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Nonradioactive *In Situ* Hybridization Using Digoxigenin-labeled Oligonucleotides

Applications to Musculoskeletal Tissues

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We have optimized a technique for* in situ *localization of specific mRNAs using digoxigenin-11-dUTP–labeled oligonucleotide probes. DNA probes were synthesized for type I and type II collagen as well as transforming growth factor-beta 1 and 2 (TGFβ1 and TGFβ2). Control experiments, such as competitive inhibition, nonsense sequence hybridization, and RNAse digestion all indicated that the technique was highly sensitive and specific. In sections of growth plate, type II collagen mRNA was predominantly expressed in the lower proliferative and upper hypertrophic zone, whereas chondrocytes in articular cartilage stained equally. These techniques then were applied to sections cut from archival pathology specimens of musculoskeletal tissues. Primitive chondrocytes in a chondrosarcoma expressed type I and type II collagen mRNA, but did not stain with the nonsense probe. Sections from an osteosarcoma, an aneurysmal bone cyst, and a neurofibroma also were investigated. The ability to use chemically synthesized oligonucleotide probes, the high resolution, and the short development times possible with this* in situ *procedure makes this technique appealing for applied research into the gene expression of normal and pathologic cellular events. (Am J Pathol 1992, 141:579–589)

In this study, we have developed a protocol for *in situ* hybridization in cartilage using digoxigenin-dUTP–labeled oligonucleotide and alkaline phosphatase/antibody detection. The digoxigenin labeling technique is very sensitive, defines the mRNA location more accurately than radioactive probes,[1] and has less nonspecific binding than biotinylated probes.[2,3] In addition, the digoxigenin labeling technique is rapid, the entire processing time taking only 3 days compared with weeks to months, as may be needed for radiolabeled probes.[4–6] Finally, the ability to use oligonucleotide probes (20 to 50 bases) that can be chemically synthesized markedly simplifies the acquisition of a probe library.[6–8]

Growth plate cartilage, with its orderly progression of maturation[9–11] and gene expression, served as an excellent model for refining this technique of *in situ* hybridization. Our laboratory has extensive experience with cartilage growth and maturation and used this experience to predict and confirm our results. Cartilage is a notoriously difficult tissue to work with, because of the high matrix content of proteoglycans and resultant nonspecific background binding. Studies using embryonic bone and cartilage have identified specific mRNA molecules using radioactive-labeled *in situ* hybridization[12–15]; however, such methods in mature cartilage have been complicated by background binding to the matrix.[16] Two previous reports have successfully demonstrated *in situ* hybridization in mature cartilage; a radioisotope-labeled probe was used to show type II collagen mRNA in a band in the proliferative and upper hypertrophic zone of the growth plate[17] and a biotinylated probe was used to show the expression of type X collagen in the hypertrophic zone.[16] Digoxigenin-labeled probes offer significantly enhanced resolution when compared with either of these traditional labeling techniques. In addition, both of these studies required the use of cloned cDNA probes, whereas in our experiments, the digoxigenin labeling was accomplished with chemically synthesized oligonucleotide probes.

This series of experiments has focused on determining the sensitivity and specificity of *in situ* hybridization in cartilage using the digoxigenin labeling technique. The

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technique has been applied to both isolated chick growth plate chondrocytes and to tissue sections from growth plate and articular cartilage. To demonstrate the broader applications of this technique, we have also applied it to fixed, embedded tissue sections of chondrosarcoma, osteosarcoma, aneurysmal bone cyst, and neurofibroma. An oligonucleotide probe complementary to both human and chicken Type II collagen mRNA was synthesized (C2), and used to develop the protocol, because this message is produced in abundance by growth plate chondrocytes. A uniquely human type II collagen probe also was synthesized (C2a). An oligonucleotide probe for type I collagen was synthesized as it was likely to be expressed in the fibrous regions of skeletal tissue but not in the cartilage. Probes for transforming growth factor-beta-1 (TGFβ1) and transforming growth factor-beta-2 (TGFβ2) were synthesized as examples of local growth regulators thought to be important in cartilage maturation. A poly-dT(deoxythymidine) probe was prepared to demonstrate total message retained in the tissue and a nonsense probe was used to evaluate nonspecific binding. These oligonucleotide probes were then used to demonstrate the sensitivity of the message detection using the digoxigenin labeling and detection technique.

Materials and Methods

Cell and Tissue Preparation

Cellular preparations consisted of chondrocytes isolated from the growth plate and articular cartilage of the distal femur and proximal tibia of 4- to 6-week-old chickens by sequential digestion with hyaluronidase, trypsin, and collagenase.[10] Cells were plated on UV-sterilized poly-L-lysine–coated glass microscope slides in Dulbecco's modified Eagle medium. After adhesion for 4 hours, the slides were fixed in 4% paraformaldehyde, in phosphate-buffered saline (PBS) pH 7.4, with RNAse inhibitor, diethylpyrocarbonate (DEPC) for 20 minutes. They then were dehydrated in sequential ethanol baths (50% to 99%) and stored frozen until use. Cartilage tissue samples were fixed in 4% paraformaldehyde-DEPC for 4 to 6 hours, paraffin-embedded, sectioned (5 μ), and dehydrated after overnight heat fixation at 60°C. Specimens from the pathology archives had been routinely fixed and paraffin embedded. Calcified tissues were decalcified for 24 hours in formic acid/formalin (Surgipath Decalcifier, Gray Lakes, IL). These were sectioned (5 μ) and heat fixed as above. The tissue preparations were cleared of paraffin with xylene for 10 minutes. The cell preparation and deparaffinized tissue sections were rehydrated in sequential ethanol baths from 99% to 50% ethanol (each for 5 minutes), before prehybridization.

Probe Selection

Oligonucleotide cDNA probes complementary to specific mRNA sequences were synthesized using cyanoethylphosphoamadite chemistry on an Applied Biosystems 380B DNA synthesizer. The efficiency of the reaction was checked using trityl detection and found to be >99%. Sequences complementary to both human and chick alpha 2 chain of type 1 collagen (C1), both human and chicken type 2 collagen (C2), a uniquely human type II collagen (C2a), transforming growth factor-beta 1 (TGFβ1), transforming growth factor-beta 2 (TGFβ2) were prepared. In addition, a nonsense probe (reverse sequence of C1 probe, designated RC) and a poly-dT probe designed to bind to the polyadenylated tail on the mRNA, were synthesized. Probe sequences were selected to minimize homology with any other known sequences by Genbank searching.

Labeling of Oligonucleotides

Digoxigenin-11-dUTP labeling of 35 picomoles of the oligonucleotide probes at the 3′ end was accomplished using the Boehringer Mannheim Biochemicals DNA tailing kit (Indianapolis, IN, catalogue #1028-707). Several modifications were made to the manufacturer's terminal transferase labeling protocol. The reaction includes the incorporation of 2.5 μl digoxigenin-11-dUTP, a 1/50 dilution of dATP, and 6 μl cobalt chloride solution, with 1 μl terminal transferase. The concentration of $CoC1_2$ was increased 2.5-fold and resulted in more equal labeling of probes. Incubation of the reaction mixture at 37°C was completed in 8 minutes. Strict time control of the labeling reaction was important because after 7 to 8 minutes, the terminal transferase may catalyze digestion of the probe.[18] Each labeled oligonucleotide was precipitated from the labeling mixture by adding 10 μl of 4.5 mol/l (molar) sodium acetate (pH 6.0), 1 μl 20 mg/ml glycogen solution, and 3 volumes of 100% ethanol, followed by incubation in a −70°C freezer for 30 minutes. Each tube was centrifuged at 12,000 rpm in a microcentrifuge tube for 10 minutes and the supernatant removed. This was repeated with 70% ethanol, and the pellet was then air dried and resuspended in 20 μl 10 mmol/l (millimolar) TRIS-HCl, 1 mmol/l ethylenediaminetetra-acetic acid (EDTA), pH 7.5, and 0.1% sodium dodecyl sulfate (SDS). By resuspending the labeled oligonucleotide in TRIS/EDTA containing 0.1% SDS, nonspecific and sequence dependent adherence to the microfuge tubes was prevented.

Labeling efficiency was measured using polyacrylamide gel electrophoresis with 16% acrylamide, and ultraviolet photography of the gel after ethidium bromide

staining of the oligonucleotide. Routine normalization of oligonucleotide labeling was performed using a spot blot assay as follows. The probes were serially diluted in 10 mmol/l TRIS-HCl, 1 mmol/l EDTA, pH 8.0, containing 50 μg/ml herring sperm (Boehringer, Indianapolis, IN). One microliter of each dilution was applied to a nylon membrane. The membranes then were incubated at 80°C for 20 minutes. The standard immunodetection procedure, as described below, was then performed on the nylon membranes, using a 40-minute incubation at room temperature with the color reagent.

Enzymatic Pretreatment

After the cells and tissues had been rehydrated, they were equilibrated in PBS for 5 minutes. Deproteinization was carried out in a 0.2 N HCl for 20 minutes at room temperature followed by digestion with proteinase K (Sigma Chemical Co., St. Louis, MO) at 1 μg/ml for cells and 10 μg/ml for tissues for 20 minutes in PBS at 37°C. Tissue sections were further digested with hyaluronidase (Sigma, St. Louis, MO) at 10 μg/ml in PBS (pH 6.0) for 20 minutes at 37°C. After digestion, all specimens were fixed in 4% paraformaldehyde containing 0.1% DEPC for 5 minutes. After rinsing with PBS three times, the slides were acetylated in 200 ml 0.1 mol/l triethanolamine-HCl buffer (pH 8.0) to which 0.5 ml of acetic anhydride (0.25%) was added.[19] After 5 minutes, 0.5 ml of acetic anhydride was added again and then the slide rinsed in 2 × SSC (.3 mol/l NaCl, .3 mol/l sodium citrate, pH 7.0).

In Situ *Hybridization*

Slides were covered with 500 μl prehybridization solution and incubated in a humidified chamber for 1 hour at room temperature. The prehybridization solution was composed of 5.0 ml deionized formamide, 2.0 ml 20 × SSC, 0.2 ml of 50X Denhardt's solution (Sigma, St. Louis, MO), 0.5 ml of herring sperm DNA (10 mg/ml, heat denatured in boiling H_2O for 10 minutes), 0.25 ml of yeast tRNA (10 mg/ml), and 2.0 ml of dextran sulfate (50% solution). The labeled probes were heated for 2 to 3 minutes in a 95° water bath before being added in a 1/50 dilution to the prehybridization solution. The slides were rinsed briefly in 2 × SSC and 40 μl of probe solution for the cell samples and 80 μl of probe solution for the tissue sections was applied to each slide. Parafilm cover slips were placed over the cell or tissue sections and the hybridization was allowed to occur overnight at 37°C, in a moisturized environment. The cover slips were removed and the slides were incubated in 2 × SSC for 1 hour at room temperature. This was followed by incubation in 1 × SSC for 1 hour at room temperature, 0.5 × SSC for 30 minutes at 37°C, and finally 0.5 × SSC for 30 minutes at room temperature. At this point in the procedure the slides were stored overnight at 4°C in buffer 1 (100 mmol/l TRIS-HCl, 150 mmol/l NaCl, pH 7.5) before immunodetection.

Antibody Binding and Color Detection

The slides were rinsed in buffer 1 (100 mmol/l TRIS-HCl and 150 mmol/l NaCl, pH 7.5) and then preblocked in 2% normal sheep serum for 30 minutes at room temperature. The excess was decanted and 1% sheep serum with a 1/500 dilution of polyclonal sheep antidigoxigenin Fab fragments conjugated to alkaline phosphatase was added (Boehringer, Indianapolis, IN). This incubation was carried out for 3 hours in a sealed humidified container at 28°C. The slides were washed in buffer 1 with shaking. The slides then were washed for 10 minutes in buffer 2 solution, again with shaking. Next, 200 to 400 μl of the freshly prepared color solution was added to each section. The color solution consisted of 67 μl 4-nitro blue tetrazolium chloride (NBT, 75 mg/ml in 70% dimethylformamide), 53 μl 5-bromo-4-chloro-3-indolyphosphate (x-phosphate, 50 mg/ml in 100% dimethylformamide), and 3.6-mg levamisole. This was added directly to 15 ml of buffer 2. The reaction was incubated for 4 to 16 hours in a light-tight box. The color reaction was stopped with 10 mmol/l TRIS-HCl, 1 mmol/l EDTA, pH 8.0, and the sections then were dehydrated in sequential ethanol baths from 70%–99%, for 4 minutes each. The slides were rinsed in xylene for 3 minutes and then cover slips applied with Permount before microscopic examination.

Results

Probe Preparation

Probes were synthesized for type I and type II collagen, transforming growth factor-β1, transforming growth factor-β2, poly-dT, and a nonsense probe. The sequences were selected from the Genbank and chosen for their homology with human and chicken nucleotide sequences. A second type II collagen sequence was selected that was specific for the human mRNA (C2a) and has been previously used and published by Sandell and Robbins.[20] Probe sequences were selected to be approximately 25 bases in length. Sequences and melting temperatures (Tm) of the probes are shown in Table 1.

Initially the probe concentrations were normalized by spectrophotometric measurement of their absorbance at 260 and 280 nm. The probes were then end labeled with digoxigenin-11-dUTP using terminal transferase. La-

Table 1. *Sequences and Melting Temperatures (Tm) of the Probes*

Probe		Base sequence	Tm°C
Type I collagen	C1	TCCGCGTATCCACAAAGCTGAGCAT	64
Type II collagen	C2	CGGGGCCCTTCTCCGTCTGACCCAGTC	72
Type II collagen	C2a	TCGCAGCCTCCTGGACATCCTGGC	70
TGF-β1	T1	TAGCACGCGGGTGACCTCCTTGGCG	72
TGF-β2	T2	CCTCCGGGGGCACCTCCTCGGGCTCGGGG	80
Nonsense probe	RC	TACGAGTCGAAACACCTATGCGCCT	62
Poly-dT	dT	TTTTTTTTTTTTTTTTTTTTTTTTTTTT	46

beled and unlabeled probes were analyzed by polyacrylamide gel electrophoresis, and the resulting bands were visualized using ethidium bromide labeling and ultraviolet photography (Figure 1). Essentially none of the labeled probe co-migrated with the unlabeled probe, suggesting 100% effectiveness of the labeling reaction. The evenly spaced bands were interpreted as incremental additions of digoxigenin-11-dUTP added to the probe by the transferase. To confirm this, the DNA on the gel was transferred to nitrocellulose paper and immunodetected using the antidigoxigenin antibodies and subsequent color reaction, as described for the spot blot procedure in Methods. The blot showed diffuse blue stains over the labeled lanes, confirming that these more slowly migrating bands do indeed reflect digoxigenin labeling (data not shown).

Examination of the gel suggested that the concentrations of the probes, both labeled and unlabeled, were not similar. This was due to the inaccuracy of absorbance as a measure of concentration for these short oligonucleotide sequences. To assure equal specific activity of probe, a spot blot procedure was carried out for each labeling as described in Methods. Probe concentrations were then normalized by comparing serial dilution spots on the membrane. Relative probe concentrations were estimated by visual comparison of the staining intensity of the spots. Probe stock solution concentrations then were adjusted until approximately equal intensities were observed on repeat blots. An example of a normalized set of probes used in the current study is shown in Figure 2.

In Situ *Hybridization of Cells in Monolayer*

The isolated chondrocytes grown on microscope slides produced a matrix and began to form lacunae. This proteoglycan and collagen matrix caused nonspecific background staining extracellularly. Nonspecific probe binding was also observed in bone and cartilage matrix in whole tissue sections. Acetylation completely eliminated this problem in the cells, and markedly reduced it in the tissue sections. Moreover, the lack of any staining when the antidigoxigenin antibody was omitted confirmed that endogenous alkaline phosphatase activity in the tissues or cells did not participate in the detection reaction, because this activity was destroyed by the fixation procedure. Furthermore, the oligonucleotide probe was rou-

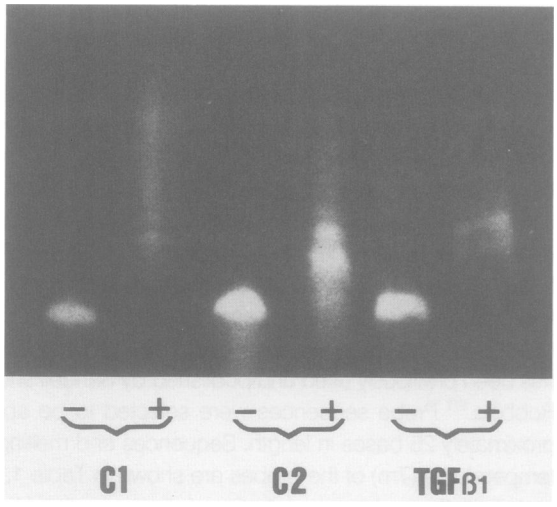

Figure 1. *Digoxigenin-labeled (+) and -unlabeled (−) oligonucleotide probes stained with ethidium bromide after electrophoresis on 16% acrylamide.*

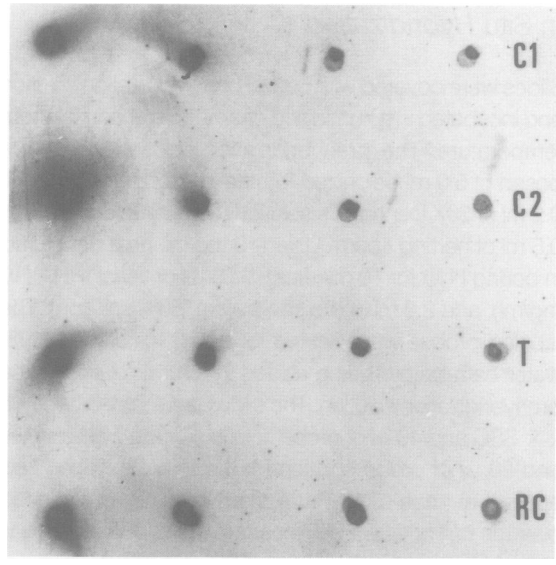

Figure 2. *Serial 10-fold dilutions of digoxigenin-labeled oligonucleotides. Type I collagen (C1), type II collagen (C2), TGF-β1 (T) and nonsense probe (RC).*

tinely omitted on one slide in every experiment to estimate background staining.

Tissue digestions to optimize probe penetration and hybridization while retaining adequate morphology were performed. Proteinase K at 1 μg/ml was found to be the optimal concentration for the cell culture specimens and 10 μg/ml was the optimal concentration for the tissue specimens. At higher concentrations of proteinase K,

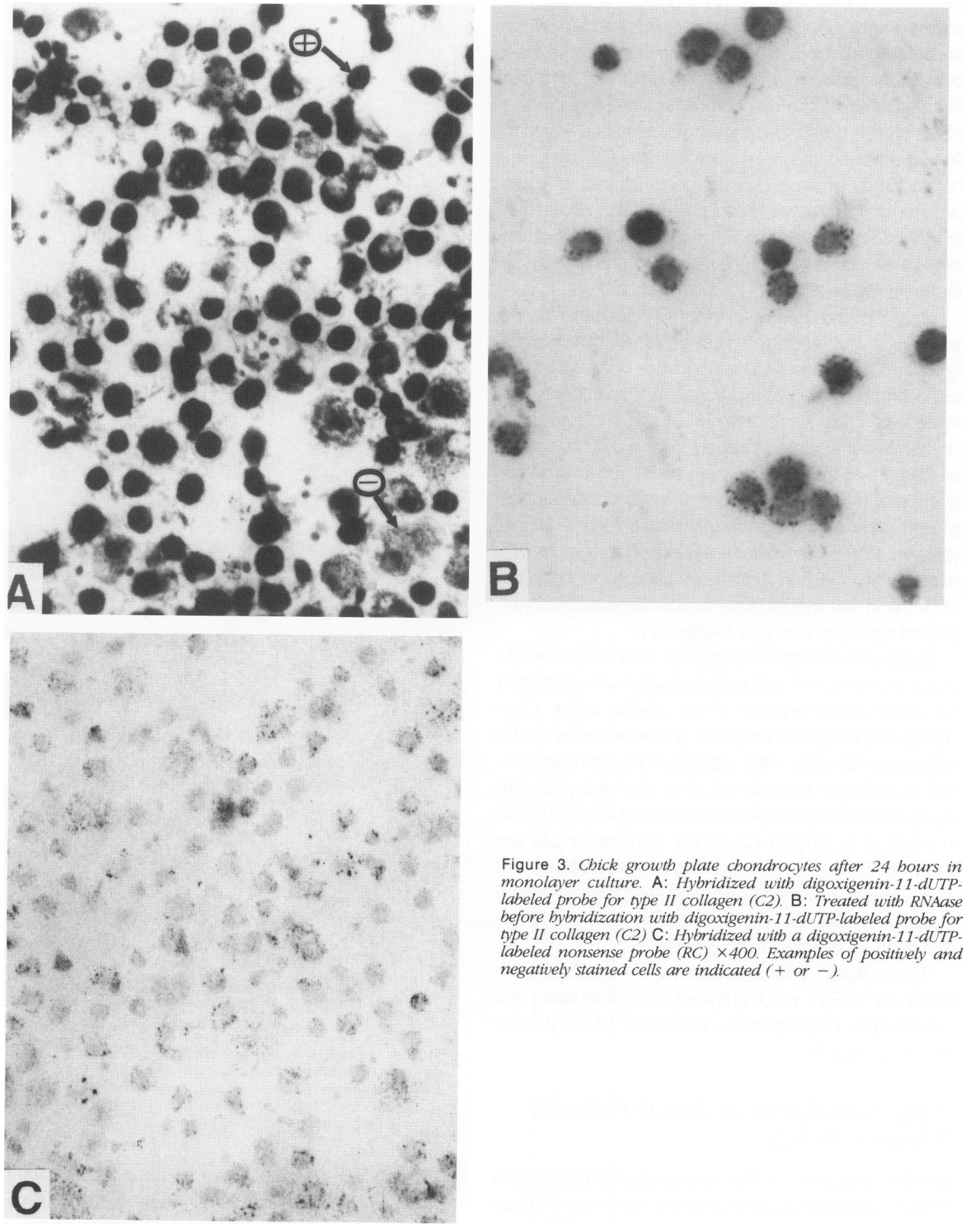

Figure 3. *Chick growth plate chondrocytes after 24 hours in monolayer culture.* A: *Hybridized with digoxigenin-11-dUTP-labeled probe for type II collagen (C2).* B: *Treated with RNAase before hybridization with digoxigenin-11-dUTP-labeled probe for type II collagen (C2)* C: *Hybridized with a digoxigenin-11-dUTP-labeled nonsense probe (RC)* ×400. *Examples of positively and negatively stained cells are indicated (+ or −).*

there was loss of cells and tissue from the slides, and a deterioration of morphology. Digestion with hyaluronidase 10 μg/ml at 37°C did improve the hybridization signal on tissue sections, but not on cell culture specimens.

Chick growth plate chondrocytes were subjected to *in situ* hybridization with type II collagen probe (C2), and the results are shown in Figure 3A-C. When no probe was added, very little background color was detected. When the cells were hybridized with type II collagen probe, dark cytoplasmic staining was observed. This indicated that the deposition of chromophore reflected actual binding of the type II probe to cytoplasmic elements. Although 90% of the chondrocytes were positive for C2, there was heterogeneity of expression as demonstrated in Figure 3A. The chondrocytes were then treated with RNAse before hybridization with the C2 probe. Almost all of the signal was eliminated by the digestion of the RNA, as shown in Figure 3B.

To assess the specificity of this hybridization, a probe with no known homology was synthesized, "reverse collagen" (RC), by reversing the sequence of the type I collagen probe. When run in parallel experiments with C2, the level of signal was extremely low and similar to specimens without any probe added (Figure 3C). This demonstrated that the binding was specific for a given sequence of mRNA. Furthermore, C1 hybridization to the growth plate chondrocytes resulted in minimal signal (data not shown), as would be expected because growth plate chondrocytes synthesize very little type I collagen. Thus the *in situ* hybridization results correlated with expected cellular phenotypic expression.

Specificity of probe hybridization was further investigated by the use of competitive studies with unlabeled C2 probe before addition of the labeled probe. Conversely, the effect of a dissimilar unlabeled probe on the subsequent binding of the labeled probe was also studied. Accordingly, the cultured chick chondrocytes were incubated with a 3000-fold excess of unlabeled C2 probe in addition to labeled C2 probe, and the results are shown in Figure 4A, with essentially complete absence of signal. Similar incubations with 3000-fold excess of unlabeled C1 with labeled C2 are shown in Figure 4B, demonstrating no effect on the C2 signal.

The cultured chondrocytes were hybridized with probes for TGFβ1, which is known to be produced in the growth plate. Heterogeneous expression of TGFβ1 was evident (Figure 5).

In Situ *Hybridization in Growth Plate and Articular Cartilage*

Slight modifications of the hybridization protocol (See Methods) enabled application of the technique to tissue sections. In general, the best preservation of mRNA in the tissue sections resulted with tissues immediately fixed in 4% paraformaldehyde; however, good results have also been obtained with standard formaldehyde fixation. Paraffin sections were processed as described above for the cells, except that more rigorous enzymatic digestions

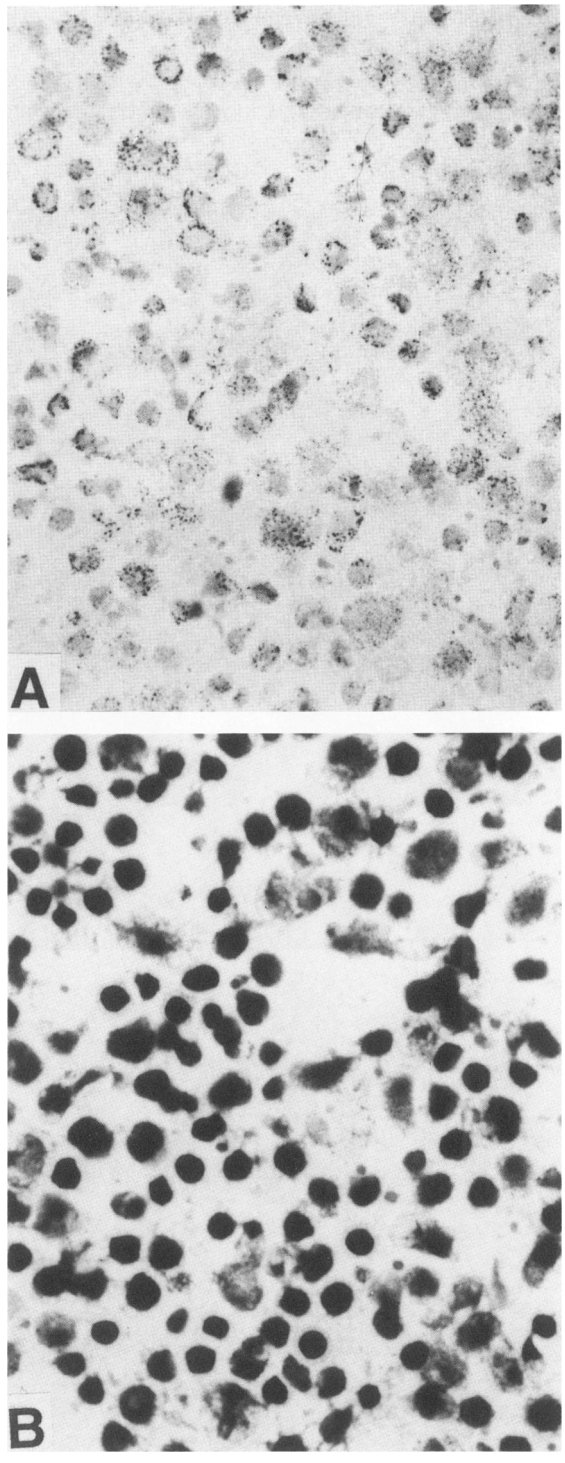

Figure 4. *Chick growth plate chondrocytes after 24 hours in monolayer culture hybridized with* **(A)** *digoxigenin-11-dUTP-labeled probe for C2 and simultaneously a 3000 fold excess of unlabeled C2 probe or* **(B)** *digoxigenin-11-dUTP-labeled probe for C2 and simultaneously a 3000-fold excess of unlabeled C1 probe 400×.*

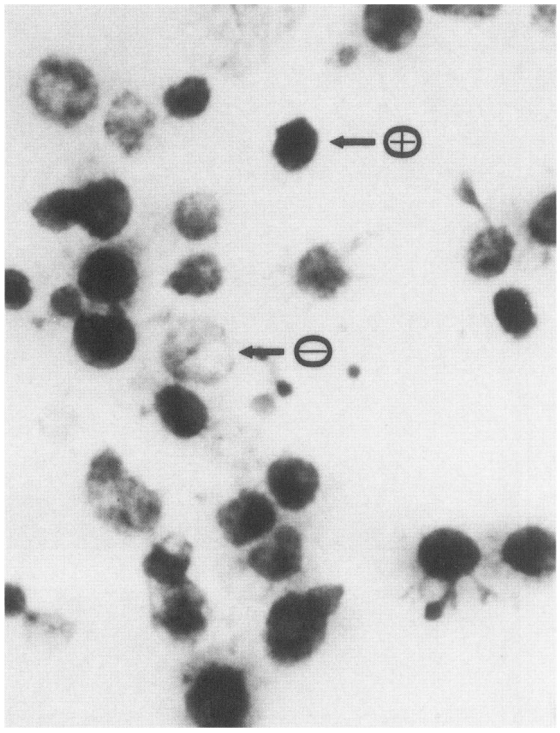

Figure 5. *Chick growth plate chondrocytes after 24 hours in culture hybridized with digoxigenin-11-dUTP-labeled probe for transforming growth factor-β (TGFβ1) ×400. Examples of positively and negatively stained cells are indicated (+ or −).*

were needed to permeabilize the cells to the probes and antibodies, presumably because of the increased matrix protein present. Frozen sections proved more difficult to retain on the slides through the hybridization procedure, and the morphology was poor.

Figure 6 shows C2 hybridization to chick articular cartilage, with constitutive expression of collagen II mRNA in essentially all the cells. Figure 7 shows chick and human growth plate sections hybridized with C2 and C2a, respectively. Although expression of the type II collagen message is present to a slight degree throughout the growth plate, the signal is maximal in the lower proliferating and upper hypertrophic zone, with a marked decrease in the lower hypertrophic and calcifying zones. No C2 signal was observed in the adjacent bone or fibrous tissue. Comparison sections probed with poly-dT shows an abundance of mRNA in all levels of the growth plate with approximately equal staining density. This suggests that poor probe penetration into the upper zones of the growth plate is not responsible for the observed distribution of signal with the type II collagen probes.

In Situ *Hybridization in Archival Pathology Specimens*

To demonstrate the broader application of this technique for investigations of surgical pathology archival tissue, we studied several skeletal neoplasms that had been routinely processed and stored as paraffin blocks for variable lengths of time. A surgical specimen of a low-grade human chondrosarcoma was hybridized with C1, C2, and the nonsense probe (RC). The results are shown in Figure 8. Both type I and type II collagen mRNA was detected in this tumor, whereas the nonsense probe demonstrated extremely low nonspecific staining. The expression of type I collagen by this cartilaginous tumor might be anticipated from its less differentiated character as compared with normal cartilage.

Figure 9 shows three archival specimens, an osteosarcoma, an aneurysmal bone cyst, and a neurofibroma. The osteosarcoma, a chondroblastic variant, was hybridized with the C2a probe. Sections of the tumor that had histologic characteristics of cartilage stained intensely for type II collagen, whereas the surrounding, more osteoblastic cells exhibited much less type II staining (Figure 9A). The aneurysmal bone cyst was hybridized with a probe for TGFβ2. This growth factor is a known autocrine regulator in bone,[21] and its presence was confirmed in the cells ringing an island of reactive bone in the aneurysmal bone cyst (Figure 9B). A neurofibroma was hybridized with a probe for type I collagen. Although most of the cells in this tumor were not stained, a subset of the cells stained intensely with the type I probe, Figure 9C. These are the fibroblastic cells in the process of producing matrix.

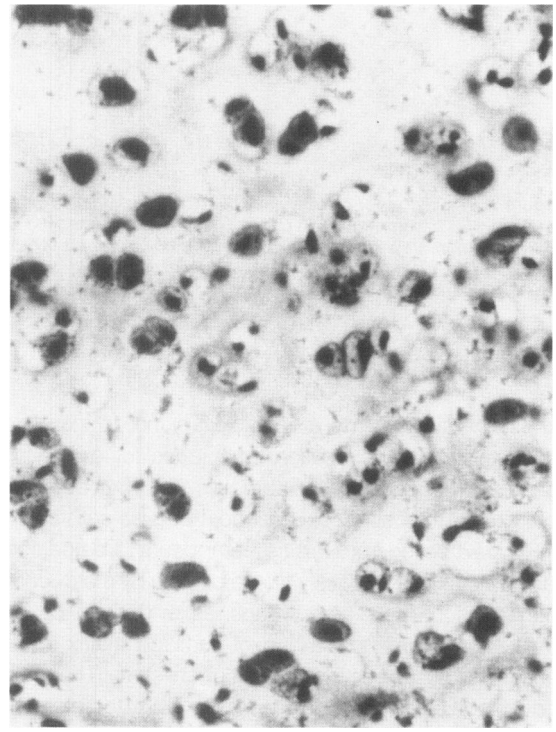

Figure 6. *Chick articular cartilage hybridized with digoxigenin-11-dUTP-labeled probe for type II collagen (C2), ×400.*

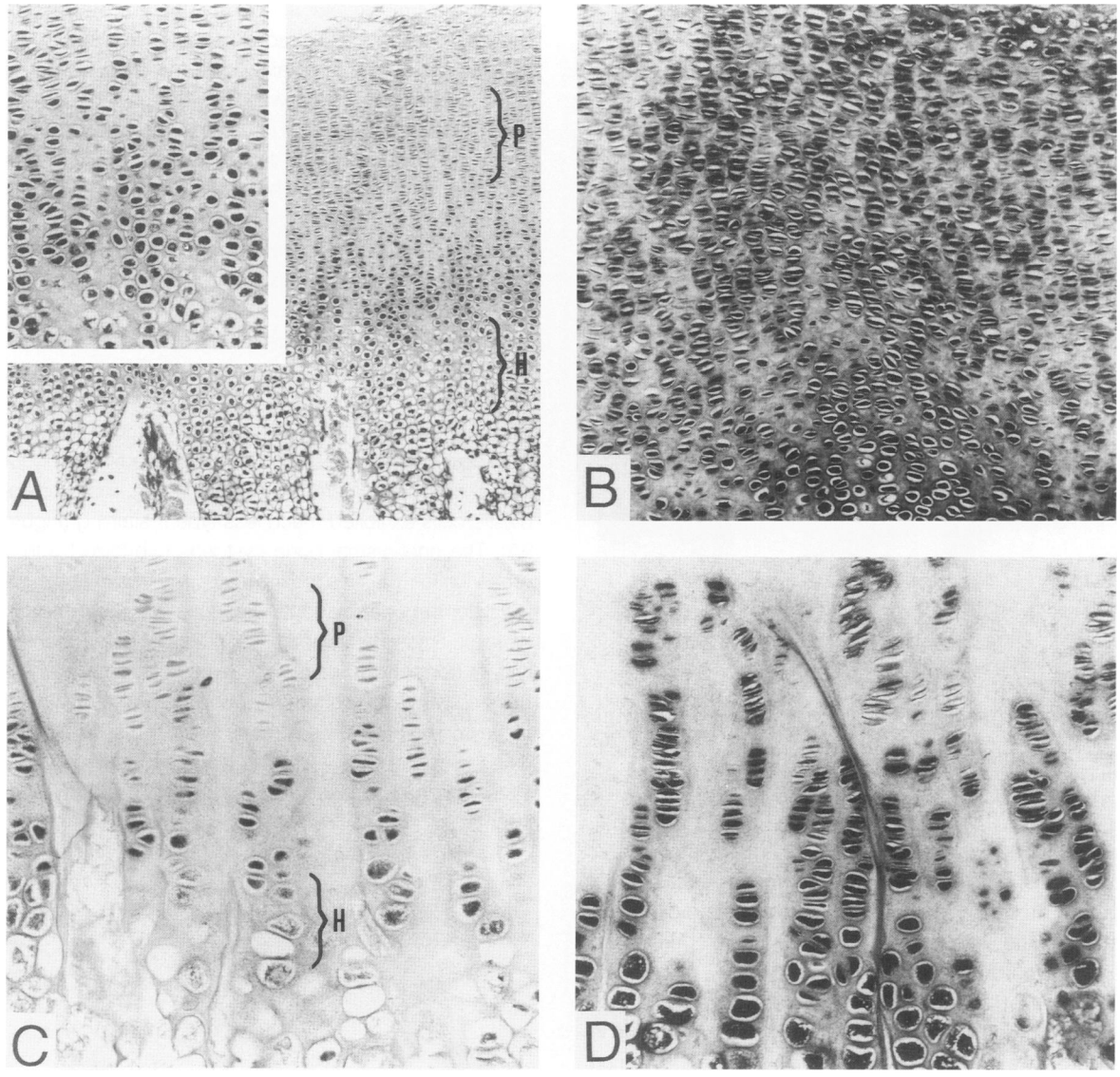

Figure 7. **A:** *Sections of chick growth plate hybridized with type II collagen (C2) probe. Signal is seen in both flattened proliferating cells (P) and rounded hypertrophic cells (H) (×100). The inset shows the junctional area between the hypertrophic and proliferating zone (×200).* **B:** *Chick growth plate hybridized with poly-dT probe. Signal is present throughout the growth plate (×200).* **C:** *Human growth plate hybridized with type II collagen probe (C2a). Again maximal signal is seen in the junctional area between the proliferating and hypertrophic zones (×200).* **D:** *Human growth plate hybridized with poly-dT probe with signal uniformly distributed throughout the growth plate cells.*

Discussion

In situ hybridization is a valuable adjunct to standard quantitative RNA extraction techniques for evaluating gene expression in tissues and cells. This may be especially true in the study of cartilage, because satisfactory mRNA extraction from this tissue appears to be particularly difficult.[13] This is likely due to binding by matrix components such as proteoglycans, as evidenced by the marked background staining of cartilage matrix in unacetylated tissue sections in the current study. The major advantage of *in situ* techniques is the ability to determine which cells in mixed populations or tissues are expressing the mRNA of interest. A previously published comparison of conventional radiolabeled hybridization with the digoxigenin technique demonstrated comparable results with the two methods.[12] The nonradioactive digoxigenin method is sensitive, enabling detection of low-level messages such as TGFβ1 and TGFβ2 in the current study. This results from the amplification of signal by the alkaline phosphatase color reaction. Also, the addition of multiple digoxigenated nucleotides to the probes by the terminal transferase allows the possible simultaneous interaction with multiple anti-digoxigenin antibody molecules. Control experiments demonstrated high specificity of the technique as well, including: (1) failure of a non-

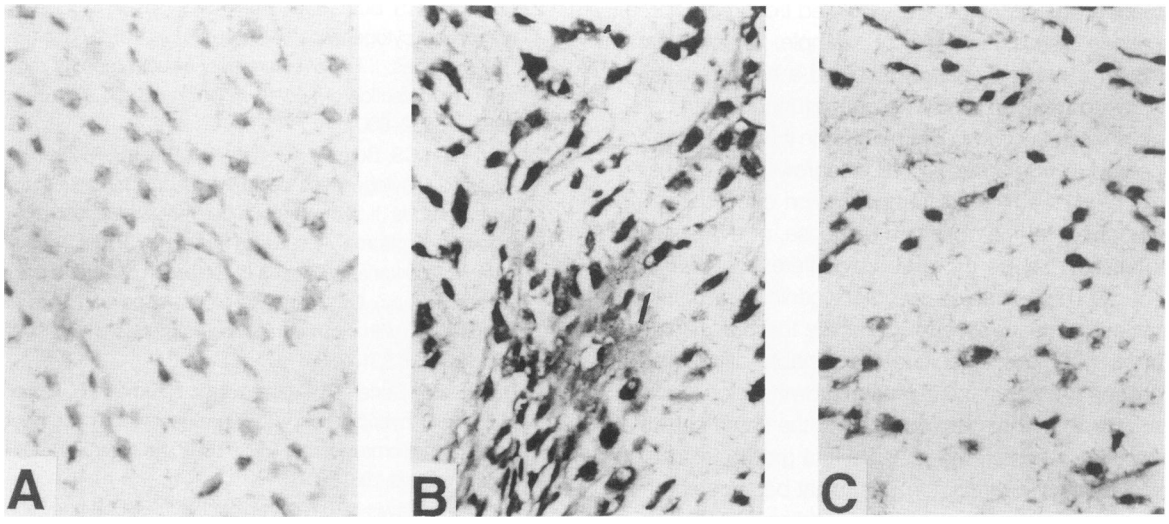

Figure 8. *Human chondrosarcoma hybridized with nonsense probe* (A), *type II collagen probe* (B), *or type I collagen probe* (C).

sense probe (RC) to exhibit signal; (2) the ability of competition with unlabeled probe to obliterate signal; and (3) lack of competition between dissimilar probes. The background staining with this technique is extremely low, an improvement over other nonradioactive methods using biotinylated probes.[16] An additional advantage is the method's speed, which facilitated the extensive optimization of the technique in the current study.

The use of synthetic oligonucleotide cDNA probes allows access to any known genetic sequences, plus the ability to include or exclude specific homologies in the probe selection. This was used to advantage in the current study to select probes with high specificity and species homologies for the tissues most used in our laboratory. The excellent stability of both the labeled and unlabeled oligonucleotide probes obviates the need for frequent probe preparation and labeling. Furthermore, we have demonstrated that under the labeling conditions optimized in the current study, the terminal transferase is essentially 100% effective in probe labeling, providing high specific activity of the probes. The simple and rapid spot blot immunoquantitation of labeled probes provides an easy method for normalization of different labeled probes within a given experiment.

Isolated chondrocytes from the whole growth plate represent a varied population of cells in different stages of maturation. Thus, the observed heterogeneity of cellular expression of message for proteins such as type II

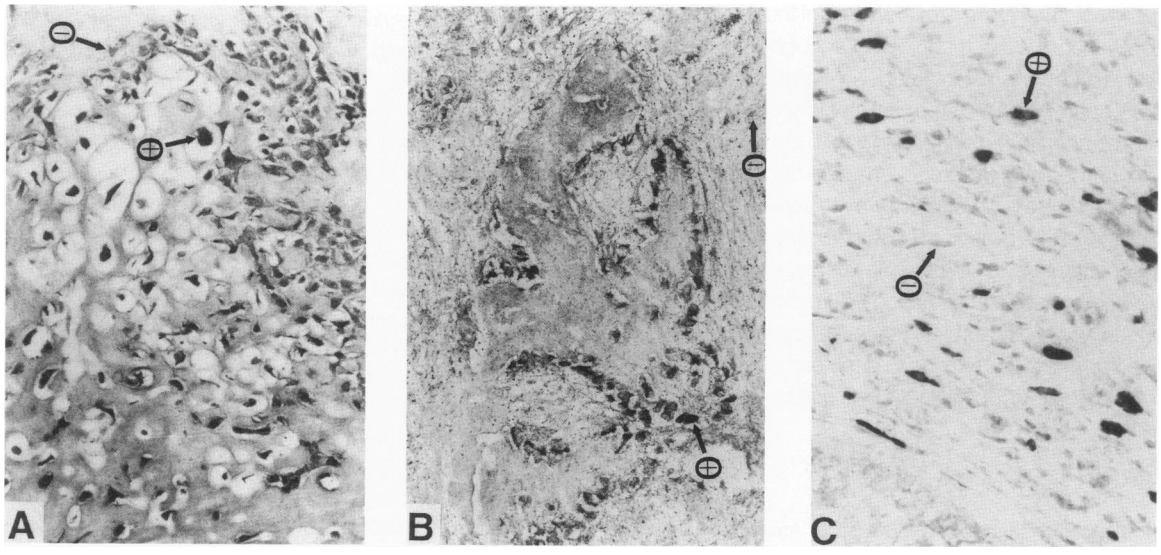

Figure 9. **A:** *Human osteosarcoma (chondroblastic variant) hybridized with human type II collagen probe (C2a) (×200).* **B:** *Reactive bone in a human aneurysmal bone cyst hybridized with TGFβ2 (×100).* **C:** *Human neurofibroma hybridized with type I collagen (×200). Examples of positively and negatively stained cells are indicated (+ or −).*

collagen was expected. Cells isolated from more homogeneous tissues, however, for example, fibroblasts and articular chondrocytes, also showed a heterogeneity of expression for type I and II collagens (unpublished data). This phenomenon may be important in the assessment of phenotypic responses of cells to growth factors using culture models. A small subpopulation of cells may be responsible for the observed response, or even opposing effects may be expressed by different cells simultaneously. The nonradioactive digoxigenin *in situ* method provides a means to readily evaluate these possibilities. Furthermore, this technique may enable differentiation between paracrine and autocrine growth factor effects in cell culture by determining whether the same cells that express a receptor and respond to a growth factor also produce the growth factor. This might be accomplished by using dual probes, combining radioactive labeled *in situ* hybridization with the digoxigenin method.

In the current study, the distribution of type II collagen mRNA in the chick growth plate was investigated using the digoxigenin *in situ* technique. Maximal expression was observed in the lower proliferating and upper hypertrophic zones. This parallels recently published reports in bovine and human growth cartilage using radioactive *in situ* techniques[16] and quantitative mRNA extraction.[22] In addition, we have previously analyzed collagen types synthesized by growth plate chondrocytes separated into maturational stages by countercurrent centrifugal elutriation. The maximal type II collagen production occurred in the middle fractions, corresponding to the late proliferating and early hypertrophic cells,[11] which is consistent with our observations with *in situ* hybridization, and supports the validity of the method.

The use of nonradioactive *in situ* hybridization to study pathologic archival materials is one of the most promising applications of this technique. Although the best results with tissues were obtained by immediate fixation in DEPC-paraformaldehyde, satisfactory results also have been obtained in our laboratory with conventionally fixed and embedded tissues even after decalcification. The poly-dT probe is an efficient way to screen archival samples for mRNA preservation. Combining the advances in oligonucleotide synthesis with digoxigenin labeling and alkaline phosphatase/antibody detection affords the opportunity to study archival tissues retrospectively, and ask specific questions relating to gene expression in a variety of pathologic or physiologic processes.

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