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In situ hybridization with polymerase chain reaction-derived single-stranded DNA probe and S1 nuclease

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Abstract A rapid and simplified protocol for in situ hybridization (ISH) with polymerase chain reaction (PCR)-derived single-stranded DNA probes and S1 nuclease revealed transcripts of bone matrix proteins on decalcified skeletal bone specimens. Mouse bone tissue was fixed with 4% paraformaldehyde, decalcified with 20% EDTA, and embedded in paraffin. Each pair of primers for reverse transcriptase –PCR was designed to amplify a 280-bp DNA fragment from the coding region of the mature protein of mouse osteonectin (ON) and a 320-bp fragment from the coding region of mouse osteopontin (OP). Initial PCR products were eluted, purified, and reamplified by unidirectional PCR in the presence of the digoxigenin (DIG)-labeled dUTP. ISH was carried out by proteinase K treatment, hybridization, and washing. The unhybridized single-stranded DNA probe was selectively removed by S1 nuclease treatment. Hybridized probes were visualized with the alkaline phosphatase-conjugated anti-DIG antibody. The transcripts of ON and OP were clearly detected on the thin sections of the decalcified bone. Because this protocol does not require cloning or in vitro transcription, reliable and stable ISH can be done in an ordinary laboratory equipped with a thermal cycler.

Introduction

In situ hybridization (ISH) is a useful and widely applied technique that shows the localization of specific nucleic acid sequences at chromosomal, cytological, and histological levels (Arrighi et al. 1970; McNicol and Farquharson 1997). Probes are usually labeled with either radioactive materials, such as ^{3}H and ^{35}S (Jacobsson 1989), or non-radioactives, such as digoxigenin (DIG; Heiles et al. 1988), biotin (Manning et al. 1975), bromodeoxyuridine (Niedobitek et al. 1988; Kitazawa et al. 1989), and T-T dimer (Koji et al. 1988). Recently DIG-labeled single-stranded RNA probes transcribed in vitro from linealized double-stranded DNA template have been most widely used (Aigner and Pette 1990; Tsukamoto et al. 1991). These RNA probes are, however, susceptible to degradation by troublesome RNase contamination. Moreover, handling of plasmid and RNA requires special laboratory techniques, space, and equipment.

For this study, we developed a simplified and efficient method for ISH, where we employed a polymerase chain reaction (PCR)-derived single-stranded DNA probe and S1 nuclease treatment. The single-stranded DNA probe generated by PCR was very stable and amply sensitive for detecting transcripts of bone matrix proteins [osteopontin (OP) and osteonectin (ON), markers for differentiating osteoblastic cell] on the decalcified bone tissue.

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Materials and methods

Reverse transcriptase (RT)–PCR and probe preparation

Total RNA was extracted from the kidneys of 14-week-old BALB/c mice (Charles River, Yokohama, Japan) with the commercially available RNA extraction system, RNAzol (Tel-Test, Friedswood, Tex., USA), according to the manufacturer's instruction, and used as a template of RT–PCR. A 320-bp cDNA fragment from the coding region of the mature protein of mouse OP and a 280-bp cDNA fragment from mouse ON were amplified by RT-PCR with rTth reverse transcriptase (Perkin-Elmer Cetus, Norwalk, Conn., USA) using the following pairs of oligonucleotide primers:

primer OP (sense);
5′-GCCTGACCCATCTCAGAAGCAGAAT-3′

primer OP (antisense);
5′-TAAGCCAAGCTATCACCTCGGCCGT-3′

primer ON (sense);
5′-AGCGTCAAGCCAAACACAAACAGCG-3′

primer ON (antisense);
5′-CATCTAGGTACAACATGGAGATTGC-3′

In the reverse transcription step, an antisense primer-primed cDNA was synthesized at 60° C for 60 min, then by both sense

and antisense primer-primed PCR amplification to obtain each DNA fragment. The cycling conditions for PCR were denaturation at 94° C for 15 s, annealing at 67° C for 15 s, and elongation at 72° C for 30 s, for a total of 35 cycles. Amplified PCR products were loaded onto 3% agarose gel, and specific DNA bands of the predicted size were cut and dissolved in NaI solution. The DNA was recovered and purified by the glass powder-mediated method using Easytrap according to the manufacturer's recommendation (Takara, Tokyo, Japan). To prepare the DIG-labeled single-stranded antisense DNA probe, the purified PCR product was subjected to unidirectional PCR with the antisense primer alone in the presence of DIG-dUTP (DIG DNA labeling mixture; Boehringer Mannheim, Mannheim, Germany) in the same PCR parameters for a total of 40 cycles. The DIG-labeled single-stranded antisense DNA was purified with Quickspin Column Sephadex-G50, Fine (Boehringer Mannheim) at a speed of 500 *g*. For negative controls, DIG-labeled sense probes were generated with sense primer-primed unidirectional PCR.

Dot-spot hybridization

To test the specificity and sensitivity of the PCR-derived single-stranded DNA probes, the denatured non-labeled cDNAs were serially diluted and spotted onto nylon membranes (Hybond N+; Amersham, Arlington Heights, Ill., USA), and then crosslinked by UV irradiation for 2 min. The membranes were hybridized, and developed under the same conditions as for ISH, described below.

Northern blot hybridization

To test the specificity and sensitivity of the PCR-derived single-stranded DNA probes, we performed three sets of the Northern blot analyses using ^{32}P-labeled, in vitro-transcribed DIG-labeled cRNA and PCR-derived single-stranded DNA DIG-labeled probes. Total RNA was extracted and purified from the MC3T3-E1 mouse osteoblastic cell line (Sudo et al. 1983) and the ST2 mouse stromal cell line (Hardy et al. 1987) by RNAzol. Of each RNA 7.5 µg was electrophoresed on a 1.2% agarose gel and transferred onto the nylon membrane (Hybond N+). The membranes were then hybridized at 60° C for 16 h and washed twice in 2×SSPE at room temperature and in 0.1×SSPE at 60° C. For the radiolabeled probe detection, the membrane was analyzed with a BAS 2000 image analyzer (Fuji, Tokyo, Japan). For DIG-labeled probe detection, the membranes were first blocked with phosphate-buffered saline (PBS), containing 1% non-fat dry milk, and then incubated with alkaline phosphatase-conjugated anti-DIG antibody for 30 min. The membranes were washed 3 times with PBS for 30 min and developed under the same conditions as for ISH, described below.

In situ hybridization

Tissue samples were excised from 8 week-old BALB/c mice and the tibiae were immediately fixed with 4% paraformaldehyde (PFA) for 2 days and decalcified with 20% EDTA for 4 days. Five-micron sections were prepared, dewaxed in xylene, and rehydrated through a series of graded ethanols. The samples were treated with 2–5 µg/ml proteinase K (Sigma, St. Louis, Mo., USA) for 10 min, refixed with 4% PFA, immersed in 0.1 M triethanolamine (pH 8) for 10 min and 0.1 M triethanolamine containing 0.25% acetic acid for 15 min, and washed in 0.1 M phosphate buffer (pH 7.4). The samples were then incubated in a hybridization medium [10 mM TRIS-HCl (pH 7.3), 1 mM EDTA, 600 mM NaCl, 0.25% sodium dodecyl sulfate, 1× Denhardt's medium, 50% (v/v) deionized formamide/pg per ml of probe DNA, and 10% dextran sulfate] at 50° C in a moist chamber for 16 h. Negative controls were prepared with either a DIG-labeled sense DNA or RNase predigestion. After hybridization, the slides were washed with 50% deionized formamide/2× SSC to remove the superfluos probe, washed further with 2× SSC and 0.2× SSC, and then treated with freshly prepared S1 nuclease [10 U/ml S1 nuclease (Promega, Madison, Wis., USA), 50 mM potassium acetate (pH 4.6), and 300 mM NaCl] at 37° C for 15 min to remove the unhybridized single-stranded DNA probe. To visualize the probe, the slides were incubated with alkaline phosphatase-conjugated anti-DIG antibody (Boehringer Mannheim) for 60 min after blocking with 1.5% non-fat dry milk in PBS for 30 min. The specimens were then washed twice with 100 mM TRIS-HCl (pH 7.5) containing 150 mM NaCl for 20 min each time, and immersed briefly in 100 mM TRIS-HCl (pH 9.5) containing 100 mM NaCl and 50 mM $MgCl_2$. Colorimetric reaction was done with nitro blue tetrazolium salt and bromo-4-chloro-3-indolyl phosphate solution (Boehringer Mannheim) in the dark for 4 h, then stopped with 10 mM TRIS-HCl (pH 8) containing 1 mM EDTA. Slides were mounted on Crystalmount (Biomedica, Foster City, Calif., USA) and analyzed under a light microscope without counterstaining.

Results

The dot-spot hybridization study with the serially diluted cDNA showed that the PCR-derived single-stranded DNA probes were of sufficient sensitivity to detect as little as 2 pg DNA in both OP and ON. Each probe hybridized specifically to the corresponding spotted DNA (Fig. 1). In Northern blot analyses, radiolabeled, in vitro-transcribed cRNA and PCR-derived single-stranded DNA probes all detected a specific 2.3-kb band of ON mRNA. PCR-derived single-stranded DNA probes showed comparative sensitivity to both radiolabeled and cRNA probes (Fig. 2). In ISH with decalcified normal mouse bone tissue when the specimens were treated with 2 µg/ml proteinase K, the transcripts of OP were detected in the cytoplasm of osteoblastic cells on the surface of the mature trabecular bone (Fig. 3b). When the concentration of the proteinase K was increased to 5 µg/ml, in an attempt to heighten the sensitivity, not only were the signals observed in the osteoblastic lineage enhanced, but the non-specific bindings to the collagen fibers of surrounding connective tissue increased (Fig. 3c). Signals were detected neither in the specimen hybridized with DIG-labeled ON sense DNA (Fig. 3 d) nor in the specimens predigested with RNase (data not shown). When we applied the treatment of S1 nuclease (10 U/ml), a specific enzyme to digest single-stranded DNA

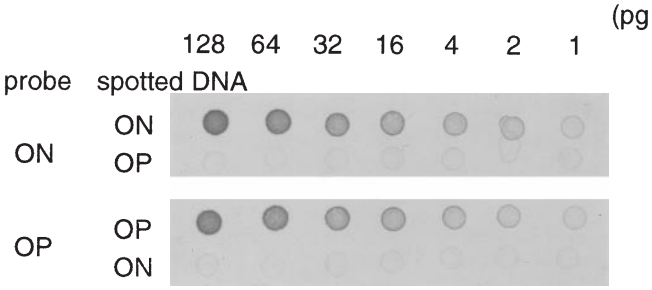


Fig. 1 Dot-spot hybridization. Between 1 and 100 pg of serially diluted target DNAs [osteopontin (OP) and osteonectin (ON)] were spotted onto the nylon membrane. Both OP and ON digoxigenin (DIG)-labeled single-stranded DNA probes hybridized efficiently and specifically to their corresponding DNA. As little as 2 pg of the target DNAs were detectable

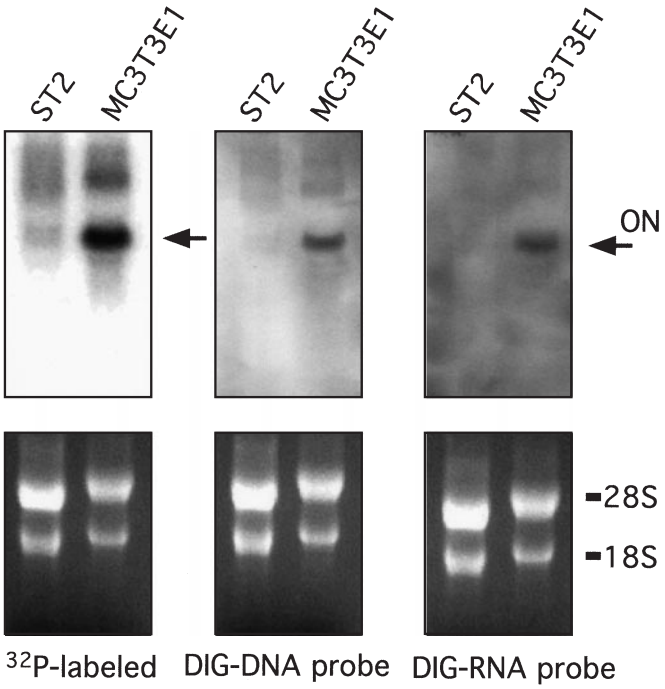


Fig. 2 Northern blot analyses. Total RNA, 7.5 µg, from the MC3T3-E1 mouse osteoblastic cell line and the ST2 mouse stromal cell line were analyzed by Northern blotting. Three sets of the membranes were hybridized with radiolabeled, in vitro transcribed, and polymerase chain reaction (PCR)-derived single-stranded DNA probes. PCR-derived single-stranded DNA probe detected specific 2.3 kb of ON mRNA and showed comparative sensitivity to other probes

and RNA, the non-specific bindings decreased significantly while maintaining the enhanced signals for the specific hybridization (Fig. 4a, c: OP, b, d: ON). As shown in Fig. 3c, the combined treatment 5 µg/ml proteinase K and S1 nuclease was effective in revealing OP mRNA in immature osteoblastic cells in the primary spongiosa of mouse diaphysis. The transcripts of ON, on the other hand, were observed mainly on the osteoblastic cell lineage in both mature trabecular and the newly formed bone of the endosteal site (Fig. 4b, c).

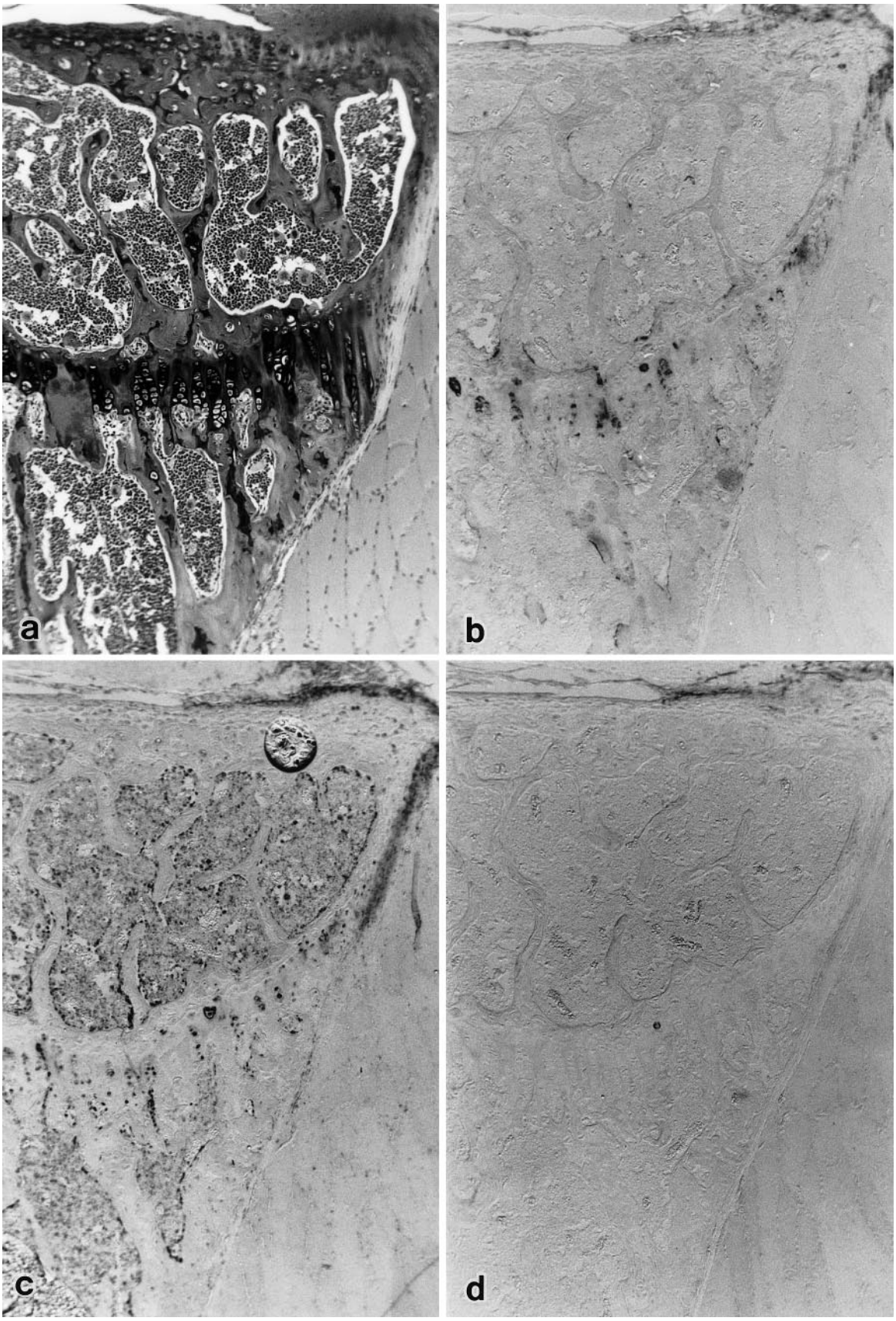
Discussion

Since biochemical detection of the transcripts by Northern blot analysis is based on the summation of homogenized materials, precise information on the frequency and characteristics of transcribing cells can be obtained only by morphological assessment of signals at the cellular level by ISH. Various kinds of nucleic acid probes have recently been introduced to enhance the specificity and sensitivity of ISH. Among these probes, the most frequently used are single-stranded cRNA riboprobes transcribed in vitro from cloned cDNA. Such cRNA probes are highly sensitive with little background staining, because non-specific bindings can be eliminated by ribonuclease treatment after hybridization. A riboprobe is, however, susceptible to nuclease digestion and requires complicated laboratory techniques including cDNA cloning in an appropriate vector plasmid, linealization with restriction endonuclease digestion, and in vitro transcription. Furthermore, an additional combination of restriction enzymes and transcriptase is necessary to synthesize the sense probe for the negative control. Double-stranded cDNA amplified either in plasmid form or directly by PCR is also often used as a hybridization probe. In this technique, however, the lack of negative control with sense DNA and the adjustment to optimize RNA–DNA rather than DNA–DNA hybridization limit the reliability and sensitivity of the ISH.

PCR, widely used in both research and diagnostic fields, has also been applied in ISH studies, generation of vector-free probes (Wu et al. 1995), and in situ PCR techniques (Nuovo et al. 1991). An asymmetric PCR has efficiently generated radiolabeled DNA probes for ISH, and Northern and Southern blotting (Scully et al. 1990). For non-isotopic labeling, DIG-dUTP has been used for PCR-derived DNA probes to demonstrate hepatitis B virus DNA in biopsied liver tissue (An et al. 1992). In our study, to synthesize the single-stranded DNA probes, we did not employ the asymmetric PCR technique because contaminating a small amount of the labeled complementary DNA fragment is inevitable. Instead, we carried out two separate steps of PCR: the first (conventional cycles) to yield a sufficient amount of the template double-stranded DNA and the second (unidirectional PCR cycles) for DIG-labeling with either the sense or antisense primer. In the second step, the amount of the labeled single-stranded DNA depended on that of the double-stranded template DNA and the number of the reaction cycles. The unidirectional reaction lineally increased the template DNA up to 30–40-fold at most. The efficiency of the probe generation was confirmed and monitored by comparing 1/10 of the template double-stranded DNA with 1/30 of the DIG-labeled single-stranded DNA purified by spin column on agarose gel electrophoresis with ethidium bromide staining. A single unidirectional PCR of 25 µl yielded an ample number of probes sufficient for at least 30 specimens of ISH. Furthermore, DIG-labeled DNA is stable for years when stored at –20° C.

We demonstrated the expression of OP and ON mRNA on the bone tissue of normal mouse. In mouse bone tissue, ON was rather ubiquitously expressed among the osteoblastic lineage up to 0.0025% of the to-

Fig. 3a–d In situ hybridization (ISH) after 2 and 5 µg/ml proteinase K treatment. Decalcified normal mouse tibiae were fixed with 4% paraformaldehyde and embedded in paraffin. **a** Hematoxylin and eosin staining of decalcified bone tissue. **b** When the specimens were treated with 2 µg/ml proteinase K, the transcripts of OP were visualized in hypertrophic chondrocytes around the growth plate (×40).**c** When the specimens were treated with 5 µg/ml proteinase K, OP signals were observed on both hypertrophic chondrocytes and osteoblastic cells in newly formed trabecular bone, although non-specific probe bindings to the bone marrow cells and collagen fibers were present (×40). **d** Negative control prepared with sense OP probe did not show significant staining (×40)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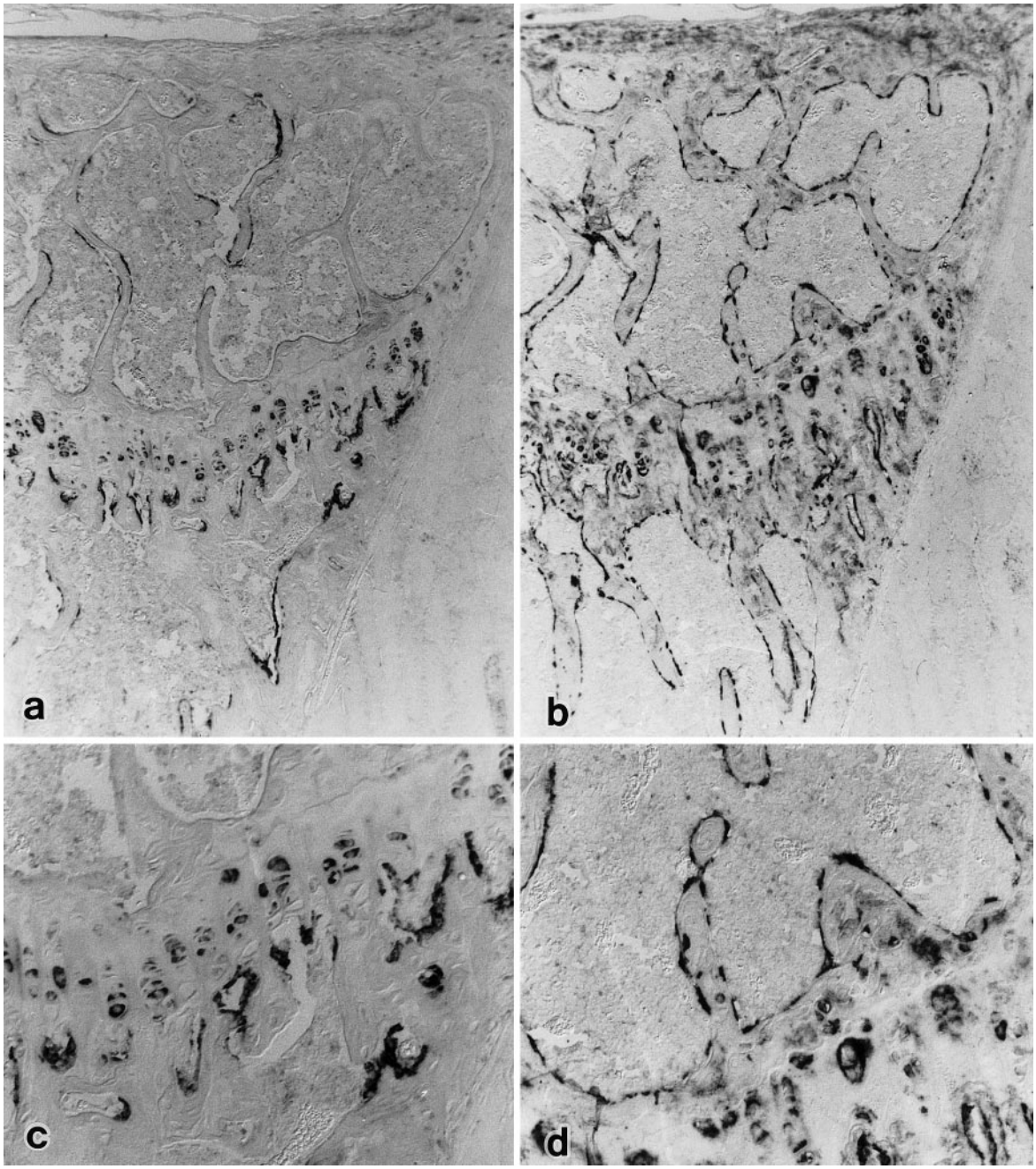

Fig. 4a–d ISH after 5 µg/ml proteinase K treatment with S1 nuclease treatment. Non-specific probe bindings decreased in OP (**a**; ×40) and ON (**b**; ×40). OP mRNA was detectable not only in the hypertrophic chondrocytes, but also in the differentiating osteoblastic cell lineage in the diaphysis (**c** ×100). ON signals were observed on osteoblastic cells in both mature and newly formed bone (**d**, ×100)

tal RNA (Ringuette et al. 1991). On the other hand, OP mRNA expression has been reported to be less than ON and restricted to the mature osteoblastic cells (Cowles et al. 1998). Single-stranded cDNA probes for OP and ON were of sufficient sensitivity and specificity to demonstrate their localization on the decalcified bone sections. To improve the sensitivity of hybridization, we treated the dewaxed tissue with 2–5 µg/ml proteinase K. This proteinase K treatment at more than 5 µg/ml for 10 min, however, increased the non-specific probe bindings to the surrounding connective tissues. Although the sensitivity and specificity of the single-stranded DNA probes were sufficiently high to detect OP mRNA expression in the hypertrophic chondrocytes, optional treatment with S1 nuclease decreased the non-specific probe bindings. As shown in Fig. 4c, the combined treatment with 5 µg/ml proteinase K and S1 nuclease contributed to demonstrating OP mRNA in immature osteoblastic cells in the primary spongiosa of mouse diaphysis where only a nominal amount of OP mRNA has been expressed (Ibaraki et al. 1992). This study demonstrated a simplified and reliable ISH protocol where with the combination of

unidirectional PCR-derived single-stranded DNA probe and S1 nuclease treatment was used.

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