We cloned a novel matrix metalloproteinase (MMP) called CMMP from cultured primary chicken embryo fibroblasts. The cDNA-derived CMMP sequence contains 472 amino acids including a putative 19-residue signal peptide and a unique cysteine in the catalytic domain, an insertion in a sequence motif that binds the structural (noncatalytic) zinc of MMPs. Strikingly, a homologously inserted cysteine is also found in Xenopus XMMP and human MMP19, two recently cloned novel members of the MMP family. Phylogenetic analysis suggest that XMMP and MMP19 represent founding members of the MMP family, whereas CMMP is related to collagenase MMPs. Bacterially produced recombinant CMMP (without the amino-terminal inhibition domain), which was autoprotoelyzed at the carboxyl-terminal domain, digested casein and gelatin. As shown by Northern blotting, CMMP mRNA of 1.8 kilobase pairs was constitutively expressed in cultured primary chicken embryo fibroblasts and up-regulated by tumor necrosis factor-α and the phorbol ester 12-O-tetradecanoylphorbol-13-acetate, but it was not regulated by interleukin-1, basic fibroblast growth factor, or retinoic acid. CMMP mRNA of 1.8 kb was also detected in the head and body of 8-day-old chicken embryos and dramatically up-regulated in 9-day-old embryos.

Degradation and remodeling of the extracellular matrix (ECM) is thought to play important morphogenic roles during growth and development. ECM degradation is largely controlled by a superfamily of zinc-dependent endopeptidases called matrix metalloproteinases (MMPs). At present, 18 different MMPs have been cloned and characterized (1–8), including a novel MMP that we cloned from the frog *Xenopus laevis* embryos (9). MMPs share similar domains with distinct structure and function, have wide and often overlapping substrate specificities, and are usually grouped as collagenases, gelatinases, stromelysins, and membrane-type MMPs.

Collagenases are the only enzymes that can cleave the triple helical regions of interstitial fibrillar collagens such as collagen types I, II, and III, the most abundant proteins in the body. The cleavage occurs at one specific peptide bond, Gly775–Ile776 (Leu776 in the α2(I)-chain) in type I collagen and leads to unfolding and denaturation of the fibrillar collagens. Gelatinases A and B, also known as 72- and 92-kDa type IV collagenases, digest denatured collagen (gelatin) and components of basement membranes, a special type of ECM of epithelial, endothelial, fat, muscle, and peripheral nerve cells (10). Stromelysins are three different MMPs with wide substrate specificity, and stromelysin-1 can degrade almost any ECM component including cartilage proteoglycans (11). Recently, four new members of the MMP family called membrane-type MMPs (MT-MMPs) were cloned (12–17). They are not secreted extracellularly but remain on the cell surface through their carboxyl-terminal transmembrane domain. Recently, however, Matsumoto *et al.* (18) characterized a secreted form of MT3-MMP encoded by an alternatively spliced mRNA. MT-MMPs degrade ECM components, proteolytically cleave and activate 72-kDa type IV collagenase, and therefore may regulate pericellular matrix degradation at the cell surface (19–21).

MMPs are produced as inactive precursors, which are activated by proteolytic removal of their amino-terminal domain, a 78–178-residue-long propeptide (22–24). Thus, it is striking that stromelysin-1 (MMP3) proteolytically activates at least five other members of the MMP family including interstitial collagenase-1 (MMP1), matrilysin (MMP7), neutrophil collagenase-2 (MMP8), 92-kDa type IV collagenase (MMP9), and collagenase-3 (MMP13), suggesting that stromelysin-1 plays a special “upstream” role in ECM degradation and remodeling (25–29). Recently, Okamoto *et al.* (30) reported that various bacterial proteinases can also activate MMP1, MMP2, and MMP9.

MMPs are expressed widely during embryogenesis, often in a highly tissue- and cell-specific pattern, suggesting distinct roles for MMPs in growth and development (17, 31–37). In adult life, MMPs are rarely expressed except in rapidly remodeling tissues such as the term placenta, menstrual endometrium, and involuting mammary glands and during wound healing and inflammation (38–42). MMPs are also thought to play a critical role in tumor growth and metastasis (43, 44) and in the progression of other diseases such as arthritis, atherosclerosis, and aneurysm (3, 45–47).

To study the role of ECM degradation and remodeling in early vertebrate development, we are cloning MMPs and tissue inhibitors of metalloproteinases from chicken embryos and cultured cells (17). Here we describe the cloning and characteriza-
tion of a novel member of the MMP family, called CMMP, from cultured primary chicken embryo fibroblasts. A cDNA-derived CMMP sequence contains 472 amino acids including a putative 19-residue signal peptide and a unique cysteine in the catalytic domain. A homologously placed cysteine is found only in Xeno-
pus XMMP and human MMP19, two recently cloned novel MMPs (5, 8, 9).

MATERIALS AND METHODS

Cells—Primary chicken embryo fibroblasts were isolated from skin dissected from the heads of day 7 embryos (17) and cultured for 5 days until confluent and then treated for 16 h with TNF (5 units/ml), TPA (100 ng/ml), IL-1 (5 units/ml), or 5×10⁻⁷ M retinoic acid (Fig. 6) before the isolation of RNA as described below.

RT-PCR with Universal MMP Primers—We synthesized two degenerate inosine-containing oligodeoxynucleotides called universal MMP primers (17). The forward primer (Y35, 20-mer 16-degenerate with five inosines), and the reverse primer (Y36, 23-mer 24-degenerate with five inosines), were expected to amplify cDNAs from all mRNAs for MMPs except that of matrilysin (MMP7) in which the second conserved sequence is altered (underlined) YAATHELGH (48). Total RNA was isolated from cultured primary chicken embryo fibroblasts treated with TPA (100 ng/ml) for 16 h by a modified acid guanidinium thiocyanate phenol-chloroform method (49, 50), and 1 mg was used for cDNA synthesis by reverse transcription as described (17) followed by PCR using primers Y35 and Y36 and 1 unit of Taq DNA polymerase (Perkin-Elmer). The PCR products were analyzed by 1% agarose gel electrophoresis in 0.5× Tris borate-EDTA buffer and visualized by EtBr-staining and UV light.

cDNA Cloning and Sequence Analysis—The PCR products were cloned into the SfiI site of pCR-Script Direct SK+ vector (Stratagene) and sequenced using the dideoxynucleotide method (Sequenase kit, version 2.0, DNA sequencing kit, U.S. Biochemical Corp.). Later, cycle sequencing was carried out using Taq DyeDeoxy (Applied Biosystems), and the samples were run on a model 373A DNA Sequencer (Applied Biosystems). Analysis of several clones with a 395-bp cDNA

![Chicken CMMP](image)

**Fig. 1. Chicken CMMP.** In the cDNA-derived amino acid sequence of 472 residues of chicken CMMP, the first bar indicates a putative signal peptide cleavage site, and the second bar shows propeptide cleavage site. The boxed residues are two conserved sequences that were used to design two degenerate universal primers Y35 and Y36 for MMP cloning and a conserved MMP sequence with a catalytically active aspartyl residue (circle). Potential sites for N-linked sugars, Asn116 and Asn123, are boxed. The broken line is a 19-amino acid-long linker peptide. The large box shows four tandem repeats of a vitronectin-like sequence. A unique valine and two “signature” Cys residues are circled. The underlined nucleotide sequence is a polyadenylation signal. At bottom, a representation of the chicken CMMP primary structure shows the signal peptide (SP), inhibition domain (ID), catalytic domain (CD), linker peptide (LP), and vitronectin-like domain (VD). Numbers refer to the first residue of each domain.

-19 1 86 248 267 453

| SP | PP | CD | LP | VD |

amino acid permutations in the two most conserved MMP sequences PRGCVPNVD and VAATVEH/FILGH, respectively. The two nucleotides in lowercase letters were added for cloning purposes (see below).

In reverse transcription-coupled PCR, these primers are expected to amplify cDNAs from all mRNAs for MMPs except that of matrilysin (MMP7) in which the second conserved sequence is altered (underlined) YAATHELGH (48). Total RNA was isolated from cultured primary chicken embryo fibroblasts treated with TPA (100 ng/ml) for 16 h by a modified acid guanidinium thiocyanate phenol-chloroform method (49, 50), and 1 µg was used for cDNA synthesis by reverse transcription as described (17) followed by PCR using primers Y35 and Y36 and 1 unit of Taq DNA polymerase (Perkin-Elmer). The PCR products were analyzed by 1% agarose gel electrophoresis in 0.5× Tris borate-EDTA buffer and visualized by EtBr-staining and UV light.

cDNA Cloning and Sequence Analysis—The PCR products were cloned into the SfiI site of pCR-Script Direct SK+ vector (Stratagene) and sequenced using the dideoxynucleotide method (Sequenase kit, version 2.0, DNA sequencing kit, U.S. Biochemical Corp.). Later, cycle sequencing was carried out using Taq DyeDeoxy (Applied Biosystems), and the samples were run on a model 373A DNA Sequencer (Applied Biosystems). Analysis of several clones with a 395-bp cDNA
insert confirmed its MMP origin yet revealed significant sequence differences including a unique cysteine (Cys\textsuperscript{174}) in the catalytic domain not found in other MMPs. Subsequently, we found a homologously placed cysteine in XMMP, a novel MMP that we recently cloned from the frog \textit{X. laevis} embryos (9), and in human MMP19 also called MMP18 (Refs. 5 and 8; see Fig. 3).

To determine a full-length nucleotide and amino acid sequence for this novel MMP, called CMMP, we used the following rapid amplification of 5'- and 3'-cDNA ends strategy. For the 5'-end of the mRNA, we used the degenerate primer Y36 and Superscript II RNase H-reverse transcriptase (Life Technologies, Inc.) and constructed a cDNA library by the anchor ligation method as described by Apte and Siebert (51). The transcriptase (Life Technologies, Inc.) and constructed a cDNA library using the 3'- and 5'-end cDNA sublibrary. The specific PCR products were amplified and sequenced to obtain the 5'-end sequence of CMMP. For the 3'-end of the mRNA, the “lock-docking” NN oligo(dt) primer was used as described in the CLONTECH 3'AmpliFinder rapid amplification of 5'- and 3'-cDNA ends kit. The first PCR was carried out with the primers from the kit and the degenerate Y35 primer to construct a 3'-end cDNA sublibrary. The specific PCR products were amplified using the 3' anchor primer and a nested specific primer derived from the chicken CMMP sequence. We have confirmed the CMMP sequence (Fig. 1) by independent cloning and sequencing of a full-length clone generated by PCR and specific primers for CMMP. Sequences were analyzed using the MacVector program (IBI/Eastman Kodak Co.). A putative signal peptide (52) was predicted using an on-line server.\textsuperscript{2} We note that the 5'-untranslated nucleotide sequence of CMMP (Fig. 1) is an open reading frame with two methionines. However, the codon context of these methionines do not fit well with the Kozak sequence [\textsuperscript{61}], and the following downstream residues would form a hydrophilic peptide, suggesting that these methionines are not used for the initiation of translation.

To search for CMMP-related sequences in the public data base, both blastn and blastx searches were performed against the nonredundant dbEST data bases in the National Center for Biotechnology Information (54). Also, in a BLAST search, the chicken CMMP amino acid sequence was compared with 215,495 protein sequences.

**Phylogenetic Tree Analysis**—Unweighted dendrograms were produced using the program MegAlign, version 3.03 for the PowerMacintosh (DNA Star, Madison, WI), using a gap penalty of 10, a gap penalty length of 10, and the CLUSTAL alignment algorithm. In Fig. 4, we used full-length MMP sequences without signal peptides that were predicted using the on-line server.\textsuperscript{2} A similar dendrogram was also constructed from MMP sequences of the catalytic domain.

**Bacterial Expression**—To produce in bacteria \textit{E. coli} an active recombinant CMMP without the signal peptide (residues 1–19) and amino-terminal propeptide (residues 20–104), we used PCR and a forward primer 5'-CGCATTATGATGTGTTACTCTCAGTAAC-3' containing an NdeI site (underlined) and a reverse primer 5'-GGAATTCATTAAACGAGTTACGTTACGTTAC-3' containing an EcoRI site (Fig. 2). The PCR product was cut with NdeI and EcoRI and cloned into pET28a bacterial expression vector (Novagen). This vector provides the amino-terminal His tag (six histidines) for CMMP. Similarly, to produce an active recombinant human collagenase-1 (MMP1) without the signal and propeptide sequence, we used PCR and a forward primer 5'-CGCATTATGATGTGTTACTCTCAGTAAC-3 containing an NdeI site and a reverse primer 5'-GGAATTCATTAAACGAGTTACGTTACGTTAC-3' containing an EcoRI site and a reverse primer 5'-CATGACGCGCGCCCAAGTTTTTTTCTGCGTGAGGAA-3' containing an \textit{NcoI} site and cloned the PCR product into pET28a bacterial expression vector. These CMMP and MMP1 constructs were confirmed by nucleotide sequencing and sequenced into the BL21(DE3)pLysS \textit{E. coli} bacterial host (Novagen). Expression of the constructs was induced by isopropyl-\textit{D}-galactopyranoside, and the His-tagged CMMP and MMP1 were isolated from inclusion bodies (55), purified using nickel affinity chromatography as described in the manufacturer’s protocol, and characterized by SDS-polyacrylamide gel electrophoresis and Coomassie Blue staining. In contrast to MMP1, which remained a full-length protein, CMMP appeared in SDS-gel electrophoresis as a major band of 29 kDa, and three minor bands of smaller size. This is significantly smaller than the calculated molecular weight of 42,190 (374 residues including six histidines), indicating autoproteolysis and carboxyl-terminal truncation of the recombinant CMMP. This autoproteolysis occurs during the refolding step in the isolation protocol (55), since in the inclusion bodies CMMP was a full-length 39-kDa polypeptide.

**Zymography**—Casein and gelatin zymography was performed as described (20). In substrate digestion analysis, 10 ng of bacterially produced recombinant CMMP or MMP1 was incubated with 15 \microg of casein or gelatin for 1 h at 37 °C or with 15 \microg of rat tail acid-soluble fibrillar type I collagen (Sigma) for 1 h at room temperature. The digestion...
products were analyzed by SDS-gel electrophoresis and staining with Coomassie Blue. In control reactions, EDTA (20 mM) was used to inhibit MMP activity.

**Northern Hybridization.—**Total RNA (30 μg) was isolated from cultured chicken embryo fibroblasts by the acid-phenol method (49, 50) and run on a 1% agarose-formaldehyde gel in 0.5 MOPS buffer. For the experiments, the cell cultures were treated with TPA (100 ng/ml), FGF (10 ng/ml), IL-1 (5 units/ml), and TNF (5 units/ml), all-trans-retinoic acid (5 μM), or TNF (5 units/ml) for 16 h before RNA extraction. To isolate total RNA from chicken embryos, the head and body were dissected from 8- and 9-day-old embryos, frozen in liquid nitrogen, and pulverized to fine powder using a pestle and mortar. After lysis in 4.5 M guanidinium isothiocyanate solution, the subsequent steps were as described (49, 50). The RNA from the agarose gel was blotted in 10 M NaCl, 0.15 M sodium citrate) to Nytran membrane. A 0.4-kb cDNA of CMMP was 32P-labeled by PCR (56) and hybridized overnight at 65 °C in 5× SSC, 5× Denhardt’s solution, 0.1 mg/ml denatured salmon sperm DNA, 0.1% SDS. For control experiments, CMMP probe was first removed in boiling distilled water containing 0.1% SDS, and the filters were then hybridized with a 967-bp chicken glyceraldehyde-3-phosphate dehydrogenase probe that was prepared from total RNA and 32P-labeled by PCR using primers 9'-ATGTTGAAGAATCGGAGTG-CAACGGA-3' (forward) and 5'-TCACCTCTTGGATCGATGACGAC-3' (reverse). The membrane was washed at 65 °C in 0.1× SSC, 0.1% SDS and exposed overnight to Kodak x-ray film.

**RESULTS AND DISCUSSION**

**Cloning and Sequence Analysis of CMMP.—**To isolate cDNA clones for chicken MMPs, we first synthesized two degenerate inosine-containing MMP primers designed from the two most conserved MMP sequences (17). The first sequence PRGCG/N/VIPD, containing an unpaired cysteine necessary for the lability of MMPs (57), is at the end of the amino-terminal propeptide that is proteolytically removed from the active MMPs. The second sequence VAA/V/HEF/ILGH is in the catalytic domain and has two of the three histidines that coordinate the catalytic zinc in MMPs. Using total RNA from cultured and TPA-treated primary chicken embryo fibroblasts, cDNA was synthesized with reverse transcriptase and random hexamer primers followed by PCR amplification using the degenerate MMP primers. As described previously (17), we obtained at least seven distinct PCR products between 350 and 900 bp. Cloning and sequencing of one of these PCR products of 395 bp confirmed that it was derived from an MMP but also revealed significant sequence differences from previously characterized MMPs (see below).

A reverse transcription-coupled PCR strategy of cDNA cloning (see “Materials and Methods”) allowed us to determine the 1817-nucleotide and complete 472-amino acid sequence of this novel MMP, which is called CMMP (C for its unique cysteine, see below). The cDNA-deduced CMMP sequence contains a potential 19-residue signal peptide with a characteristic hydrophobic profile but no other significant hydrophobic segments, suggesting that CMMP is a secretory protein. CMMP has an amino-terminal propeptide domain of 85 amino acids with the conserved PRCGVPD motif (Fig. 1); deletion of this domain generates a proteolytically active CMMP (Fig. 5). The chicken CMMP sequence has two potential sites for N-linked sugars, Asn116 and Asn128, both in the catalytic domain, and five cysteines. Cys97, Cys286, and Cys472 (CMMP numbering) are conserved in all MMPs, whereas Cys174 and Cys388 characterize CMMP (see below). Excluding the signal peptide and any posttranslational modifications, CMMP has a calculated molecular weight of 51,047.

The catalytic domain of CMMP contains a number of unique amino acids (Arg144, Ala153, His173, Cys174, Arg176, and Phe214) not found in other MMPs and a unique Val224 residue (underlined) in the conserved catalytic zinc-binding motif VAA/V/HEF/ILGH. Except for human MMP19, which also has valine (5, 8), other MMPs have other hydrophobic residues (phenylalanine, isoleucine, or leucine) in this position. Of note is that we used the catalytic zinc-binding motif of the consensus sequence VA(A/V)HE(F/I/L)GH to design the degenerate MMP primer Y36 (see “Materials and Methods”). However, the Y36 primer sequences do not cover codons for the unique valine found in CMMP, indicating that our experimental conditions tolerated a single base pair mismatch (in this case C versus G) for MMP cloning by PCR.

There is a conserved MMP sequence DDJSIQHG at the end of the catalytic domain of CMMP (Fig. 1, boxed). As shown by Hirose et al. (58), mutation of the aspartic acid (underlined) to glycine in neutrophil collagenase-2 (MMP8) completely abol-
ished its proteolytic activity on casein and native fibrillar type I collagen. This aspartyl residue presumably has a conformational role; as shown by crystal structure studies (59), it forms hydrogen bonds with the so-called Met turn residues Leu$^{214}$ and Met$^{215}$ (59). Met turn is a characteristic motif ALMY (with variations but an absolutely conserved methionine) found in all metzincin family of metalloproteinases including MMPs (60); in CMMP, it is 238ALMF (Fig. 1).

In MMPs, the catalytic domain is linked to the carboxyl-terminal domain via a linker peptide, a 17–72-residue-long proline-rich sequence and most pronounced in 92-kDa type IV collagenase (9, 61). The only exceptions are Xenopus XMMP, which has no linker peptide (9), and matrilysin (MMP7) lacks the linker peptide and carboxyl-terminal domain altogether (48). In CMMP, the linker peptide is 19 residues long with four prolines (broken line in Fig. 1). A crystal structure study of porcine collagenase-1 (MMP1) has shown that the linker peptide, in this case a 17-residue sequence GSPENPVQPSGPQT-PQV, is a highly exposed peptide without any regular structure (61). The linker peptide appears to be the most variable sequence domain of MMPs and plays a role in MMP activity and substrate specificity (see Ref. 61 and references therein).

Similar to other MMPs (62), the carboxyl-terminal domain of CMMP is composed of four tandem repeats of a sequence related to vitronectin, a major cell adhesion protein in plasma and tissues (63). In CMMP, the four repeats are 41–49 amino acids in length, are 36–45% identical, and show 20–38% amino acid identity to a rat vitronectin sequence between residues 204 and 251, suggesting evolution from a common ancestral origin (Fig. 2A). The crystal structure details of porcine collagenase-1 (MMP1) revealed that the four vitronectin-like repeats are folded as four perpendicular sheets forming a so-called four-bladed β-propeller. In each repeat, about half of the amino acids are hydrogen-bonded in four-anti-parallel β-strand configuration (Fig. 2B; for details, see Ref. 61). In CMMP, the vitronectin-like repeats and β-strand residues are 50% identical to those of porcine MMP1 (Fig. 2B); therefore, we predict a four-bladed β-propeller structure for the carboxyl-terminal domain of CMMP as well.

**A Unique Cysteine in the Catalytic Domain of CMMP**—There is a unique cysteine in the catalytic domain of CMMP (Fig. 1, circled). This is interesting because previously no other MMPs were found to have cysteine in the catalytic domain. MMP2 and MMP9, however, contain a fibronectin-like domain of 176 residues with 12 cysteines, inserted into the catalytic domain in front of the active site zinc-binding motif. Strikingly, a homologously placed cysteine is also found in Xenopus XMMP and human MMP19, two recently cloned novel members of the MMP family (Fig. 3). XMMP has 604 amino acids including a putative signal peptide of 22 residues, and interestingly, the XMMP gene is transiently expressed during early development. It is first activated in gastrula and neurula embryos (49). The crystal structure of porcine collagenase-1 (MMP1) reveals that the four vitronectin-like repeats are folded as four perpendicular sheets forming a so-called four-bladed β-propeller. In each repeat, about half of the amino acids are hydrogen-bonded in four-anti-parallel β-strand configuration (Fig. 2B; for details, see Ref. 61). In CMMP, the vitronectin-like repeats and β-strand residues are 50% identical to those of porcine MMP1 (Fig. 2B); therefore, we predict a four-bladed β-propeller structure for the carboxyl-terminal domain of CMMP as well.

**Cloning of a Novel MMP from Chicken**—There is a unique cysteine in the catalytic domain of CMMP (Fig. 1). This is interesting because previously no other MMPs were found to have cysteine in the catalytic domain. MMP2 and MMP9, however, contain a fibronectin-like domain of 176 residues with 12 cysteines, inserted into the catalytic domain in front of the active site zinc-binding motif. Strikingly, a homologously placed cysteine is also found in Xenopus XMMP and human MMP19, two recently cloned novel members of the MMP family (Fig. 3). XMMP has 604 amino acids including a putative signal peptide of 22 residues, and interestingly, the XMMP gene is transiently expressed during early Xenopus development. It is first activated in gastrula and neurula embryos, and then down-regulated in pre-tail bud embryos (9). Human MMP19 has 508 amino acids and is mainly expressed in placenta, lung, pancreas, ovaries, spleen, and intestine (8). Of note is that Cossins et al. (5) also cloned a novel human MMP (identical to MMP19) and called it MMP18. This is confusing, since Strovil et al. (6) had previously cloned Xenopus collagenase-4, which they named MMP18. So far, we have no evidence for human or mouse homologs of CMMP (see below) and therefore do not suggest any number for CMMP. Indeed, we suggest that the future numbering of novel MMPs should follow the discovery and characterization of human MMPs.

Of note is that the homologously placed cysteine in the catalytic domain of CMMP, XMMP and MMP19 is an apparent insertion in a conserved sequence that constitutes the structural (noncatalytic) zinc-binding motif of MMPs, about 20 residues upstream of the second (catalytic) zinc-binding motif (Fig. 3). This conserved sequence also contains six residues that coordinate a structural calcium of MMPs (Fig. 3).

At present, the biological significance, if any, of the unique cysteine in the catalytic domain of CMMP, XMMP, and MMP19 is not known. Its sequence context in these MMPs is unrelated to the consensus sequence of the active site of cysteine proteinases (64), indicating that it has no catalytic function. However, it is possible that it forms intermolecular disulfide bonds. This suggestion is based on our zymogram studies, which consistently have shown monomeric and higher molecular weight forms of CMMP (Fig. 5) as well as XMMP (not shown).

CMMP has another unique cysteine, Cys$^{308}$, in the third vitronectin-like motif (Fig. 1, circled). Only a few other MMPs have cysteines in the vitronectin-like motifs that constitute the carboxyl-terminal domain important in the substrate specificity of MMPs. A sea urchin hatchling enzyme (or envelysin), an MMP with collagenolytic activity, has two cysteines in the second vitronectin-like motif (65). Bovine and porcine collagenase-1 (MMP1) have one cysteine in the third vitronectin-like motif (see Fig. 2B), and 92-kDa type IV collagenase (MMP9) has one cysteine in the fourth vitronectin-like motif (62). So far, no role has been described for these cysteines. The two other cysteines of CMMP, Cys$^{296}$ at the beginning and Cys$^{472}$ at the end of the carboxyl-terminal domain (Fig. 1), are conserved in all MMPs and linked together to form an intramolecular disulfide bond.

**Phylogenetic Tree of MMPs**—The chicken CMMP sequence appears significantly diverged from other MMPs but is most related to the human collagenases (MMP1, MMP8) with an overall amino acid identity of 52%. This suggests that CMMP is not a homolog of previously characterized MMPs but represents a novel member of the MMP family. In comparison, chicken 72-kDa type IV collagenase (MMP2) and MT3-MMP are 84 and 89% identical with their human homologs, respectively (17, 66).

Interestingly, in a phylogenetic tree of MMPs (Fig. 4), XMMP lies at the very bottom, providing an outlier from which the branching and MMP evolution begins. MMP19 appears along...
one of the earliest branching points together with stromelysin-3 (MMP11) and MT4-MMP, whereas CMMP together with MMP1 and MMP8 form the top of the tree. Similar evolutionary relationships were also evident by constructing the phylogenetic tree from the catalytic domains of MMPs (not shown).

With respect to the conserved unique cysteine, this scenario implies that it was present in an ancestral MMP, which gave rise to XMMP, and then was lost in further MMP evolution and again inserted into MMP19 and CMMP. One wonders what might be the selective pressure to maintain the unique cysteine in the catalytic domain of these three MMPs.

Further sequence comparison indicates that CMMP, XMMP, and MMP19 are not orthologs of different species but novel members of the MMP family. The major differences include the following: XMMP lacks a linker peptide (9), while CMMP has a 19-residue and MMP19 a 32-residue linker peptide, which is unusually rich in acidic residues. XMMP has a 37-amino acid-long insertion domain at the end of the propeptide, followed by an RRKR motif. As shown by Pei and Weiss (19, 23), this motif begins with the sequence81FVLTE. In SDS-gel electrophoresis, when indicated, digestions were without (-) or with (+) EDTA to inhibit the enzyme activity. In A and B, digestion products were analyzed by 12 and 10% SDS-polyacrylamide gel electrophoresis, respectively. C and D, casein and gelatin zymography of bacteria produced recombinant CMMP and MMP1. The arrowheads indicate the position of proteolytic activity of 29- and 95-kDa bands (clearly visible in original gels) for CMMP, and 39 kDa for MMP1. Zymograms were run in 10% SDS-gel electrophoresis. E, digestion of native fibrillar type I collagen analyzed in 8% SDS-gel electrophoresis. In the CMMP lane (which shows no digestion), the arrowheads indicate the monomeric a1- and a2-chains of type I collagen and their cross-linked b1- and b2-dimers and γ trimers. In the MMP1 lane (digestion), the asterisks show the specific three-quarter products of type I collagen, which are also produced from the cross-linked b dimers. The one-quarter products have been run out from this gel. In each panel, the M lane shows the migration of molecular weight markers.

To identify homologous sequences in other species, we searched (59) the 1817-nucleotide and 472-amino acid sequence of CMMP in the EST data bases including those of human and mouse. However, the only ESTs we could find were all derived from previously cloned MMPs. This is interesting, especially because there are already 964,050 ESTs from humans and 301,508 ESTs from mice (as of March 13, 1998). Since these ESTs are derived from a variety of tissues, mainly from adult tissues, it is reasonable to propose that the CMM gene is expressed preferentially during early development or in a few select tissues and organs.

**Characterization of CMMP Activity**—To study the proteolytic activity of CMMP, we produced in E. coli bacteria a recombinant form of CMMP without the 85-residue amino-terminal inhibitory propeptide. For protein purification purposes, six histidines (His tag) were added to the amino-terminal end of CMMP, which begins with the sequence86YGVTL (Fig. 1). After isolation and purification by nickel affinity chromatography, the His-tagged CMMP migrated in SDS-gel electrophoresis as a major band of 29 kDa and three minor bands of smaller size (not shown). This is significantly smaller than the calculated molecular weight 42,190 (374 residues including six histidines), indicating autoproteolysis and carboxyl-terminal truncation of the recombinant CMMP. We made a similar His-tagged recombinant construct of the human collagenase-1 (MMP1) without the 80-residue amino-terminal propeptide. It begins with the sequence88FVLTE. In SDS-gel electrophoresis, it migrated as a major band of 42 kDa (not shown), which agrees with the calculated molecular weight of 43,465 (376 residues including six histidines).

As shown in (Fig. 5), the bacteria produced recombinant CMMP-digested gelatin and casein with an efficiency compa-
Cloning of a Novel MMP from Chicken

The manuscript describes the cloning of a novel metalloproteinase (MMP) from chicken primary embryo fibroblasts. The MMP, named CMMP, was purified and its mRNA expression was studied using Northern blotting.

**CMMP mRNA Expression**

In Northern blotting (Fig. 6), CMMP mRNA of 1.8 kb was detected in the head but up-regulated in the body between days 8 and 9 of chicken embryo development (Ref. 17; data not shown).

**Why Are There So Many MMPs?**—Recent cloning of four different MT-MMPs (12–17) and five novel members of the MMP family called MMP19, porcine enamelysin, chicken CMMP, Xenopus collagenase-4, and XMMP (Refs. 5–9 and this report) brings the current number of different MMPs to 19. Why are there so many different MMPs? This may reflect their highly tissue- and cell-specific expression pattern, in particular during embryonic growth and development. For example, a recently cloned MMP from porcine called enamelysin is expressed exclusively in the tooth's enamel organ (7). Human collagenase-2 (MMP8) is expressed only in neutrophils and cartilage articular chondrocytes (67, 68). High expression of mRNA for the 92-kDa type IV collagenase and MT1-MMP genes is detected in the cells of osteoclast lineage during development (35–37). Expression of the mouse interstitial collagenase, now known to be collagenase-3 (MMP13) (see Ref. 29), is restricted to skeletal tissues; it is expressed in hypertrophic chondrocytes in the growth plates and in osteoblasts and capillary endothelial cells of developing bones (32, 33). Our in situ mRNA hybridization studies on the chicken MT3-MMP showed that it is expressed at high levels in several embryonic tissues such as neural tube, dorsal root ganglia, respiratory epithelium, and in developing cartilage and muscle (17).

It is also possible that the large number of MMPs reflects the complex molecular details of the ECM in tissues and organs. Indeed, more than 80 genes encode the various ECM components in human, of which 34 encode the collagen family of proteins. However, many MMPs are broad spectrum enzymes with overlapping substrate specificities that probably degrade the same ECM components with comparable specificity and kinetics. Therefore, it is possible that the large number and apparent redundancy of MMPs suggest a combinatorial role of MMPs, perhaps in concert with other extracellular proteinases such as plasmin/plasminogen activators, in development, tissue remodeling, or disease.

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Note Added in Proof—We have now found that the bacteria produced and activated full-length CMMP, and XMMP, cleave native fibrillar type I collagen into the characteristic three-quarter and one-quarter cleavage products from native fibrillar type I collagen, whereas CMMP was unable to cleave type I collagen (Fig. 5E). However, because of its partial carboxyl-terminal truncation, which would abolish collagenolytic activity, if any, these data are not conclusive and must await the purification and analysis of full-length CMMP.

In conclusion, we have purified and characterized a novel MMP from chicken, called CMMP, which differs from the known MMPs in both its substrate specificity and its tissue distribution. The cloning of this enzyme and the development of specific antibodies against it will allow a more detailed analysis of its role in development and tissue remodeling.
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