand in mouse and man are individually listed in Table 2 and marked in Fig. 3. A cluster of regulatory elements is recognized in both species in the middle of the first intron. The interspecies comparisons revealed that only a few of the elements were conserved and most of these sequences were located in the cluster region. Three conserved enhancer-type sequences included two elements classified as general enhancers (the NF-3-binding factor and fos BLE-1) and a liver-associated enhancer element (LTF-E-Site). β-Interferon silencer B element was found twice in this region. In addition, one ATF element, conferring cyclic AMP inducibility in viral and cellular genes and an LF-A1 motif, an element reported to bind liver-specific factors, were found in this region [26]. These well-conserved consensus elements are marked in Fig. 3.

To conclude, we have tried here to identify homologies to already known motifs regulating transcription in the promoter and first intron of COL2A1 gene. The significance of the motifs found was evaluated on the basis of their evolutionary conservation. Only a few enhancer and silencer motifs could be unequivocally identified on the basis of this assumption.

Cartilage, however, is unique among connective tissues in having a collagen composition very different from all others. This could indicate the presence of specific hitherto uncharacterized transcription factors in chondrocytes regulating the cartilage-specific genes. Considering the very restricted expression pattern of the COL2A1 gene, it is also conceivable that novel cartilage-specific regulatory motifs exist in the conserved regions of the promoter and/or the first intron of this gene. Therefore we

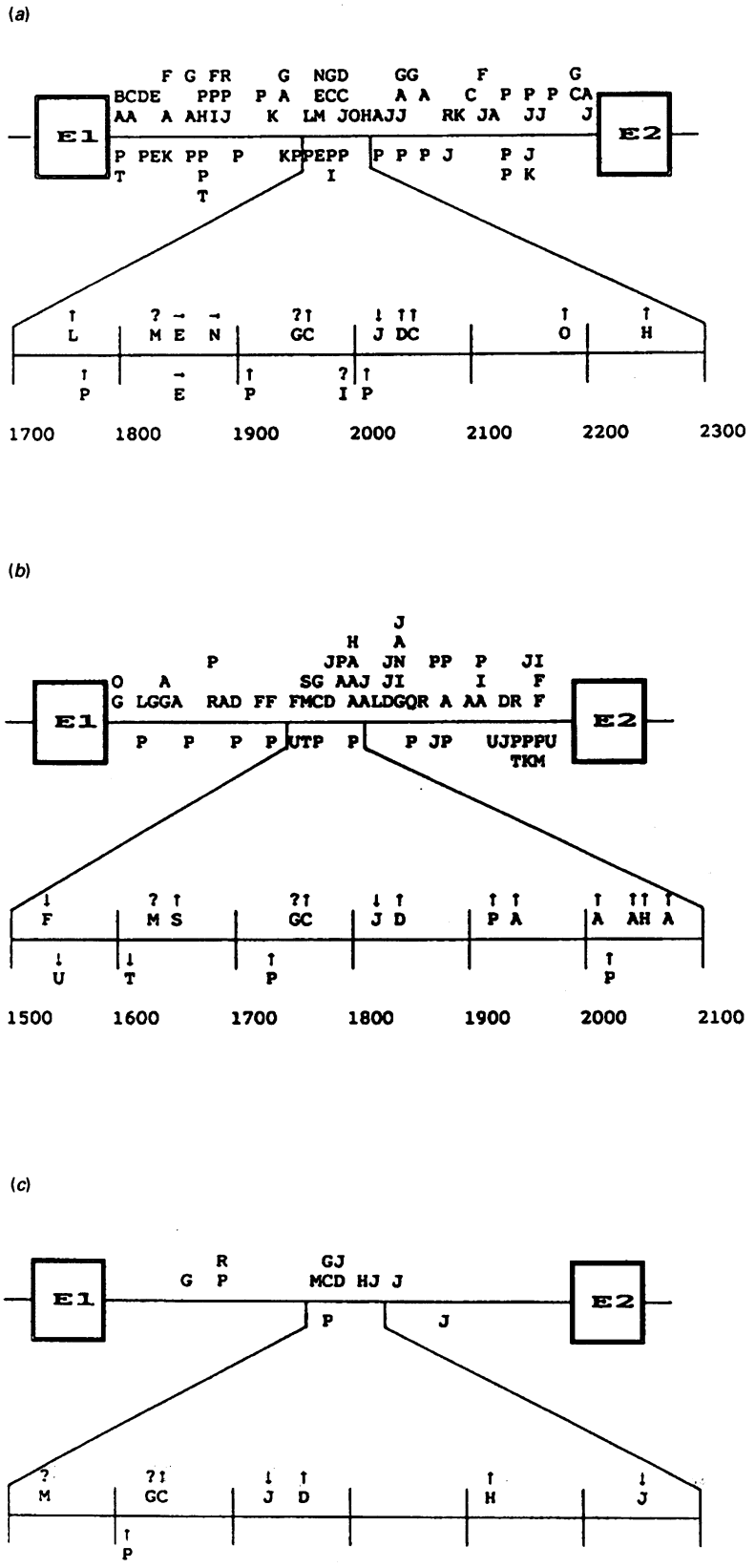


Fig. 3. Transcription control sequences found in the first intron of COL2A1 gene

The homologies (allowing maximally one mismatch) to the elements listed in Table 2 are marked schematically in the human (*a*) and mouse (*b*) maps. Conserved elements are shown separately in (*c*). The region in the middle of the first intron revealing a cluster of these elements is magnified below each schematic presentation. In these regions the reported character of each element is also given: ↑ = enhancer, ↓ = silencer, → = promoter element, ? = not known.

conclude that the conserved regions described here are good candidates when searching for binding sites of cartilage-specific transcription factors in the COL2A1 gene.

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