Localization of Types I, II, and III Collagen mRNAs in Developing Human Skeletal Tissues by In Situ Hybridization

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Abstract. Paraffin sections of human skeletal tissues were studied in order to identify cells responsible for production of types I, II, and III collagens by in situ hybridization. Northern hybridization and sequence information were used to select restriction fragments of cDNA clones for the corresponding mRNAs to obtain probes with a minimum of cross-hybridization. The specificity of the probes was proven in hybridizations to sections of developing fingers: osteoblasts and chondrocytes, known to produce only one type of fibrillar collagen each (I and II, respectively) were only recognized by the corresponding cDNA probes. Smooth connective tissues exhibited variable hybridization intensities with types I and III collagen cDNA probes.

The technique was used to localize the activity of type II collagen production in the different zones of cartilage during the growth of long bones. Visual inspection and grain counting revealed the highest levels of proα1(II) collagen mRNAs in chondrocytes of the lower proliferative and upper hypertrophic zones of the growth plate cartilage. This finding was confirmed by Northern blotting of RNAs isolated from epiphyseal (resting) cartilage and from growth zone cartilage.

Analysis of the osseochondral junction revealed virtually no overlap between hybridization patterns obtained with probes specific for type I and type II collagen mRNAs. Only a fraction of the chondrocytes in the degenerative zone were recognized by the proα1(II) collagen cDNA probe, and none by the type I collagen cDNA probe. In the mineralizing zone virtually all cells were recognized by the type I collagen cDNA probe, but only very few scattered cells appeared to contain type II collagen mRNA. These data indicate that in situ hybridization is a valuable tool for identification of connective tissue cells which are actively producing different types of collagens at the various stages of development, differentiation, and growth.

THE majority of total body collagen is found in the specialized connective tissues of the skeletal system in the form of the fibrillar collagens of types I, II, and III. Each of these collagens are synthesized as procollagens containing three proα-chains. Type I collagen is a heterotrimer of two α1(I) and one α2(I) chains, while types II and III collagens are homotrimers of α1(II) and α1(III) chains, respectively. All these chains share considerable homology both at the level of amino acids, mRNAs, and gene structure (5, 17, 19). Additionally, at least nine other collagen types with variable functions have been characterized (5).

The development and growth of the skeletal tissues is a complicated process involving a number of changes in the expression of collagen genes (29, 34). This is exemplified by the growth of long bones which occurs in the growth plate areas by chondrocyte division and deposition of cartilage matrix, containing type II collagen fibers. Having terminally differentiated, the hypertrophic chondrocytes degenerate, the extracellular matrix becomes calcified, and is finally invaded by blood sinusoids, reassembled, and replaced by bony matrix containing type I collagen (29). Biochemical analyses of the many spatially and temporally controlled changes have been difficult to perform and most of our current knowledge of these processes comes from studies using histochemical techniques (12, 26, 35), various in vitro models of chondrogenesis (15, 16), and bone induction models (24). The sequential expression of chick type I and II collagen genes has recently been studied by nucleic acid hybridization during in vitro chondrogenesis (15, 16), and by in situ hybridization in developing chick skeleton (14). The availability of cDNA probes for human fibrillar collagens has now made it possible to perform similar studies on human tissues. In situ hybridization is particularly useful for studies on accumulating proteins, such as collagen, because newly synthesized matrix cannot be distinguished from older matrix which makes localization of metabolically active cells difficult. Such information is not only important for the understanding of human development, but also for studies on the many hereditary diseases (osteochondrodysplasias) affecting the human skeletal system (27).

In this study we report the localization of type I, II, and III collagen mRNAs in developing human long bones and associated structures, with emphasis on the various zones of cartilage and the osseochondral junction. The high degree of

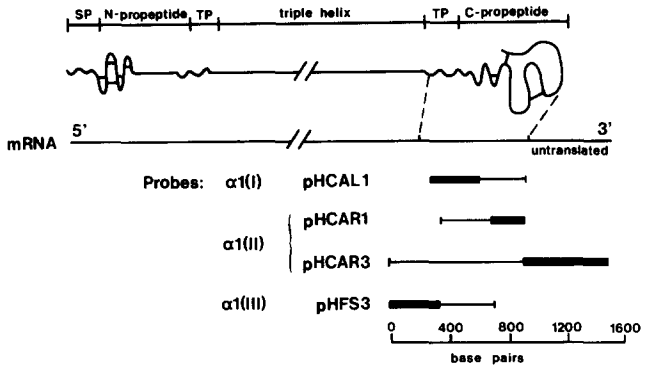

Figure 1. Schematic presentation of a preproα-chain of a procollagen molecule, its corresponding mRNA, and the hybridization probes used in this study. The various domains of the preproα-chain are shown above (*SP*, signal peptide; *TP*, telopeptide). The human cDNA probes, constructed in our laboratory, are shown in respect to their position relative to the mRNA. The regions of the cDNA inserts used for in situ hybridization probes are shown as wide black bars; the thin lines indicate regions not used for probes. At the bottom of the figure is the size scale for the cDNA clones.

homology between the different collagens at nucleotide level required careful selection of probe fragments and washing conditions to minimize the cross-hybridization to other procollagen mRNAs. This is particularly important during in situ hybridization as has recently been discussed in detail by Hayashi et al. (14).

Materials and Methods

Preparation of Tissue Sections

Sections from fingers of 15–18-wk-old human fetuses from therapeutic abortions were fixed in 10% buffered formalin at room temperature for 24 h. The samples were dehydrated in rising ethanol series, cleared in xylene, and embedded in paraffin. 5-μM sections were cut and transferred to acetylated microscope slides using 1% Elmer's glue (Borden, Inc., Columbus, OH) in the flotation bath. For this purpose the slides were cleaned and treated with Denhardt's solution (8) followed by acetylation as described by Brahic and Haase (3). Before hybridization, the slides were baked at 60°C overnight to ensure firm adherence of the samples to the slides (9). The slides were deparaffinized by washing twice in xylene for 10 min, twice in 100% ethanol, and once in 70% ethanol for 5 min.

Preparation of cDNA Probes

The recombinant DNA clones used in this study were: pHCAL1, containing an insert complementary to human proα1(I) collagen mRNA (38), pHCAR1 (10) and pHCAR3 (Elima, K., unpublished data) for human proα1(II) collagen mRNA, and pHFS3 (Sandberg, M., and E. Vuorio, unpublished data) for human proα1(III) collagen mRNA. The nucleotide sequences of these cDNA clones were compared and 200–550 base pair (bp) fragments exhibiting the lowest degree of homology were selected for probes. The fragments were released by restriction endonucleases, subjected to electrophoresis on 1.0% agarose gels, and collected by binding to DEAE membrane (Schleicher & Schuell, Inc., Keene, NH). Similarily the 396-, 344-, and 298-bp HinfI fragments of pBR322 were isolated for negative control probes. The fragments were nick-translated to a specific activity of $\sim 1 \times 10^8$ cpm/μg using ^{35}S-dATP (Amersham Corp., Arlington Heights, IL). The lengths of the nick-translated fragments varied between 550 and 80 bp (with an average between 200 and 100 bp) as determined by electrophoresis on 2% alkaline agarose gels.

Hybridization

The prehybridization treatments of the sections were slightly modified from those described earlier (3, 31). Deparaffinized sections were rehydrated in PBS (10 min) and pretreated by sequential immersion in 0.2 N HCl (20 min); 2× SSC (1× SSC [standard saline citrate] = 0.15 M NaCl, 0.015 M trisodium citrate) (twice for 3 min); 1 μg/ml proteinase K in 10 mM Tris–HCl, 2 mM $CaCl_2$, pH 7.4, at 37°C (15 min); 2 mg/ml glycine in PBS (twice for 3 min); freshly prepared 0.25% acetic anhydride in 0.1 M triethanolamine, pH 8.0 (10 min). The slides were rinsed twice in distilled H_2O and dehydrated by sequential 5-min immersions in 70 and 95% ethanol, and air dried. The hybridization mixture contained 0.1 μg/ml ^{35}S-labeled probe, 10 mM dithiothreitol (DTT), 1 mg/ml BSA, 0.6 M NaCl, 50% deionized formamide, 10% (wt/vol) dextran sulfate, 0.2 mg/ml sonicated calf thymus DNA, 0.02% (wt/vol) Ficoll, 0.02% (wt/vol) polyvinyl pyrrolidone, 10 mM Tris (pH 7.4), 0.5 mM EDTA (3, 31). The mixture was heated to 100°C for 1 min and cooled on ice. Aliquots of 20–60 μl (depending on sample size) were applied onto each slide, covered with siliconized coversips, and sealed with rubber cement. The slides were hybridized at 42°C for 50 h.

After hybridization the coverslips were removed and the tissue sections were washed using a modification of the procedure described by Moench et al. (21): twice for 5 min in 0.5× SSC with 1 mM EDTA and 10 mM DTT; twice for 5 min in 0.5× SSC with 1 mM EDTA; 10 min in 50% formamide, 0.15 M NaCl, 5 mM Tris (pH 7.4) and 0.5 mM EDTA; 4 × 5 min in 0.5× SSC at 55°C, followed by 5 min at room temperature in 0.5× SSC. The slides were then dehydrated in 70 and 95% ethanol with 0.3 M ammonium acetate, and air dried.

Detection of Bound Probe

Autoradiography was performed by dipping slides into Kodak NTB-3 nuclear track emulsion melted at 40°C and diluted 1:1 with 0.6 M ammonium acetate. After drying horizontally at room temperature for 5 h, the slides were exposed to desiccant-containing boxes at 4°C for 4–9 d. The exposed slides were developed in Kodak D-19 developer for 2.5 min at 15°C, rinsed, fixed for 5 min, stained with Gill's hematoxylin No. 1 (Sigma Chemical Co., St. Louis, MO), dehydrated, and mounted with Permount (Fisher Scientific Co., Fair Lawn, NJ).

RNA Extraction and Northern Blots

Total RNA was prepared from epiphyseal cartilages and growth plates of long bones as described earlier (10, 25). These RNAs were fractionated by electrophoresis on 0.75% agarose gels after denaturation with glyoxal and DMSO (32). One set of samples was stained to visualize the rRNAs, the other set was transferred by blotting to Pall Biodyne membrane. The prehybridizations, hybridizations with nick-translated (^{32}P-dCTP-labeled) plasmid DNAs, washes, and autoradiography were performed as described earlier (32).

Results

Selection of cDNA Probes

Our earlier experience from Northern hybridizations with types I, II, and III collagen mRNAs and their respective cDNA probes clearly pointed out the importance of stringent washing conditions to eliminate nonspecific hybridization (10, 36). All the four different proα-chains of these three collagens share a high level homology both in the triple-helical domain and in the COOH-propeptide domain. As the nucleotide sequences of all the four mRNA (cDNA) sequences for the domains covered by our probes are known (1, 2, 6, 10, 18), it was possible to align these sequences and to select restriction fragments that exhibited minimal homology. The insert of clone pHCAL1 (38) which contains a 670-bp XhoI-EcoRI fragment of human proα1(I) collagen cDNA was recloned between the SalI and EcoRI sites of plasmid pUC8 (to facilitate easier removal of the insert). To obtain the hybridization probe, the clone was digested with PvuII and PstI to release the 372-bp probe fragment (Fig. 1). Clone pHCAR1, containing a 580-bp PstI-EcoRI fragment of human proα1 (II) collagen cDNA in pBR322 (10), was digested with PvuII and EcoRI to give a 224-bp probe (Fig. 1). Later,

Table I. Homologies between the Nucleotide Sequences of the Hybridization Probes and the Corresponding Areas of Human Procollagen mRNAs (cDNAs)

Probe	Length	mRNA			
		α1(I)	α2(I)	α1(II)	α1(III)
		%	%	%	%
pHCAL1					
XhoI-PvuII	372 bp	100	64	74	59
pHCAR1					
PvuII-EcoRI	224 bp	73	69	100	68
pHFS3					
XhoI-PstI	295 bp	51	51	47	100

a larger cDNA clone pHCAR3, covering over 400 bp of 3′-nontranslated sequences, was constructed (Elima, K., unpublished data). The nucleotide sequence of the nontranslated region (sequenced previously from a genomic clone) shows virtually no homology with the other mRNAs (30). Therefore, the 550-bp EcoRI–EcoRI fragment of pHCAR3 was used as the probe for type II collagen mRNA in the later experiments. Recombinant plasmid pHFS3 was constructed by cloning a 700-bp XhoI–EcoRI fragment of human proα1(III) collagen cDNA in pUC8 between the single SalI and EcoRI sites (Sandberg, M., unpublished data). The 295-bp PstI–PstI fragment was used as the probe (Fig. 1). The homologies between these probes and the procollagen mRNAs are calculated in Table I. None of the probes contains the 87-bp sequence in the middle of the COOH-propeptide domain which is virtually identical in all the mRNAs for fibrillar collagens (1, 2, 18). Use of cDNA fragments containing this sequence for in situ hybridization probes resulted invariably in considerable cross-hybridization to other procollagen mRNAs (data not shown).

Localization of mRNAs in Different Connective Tissues

Several methods for preparing sections for in situ hybridization were tested by comparing the hybridization patterns to sections containing various types of connective tissues. The short washing conditions at higher temperatures described here were found to provide the desired stringency better than the longer washes at room temperature used in many earlier protocols (3, 13). The advantage of using sections of developing human long bones and their surrounding tissues (sections of fingers of 15–18-wk fetuses) was the presence of tissues known to produce only one type of fibrillar collagen; e.g., bone (type I) and cartilage (type II) in the same section. These tissues served as internal controls during optimization of the hybridization conditions. Shown in Fig. 2 are serial sections of one tissue sample hybridized with probes for the three different fibrillar collagen types and with pBR322 DNA fragments (serving as an additional negative control). At low magnification, the grains were best visualized by dark field imaging. Type I collagen mRNA is found in most tissues except cartilage; it is most abundant in developing bone (particularly in periosteal osteoblasts), in perichondrium, and in structures which will eventually become tendons, ligaments, and synovial tissue. Type II collagen mRNA is detected in the chondrocytes of cartilage, and in some experiments also in the cambial (inner) layer of the periosteum (Fig. 2, *c* and *d*). Type III collagen mRNA is not present in cartilage and bone, but is seen co-distributed with type I collagen mRNA in the other tissues. Their relative amounts, however, appear to be quite different: proα1(III) collagen mRNA is most abundant in skin, and a relatively strong signal is also seen in developing synovial tissue. In developing tendons and ligaments the proportion of type III collagen mRNA is considerably lower.

Quantitation of Type II Collagen mRNA Levels in Developing Cartilage

Our attention was drawn by the uneven distribution of grains in the epiphyseal cartilage after hybridization with the proα1(II) collagen cDNA probe. Therefore, grain counting through the various cartilaginous zones of the growth plate and epiphysis was performed (Fig. 3). The results clearly show that the highest number of grains per chondrocyte are seen in the cells of the upper hypertrophic layer. As the cell density is higher in the proliferative cell zone, the amount of type II collagen mRNA per unit area of cartilage peaks somewhat more distally. To ascertain this finding of zone-dependent variation of type II collagen mRNA in developing human cartilage, the growth plate areas of knee and elbow joints of one 16-wk fetus were dissected away from the rest of the epiphyseal cartilage and total RNA was extracted from both tissues. Northern hybridization with pHCAR3 probe revealed that the growth zone cartilage contains ~10-fold more type II procollagen mRNA (per total RNA) than the resting cartilage (Fig. 4).

Attention was also given to the osseochondral junction where sudden changes in the type of procollagen mRNA were observed (Fig. 5). Some of the chondrocytes of the degenerative cell zone still contained detectable amounts of type II collagen mRNA. None of these cells were seen to contain type I collagen mRNA. In the mineralizing zone, however, a majority of the (osteoblastic) cells gave a positive signal with the type I collagen probe. Interestingly, a few scattered cells were found to contain type II collagen mRNA.

Discussion

Localization of tissues and areas where metabolic activity is high has been particularly difficult with respect to collagen synthesis, as these proteins accumulate in the extracellular matrix and exhibit very long half-lives. To determine the metabolically active cells in developing human skeletal connective tissues we have used an in situ hybridization technique. The specificity of the probes and the conditions described in this paper is best exemplified by the patterns shown in Fig. 2. The best control tissue is cartilage (which contains no type I or III collagen): practically no cross-hybridization is detectable between the cartilage-specific type II collagen mRNA and the cDNA probes for type I and III collagen mRNAs. Small amounts of type I collagen mRNA have been detected in chondrocytes during in vitro chondrogenesis (15, 16) and in total RNA extracted from epiphyseal cartilages (10, 36). The latter is most likely due to contaminating perichondrium which is here shown to contain considerable amounts of type I collagen mRNA. Our experiments failed to detect any type I collagen mRNA in chondrocytes. Type I and III collagen mRNAs are mostly

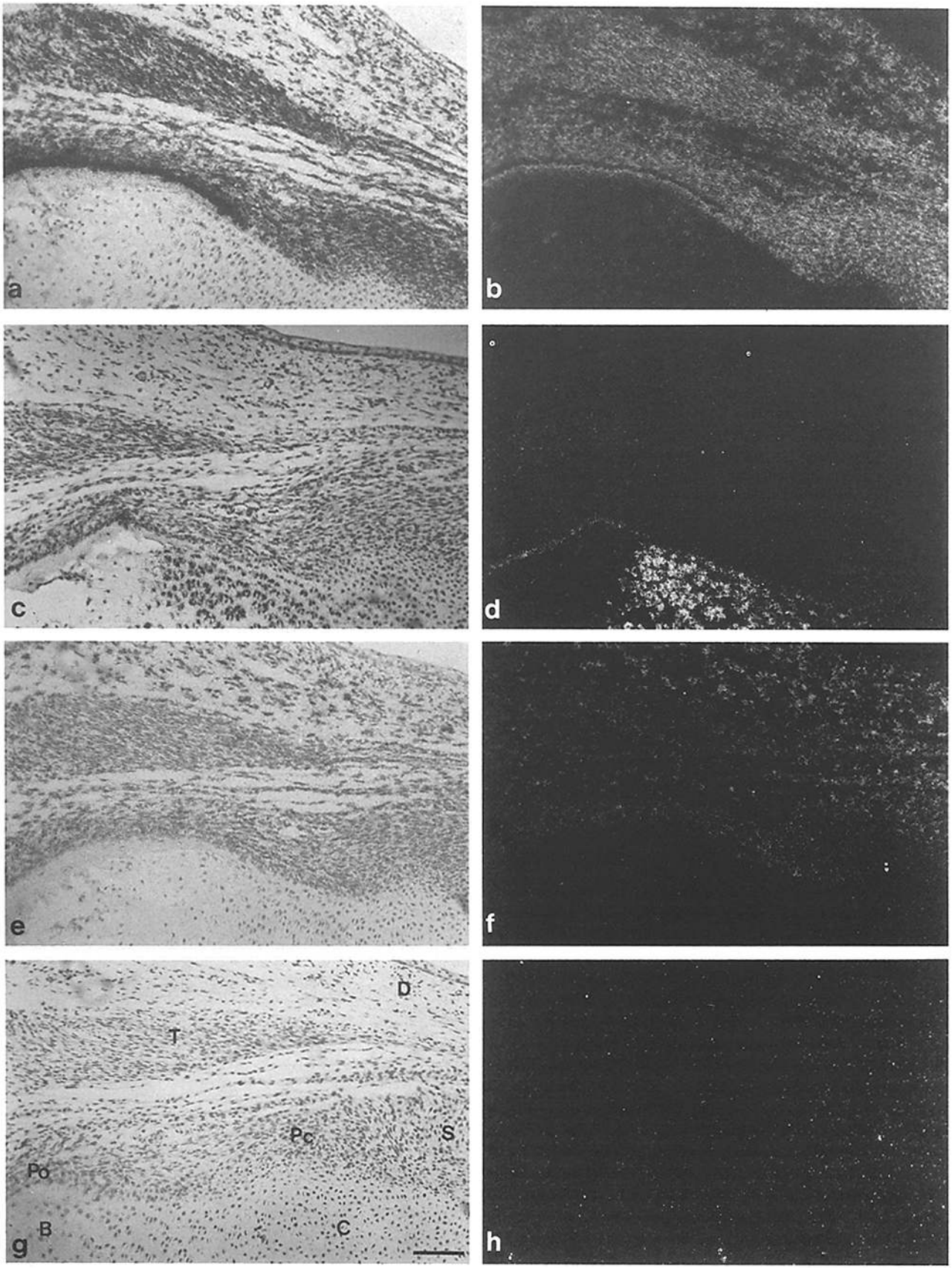

Figure 2. Localization of types I, II, and III collagen mRNAs in paraffin sections containing a portion of finger from a 16-wk fetus. Serial sections were hybridized with cDNA probes for type I (*a* and *b*), type II (*c* and *d*), and type III (*e* and *f*) collagen mRNA. Restriction fragments of plasmid pBR322 were used as negative controls (*g* and *h*). In situ hybridization was performed using ^{35}S-labeled probes and

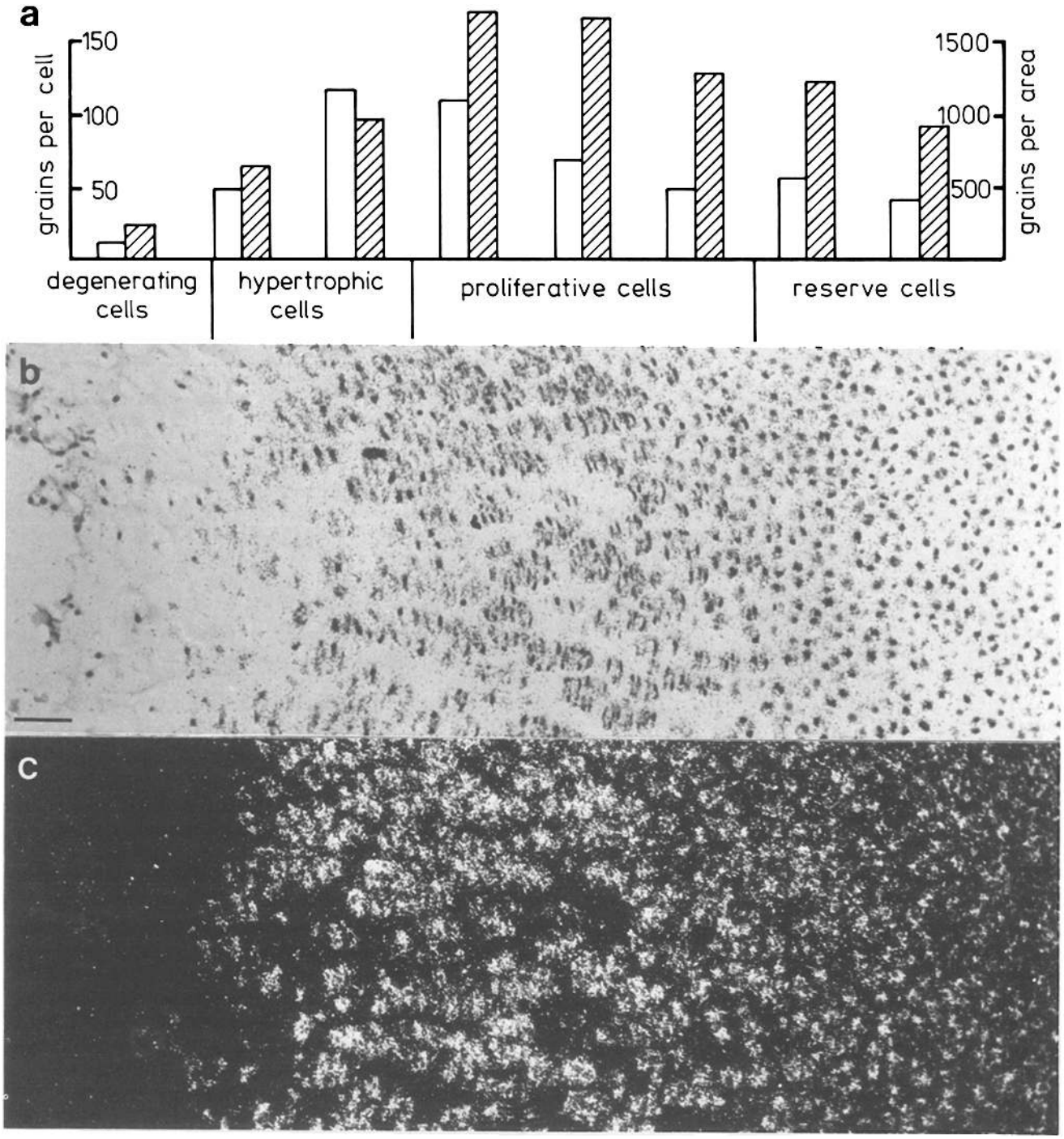

Figure 3. Localization and quantitation of type II collagen mRNA in the different zones of cartilage. Sections containing epiphyseal and growth cartilage of phalangeal long bones were hybridized using the 224-bp PvuII–EcoRI fragment of pHCAR1 as the probe. Grain counting was performed by two independent individuals in five different views of the sections. Altogether over 23,000 grains were counted. Their distribution is shown in *a*. Open bars represent the number of grains per cell in the given zone; hatched bars represent the number of grains per unit area of the cartilage. *b* shows a photomicrograph of the autoradiogram of one of the sections analyzed and *c* shows the dark field image of the corresponding section. Bar, 50 μm.

stringent washing conditions as described in Materials and Methods. Autoradiography was performed for 9 d. In addition to the photomicrographs (*left*), dark field images of the same field (*right*) are shown for easier grain localization at this low magnification. The structures are identified (in *g*) as follows: *B*, bone; *C*, cartilage; *D*, dermis; *Pc*, perichondrium; *Po*, periosteum; *S*, synovial tissue; *T*, structures which are developing into tendons and ligaments. Bar, 100 μm.

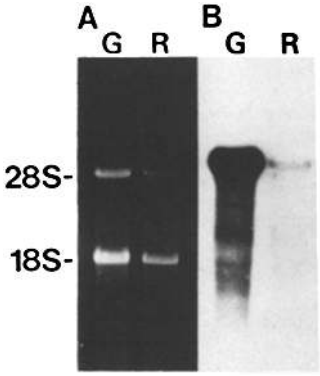

Figure 4. Hybridization of Northern blots containing total RNA extracted from resting epiphyseal cartilages (*R*) and growth plate zones (*G*) with a cDNA probe for human type II collagen mRNA. Aliquots of 12.5 μg of total RNA were subjected to electrophoresis under denaturing conditions. One part of the gel was stained with ethidium bromide (*A*) to visualize the rRNAs (marked on the left); the other was transferred to Pall Biodyne membrane and hybridized with nick-translated ^{32}P-labeled pHCAR3 DNA. The autoradiogram is shown in *B*.

co-distributed, but their ratios vary considerably from tissue to tissue. No type III collagen mRNA is seen in bone or cartilage, and only a little is seen in developing periosteum, perichondrium, tendon, and ligaments. The highest levels of proα1(III) collagen mRNAs are seen in skin, blood vessels, and synovial tissue. These results are in good agreement with earlier protein chemical findings (4, 5, 7).

We feel that in situ hybridization promises to become a powerful tool to study complex developmental processes. We have selected cartilage development and bone formation to exemplify this approach (Fig. 3). Earlier histochemical evidence has suggested that type II collagen is distributed rather homogenously throughout the cartilage zones (26, 34, 35). In studies on embryonic chick tibiotarsus, the highest synthetic rates of collagen were detected in cells cultured from the lower proliferative zone, while those from the hypertrophic zone exhibited an overall reduction in collagen synthesis (28) with a partial switch towards type I collagen production (12). In the present study, the highest cellular levels of type II procollagen mRNA were detected in chondrocytes in the lower proliferative and the upper hypertrophic layers of the growth plate (Fig. 3). Throughout the resting or reserve zone cartilage, the type II collagen mRNA levels were clearly lower and quite evenly distributed. The cells in the hypertrophic zone have also been shown to be active in production of both type X collagen (26) and chondrocalcin, a calcium-binding protein (22) which has recently been shown to be another product of the type II collagen gene. Biochemical evidence strongly suggests that chondrocalcin is identical with the COOH-propeptide of type II procollagen (33). Our cDNA probe covers this very sequence and therefore, even if alternative splicing was used to generate a specific chondrocalcin mRNA, our probe would also detect the mRNA coding for this protein. Northern hybridization was performed to confirm the elevated level of type II procollagen mRNA in growth cartilage and to show that the pHCAR3 probe only recognized the single (5.4 kb) mRNA species for human proα1(II) collagen mRNA (Fig. 4). No sign of a smaller mRNA (for chondrocalcin) was detected. The apparent discrepancy in the levels of activation of the type II collagen expression between in situ hybridization (approximately threefold) and Northern hybridization (approximately 10-fold) may have many explanations. In situ hybridization was performed with phalangeal long bones while the RNA extraction for the Northern blots was from larger bones with a somewhat different developmental stage. A possible decrease in total cellular RNA levels in hypertrophic chondrocytes could result in apparently elevated levels of type II collagen mRNA when expressed per total RNA.

Contrary to immunological observations of type I collagen synthesis in hypertrophic chondrocytes (12, 35), our experiments failed to detect any type I collagen mRNA in this zone (Fig. 5). There was hardly any overlap between the zones of type I collagen mRNA and of type II collagen mRNA-containing cells; this suggests that basically all chondrocytes in our preparations had degenerated before they came in contact with the zone of osteoblasts. The exact nature of the few cells containing type II collagen mRNA in the ossifying zone remains uncertain. We would like to suggest that they represent surviving chondrocytes.

In some experiments (such as shown in Fig. 2, *c* and *d*), the proα1(II) collagen cDNA probe also hybridized to cells in the inner (cambial) layer of the periosteum. Although cross-hybridization to type I procollagen mRNAs may partially explain this finding, we cannot rule out the possibility that small amounts of type II collagen mRNA exist in the cambial cells; possibly as a sign of their suggested capacity to differentiate into chondrocytes during callus formation (20). This result does not mean that the cells are using the mRNA to produce type II collagen. Studies on in vitro chondrogenesis have, for example, suggested that type II collagen mRNA is present in prechondrogenic cells in low amounts before they start producing type II collagen (15, 16). On the other hand, a number of studies, in vivo and in vitro, have shown that collagen production is regulated predominantly at transcriptional level (15, 16, 23, 25, 37, 38), although modulation at translational level also occurs (11). Our results also support transcriptional level as the main control point in collagen synthesis. The usefulness of in situ hybridization for the localization of metabolically active cells is based on this fact.

In summary, the in situ hybridization technique is a new powerful tool for developmental studies on human tissues, which are difficult to study using the conventional biochemical techniques due to the small amount of starting material and lack of relevant in vitro models. The expression of minor cartilage collagens and other cartilaginous proteins can also be studied in this model once cDNA probes become available. The fact that old paraffin blocks at pathology departments can now be reexamined by in situ hybridization greatly increases the usefulness of the technique in studies on human cartilage diseases.

Introduction of the in situ hybridization techniques in our biochemical laboratory required the help of many individuals. We gratefully acknowledge the assistance and advice of Drs. Stina Syrjänen, Mikko Niemi, Martti Parvinen, Lauri J. Pelliniemi, and Heikki Aho. The expert technical assistance of Leena Simola, Merja Haapanen, and Tuula Oivanen is gratefully acknowledged.

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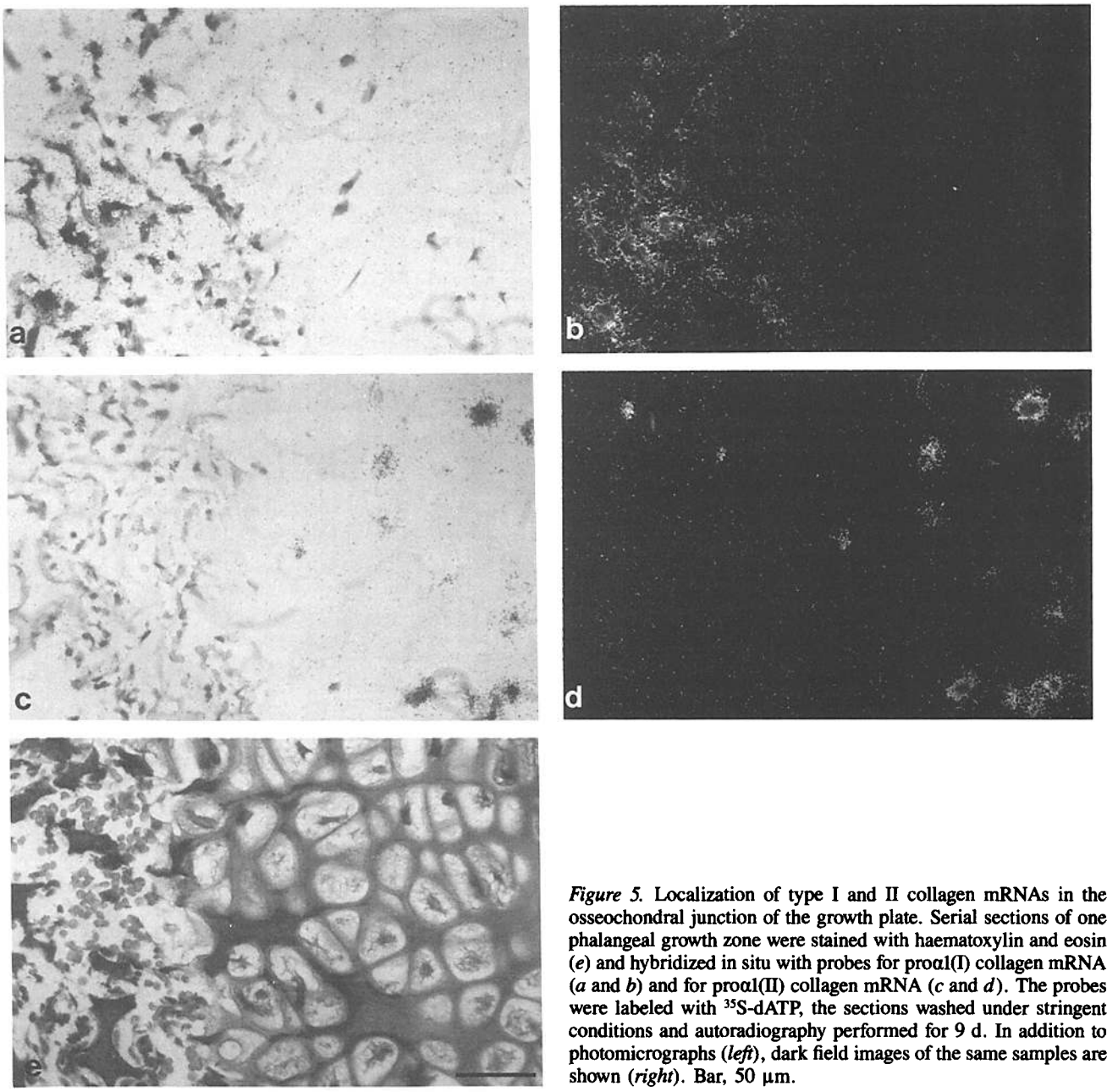

Figure 5. Localization of type I and II collagen mRNAs in the osseochondral junction of the growth plate. Serial sections of one phalangeal growth zone were stained with haematoxylin and eosin (*e*) and hybridized in situ with probes for proα1(I) collagen mRNA (*a* and *b*) and for proα1(II) collagen mRNA (*c* and *d*). The probes were labeled with ^{35}S-dATP, the sections washed under stringent conditions and autoradiography performed for 9 d. In addition to photomicrographs (*left*), dark field images of the same samples are shown (*right*). Bar, 50 μm.

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