# Differential Localization of mRNAs of Collagen Types I and II in Chick Fibroblasts, Chondrocytes, and Corneal Cells by In Situ Hybridization Using cDNA Probes

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*Abstract.* We have employed a highly specific in situ hybridization protocol that allows differential detection of mRNAs of collagen types I and II in paraffin sections from chick embryo tissues. All probes were cDNA restriction fragments encoding portions of the C-propeptide region of the pro $\alpha$-chain, and some of the fragments also encoded the 3′-untranslated region of mRNAs of either type I or type II collagen.

Smears of tendon fibroblasts and those of sternal chondrocytes from 17-d-old chick embryos as well as paraffin sections of 10-d-old whole embryos and of the cornea of 6.5-d-old embryos were hybridized with $^3$H-labeled probes for either type I or type II collagen mRNA. Autoradiographs revealed that the labeling was prominent in tendon fibroblasts with the type I collagen probe and in sternal chondrocytes with the type II collagen probe; that in the cartilage of sclera and limbs from 10-d-old embryos, the type I probe showed strong labeling of fibroblast sheets surrounding the cartilage and of a few chondrocytes in the cartilage, whereas the type II probe labeled chondrocytes intensely and only a few fibroblasts; and that in the cornea of 6.5-d-old embryos, the type I probe labeled the epithelial cells and fibroblasts in the stroma heavily, and the endothelial cells slightly, whereas the type II probe labeled almost exclusively the epithelial cells except for a slight labeling in the endothelial cells. These data indicate that embryonic tissues express these two collagen genes separately and/or simultaneously and offer new approaches to the study of the cellular regulation of extracellular matrix components.

In situ hybridization is a technique developed to detect the formation of nucleic acid hybrid molecules (DNA/DNA, DNA/RNA, RNA/RNA) between the target nucleic acid immobilized in cyto-histological preparations and the labeled probe polynucleotide containing a complementary sequence. Because of the specificity of the hybridization reaction under conditions that allow a precise cytological localization, this technique has been a valuable tool for mapping specific DNA sequences on chromosomes (10, 26). Recently, with the availability of cDNA clones encoding a variety of unique mRNA sequences and the increase in sensitivity and specificity of the technique, in situ hybridization has become an essential tool for detecting the location and abundance of RNA transcripts of interest in cells and tissue sections (3, 7, 11, 14, 31).

Application of in situ hybridization to matrix-producing cells has been limited (6, 29, 32). In vertebrates, there are at least ten different types of collagens composed of polypeptides that are the products of more than ten different genes (2, 22). All collagen polypeptides share common features in their basic molecular structure. Type I and type II procollagens contain highly conserved amino acid sequence domains (9, 24). Nucleotide sequences of cDNAs coding for these two collagen pro $\alpha$-chains show a high degree of homology (9, 30). This high degree of homology may lead to serious problems of cross-hybridization when type I and type II collagen cDNAs are used for in situ hybridization, unless special precautions are taken to avoid such cross-hybridization.

Moreover, a switch of collagen types from type I to type II occurs during chondrogenesis and vice versa in cultured chondrocytes (34). For example, fluorescence staining with antibodies to type I collagen is demonstrable in the extracellular matrix of chick precartilaginous limb bud mesenchyme and is increased in intensity in the cartilage blastema until early stage 24 (15). At late stage 24, the first staining with antibodies to type II collagen appears in the cartilage blastema. In succeeding stages the staining for type II collagen of the cartilage anlage is enhanced, while that for type I collagen disappears from the core of the blastema and eventually remains only in the perichondrium. Conversely, in monolayer

culture of mature chondrocytes, transition from type II to type I collagen synthesis occurs with time in culture. In either case of transition of collagen types, immunofluorescence double staining of tissues at certain stages demonstrates two types of collagen in individual cells. Also, in the developing chick eye, the 5-d primary cornea stroma before invasion by fibroblasts stains uniformly with antibodies to collagen types I and II (17, 33), suggesting the simultaneous production of the two types of collagen by corneal epithelium at this stage.

In this report we describe our results of in situ hybridization studies of chick tendon, cartilage, corneal epithelium, and corneal fibroblasts using cDNA fragments specific for type I and type II collagens. We show how specific hybridizations, with a minimum of cross-hybridization, can be obtained by a careful selection of probes and hybridization conditions. Our results also demonstrate the virtual absence of type I collagen mRNA in chondrocytes in situ, a level of type II mRNA which is below the detection limit in fibroblasts, and high levels of both type I and type II collagen mRNAs in corneal epithelial cells at the time of synthesis of the corneal stroma. Thus, molecular models for regulation of type I and type II collagen gene transcription must be able to account for the independent control of the expression of the two collagen types in different cells.

## Materials and Methods

### Preparation of Cells and Tissue Sections

Fibroblasts were isolated from 17-d-old embryonic chick leg tendons and chondrocytes were from the sternal cartilages by digestion with collagenase–trypsin as detailed previously (27). Cells ($2.5 \times 10^5$/10 μl Krebs medium) were spread in a circular area 7 mm in diameter on subbed microscope slides. Subbed slides were prepared by incubating microscope slides overnight at 68°C in 3 × standard saline-citrate buffer (SSC)[1] (1 × SSC is 0.15 M NaCl, 0.015 M trisodium citrate, pH 7.0) containing 1 × Denhardt's mixture (0.02% each of Ficoll [Sigma Chemical Co., St. Louis, MO, type 400], polyvinylpyrrolidone [Sigma Chemical Co., PVP-40], and bovine serum albumin [BSA] [Sigma Chemical Co., A 4378]) (8) and, after draining, by fixing for 20 min in ethanol/acetic acid, 3:1 (3). Cells were dried in air, fixed in a 4% formaldehyde (Polysciences, Inc., Warrington, PA, methanol free, E. M. grade.) in 0.01 M phosphate-buffered saline (PBS) at room temperature for 30 min, washed three times in PBS for 5 min each, dehydrated twice in 70% ethanol, once in 95% ethanol for 5 min each, and dried in air. The cells may be stored at 4°C for at least 1 mo.

10-d-old whole chick embryos and the cornea of 6.5-d-old embryos (stage 30) (15) were fixed in the same formaldehyde fixative for 1 h at room temperature. They were washed three times in PBS for 5 min each, dehydrated in a graded series of ethanol, cleared in xylene, and embedded in paraffin. Sections were cut at 5 μm, mounted on subbed microscope slides, dried thoroughly for 3 h on a slide warmer at 42°C, and stored at 4°C.

Before use, the slides were thoroughly deparaffinized by placing them in a staining jar and heating in an oven at 60°C for 1 h. Melted paraffin was removed by adding fresh xylene to the hot staining jar which was then placed under a ventilation hood for 30 min with occasional shaking. The slides were washed in two changes of fresh xylene for 15 min each, two changes of 100% ethanol for 5 min each, and dried in air for 10 min. The slides were then immersed in the formaldehyde fixative for 10 min at room temperature to ensure that the deparaffinized sections attach firmly to the coating of the subbed slides, and then washed in three changes of PBS for 5 min each. The slides were dehydrated in two changes of 70% ethanol, once in 95% ethanol for 5 min each, and dried in air before processing for prehybridization treatment.

### Preparation of cDNA Probes

To select a probe showing a minimum degree of cross-hybridization for each type of collagen RNA, we have compared different restriction fragments of types I and II collagen cDNAs. These fragments included the following (Fig. 1): from pYN535, a pro α2(I) specific recombinant plasmid (23), three restriction fragments (a 760-base-pair [bp] PstI-PvuII fragment, a 230-bp PstI-BamHI fragment, a 530-bp BamHI-PvuII fragment); from pro α1(II) specific clones, one fragment of pYN509 (24) (a 320-bp PstI-BamHI fragment), and one fragment of pYN2142 (24) (a 603-bp BamHI-PvuII fragment). As a control, we used a 1,055-bp PstI-PstI fragment containing the 782-bp PstI-HindIII fragment of pBR322 linked to the 273-bp HindIII-PstI fragment of SV40 DNA.

Double-stranded probes were labeled with either α-$^{32}$P or $^3$H using a conventional nick translation protocol (28) with minor modifications. $^{32}$P-labeled nucleotides (dATP, dTTP, dCTP, dGTP) were purchased from Amersham Corp. (Arlington Heights, IL) and each had a specific activity of ~800 Ci/mmol. $^3$H-labeled dTTP with a specific activity of 95–100 Ci/mmol was purchased from New England Nuclear (Boston, MA).

For the labeling of cDNA with $^{32}$P, 10 μCi each of the four different $^{32}$P-labeled deoxynucleoside triphosphates were lyophilized in an Eppendorf tube. To the tube the following components were added to give final concentrations of the nick translation buffer (50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 10 mM β-mercaptoethanol, 50 μg/ml BSA [Miles Scientific, Naperville, IL]), a mixture of unlabeled deoxynucleoside triphosphates (0.1 μM each of dATP, dCTP, dTTP, and dGTP), 0.03 μg/ml DNase I (Boehringer Mannheim Biochemicals, Indianapolis, IN), and 1–7 μg/ml DNA fragments. The mixture was incubated at 37°C for 15 min. After adding 5 U of *Escherichia coli* DNA polymerase I (Boehringer Mannheim Biochemicals), the mixture was incubated at 14°C for 2 h. After the reaction was terminated by adding EDTA, the sample was fractionated by chromatography on a Sephadex G-50 column and DNA was precipitated by adding 20 μg sonicated salmon sperm DNA, NaCl, and ethanol. The DNA sample was pelleted by centrifugation, washed with 70% ethanol, dried, and suspended in $H_2O$. The final sample had a specific activity of ~1–5 × $10^8$ cpm/μg DNA.

For the labeling with $^3$H, the reaction mixture contained 100 μCi of lyophilized $^3$H-dTTP, nick translation buffer, cold nucleotide-mix (30 μM each of dATP, dGTP, dCTP), 20–40 μg/ml of the DNA fragments, 1 μg/ml DNase I, and 15 U/50 μl *E. coli* DNA-polymerase I. The mixture was incubated at 14°C for 2 h, fractionated, and precipitated as above. The final sample usually had a specific activity of 1–2 × $10^7$ cpm/μg DNA.

### Northern Blotting

RNAs from 17-d-old chick embryo calvaria and from sterna were electrophoresed on a 0.8% agarose gel containing formaldehyde (18). After the RNA was

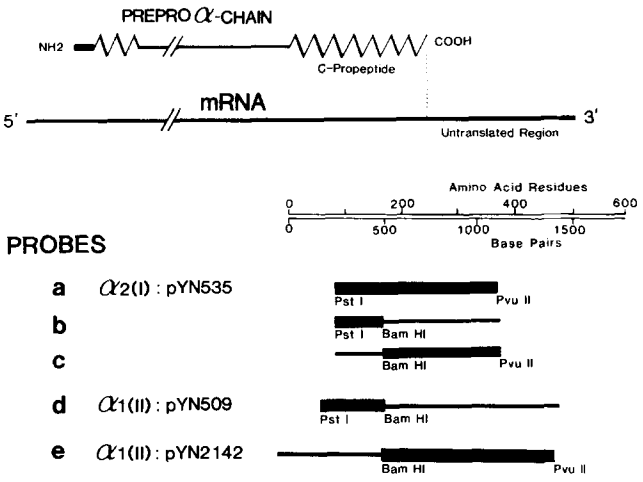



*Figure 1.* Diagram showing different restriction fragments of collagen cDNAs. pYN535 (a pro α2[I]-specific cDNA), pYN509, and pYN2142 (pro α1[II]-specific cDNAs) are shown in their positions relative to the procollagen mRNA. Regions used as hybridization probes are indicated by wide black bars; the thin lines indicate regions of the cDNA inserts that were not included with the probes. The direction of transcription is from left to right, as indicated by 5′ and 3′. Numbers refer to nucleotides per amino acids in the C-propeptide, the 3′-untranslated region, and the poly(A) region, while zero refers to the C-protease cleavage site. At the top of the figure, the positions of the different domains within the pre-pro α-chain are indicated relative to the mRNA.

1. *Abbreviation used in this paper:* SSC, standard saline-citrate buffered solution.

blotted onto nitrocellulose, the filters were baked and prehybridized in a solution containing 50% formamide (Bethesda Research Laboratories, Bethesda, MD), 0.1% sodium dodecyl sulfate, 2 × SSC, 1 × Denhardt's mixture (8), and 50 μg/ml salmon sperm DNA (Sigma Chemical Co.) at 45°C for 6 h. The $^{32}$P-labeled DNA probes were denatured by being boiled for 2 min and quickly chilled. The nitrocellulose filter was hybridized in the above prehybridization solution by adding $^{32}$P-labeled, denatured DNA probes at 45°C for 24 h. The filter was subsequently washed first in 2 × SSC at room temperature and then in 2 × SSC, 1 × SSC, 0.5 × SSC, and 0.2 × SSC at 50°C for 10 min each. After drying completely, the filter was exposed to x-ray film for autoradiography.

### *In Situ Hybridization*

Our hybridization procedures were basically the same as those described by Hafen et al. (14), but differed in the following steps: (*a*) we eliminated the prehybridization treatment with 0.2 N HCl and heating at 70°C in 2 × SSC; (*b*) we included the acetylation step of Hayashi et al. (16), and (*c*) we compared two different concentrations of NaCl and temperatures of the hybridization medium.

Prehybridization treatment of cell smears and tissue sections was done by covering the sample with predigested pronase at a final concentration of 0.25 mg/ml in 50 mM Tris-HCl, pH 7.6, containing 5 mM EDTA for 10 min at room temperature. Predigestion of pronase (Boehringer Mannheim Biochemicals) was carried out as described by Hafen et al. (14). The slides were washed in PBS containing 2 mg/ml glycine for 30 s, twice in PBS for 30 s each, and fixed in 4% formaldehyde in PBS for 20 min. Then the slides were washed twice in PBS containing 2 mg/ml glycine for 5 min each, immersed in 0.1 M triethanolamine buffer (pH 8.0) for 5 min, and incubated in a freshly prepared mixture of 0.25% acetic anhydride in the triethanolamine buffer for 10 min (16). To prepare the mixture, acetic anhydride was added within a few seconds before use. The slides were washed twice in 2 × SSC for 5 min each, dehydrated twice in 70% ethanol for 5 min each, in 95% ethanol for 5 min, and dried in air under sterile conditions.

The sterile hybridization mixture contained the labeled cDNA probe (2 μg/ml), yeast t-RNA (500 μg/ml), salmon sperm DNA (80 μg/ml), 50% formamide, 10 mM Tris-HCl, pH 7.0, 0.15 M or 0.3 M NaCl, 1 mM EDTA (pH 7.0), and 1 × Denhardt's mixture (8), with or without 10% dextran sulfate (Pharmacia P-L Biochemicals, Piscataway, NJ). The complete hybridization mixture was heated for 3 min at 80°C to denature the probe DNA (4) and chilled quickly in ice-water. 20 μl of the mixture was spread over the pretreated dry sample on a microscope slide. The sample was covered with a sterile 22 × 22 mm cover glass (Corning Glass Works, Corning, NY) and its edges were sealed with a 1:1 mixture of rubber cement (Carter's Ink Co., Waltham, MA) and petroleum ether. The slides were incubated by placing them on an aluminum cake pan (G & S Metal Products Co., Inc., Cleveland, OH) floating on a water bath (12) at 35° or 45°C for 18–20 h. After hybridization, the rubber cement was peeled off with fine forceps and the cover glass was removed by immersing the slides in 2 × SSC at room temperature. The slides were washed further in three changes of 2 × SSC for 10 min each at room temperature, 0.5 × SSC for 10 min at 35° or 50°C, and three changes of 0.1 × SSC for 10 min each at 35° or 50°C, unless otherwise mentioned. Afterwards the slides were dehydrated in two changes of 70% ethanol and 95% ethanol for 5 min each and dried in air. The slides were immersed in Kodak NTB-2 nuclear track emulsion diluted 1:1 with 0.6 M ammonium acetate, pH 7.0 (3), unless otherwise specified, and kept at 43°C, and the back of the slide wiped free of emulsion. The slide was placed for 5 min on a cold stainless steel plate laid horizontally on crushed ice and then dried at room temperature for 1 h. Exposure was carried out in a partially evacuated dessicator over a Drierite dessicant (W. A. Hammond Drierite Co., Xenia, OH) for 3–10 d at 4°C. The exposed slides were developed in Kodak D-19 developer for 2.5 min at 18°C. The sections were stained for 1 min with Harris' alum hematoxylin (Harleco, Gibbstown, NJ) and examined in the light microscope.

In this paper "standard conditions" refer to a hybridization mixture containing 0.3 M NaCl and a temperature of hybridization and subsequent washing at 35°C. "Stringent conditions" refer to a mixture containing 0.15 M NaCl and a temperature of hybridization at 45°C and of washing at 50°C. In some cases dextran sulfate was omitted from the stringent hybridization mixture.

## *Results*

### *cDNA Probes versus RNA Probes*

We began this study by using single-stranded RNA probes which were prepared by cloning cDNA restriction fragments into the pSP6 vector and by synthesizing RNA using SP6 RNA polymerase (5, 13). RNA probes have the advantage over nick translated cDNA probes in that they can be labeled to a higher specific activity and have been used successfully by Angerer and his associates (7, 20) in their studies on sea urchin embryo RNAs. However, for the probe sequences used in our studies, RNA probes gave high levels of cross-hybridization. With RNA probes the stringency required to obtain a differential labeling between type I and type II collagen mRNAs was such (hybridization and washing at 68°C) that the overall signal was reduced and many cells detached from slides. Since in situ hybridization requires retention of cells or tissue sections on slides and preservation of morphology, the whole procedure has to be carried out at a reasonably low temperature. The less stable nature of DNA/RNA hybrids formed with DNA probes has the advantage over the more stable RNA/RNA duplex (35) of being melted at a lower temperature. cDNA probes can also be prepared quickly and reproducibly by the well-established nick translation technique (28) which is convenient when numerous probes are to be screened. Therefore, in the present study only cDNA probes were used.

### *Selection of Restriction Fragments*

Calvarial osteoblasts and tendon fibroblasts synthesize type I collagen but not type II, whereas sternal chondrocytes synthesize normally type II collagen but not type I (19). Thus a specific type I probe should exclusively hybridize to the osteoblast or fibroblast mRNA and type II probe to chondrocyte mRNA. In Northern blot hybridization analyses three restriction fragments of pYN535 (PstI-PvuII, PstI-BamHI, BamHI-PvuII)-specific for pro α2(I), a pYN509 fragment (PstI-BamHI) and a pYN2142 fragment (BamHI-PvuII), both specific for pro α1(II), were compared for specific hybridization to mRNAs isolated from calvaria and sterna. All three fragments of pYN535 hybridized quite selectively to calvarial RNA with a slight cross-hybridization to sternal cartilage RNA (data not shown). The pYN509 fragment hybridized

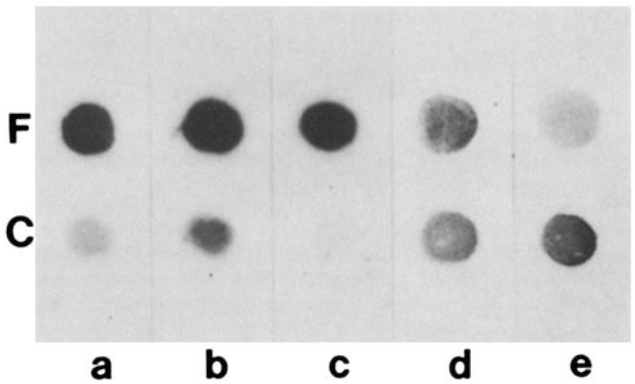



*Figure 2.* Hybridization of $^{32}$P-labeled restriction fragments of collagen cDNAs to chicken tendon fibroblasts (*F*) and sternal chondrocytes (*C*). The probes used were the same as shown in Fig. 1 and are arranged in the same order. Cells were processed for in situ hybridization with $^{32}$P-labeled probes under standard conditions (see Materials and Methods) and exposed to x-ray film for autoradiography. Note the differences in the degree of cross-hybridization among fragments (*a–c*) of pYN535 to sternal chondrocytes (*C*), and of the type II probes (*d* and *e*) to fibroblasts (*F*).

strongly to sternal RNA but also considerably to calvarial RNA. The pYN2142 fragment hybridized strongly to sternal RNA, producing much less cross-hybridization to calvarial RNA than did the pYN509 fragment.

Subsequently, cell smears of fibroblasts (F) and chondrocytes (C) were hybridized with the $^{32}$P-labeled five fragments of cDNAs mentioned above and exposed to an x-ray film for autoradiographic evaluation. The autoradiography demonstrated (Fig. 2) a similar pattern of specificity in hybridization for each probe as seen by Northern blotting but with a better definition of small differences in labeling. Here the degree of specific hybridization of the three fragments of pYN535 to

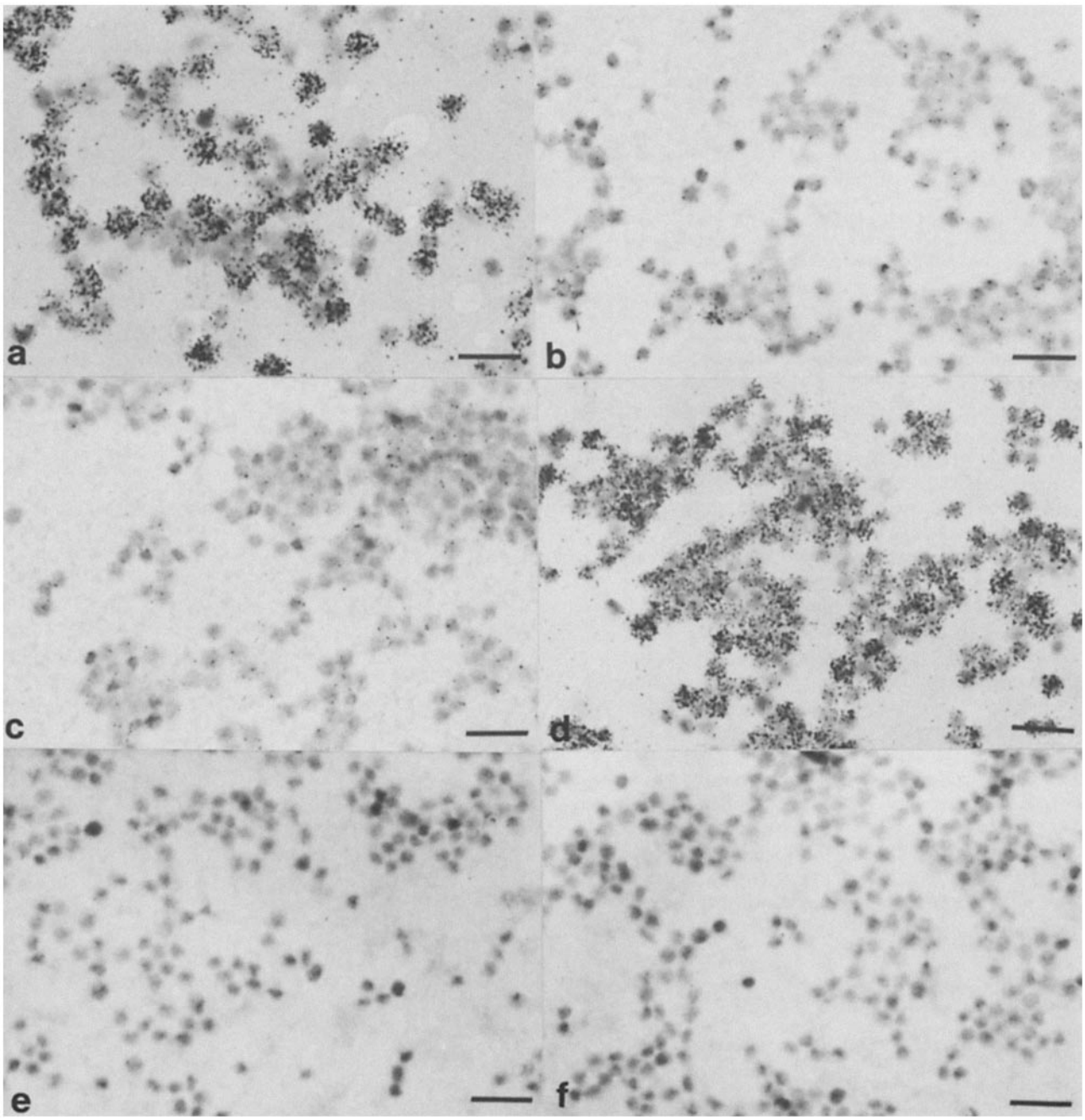



*Figure 3.* Autoradiographs of tendon fibroblasts and sternal chondrocytes isolated from 17-d-old chick embryos. Cells were processed for in situ hybridization with $^{3}$H-labeled probes under stringent conditions (see Materials and Methods) without dextran sulfate. The probes used were the PstI-PvuII fragment of pYN535-specific for type I collagen mRNA, and the BamHI-PvuII fragment of pYN2142-specific for type II collagen mRNA. The slides were exposed to nuclear emulsion for 6 d. (*a*) Fibroblasts hybridized to the type I collagen probe. Note many silver grains over fibroblasts. There is a considerable variation in the grain number per cell. Cytoplasmic localization of silver grains is indicated in cells containing a small number of grains. (*b*) Chondrocytes hybridized to the type I collagen probe. Note few or no silver grains over cells. (*c*) Fibroblasts hybridized to the type II probe, showing few or no silver grains. (*d*) Chondrocytes hybridized to the type II probe. Note that most of the cells are labeled. (*e*) Fibroblasts hybridized to the control probe (see Materials and Methods), showing no labeling. (*f*) Chondrocytes hybridized to the control probe, showing no silver grains. Bars, 20 $\mu$m.

fibroblast (F) RNA was shown to be in the increasing order of PstI-BamHI (*b*), PstI-PvuII (*a*), and BamHI-PvuII (*c*). Based on these results, we chose BamHI-PvuII fragment of pYN535 as probe for pro α2(I) mRNA and used in our recent work (Fig. 5). Our earlier preparations (Figs. 3 and 4) were, however, reacted with PstI-PvuII fragment because BamHI-PvuII fragment was not available at that time. As probe for pro α1(II) mRNA, we chose the BamHI-PvuII fragment of pYN2142 (*e*).

No labeling occurred when fibroblast smears were treated with 2 mg/ml preboiled RNase A (Worthington Scientific Div., Cooper Biomedical, Inc., Malvern, PA) (21) for 1 h at room temperature before hybridization with the probe for pro α2(I) (data not shown), implying that the hybridization with the probe was dependent on the presence of RNA in cells.

### *Conditions of In Situ Hybridization*

Stringency of the hybridization condition was optimized by microscopic comparison of autoradiographic labeling of isolated fibroblast or chondrocyte smears that had been hybridized with $^3$H-labeled probes for either type I or type II collagen mRNAs or with the similarly labeled control probe prepared from pBR322 DNA. Under the standard conditions, the probe for type I collagen mRNA labeled fibroblasts intensely and chondrocytes only slightly, whereas the probe for type II collagen mRNA labeled chondrocytes strongly and, to some degree, fibroblasts. Under the stringent conditions, the specificity of hybridization was further increased, but with some reduction of the overall signal. Here the type I probe labeled fibroblasts almost exclusively and the type II probe labeled only chondrocytes.

Fig. 3 illustrates autoradiographs of fibroblasts and chondrocytes labeled under the stringent conditions, without dextran sulfate in the hybridization mixture. All slides were exposed to nuclear emulsion for 6 d. Fibroblasts labeled with the type I probe (PstI-PvuII fragment) (Fig. 3 *a*) were covered with silver grains but with a considerable variation in grain number per cell. Cytoplasmic localization of silver grains was indicated in cells containing a small number of grains. Fibroblasts exposed to the type II probe (Fig. 3 *b*) contained few or no silver grains. In contrast, chondrocytes exposed to the type I probe (Fig. 3 *c*) showed few or no silver grains, whereas those hybridized with the type II probe (Fig. 3 *d*) contained many silver grains over almost all cells. With the control probe there were practically no silver grains over the fibroblasts (Fig. 3 *e*) or chondrocytes (Fig. 3 *f*).

### *Localization in Chick Embryo Tissues*

Paraffin sections from 10-d-old whole chick embryos were processed for in situ hybridization with the probes under the same stringent conditions as were done for the cell smears, but in the presence of dextran sulfate. Autoradiographs after the exposure to nuclear emulsion for 10 d revealed that in the scleral cartilage with its surrounding fibroblastic sheet, the type I probe (Fig. 4 *a*) labeled most fibroblasts and only a few chondrocytes, whereas the type II probe (Fig. 4 *b*) labeled chondrocytes and few fibroblasts. Similarly in the limb, the type I probe (Fig. 4 *c*) labeled mostly perichondrial fibroblasts and the type II probe (Fig. 4 *d*) chondrocytes. Cytoplasmic localization of silver grains were obvious in the chondrocytes. Sections of the cornea from 6.5-d-old chick embryos were subjected to in situ hybridization under the same conditions but using the BamHI-PvuII fragment (see Selection of Restriction Fragment) as the type I probe. These sections were washed at 45°C and exposed to the undiluted nuclear emulsion for 10 d. Autoradiographs demonstrated that the type I probe (Fig. 5 *a*) labeled heavily the epithelial cells and fibroblasts in the stroma, and slightly the endothelial cells. In contrast, the type II probe (Fig. 5 *b*) labeled almost exclusively the epithelial cells except for a slight labeling in the endothelial cells.

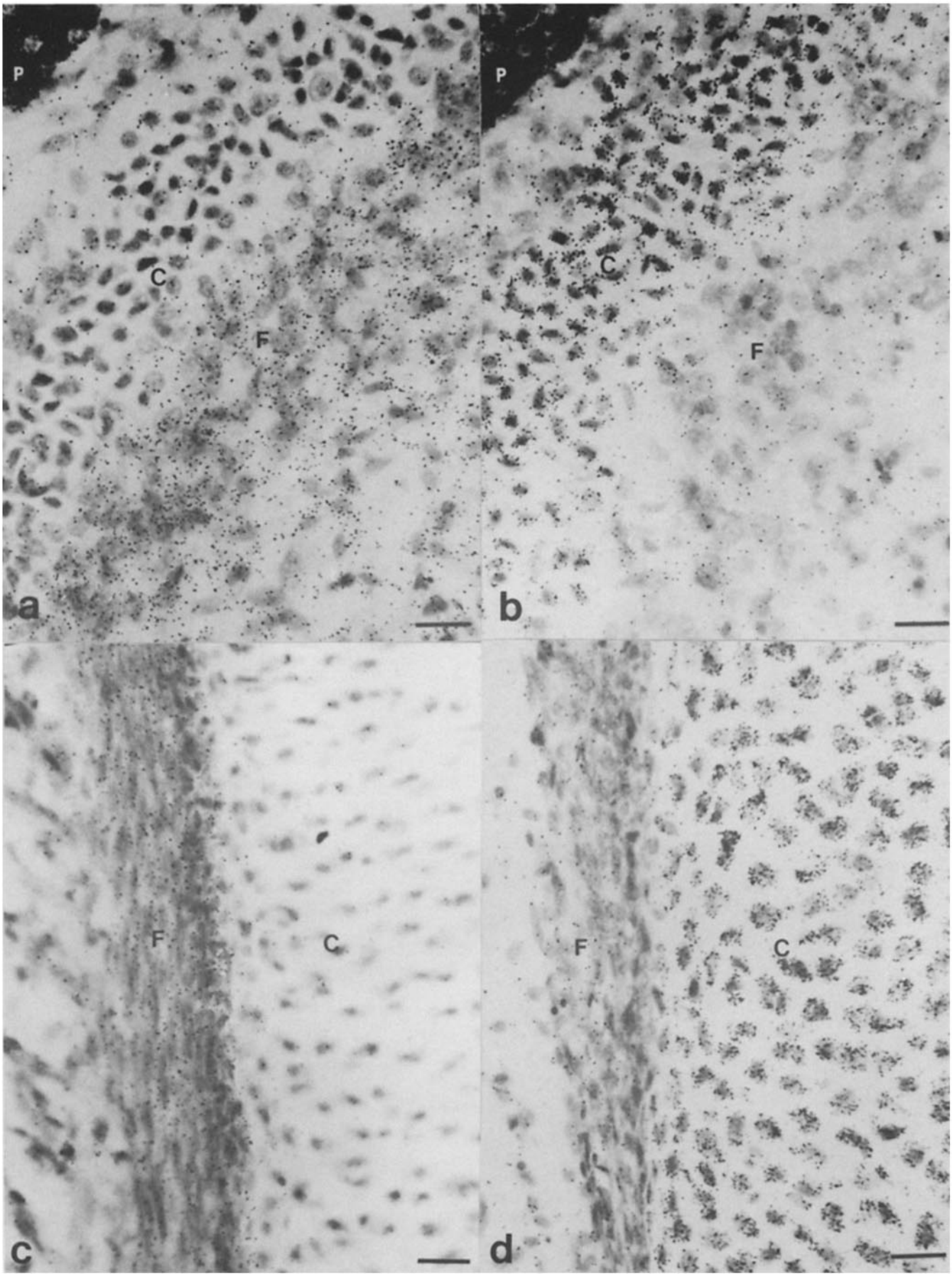
## *Discussion*

### *Requirements for Specific Hybridization*

***Specificity of Restriction Fragments.*** All five restriction fragments of cDNAs tested were complementary to portions of the C-propeptide region of pro α-chain, and three of the fragments were also complementary to the 3′-end untranslated region of mRNAs of either type I or type II collagen (Fig. 1). A comparison of the pro α2(I) C-peptide sequence with that of the pro α1(II) C-peptide indicates that two domains, one at the amino terminus of the C-peptide and one around amino acid residue 125 shows a high degree of variability. In the remaining part of the sequence the C-peptides contain several domains of highly conserved amino acid sequences separated by moderately conserved sequence domains (24).

To examine how probe specificity correlates with nucleotide sequence homology, we compared sequences among the chicken pro α1(II), pro α1(I), and pro α2(I) cDNAs for each region of the sequences that is covered by each restriction fragment tested as probe for in situ hybridization (9, 30). The following results were obtained (Table I): (*a*) the probe for pro α2(I), the insert of pYN535, contained a sequence that was homologous with that of pro α1(II) in the range of 45–68%, varying in the degree of homology among three restriction fragments examined; and (*b*) in the probes for pro α1(II), the PstI-BamHI fragment of pYN509 showed 76% sequence homology and the BamHI-PvuII fragment of pYN2142

*Figure 4.* Paraffin sections from a 10-d-old whole chick embryo. Serial sections were processed for in situ hybridization using the same conditions as in Fig. 3, except that dextran sulfate was included in the hybridization mixture. Slides were exposed to nuclear emulsion for 10 d. (*a*) An area including the scleral cartilage with its surrounding fibroblast sheet. Hybridization was with the type I probe. Note a number of silver grains over fibroblasts (*F*) and few or no grains over chondrocytes (*C*). (*P*) indicates a portion of the pigment epithelium. (*b*) Area comparable with that of *a*, showing hybridization to the type II probe. Note numerous silver grains over chondrocytes (*C*) but only few over fibroblasts (*F*). (*c*) An area of the forelimb cartilage and its surrounding fibroblasts. Hybridization was with the type I probe. Note many silver grains in the fibroblast (*F*) area and a few over chondrocytes (*C*). (*d*) A comparable area with that of *c* but hybridized to the type II probe. Note a number of silver grains over most of the chondrocytes (*C*) and a few over fibroblasts (*F*). Bars, 20 μm.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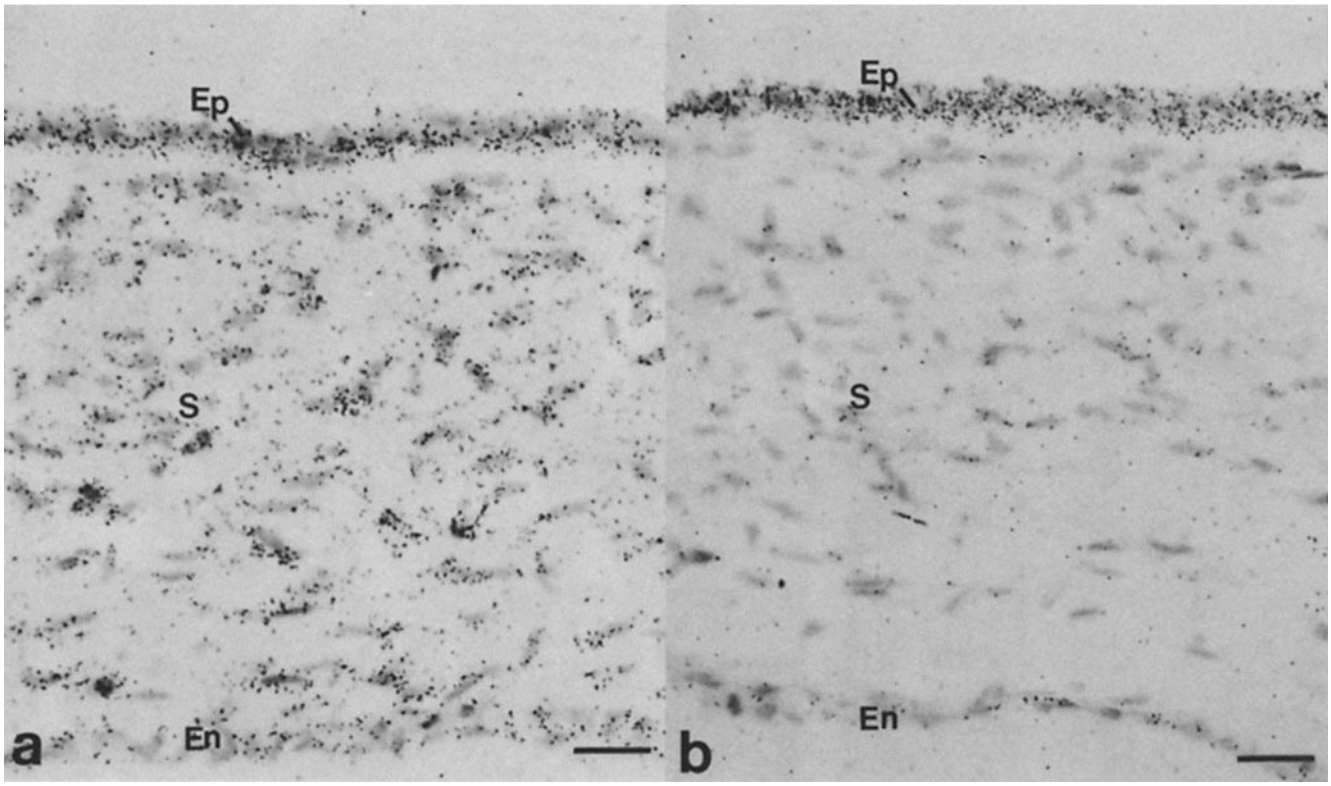


*Figure 5.* Paraffin sections of the cornea from a 6.5-d-old chick embryo. Serial sections were processed for in situ hybridization with the same conditions as in Fig. 4, except that the probe for type I collagen mRNA was the BamHI-PvuII fragment (see text) and the washing temperature was 45°C. The slides were exposed to undiluted nuclear emulsion for 10 d. (*a*) Hybridized to the type I probe. Note numerous silver grains over the epithelial cells (*Ep*) and most of the fibroblasts in the stroma (*S*), and a small number of grains over endothelial cells (*En*). (*b*) Hybridized to the type II probe. Note accumulation of silver grains almost exclusively over the epithelial cells (*Ep*) and a few grains over the endothelial cells (*En*). Bars, 20 μm.

*Table I. Homology in Nucleotide Sequence between Different Procollagen cDNAs within the Regions Used as Hybridization Probes*

| | Pro α2 (I) cDNA | | | Pro α1 (II) cDNA | |
|---|---|---|---|---|---|
| | pYN535 | | | pYN509 | pYN2142 |
| cDNAs compared | PstI-PvuII | PstI-BamHI | BamHI-PvuII | PstI-BamHI | BamHI-PvuII |
| | % | % | % | % | % |
| Pro α2 (I) | 100 | 100 | 100 | 76 | 55 |
| Pro α1 (I) | 61 | 73 | 54 | 65 | 45 |
| Pro α1 (II) | 52 | 68 | 45 | 100 | 100 |

showed 55% sequence homology with pro α2(I). As expected, probes showing extensive sequence homology had a tendency to produce cross-hybridization when compared by Northern blotting and x-ray film autoradiography of cell smears. It appears that if a probe is derived from a region showing over 70% sequence homology with other collagens, substantial cross-hybridization occurs with the hybridization conditions we have employed.

### *Optimization of Hybridization Procedures*

Although selected probe fragments gave a highly specific hybridization when evaluated using x-ray film autoradiography of cell smears, close examination under a microscope of autoradiographs of cells hybridized with $^3$H-labeled probes revealed a slight yet definite labeling due to cross-hybridization. Raising the stringency of hybridization and washing conditions as discussed above largely eliminated this degree of cross-hybridization.

We have eliminated two commonly used steps in the prehybridization treatment. First, we eliminated treatment with 0.2 N HCl for 20 min at room temperature, a step used by some workers (3, 14) to remove basic proteins, because it reduced autoradiographic signals in our preparations. Pardue and Gall (26) experienced a similar reduction of the signal when they used 0.2 N HCl for 30 min before hybridization to DNA. Recently Gee and Roberts (11) noted that 0.2 N HCl treatment for 10 min enhanced the autoradiographic signal, whereas 0.5 N HCl treatment for 10 min decreased the signal. The second step we omitted was treatment with 2 × SSC for 30 min at 70°C first used by Bonner and Pardue (1) to maintain chromosome morphology and enhance RNA hybridization. We eliminated this treatment by weighing the degree of signal enhancement achieved against possible structural damage to tissue sections by the heat treatment. However, we retained pronase treatment (4, 14) in our prehybridization treatment as this enzyme digestion always resulted in marked enhancement of the signal.

## *Localization of Collagen mRNAs by In Situ Hybridization versus Localization of Collagens by Immunohistochemistry*

With the use of monoclonal antibodies and immunohistochemistry, specific patterns of distribution of different types of collagens in tissues have been demonstrated (19). Type I collagen is present in tendons and fibroblasts (25), the perichondrium and surrounding connective tissues, and type II collagen in the cartilage matrix (19). Therefore, detection of type I collagen mRNA in fibroblasts and type II mRNA in chondrocytes by in situ hybridization was expected. In their immunohistochemical study of the chick cornea, Hendrix et al. (17) found that in the 5-d-old embryo, the primary corneal stroma stained uniformly for collagen types I and II and in the 7-d embryo, type I collagen became the predominant collagen within the stroma, whereas type II became progressively localized in subepithelial and subendothelial regions. The present hybridization results suggest that in the early chick cornea the epithelial and, to a lesser degree, the endothelial cells, contribute to the synthesis of both collagen types I and II in addition to the production of type I collagen by the stromal fibroblasts.

While the immunohistochemical studies demonstrate collagen that have already been accumulated in tissues and indicate the sites of either biosynthesis, storage, or uptake (11) of collagen, the in situ hybridization technique obviously can help determine when specific cells accumulate mRNAs for specific collagens. The in situ hybridization technique would thus be an essential tool for studies of the rapidly modulated expression of collagen genes during embryonic development.

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