Generation of multiple transcripts from the chicken *chondromodulin-I* gene and their expression during embryonic development

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Abstract Chondromodulin-I (ChM-I) is an angiogenesis inhibitor isolated from fetal bovine cartilage. Here, we report the nucleotide sequence of chicken *ChM-I* cDNA. Chicken mature ChM-I had a significantly larger N-terminal hydrophilic domain than its mammalian counterparts. Chicken embryos expressed multiple transcripts (3.3, 2.0 and 1.7 kb in size) due to the alternative utilization of polyadenylation signals, whereas only the 1.7 kb transcripts were detected in mammals. Although confined to cartilage and eye at a later stage of development, whole-mount in situ hybridization revealed the expression of *ChM-I* mRNA in somites, heart, bronchial arches, roof plate, retina and limb buds. The expression pattern of the gene suggests a role for ChM-I in the morphogenesis during embryonic development.

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Key words: Chondromodulin-I; Angiogenesis inhibitor; Cartilage; Morphogenese

1. Introduction

Chondromodulin-I (ChM-I) is a 25 kDa glycoprotein originally purified from bovine epiphyseal cartilage on the basis of its growth-promoting activity for cultured chondrocytes [1]. Proteoglycan synthesis in growth plate chondrocytes and colony formation of chondrocytes in agarose culture were stimulated by ChM-I [2]. ChM-I also stimulated the growth of osteoblasts in vitro [3]. Recently, we demonstrated that ChM-I inhibits the growth of cultured vascular endothelial cells in vitro, as well as angiogenesis in vivo [4–6]. During skeletogenesis, the expression of *ChM-I* mRNA was restricted to the avascular zone of cartilage in the developing bone rudiments [4,7]. The level of *ChM-I* mRNA in primary chondrocytes was regulated by a variety of local growth factors [8]. When added to de-mineralized bone matrix, exogenous ChM-I protein inhibited the replacement of cartilage by bone in vivo [7]. These previous observations indicate that ChM-I plays a regulatory role in the bone formation.

Interestingly, we recently found that mouse embryos expressed the *ChM-I* transcripts at a high level even in the stages prior to cartilage formation [7], implying that the gene plays a role in the early embryonic development. Chicken embryos are more amenable to developmental analysis and manipulation in comparison with mice. In the present study, we determined the nucleotide sequence of chicken *ChM-I* cDNA isolated from the day 10 chicken embryo cDNA library. The cDNA was used as a hybridization probe for Northern blot and in situ hybridization analysis. In addition to the 1.7 kb transcripts [1,7,8], we found that the larger transcripts for *ChM-I* (3.3 and 2.0 kb in size) were expressed in the avian tissues due to the multiple polyadenylation signals in the 3′-untranslated region of the gene. Moreover, the gene was evidently expressed in association with a variety of embryonic structures prior to cartilage formation.

2. Material and methods

2.1. Rapid amplification of cDNA ends (RACE)

According to the manufacturer's instructions, the RACE was performed using the 3′-RACE system (Gibco BRL, Gaithersburg, MD, USA). First-strand cDNA was generated using the adapter primer that was oligo(dT)-engineered. Aliquots of 1/20 of the cDNA were used as a template for the amplification with the anchor primer complementary to the specific 5′-sequence of the adapter primer and the primer P1 (5′-CATCGGGGCCTTCTACTTCT-3′) that was designed on the basis of the conserved sequence between human and bovine *ChM-I* cDNA [1,6]. For the 3′-RACE of the longest transcript, cDNA was amplified using the nested anchor primer and the primer P2 (5′-GACAACCTCATGCAGAATGC-3′) that was nested downstream of the primer P3 (5′-CTACGGAAGGTACTGAGCTGAAG-3′). The amplified PCR products were subcloned into pCRII (Invitrogen, San Diego, CA, USA). For 5′-RACE, adapter-ligated double-stranded cDNAs were prepared using a Marathon cDNA Amplification kit (Clontech, Palo Alto, CA, USA) according to the manufacturer's instructions. The cDNA was amplified using an anchor primer and the primer P3 (5′-CGGTGATGCCAATCTGGAAATC-3′). The amplified PCR products were subcloned into pCRII.

2.2. Screening of the day 10 chicken embryo λgt11 cDNA library

A λgt11 cDNA library constructed from whole day 10 white leghorn embryos was purchased from Clontech for isolation of chicken *ChM-I* cDNA clones. A total of 5×10^5 independent recombinant phages was initially screened with a partial cDNA clone (RCI1) obtained from the 3′-RACE. The hybridization-positive clones were isolated by the repeated plaque purification. The inserts isolated from the phage clones were subcloned into the *Eco*RI site of pBluescript II SK(+) (Stratagene, La Jolla, CA, USA). The nucleotide sequence of the inserts was determined using the Big Dye Terminator Cycle Sequencing FS Ready Reaction kit with ABI 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Both cDNA strands were sequenced using the standard sequencing primers and gene-specific primers. The obtained DNA sequences were compiled and analyzed by GeneWorks computer programs (IntelliGenetics, Tokyo, Japan).

2.3. Northern hybridization

Total RNA was prepared from sterna of chicken day 20 embryos by a single step method, according to Chomczynski and Sacchi [9]. For Northern blot analysis, 20 μg of the total RNA was denatured with 6% formaldehyde, fractionated by 1% agarose gel (SeaKem GTG, FMC Bioproducts, Rockland, ME, USA) electrophoresis and transferred onto Nytran membranes (Schleicher and Schuell, Dassel, Germany) with Turboblotter (Schleicher and Schuell). Hybridization was performed overnight at 42°C with an appropriate cDNA probe labelled with [α-^{32}P]dCTP (10^6 cpm/ml) in a solution containing 50% formamide, 6×SSPE (0.9 M NaCl, 20 mM NaH_2PO_4, 6 mM EDTA at pH 7.4), 0.1% bovine serum albumin (Calbiochem-Novabiochem,

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La Jolla, CA, USA), 0.1% Ficoll 400 (Pharmacia, Sweden), 0.1% polyvinylpyrrolidone (Wako Pure Chemical, Osaka, Japan), 0.5% SDS and 100 μg/ml denatured salmon sperm DNA. The cDNA probes were labelled with a [α-^{32}P]dCTP (Amersham, UK) by the random-primer method using a BcaBEST labelling kit (Takara, Otu, Japan) using the appropriate cDNA fragments: the 1.0 kb *Eco*RI fragment of RCI1, the 0.35 kb fragment amplified from RCI1 (probe A) and the 0.8 kb fragment amplified from LCI33-2-3 (probe B), as the probes for *ChM-I* mRNA. The 1.15 kb *Bam*HI-*Pvu*II fragment of pYN509 was used as a probe for chicken type II collagen [10]. After hybridization, filters were washed for 30 min at 55°C in 2×SSPE, 0.1% SDS, then washed for 30 min at 55°C in 0.1×SSPE, 0.1% SDS and exposed to BIOMAX film (Eastman Kodak, Rochester, NY, USA) at −80°C.

2.4. In situ hybridization

Chicken embryos were obtained by incubation of fertilized white leghorn eggs and staged according to Hamburger and Hamilton [11]. For whole-mount in situ hybridization, the antisense and sense cRNA probes were transcribed from RCI1 with a DIG RNA labelling kit (Boehringer Mannheim, Mannheim, Germany). Chicken embryos were fixed in 4% paraformaldehyde (PFA) resolved in phosphate-buffered saline (PBS) and gradually transferred into methanol by exchange of the PFA/PBS against the increasing concentrations of methanol/PBS. After rehydration, the embryos were incubated with the antisense cRNA probes. Hybridization was performed at 70°C overnight and the embryos were washed under conditions of high stringency. Hybridization was detected immunohistochemically by an alkaline phosphatase-conjugated antibody to obtain purple deposits at the site of hybridization using a nucleic acid detection kit (Boehringer Mannheim). Appropriate control experiments were performed to exclude false-positive staining due to the endogenous alkaline phosphatase activity and they were negative.

3. Results and discussion

To isolate the chicken *ChM-I* cDNA, we extracted total RNA from sternum of stage 45 (day 19) chicken embryos and performed 3′-RACE by anchor primer and the primer P1 designed on the basis of the nucleotide sequence conserved between bovine and human [1,6]. One clone (RCI1) encoded 3/4 of the coding region and the 3′-untranslated region including the polyadenylation signal (AATAAA) followed by a poly(A)-tail (Fig. 1A). Using this clone as a probe, we screened the day 10 chicken embryo λgt11 cDNA library. 30 Positive clones were identified from 3×10^5 recombinant phage clones. Six clones were randomly selected for purification and subcloning for further analysis (Fig. 1A). Since all the clones obtained from the cDNA library lacked the 5′-region of the cDNA, we performed 5′-RACE and obtained the clone containing the initial codon.

Three clones (LCI1-3, LCI10-6 and LCI33-2-3) contained approximately 1.6 kb of the 3′-untranslated region with three polyadenylation signals (AATAAA) (Fig. 1A). In addition to that at position 1196 (PA1), a signal was present at position 1466 (PA2) and at position 2362 (PA3), respectively. One clone (LCI2-1) is the short form containing only PA1 followed by a poly(A)-tail. The position of the polyadenylation signal is identical to that of clone RCI1. Clone LCI5-1 contained one polyadenylation signal at the same position but lacked the poly(A)-tail. Clone LCI8-1 contained two polyadenylation signals without a poly(A)-tail. Three clones (LCI1-3, LCI10-6 and LCI33-2-3) contained the 3′-untranslated regions (approximately 1.6 kb in size) with three polyadenylation signals. We performed the 3′-RACE experiment to determine if there was any additional 3′-untranslated sequence. The 3′-RACE provided an additional downstream sequence of approximately 70 bases, but no additional polyadenylation signal was found (Fig. 1A).

Nucleotide and deduced amino acid sequences of chicken *ChM-I* cDNA were assembled (Fig. 1B). Chicken *ChM-I* cDNA contained a 1041 bp open reading frame harboring 347 amino acid residues for ChM-I precursor protein. Initial and terminal codons were found at positions 3 and 1044, respectively. The nucleotide sequence in the coding region was 72% identical with that of the bovine counterpart. The mature ChM-I sequence was preceded by a processing signal (RERR), which was completely conserved between chicken

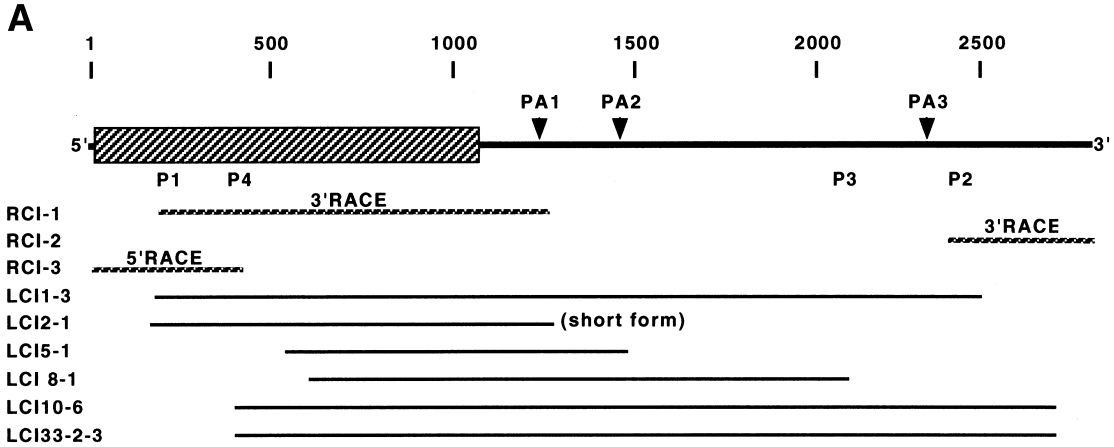

Fig. 1. Molecular cloning of the chicken *ChM-I* cDNA. (A) Cloning strategy. The top bar depicts the structure of chicken *ChM-I* cDNA. The part of the cDNA corresponding to the coding region is represented by a box. The polyadenylation sites (AATAAA) are marked by arrowheads. The primer P1, which is designed on the basis of the nucleotide sequences conserved between bovine and human, was used for 3′-RACE of the short form transcripts. The cDNA clones (LCI1-3, LCI2-1, LCI5-1, LCI8-1, LCI10-6 and LCI33-2-3), which were obtained from the chicken day 10 embryo library by using RCI1 as a probe, are shown below. The 3′-end of the nucleotide sequence derived from the longest transcripts was amplified by 3′-RACE. The 5′-end of the cDNA was obtained by 5′-RACE. (B) Nucleotide sequence of chicken *ChM-I* cDNA with the deduced amino acid sequence. The nucleotide sequence is given to the left and the right sides of each line. The putative transmembrane domain is underlined and the putative processing signal is double-underlined. The mature portion of the deduced amino acids is boxed. The asterisks indicate the potential N-linked glycosylation site. Cysteine residues are encircled. The consensus sequence of polyadenylation sites (AATAAA) is indicated in bold and the poly(A)-tail is given. This sequence has been deposited in the GenBank database under accession number AF027380.

B

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1    CCATGGCCGA GGGCTCGGAG AAGGTACCCA TCGCCCGAGC GGGGCCCGAA GACGTGGAGC AGGGCCTGCC  70
       M  A  E  G  S  E  K  V  P  I  A  R  A  G  P  E  D  V  E  Q  G  L  P
71   GCCCGCATAT ACGGCAGCAG TGCCCCCTCC GGGGCCAGGG CGGCTGCTGA AGGCAGGGGC CACAGTGCTC 140
       P  A  Y  T  A  A  V  P  P  P  G  P  G  R  L  L  K  A  G  A  T  V  L
141  ATCGCTGGGG CCCTTCTGCT GCTGGCTGGG GCCATCGGGG CCTTCTACTT CTGGAAAGCC ACTGAGAGGC 210
       I  A  G  A  L  L  L  L  A  G  A  I  G  A  F  Y  F  W  K  A  T  E  R  Q
211  AGGTCTACAA CGTTCACTAC ACCATGAGCA TTAATGGAAA AGTGCAAGAC GGATCGATGG AAATAGATGC 280
       V  Y  N  V  H  Y  T  M  S  I  N  G  K  V  Q  D  G  S  M  E  I  D  A
281  CGGAAACAAC TTAGAGACAT TCAAAACAGG AAGCGGGAGT GAAGAGGCTG TTGAAGTTCA TGATTTCCAG 350
       G  N  N  L  E  T  F  K  T  G  S  G  S  E  E  A  V  E  V  H  D  F  Q
351  ATTGGCATCA CCGGCATCCG ATTTGCTGGA GGAGAGAAGT GTTACATCAA GGCCCAGCCC AAAGCTCGTG 420
       I  G  I  T  G  I  R  F  A  G  G  E  K  C  Y  I  K  A  Q  P  K  A  R  V
421  TGCCTGAGGT GGATGCCATG ACCAAGGCCA GCCTGTCCTC TGACCTGGAA GATGAAATCA TGCCTGTTAG 480
       P  E  V  D  A  M  T  K  A  S  L  S  S  D  L  E  D  E  I  M  P  V  R
491  ATTTGACGAA AACTCTCTTA TCTGGGTGGC TGCAGACGAG CCCATCAAGC ACAACGGTTT CCTAAGCCCC 560
       F  D  E  N  S  L  I  W  V  A  A  D  E  P  I  K  H  N  G  F  L  S  P
561  AAAATTTTAG AGCTTTGTGG GGATCTTCCG ATTTTCTGGC TTCGACCTAC ATATCCCAAA GATAAACAAA 630
       K  I  L  E  L  C  G  D  L  P  I  F  W  L  R  P  T  Y  P  K  D  K  Q  R
631  GAGAAAGGAG AGAAATGAAG AGAAATAAAC GCCAGTCCGA GTCAAACTTC GATGCAGAAC ACAGAGCAGC 700
       E  R  R  E  M  K  R  N  K  R  Q  S  E  S  N  F  D  A  E  H  R  A  A
701  TGCTGCTGAA GAGGTGAACA CCAGGTCCAC CCCCACCCAG CTGACTCAAG AGCTTGGCCC CCAGTCGAAT 770
       A  A  E  E  V  N  T  R  S  T  P  T  Q  L  T  Q  E  L  G  P  Q  S  N
771  GAAACCAGGC CAATGCAGCA AGAAAGCGAT CAAACACTTA ACCCAGACAA CCCATACAAC CAACTGGAAG 840
       E  T  R  P  M  Q  Q  E  S  D  Q  T  L  N  P  D  N  P  Y  N  Q  L  E  G
841  GTGAAGGGAT GGCTTTTGAC CCCATGCTGG ACCACCTGGG CGTGTGCTGC ATCGAGTGCA GGCGAAGTTA 910
       E  G  M  A  F  D  P  M  L  D  H  L  G  V  C  C  I  E  C  R  R  S  Y
911  CACGCAGTGC CAGAGGATCT GTGAGCCTCT GCTGGGCTAC TACCCGTGGC CATACAACTA CCAGGGCTGC 980
       T  Q  C  Q  R  I  C  E  P  L  L  G  Y  Y  P  W  P  Y  N  Y  Q  G  C
981  CGCACCGCCT GCCGCATCAT CATGCCCTGC AGCTGGTGGG TCGCTCGCAT CATGGGCGTT GTGTGAGAGA 1050
       R  T  A  C  R  I  I  M  P  C  S  W  W  V  A  R  I  M  G  V  V
1051 GCTTTGGGCT CCTGGAGGAA GCCAGCAGAA GCCTGACAAC GCCAAACTGA TGAAAGACTC CGACCAAAGC 1120
1121 AGCATGGCCA ATCCTGGCTT CTAAATCCAA ACGCGCGTTA CTGTGCTAAA AGCAAGGGCA GGTATTCTGA 1190
1191 GTGTAAATAA ACTCTTAAAT TAAGGTGCCA TTAGCTTTAC CAATGACAAA GAGTATCCTA GCGGGCGCTG 1260
1261 AATTGCAAAC TAAGAAACCC CAGAACAGAC AACACTAACT TCGCTCACAA ACTGGTTTAG CAGTCTAGAA 1330
1331 GCTTGCTCAT AAATCCAAAT TCTGACTTTC ATAACAAAAA CCTCTCTCTG AGCTAGACGT GAAAATTTAT 1400
1401 GTCCAATTAG GCAACTAGCT AAACTTGTAC ACTTGGGAAT ACCTTTCATC CACGTAGCTT ACCTAAATAA 1470
1471 ATCACAAGTT AAGTCGCTCC CCGTTGCTAT CCGTACAGGG CTGCCAGGTC ATTATGAATG GTTATAAACC 1540
1541 AATTACGCAT ACCTTTCCCA CAAAGCTGCT CCAGGGGACA TGGGAAAGCT CACATCGTGT TTAATCAGAA 1610
1611 ACAAACGGCA GGAAGAGGGG CTGTGGGATG GAATTTGTGT GGCTGCAGAA TCAAAACCCT TCGCACTCCC 1680
1681 AACATCAAAG CTGCCCCGGC ATCAAGAATC TTGTATCCAT GATACCACTG CAGTGTCTGA CAACTATCTG 1750
1751 CCATCCAGCG ACTACAGCAA AAGTATTTAC ACCCAACTGC AGGCCAAAGA GTCTCAAGAC CCTTCCGAAC 1820
1821 CTACGAGAGC CACTGTTATT TATAAACCCT TGAACTAGAC ACTTCTGACA CAAGATTACA ACCCCTCCTT 1890
1891 ATTCCACATG GCATAGCGGT TGTTCGTGCC AGCTCCTTGG CCTTCGCTGG AGCTAACCTC TGTTCCCTAC 1960
1961 AAGAGAGATT GCTTACTGGT AAATGAGAGG AGAAAAAGAT TTTAACCTCA CCCTTGTTGC CCAGGCAAAC 2030
2031 TCTGCAATTA GAGGGATTGA TCCTATTATG TACTAAATCT CTACGGAAGG TACTGAGCTG AAGATGGTTG 2100
2101 ATTAGGATTC CCAGTCTTTG AACAAAATTA GAAGTATTAT ACTGCCTTCA TCCATACGAC ATCCTCAGCT 2170
2171 CCACCCCTGA GTTCACGGAG CCATCTACAC AGGCATGATA AACAAAAAGG TCCAATCTTT TAAGGTTCAC 2240
2241 TTTCTGAAGC ATTCATTTTA GTGACTAAAC AGCTCCCGAG ATGTTGCAAG CCTAGTAATG TTATTAGCAC 2310
2311 TGCATCACAC AGAAAAGAGG AATTAGTTTG ACATAATCGT GGCTTAAAGA AAATAAAGAC AACCTCATGC 2380
2381 AGAATGCTAA ACAGCGTATC TAAAAAGCAA AGTGTTAAAT GAGAGAAGAT GAGAATAGAG TTCAGTATTT 2450
2451 TGTCCTATTG TTATTATAGA TAAGCTTCAC CTTCCCTTAA TCTCAAGAAC AAACTTGCTT TGAAATACTC 2520
2521 CAACTGGAAA CTTCTTAGAG TGGGATTTCA AGGCCTGTTA CATTTGAGGT TTTTGCTCTT TTGAAAGCAA 2590
2591 ATGACCTTGA TCATTATGTT GTTTTAGAAG TACGCCATTC ATATGCTTAG AAAAATCATT CTTTCTTCTT 2660
2661 GGGGTTTTTT TTTTTTAATT TTATTTTTTA CTGCTGATTA AACAAATACA TTAAAAATAG AAAAAAAAAA 2730
2731 AAAAAAAAAA AAAAAA                                                          2749
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Fig. 1 (*continued*).

and other mammalian homologues (Fig. 2) [1,6–8]. The possible transmembrane domain in the precursor was also conserved. Most amino acid substitutions and insertions were found in the N-terminal hydrophilic domain of the mature ChM-I (Fig. 2). However, the *N*-glycosylation site (NXT) in this domain was conserved among all of the cloned homologues (Fig. 2). In contrast to the N-terminal domain, the C-terminal 2/3 of the mature ChM-I was well conserved be-

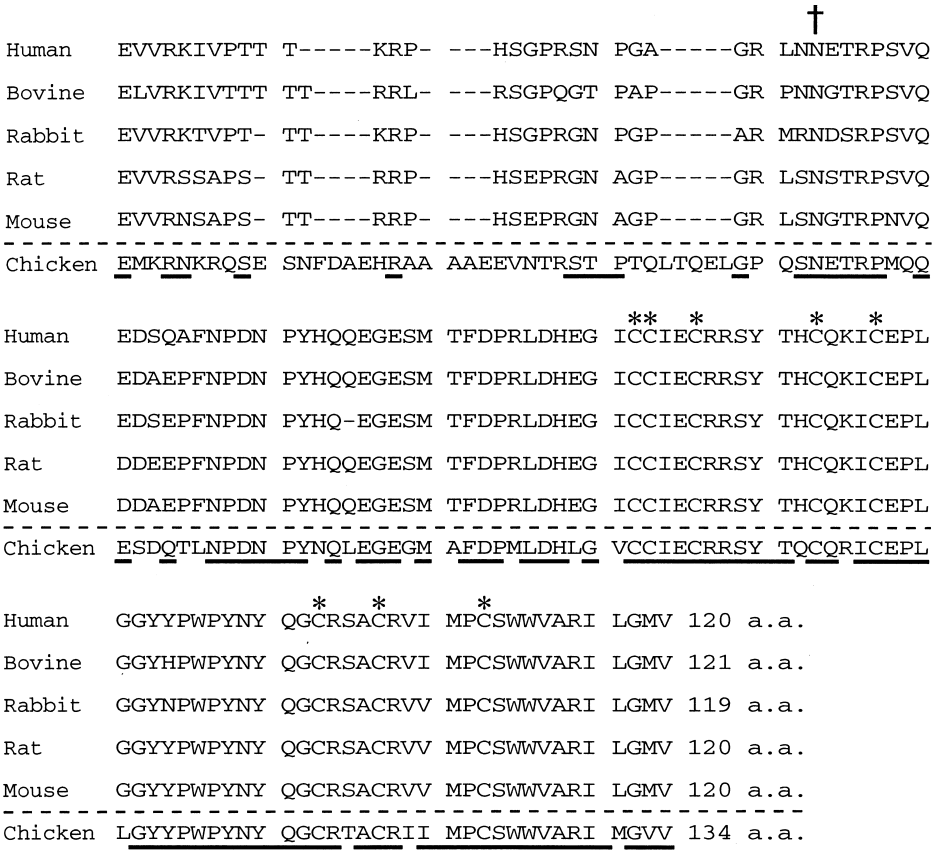

Fig. 2. Amino acid sequence comparison of chick mature ChM-I with those of mammalian species. Conserved amino acid residues are underlined. Positions of Cys residues and the potential N-linked glycosylation site are denoted by (*) and (†), respectively.

tween the species, including all eight Cys residues in this domain (Fig. 2).

In mammals, the 1.7 kb transcript was predominantly expressed in cartilage [1,7,8]. However, three possible polyadenylation signals were found within the 3′-untranslated region of chicken *ChM-I* cDNA (Fig. 1B), whereas other mammalian cDNAs have only one. Consistent with this finding, Northern blotting with probe A, covering a portion of the coding sequence, revealed three transcripts with approximate sizes of 1.7, 2.0 and 3.3 kb in day 20 chicken sternal cartilage (Fig. 3A). Probe B containing the downstream polyadenylation signal PA3 detected only the 3.3 kb transcripts (Fig. 3A), suggesting that the multiple *ChM-I* transcripts in chicken cartilage were generated by the alternative utilization of polyadenylation signals in the 3′-untranslated region. As in mammalian tissues [1,7], the *ChM-I* gene was expressed in a tissue-specific manner in cartilage and eye in the Northern blot analysis of stage 45 (day 20) chicken embryos (Fig. 3B). In both tissues, the major transcripts were those of 3.3 kb in size, but all three transcripts were detected. We did not detect the expression of *ChM-I* mRNA in liver, heart, intestine or brain at this stage of development (Fig. 3B).

In the developing cartilaginous bone rudiments of chicken embryos at stage 41 (day 15), *ChM-I* transcripts were present in the avascular zone of cartilage, but absent in the late-hypertrophic and calcified zone (data not shown), consistent with previous observations in mammals [4,7]. The expression of the *ChM-I* gene was augmented in a manner dependent on chondrogenic differentiation and was abolished upon the application of de-differentiation signals to chondrocytes, such as by treatment with parathyroid hormone or parathyroid hormone-related peptide [7,8]. Recently, chicken *ChM-I* cDNA was identified as a cartilage-specific gene markedly down-regulated by retinoic acid (U. Dietz, personal communication, GSF-Research Center, Munich, Germany). Retinoic acid causes a rapid de-differentiation of chondrocytes in vitro [12]. Taken together, the expression of the *ChM-I* gene was closely associated with acquiring the mature phenotype of cartilage that exhibited a powerful anti-angiogenic property.

Northern blot analysis revealed that the expression of the *ChM-I* gene is restricted to cartilage and eye at the later stage of development (Fig. 3A). However, whole-mount in situ hybridization clearly indicated that *ChM-I* was expressed at the stages of development preceding cartilage formation in chicken embryos (Fig. 4). We detected the expression of *ChM-I* mRNA in various non-cartilaginous structures such as somites, limb buds, bronchial arches, heart, eye and roof plate of neural tube. The pattern of expression suggested that ChM-I plays a role in morphogenesis during the early development of embryos. The pattern of expression in limb buds was of most interest in the light of vascular development prior to cartilage formation. Limb bud development begins when mesenchymal cells proliferate from the somitic layer of the limb field lateral plate mesoderm around stage 17 [13]. In association with the limb bud outgrowth, the vascular system is developed by the sprouting angiogenesis from the aorta. However, an avascular peripheral zone of mesoderm remains beneath the apical ectodermal ridge [13]. The expression of

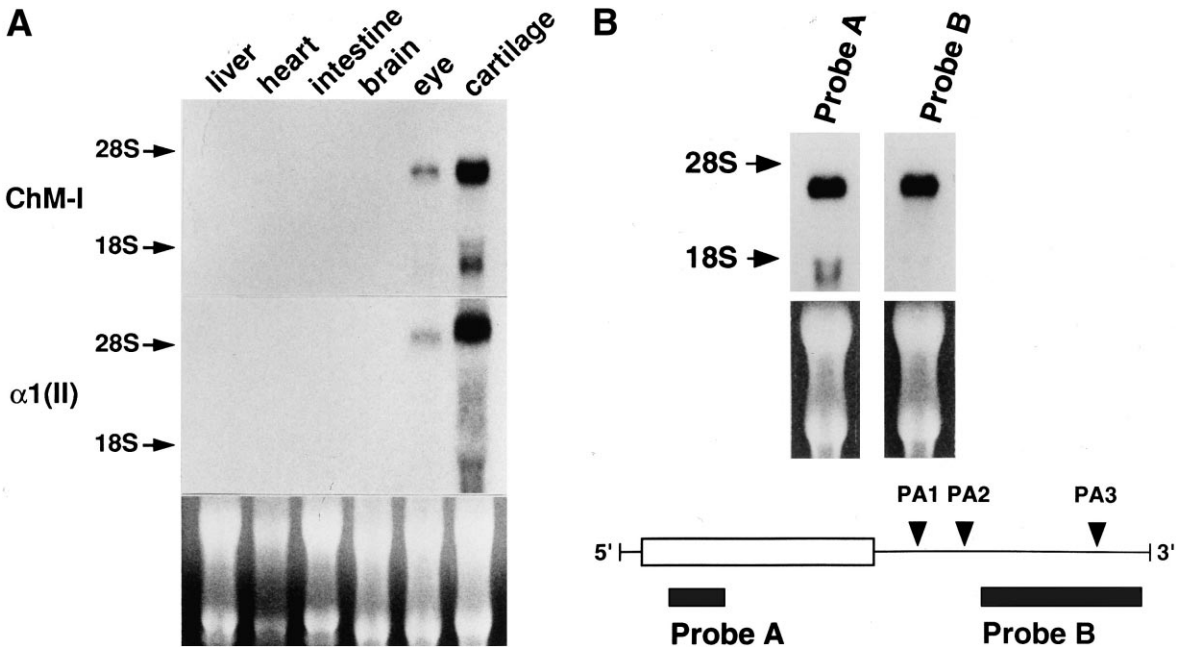

Fig. 3. Northern blot analysis of *ChM-I* mRNA in chick embryos. (A) Alternative utilization of polyadenylation signals. Total RNA was isolated from sterna of day 20 chicken embryos and 20 μg of RNA was loaded in each lane. Hybridization probes (probe A and probe B) are shown as shadowed boxes in the bottom panel where the coding region is boxed. All three transcripts (3.3, 2.0 and 1.7 kb in size) were recognized with probe A, while the 3.3 kb transcript was visualized with probe B. (B) Total RNA (20 μg) was isolated from the indicated tissues of stage 45 (day 20) chicken embryos and probed with a chicken *ChM-I* cDNA clone (probe A) and a rat type II collagen cDNA. Equivalent loading of total RNA was verified with ethidium bromide staining. The positions of 28S and 18S ribosomal RNA are indicated.

ChM-I mRNA was first recognized in this avascular zone in limb buds at stage 21 (Fig. 4), whereas no limb expression of *ChM-I* mRNA was detected at stage 19 (data not shown). Then, the ChM-I expression was detected at the center of limb buds (Fig. 4, inset) when vascular regression took place during the mesenchymal condensation prior to cartilage formation [14]. These results suggest that ChM-I participates in the formation of an avascular zone in the limb mesoderm and during the precartilaginous vascular regression.

Interestingly, a high level of *ChM-I* mRNA was detected in the upper part of the developing heart at stage 21 (Fig. 4), while there was no expression of *ChM-I* mRNA in the heart

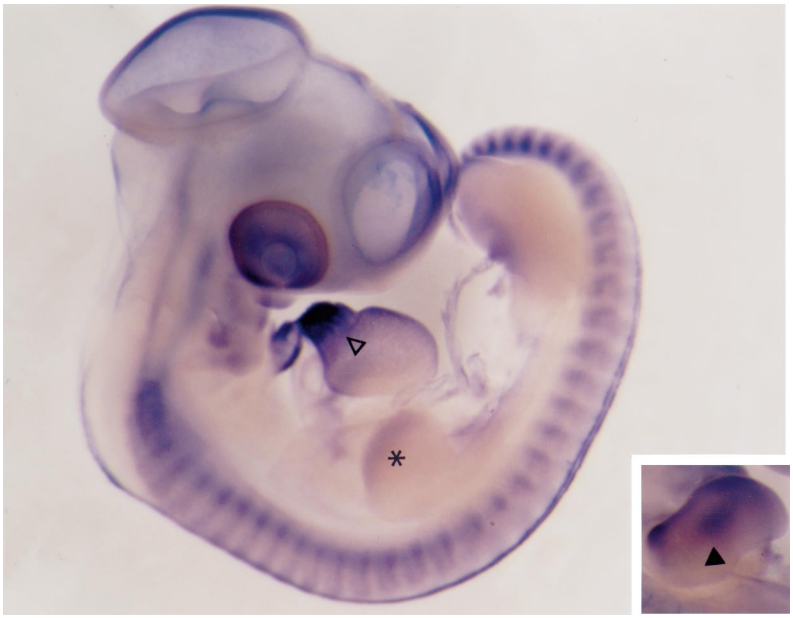

Fig. 4. Whole-mount in situ hybridization of *ChM-I* mRNA during embryogenesis. The expression of the *ChM-I* gene was detected in eye, bronchial arches, heart, somites, limbs and roof plate in the chicken embryo at stage 21. Avascular peripheral mesoderm (*) and atrium and truncus arteriosus (▷) are denoted. A forelimb at stage 24 is shown in the inset. Cartilaginous condensation (▲) at the center of the limb bud is denoted.

at stage 45 (Fig. 3B). In chicken, endocardium derived from splanchnic mesoderm fuses to form a single pumping heart tube at stage 9 [13]. Around this stage, we could not detect the expression of *ChM-I* mRNA in the heart (data not shown). At stage 18, the heart is a two-chambered tube, with one atrium and one ventricle [13]. However, at the stage where the partitioning of the tube into a distinctive atrium and ventricle occurs, *ChM-I* is expressed in sinus venosus, endocardium, truncus arteriosus and atrio-ventricular cushion (U. Dietz, personal communication, GSF-Research Center, Munich, Germany). This partitioning of the tube is accomplished when cells detach from the endocardium and enter the hyaluronate-rich 'cardiac jelly' to form the endocardial cushion. The present hybridization data suggest an involvement of ChM-I protein in this process as one of the bioactive components in 'cardiac jelly'. This possibility will be tested by the immunochemical analysis using anti-chicken ChM-I antibody.

The expression of ChM-I was associated with vascular regression in limb buds as well as avascular tissues such as cartilage and eye (Fig. 3), indicating its functional role in angiogenic switching of tissues [7]. Molecular cloning of chicken ChM-I enabled us to determine its expression pattern at earlier stages of development (Fig. 4). It is especially intriguing to clarify the functional role of ChM-I expressed in somites, bronchial arches and neural tube at the early developmental stages prior to establishment of the vascular system in embryos. Experiments for the generation of ChM-I null mutant mice are now underway. Moreover, by comparison of the chicken ChM-I sequence with those of mammalian counterparts, it was suggested that the N-terminal hydrophilic domain of ChM-I might be dispensable for its anti-angiogenic action [6]. Thus, recombinant ChM-I lacking the N-terminal domain was expressed. Evaluation of its bioactivity is now underway in vitro and in vivo.

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