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Molecular Cloning of Mouse and Bovine Chondromodulin-II cDNAs and the Growth-Promoting Actions of Bovine Recombinant Protein[1]

Chisa Shukunami,* Jun Kondo,† Hiromichi Wakai,† Kazuhiro Takahashi,† Hiroyuki Inoue,‡ Akihito Kamizono,† and Yuji Hiraki*,[2]

*Department of Molecular Interaction and Tissue Engineering, Institute for Frontier Medical Sciences, Kyoto University, Kyoto 606-8507; †Research Center, Mitsubishi Chemical Corporation, Kanagawa 227-8502; and ‡Department of Orthodontics, Osaka University Faculty of Dentistry, Suita, Osaka 565-0871

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We previously determined the complete primary sequence of a heparin-binding growth-promoting factor, chondromodulin-II (ChM-II), which stimulated the growth of chondrocytes and osteoblasts in culture. Bovine ChM-II was a 16-kDa basic protein with 133 amino acid residues and exhibited a significant sequence similarity to the repeats of the chicken *mim-1* gene product. Here we report the nucleotide sequences of bovine and mouse ChM-II cDNAs. The cDNAs each contained an open-reading frame corresponding to the ChM-II precursor with 151 amino acid residues. The N-terminus of the precursor included a secretory signal sequence of 18 amino acids prior to the mature ChM-II sequence. Unlike MIM-1, there was no repeat structure in the precursor protein, indicating that ChM-II was encoded as a gene product distinct from MIM-1. We then expressed recombinant bovine ChM-II protein which was purified to homogeneity. The recombinant protein stimulated the growth of rabbit growth plate chondrocytes, mouse MC3T3-E1 cells and rat UMR-106 osteoblastic cells *in vitro*.

Key words: bone, cartilage, chondromodulin-II, growth factor, recombinant protein.

The growth of chondrocytes is regulated by a variety of local growth and differentiation factors. Among them, fibroblast growth factors (FGF) are the most powerful mitogen for chondrocytes and stabilize the phenotypic expression of these cells (*1, 2*). FGF-2 markedly stimulated the anchorage-independent growth of chondrocytes in agarose culture *in vitro* (*3*). Transforming growth factor-β (TGF-β) and bone morphogenetic protein (BMP) stimulate the synthesis of cartilage-matrix in chondrocytes, but these have less effect on the proliferation of the cells than does FGF-2 (*4, 5*). However, these factors synergistically stimulated the proliferation of chondrocytes in the presence of FGF-2, while they maintained the synthetic activity of cartilage-matrix during the rapid growth of the cells (*4, 5*). The colony formation of chondrocytes was also synergistically stimulated by TGF-β or BMP in the presence of FGF-2 (*6*).

In addition to these well-characterized molecules, there are several other tissue-specific macromolecules in cartilage that act synergistically with FGF-2 to stimulate the growth of chondrocytes. In fetal bovine cartilage extracts, we found a 25-kDa cartilage-specific glycoprotein, chondromodulin-I (ChM-I), which stimulated the growth and colony formation of chondrocytes in culture (*6, 7*). Chondromodulin-II (ChM-II) was also isolated from the heparin-binding components in the extracts (*8*). Bovine ChM-II stimulated the growth of chondrocytes and osteoblasts *in vitro* (*9*). In contrast to ChM-I, which was found to be a growth inhibitor for vascular endothelial cells (*10, 11*), ChM-II exhibited no inhibitory action on the growth of cultured endothelial cells.

We determined the complete primary amino acid sequence of ChM-II (*8*). Bovine ChM-II is a 16-kDa basic protein with 133 amino acid residues which shares no sequence similarity to ChM-I (*8*). However, ChM-II showed a significant sequence similarity to the repeats 1 and 2 of the chicken *mim-1* gene product (*12*). Ness and coworkers demonstrated that chicken MIM-1 protein was stored in the granules of normal bone marrow promyelocytes as a 35-kDa form by Western blotting and immunocytochemistry using MIM-1 antibody (*12*). These observations suggested that ChM-II may be the bovine counterpart of chicken MIM-1. In the present study, we successfully isolated

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[2] To whom correspondence should be addressed. Tel/Fax: +81-75-751-3808, E-mail: hiraki@frontier.kyoto-u.ac.jp

Abbreviations: αMEM, alpha modified Eagle's medium; BMP, bone morphogenetic protein; ChM-I, chondromodulin-I; ChM-II, chondromodulin-II; FGF, fibroblast growth factors; IPTG, β-D-thiogalactopyranoside; MEM, Eagle's minimum essential medium; PBS, phosphate-buffered saline; PMS, phenazine methosulfate; RACE, rapid amplification of cDNA ends; TGF-β, transforming growth factor-β; XTT, 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)-carbonyl]-2*H*-tetrazolium hydroxide.

bovine and mouse ChM-II cDNAs and demonstrated that ChM-II was encoded in a gene distinct from MIM-1. In addition, the growth-stimulating activity of ChM-II was confirmed by the treatment of chondrocytes and osteoblastic cells with the recombinant protein.

MATERIALS AND METHODS

Cloning of Bovine and Mouse ChM-II cDNAs—A frozen fetal bovine whole embryo (4 cm in length) was homogenized in 6 M guanidine isothiocyanate. Total RNA was isolated by centrifugation of the homogenate over a cushion of 5.7 M CsCl (*13*). Poly(A)$^+$ RNA was prepared by affinity chromatography using oligo(dT)-cellulose, then reverse-transcribed at 37°C for 40 min into cDNA by AMV Reverse Transcriptase (Toyobo, Osaka) with annealed anchor primers. Nested degenerative primers were used in the sequential polymerase chain reactions (PCR). The degenerative sense primer corresponding to the amino acid sequence near the N-terminus of ChM-II (Pro-Trp-Ala-Ile-Ile-Cys-Ala) and the antisense oligonucleotide primer corresponding to the internal amino acid sequence (His-Ile-His-Ile-Glu-Asn-Cys-Asp-Leu) were synthesized (*8*). The PCR was then performed in a 100-μl reaction volume [10 mM Tris, pH 8.3; 50 mM KCl; 1.5 mM $MgCl_2$; 0.01% gelatin; 50 mM each of the four deoxynucleoside triphosphates; and 1 unit of Taq DNA polymerase (Takara, Shiga)] with fetal bovine embryo cDNA as a template. The amplification conditions were 30 cycles of 1 min at 94°C, 90 s at 48°C, and 3 min at 72°C after the initial denaturation for 10 min at 94°C, followed by a final 10 min incubation at 72°C. One microliter of reaction mixture was used as a template for the nested PCR with the degenerative sense primer corresponding to the internal amino acid sequence (Cys-Asp-Gly-His-Gly-Cys) and an antisense primer corresponding to the internal amino acid sequence (Lys-Val-Tyr-Pro-Gly-Ile-Gln) of ChM-II (*8*). A DNA fragment of 297 nucleotides in length was amplified and subcloned into the *Sma*I site of pUC18. The 3′-end of bovine ChM-II cDNA was determined by the 3′-nested rapid amplification of cDNA ends (RACE), which was performed using a Marathon cDNA Amplification Kit (Clontech, Palo Alto, CA). First-strand cDNA was synthesized from 2 μg of bovine whole embryo poly(A)$^+$ RNA according to the manufacturer's instructions. The gene-specific primers were designed on the basis of the nucleotide sequence of the above-mentioned ChM-II cDNA fragment. The nested PCR yielded an amplification product (approximately 200 nucleotides in length) which was then subcloned into pCRII vector (Invitrogen, San Diego, CA). The entire coding region of ChM-II cDNA was obtained by the 5′-RACE. The gene-specific primers (5′-AGATCGGCTTGTCCTCCAT-3′ and 5′-AGAAGATGGC-TGCCTTTTTAGA-3′) were designed on the basis of the nucleotide sequence of the above 3′-RACE product. The nested PCR yielded an amplification product (approximately 600 nucleotides in length) which was subcloned into pCRII. The nucleotide sequences were determined by use of an automated DNA sequencer 373A (Applied Biosystems, Foster City, CA). Universal and reverse primers of M13 and a set of consecutive inner primers were used for the sequencing from the plasmid vectors. The DNA sequence assembly, analysis, and translation were performed using the Genetix Mac 8.0 software package (Software Development, Tokyo).

For identification of mouse ChM-II cDNA, Marathon-Ready cDNA of day-17 mouse embryo was purchased from Clontech. Five microliters of the cDNA was used to amplify a partial cDNA clone. The PCR conditions were 94°C for 30 s, 50-60°C depending on the primers for 30 s, 72°C for 2 min for 35 cycles, and final extension at 72°C for 10 min. A primer set was designed on the basis of the nucleotide sequence of bovine ChM-II cDNA: primer 1, 5′-ATATGT-GCTGGCAAGTCTTCCAATG-3′; primer 2, 5′-ATAAGG-TTTCTCCTGGCCCA-3′. A 174-bp cDNA fragment was amplified (Fig. 1A). For 3′-RACE and 5′-RACE, Marathon-Ready cDNA of mouse liver (Clontech) was used as templates. The 3′-end of ChM-I cDNA was amplified using an anchor primer and the nested primer 3 (5′-GTATGCACC-ATTCACTGGGAAG-3′) downstream of primer 1. The 5′-end of the cDNA was amplified using an anchor primer and the nested primer 4 (5′-ATAGCTGTCACACGTCCGGA-TC-3′) upstream of primer 5 (5′-TGTACATGCGACTGG-ATGCCC-3′) (Fig. 1A). The amplified PCR products were subcloned into pCRII-TOPO vector (Invitrogen). Alternatively, a λgt11 cDNA mouse liver library (Clontech) was also screened by use of the 174-bp DNA fragment of mouse ChM-II cDNA generated by PCR. The hybridization-positive phage clones were isolated by the repeated plaque purification. Thirty-six positive clones were identified from 5×10^5 recombinant phage clones. The inserts isolated from the clones were subcloned into the *Eco*RI site of pBlue-Script SK(+) (Stratagene, La Jolla, CA). The nucleotide sequence of the inserts was determined using a Dye Terminator Cycle Sequencing Kit with an ABI 310 Genetic Analyzer (Applied Biosystems, Foster City, CA). Both cDNA strands were sequenced using standard sequencing primers and gene-specific primers. The DNA sequences obtained were compiled and analyzed by use of the Gene Works computer software program (IntelliGenetics, Tokyo). The nucleotide sequence of the inserts from the recombinant phage clones was completely matched with that determined by the above-mentioned PCR cloning from mouse embryo cDNA.

Northern Blot Hybridization—Total RNA was prepared from a variety of tissues of 4-week-old male DDY mice, including rib cartilage, and from isolated liver cells of male Wistar rats by a single-step method according to Chomczynski and Sacchi (*14*). Parenchymal hepatocytes were isolated from male Wistar rats weighing 180 g by *in situ* perfusion of the liver with collagenase and by subsequent centrifugation, essentially as described (*15, 16*). Residual non-parenchymal cell-enriched fraction was also prepared. Total RNA (20 μg) was denatured with 6% formaldehyde, fractionated on 1% agarose gel (SeaKem GTG, FMC Bioproducts, Rockland, ME), and transferred onto Nytran membranes (Schleicher & Schuell, Dassel, Germany) with a Turboblotter (Schleicher & Schuell) apparatus. Hybridization was performed overnight at 42°C with an appropriate probe (10^6 cpm/ml) in solutions containing 50% formamide, 6×SSPE (2×SSPE contains 0.3 M NaCl, 20 mM NaH_2PO_4, 2 mM EDTA at pH 7.4), 0.2% BSA, 0.2% Ficoll 400, 0.2% polyvinylpyrrolidone, 0.1% SDS, and 100 μg/ml denatured salmon sperm DNA. The 174-bp mouse ChM-II cDNA fragment amplified by PCR was labeled with [α-^{32}P]-dCTP with a BcaBEST Labeling kit (Takara) and used as a hybridization probe. The filters were washed three times

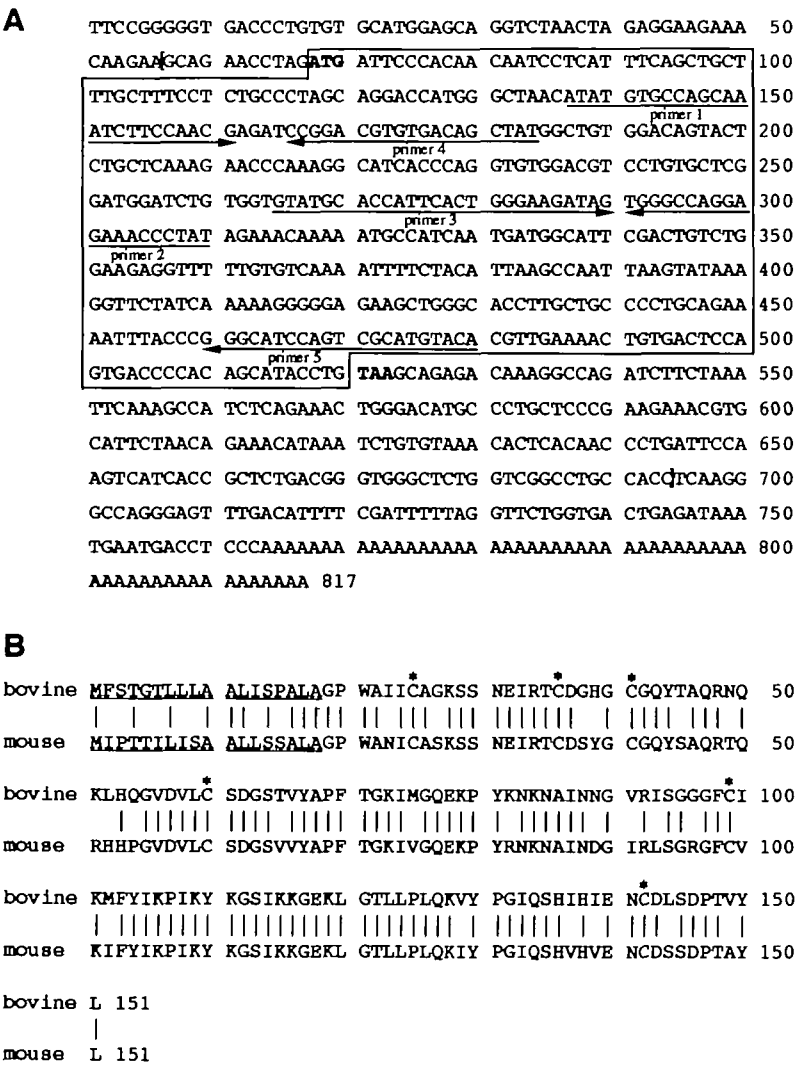

Fig. 1. **Nucleotide sequence of mouse ChM-II cDNA and its deduced amino acid sequence.** In (A), the nucleotide sequence of mouse ChM-II cDNA is shown. The coding sequence for mouse ChM-II precursor protein is boxed, and the initiation codon (ATG) and stop codon (TAA) are shown in bold letters. Arrows indicate the nucleotide sequences corresponding to oligonucleotide primers synthesized for the PCR-cloning. The primers 1 and 2 were designed on the basis of bovine ChM-II cDNA sequences in the initial amplification of the 174-bp mouse ChM-II cDNA fragment, as described in "MATERIALS AND METHODS." The nucleotide sequence of the inserts isolated from a λgt11 mouse liver cDNA library was completely matched with that determined by the PCR-cloning. In (B), the amino acid sequences of bovine and mouse ChM-II deduced from the corresponding cDNA sequences are compared. The signal peptide sequences are underlined. The asterisks indicate the positions of cysteine residues.

for 15 min at 55°C in 0.1×SSPE and 0.1% SDS, then exposed to Kodak X-OMAT film at −80°C with an intensifying screen Cronex lightening plus (DuPont).

Expression and Purification of Recombinant Met-bChM-II—Using the ChM-II cDNA clone obtained by 5′-RACE as a template, the coding region of ChM-II cDNA was modified by PCR to introduce a *Nco*I site and a *Bam*HI site at each end. The PCR product amplified by the following primer set was inserted into pT3Blue T-vector (Novagen, Madison, WI). The sense and antisense oligonucleotide primers were 5′-CCTGTATTCCATGGGCCCTTGGG-3′ and 5′-CCTGGATCCTATAGGTAGACAGTA-3′, respectively. The sense primer was designed so that it contained the initiation methionine codon. The *Nco*I-*Bam*HI fragment of the plasmid was inserted into pET15b (Novagen), which was introduced into *Escherichia coli* BL21(DE3) for the expression of recombinant Met-bChM-II protein.

For the preparative expression of recombinant Met-bChM-II protein, the transformed cells were inoculated in LB medium (1 liter) containing 50 μg/ml ampicillin. The expression of Met-bChM-II was induced by the addition of 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) for 3 h. Cells from a 1-liter fermentation were suspended in phosphate-buffered saline (PBS) and sonicated three times for 4 min each on ice. The cell lysate was centrifuged at 10,000×*g* for 10 min, and the precipitates were dissolved in PBS containing 6 M urea. The solution was applied to a DEAE Cellulofine (Seikagaku, Tokyo) column, and then Met-bChM-II was recovered in the pass-through fraction. This fraction was applied to a heparin Toyopearl (Tosoh, Tokyo) affinity column that had been equilibrated with PBS containing 0.03% 3-[(3-cholamidopropyl)dimethyl ammonio]-1-propanesulfonate. The Met-bChM-II was eluted with the same buffer containing 1.2 M NaCl. The recombinant Met-bChM-II was finally purified by reverse-phase high performance liquid chromatography (RP-HPLC) on a YMC C4 column that had been equilibrated with 25% acetonitrile in 0.08% trifluoroacetic acid and was developed with a linear gradient of 25–40% acetonitrile in 0.08% trifluoroacetic acid. The purified protein was analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) in 15% gel and visualized by staining with Coomassie Brilliant Blue.

Cell Culture and Bioassay—Chondrocytes were isolated from the growth plate cartilage of the ribs of young male New Zealand rabbits as previously described (*5*). The

isolated cells (10^4 cells/well) were inoculated onto 96-microwell plates and cultured in 0.1 ml of Eagle's minimum essential medium (MEM) containing 10% fetal bovine serum (FBS) at 37°C under 5% CO_2 in air. The culture medium was renewed every other day. When the culture became confluent, the cells were preincubated in MEM containing 0.3% FBS for 24 h. The medium was then replaced with MEM supplemented with 0.3% FBS and ChM-II in the presence or absence of 1 ng/ml recombinant human FGF-2 (R&D Systems, Minneapolis, MN). The cells were incubated for 22 h, then labeled with 13 μCi/ml [^{3}H]-thymidine (Amersham, Buckinghamshire, UK) for a final 4 h. Radioactivity incorporated into the DNA was measured in a scintillation spectrometer (*5*).

The cellular growth of MC3T3-E1 cells was assayed by the XTT-microculture tetrazolium assay with some modification (*17*). Mouse MC3T3-E1 cells (4×10^3 cells/well) were plated in 96-multiwell plates and grown to subconfluency in alpha modified Eagle's medium (αMEM) containing 10% FBS at 37°C in 5% CO_2 in air (*18*). The medium was replaced with 100 μl of αMEM containing 0.3% FBS, and the cells were incubated for 24 h. The cells were then rinsed once with αMEM containing 0.1% bovine serum albumin (BSA, Sigma, St. Louis, MO), then incubated in αMEM containing 0.1% BSA and test samples for another 48 h. The culture medium was then replaced with 100 μl of αMEM and 50 μl of XTT assay solution which contained 1 mg/ml 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2*H*-tetrazolium hydroxide (XTT, Sigma) and 25 μM phenazine methosulfate (PMS, Sigma) (*17*). The culture supernatant was recovered after incubation at 37°C for 24 h, and absorbance at 490 nm was determined. Rat UMR-106 osteosarcoma cells were maintained in DMEM containing 10% FBS, as described (*19*). The cells (6×10^3 cells/well) plated in 96-multiwell plates were grown to subconfluency and similarly used for an XTT assay as described above.

RESULTS AND DISCUSSION

Chondromodulin-II was first identified in the heparin-binding components extracted from fetal bovine epiphyseal cartilage as a growth-stimulating activity on cultured growth plate chondrocytes (*20*). We previously reported the complete amino acid sequence of bovine chondromodulin-II (bChM-II) protein (*8*). Using the degenerative oligonucleotide primers, PCR successfully amplified a bChM-II cDNA fragment of 297 base pairs corresponding to the internal amino acid sequence of bovine ChM-II from Cys^{18} to Gln^{116} from reverse-transcribed cDNAs isolated from fetal bovine whole embryo (*8*). The full-length bChM-II cDNA was isolated by the 3′- and 5′-RACE methods. The nucleotide sequence is available from the GenBank data base with the accession number D89011. The bChM-II cDNA (720 base pairs in length) contained an open reading frame that accommodated the ChM-II precursor protein with 151 amino acid residues. The deduced amino acid sequence of the protein contained the N-terminal hydrophobic signal sequence of 18 amino acids (Fig. 1B). Following the signal sequence, the amino acid sequence deduced from the cDNA completely matched that determined by the protein sequencing of ChM-II purified from bovine cartilage (*8*). Northern blot analysis indicated that ChM-II mRNA was detected as the transcripts of approximately 1 kb in size in poly(A)$^+$ RNA prepared from bovine whole embryos (data not shown). No ChM-II mRNA was detected in cartilage or in long bone.

During purification, we noted that ChM-II was a relatively abundant growth factor in cartilage, as suggested by the estimated ChM-II content in cartilage (approximately 0.2 μg/g tissue) (*8*). Thus, in the initial stage of the study, we assumed that ChM-II mRNA was expressed in cartilage. However, we failed to isolate ChM-II cDNA from the fetal bovine cartilage cDNA library that was previously used in the cloning of ChM-I cDNA (*7*). These observations indicate that cartilage cannot be a source of ChM-II. On the basis of the nucleotide sequence of bChM-II cDNA, we synthesized oligonucleotide primers and tried to amplify the mouse counterpart of ChM-II cDNA from Marathon-Ready cDNA prepared from day-17 mouse embryo (Clontech). A 174-bp cDNA fragment was successfully isolated. Since the preliminary Northern blot analysis using the 174-bp cDNA as a probe suggested that transcripts for the ChM-II gene were specifically expressed in liver, we determined the nucleotide sequence of the full-length mouse ChM-II (mChM-II) cDNA amplified by the 3′- and 5′-RACE methods from mouse liver Marathon-Ready cDNA (Fig. 1). The mChM-II cDNA also contained an open reading frame for the protein with 151 amino acid residues. As shown in Fig. 1B, the deduced amino acid sequences of bovine and mouse ChM-II precursor proteins contained the N-terminal hydrophobic signal sequence of 18 amino acids, indicating that ChM-II is potentially a secreted protein. Following the signal sequence, the amino acid sequence deduced from the bChM-II cDNA completely matched that determined by the sequencing of bovine ChM-II protein purified from bovine cartilage (*8*). The deduced amino acid sequence of the mature mChM-II domain was about 85% identical to that of bChM-II.

As mentioned above, we failed to isolate ChM-II cDNA from the fetal bovine cartilage cDNA library. By Northern blot analysis, no ChM-II mRNA was detected in mouse primary cultured chondrocytes or in mouse osteoblastic MC3T3-E1 cells (data not shown). However, as shown in Fig. 2A, ChM-II mRNA (1.0 kb in size) was readily detected in liver in a tissue-specific manner, but was not detected in cartilage. Similarly, we detected the expression of ChM-II transcripts in total RNA prepared from rat liver (Fig. 2B). Then, we isolated parenchymal hepatocytes by *in situ* perfusion from rat liver by the reported method (*15, 16*). Isolated parenchymal cells expressed a readily detectable level of ChM-II mRNA. Only a marginal level of mRNA was detected in the residual non-parenchymal cell-enriched fraction (Fig. 2B), indicating that parenchymal hepatocytes were the major expression source for ChM-II. These observations suggest that mature ChM-II protein processed in liver is secreted into blood. We previously isolated ChM-II protein from epiphyseal cartilage or costal cartilage (*8*). We then raised a monoclonal antibody against bovine ChM-II. Owing to its significant cross-reactivity with other proteins, this antibody could not be used for immunocytochemistry, but it was useful to identify ChM-II protein in the cartilage extracts by Western blotting. We examined the presence of ChM-II by Western blotting in the extracts from various tissues including lung, brain, heart, kidney, and pancreas as well as liver, but failed to

detect it in any of these extracts (J. Kondo, unpublished data). Our preliminary *in situ* hybridization study revealed that periosteum surrounding cartilaginous bone rudiments gave positive hybridization signals for ChM-II mRNA (Y. Hiraki, unpublished data), indicating that the periosteum is one of the sources of ChM-II, which was then translocated and accumulated in cartilage. We cannot, however, rule out the possibility that mature ChM-II protein accumulated in cartilage is supplied by liver through the blood stream, although cartilage is a well-known avascular tissue.

The amino acid sequence of bovine ChM-II exhibited a significant homology to repeat 1 and repeat 2 in the *mim-1* gene product (*8*). Comparison of ChM-II with MIM-1 by a Harr plot analysis clearly indicated that the 35-kDa MIM-1 protein was composed of two amino acid stretches from Arg^{31} to Leu^{165} and from Ala^{180} to Leu^{315} (Fig. 3). The *mim-1* gene is specifically expressed in promyelocytes in chicken, and its transcriptional activation is directly regulated by the Myb protooncogene product in association with other transcriptional activators (*12, 21, 22*). As previously reported (*12*), Western blotting and immunocytochemistry revealed that chicken MIM-1 protein is stored in the granules of normal bone marrow promyelocytes as a 35-kDa form. Moreover, MIM-1 protein is also stored as a 16-kDa form generated by a possible proteolytic cleavage of the protein. Therefore, there was a possibility that ChM-II is derived from the bovine counterpart of chicken *mim-1* gene. However, the nucleotide sequence analysis of bovine and mouse ChM-II cDNAs clearly indicated that ChM-II was encoded in a gene distinct from *mim-1*. The codon corresponding to the C-terminal leucine residue was directly followed by a stop codon (Fig. 1A), while the putative cleavage products of MIM-1 have an extension of 11 amino acids at their C-terminus (*12*).

We previously demonstrated that ChM-II purified from bovine cartilage stimulated the growth of chondrocytes and osteoblasts *in vitro* (*8, 9*). To confirm the bioactivities of ChM-II, we constructed a vector by addition of the ATG initiation codon prior to the coding region corresponding to the mature form of bovine ChM-II for the expression of the recombinant Met-bChM-II protein in *E. coli*. The construct was introduced into *E. coli* JM-109, and the expression of the recombinant protein was induced. The expressed recombinant Met-bChM-II was finally purified to homogeneity after the successive chromatographic procedures (Fig. 4). The identity of the recombinant protein was confirmed by N-terminal amino acid sequencing. The resultant Met-bChM-II was 16 kDa in size on SDS-PAGE and was indistinguishable from the naturally occurring bovine ChM-II derived from cartilage (Fig. 4).

Chondrocytes were isolated from the growth plate cartilage of rabbit ribs, and grown to confluency in the presence of 10% FBS (*8*). Quiescent monolayers of chondrocytes

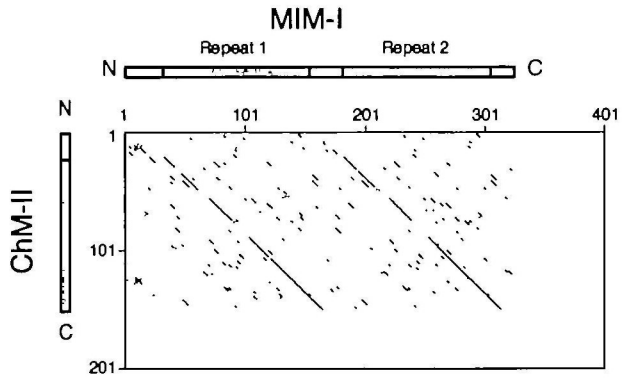

Fig. 3. **Harr plot analysis of the amino acid sequence similarity between chicken MIM-1 and bovine ChM-II.** Dot matrix analysis of the structure of bChM-II was made with a Harr plot program (*26*). The amino acid sequence of bChM-II was compared with that of chicken MIM-I, and dots were placed at positions of identity.

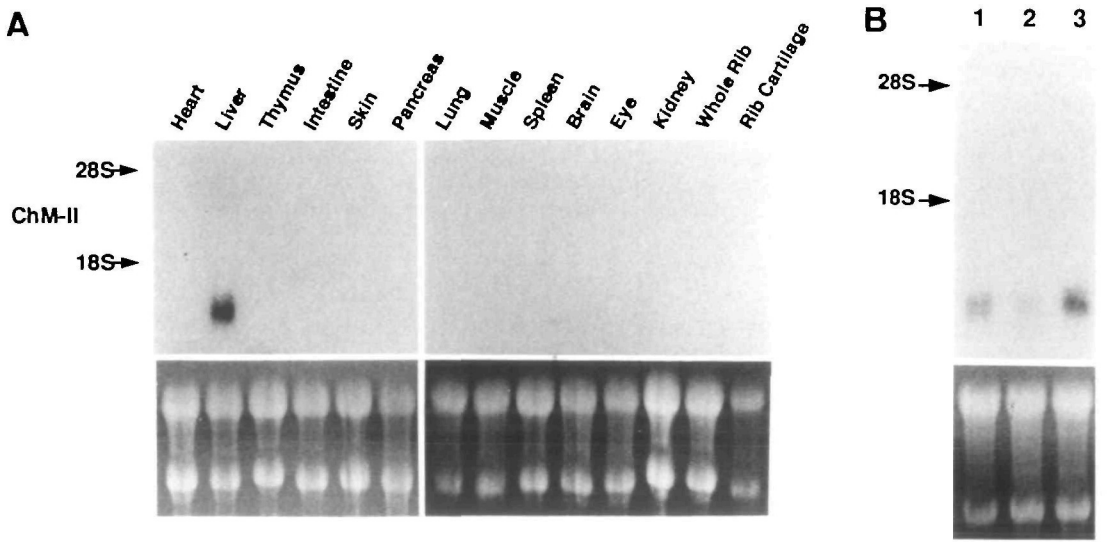

Fig. 2. **Northern blot analysis of ChM-II mRNA.** In (A), total RNA (20 μg) isolated from the indicated tissues of 4-week-old male DDY mice was hybridized with a mouse ChM-II cDNA probe. In (B), total RNA was isolated from male Wistar rat liver (lane 1), the non-parenchymal cell-enriched fraction isolated from rat liver (lane 2), and isolated rat parenchymal hepatocytes (lane 3). Each lane contained 20 μg of total RNA. The transferred filter was hybridized with a mouse ChM-II cDNA probe. The equivalent loading of each RNA was verified by ethidium bromide staining. The positions of 28S and 18S ribosomal RNAs are indicated.

were treated with Met-bChM-II. The recombinant protein clearly stimulated DNA synthesis in chondrocytes in a dose-dependent manner (Table I). The DNA synthesis of cells increased approximately twofold at the optimal dose of the protein. Met-bChM-II markedly stimulated DNA synthesis in the chondrocytes in the presence of FGF-2, similarly to the naturally occurring ChM-II (Table I). In accord with previous findings (*9*), Met-bChM-II also stimulated the growth of osteoblastic cells (Table II). The growth of rat and mouse osteoblastic cells was stimulated by Met-bChM-II in a dose-dependent manner, to an extent similar to that seen in cells stimulated by purified cartilage-derived ChM-II. When the dose-response curves were compared (Tables I and II), ChM-II induced a significant growth-response at a lower dose (10–30 ng/ml) in osteoblasts than in chondrocytes. In agreement with the previous observations (*9*), recombinant Met-bChM-II did not stimulate the growth of fibroblasts. These results may indicate that ChM-II plays a more important role in the growth regulation of bone than that of cartilage.

In parallel with our study on the purification and structural determination of bChM-II (*8*), Yamagoe and coworkers reported purification of a novel human neutrophil chemotactic factor LECT2 from the culture medium of the phytohemagglutinin (PHA)-activated human T-cell leukemia SKW-3 cells (*23*). They reported the partial sequences of 98 amino acid residues in LECT2, which included the N-terminal 54 amino acid stretch and exhibited a significant sequence homology with chicken MIM-1 (*23*). Interestingly, all the reported partial sequences of human LECT2 were identical to the portions in our full primary sequence of bovine ChM-II (available from PIR data base under the accession number JH0270) (*8*). Recently, they reported the nucleotide sequences of bovine and human LECT2 cDNAs (*24*). Their results clearly indicated that the original LECT2 protein was derived not from human T-cell leukemia cells but from fetal bovine serum supplemented in the culture medium. Although ChM-II and LECT2 were purified on the basis of different biological activities, they are identical with each other (*8, 24*). Mouse ChM-II/LECT2 shared a 87% sequence identity with the human counterpart (*25*).

Unlike the human gene utilizing two polyadenylation signals (*24*), a single 1.0-kb ChM-II/LECT2 mRNA was expressed in bovine and mouse liver in a tissue-specific manner. Contrary to their earlier expectation, Yamagoe and coworkers found that PHA-activated SKW-3 cells expressed only a few copies of ChM-II/LECT2 transcripts per cell. No ChM-II/LECT2 expression was detected in the leukemia cells activated with lipopolysaccharide or phorbol myristate acetate (*24*), indicating that the gene expression occurs independently of T-cell activation. Although liver contains hematopoietic cells such as T-cells, Kupffer cells, and NK cells, our data strongly suggested that parenchymal hepatocytes are a major source of ChM-II/LECT2. The production of recombinant ChM-II protein will facilitate examination of whether ChM-II participates in the regulation of bone metabolism *in vivo*. It is also interesting to study the biological functions of ChM-II/LECT2 protein

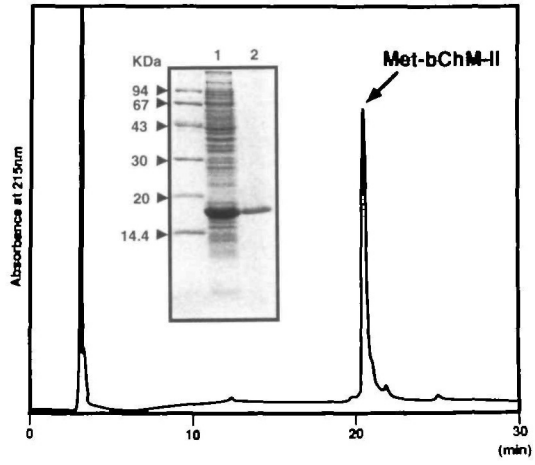

Fig. 4. **Elution profile of purified recombinant Met-bChM-II.** The purified recombinant bChM-II protein was eluted by RP-HPLC from a YMC C4 column that had been equilibrated with 25% acetonitrile in 0.08% trifluoroacetic acid and developed with a linear gradient 25–40% of acetonitrile in 0.08% trifluoroacetic acid. Inset shows the results of the SDS-PAGE analysis of the crude cell lysate after IPTG induction (lane 1) and purified Met-bChM-II (lane 2) on a 15% polyacrylamide gel.

TABLE I. **Effect of recombinant Met-bChM-II on DNA synthesis of rabbit growth plate chondrocytes in the presence and absence of FGF-2.**

Addition	[^{3}H]Thymidine incorporation	
	(dpm/well)	(% of control)
None	912±257	100± 28
FGF-2, 1 ng/ml	3,731±602	409± 66
Met-bChM-II, 30 ng/ml	1,046±148	115± 16
60 ng/ml	1,208±258	132± 28
300 ng/ml	1,455±228	160± 25
Met-bChM-II, 1 μg/ml	1,548±280	170± 31
Met-bChM-II + FGF-2	5,904±986	647±108
Cartilage-derived bChM-II, 1 μg/ml	1,649±361	181± 40
bChM-II + FGF-2	5,863±715	643± 78

Confluent rabbit growth plate chondrocytes were preincubated in DMEM containing 0.3% FBS for 24 h. The medium was then replaced with DMEM supplemented with 0.3% FBS and test samples. Cells were labeled with [^{3}H]thymidine from 22 to 26 h after the addition of the samples. Values are means±SD for triplicate samples.

TABLE II. **Effects of recombinant Met-bChM-II on the growth of mouse MC3T3-E1 and rat UMR106 osteoblastic cells.**

Addition	Absorbance at 490 nm (% of control)	
	MC3T3-E1 cells	UMR-106 cells
None	100± 4	100± 6
Met-bChM-II		
3 ng/ml	168±18	186±22
10 ng/ml	179±34	210±19
30 ng/ml	203±22	226±21
100 ng/ml	212±21	243± 1
Cartilage-derived bChM-II		
30 ng/ml	198±18	215± 1

Mouse MC3T3-E1 cells and rat UMR-106 cells were grown to subconfluency in αMEM and DMEM containing 10% FBS, respectively. When cells reached subconfluency, the medium was changed to culture medium containing 0.3% FBS. After 24 h, the wells were washed with medium containing 0.1% FBS. Cartilage-derived bChM-II or recombinant Met-bChM-II was then added to the cell culture. After 48 h, XTT assay solution was added to each well. After incubation for 24 h, the supernatant was recovered and absorbance at 490 nm was measured. Values are means±SD for quadruplicate samples.

during bone marrow hematopoiesis, even though its chemotactic activity was several times lower than typical chemotactic factors such as IL-8 (*24*). Studies along this line are now underway.

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