Construction and characterization of type II collagen complementary deoxyribonucleic acid clones

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ABSTRACT

The mRNA for type II collagen was purified from embryonic chick sternum or from purified sternal chondrocytes with guanidine thiocyanate as the extractant. Double-stranded cDNAs to procollagen mRNAs from sternum were synthesized and dC-tailed. After annealing with PstI-cleaved, dG-tailed pBR322, this DNA was used to transform *Escherichia coli* X1776. Transformed colonies were screened by colony hybridization to type I and II collagen cDNAs. Clones that preferentially hybridized to type II cDNA were characterized further. Four such cDNA clones, pCgII-2, 3, 10 and 12, with inserts of 400, 320, 260 and 750 bp, have been identified as type II collagen cDNA clones by several criteria, including their preference for hybridizing with type II rather than type I collagen mRNAs in hybrid-selected translation experiments.

INTRODUCTION

The collagens present in avian and mammalian organisms have recently been found to consist of at least 5 different types (1,2). Since all collagens consist of 3 polypeptide chains wound around each other in a helix, and since in some collagen types the 3 chains are not identical, at least 9 genes are required to code for these collagens (1,2). These genes are expressed in specific tissues at specific times in embryonic development, but at present very little is known about the mechanisms responsible for regulating the expression of these genes.

Our own studies have been directed at the shift in collagen gene expression that occurs when chick embryo sternal chondrocytes are cultured in the presence of 5-bromodeoxyuridine. When first isolated, these chondrocytes synthesize the type II collagen characteristic of cartilage which has the chain composition [pro α 1(II)]₃. After 6 to 7 days of culture in the

presence of bromodeoxyuridine, the cultures no longer synthesize type II collagen, but now make instead type I collagen, the ubiquitous collagen found in bone, tendon, skin and elsewhere (2), which has the chain composition $[\text{pro}\alpha 1(\text{I})]_2\text{pro}\alpha 2(\text{I})$, as well as $[\text{pro}\alpha 1(\text{I})]_3$. Measurements of collagen mRNA levels by cell-free translation have established that the levels of translatable mRNAs parallel the synthetic activity of the intact cells (3), during this shift from type II to type I collagen synthesis. Further analysis, however, of the changes in mRNA levels that occur in this system requires the availability of cloned cDNAs that are complementary to the type II mRNA and to the mRNAs for the 2 kinds of type I chains. In addition, cloned cDNAs are needed as probes for isolation of the corresponding genes, a step which appears to be essential for understanding the regulation of these genes. Several laboratories have reported the cloning of type I cDNAs (4-7), as well as their subsequent use to isolate type I genes (e.g. 8-10), but at the time the present experiments were begun, no type II cDNA clones had been described. This paper describes the isolation and characterization of 4 such collagen type II cDNA clones. While this work was in progress, Upholt and his colleagues reported the isolation of two type II cDNA clones (11). The structure and orientation of our clones confirm this previous report. In addition, the "hybrid-selected translation" experiments reported here add important evidence in support of the conclusion that these cloned sequences represent type II collagen cDNAs. In these experiments, the cDNA clones were found to hybridize selectively with type II collagen mRNA, which could be identified by its cell-free translation product.

METHODS

RNA purification

The sterna or skull frontal bones from 17-day virus-free chick embryos (Spafas, Norwich, CT) were dissected free of adhering tissue, collected in liquid N_2 , and then either used directly or stored in an ultrafreezer at -76°C until needed. RNA extraction was performed by the procedure of Chirgwin et al. (12), with 10 dozen sterna or skull frontal bones per 25 ml of

guanidine thiocyanate buffer. The yield of total sternum or calvaria RNA per dozen embryos was, respectively, approximately 8 and 30 A₂₆₀ units, or 0.33 and 1.25 mg. The RNA was isolated from chondrocytes purified as "floaters" (13) or from cultures of these chondrocytes in the presence of bromodeoxyuridine as previously described (3).

The poly(A)+ RNA of sternum or calvaria was obtained by affinity chromatography on a column of poly (U)-Sephadex G-10 (14). Sucrose gradient fractionation of the poly(A)+RNA (15) was performed on 5 to 20% linear gradients in 0.01 M Hepes pH 7.5, 0.005 M EDTA. The RNA was heated to 60°C for 5 min, cooled, layered on the gradients and centrifuged for 12.5 hours at 32,000 rpm at 2°C (Beckman SW41 rotor).

Collagen mRNAs were assayed during purification by the previously described procedures (3) of translation in the nuclease-treated reticulocyte lysate system (16) in the presence of [³H] proline followed by gel electrophoresis and autoradiography. Before adding the RNA sample to the translation system, it was incubated for 5 min at 22°C in the presence of 4 mM methylmercuric hydroxide.

Cloning of type II collagen cDNA

Ten µg of the sternum poly(A)+RNA present in the 4 heaviest fractions from the second sucrose gradient (Fig. 1) were used as the template for the synthesis of the type II collagen cDNA for cloning. Double-stranded cDNA was synthesized (17), treated with S1 nuclease (17), and tailed with dC residues (18), prior to annealing with PstI-cleaved pBR322 that had been tailed with dG residues (18). After transformation of E. coli strain λ 1776 (17), tetracycline-resistant colonies were screened for the presence of type II collagen cDNA sequences by colony hybridization (17) with procollagen cDNA probes.

Single-stranded, ³²P-labeled type II cDNA was prepared from purified-chondrocyte collagen mRNA and type I cDNAs were prepared from 17-day calvaria collagen mRNAs; in addition, cloned cDNAs for the 2 type I chains proα1(I) and proα2(I), were prepared from, respectively, the plasmids pCg54 and pCg45 (6,4), which were a generous gift from Dr. Helga Boedtker. The proα2(I) cDNA was excised from pCg45 by HindIII digestion and an 1100 bp

fragment of pro α 1(I) cDNA was released from pCg54 by digestion with KpnI and Hind III. The cDNAs were separated from the vector DNAs by electrophoresis in low melting point agarose and were ³²P-labeled by nick translation (19).

Plasmid DNA was isolated for preliminary characterization (e.g. Fig. 2) by a "mini" plasmid preparation that was a minor modification of Holmes and Quigley's procedure (20). Larger-scale, "maxi" preparations followed the procedure of Birnboim and Doly (21).

Gel electrophoresis and hybridizations of DNA and RNA

Agarose gel-electrophoresis of DNA was carried out by standard procedures (19). After Southern-blotting (22) hybridization with DNA probes was performed under the conditions used for colony hybridization (17). RNA was electrophoresed under denaturing conditions in 0.83% agarose gels containing 2.2 M formaldehyde (23). After transfer to nitrocellulose (22) it was hybridized with DNA probes under sufficiently stringent conditions to prevent cross-hybridization between type I mRNAs and type II cDNAs or between type II mRNAs and type I cDNAs. We are indebted to Stephen Saxe for providing us with this hybridization procedure. After pre-hybridization under the conditions of Thomas (24), hybridization was performed at 60°C overnight in 3.75-x SSC (19), 60% formamide, 37.5 mM sodium phosphate buffer, pH 6.5, 0.75-x Denhardt's solution (19) 10 µg/ml denatured salmon sperm DNA, 0.1% sodium dodecyl sulfate and 0.1 g/ml dextran sulfate. The indicated plasmid DNAs, labeled with ³²P by nick-translation (19), were added after heat denaturation (19). After hybridization, the filters were washed as described (24) but with 4 changes of each buffer at 55°C, and were then dried and autoradiographed.

Hybridization of RNA "dot-blots" on nitrocellulose paper with ³²P-labeled cloned cDNAs (Fig. 4), was performed by the method of Thomas (24) with minor modifications. After autoradiography, under conditions where the density on the film was directly proportional to radioactivity, the relative densities of the spots were calculated after scanning on a recording densitometer.

Hybrid-selected translation

Plasmid DNA after linearization with Eco R1, was spotted on dry nitrocellulose (SS-BA85) and subsequently treated as described by Patterson (25). The sternum poly(A)+RNA was dissolved in freshly deionized formamide at a concentration of 0.2 $\mu\text{g}/\text{ml}$ (lanes 3-5, Fig. 5A) or 0.24 $\mu\text{g}/\text{ml}$ (lanes 7-9, Fig. 5A). After heating for 1 min at 60°C, the RNA was quick-chilled for 30 sec in dry ice-methanol and 25 μl were then added to the tubes containing the DNA-nitrocellulose sample. This was followed by 25 μl of double-strength hybridization mix, which contained all the components but the RNA and formamide. Hybridization and washing conditions were those of Patterson (25) in the experiment shown in Fig. 5A, lanes 3-5. Similar results were obtained when prehybridization, hybridization and washing were done under the conditions described by Griffin-Shea et al. (26), as in the experiment shown in Fig. 5A, lanes 7-9. The hybridized RNA was eluted with 200 μl H_2O as described by Ricciardi et al. (27) except (1) the time at 100°C was reduced to 35 sec, (2) no carrier tRNA was added since it appeared to inhibit translation of the mRNA and (3) the pelleted RNA was washed once with 95% ethanol. Control experiments indicated that these elution, precipitation, and washing conditions appeared to give maximal recovery of translatable RNA; 0.2 μg of poly(A)+sternum RNA could be precipitated quantitatively from 0.3 ml H_2O under these conditions.

Restriction maps

The type II collagen inserts were excised from the vector as described in the text and were isolated by electrophoresis in low melting-point agarose (1-1.5% agarose, depending on the size of the inserts). The incubations with restriction endonucleases were performed as recommended by the supplier, New England Biolabs. The DNA fragments were electrophoresed in 8% polyacrylamide gels (19) and size markers were included (Hae III-digested $\phi\text{X}174$ and HinfI-digested pBR322).

RESULTS

Isolation of type II procollagen mRNA

Chick embryonic sternum was chosen as the starting

Figure 1. Assay of procollagen mRNAs by gel electrophoresis of cell-free translation products. Lanes 2 through 8 represent the translation products of poly (A)+ sternum RNA fractionated on a sucrose gradient (see Methods). 1-ml fractions extending from fraction 1 (bottom) to fraction 12 (top) were collected and the RNA was recovered by ethanol precipitation. Lane Ts represents the translation products of total sternum RNA before fractionation, and Tc represents the translation products of total, unfractionated calvaria (frontal bone) RNA. Lane Hf represents the translation products of "floater" (purified-chondrocyte) RNA after the 5 heaviest gradient fractions were sedimented on a second gradient and the four heaviest fractions were pooled prior to translation. Lane Tf shows the translation products of total "floater" RNA, before fractionation. The biosynthetic precursors of collagen alpha chains are called pro α chains (1); this term is used here to represent the initial cell-free translation products.

material for the isolation of type II collagen mRNA since it is known to be a relatively rich source of type II collagen (28). It has the disadvantage, however, that it also contains Type I collagen mRNAs, which are synthesized by the perichondrium that surrounds the inner cartilaginous tissue.

When sternum RNA is isolated by guanidine thiocyanate extraction (12), the yield of type II collagen chains in the cell-free translation assay is one-third of the total collagen pro α chains (Fig. 1, lane Ts). It is not known, however, whether this figure accurately represents the level of type II mRNA in this preparation, because of the possibility that the

type I and II collagen mRNAs are translated with different efficiencies. For comparison, the translation products of total calvaria (skull frontal bone) RNA, which contains only type I collagen mRNAs, are shown in lane Tc.

The effectiveness of the further purification procedure for collagen mRNAs is shown for sternum RNA by sucrose-gradient fractions 2 and 3 of Fig. 1 (lanes 2 and 3) where it can be seen that the heavy collagen Type I and II mRNAs are much enriched relative to lighter mRNAs. This mRNA (fractions 1 through 4, pooled) was used as the template for the synthesis of cDNAs for cloning.

A type II collagen mRNA preparation virtually free of type I mRNA (Fig. 1, lane T_f) can be prepared by using as starting material isolated chondrocytes that have been purified free from the fibroblasts in the sternum perichondrium by enzymatic digestion of the sternum, followed by selection of "floaters" (3,13). In this case also, guanidine thiocyanate extraction (3) appeared to be essential to obtain undegraded type II mRNA. Further purification of this RNA gives a sample considerably enriched in type II collagen mRNA (Fig. 1, lane H_f). Although this RNA would presumably be a preferred starting material for preparing and cloning type II cDNA, it was not available as early as the total sternum RNA and so was not used in constructing the clones described in this report. The collagen mRNA preparation from purified chondrocytes was, however, very useful in providing a template for the synthesis of type II collagen cDNA free from type I sequences, that could be used to distinguish type II from type I collagen cDNA clones. Initial identification of type II collagen clones by colony and Southern-blot hybridizations.

To increase the probability of isolating a type II, rather than a type I, collagen cDNA clone, approximately 1500 colonies were screened with both type I and type II cDNAs, and 60 clones that hybridized strongly to type II cDNAs but weakly or not at all to type I cDNAs, were characterized further. The preferential hybridization of the DNA of the selected clones to type II collagen cDNAs was re-examined after electrophoresis and Southern blotting of the isolated plasmid DNAs. The plasmid DNAs

Figure 2. Hybridization of the DNA of the 4 putative type II collagen clones after electrophoresis to 32 P-cDNAs corresponding to type I and II collagen mRNAs. After electrophoresis in 1% agarose gels the DNAs were transferred to nitrocellulose and hybridized to: (A) 32 P-cDNA made by reverse transcription of a preparation enriched for type II collagen mRNA from purified chondrocytes; (B) cloned pro α 1(I) cDNA obtained by isolating the cDNA insert from pCg54; and (C) cloned pro α 2(I) cDNA consisting of the insert from pCg45. The inserts were 32 P-labeled by nick translation. Lanes 1, 2 and 3 contained the DNA from, respectively, pCg45, pCg54 and pBR322. Lanes 4-7 contained the DNA from the putative type II clones; in order, pCgII-12, pCgII-3, pCgII-2 and pCgII-10. All lanes in A are from an overnight exposure of the autoradiogram, in B and C, lanes 1 and 2 are from a 40-min autoradiogram and lanes 3 through 7 from a 6-hr exposure. At 40 min, the hybridization of pCgII-12 to pCg54 (B, lane 4) is barely visible (not shown), and at 6 hr, extensive cross-hybridization between pCg54 and pCg45 sequences becomes visible (B, C, lanes 1 and 2, not shown).

isolated from the 4 clones described in this report hybridize strongly with type II collagen cDNA as shown in Fig. 2A, lanes 4 through 7. The presence of 3 bands is due to the presence of super-coiled, nicked circular, and linear molecules. These DNAs do not hybridize with pro α 2(I) cDNA (Fig. 2C, lanes 4-7) nor with pro α 1(I) cDNA (Fig. 2B, lanes 5-7), with the exception of pCgII-12 which showed an intermediate level of hybridization with

pCg54 (Fig. 2B, lane 4). These hybridization conditions are sufficiently non-stringent that some cross-hybridization occurs between different collagen cDNAs -- for example, pCg54 and pCg45 sequences could be seen to cross-hybridize on longer exposure of the autoradiograms of Fig. 2B and 2C (not shown). The ability of pCgII-12 to cross-hybridize with pCg54, but not with pCg45 is consistent with the greater homology known to exist between $\alpha 1(I)$ and $\alpha 1(II)$ chains than between either chain and $\alpha 2(I)$ (2). The absence of cross-hybridization of the other 3 clones with pCg54 is consistent with the results of further analysis (Fig. 6) which showed that these clones contained only sequences that are downstream from the regions of pCg54 that would be expected to be homologous.

The putative type II collagen cDNA clones hybridize with type II, but not type I, collagen mRNAs

Since the 4 putative type II collagen cDNA clones were selected primarily because in both colony and Southern blot hybridizations they hybridized well to the cDNA prepared from a collagen-enriched mRNA fraction isolated from purified chondrocytes, it was important to find out whether they contained type II collagen cDNA sequences or cDNA sequences derived from other mRNAs that are present in chondrocytes. Therefore the RNA from sternum was electrophoresed on agarose gels and after transfer to nitrocellulose was hybridized separately with each of the putative type II cDNAs, which were labeled by nick-translation in the presence of [α -³²P]dGTP. Each of the 4 plasmids described here was found to hybridize to a single RNA species with the expected mobility for a collagen mRNA, similar to that exhibited by pro $\alpha 1(I)$ collagen mRNA, just slower than chicken 27s rRNA (data not shown).

This result could be extended to show that under sufficiently stringent hybridization conditions, the 4 putative type II cDNA clones hybridized only to the collagen mRNA from purified chondrocytes (Fig. 3, lanes 3, 5, 7 and 9) and not to the type I collagen mRNAs from skull frontal bones (Fig. 3, lanes 4, 6, 8 and 10). The presence of type I mRNA in the frontal bone samples is confirmed by the hybridization of this RNA to pCg54 DNA (lane 2) which contains cloned pro $\alpha 1(I)$ cDNA

Figure 3. Hybridization of gel blots of electropherograms of total RNA from purified chondrocytes (odd-numbered lanes) or from frontal bones (even-numbered lanes) with nick-translated pCg54 (lanes 1 and 2) or with each of 4 pCgII clones (lanes 3 through 10). 7.5 μ g of chondrocyte RNA or 4.5 μ g of frontal bone RNA were used per lane and, as a size marker, 7.3 μ g of ribosomal RNA from chondrocytes (unbound fraction from poly(U) Sephadex chromatography) were run in an additional lane and visualized with ethidium bromide staining; the positions of the large ribosomal RNA (27S in chickens) and the 18S ribosomal RNA are indicated. Lanes 3 and 4 were hybridized with nick-translated pCgII-2; lanes 5 and 6 with pCgII-10; lanes 7 and 8 with pCgII-3; and lanes 9 and 10 with pCgII-12.

sequences. The absence of hybridization between pCg54 and type II mRNA (lane 1) is consistent with the lack of occurrence of cross-hybridization under these conditions. The major hybridizing RNA species in lane 2 has the mobility reported for pro α 1(I) mRNA, corresponding to 5.3 kb (29,30), and the previously reported (29,30) minor pro α 1(I) mRNA species of about 7.2kb is also present. The major type II mRNA species (lanes 3, 5, 7 and 9) also appears to be approximately 5.3 kb in size. In summary, the putative type II cDNA clones hybridized only to type II collagen mRNA and did not hybridize to type I collagen mRNAs.

Figure 4. Relative hybridizations of pCgII-2 (+—+) and of cloned pro α 1(I) cDNA, pCg54, (⊕—⊕) to equal amounts of RNA from chondrocytes exposed to bromodeoxyuridine for the indicated number of days. Purified sternal chondrocytes were cultured in the presence of bromodeoxyuridine (3) and total RNA was isolated from a single series of plates on days 0, 3, 4, 5, 6, and 7. Equal amounts of RNA (5 μ g in 5 μ l) were spotted onto nitrocellulose (24) and were hybridized with nick-translated, 32 P-labeled pCgII-2 or pCg54. The relative extent of hybridization was measured by densitometry of autoradiograms, and the maximum observed hybridization with each probe was arbitrarily assigned the value of 100%. Further hybridizations under more stringent conditions (unpublished observations) indicate that the initial levels of hybridization of pCg54, and the day 6 and 7 levels of hybridization of pCgII-2 are inflated by cross-hybridization of pro α 1(II) and pro α 1(I) mRNAs and the corresponding cDNAs.

Cultured chondrocytes induced to shift from type II to type I collagen synthesis show a corresponding decrease in RNA complementary to the type II cDNA clones

Another way to identify the putative type II clones was based on observations that chondrocytes treated with bromodeoxyuridine shift from synthesizing type II to type I collagen and that this shift is accompanied by corresponding changes in the levels of translatable collagen mRNAs (3). When "dot-blots" containing equal amounts of RNA from cultures exposed for increasing times (0 to 7 days) to bromodeoxyuridine were

hybridized to the ³²P-labeled putative type II collagen cDNAs, in each case decreasing amounts of hybridization occurred as the cultures were exposed for increasing times to bromodeoxyuridine (Fig. 4). In contrast, over the same time period, the amounts of RNA complementary to pro α 1(I) cDNA or pro α 2(I) cDNA increased, as shown in Fig. 4 for pro α 1(I) cDNA. Measurements of the collagen chains synthesized by these identical cultures confirmed that these cultures exhibited the usual bromodeoxyuridine-induced shift from type II to type I collagen synthesis (3) with a similar time-course as shown for the mRNAs in Fig. 4. The behavior predicted for type II or type I cDNAs in this system is clearly very different; all 4 of the pCgII clones exhibit the behavior predicted for type II cDNA sequences.

Characterization of clones by hybrid-selected translation

To confirm that these 4 clones contained sequences complementary to type II collagen mRNA, the DNA from each clone was immobilized on nitrocellulose filters and hybridized with total poly(A)+RNA from sternum. The selected RNAs were identified by in vitro translation. Sternum was chosen as the source of the RNA, since it contains both type I and type II RNAs, and therefore the preference of a particular plasmid DNA for type I or II mRNAs could be observed. The results (Fig. 5) show that the 4 clones described here select pro α 1(II) mRNA from sternum RNA, preferentially over pro α 1(I) mRNA, whereas a pro α 1(I)cDNA clone, pCg54, included for comparison, preferentially selects pro α 1(I) mRNA. Although neither type of cDNA selected appreciable amounts of pro α 2(I)mRNA, these hybridization conditions were less stringent than those used in the RNA gel electrophoresis experiments and did allow some cross-hybridization between the 4 pro α 1(II) cDNA clones and pro α 1(I) mRNA and between the pro α 1(I) cDNA of pCg54 and pro α 1(II) mRNA. The ratio of pro α 1(II) mRNA to pro α 1(I) mRNA in this sternum RNA sample was approximately 1.6, as assayed by cell-free translation and separation of the collagen translation products as in Fig. 5. In the experiment shown in lanes 1-5 of Fig. 5, selection by the plasmids pCgII-2 or pCgII-10 (lanes 3 and 4, respectively) increased this ratio to, respectively, 6.5 and 7.9 (Fig. 5B); selection by pCg54 (lane 5) decreased this ratio from 1.6 to

0.69. The experiment shown in lanes 6-9 of Fig. 5A shows that the plasmids pCgII-3 and pCgII-12 (lanes 8 and 9, respectively) also select pro α 1(II) mRNA preferentially over pro α 1(I) mRNA, in contrast to the plasmid pCg54 (lane 7). To summarize these results, the 4 clones described in this paper preferentially select pro α 1(II) mRNAs under conditions in which the pro α 1(I) cDNA, pCg54, preferentially selects pro α 1(I) mRNA. These observations show not only that these 4 clones contain collagen cDNA sequences, but that these sequences are complementary to pro α 1(II) mRNA rather than to pro α 1(I) mRNA.

Physical structure of 4 collagen type II cDNA clones

The restriction map of pCgII-12 is shown in Fig. 6 in the same 5' to 3' orientation as the mRNA, as deduced from sequencing data (see below). The overlap of the 3 smaller clones with pCgII-12 is also shown. The restriction maps were determined on the type II collagen cDNAs purified free from the pBR322 DNA, except in the case of pCgII-10 where loss of one of the 2 Pst 1 sites expected to flank the inserted cDNA sequences made it necessary to isolate an Eco R1-Pst 1 fragment that contained pBR322 as well as collagen cDNA sequences.

The alignment of the small inserts with pCgII-12 was also confirmed by digesting the isolated insert of pCgII-12 with Hinf I or Hpa II and separating the restriction fragments by gel electrophoresis. Subsequent Southern blotting and hybridization with nick-translated, ³²P-labeled pCgII-2,3 or 10 gave results consistent with the alignments shown in Fig. 6. None of the 3 plasmids hybridized with the 5'-Hinf I or Hpa II fragments of pCgII-12. All 3 plasmids hybridized to the 3'-Hinf I fragment of pCgII-12 and to the adjoining small Hinf I fragment. Plasmids pCgII-2 and 3 also hybridized to the Hinf I piece immediately 5' to the small Hinf I fragment, but pCgII-10 did not. All 3 plasmids hybridized with the DNA fragments in the gel band which contained the co-migrating 3' and internal Hpa II fragments of pCgII-12, as predicted from the presence of the 3' Hpa II fragment in this band.

The restriction enzymes used to obtain the map of pCgII-12 shown in Fig. 6 include all those mapped by Upholt and coworkers on their two collagen type II cDNA clones, pCAR1 and

Figure 5. Identification of type II clones by hybrid-selected translation. A, Poly (A)⁺ sternum RNA was hybridized with, respectively, pCgII-2, pCgII-10 and pCg54, lanes 3, 4 and 5, and with pCg54, pCgII-3 and pCgII-12, lanes 7, 8 and 9. The selected RNA was translated in the presence of [³H]proline (see Methods) and the [³H]-translation products were separated by slab gel electrophoresis and visualized by fluorography. Lanes 1-5 represent one experiment and lanes 6-9 another; in each experiment, the translations were performed at the same time with the same batch of nuclease-treated lysate and the products were electrophoresed on the same slab gel. Lanes 1 and 6 represent the translation products obtained in the absence of added RNA and lane 2 represents the translation

products from 1 μ g of poly(A)⁺ sternum RNA. The bands corresponding to the two type I chains and to the single type II chain, pro α 1(II), are indicated. The evidence for the identification of these bands has been presented earlier (3). The faint band running between pro α 1(I) and pro α 1(II) (most evident in lane 7) has not been identified. Lanes 2 and 9 were exposed for 3 days and the remaining lanes for 26 days. B, the densitometer tracings for lanes 3-5 are shown along with the ratios of pro α 1(II) to pro α 1(I) chains, obtained by cutting out and weighing the peaks.

pCAR2 (11), except Hha I. In addition, the sites for BstN 1, Rsa I and Sau3A I were mapped on pCgII-12. No cleavage sites for Alu I, Bam HI or Sal I were found. Comparison of the restriction maps place the 5' end of pCgII-12 near the middle of pCAR1 and place its 3' end approximately 50 bp downstream from the 3' end of pCAR2. Within the region of overlap, the restriction maps are in good agreement.

Preliminary sequence data have been obtained by Philip Mason (personal communication) for the two 5'-Sau3A I fragments (87 and 77 bp long, respectively), and for the small Hinf I fragment (61 bp) and the adjoining 5'-Hinf I fragment (209 bp). These sequences correctly predict all the restriction sites that we found in these fragments, including the 3 Hha I sites that we did not map but that were mapped by Upholt for pCAR2 (11). The sequence data also predict the 2 Hpa II sites separated by 12 bp shown in Fig. 6, rather than the single Hpa II site previously mapped to this region (11). The sequences of the 2 Sau3A I fragments show that they code for a peptide that is very homologous to the carboxyl-propeptide of chick collagen pro α 1(I) and pro α 2(I) chains (32). The homology begins at the 5' end of the 87 bp Sau3A I fragment with a codon (ATC) for isoleucine, corresponding to the ATC codon beginning at position 616 in pro α 1(I) cDNA (32) and to the CTA codon for leucine beginning at position 580 in pro α 2(I) cDNA (32) and extends downstream for at least 42 amino acid residues, through the Leu-Pro-Ile sequence. This region contains both the Cys and the Trp residue which are found in the homologous region of both type I chains and are underlined in ref. 32, as conserved amino acids that may be important to function. The absence of the Hinf I site found in pro α 1(I) cDNA in this region is due to the change of an AGT codon

Figure 6. Restriction cleavage maps of collagen type II cDNA plasmids. The 3 smaller inserts are aligned under pCgII-12 to indicate the areas of overlap. In the case of the 3 smaller plasmids, only sufficient sites to orient these plasmids relative to pCgII-12 were mapped. The apparent absence of a site expected from the overlap with pCgII-12 indicates only that data for the location of that site were not obtained. The evidence for a pair of Hpa II sites separated by 12 bp in pCgII-12 comes from the DNA sequencing data; the resolution of the restriction mapping was sufficient only to indicate that an Hpa II site was present in this area. Similarly, restriction mapping showed the presence of a BstNI site in the region where 3 BstNI sites separated by, respectively, 27 and 9bp are mapped; the presence of these 3 sites is also based on DNA sequencing data.

for serine to an AAA codon for lysine in the pro α 1(II) cDNA sequence. The 5' to 3' orientation of the mRNA required by the sequence data as shown in Fig. 6 is in agreement with the earlier report (11).

DISCUSSION

The evidence that the 4 clones described here contain type II collagen cDNA sequences can be summarized as follows. In colony hybridization, these clones hybridized better to the type II cDNA prepared from collagen mRNA from purified chondrocytes than to the type I cDNAs prepared from collagen mRNAs from calvaria. The plasmid DNA isolated from these clones also

hybridized strongly to type II cDNA. Sufficiently stringent hybridization conditions were found to prevent cross-hybridization between type I cDNAs and type II mRNAs or between type II cDNAs and type I mRNAs. Under these conditions, the 4 cloned cDNAs described here hybridized to an RNA species from purified chondrocytes that has the size expected for collagen mRNA, but they did not hybridize to the type I collagen mRNAs from calvaria. The 4 cloned cDNAs are also complementary to an RNA species in chondrocytes that decreases on exposure of cultures to bromodeoxyuridine with the same time-course as the decrease of type II collagen mRNA. Hybrid-selected translation experiments showed that these clones specifically selected collagen mRNAs from total sternum poly(A)+RNA, and that they select the mRNA for type II collagen in preference to the mRNAs for type I collagen.

The restriction map of the largest of these clones, pCgII-12, indicates that it is probably identical, in its area of overlap, with the two clones previously described by Upholt and coworkers (11). The partial DNA sequence data show that the sequence of pCgII-12 is very homologous to, but not identical with, the sequences of the corresponding regions of chick $\text{pro}\alpha 1(\text{I})$ and $\text{pro}\alpha 2(\text{I})$ mRNA (31).

These cloned type II collagen cDNAs should prove useful in clarifying the mechanisms of control of type II collagen gene expression during the bromodeoxyuridine-induced shift to type I collagen synthesis, as well as in other situations where the expression of this gene is changing.

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