DEVELOPMENTAL DYNAMICS 216:233–243 (1999)

Spatio-Temporal Distribution of Chondromodulin-I mRNA in the Chicken Embryo: Expression During Cartilage Development and Formation of the Heart and Eye

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ABSTRACT **To define genes specifically expressed in cartilage and during chondrogenesis, we compared by differential display-polymerase chain reaction (DD-PCR) the mRNA populations of differentiated sternal chondrocytes from chicken embryos with mRNA species modulated in vitro by retinoic acid (RA). Chondrocyte-specific gene expression is downregulated by RA, and PCR-amplified cDNAs from both untreated and RA-modulated cells were differentially displayed. Amplification products only from RNA of untreated chondrocytes were further analyzed, and a cDNA-fragment of the chondromodulin-I (ChM-I) mRNA was isolated. After obtaining full length cDNA clones, we have analyzed the mRNA expression patterns at different developmental stages by RNase protection assay and in situ hybridization. Analysis of different tissues and cartilage from 17-day-old chicken embryos showed ChM-I mRNA only in chondrocytes. During somitogenesis of the chicken embryo, ChM-I transcripts were detected in the notochord, the floor and the roof plate of the neural tube, and in cartilage precursor tissues such as the sclerotomes of the somites, the developing limbs, the pharyngeal arches, the otic vesicle, and the sclera. ChM-I continued to be expressed in differentiated cartilages derived from these tissues and also in noncartilaginous domains of the developing heart and retina. Thus, in the chicken, the expression of ChM-I is not restricted to mature cartilage but is already present during early development in precartilaginous tissues as well as in heart and eye.** ***Dev Dyn 1999;216:233–243.*** **© 1999 Wiley-Liss, Inc.**

Key words: chondrogenesis; retinoic acid; differential display; chondromodulin-I; development; vascularization

INTRODUCTION

The development of a large part of the vertebrate skeleton—including the vertebral column, the pelvis, and the appendicular skeleton—proceeds through endochondral ossification. Pluripotent mesenchymal cells condense into avascular nodules, differentiate into chondrocytes, and form the cartilage models of future bones by depositing a cartilage-specific extracellular matrix. Subsequently, the chondrocytes transit through a cascade of several distinct stages of differentiation before the initially avascular cartilage is invaded by blood vessels and remodeled into bone. Thus, a finely tuned regulation between cartilage growth and angiogenesis comprises positive and negative control factors that are pivotal for the first deposition of cartilaginous elements as well as for the proper replacement of cartilage by bone during endochondral ossification. The molecular mechanisms can be studied in vitro in several culture systems developed recently (for review, see Cancedda et al., 1995).

In long-term monolayer culture or after repeated passage, the chondrocytes characteristically lose their morphological and biochemical cartilage phenotype, and retinoic acid (RA) at high doses (10^{-6}M) strongly accelerates this development. RA causes the cells to assume several properties of mesenchymal chondrogenic cells. Instead of retaining their typical polygonal shape, they become fibroblast-like and switch their expression of collagen genes from cartilage types II, IX, X, and XI towards those of mesenchymal cells, e.g., collagens I, III, and V (Vasan and Lash, 1975; Shapiro and Poon, 1976; Pacifici et al., 1980; Yasui et al., 1986; Benya and Padilla, 1986; Horton et al., 1987; Dietz et al., 1993). However, in cells derived from cartilage at more advanced stages of late differentiation, RA can enhance chondrocyte hypertrophy and mineralization (Oettinger and Pacifici, 1990; Pacifici et al., 1991).

Differential display of mRNA expression in untreated and RA-modulated bovine articular chondrocytes by polymerase chain reaction (DD-PCR; Liang and Pardee, 1992) has recently been employed to recognize genes specifically expressed in cartilage (Dietz and Sandell, 1996). Based on RA-dependent downregulation, this study identified a novel gene product designated CD-RAP/MIA, which is expressed in cartilage and

Grant sponsor: Deutsche Forschungsgemeinschaft; Grant sponsor: Interdisciplinary Clinical Research Center (IZKF) at the University of Münster; Grant number: C4; Grant sponsor: Deutsche Forschungsgemeinschaft; Grant number: Br 1497/1–2,3.

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Received 20 April 1999; Accepted 21 July 1999

several tumors and is not expressed in other normal tissues (Blesch et al., 1994; Bosserhoff et al., 1997). Thus, RA modulation is now an established means of detecting chondrocyte-specific gene expression.

In the present study, an additional mRNA in chick embryo chondrocytes was identified by RA downregulation. Cloning and sequencing revealed that the mRNA encodes chondromodulin-I (ChM-I). In a previous search for components promoting chondrocyte growth, ChM-I was isolated from fetal bovine cartilage, and the protein binds to heparin with high affinity (Hiraki et al., 1991). The cartilage-specific glycoprotein is synthesized as a transmembrane precursor with a molecular mass of 25 kDa and is converted to the mature molecule by cleavage at a predictable processing site (Hiraki et al., 1997a). The carboxyterminal portion of the molecule is then secreted into the extracellular matrix. ChM-I expression has been described so far mainly in cartilage (Hiraki et al., 1991; Hiraki et al., 1999; Shukunami et al., 1999) but was also shown in other tissues such as thymus and eye. Until now, there were no detailed data available about the spatiotemporal ChM-I mRNA distribution during embryonic development and chondrogenesis. Here we provide this information in the developing chicken. We have analyzed ChM-I expression in chicken embryos, starting from very early developmental stages through the formation of the first differentiated cartilages. Our data show that ChM-I mRNA is not only expressed in chondrocytes but is also detectable in cartilage precursor tissues well before overt chondrogenesis begins. In addition, ChM-I is also expressed during the formation of some noncartilaginous tissues. The current study suggests that ChM-I may function in early development before the onset of chondrogenesis and during cartilage formation as well as in other tissues such as the heart and the eye.

RESULTS

Identification and Cloning of Chondromodulin-I mRNA

A partial cDNA sequence of chicken ChM-I mRNA was identified by differential display-PCR (DD-PCR) comparing the mRNA populations of RA-modulated and untreated sternal chondrocytes from chicken embryos as described by Dietz and Sandell (1996). A cDNA-fragment of the ChM-I mRNA with a length of 262 bp (DD-13) was selectively amplified from the RNA population of untreated chondrocytes and cloned into a plasmid vector.

To clone the full-length cDNA we screened a stage-22 chicken embryonic cDNA-library. After screening of 4×10^5 recombinant phage clones from an oligo(dT) primed lambda Zap cDNA library with the radiolabeled DD-13 cDNA as a probe, eight positive clones were identified. The largest clone carried a cDNA-insert of 2,412 bp and was analyzed by sequencing. Alignment of the DD-13 cDNA with the phage clone showed that the DD-13 sequence corresponds to nucleotide 1,217 to 955 upstream of the polyA-tail.

The oligonucleotide 5′-CG(dT_{12}), which was used for reverse transcription of the DD-13 cDNA, appearently hybridized to the sequence 5′-GCAAAA-3′ within the mRNA molecule and not to the polyA-tail.

The size of the complete ChM-I mRNA, as predicted from Northern blot hybridization, was approximately 3 kb (data not shown). When compared with the bovine sequence described by Hiraki et al. (1991) our phage clones were still missing the coding region for 112 amino acids and the 5′ untranslated region. To clone the complete molecule we performed 5′-RACE (rapid amplification of cDNA ends), which yielded additional sequence information of 339 bp. The cloning strategy and the combined cDNA information with the deduced amino acid sequence is shown in Fig.1.

Including the 22 bp polyA-tail, we identified 2,751 bp of the chicken ChM-I mRNA, containing three basepairs of the 5′-UTR (untranslated region), the coding region, and the 3′-UTR. By inspection of the nucleotide sequence we found an open reading frame of 1,041 bp that encodes 347 amino acids. Comparison of the chicken and the bovine sequences shows that there is an insertion of 17 additional amino acids directly behind the cleavage signal, which does not align with the bovine homologue (boxed in Fig.1B). The homology within the coding region between the chicken and bovine ChM-I is 72% at the DNA level and 68% at the amino acid level.

Expression Analysis of ChM-I mRNA by RNase Protection Assays

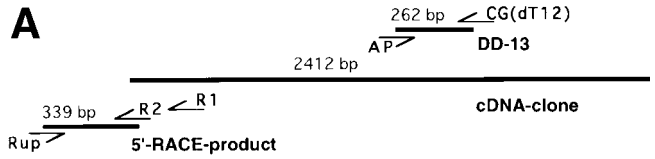
For the first expression analysis, total RNA was isolated from different tissues such as brain, heart, kidney, liver, lung, muscle, and skin of 17-day-old chicken embryos, and the presence of ChM-I mRNA was examined by RNase protection assays.

To confirm the downregulation of ChM-I mRNA levels in chondrocytes by RA, samples of total RNA from untreated and RA-modulated cells were used in the reactions. The probe for the hybridization was a ^{32}P-labeled antisense RNA transcribed from the cloned DD-13 fragment. To control for the specificity of the assay, the reaction was also performed with hybridization to tRNA.

After RNase treatment and analysis through a 6% sequencing gel, hybridization products were detected by autoradiography. Protected fragments were detected only in total RNA samples from untreated chondrocytes and not in any of the other samples (Fig. 2). This analysis confirmed that ChM-I mRNA is downregulated by RA and indicated that ChM-I expression is chondrocyte-specific during late stages (day 17) of embryonic development.

Analysis of ChM-I mRNA Expression by In Situ Hybridization

To investigate the temporal and spatial distribution of the ChM-I mRNA during chondrogenesis and in



B

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GTGCCCCCTCCGGGGCCAGGGCGGCTGCTGAAGGCAGGGGCCACTGTGCTCATCGCTGGGGCCCTTCTGCTGCTGGCTGGGGCCATCGGT     180
 V  P  P  P  G  P  G  R  L  L  K  A  G  A  T  V  L  I  A  G  A  L  L  L  L  A  G  A  I  G      60

GCCTTCTACTTCTGGAAAGCCACTGAGAGGCAGGTCTACAACGTTCACTACACCATGAGCATTAATGGAAAAGTGCAAGACGGATCGATG     270
 A  F  Y  F  W  K  A  T  E  R  Q  V  Y  N  V  H  Y  T  M  S  I  N  G  K  V  Q  D  G  S  M      90

GAAATAGATGCCGGAAACAACTTAGAGACATTCAAAACAGGAAGCGGGAGTGAAGAGGCTGTTGAAGTTCATGATTTCCAGATTGGCATC     360
 E  I  D  A  G  N  N  L  E  T  F  K  T  G  S  G  S  E  E  A  V  E  V  H  D  F  Q  I  G  I     120

ACCGGCATCCGATTTGCTGGAGGAGAGAAGTGTTACATCAAGGCCCAGCCCAAAGCTCGTGTGCCTGAGGTGGATGCCATGACCAAGGCC     450
 T  G  I  R  F  A  G  G  E  K  C  Y  I  K  A  Q  P  K  A  R  V  P  E  V  D  A  M  T  K  A     150

AGCCTGTCCTCTGACCTGGAAGATGAAATCATGCCTGTTAGATTTGACGAAAACTCTCTTATCTGGGTGGCTGCAGACGAGCCCATCAAG     540
 S  L  S  S  D  L  E  D  E  I  M  P  V  R  F  D  E  N  S  L  I  W  V  A  A  D  E  P  I  K     180

CACAACGGTTTCCTAAGCCCCAAAATTTTAGAGCTTTGTGGGGATCTTCCGATTTTCTGGCTTCGACCTACATATCCCAAAGATAAACAA     630
 H  N  G  F  L  S  P  K  I  L  E  L  C  G  D  L  P  I  F  W  L  R  P  T  Y  P  K  D  K  Q     210

AGAGAAAGGAGAGAAATGAAGAGAAATAAACGCCAGTCCGAGTCAAACTTCGATGCAGAACACAGAGCAGCTGCTGCTGAAGAGGTGAAC     720
 R  E  R  R  E  M  K  R  N  K  R  Q  S  E  S  N  F  D  A  E  H  R  A  A  A  A  E  E  V  N     240

ACCAGGTCCACCCCCACCCAGCTGACTCAAGATCTTGGCCCCCAGTCGAATGAAACCAGGCCAATGCAGCAAGAAAGCGATCAAACACTT     810
 T  R  S  T  P  T  Q  L  T  Q  D  L  G  P  Q  S  N  E  T  R  P  M  Q  Q  E  S  D  Q  T  L     270

AACCCAGACAACCCATACAACCAACTGGAAGGTGAAGGGATGGCTTTTGACCCCATGCTGGACCACCTGGGCGTGTGCTGCATCGAGTGC     900
 N  P  D  N  P  Y  N  Q  L  E  G  E  G  M  A  F  D  P  M  L  D  H  L  G  V  C  C  I  E  C     300

AGGCGAAGTTACACGCAGTGCCAGAGGATCTGTGAGCCTCTGCTGGGCTACTACCCGTGGCCATACAACTACCAGGGCTGCCGCACTGCC     990
 R  R  S  Y  T  Q  C  Q  R  I  C  E  P  L  L  G  Y  Y  P  W  P  Y  N  Y  Q  G  C  R  T  A     330

TGCCGCATCATCATGCCCTGCAGCTGGTGGGTCGCTCGCATCATGGGCGTTGTGTGAGAGAGCTTTGGGCTCCTGGAGGAAGCCAGCAGA    1080
 C  R  I  I  M  P  C  S  W  W  V  A  R  I  M  G  V  V  *                                       348

AGCCTGACAACGCCAAACTGATGAAAGACTCCGACCAAAGCAGCATGGCCAATCCTGGCTTCTAAATCCAAACGCGCGTTACTGTGCTAA    1170
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GAGTCTCAAGACCCTTCCGAACCTACGAAAGCCACTGTTATTTATAAACCCTTGAACTAGACACTTCTGACACAAGATTACAACCCCTCC    1890
TTATTCCACATGGCATAGCGGTTGTTCGTGCCAGCTCCTTGGCCTTCACTGGAGCTAACCTTCTGTTCCCTACAAGAGAAATTGCTTACT    1980
GGTAAATGAGAGGAGAAAAAGATTTTAACCTCACCCTTGTTGCCCAGGCAAACTCTGCAATTAGAGGGATTGATCCTATTATGTACTAAA    2070
TCTCTACGGAAGGTACTGAGCTGAAGATGGTATGACTTAGGATTCCCAGTCTTTGAACAAAATTAGAAGTATTATACTGCCTTCATCCAT    2160
ACGACATCCTCAGCTCCACCCCTGAGTTCACGGAGCCATCTACACAGGCATGATAAACAAAAAAGGTCTAATCTTTTAAGGTTCACTTTCT    2250
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GCTGATTAAACAAATACATTAAAAATGGCAAAAAAAAAAAAAAAAAAAAAA                                            2751
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Fig. 1. **A:** Isolated cDNA-fragments of chicken ChM-I mRNA derived from a stage-22 chicken embryonic cDNA-library or PCR amplification. The sequences of the indicated primers are described under Material and Methods. **B:** Chicken ChM-I cDNA sequence and translation into protein. This sequence is deposited in the GenBank database under accession number AF138280. The ChM-I sequence shown starts from the poly-A tail of the mRNA and covers the 3′ UTR, the coding region, and 3 bp of the 5′ UTR. The cleavage signal for the mature protein is underlined, and amino acids unique to the chicken ChM-I sequence are boxed.

cartilage, we performed in situ hybridization of whole mounts or sectioned chicken specimens from day 1½ (stage 10) to day 6 of development.

These studies showed that ChM-I mRNA expression begins before the onset of chondrocyte differentiation. ChM-I transcripts were present in the notochord and

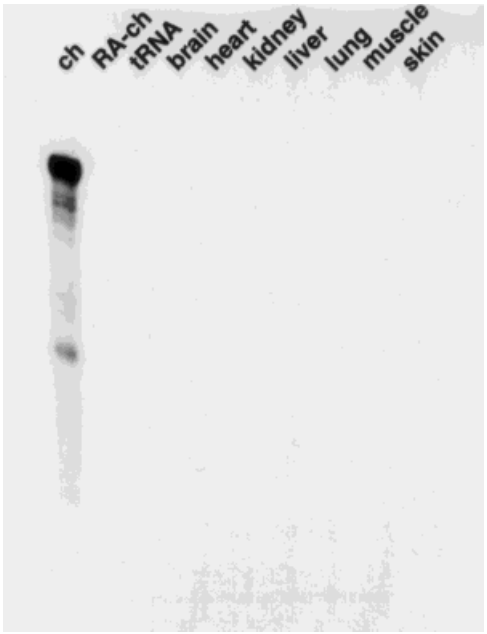

Fig. 2. RNase protection of total RNA from cultured chodrocytes and tissues of 17-day-old chicken embryos. Antisense RNA labeled with ^{32}P was transcribed from the DD-13 cDNA-fragment and hybridized to 10µg of each total RNA from untreated and RA-modulated chondrocytes, as well as several tissues such as brain, heart, kidney, liver, lung, muscle, and skin. After digestion with RNases A and T1 the samples were electrophoresed through a 6% sequencing gel. Protected fragments were detected by autoradiography. Exposure time to x-ray films shown in the figure was 5 hours. Protected fragments were detected only in the hybridization reaction containing RNA from untreated chondrocytes.

other embryonic structures that give rise to cartilage-forming tissues. We investigated embryos as early as stage 10, in which no ChM-I mRNA could be detected (data not shown). The first significant staining with a ChM-I specific antisense riboprobe occurred in the somites and the notochord of a stage-13 embryo hybridized as whole mount (Fig. 3A,D). Therefore, the onset of ChM-I expression during chicken development must occur between stage 10 and stage 13.

In the stage-13 embryo, ChM-I expression was detected within the more developed somites at the anterior part of the embryo, whereas in the five most recently formed somites and the unsegmented paraxial mesoderm, ChM-I mRNA was not detected (Fig. 3A,D).

Within the somites, the expression domain is restricted to the anterior medial part, which can be seen on the embryo at higher magnification in Fig. 3D. ChM-I expression starts anterior to the von Ebner's fissure and remains localized to the anterior as mRNA expression increases in the more differentiated somites. Transversal sections through embryos show that this expression domain is restricted to the sclerotome (Fig. 3E,F).

At later stages (day 4), transcription of ChM-I mRNA occurs within the entire developing vertebral column (Fig. 3B). In the 4-day-old embryo, additional expression domains appear in the floor and roof plate of the neural tube, whereas expression in notochord has vanished (Fig. 3F).

At day 5, the vertebrae of the chicken embryo become cartilaginous, and in situ hybridization of sections through the vertebral column of a 6-day-old embryo show that ChM-I mRNA continues to be expressed in the differentiated cartilage (Fig. 3C). During development of the cartilages of the head and neck region in the 4-day-old embryo, ChM-I mRNA was detected in the pharyngeal arches I–III and the otic vesicle (Fig. 4A).

The first pharyngeal arch that divides into two parts forms the maxilla and the mandible and will give rise to the palato-quadrate in the upper jaw and to Meckel's cartilage in the lower jaw, respectively. ChM-I is strongly expressed in these cartilages and in other cartilages in the head such as the nasal capsule and the basal plate of the chondrocranium (Fig. 4B,C).

The ChM-I–specific signals in the second and third pharyngeal arch and in the otic vesicle (4A,C), mark further tissues that differentiate into cartilaginous structures.

In the limbs, the first detectable ChM-I expression appears at the bud stage of a 4-day-old (stage 22) embryo as a dot-like structure and correlates well with the location of the first chondrogenic condensations (Fig. 5A). In chicken development, the precursor tissues that form the appendicular skeleton start to differentiate into cartilage at around day 6. Then also the differentiated cartilaginous structures in the developing limbs are extensively stained with ChM-I antisense riboprobes (Fig. 5B), in a manner similar to that described above for cartilage of the developing vertebral column.

A further tissue that expresses ChM-I and undergoes chondrification is the sclera (Bellairs and Osmond, 1998). We detected ChM-I mRNA at the precartilaginous state in the sclera of sections through the eye of 6-day-old embryos (Fig. 6A). On these sections, as well as on sections through the eye of 4-day-old embryos, we also identified a ChM-I expression domain in the retina (Fig. 6B,C). This expression domain is not evenly distributed over the retina; instead, it is most abundant within the peripheral part adjacent to the lens.

Another noncartilaginous organ in which we detected ChM-I transcripts is the developing heart. On a section through a 4-day-old embryo, ChM-I-specific expression was present in the truncus venosus, the endocardium, and the sinus venosus (Fig. 7A). In 6-day-old embryos the atrioventricular cushions have fused to each other and to the interatrial septum, and the atrium becomes divided into two compartments. At this stage, expression of ChM-I from the endocardium is not seen anymore, but transcripts are clearly detected in cells of the atrioventricular cushions (Fig. 7B).

DISCUSSION

One of the distinct properties of chondrocytes in vitro is the instability of the differentiated phenotype. In

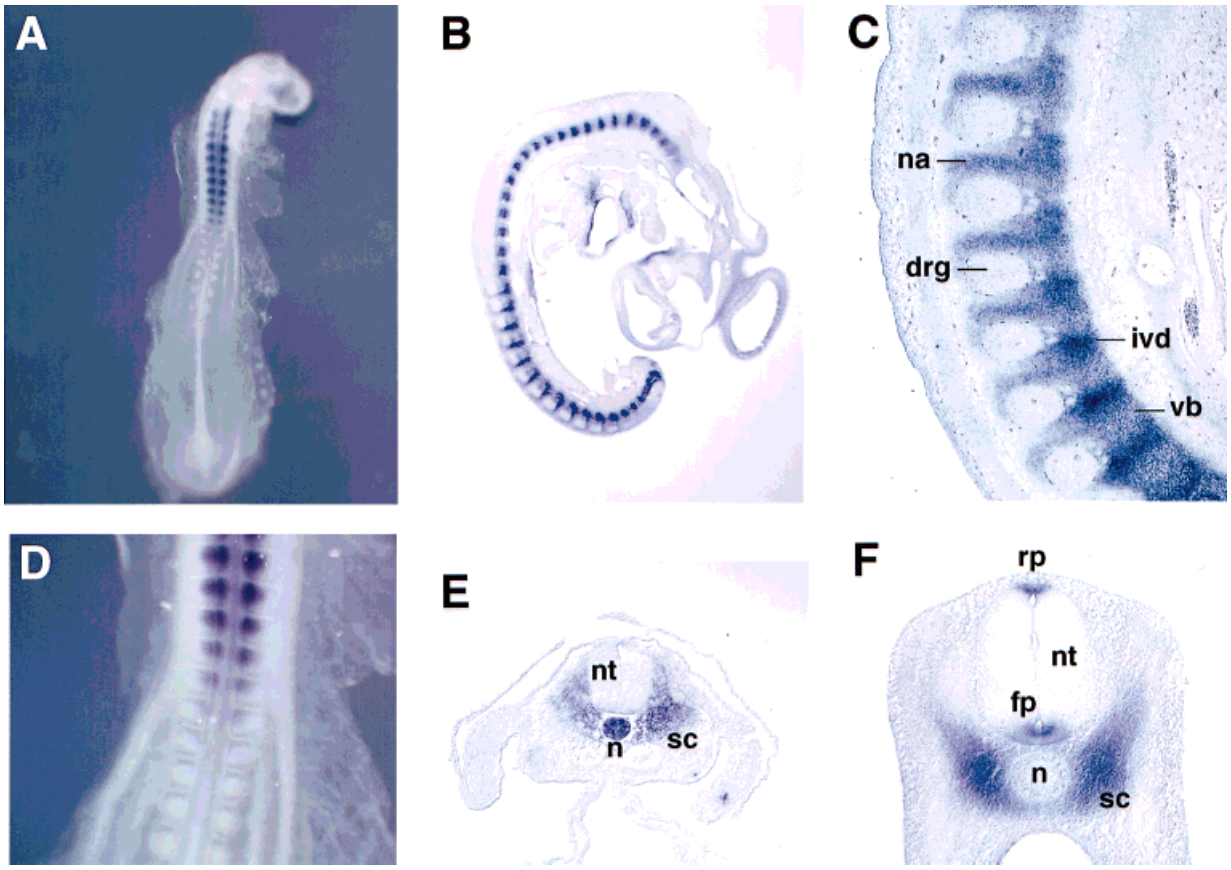

Fig. 3. ChM-I mRNA expression patterns along the developing vertebral column. **A, D:** Expression of ChM-I mRNA in a stage-13 chicken embryo. As shown in A, at this stage ChM-I mRNA is highly expressed in the more developed anterior somites but not in the paraxial mesoderm or the most recently formed somites. Somitic expression is restricted to the anterior medial part of the sclerotomes (D,E,F). At day 3 of development, ChM-I mRNA can be detected in the notochord (n) as shown in **E** and is absent at day 4 (**F**). **B** and F are sections through paraffin-embedded 4-day-old embryos (stage 22) that were hybridized as whole mounts. At this stage, ChM-I mRNA is expressed along the entire developing vertebral column (B) and in the floor plate (fp) and roof plate (rp) of the neural tube (nt) (F). **C** shows a sagittal section through the vertebral column of a 6-day-old embryo. Positive staining with the ChM-I–specific probe is visable in the neural arches, the vertebral bodies, and the intervertebral discs. drg, dorsal root ganglion; ivd, intervertebral disk; na, neural arch; sc, sclerotome; vb, vertebral body.

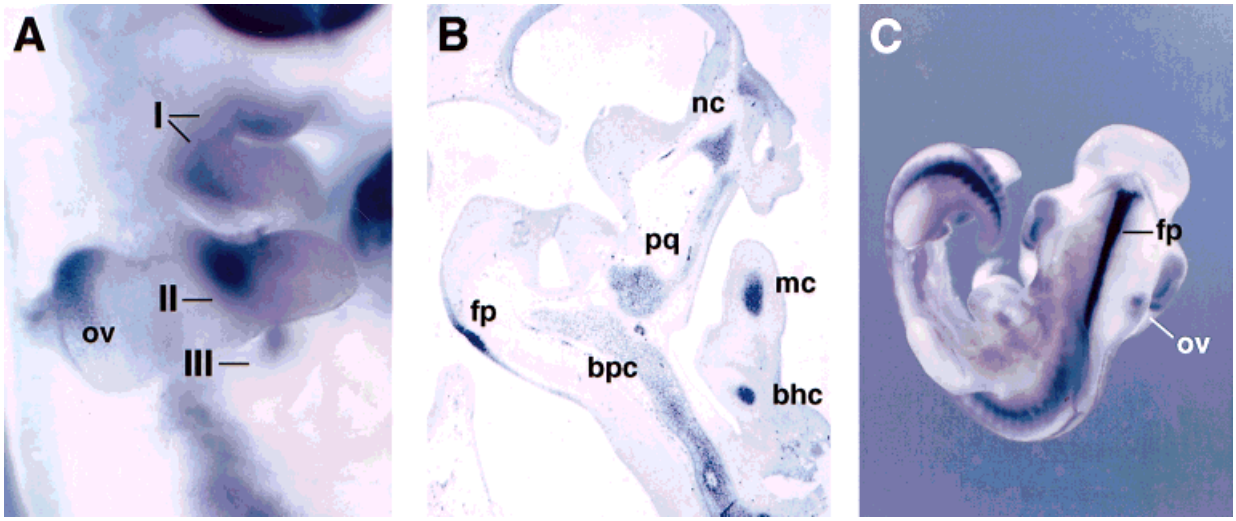

Fig. 4. Detection of ChM-I mRNA in precartilaginous structures and cartilages of the head. **A:** Whole mount in situ hybridization of a 4-day-old embryo. Hybridization signals with the ChM-I antisense RNA probe can be seen in the pharyngeal arches I, II, III, and in the otic vesicle (ov). **B** shows ChM-I mRNA expression on a section through the head of a 6-day-old embryo. Specific signals were detected in the nasal capsule (nc), the palato-quadrate (pq), the basal plate of the chondrocranium (bpc), Meckel's cartilage (mc), basihyal cartilage (bhc), and the floor plate of the neural tube (fp). **C** shows a 4-day-old embryo hybridized as whole mount that demonstrates the floor plate (fp) expression domain, which reaches as far as to the isthmus between the metencephalon and mesencephalon. Further expression domains to notice are in the eye, the otic vesicle (ov), and the developing vertebral column.

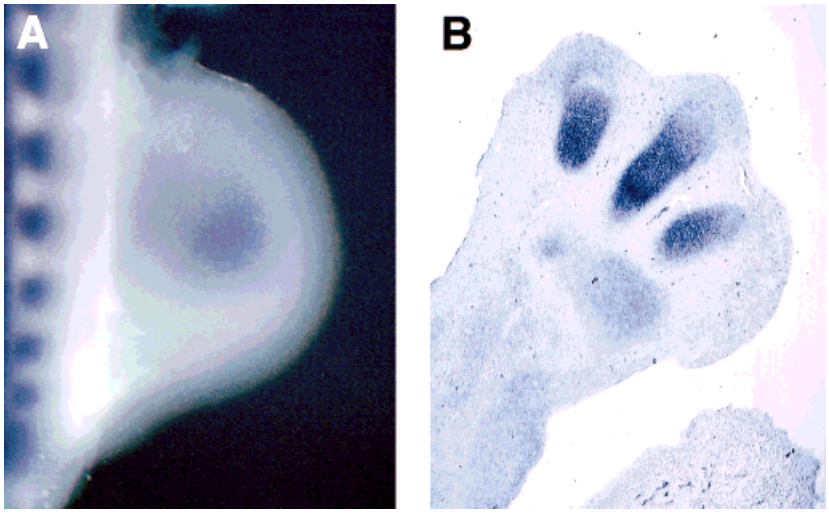

Fig. 5. ChM-I mRNA in the developing hindlimb. **A:** A 4-day-old embryo (stage 22) hybridized as whole mount shows ChM-I expression in the center of the limb bud. The section through the hindlimb of a 6-day-old embryo (**B**) shows ChM-I transcripts in the cartilaginous anlagen of the developing digits.

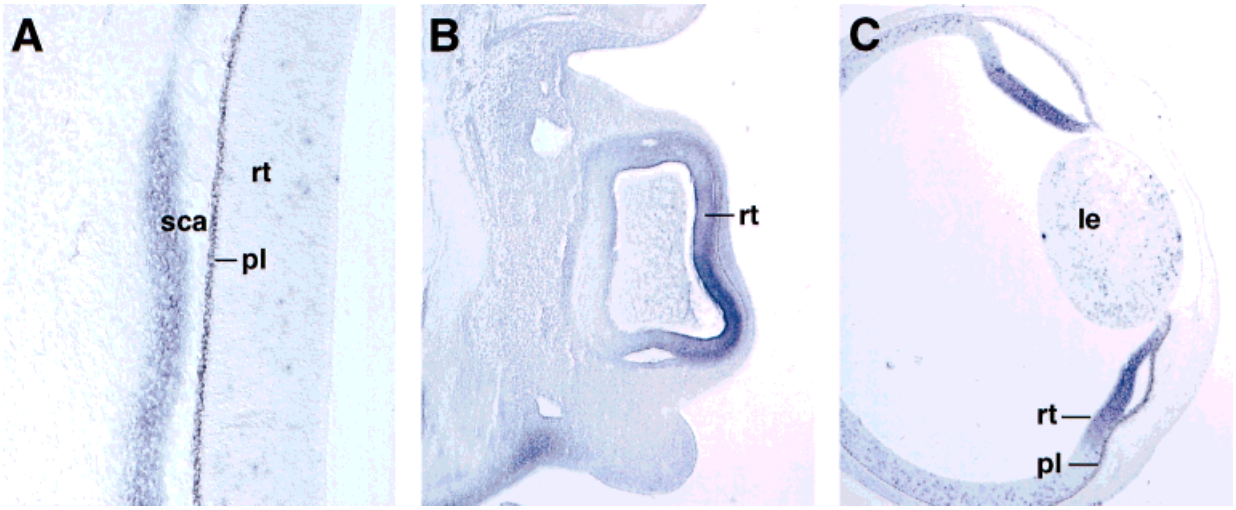

Figure 6. (Legend on following page.)

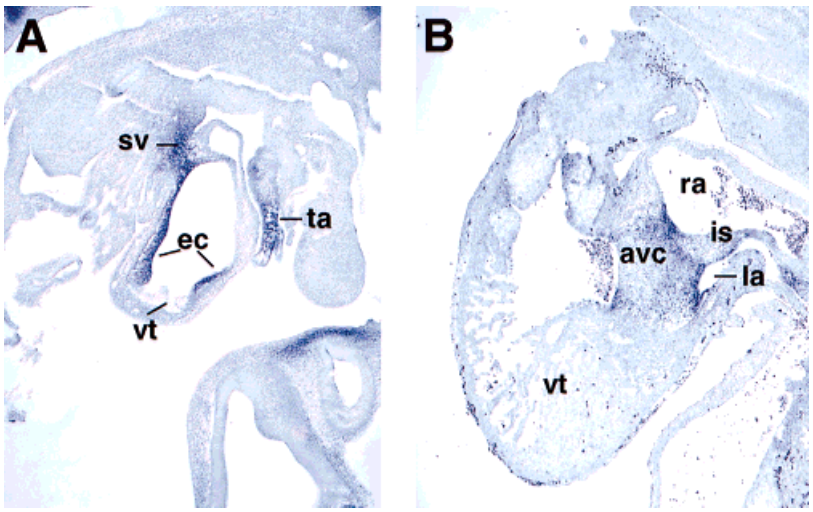

Figure 7. (Legend on following page.)

culture, the phenotype of chondrocytes is easily lost, particularly when exposed to RA. As a result, they cease to produce macromolecules typically found in cartilage matrix, including the proteoglycan aggrecan, collagens of types II, IX, and X, and the cartilage version of collagen XI. Instead, they express a repertoire of matrix components resembling that of chondrogenic mesenchymal cells, such as collagens I, III, and V. Although known for decades (Abbot and Holtzer, 1968), the biological significance of this phenomenon, also designated "dedifferentiation," still is poorly understood. For this reason, we have established a search by differential display PCR (DD-PCR) for genes that are distinctly regulated in modulated and differentiated chondrocytes, respectively. We have previously identified CD-RAP/MIA as a gene product downregulated in RA-modulated chondrocytes. Here, we have shown that ChM-I expression is similarly affected by RA-induced phenotypic alteration. This led to the cloning and structural characterization of the cDNA for the chicken homologue of ChM-I.

ChM-I was previously isolated and described from bovine, mouse, and human cartilage (Hiraki et al., 1991; Hiraki et al., 1999; Shukunami et al., 1999). The protein consists of a hydrophilic N-terminal domain and a hydrophobic C-terminal domain. The molecule becomes cleaved at a predicted processing site, and the C-terminal domain is secreted into the extracellular matrix. Among the species, the molecule is highly homologous, especially within the C-terminal domain, which contains eight conserved cystein residues.

To find a function for ChM-I, the bovine and human proteins were purified from cartilage or isolated as recombinant protein, respectively, and tested in vitro. The proteins from both organisms stimulated the proteoglycan synthesis of cultured chondrocytes and inhibited tube morphogenesis of cultured vascular endothelial cells (Hiraki et al., 1997a; Hiraki et al., 1997b; Hiraki et al., 1999). Therefore, a possible role for ChM-I as an antiangiogenic factor was suggested and further investigated in different in vivo systems.

When recombinant human ChM-I was applied to the chorioallantoic membrane of chicken embryos, there was an evident loss of the fine capillary networks (Hiraki et al., 1999). In nude mice demineralized bone matrix was implanted into the fascia of the back muscle to induce ectopic bone formation. There, administration of exogenous ChM-I caused an inhibition of vascular invasion into the newly formed cartilage precursor (Shukunami et al., 1999). These observations provided strong evidence that ChM-I, together with other molecules (Moses et al., 1992; Hidai et al., 1998), has the potential to counteract potent angiogenic factors present in cartilage such as FGF-2 and vasacular endothelial growth factor (VEGF) (Gonzalez et al., 1990; Harada et al., 1994) and thus can retain the avascularity of the tissue. Interestingly, ChM-I is not expressed within the deeper hypertrophic region of cartilage (Hiraki et al., 1999) where the invasion of the vascular system and a switch to the angiogenic status occurs.

To investigate the tissue distribution of ChM-I mRNA, different bovine and human tissues were tested (Hiraki et al., 1991; Hiraki et al., 1999). ChM-I transcripts were not detected in tissues other than cartilage, which indicated that the expression was chondrocyte-specific and agrees with our analysis of different tissues and chondrocytes from 17-day-old chicken embryos. However, in tissues of 4-week-old mice, ChM-I mRNA has been detected in the thymus and the eye (Shukunami et al., 1999). Analysis of RNA from total mouse embryos showed expression of ChM-I mRNA already present from day 7 post coitum (pc). Until now, there has been no detailed information about the spatiotemporal distribution of ChM-I mRNA during embryogenesis. The current study has provided an extensive analysis of ChM-I mRNA expression in the developing chicken embryo.

Expression of ChM-I During Somite Formation and Chondrogenesis

We analyzed ChM-I mRNA patterns in chicken embryos from day 1½ to day 6 of development, and our findings support the conclusion that ChM-I is expressed well before overt formation of cartilage. ChM-I mRNA was detected in several embryo-specific structures, most of which give rise to cartilage derivatives later on. Similar to previous studies investigating the developmental distribution of other molecules of the cartilage extracellular matrix (ECM) such as aggrecan, α1(II), α1(IX), and α1(XI) collagen (Cheah et al., 1991; Hayashi et al., 1992; Sandell et al.1994; Schwartz et al., 1996; Sugimoto et al., 1998), we detected ChM-I expression within the notochord.

During vertebral development the notochord plays an important role by secreting molecules such as sonic hedgehog, which induces Pax1 expression followed by sclerotome formation (Borycki et al., 1998). Subsequently, Pax1-positive sclerotomal cells migrate towards the notochord and form the perinotochordal tube (Wallin et al., 1994; Ebensperger et al., 1995). Cartilage ECM molecules may control these migratory processes in synergy and therefore might be expressed from the notochord in a temporal manner, as demonstrated in this study for ChM-I mRNA.

Fig. 6. ChM-I expression domains in the developing eye. **A** shows ChM-I mRNA in the sclera (sca) of a 6-day-old chicken embryo. At this stage chondrification of the sclera has not yet started and the tissue is still at a precartilaginous state. **B** and **C** show expression in the retina on a frontal section through the head of a 4-day-old embryo, hybridized as whole mount (B) and on an eye section of a 6-day-old embryo (C). Hybridization signals in B and C appear to be restricted to the peripheral part of the retina. Dot-like signals are also seen within all other parts of the retina and the lens (le). pl, pigment layer of the retina; rt, retina.

Fig. 7. ChM-I mRNA expression in the developing heart. **A** shows an enlargement of the heart region of a 4-day-old embryo taken from the section shown in Fig. 1B. At this stage ChM-I mRNA is expressed in the endocardium (ec), the truncus arteriosus (ta), and the sinus venosus (sv). **B** shows a section through the heart of a 6-day-old embryo. At this stage, the atrioventricular cushions have fused to the interatrial septum and the atrium is devided into two compartments. ChM-I hybridization signals are evident in cells of the atrioventricular cushions (avc). is, interatrial septum; la, left atrium; ra, right atrium; vt, ventricle.

Several other embryonic tissues contributing to cartilaginous structures—such as the sclerotomes of the somites, the otic vesicle, the sclera, and the mesenchymes of the pharyngeal arches and the limb buds—showed ChM-I expression as well as their cartilaginous derivatives. Different possibilities arise for the function of ChM-I within these tissues. ChM-I was recently shown to stimulate the growth of cultured chondrocytes (Hiraki et al., 1997a) and may have the same effect on the condensing mesenchymal cells. The factor may also induce differentiation into chondrocytes. Thus, ChM-I and cartilage matrix macromolecules may complement the role of molecules such as N-cadherin or N-CAM, which are factors known to be important for the condensation of chondroprogenitor cells into nodules (Oberlender and Tuan, 1994; Hall and Miyake, 1995).

Our data do not favor the theory that ChM-I functions to induce mesenchymal condensations or chondrogenesis during chicken embryogenesis, which is well supported by in vitro studies of the ATDC5 cell line. ATDC5 is an embryonal carcinoma-derived cell line that undergoes chondrogenic differentiation through a cellular condensation stage. ATDC5 cells can encompass the differentiation steps of chondrogenesis from mesenchymal condensation to calcification (Shukunami et al., 1996; Shukunami et al., 1997).

ChM-I transcripts are undetectable in undifferentiated ATDC5 cells and are first expressed upon chondrogenic differentiation (Shukunami et al., 1999). In consideration of these findings and of functional studies on the ChM-I protein, we suggest that during chicken embryogenesis, ChM-I actually might function to enhance chondrocyte proliferation and to inhibit vasculogenesis in places of cartilage formation.

Vascularization of the chicken embryo is well investigated in the developing limb, where avascular regions are formed in the place of chondrogenic condensations. Hallmann et al. (1987) found that zones of avascularity always developed earlier than did cartilage, and progressing avascularity in the digital regions is followed by increased staining for cartilage antigens in the same areas. These studies demonstrated that blood vessels disappear in predictable areas before the overt differentiation of cartilage, and as shown here, ChM-I expression is actively going on in these regions.

In contrast, overexpression of vascular endothelial growth factor (VEGF) within the limb buds of chicken embryos resulted in hypervascularization, which was reflected by an increase in vascular density. However, although the ectopic VEGF expression also occurred in cartilage, the tissue was resistant to invasion of endothelial cells and was not prematurely vascularized (Flamme et al., 1995). Apparently, factors such as ChM-I are present, and they are conteracting angiogenic molecules such as VEGF to prevent vascularization of growing cartilage.

Expression of ChM-I in the Neural Tube

The relevance of ChM-I expression in the floor and roof plate of the neural tube is more difficult to understand, but such expression may be important for angiogenesis.

In vertebrates, angioblastic differentiation is established from the mesoderm, and subsequent growth of the blood vessels is achieved by endothelial cell migration and proliferation (for review see Risau and Flamme, 1995). Also, in the somites, high angiogenic potential and the generation of angioblasts has been described (Wilting et al., 1995). Angioblasts originated from the somites contribute to the formation of the perineural vascular plexus. There, the migrating angioblasts populate only the ipsilateral side of the body. The only place where angioblasts cross the median plane is the dorsal perineural vascular plexus. Further, invasion of the first ventral sprouts into the neural tube occurs strictly ipsilaterally and is preceeded by the presence of angiogenic signals as shown by the expression of VEGF in the ventral aspect of the neural tube (Aitkenhead et al., 1998). In contrast to signalling molecules such as VEGF, secretion of ChM-I from the floor and roof plate, as well as from the notochord, could serve as an antagonist of angiogenic activities. It might be involved to prevent angioblasts from crossing the median plane of the embryo or in the temporal regulation of vasculogenesis, respectively. As during endochondral bone formation, a well coordinated swich between angiogenic and antiangiogenic events might be required for correct vasculogenesis. Future studies will investigate the distribution of ChM-I protein secreted from the floor and roof plate.

Expression of ChM-I in Heart and Eye

In the developing heart, ChM-I mRNA is expressed in the endocardium, which will form the inner lining of the organ. Cells from this layer detach and invade the initially acellular cardiac jelly, and from there, the atrioventricular cushions are formed. Participation of ChM-I in this process can be assumed because it is still expressed in the atrioventricular cushions, but future studies are required to investigate this in more detail.

ChM-I expressed in the retina might affect the retina itself or the development of neighboring compartments such as the vitreous body. In the human eye, the primary vitreous contains hyaloid vessels, which form the vascular capsule of the lens. The avascular secondary vitreous arises between the primary vitreous and the retina. There, secretion of ChM-I from the retina into the secondary vitreous could prevent the invasion of hyaloid blood vessels and retain the avascularity of the secondary vitreous. Schimdt and Flamme (1998) reported on the viral overexpression of VEGF within the retina of chicken embryos. Despite heavy infection and long-term overexpression of VEGF, they did not observe ectopically induced vascularization of the retina. Only the adjacent choroid and the pecten oculi became hypervascularized. Unlike the retina in mammals, the retina in birds is avascular (Sillman 1973).

The spatiotemporal expression of ChM-I mRNA during chicken embryonic development raises the question whether ChM-I might function early in developmental processes, before the onset of cartilage differentiation.

According to the data on previously published functional studies of ChM-I, a role as a regulator of various angiogenic processes in the chicken embryo is likely. To support this assumption, future studies will be necessary to investigate ChM-I protein expression in chicken embryos and to correlate the protein expression patterns with the mRNA expression patterns presented here. In addition, further crucial infomation will come from the analysis of ChM-I knock out mice. Such studies are in progress.

EXPERIMENTAL PROCEDURES

Chondrocyte Cell Culture

Chondrocytes from bovine articular cartilage were prepared as described by Kuettner et al. (1982). Sternal chondrocytes from 17-day-old chicken embryos were isolated by treatment of the sternae with 0.2% trypsin for 30 minutes at 37°C, followed by two washes with phosphate-buffered saline (PBS) and digestion with 0.1% collagenase overnight in Ham's F12 medium. The released chondrocytes were harvested by centrifugation and plated onto 100-mm tissue culture dishes (Falcon, Becton Dickinson, Franklin Lakes, NJ) in monolayer culture at a density of 1×10^{-5} cells/cm^2. The cells were cultured in Ham's F12 medium supplemented with 10% fetal calf serum (FCS), 100 mg/l ascorbic acid, 100 U/l penicillin, and 100 mg/l streptomycin in a humified atmosphere at 37°C containing 5% CO_2. For modulation of the chondrocytes, all-*trans*-RA was dissolved in 100% ethanol at a concentration of 1 mg/ml and added to the culture medium at a final concentration of 3×10^{-6} M over a period of 3 days.

RNA Isolation

For isolation of total RNA from chondrocytes, the cells were scraped from the cell culture dishes, harvested by centrifugation, and dissolved in lysis buffer (4 M guanidinium thiocyanate, 25 mM sodium citrate, 0.5% sodium-sarcosyl, and 0.7% β-mercaptoethanol). For isolation of total RNA from different soft tissues, a 5-fold volume of lysis buffer was added to the tissue pieces, followed by homogenization. Total cellular RNA was then isolated by cesium chloride centrifugation according to the method of Chirgwin et al. (1979).

Differential Display

Differential display-PCR and reamplification of cDNA fragments after polyacrylamide gel electrophoresis (PAGE) was performed using the instructions of the RNAmap kit from Gene Hunter Co. (Brookline, MA). For cDNA synthesis 0.1 μg total RNA from chondrocytes cultured with or without RA was reverse transcribed with a 5′-T_{12}GC-3′ oligonucleotide (RT-primer). For PCR amplification the RT-primer and 12 different arbitrary 10 mer primers were used: 5′-GGTACTCCAC-3′ (1), 5′-GTTGCGATCC-3′ (2), 5′-GCAATCGATG-3′ (3), 5′-CCGAAGGAAT-3′ (4), 5′-GGATTGTGCG-3′ (5), 5′-CGTGGCAATA-3′ (6), 5′-TAGCAAGTGC-3′ (7), 5′-CAGACCGTTC-3′ (8), 5′-TGCTGACCTG-3′ (9), 5′-AGTTAGGCAC-3′ (10), 5′-AGGGCCTGTT-3′ (11), 5′-CGTCAGTGAC-3′ (12).

Twenty eight differentially displayed amplification products were choosen for further analysis.

RNase Protection Assay

Radiolabeled–antisense RNA was transcribed from the DD-13 cDNA fragment cloned into pGem11zf(+). After linearization by restriction digest with EcoRI, antisense RNA was transcribed in vitro with Sp6 RNA-polymerase. For radioactive labeling, ^{32}P-uridine triphosphate (UTP) (Amersham, Braunschweig) was added to the reaction mixture. Labeled RNA was resuspended in 100 μl hybridization buffer (80% formamide, 40 mM 1,4-piperazinediethanesulfonic acid (PIPES), pH 6.4, 400 mM NaCl, and 1 mM EDTA). Five micrograms of total RNA from chondrocytes and different soft tissues were added to 30 μl of hybridization buffer containing 5×10^5 cpm of the radiolabeled antisense RNA. The samples were incubated for 5 minutes at 85°C, and hybridized at 37°C for 16 hours. For removal of excess probe and unhybridized RNA, 350 μl of ribonuclease digestion buffer (10 mM Tris-HCl, pH 7.5, 300 mM NaCl, 0.5 mM EDTA, pH 8.0, 40 mg/ml RNase A, 2 mg/ml RNase T1) were added to the hybridization mixtures and incubated for 30 minutes at 37°C. The reaction was stopped by adjustment to 0.5% sodium dodecyl sulfate (SDS) and digestion with proteinase K (125 mg/ml) for 15 minutes at 37°C. The samples were phenol extracted, ethanol precipitated, and resuspended in 10 μl RNA loading buffer (80% formamide, 20 mM EDTA, 0.1% bromphenol blue, 0.1% xylene cyanol FF). Protected fragments were heated to 85°C for 5 minutes, electrophoresed through a 6% sequencing gel, and detected by autoradiography after exposure to Hyperfilm x-ray films.

cDNA Library Screening

Chicken ChM-I cDNA was isolated from a oligo(dT) primed lambda Zap library constructed from RNA of stage-22 chicken embryos. Recombinant phage clones were plated on 15-cm agar plates at a density of 50,000 clones per dish. The bacteriophages were transfered to Hybond-N membranes (Amersham) and prepared for hybridization according the manufacturer's instructions.

The membranes were hybridized in 50% formamide, 5 × SSC, 5 × Denhardt's solution, and 100 μg/ml salmon sperm DNA for 16 hours at 42°C in a hybridization oven. After hybridization, the filters were washed twice with 2 × SSC for 5 minutes at room temperature and two times with 2 × SSC/0.1% SDS for 30 minutes at 65°C. The washed membranes were exposed to x-ray films (Kodak) overnight at −20°C with an intensifying screen, and an average of one positive clone per 50,000 phages was detected. The phage clones were transferred into pBluescript plasmids by superinfection with a helper phage (Ex-Assist, Stratagene, La Jolla, CA) following the manufacturer's protocol.

5′-RACE

The 5′ region of the chicken ChM-I mRNA was identified by 5′-RACE using a commercially available Kit (GIBCO/BRL, Karlsruhe). Reverse transcription of

total RNA isolated from sternal chondrocytes of 17-day-old chicken embryos was initiated from a specific primer with the sequence 5′-CCTCAGGCACACGAGCTTTGG-3′ (R1). The sequence was derived from the most 5′ reaching cDNA-fragment isolated from the stage-22 chicken embryonic cDNA library. After reverse transcription, a C-tail was attached to the 5′ ends of the cDNAs using terminal transferase to catalyze the reaction. PCR amplification was performed with a primer containing the sequence 5′-GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG-3′ (Rup) and a ChM-I–specific nested primer with the sequence 5′-CTCCTCCAGCAAATCGGATGC-3′ (R2). The reactions were performed according to the manufacturer's instructions, and amplification products were cloned into pBluescriptSK(+).

DNA Sequencing

The sequence of all cDNA fragments derived from PCR amplification (DD-PCR, 5′-RACE) or screening of a cDNA library was determined on both strands by fluorescent dideoxy sequencing. For analysis of shorter DNA-stretches an ABI-PRISM dye terminator cycle sequencing kit (Perkin Elmer, Foster City, CA) was used, and the labeled fragments were analyzed on an ABI-373 instrument. For longer readings cDNAs were sequenced by a company (MWG-Biotech, Ebersberg).

In Situ Hybridization of Whole Mounts and Sections

In situ hybridization of sectioned chicken embryos or whole mounts was performed with digoxygenin-labeled antisense riboprobes transcribed from the DD-13 cDNA fragment.

Chicken embryos at different stages were fixed overnight in 4% paraformaldehyde (PFA) resolved in PBS and embedded in paraffin wax or gradually transferred into methanol by exchange of the PFA/PBS against increasing concentrations of methanol/PBS. Embryos were stored in methanol at −20°C.

For in situ hybridization of sections, paraffin-embedded embryos were cut at 7 mm and mounted onto microscope slides. The sections were hybridized with digoxygenin-11-UTP labeled antisense riboprobes that were transcribed with Sp6 RNA-polymerase from the DD-13 cDNA-fragment cloned into pGem11zf(+), after linearization of the plasmid with EcoRI.

In situ hybridization was performed according to Jostarndt et al. (1994) with some modifications.

Paraffin sections were dewaxed and rehydrated, fixed in 4% PFA/PBS for 30 minutes, digested with 10 µg/ml proteinase K in 20 mM Tris/HCl, pH 7.0, 1 mM EDTA for 10 minutes and refixed in 4% PFA/PBS for 20 minutes. The hybridization solution was 50% formamide, 75 mM NaCl, 10 mM PIPES, pH 6.8, 1 mM EDTA, pH 8.0, 0.05% heparin, 0.1% bovine serum albumin (BSA), 1% SDS, and 100µg/ml tRNA, and contained the digoxygenin-11-UTP labeled riboprobe at a concentration of 1µg/ml. Hybridization was carried out overnight under a parafilm cover at 60°C. The next day, the parafilm was floated off in 5 × SSC, followed by a 40-minute wash in 0.5 × SSC/20% formamide at 60°C and a 15-minute wash in NTE (0.5 M NaCl, 10 mM Tris/HCl, pH 7.0, 5 mM EDTA). The sections were then treated with 10 µg/ml RNase A in NTE for 30 minutes at 37°C and then washed in NTE for 15 minutes without RNase A, followed by 0.5 × SSC/20% formamide for 30 minutes at 60°C and 2 × SSC for 30 minutes. Antibody incubation and detection were performed according to the manufacturer's descriptions (Boehringer, Mannheim) and Jostarndt et al. (1994). Sections were mounted with coverslips in Kaiser's glycerol gelatine (Merck, Darmstadt) and photographed under a Zeiss Axioplan microscope.

For whole mount in situ hybridization, embryos were rehydrated from the methanol and incubated with the same riboprobes described above for the analysis of sections. The procedure was performed according to Wilkinson (1992) and Rosen and Beddington (1993) with the modifications described by Spörle and Schughart (1998). For documentation, embryos were mounted on a thin layer of agarose under a dissecting microscope (Leica, Bensheim) in darkfield illumination. The embryos were documented with a high-resolution CCD color camera (Fujix HC-2000, Fuji) connected to an Apple PowerMacintosh.

Some of the embryos were embedded in paraffin after the hybridization procedure, cut at 7µm sections and photographed under a Zeiss Axioplan microscope.

ACKNOWLEDGMENTS

We are grateful to Dr. C. Shukunami and Dr. Y.Hiraki for communication, the open discussion of their data, and comments on the manuscript. We also thank Dr. H. Peters for his expert scientific advice, Dr. K. Imai and S. Sachdev for reading of the manuscript, and Dr. N. Sakai for discussion and organization of scientific communication. Deutsche Forschungsgemeinschaft grant Br 1497/1–2,3 was given to P.B.

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