Cloning of a Retinoic Acid-sensitive mRNA Expressed in Cartilage and during Chondrogenesis*

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Retinoic acid (RA) is known to play a role in various aspects of skeletal development in vivo, including morphogenesis, growth plate maturation, and apoptosis. In cell culture, RA treatment of chondrocytes suppresses the differentiated phenotype characterized by production of type II collagen and aggrecan. In an effort to discover molecules involved in regulation of the chondrocyte phenotype or related to developmental processes such as chondrogenesis, mRNAs from bovine chondrocytes cultured with and without RA were amplified by reverse transcription-polymerase chain reaction (PCR) and compared by differential display. PCR products whose expression was inhibited by RA treatment were cloned. One cDNA encodes a molecule we call cartilage-derived retinoic acid-sensitive protein (CD-RAP), and its properties are described here. The full-length bovine CD-RAP mRNA was cloned after amplification by the rapid amplification of cDNA ends procedure, and a part of the rat CD-RAP mRNA was amplified by reverse transcription-PCR using sequence-specific primers. The bovine CD-RAP mRNA contains an open reading frame of 130 amino acids. CD-RAP mRNA expression, as determined by Northern blot analysis and in situ hybridization, was present only in cartilage primordia and cartilage. The inhibition of CD-RAP mRNA expression by RA in vitro was time- and dose-dependent and was tested over concentrations from 10⁻⁸ to 10⁻⁶ M. Southern blot analysis of genomic DNA indicated that CD-RAP was encoded by a single copy gene and that no other genes were closely related. What appears to be the human homologue of CD-RAP was recently isolated and cloned from a melanoma cell line and shown to function as a growth inhibitory protein (Blesch, A., Boberhoff, A.-K., Apfel, R., Behl, C., Hessdoerfer, C., Schmitt, A., Jachimcza, P., Lottspeich, F., Buettner, R., and Bogdahn, U. (1994) Cancer Res. 54, 5695-5701). Neither CD-RAP nor this protein showed any homology to known proteins. We speculate that, in vivo, CD-RAP functions during cartilage development and maintenance.

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The abbreviations used are: RA, retinoic acid; PCR, polymerase chain reaction; CD-RAP, cartilage-derived retinoic acid-sensitive protein; RACE, rapid amplification of cDNA ends; MOPS, 4-morpholinepropanesulfonic acid; PIPES, 1,4-piperazinediethanesulfonic acid; bp, base pair(s).

EXPERIMENTAL PROCEDURES

Materials—General laboratory chemicals, RNase A, and RNase T1 were from Sigma. Dulbecco's modified Eagle's medium, restriction enzymes, and the random priming kit were from Life Technologies, Inc. pGem11Zf', reverse transcriptase, and the pdy(A) tract kit were from Promega. Hybond N paper was purchased from Amersham Corp. The RACE kit was from CLONTECH (Palo Alto, CA). Differential display PCR was performed using primers from Gene Hunter Co. (Brookline, Mass.)
MA), [γ-32P]dCTP (3000 Ci/mmole) and [γ-32P]dATP (5000 Ci/mmole) were from Amersham Corp., and [γ-35S]UTP (800 Ci/mmole) was from DuPont NEN. PBluescript SK(+) doning vector was from Stratagene (La Jolla, CA). Pronase was from Calbiochem. Collagenease (Class 2) was from Worthington. Fetal calf serum was from Hyclone Laboratories (Logan, UT). dNTPs were from Pharmacia Biotech Inc.

Chondrocyte Culture—Chondrocyte cultures were obtained from bovine articular cartilage as described by Kuenttner et al. (17). The isolated cartilage was digested in 1% in 0.75% Pronase in Dulbecco's modified Eagle's medium at 37°C, followed by two washes with phosphate-buffered saline. The cartilage was then digested by incubation with 0.15% collagenase for 5 h at 37°C in Dulbecco's modified Eagle's medium containing 150 mM NaCl, 0.5% sodium citrate, 20 mM MOPS, 5 mM sodium acetate, and 1 mM EDTA. Followed by incubation with 0.02% Ficoll 400, 0.02% polyvinylpyrrolidone, and 0.02% bovine serum albumin. For analysis of RNAs, the membrane in a Stratalinker (Stratagene). For analysis of RNAs, transfer of RNA to Hybond N nylon membranes was carried out by capillary blotting with 20 × SSC, 50% deionized formamide, 20% dextran sulfate, 20 mM dithiothreitol, 1 pg/ml tRNA, 0.7% sodium citrate). After transfer, the nucleic acids were cross-linked to the membrane in a Stratalinker (Stratagene). For analysis of RNAs, cDNA probes were labeled with [γ-32P]dCTP by random priming and hybridized in 50% formamide, 5 × SSC, 5 × Denhardt's solution. The reaction was incubated for 2 h at 42°C. The mRNA template was then removed by digestion with RNase A (5 μg/ml) for 30 min at 37°C. After extraction with phenol/chloroform, the nucleic acids were precipitated by centrifugation and redissolved in 10 μl of formamide loading dye (80% formamide, 10 ml EDTA, 1 mg/ml xylene cyanol FF, and 1 mg/ml bromphenol blue). Half of the reaction was heated for 5 min at 95°C and analyzed by electrophoresis through a 6% sequencing gel. The extended cDNA products were detected by autoradiography.

In Situ Hybridization—Mouse tissue at 13.5 days gestation was prepared for in situ hybridization as described by Sandell et al. (21). Tissue was hybridized with antisense riboprobes of CD-RAP and type IIIA procollagen mRNA. CD-RAP antisense RNA was transcribed from a mouse cDNA fragment cloned into pGem12Z(+) (plasmid), which is described above. The plasmid was linearized by restriction with EcoRI before in vitro transcription. For hybridization, 1 μg of the type IIIA procollagen mRNA, cDNA from a segment spanning exons 1 and 2 was amplified via PCR. The fragments were designed within rat and chick cDNAs, and inserted into the corresponding sites of pGem3Zf(-). The total length of the cDNA insertion was 266 bp. It contained 59 bp of exon 1 and 207 bp of exon 2. Antisense RNAs were transcribed in vitro using Sp6 RNA polymerase in the presence of [γ-35S]UTP. Hybridizations were carried out at 40°C overnight with antisense riboprobes with a specific activity of 3–5 × 108 cpm/μg of RNA. Hybridization solutions (50% formamide, 20% dextran sulfate, 20 μM dithiothreitol, 1 mg/ml RNA, 300 mM NaCl, 10 mM Tris-Cl, pH 7.4, 10 mM NaPO4, pH 6.4, 5 mM EDTA, 0.02% Ficoll 400, 0.02% polyvinylpyrrolidone, and 0.02% bovine serum albumin). Slides were washed according to the procedure of Ausbel et al. (19), followed by exposure to Beta-Max autoradiographic film for 3 days and analysis with an MCID image analysis system. RESULTS

Identification of New Chondrocyte Molecules by Differential Display—Chondrocytes were isolated from bovine articular cartilage and plated on tissue culture dishes at a density of 104 cells/cm2. After 4 days of culture, all of the cells were adhered as monolayers. They expressed mRNA for characteristic extracellular matrix molecules such as type II collagen, aggrecan, and link protein, which were used as markers for the normal chondrocyte phenotype (22). The cells were then treated with RA at a concentration of 3 × 10−6 M for 6 days to modulate the chondrocyte-specific gene expression. At this time, synthesis of cartilage-characteristic type II and XI collagens was not detected (data not shown). Messenger RNA from these cells was
used for differential display by the method of Liang and Pardee (23). We isolated a total of 120 cDNA fragments that appeared only in the display of mRNA from untreated chondrocytes. Six of the 120 cDNA fragments were amplified from independent mRNAs that were down-regulated by RA. One of them was derived from an mRNA encoding a protein that was recently identified as melanoma growth inhibitory activity (EMBL accession number X75450). The expression of this molecule was detected only in a subset of melanoma cell lines, but not in any normal tissue (16). The sequences of the two primers that amplified the molecule for the original display were 5'-TTTTTTTTTTTNG-3' for the downstream PCR primer and 5'-GCAATCGATG-3' for the upstream PCR primer (shown in Fig. 1). Reverse transcription-PCR using these primers amplified a cDNA fragment of 100 bp in length. Northern blot analysis confirmed that the mRNA, which was the original template for the reaction, could be down-regulated by RA in vitro (Fig. 2A). Because of the sensitivity of the expression of this mRNA to RA, it was called CD-RAP.

Full-length Cloning by RACE—From the results of the Northern blot hybridizations, it was determined that the full-length mRNA is a small molecule ~500 bases in length (Fig. 2A). RACE was used to amplify the full-length mRNA to its 5'-end (Fig. 2B). For RACE, we created a specific primer based on DNA sequence information derived from the 100-bp cDNA fragment we had cloned after differential display. This primer was used for reverse transcription of the CD-RAP mRNA. After synthesis of the cDNA, the mRNA template was hydrolyzed with alkaline solution, and the 3'-end of the cDNA, which represents the 5'-end of the mRNA, was ligated to an anchor oligonucleotide using T4 RNA ligase. A primer complementary to the anchor oligonucleotide and a nested downstream primer were used for PCR. The oligonucleotides used for reverse transcription and RACE are shown in Fig. 1. After 35 cycles in a thermocycler, an aliquot of the reaction was analyzed by a 2% agarose gel. Three distinct bands could be detected after agarose gel electrophoresis. Two of them migrated close together and were ~500 bp long. This coincided with the expected size of the full-length CD-RAP mRNA (Fig. 2B).

The cDNA fragments in the 500-bp range were purified from the agarose gel and cloned into pBluescript II SK(+) as 1-88 primer extension product. Nine individual clones were analyzed by dideoxy sequencing. All of them represented cDNA fragments amplified from the CD-RAP mRNA. The clone with the longest cDNA insert was used for further analysis. The sequence information from this clone, the full-length CD-RAP mRNA would be 548 bp long.

Primer Extension Analysis—The 5'-end of the CD-RAP mRNA was confirmed by primer extension analysis. A new primer with a length of 35 nucleotides was synthesized for the primer extension experiments (see Fig. 1). This primer started 88 nucleotides downstream of the sequence that appeared to be

![Fig. 1. Diagram showing CD-RAP cDNA primers and products.](image)

The sequences of the primers are shown under "Experimental Procedures."

After ligation of the anchor primer to the 3'-end of the cDNA, PCR amplification was performed with primers 4 and 5 (see Fig. 1). An aliquot of the reaction was analyzed through a 2% agarose gel. Lane 1, molecular weight marker x x HaeIII fragments; lane 2, PCR products after amplification of one-one hundredth of the anchor ligation reaction; lane 3, PCR products after amplification of one-tenth of the anchor ligation reaction. The size of DNA fragments is indicated in kilobases.

![Fig. 2. A, detection of CD-RAP mRNA by Northern blot analysis. 5 μg of total RNA isolated from chondrocytes grown for 4 days in monolayer (lane 1) or treated for an additional 6 days with RA (lane 2) were electrophoresed through a 1% agarose gel and analyzed by Northern blot hybridization with a radiolabeled CD-RAP cDNA probe, followed by autoradiography. B, RACE of the full-length CD-RAP cDNA. mRNA from chondrocytes was reverse-transcribed with primer 3 (see Fig. 1). After ligation of the anchor primer to the 3'-end of the cDNA, PCR amplification was performed with primers 4 and 5 (see Fig. 1). An aliquot of the reaction was analyzed through a 2% agarose gel. Lane 1, molecular weight marker x x HaeIII fragments; lane 2, PCR products after amplification of one-one hundredth of the anchor ligation reaction; lane 3, PCR products after amplification of one-tenth of the anchor ligation reaction. The size of DNA fragments is indicated in kilobases.](image)

![Fig. 3. Definition of the 5'-end of the CD-RAP mRNA by primer extension analysis. Primer 8 (see Fig. 1) was radiolabeled with [γ-32P]ATP and used for reverse transcription of 1 μg of chondrocyte mRNA. The extended products were analyzed through a 6% sequencing gel and detected by autoradiography. In lane 1, the radiolabeled oligonucleotide alone was loaded. Lane 2 shows the cDNA products extended from the radiolabeled primer. The determination of the length of the synthesized cDNAs, a dideoxy sequencing reaction was loaded next to the primer extension reaction.](image)

The 5'-end of the mRNA based upon the analysis of the RACE product. The primer was end-labeled with [γ-32P]ATP and annealed to 1 μg of mRNA. The mRNA was then reverse-transcribed in the presence of actinomycin D to avoid self-priming. The products of the primer extension reaction were analyzed by electrophoresis through a 6% sequencing gel and detected by autoradiography. In lane 1, the radiolabeled oligonucleotide alone was loaded. Lane 2 shows the cDNA products extended from the radiolabeled primer. The determination of the length of the synthesized cDNAs, a dideoxy sequencing reaction was loaded next to the primer extension reaction.
autoradiography (Fig. 3). For determination of the length of the extended products, a deoxy sequencing reaction was run next to the primer extension reaction. After autoradiography, two major extension products could be detected: a predominant band at 88 nucleotides and a minor band at 117 nucleotides (Fig. 3). The smaller band matched the size we expected for the full-length CD-RAP mRNA. The larger extended product indicates the potential presence of an alternative transcription start site, 29 bases farther upstream. Additional bands that yielded very weak signals were most likely premature termination products.

Fig. 4 shows the complete cDNA sequence of the CD-RAP clone. The bovine sequence spans the complete mRNA molecule from the 5′-start site to the poly(A) tail. A cDNA encoding a protein responsible for melanoma inhibitory activity (16) appears to be the human homologue of CD-RAP, and its sequence is shown in Fig. 4. No other homologous proteins were found in the data bank. The CD-RAP cDNA sequence revealed an open reading frame of 130 amino acids encoding a protein with a typical hydrophobic leader sequence followed by a unique domain protein. No linkage sites for N-linked carbohydrate (Asn-Gly-Ser/Thr) or O-linked carbohydrate (Ser-Gly) were found. The protein included four cysteine residues.

To screen for tissue specificity, a rat cDNA clone was prepared using the primers shown in Fig. 1. This cDNA probe was amplified by reverse transcription-PCR from total RNA isolated from skeletal cartilage of a fetal rat. The primers for reverse transcription and PCR were chosen from identical sequences in bovine and human CD-RAP cDNAs. Fig. 4 compares this 322-bp rat cDNA fragment with bovine sequence. The DNA conservation between these species is 85.5% for human and bovine, 90% for human and rat, and 87% for rat and bovine.

Southern Blot Analysis—Southern blot analysis was used to assess copy number of the CD-RAP gene and whether closely...
related genes could be found. 5 μg of bovine genomic DNA were digested with the restriction endonuclease EcoRI or PstI. The digested DNAs were separated by electrophoresis through a 0.7% agarose gel and blotted onto nylon membranes. After hybridization and autoradiography, a single band was detected, indicating that CD-RAP is present as a unique gene (Fig. 5). When the blot was probed under low stringency conditions, no additional bands were observed, indicating that there are no other closely related genes in the genome. Similar results were obtained when the rat probe was tested on rat genomic DNA (data not shown).

Tissue-specific Expression of CD-RAP mRNA—To investigate the tissue distribution of CD-RAP mRNA, total RNAs from different rat tissues such as heart, lung, liver, kidney, skeletal muscle, and spleen were used for Northern blot hybridization. As a positive control, we included total RNA from skeletal muscle, and spleen were used for Northern blot hybridization. After exposure to x-ray films for up to 2 weeks, a hybridization signal was detected only in total RNA from the rat fetal skeletal cartilage (Fig. 6A). To determine whether the absence of CD-RAP mRNA was related to a developmental stage, we tested for CD-RAP mRNA expression in a similar spectrum of fetal bovine tissues. In addition, RNA from different cartilages was analyzed, including the vertebral column, knee, and shoulder. Northern blot analysis showed the presence of CD-RAP mRNA in total RNA isolated from all of the cartilaginous tissues, but from none of the other tissues that were tested (Fig. 6B).

To confirm the tissue distribution of CD-RAP mRNA, in situ hybridization was performed in a mouse embryo. Sections of tissues from a 13.5-day embryo were used. A representative set of hybridizations is shown in Fig. 7. A probe for the mRNA of type II procollagen was used to localize cartilage primordia and to confirm the developmental stage of the mouse embryo. Comparison of the two hybridizations shows that CD-RAP mRNA is colocalized with the type II procollagen mRNA in the cartilage primordia of the developing vertebral column, ribs, cranium, and nasal septum. Interestingly, no expression of CD-RAP mRNA occurred in the non-cartilaginous tissues of the otic vesicle, where type II collagen expression is abundant.

Regulation of Gene Expression by RA—Since the concentration of RA originally used for the modulation of chondrocytes was much higher than physiological concentrations, we examined the effect on gene expression of lower RA concentrations (Fig. 8A). The effects of three different RA concentrations (3 × 10⁻¹₀, 3 × 10⁻⁷, and 3 × 10⁻⁸ M) were tested. This represents a range from more physiological concentrations (3 × 10⁻⁸ M) to concentrations routinely used for dedifferentiation of chondrocytes in vitro (3 × 10⁻⁶ M). During culturing with RA, the morphology of the chondrocytes changed from a polygonal to a fibroblast-like cell shape as described previously (11). Total RNA was isolated after 2, 4, and 6 days of RA treatment. Under these conditions, CD-RAP mRNA expression showed dose- and time-related down-regulation by RA at all concentrations tested (Fig. 8A). In cells treated for 2, 4, and 6 days with the ethanol carrier only, expression of CD-RAP mRNA did not significantly change. The weaker signals after 4 and 6 days of ethanol treatment in Fig. 8B (lanes 3 and 4) are due to different amounts of total RNA loaded onto the gel. This is shown by the levels of elongation factor 1 mRNA detected after hybridization of the same blots with an elongation factor 1-specific cDNA probe (Fig. 8B, ELF1).

**DISCUSSION**

An mRNA encoding a cartilage protein, CD-RAP, has been cloned from cultured chondrocytes. It is down-regulated by RA in vitro in a time- and dose-dependent manner. The CD-RAP mRNA encodes a unique 130-amino acid protein containing a potential signal peptide, four cysteine residues, no obvious processing sites, and no sites of N- or O-linked glycosylation. We have characterized the full-length mRNA, demonstrated the start site of transcription, and showed that this gene is...
in chick limbs (25, 26) possibly through the induction of alkaline phosphatase, and osteocalcin synthesis followed by mineralization and aggrecan. RA-induced stimulation of type X collagen, aldrate extracellular matrix molecules such as type II collagen during growth plate maturation (28). In the growth plate, one differentiation of chondrocytes to hypertrophic chondrocytes was observed in rat fetal skeletal cartilage and in all bovine fetal tissues; however, abundant expression was synthesized only by chondrocytes and chondroprogenitor cells. No expression was detected in RNA from various other adult rat or fetal bovine tissues; however, abundant expression was observed in rat fetal skeletal cartilage and in all bovine fetal cartilages tested. In embryonic tissue, CD-RAP mRNA is expressed in the cartilage primordia of 13.5-day mouse embryos. At this time, the cartilage primordia of the skeleton begins to form. Our results indicate that CD-RAP mRNA is synthesized by cells in the cartilage primordia and in differentiated cartilage. In the mouse embryo, expression of CD-RAP mRNA is similar to that of type II procollagen mRNA (21, 24). Current studies are underway to determine the temporal sequence of expression of type II procollagen splice forms and CD-RAP.

Previous studies have indicated that RA is potentially involved in cartilage differentiation. Although not itself a morphogen in vivo, RA is a capable of initiating pattern formation in chick limbs (25, 26) possibly through the induction of hox genes (27) in mesenchymal cells. RA can further stimulate the differentiation of chondrocytes to hypertrophic chondrocytes during growth plate maturation (28). In the growth plate, one role of RA may be to suppress the expression of typical chondrocyte extracellular matrix molecules such as type II collagen and aggrecan. RA-induced stimulation of type X collagen, alkaline phosphatase, and osteocalcin synthesis followed by mineralization and apoptosis may be important in vivo for development of the hypertrophic chondrocyte phenotype (29). The RA-dependent differences of CD-RAP mRNA levels in vitro may provide information relevant to understanding cartilage differentiation.

The cDNA we have characterized is almost certainly the bovine version of a molecule recently characterized in a melanoma cell line (16). Blesch et al. (16) showed that the expressed protein has growth inhibitory activity on certain melanoma cell lines. They found its expression limited to these melanoma cells and a neuroepithelial cell line. Addition of melanoma growth regulatory protein to the culture medium caused melanoma cells to round up and to decrease their incorporation of [3H]thymidine. It is not clear how these activities could relate to chondrogenesis; however, a change in cell shape and decrease in proliferation are characteristics of the condensation phase of chondrogenesis. The relationship between the expression of CD-RAP in malignant cells and normal physiological expression by chondrosarcomas remains to be examined. Preliminary studies indicate the presence of CD-RAP mRNA in rat and human chondrosarcomas. Further studies will be needed to clarify the role of CD-RAP in the development and maintenance of cartilage.

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