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Construction and Partial Characterization of Recombinant cDNA Clones for Chicken Type I Collagen Messenger RNAs

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Messenger RNAs for the α1 and α2 chains of type I procollagen were partially purified from total embryonic chicken calvaria using gel chromatography on Sepharose 4B and used to construct recombinant cDNA clones corresponding to both mRNAs. Restriction site mapping, nucleotide sequencing and hybridization to RNA blots were used to show that clones pCAL1 and pCAL2 contain inserted sequences corresponding to the mRNAs for chicken α1 and α2 procollagen chains, respectively.

The collagens are a group of proteins with important structural functions in the extracellular matrix. They also play a central role during embryonic development in the morphogenesis of tissues.[1] Cartilage differentiation, for example, involves a switch from type I to type II collagen synthesis when mesenchymal cells differentiate into chondrocytes.[2,3] Type I collagen consists of two identical α1(I) chains and one α2(I) chain whereas type II collagen is composed of three identical α1(II) chains. In an earlier study we have identified mRNAs expressed in differentiating chicken cartilage by characterization of their cell-free translation products.[4] In order to extend these studies we have prepared cDNA clones for the cartilage specific (type II) collagen.[5] In the present report we describe the construction and characterization of cDNA clones corresponding to the mRNAs for the α1 and α2 chains of chicken type I collagen; these were needed to study the regulation of type I collagen production during cartilage differentiation. Recombinant cDNA clones for these collagen mRNAs have been described earlier by others.[6–9]

EXPERIMENTAL

Materials. Escherichia coli strain HB 101 and plasmid vector pBR322 (given to us by Murray Rabinowitz and Tom Christenson, The University of Chicago) were used in the transformation experiments. Radioisotopes were obtained from Amersham International, Amersham, U.K., reverse transcriptase from Joseph Beard, Life Sciences, Inc., St. Petersburg, FL, U.S.A., S1 nuclease from Sigma Chemicals, St. Louis, MO, U.S.A. and the deoxynucleotide triphosphates and terminal transferase from P–L Biochemicals, Milwaukee, WI, U.S.A. Restriction enzymes, DNA polymerase I, plasmid M13mp9 and nick-translation kits were obtained from BRL, Bethesda, MD, U.S.A., and T4 ligase was a gift from Kan Agarwal (The University of Chicago).

Purification and identification of RNA. Total RNA was purified from 16-day chicken embryonic calvaria and from 17-day chicken embryonic sterna by the quanidine extraction method as described earlier.[4] Total RNA from calvaria (approximately 1 mg) was further separated into bound and unbound fractions on a 2.24×34 cm column of Sepharose 4B.[10] The degree of enrichment for type I procollagen sequences in the bound fraction was estimated by gel electrophoresis of the cell-free translation

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products directed by the partially purified RNAs in a wheat germ system.[4]

Synthesis of double-stranded cDNA. The bound fraction from the Sepharose 4B column was used as a template for the synthesis of cDNA using avian myeloblastosis virus reverse transcriptase under conditions described earlier.[5] The second strand was synthesized using *E. coli* DNA polymerase I (EC 2.7.7.7.) at 130 U/ml as described earlier.[11] About 0.5 μg of double-stranded cDNA was synthesized per 100 μg of fractionated RNA (which consists primarily of 27 S rRNA).

The cDNA and remaining RNA were digested with 450 U/ml of S1 nuclease (EC 3.1.30.1) at 37 °C for 60 min at a concentration of 0.4 mg/ml of nucleic acid in 0.2 M sodium acetate (pH 4.5)–0.475 M NaCl–1 mM $ZnCl_2$. For the construction of *Eco*RI–*Bam*HI inserts, digestion with S1 was omitted.

Construction of recombinant plasmids by homopolymer tailing. Terminal deoxynucleotidyl transferase (EC 2.7.7.31) was used to add dC-homopolymer tails to the 3'-ends of S1 nuclease-digested cDNA.[12] Double-stranded cDNA at a concentration of 5 μg/ml was tailed in the presence of 1 mM $CoCl_2$ with dCTP (2.5 μM) using 400 U/ml terminal transferase. *Pst*I (EC 3.1.23.31) cleaved pBR322 at 20 μg/ml was tailed as above with dGTP (2.5 μM) and 200 U/ml terminal transferase.

Tailed cDNA was size fractionated by sedimentation through a 3–22 % (w/v) sucrose gradient containing 100 mM NaCl–10 mM Tris–HCl (pH 8.5)–1 mM EDTA at 84 000 *g* ($r_{av.}$ 8.35 cm) for 18 h at 5 °C (Beckman SW 50.1 rotor at 30 000 rpm). Fractions containing cDNA with an average size larger than 1000 base pairs were pooled and co-precipitated with an equimolar amount of tailed pBR322.

The precipitate containing approximately 150 ng of pB322 and 30 ng of double-stranded cDNA was dissolved in 100 μl of annealing buffer: 100 mM NaCl–10 mM Tris–HCl (pH 7.5)–1 mM EDTA, heated to 65 °C for 1 h and allowed to cool slowly to room temperature.

Construction of recombinant plasmids using defined restriction fragments. The reactions were performed essentially as described by Sobel *et al.*[6] An aliquot of double-stranded cDNA was taken prior to the S1 nuclease step and digested with restriction enzyme *Bam*HI (EC 3.1.23.6) followed by digestion with *Eco*RI (EC 3.1.23.13). A similar digestion was performed on 1 μg of pBR322. The large pBR322 fragment was purified from the smaller 375 bp fragment by passing the reaction mixture through a Sepharose 4B column. The precipitated plasmid and cDNA were ligated using 13 U/ml of T4 ligase (EC 6.5.1.1) at 14 °C for 20 h, extracted with phenol-chloroform and precipitated. The precipitate was taken up in 100 μl of annealing buffer and used for transformation.

Transformation. Transformations were done by the method of Mandel & Higa[13] using 50 mM $CaCl_2$. After a 2 min heat shock the homopolymer-tailed recombinants were plated on tetracycline containing plates, and colonies resistant to tetracycline were subsequently screened for loss of ampicillin resistance. Bacteria transformed with plasmids containing the insert between the *Bam*HI and *Eco*RI sites were plated on ampicillin plates and further screened for loss of tetracycline resistance. All experiments were performed in accordance with the National Institutes of Health guidelines for recombinant DNA research.

Colony hybridization. Clones containing plasmids with inserts were grown on Whatman 540 filter papers on the respective selective plates and the plasmids were amplified by transfer to chloramphenicol plates after 7 h. After processing[14] the filters were prehybridized for 16 h at 65 °C in 0.9 M NaCl–90 mM sodium citrate–0.02 % bovine serum albumin–0.02 % polyvinylpyrrolidone–0.02 % Ficoll (Pharmacia). ^{32}P-labeled cDNA probes were prepared with reverse transcriptase using partially purified calvaria RNA as template. Hybridizations were carried out for 20 h at 65 °C in the prehybridization buffer containing 100 μg/ml sonicated calf thymus DNA. The washed filters were autoradiographed at −70 °C with Kodak X-Omat film using intensifying screens.

Purification and analysis of plasmid DNA. Plasmid DNA was prepared from selected clones using the cleared lysate method.[15] Reaction conditions for restriction enzyme digestions were as recommended by the supplier. DNA samples were electrophoresed in agarose gels containing 10 mM sodium phosphate, pH 7.5, and 0.5 μg/ml ethidium bromide or in polyacrylamide gels containing 40 mM Tris–HCl (pH 8.3)–20 mM sodium acetate–2 mM EDTA.

After liberation with restriction enzymes the inserts were electrophoresed in acrylamide gels and purified.[16] DNA sequencing was done either by the method of Maxam & Gilbert[17] using purified restriction fragments or by the dideoxy method[18] using inserts recloned into M13mp9.

RNA gel transfers. RNA samples were denatured with glyoxal and electrophoresed in 0.75 % agarose gels.[19] RNAs were transferred to nitrocellulose filters and prehybridized as described earlier.[20] Whole plasmids or purified restriction fragments were labeled by nick-translation with

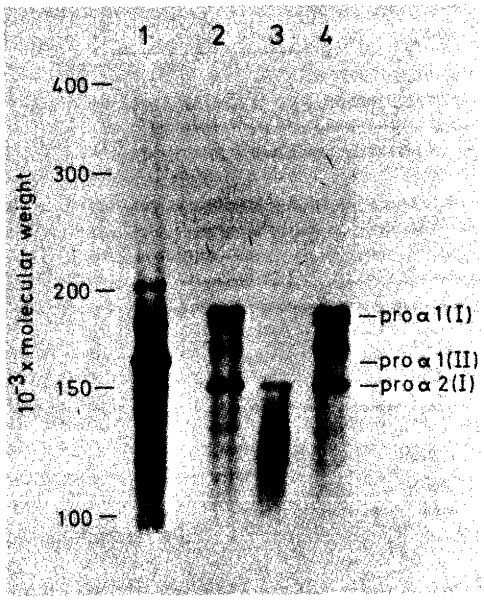

Fig. 1. Cell-free translation of calvarial RNA fractionated on Sepharose 4B. RNAs were translated in a wheat germ system using [^{35}S]methionine to label the products which were subsequently fractionated on a 5 % polyacrylamide gel. The major bands are marked as previously identified.[4] Lane 1, total sternal RNA, lane 2, total calvarial RNA, lane 3, unbound fraction from the Sepharose 4B column and lane 4, bound fraction from the column. A scale of molecular weights is shown on the left.

[^{32}P]dCTP to a specific activity of about 10^7 cpm/μg and then used as probes. The filters were hybridized for 16–20 h, washed and autoradiographed.[20]

RESULTS AND DISCUSSION

Partial purification of type I procollagen mRNAs. Chicken embryonic calvaria were chosen as the tissue source for the isolation of type I procollagen mRNAs because they are known to contain large amounts of these mRNAs and insignificant amounts of other collagen mRNA.[4] Characterization of RNAs isolated from calvaria, sterna and the bound and unbound fractions from the Sepharose 4B column was performed by analysis of cell-free translation products by polyacrylamide electrophoresis in the presence of SDS. The major cell-free translation products of calvarial and sternal RNA have earlier been identified as the α1 and α2 chains of type I procollagen and the α1 chain of type II procollagen, respectively. As shown in Fig. 1 the mRNA for pro α1(I) collagen binds well to Sepharose 4B under the conditions used and is virtually absent in the unbound fraction. About one half of the pro α2(I) collagen mRNA is also found in the bound fraction whereas all other mRNAs are enriched in the unbound fraction. Most of these cannot be seen in Fig. 1 as they have been electrophoresed off the gel to obtain good resolution of large translation products.

Transformations and screening of cDNA clones. Double-stranded cDNA was prepared from the partially purified pro α1(I) collagen mRNAs and inserted into the *Pst*I site of pBR322 using homopolymer tails. Transformation of *E. coli* strain HB 101 with these plasmids yielded 5 colonies which were both tetracycline resistant and ampicillin sensitive. ^{32}P-labeled cDNA prepared from partially purified (bound fraction) RNA was used as a probe in colony hybridization and one of the hybridizing clones, pCAL1, was selected for further characterization.

The procedure of Sobel *et al.*[6] was used to obtain a recombinant cDNA clone corresponding to the α2(I) procollagen mRNA. This procedure involves digesting double-stranded cDNA prepared from type I procollagen mRNA with *Eco*RI and *Bam*HI and inserting the resulting fragments into pBR322 which has been similarly digested. The subsequent transformation yielded several hundred transformants, over 90 % of which contained a 190 bp insert, as expected. One of the clones, pCAL2, was taken for further characterization.

Characterization of inserted sequences. Cleavage of plasmids pCAL1 and pCAL2 with various restriction enzymes yielded the maps shown in Fig. 2. Plasmid pCAL1 contains an insert with 675 bp and pCAL2 an insert with 190 bp. Restriction site mapping of pCAL1 shows a pattern which, for all the enzymes studied, agrees with the maps of other cDNA clones corresponding to pro α1(I) collagen mRNA: pCg1 and pCg54[7] and pCOL3.[9] Sequencing of pCAL1 has been performed at both ends; the clone begins with base number 245 and extends to approximately base number 864 numbered by Fuller & Boedtker.[21] Based on the end sequences, the

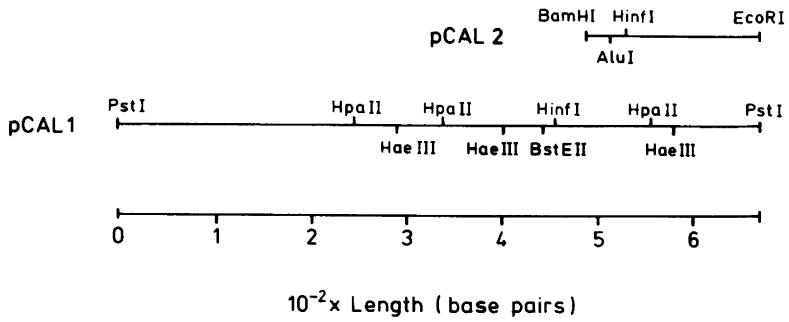

Fig. 2. Restriction maps of pCAL1 and pCAL2. The maps were deduced from single and double digestions of the hybrid plasmids. The *PstI* sites at the ends of pCAL1 represent regenerated *PstI* sites of pBR322 and are not present in the mRNA. In contrast, *Eco*RI and *Bam*HI sites at the ends of pCAL2, which represent the ligation sites, are present in the mRNA.

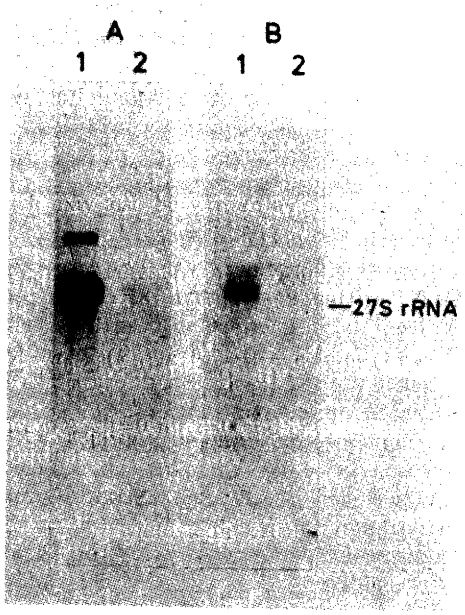

Fig. 3. Hybridization of pCAL1 and pCAL2 to calvarial and sternal RNA transferred to nitrocellulose filters. Total RNA (15 μg) from chicken calvaria (lane 1) and sterna (lane 2) were denatured with glyoxal, electrophoresed on 0.75 % agarose gels and transferred to nitrocellulose. Denatured chicken rRNAs and bacteriophage λ DNA digested with *Hind*III and *Eco*RI were used as size markers (not shown). The hybridization pattern obtained with nick-translated pCAL1 is shown in panel A and that obtained with pCAL2 in panel B.

length of the cloned insert and the restriction site map pCAL1 appears to be colinear with the mRNA unlike pCg54 and pCg26.[21] The clone corresponds to mRNA coding for most of the carboxyterminal extension peptide of the pro α1(I) collagen and to some 3′-untranslated mRNA sequences.

As expected plasmid pCAL2 contains the same restriction sites as pCOL1 which was constructed similarly.[6] Sequencing of pCAL2 from the *Eco*RI site using the dideoxy technique has shown that the first 96 bases are identical with those previously published.[21,22] pCAL2 corresponds to mRNA coding for 63 amino acids in the C-terminal extension peptide for pro α2(I) collagen.

Further characterization of the inserted sequences was performed by hybridizing nick-translated plasmids to RNA blots containing various RNA preparations. Fig. 3 shows hybridization of pCAL1 and pCAL2 to RNA from calvaria and sterna. pCAL1 hybridizes strongly to a band at 5.0 kilobases and also to a minor band at 7.2 kilobases in RNA from calvaria, whereas only a trace of hybridization is seen to sternal RNA in the area of the smaller band. Similarly pCAL2 hybridizes to bands at 5.2 and 5.7 kilobases in calvaria RNA and weakly to sternal RNA. These size estimates for the mRNAs agree well with those published by others.[23,24]

The data presented demonstrate that pCAL1 and pCAL2 are plasmids containing cDNA sequences corresponding to chicken pro α1(I) and pro α2(I) collagen mRNAs, respectively.

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