Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 8633–8637, September 1993
Developmental Biology

Cartilage homeoprotein 1, a homeoprotein selectively expressed in chondrocytes

(chondrogenesis/homeodomain)

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Communicated by J. E. Rall, June 7, 1993 (received for review April 13, 1993)

ABSTRACT **We identified a rat cDNA that encodes cartilage homeoprotein 1 (Cart-1). The deduced amino acid sequence of Cart-1 contains a paired-type homeodomain. Northern blot hybridization and RNase protection assay revealed that Cart-1 RNA was present at high levels in a well-differentiated rat chondrosarcoma tumor and in a cell line derived from this tumor. Cart-1 RNA was detected in primary mouse and rat chondrocytes but not in various fibroblasts including mouse 10T½ cells, NIH 3T3 cells, BALB 3T3 cells, and rat skin fibroblasts. It was also undetectable in mouse C2 myoblasts, S194 myeloma cells, and embryonic stem cells. Cart-1 RNA was present at a very low level in testes but was not detected in other soft tissues of 8-week-old rats. *In situ* hybridization of rat embryos between 14.5 and 16.5 days post coitum revealed relatively high levels of Cart-1 RNA in condensed prechondrocytic mesenchymal cells and in early chondrocytes of cartilage primordia. The levels of Cart-1 RNA were lower in mature chondrocytes. No hybridization was observed in brain, spinal cord, heart, spleen, gastrointestinal tract, liver, and muscle. We speculate that Cart-1 has a role in chondrocyte differentiation.**

In vertebrates, bones form through either an endochondral or an intramembranous mechanism. In endochondral bone formation, a cascade of events occurs, including the condensation of mesenchymal cells in which the expression of cartilage-specific genes is gradually switched on, the differentiation of the condensed mesenchymal cells into mature chondrocytes as cartilage-specific extracellular matrix accumulates, and the further differentiation of the mature chondrocytes into hypertrophic chondrocytes (1). The hypertrophic chondrocytes are later replaced by invading osteoblast precursors. Recent evidence has indicated that bone morphogenetic proteins, which are members of the transforming growth factor β superfamily of cytokines, can cause cartilage and bone formation both *in vitro* and *in vivo* (2, 3). Because transcription factors control numerous aspects of development and cellular differentiation (4, 5), one can postulate that specific transcription factors probably also play important roles in determining the lineage specificity of prechondrocytic mesenchymal cells and in further controlling the onset of cartilage-specific gene expression.

Homeoproteins play major roles as transcription factors during embryonic development (6–9). These proteins can be subdivided into different classes based on their sequence homology in the homeodomain (10). *Hox* genes are arranged in four clusters in mammalian genomes. The spatial and temporal expression of individual members of these clusters along the anterior–posterior axis of the embryo controls the segmentation process during early development (9). Other types of homeoproteins, such as HNF1 in liver (11), TTF1 in thyroid (12), and Pit1 in pituitary gland (5), participate in the regulation of tissue-specific gene expression. Still other homeoproteins including MHox (13), S8 (14), Mox1, and Mox2 (15) show an expression pattern that is restricted to mesenchymal cells and their derivatives. However, to our knowledge, no homeoproteins selectively expressed in the chondrocytic lineage have been described.

Here we report the identification and characterization of a cDNA that encodes cartilage homeoprotein 1 (Cart-1).§ Cart-1 is selectively expressed in chondrocytes. *In situ* hybridization of rat embryos revealed that the Cart-1 RNAs were present at high levels in the condensed mesenchymal cells that were the precursors of mature chondrocytes.

MATERIALS AND METHODS

RNA Isolation. Total RNA from different tissues and cell lines was purified by guanidine thiocyanate/cesium chloride centrifugation and phenol/chloroform extraction. Poly(A)$^+$ RNA was isolated by one or two rounds of chromatography over type II oligo(dT)-cellulose (Collaborative Research).

Molecular Cloning of Cart-1. First-strand cDNA was synthesized using a poly(A)$^+$ RNA preparation from a rat chondrosarcoma tumor with avian myeloblastosis virus reverse transcriptase and a dT-adaptor. This dT-adaptor contained the *Not* I recognition sequence followed by 18 dT residues, 5′-AACCCGGCTCGAGCGGCCGCTTTTTT-TTTTTTTTTTTT-3′ (underlined residues are for a *Not* I recognition site). Rapid amplification of cDNA ends by the polymerase chain reaction (PCR) was performed with first-strand cDNA using a Hom primer and a Not primer. This Hom primer, 5′-A(G)TC(G)AAG(A)A(G)TG(C/A/T)TGGT-TC(T)CAG(A)AA-3′, was derived from the 7-aa consensus sequence V(I)KV(I)WFQN present in helix 3 of homeodomains. The Not primer, 5′-AACCCGGCTCGAGCGGC-CGCTTTT-3′, was identical to the 5′ portion of dT-adaptor. The PCR products were digested with *Not* I and then cloned into Bluescript IIKS+ (Stratagene) between the *Eco*RV and *Not* I sites. Individual colonies that hybridized with a Hom probe were chosen for sequencing. This Hom probe, 5′-A(G)TC(G)AAG(A)A(G)TG(C/A/T)TGGTTC(T)CA-G(A)AAT(C)A(C)G(A)G(A)A(C)GG(A)-3′, was derived from the 9-aa consensus sequence V(I)KV(I)WFQNR(K)R present in helix 3 of homeodomains. A 700-bp Cart-1 cDNA fragment, located mostly 3′ to the homeobox, was obtained and used to screen a rat chondrosarcoma cDNA library under conditions of high stringency. The cDNA clones that hybrid-

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Abbreviations: Cart-1, cartilage homeoprotein 1; p.c., post coitum.

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§The sequence reported in this paper has been deposited in the GenBank data base (accession no. L14018).

ized were sequenced by the dideoxynucleotide termination method (16).

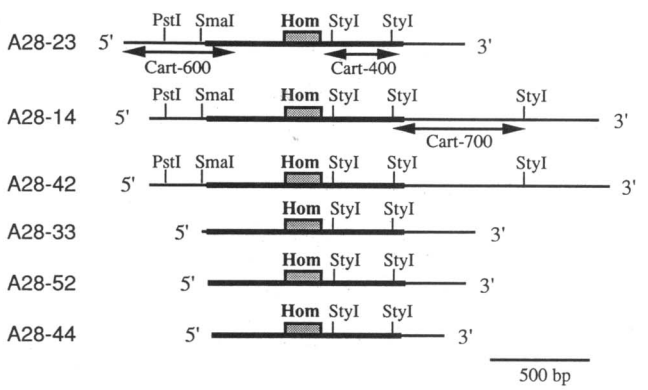
Northern Blot Hybridization. About 5–10 μg of poly(A)$^+$ RNA from various cell lines or tissues was fractionated on a formaldehyde-agarose gel and transferred to GeneScreen-*Plus* membranes (Schleicher & Schuell). Prehybridization was performed in 5× SSPE/10× Denhardt's solution/denatured sheared herring sperm DNA (200 μg/ml)/0.5% SDS at 60°C overnight (1× SSPE = 0.18 M NaCl/10 mM phosphate, pH 7.4/1 mM EDTA). Hybridization was performed in 5× SSPE/5× Denhardt's solution/denatured sheared herring sperm DNA (200 μg/ml)/0.2% SDS and DNA probe at 10^6 cpm/ml labeled with [α-^{32}P]dCTP at 60°C overnight. Final washing was done in 0.2× SSPE/0.1% SDS at 60°C until the background was reduced.

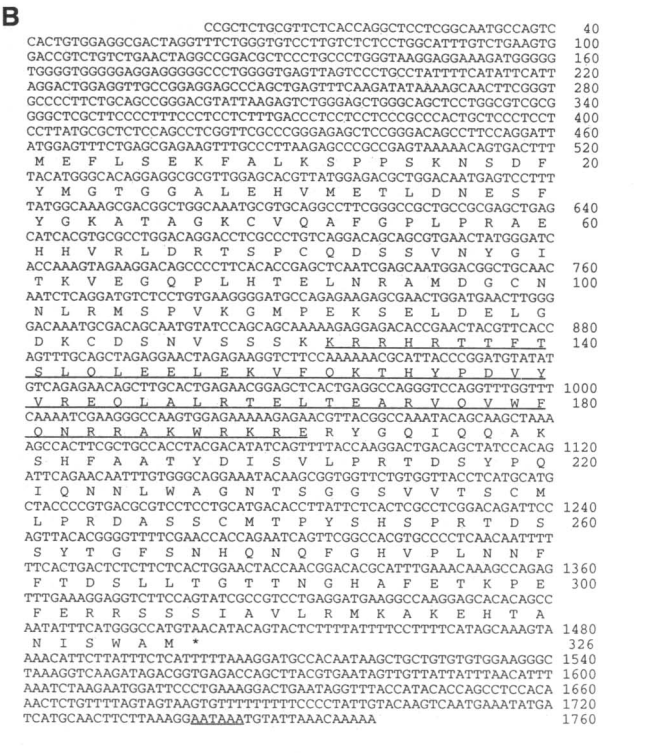
RNase Protection Assay. Plasmid Cart-300 was constructed by inserting a 307-bp *Sty* I fragment from A28-23 (Fig. 1*A*) into the *Eco*RV site of pBluescript IIKS+. Plasmid mCart-200 was created by inserting a PCR-generated mouse cDNA fragment (corresponding to nt 568–788 of the rat cDNA sequence of Fig. 1*B*) into the *Eco*RV site of Bluescript KSII+. Cart-300 DNA and mCart-200 DNA were linearized by digestion with *Eco*RI and *Xho* I, respectively. Antisense ribonucleotide probes were synthesized with T3 RNA polymerase for Cart-300 DNA and with T7 RNA polymerase for mCart-200 DNA using [α-^{32}P]UTP. Total RNA (10 μg) from each tissue and cell type was used for hybridization. The assays were performed using the RPAII ribonuclease protection kit (Ambion, Austin, TX).

***In Situ* Hybridization.** Plasmid Cart-600 was constructed by inserting a 659-bp fragment located at the 5′ end of A28-23 into Bluescript IIKS+ between *Xho* I and *Eco*RV sites. Plasmid Cart-400 was created by inserting a PCR-generated fragment covering the sequence 3′ to the homeodomain (bp 1016–1443, Fig. 1*B*) into the *Eco*RV site of Bluescript IIKS+. Plasmid Cart-700 was created by inserting a 700-bp *Sty* I DNA fragment from A28-14 into the *Eco*RV site of Bluescript IIKS+. *In situ* hybridization was performed on sections from rat embryos at different stages with antisense RNAs synthesized from Cart-600, Cart-400, and Cart-700 and labeled with uridine 5′-[α-[^{35}S]thio]triphosphate. Pregnant Sprague–Dawley rats were sacrificed on various days post coitum (p.c.) and the embryos were fixed in 10% (vol/vol) formalin or 4% (wt/vol) paraformaldehyde in phosphate-buffered saline for 24 hr; the embryos were then dehydrated in a series of increasing concentrations of ethanol. The dehydrated embryos were cleared in xylenes before being embedded in paraplast. The embedding was performed at 60–65°C with three changes of paraplast during a 2-hr period. Six- to 8-μm-thick sections were mounted onto either silane-coated slides (Histology Control Systems, Glen Head, NY) or Superfrost Plus slides (Fisher Scientific). *In situ* hybridization was then performed as described by Wilkinson *et al.* (22) and Sassoon *et al.* (23). Hybridization was performed at 48–53°C for 12–16 hr. Two 30-min high-stringency washes were performed with 2× standard saline citrate/50% (vol/vol) formamide at 60–65°C. Autoradiography was carried out using NTB-2 Kodak emulsion, and the slides were exposed for 7–14 days at 4°C. Microphotographs were taken using both light- and dark-field optics.

RESULTS

Molecular Cloning of Cart-1 cDNA. A cDNA fragment, which contained 700 bp mostly located 3′ to a sequence coding for a homeodomain helix 3-like segment, was obtained by rapid amplification of cDNA ends by PCR and used to screen a rat chondrosarcoma cDNA library. Several cDNA clones of 1–2.5 kb were isolated, and their overlapping regions were determined by restriction enzyme digestion and DNA sequencing (Fig. 1*A*).





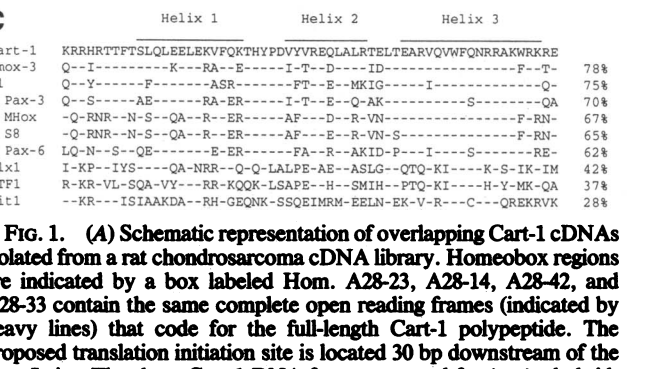


FIG. 1. (*A*) Schematic representation of overlapping Cart-1 cDNAs isolated from a rat chondrosarcoma cDNA library. Homeobox regions are indicated by a box labeled Hom. A28-23, A28-14, A28-42, and A28-33 contain the same complete open reading frames (indicated by heavy lines) that code for the full-length Cart-1 polypeptide. The proposed translation initiation site is located 30 bp downstream of the *Sma* I site. The three Cart-1 DNA fragments used for *in situ* hybridization on rat embryo sections are indicated by horizontal bars with arrows. (*B*) Nucleotide sequence of clone A28-23 and deduced amino acid sequence of Cart-1. The homeodomain and putative polyadenylylation signal are underlined. (*C*) Amino acid sequence comparison of the rat Cart-1 homeodomain with other homeodomains. The percentage of amino acid sequence identity with Cart-1 is indicated on the right. smox-3 was isolated from *Schistosoma mansoni* (17); Al is a *Drosophila* homeoprotein (18); m Pax-3 (19), m MHox (13), m S8 (14), m Pax-6 (20), Dlx1 (21), TTF1 (12), and Pit1 (5) are mouse homeoproteins. Dashes represent amino acid residues identical to those of Cart-1.

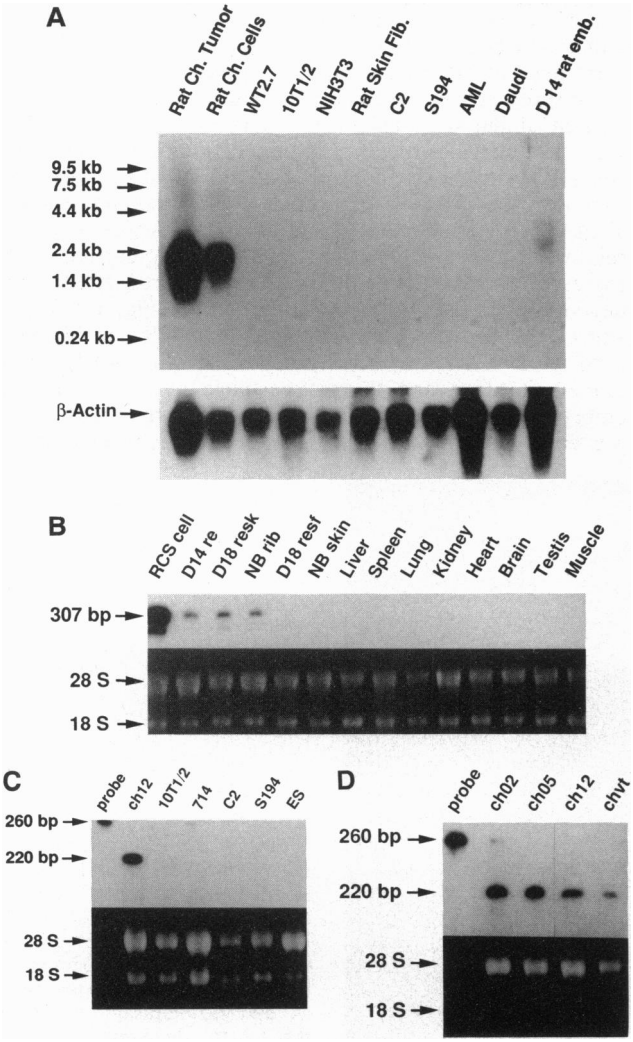


FIG. 2. (*A*) Northern blot hybridization of Cart-1 RNA. The 700-bp DNA fragment, generated by rapid amplification of cDNA ends by the PCR, located 3′ to the Cart-1 homeobox was hybridized to poly(A)$^+$ RNA under moderately stringent conditions. Lanes: Rat Ch. Tumor, rat chondrosarcoma tumor; Rat Ch. Cells, a cell line derived from the rat chondrosarcoma tumor; WT2.7, a mouse chondrosarcoma-derived fibroblastic cell line that did not express detectable levels of proα1(II) collagen RNA; 10T½, mouse C3H 10T½ cell line; NIH 3T3, mouse NIH 3T3 cell line; Rat Skin Fib., rat skin fibroblasts; C2, mouse C2 myoblasts; S194, mouse S194 myeloma cells; AML, human acute myelocytic leukemia blood sample; Daudi, a human B-lymphoma cell line; D14 rat emb., 14-day-old rat embryos. Approximately 10 μg of RNA was used for the rat chondrosarcoma tumor and the 14-day-old rat embryos, $\approx$5 μg of RNA was used for all other samples. The same membrane was stripped and rehybridized with ^{32}P-labeled human β-actin cDNA probe and is shown below the Cart-1 blot. (*B*) RNase protection assay using an antisense RNA probe generated from Cart-300 with RNAs from different rat tissues and cell types. Lanes: RCS cell, a rat chondrosarcoma cell line; D14 re, 14-day-old rat embryos; D18 resk, 18-day-old rat embryo skeletal tissues; NB rib, newborn rat rib cartilage; D18 resf, a mixture of soft tissues from 18-day-old rat embryos; NB skin, newborn rat skin. Liver, spleen, lung, kidney, heart, brain, testis, and muscle are tissues from 8-week-old rats. (*C*) RNase protection assay using antisense RNA probe generated from mCart-200 and RNAs from different mouse cells. Lanes: ch12, primary mouse chondrocytes cultured as a monolayer for 12 days; 10T½, mouse 10T½ cells; 714, a mouse fibroblast subclone derived from BALB 3T3 cells; C2, a mouse myoblast cell line; S194, a mouse myeloma cell line; ES, a mouse embryonic stem cell line. (*D*) RNase protection assay using an antisense RNA probe generated from mCart-200 with RNAs isolated from primary mouse chondrocytes in monolayer cultures. Lanes: ch02, primary mouse chondrocytes cultured for 2 days; ch05, primary mouse chondrocytes cultured for 5 days; ch12, primary mouse chondrocytes cultured for 12 days; chvt, primary mouse chondrocytes transformed with a retrovirus containing the simian virus 40 large tumor antigen gene. To control the RNA levels in *B–D*, each RNA sample (5 μg) was electrophoresed in formaldehyde/agarose gel and stained with ethidium bromide; these gels are shown below the RNase protection assays.

Fig. 1*B* presents the DNA sequence and deduced amino acid sequence of clone A28-23. The DNA sequence had a single long open reading frame of 328 aa and a long 5′ untranslated sequence of 460 bp. The DNA sequences of the other clones that were sequenced all had the same open reading frame. Clones A28-14 and A28-42 contained longer 3′ untranslated regions, perhaps because of differential usage of polyadenylylation signals. The sequence surrounding the proposed translation initiation codon was in good agreement with the translation initiation consensus sequence (24). The 5′ untranslated region contained three additional AUGs that were all closely followed by in-frame termination codons.

The sequence between aa 132 and 191 was homologous to other homeodomains. As shown in Fig. 1*C*, this sequence had a relatively high degree of sequence identity with the homeodomain of smox-3 protein of *S. mansoni* (17) (78%) and with that of the *Drosophila* aristaless protein (18) (75%). It showed somewhat lesser sequence identity with the homeodomains of the mouse MHox (13) and S8 (14) polypeptides, two closely related homeoproteins that display a broad expression in mesenchyme during embryonic development.

Selective Expression of Cart-1 RNA in Chondrocytic Cells. Northern blot hybridization showed that a cDNA probe containing 700 bp mostly 3′ to the homeobox specifically hybridized with one major 2.2-kb RNA species that was present in the rat chondrosarcoma tumor from which Cart-1 was cloned, in the cell line derived from the tumor, and at low concentrations in 14-day-old rat embryos. No hybridization signal was detected in RNAs from WT2.7 cells, 10T½ cells, mouse NIH 3T3 cells, rat skin fibroblasts, mouse C2 myoblasts, S194 mouse myeloma B cells, human acute myelocytic leukemia cells, and human B-lymphoma Daudi cells (Fig. 2*A*). WT2.7 was a mouse chondrosarcoma-derived fibroblastic cell line (25) in which we were unable to detect any RNA for the chondrocyte-specific proα1(II) collagen by Northern blot hybridization (data not shown).

We also used the more-sensitive RNase protection assays to examine the expression pattern of Cart-1 in various rat tissues and cell types. These included a rat chondrosarcoma cell line that was derived from the chondrosarcoma tumor and displays a chondrocytic phenotype; day 14 rat embryos; day 18 rat embryo skeletal tissues; newborn rat rib cartilage; day 18 rat embryo soft tissues; newborn rat skin; and liver, spleen, lung, kidney, heart, brain, muscle, and testes from 8-week-old rats (Fig. 2*B*). Interestingly, Cart-1 RNA was detected in the rat chondrosarcoma cell line, day 14 rat embryos, day 18 rat embryo skeletal tissues, and newborn rat rib cartilage. A very low level of expression was detected in testes after longer autoradiography. Cart-1 RNA was not detected in other tissues. We also examined the levels of Cart-1 RNA in various mouse cell types by RNase protection assays, using as probe antisense RNA generated from mCart-200. As shown in Fig. 2*C*, Cart-1 RNA was detected only in primary mouse chondrocytes, but not in WT2.7 cells, 10T½ cells, 714 cells (a subclone of the BALB 3T3 cell line), C2 myoblasts, S194 myeloma cells, and embryonic stem cells. The tissue distribution of Cart-1 RNA in 4-week-old mice was identical to that of 8-week-old rats (data not shown). Fig. 2*D* shows that primary mouse rib chondrocytes cultured *in vitro* for various periods of time still expressed Cart-1 RNA. Mouse chondrocytes infected with a recombinant retrovirus

containing the simian virus 40 large tumor gene under the control of the mouse sarcoma virus long terminal repeat (26) also contained low levels of Cart-1 RNA. We concluded from these experiments that the Cart-1 gene was selectively expressed in chondrosarcoma tumor and cells derived from this tumor, uncultured chondrocytes, cultured chondrocytes after several passages, and even in transformed dedifferentiated chondrocytes in which no proα1(II) collagen RNA was detected.

Expression of Cart-1 RNA in Rat Embryos. To obtain additional knowledge regarding the cell specificity of Cart-1 expression during embryonic development, we examined the distribution of Cart-1 RNA by *in situ* hybridization of rat embryos 14.5–16.5 days p.c. Rat embryonic development in this period is comparable to mouse embryonic development 13–15 days p.c. The 14.5-day-p.c. rat embryos represent a developmental stage during which chondrocytes are active in depositing cartilage-specific extracellular matrix. ^{35}S-labeled antisense RNA probes from Cart-600, Cart-400, and Cart-700 were used in these experiments. *In situ* hybridization with these three probes revealed identical patterns of hybridization on similar sections, strongly suggesting that the signals were not due to nonspecific binding. In addition, we also used sense RNA probes as controls that did not show detectable hybridization above background levels (data not shown). These experiments showed that Cart-1 RNA was present at relatively high concentrations in developing cartilage. The representative examples in Fig. 3 show Cart-1 RNA in the developing ribs of a 14.5-day-p.c. embryo (Fig. 3 *A* and *G*) and a 16.5-day-p.c. embryo (Fig. 3 *B* and *H*), in Meckel's cartilage of a 14.5-day-p.c. embryo (Fig. 3 *C* and *I*), in the cricoid cartilage and thyroid cartilage of a 16.5-day-p.c. embryo (Fig. 3 *D* and *J*), in the nasal septum of a 16.5-day-p.c. embryo (Fig. 3 *E* and *K*), and in the iliac primordium of

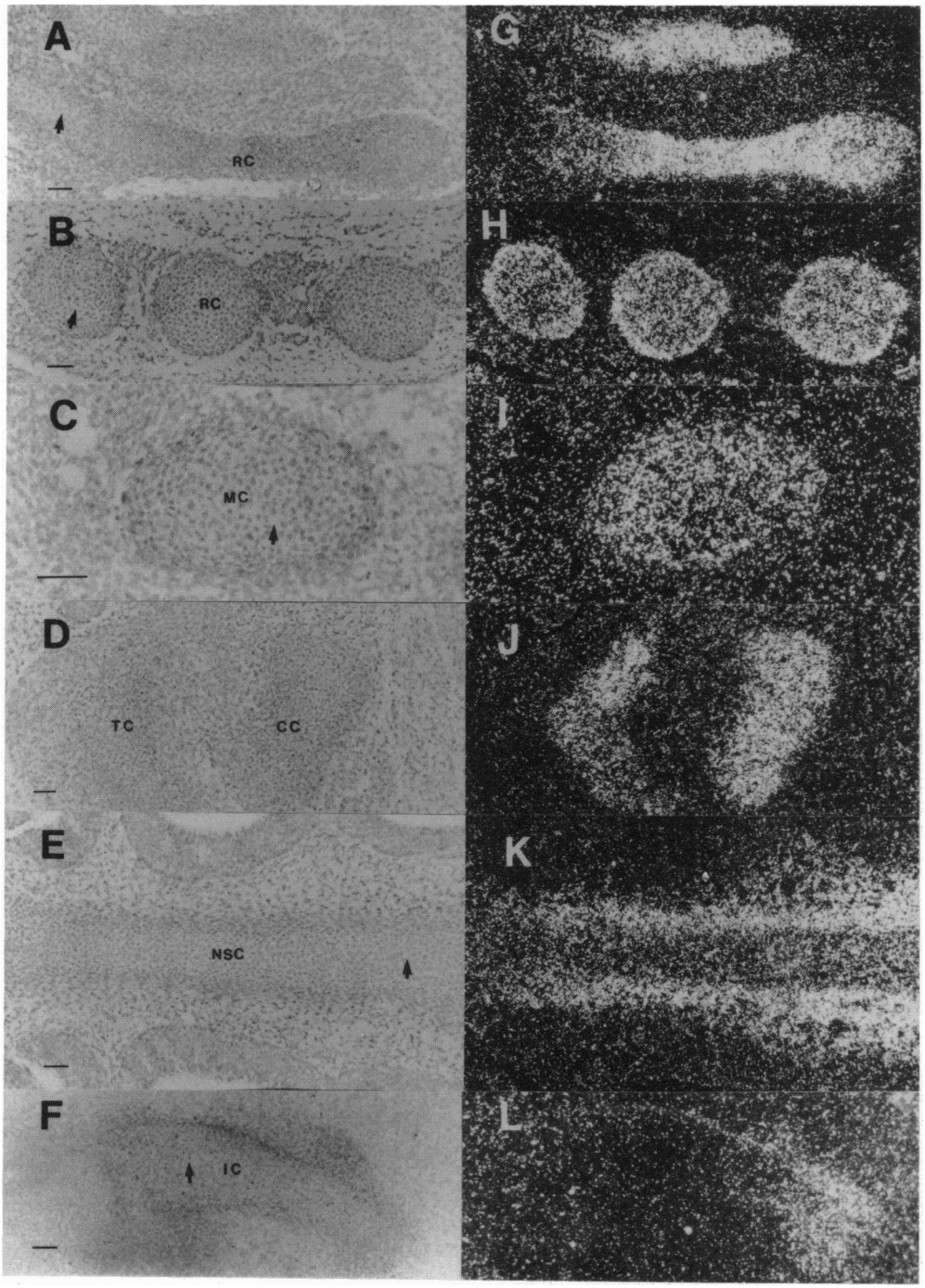


FIG. 3. *In situ* hybridization of rat embryo sections using antisense RNAs from Cart-400 (*G–K*) or Cart-600 (*L*). (*A–F*) Bright-field microphotographs. (*G–L*) Dark-field micrographs of the sections in *A–F*, respectively. (*A* and *G*) Parasagittal section through the developing ribs of a 14.5-day-p.c. rat embryo. Cart-1 RNA levels were higher in the condensed mesenchymal cells (prechondrocytes) than in mature chondrocytes (indicated by arrow). (*B* and *H*) Cross-section of ribs of a 16.5-day-p.c. rat embryo. (*C* and *I*) Meckel's cartilage of 14.5-day-p.c. rat embryo. Cart-1 RNA levels were higher in the periphery than in the center, where the chondrocytes were more mature than those in the periphery. (*D* and *J*) Thyroid cartilage and cricoid cartilage from a 16.5-day-p.c. rat embryo sagittal section. (*E* and *K*) Cross-section through the nasal septum cartilage of a 16.5-day-p.c. rat embryo. Cart-1 RNA levels were higher in prechondrocytes surrounding the relatively mature chondrocytes (arrow). (*F* and *L*) Iliac cartilage from a cross-section through the pelvis of a 15-day-p.c. rat embryo. Cart-1 RNA levels were higher in the less mature chondrocytes or prechondrocytes than in the mature chondrocytes (indicated by arrow). RC, rib cartilage; MC, Meckel's cartilage; TC, thyroid cartilage; CC, cricoid cartilage; NSC, nasal septum cartilage; IC, iliac cartilage. (Bars = 100 μm.)

a 15-day-p.c. embryo (Fig. 3 *F* and *L*). The levels of Cart-1 RNA were lower in mature chondrocytes indicated by arrows in Fig. 3. We also noted that Cart-1 RNA was absent from a number of tissues, including brain, spinal cord, heart, muscle, skin, liver, spleen, and the gastrointestinal system. These findings indicated that the Cart-1 gene was selectively expressed in chondrocytes, that it was expressed at relatively high levels in prechondrocytes and early chondrocytes, and that the levels of Cart-1 RNA were lower in mature chondrocytes.

DISCUSSION

We identified a rat cDNA that encodes a homeoprotein, Cart-1. The deduced amino acid sequence of the Cart-1 homeodomain shows 78% sequence identity to that of smox-3 (17), a homeoprotein from *S. mansoni*, and 75% sequence identity to the homeodomain of the *Drosophila aristaless* protein (18). The Cart-1 homeodomain also shows substantial sequence identity with Pax-3 (19), MHox (13), and S8 (14) but shows significantly less identity with other homeodomains (Fig. 1*C*). This suggests that Cart-1 may belong to the paired (Prd) family of homeoproteins, although in Cart-1 glutamine replaces a conserved serine at position 9 in helix 3 of the homeodomain. Outside the homeodomain, no other sequence motifs found in other homeoproteins or in other transcription factors were identified in Cart-1.

In overlapping cDNAs that we cloned, we found no evidence for alternatively spliced Cart-1 RNAs. In three cDNAs, relatively large 5′ untranslated regions were identified that represented variously extended cDNAs corresponding to the same mRNA. The 5′ untranslated sequence shown in Fig. 1*B* is also part of a single exon of >600 bp in a genomic DNA clone (data not shown).

Both Northern blot hybridization and RNase protection assays showed a unique pattern of expression of Cart-1 RNAs in various tissues and cell types. Cart-1 RNA was found in primary chondrocytes, a highly differentiated rat chondrosarcoma tumor from which the Cart-1 cDNAs were isolated, and a cell line that was derived from this tumor. Cart-1 RNA was not detected in other tissues or cell types, although a low level of Cart-1 mRNA was detected in testes by RNase protection assays. The low-level expression of Cart-1 in testes raises the question about its possible role in this tissue. It will be necessary to determine in which cells of the testes Cart-1 is expressed and also whether the Cart-1 polypeptide is present in this tissue. It is interesting to note that chondrocytes transformed with the simian virus 40 large tumor antigen still expressed low levels of Cart-1 RNA even after numerous passages when proα1(II) collagen RNA was no longer detected in these cells. *In situ* hybridization of rat embryos at 14.5–16.5 days p.c. showed relatively high levels of Cart-1 RNA in a number of cartilages and precartilaginous primordia. In each case, high RNA levels appeared to be present in condensed mesenchymal cells and early chondrocytes, whereas the levels of Cart-1 RNA were lower or undetectable in more mature chondrocytes. It should be noted that it is difficult to detect low RNA levels by *in situ* hybridization and that this method is less sensitive than the RNase protection assay, which detected Cart-1 RNA in rib chondrocytes from newborn rats and mice. Moreover, it is also possible that the Cart-1 polypeptide persists in cells and continues to exert its effects despite the decreased RNA levels.

Identifying Cart-1 expression in early development might help trace and identify the cells that are presumably committed to the chondrocytic lineage during early embryonic development. Chondrogenesis is a multistep pathway that includes the fate determination of mesenchymal cells to become prechondrocytes, their condensation in cartilage primordia, the activation of genes encoding the chondrocyte-specific components of the cartilage extracellular matrix, the formation of a growth plate, and the final maturation of chondrocytes into hypertrophic chondrocytes. We speculate that Cart-1 may play a role in this pathway, probably in an early step. Additional regulatory molecules probably also play important roles in this pathway, either in conjunction with Cart-1 or in different steps. Inhibition of Cart-1 gene expression in differentiating chondrocytes and in developing mouse embryos should help us understand the function of Cart-1.

The rat chondrosarcoma tumor was a kind gift from Dr. Richard Mayne (University of Alabama, Birmingham, AL). The rat chondrosarcoma cell line was a gift from Dr. James Kimura (Henry Ford Hospital, Detroit, MI). We are grateful to Rebecca Reiter, Li Huang, and Karen Niederreither for assistance and advice. We thank Drs. Richard Behringer and Eric Olson for their comments on this manuscript. We thank Dr. Veronique Lefebvre for RNA samples from cultured chondrocytes and Dr. Rena d'Souza for rat embryo sections. We are grateful to Patricia McCauley for editorial assistance. This work was supported by National Institutes of Health Grant AR 40335 (B.d.C.) and Grant HD 05505 (M.S.), and DNA sequencing was performed with the support of National Cancer Institute Core Grant CA 16672.

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