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Assessment of decalcifying protocols for detection of specific RNA by non-radioactive in situ hybridization in calcified tissues

Abstract For the best performance of in situ analysis of specific RNA expression in calcified tissues, it is necessary to choose an appropriate protocol to decalcify the tissues. We evaluated the usefulness of various acid-based decalcifying reagents with reference to 28 S rRNA staining by in situ hybridization using a thymine-thymine dimerized oligonucleotide probe. The reagents evaluated were 10% nitric acid, 10% HCl, 5% formic acid, 5% trichloroacetic acid, Morse's solution, Plank-Rychlo's solution, and K-CX solution, all of which are commonly used to decalcify tissues, and their effects on retention of morphology and RNA were compared with EDTA-based solutions. When normal mouse mandible was used as a model tissue, well-preserved morphology of ameloblasts was obtained from sections decalcified with Morse's solution, 10% HCl, Plank-Rychlo's solution, and K-CX solution, and best retention of 28 S rRNA was obtained with 5% formic acid and Morse's solution. We recommend Morse's solution to decalcify tissues to be processed for the rapid analysis of specific RNA expression. Indeed, we detected specific mRNAs strongly in sections treated with Morse's solution, and quantitative analysis showed that the ratio of signal intensities of 28 S rRNA and the specific mRNAs correlated with each other depending on decalcifying solutions.

Introduction

In calcified tissues such as bone and tooth, there are inherent difficulties in the process of preparation of histo-

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logical sections for in situ hybridization (ISH) analysis due to extensive mineralization. To overcome these difficulties, the main approach is to conduct ISH using histological sections obtained from paraffin-embedded tissues after decalcification. This method provides more opportunity to perform ISH of calcified tissues in many laboratories, however, one must optimize the conditions of decalcification because of its degrading effect on RNA. Although it is important to explore the effects of various decalcifying reagents on RNA preservation, only a limited number of basic experiments have been reported. Walsh et al. (1993) compared the effect of three decalcifying agents including 6% nitric acid, 20% EDTA, and buffered formic acid, while Arber et al. (1997) tested three different commercially available decalcifying agents, an EDTA-based solution and two HCl-based solutions. Both used poly-d(T) oligonucleotide as a probe and concluded that EDTA-based solution preserves substantial amounts of mRNA. Since EDTA-based solution needs a long duration to complete decalcification, more extensive studies have been required to develop a rapid and suitable decalcifying protocol for ISH analysis. This is an important issue especially in the field of surgical pathology, where minimal incubation duration, good preservation of morphology, and good retention of RNA integrity are always required.

Recently, we developed an ISH system to evaluate the levels of hybridizable RNA in tissue sections (Yoshii et al. 1995) using an oligonucleotide (oligo-DNA) complementary to a part of the 28 S ribosomal RNA (rRNA). This system seemed to be superior to the method using poly d(T) oligonucleotide probe because the probe has no guanine-cytosine residues, resulting in an unusually low melting temperature. The ISH signals for 28 S rRNA were well correlated with those for many specific mRNAs in tissues of various species.

In the present study, we investigated the appropriate acid-based decalcifying agents for ISH analysis of specific RNA expression. The agents assessed were seven kinds of acid-based solutions including commercially available decalcifying agents and their effects on reten-

Table 1 Time required for decalcification of mouse mandibles, optimal concentration of proteinase K (*PK*), and average pixels of 28 S rRNA on ameloblasts and osteoblasts

Decalcifying solution	Time required for decalcification (days)	Optimal concentration of PK (µm/ml)	Relative amounts of 28 S rRNA	
			Ameloblast	Osteoblast
10% EDTA (pH 7.4)	7	10	9840±766	4132±367
10% EDTA/TRIS-HCl (pH 7.4	7	10	9420±1021	4207±415
10% EDTA/glycerol (pH 7.4)	8	10	10105±1215	3759±355
5% formic acid	1	5	8005±662	3988±401
Morse's solution	1	5	9455±881	4171±377
10% HCl	1	5	3370±271	1145±142
Plank-Rychlo's solution	1	5	5352±462	2247±165
K-CX solution	1	5	2804±196	1177±158
5% trichloroacetic acid	1	5	5619±428	1833±202
10% nitric acid	1	5	3532±422	1554±96

tion of morphology and RNA were compared with reference to EDTA-based solutions. We first evaluated the agents by morphological details and 28 S rRNA staining of ameloblasts and osteoblasts in mandibles and then confirmed the reliability of the selected acid-based decalcifying agents by detection of the specific mRNAs in osteoblasts. We demonstrate here that Morse's solution is the favored choice for rapid decalcification for ISH analysis in calcified tissues. Moreover, our quantitative analysis shows that the increasing or decreasing ratio of signal intensities of 28 S rRNA and specific mRNAs correlate well with each other depending upon the decalcifying solutions used. It suggests that the ratio of relative amounts of 28 S rRNA between two arbitrary decalcifying solutions may be reliable as a standard in retrospective studies, which request metaphysical recovery of signal intensities of mRNA decreased by inappropriate decalcifying solutions.

Materials and methods

Tissue preparation

Ten-week-old ICR mice weighing 32–40 g were anesthetized by diethyl ether and perfused through the heart with 0.01 M phosphate-buffered saline (PBS, pH 7.2) followed by freshly prepared 4% paraformaldehyde (PFA) in PBS (pH 7.2). After this, the mandibles were dissected out and immersed further in 4% PFA for 16 h at 4°C. The mandibles were then decalcified with different solutions listed below for the indicated duration as determined by transmission of soft X-ray. After decalcification was complete, the processed mandibles were embedded in paraffin by a routine procedure, sectioned into 6-µm slices, and used for ISH.

Decalcifying protocols

Acid-based decalcifying solutions used in this experiment are 10% nitric acid, 10% HCl, 5% formic acid, 5% trichloroacetic acid (TCA), Morse's solution (10% sodium citrate and 22.5% formic acid; Morse 1945), Plank-Rychlo's solution (0.3 M aluminium chloride, 3% HCl, and 5% formic acid; Plank and Rychlo 1952), and K-CX solution (Fujisawa Pharmaceutical, Tokyo, Japan). As references, 10% EDTA (pH 7.4), 10% EDTA/TRIS-HCl (pH 7.4), and 10% EDTA with 0.07% (w/v) glycerol (pH 7.4) were selected (Table 1). These solutions are commonly used for decalcification in many laboratories. The tissues decalcified with inorganic acid (10% nitric acid, 10% HCl, and K-CX solution) were neutralized

by sodium sulfate for 12 h before dehydration by an ethanol-xylene series.

Preparation of probes

Oligo-DNA complementary to a part of 28 S rRNA was synthesized together with additional 3 ATT repeats at the 3' end, and haptenized by ultraviolet irradiation, as detailed previously (Yoshii et al. 1995). One-kilobase cDNA fragments of rat osteopontin (OP; Singh et al. 1992) which encode the OP sequence from 116 to 1069 and 0.3-kb cDNA fragments of rat osteocalcin (OC; Hashimoto et al. 1997) were generous gifts from Dr. H. Sakai (Department of Dental Pharmacology, Nagasaki University School of Dentistry, Japan). All of the cDNAs were thymine-thymine (T-T) dimerized by irradiation at a dose of 12 kJ m^{-2} as described previously (Koji and Nakane 1996). In order to increase accessibility of target mRNA to probe DNA in tissue sections, the size of the cDNAs was reduced to about 100–200 bp by DNase I digestion according to a previous study (Koji and Nakane 1990). As a control probe, T-T dimerized λDNA (also irradiated at a dose of 12 kJ m^{-2} and treated by DNase I) was used.

In situ hybridization

The procedures for ISH with T-T dimerized oligo-DNA probe and cDNA probes were described previously (Yoshii et al. 1995). Briefly, paraffin sections are deparaffinized and rehydrated with a toluene-ethanol series. After incubation for 15 min with 0.3% H$_2$O$_2$ in methanol to block endogenous peroxidase activity, these sections were treated with 0.2 N HCl and digested with various concentrations of proteinase K for 15 min at 37°C. After postfixation with 4% PFA in PBS (5 min), the sections were immersed in 2 mg/ml glycine in PBS (30 min) and kept in 40% deionized formamide in 4×SSC until used for hybridization. Hybridization was carried out at 37°C for 12–16 h with T-T dimerized oligo-cDNA for 28 S rRNA or 42°C for 12–16 h with cDNAs dissolved in the hybridization medium. After repeated washings, the T-T dimers were incubated with mouse monoclonal anti-T-T IgG (Kyowa Medex, Tokyo, Japan) followed by HRP-conjugated goat anti-mouse IgG. The sites of peroxidase activity were visualized by 3,3'-diaminobenzidine/4-HCl and H$_2$O$_2$ in the presence of nickel and cobalt ions as a chromogen solution (Adams 1981). In the case of rat OP and OC cDNAs, the signals were enhanced by a catalyzed signal amplification system with biotinylated tyramine (Koji et al. 1997).

Fig. 1A–J In situ hybridization (ISH) for 28 S rRNAs in incisor ▶ ameloblasts of mouse mandibles. The sections were decalcified with 10% EDTA (**A**), 10% EDTA/Tris-HCl (**B**), 10% EDTA with glycerol (**C**), 5% formic acid (**D**), Morse's solution (**E**), 10% HCl (**F**), Plank-Rychlo's solution (**G**), K-CX solution (**H**), 5% TCA (**I**), and 10% nitric acid (**J**). Original magnification ×132
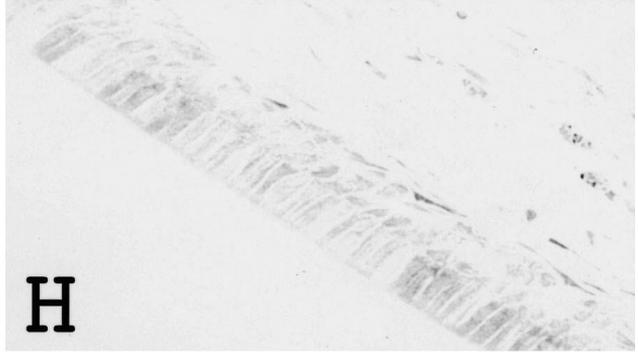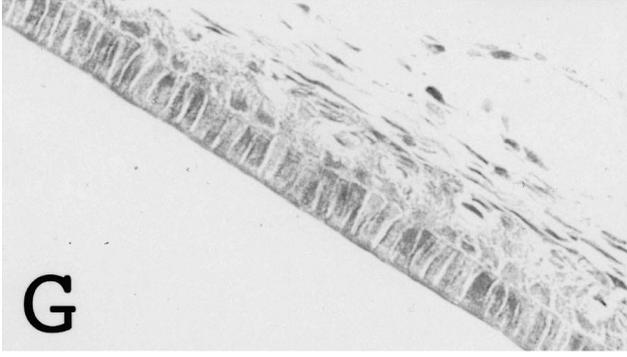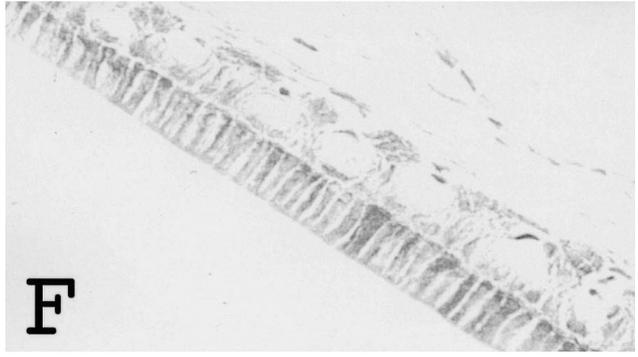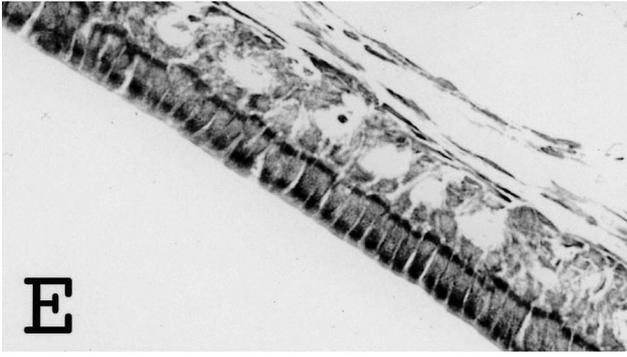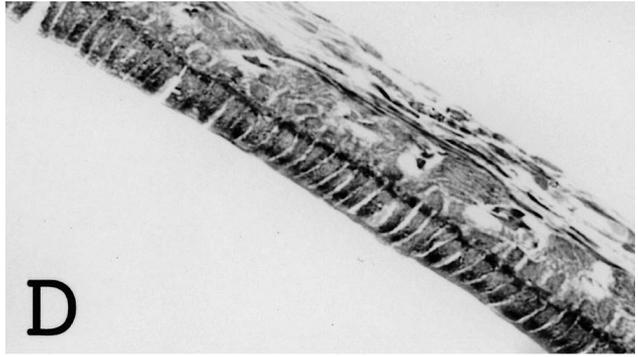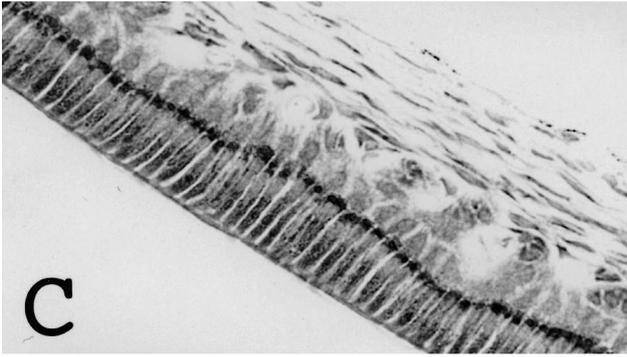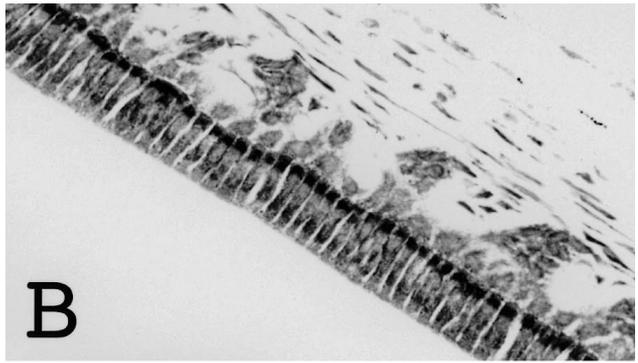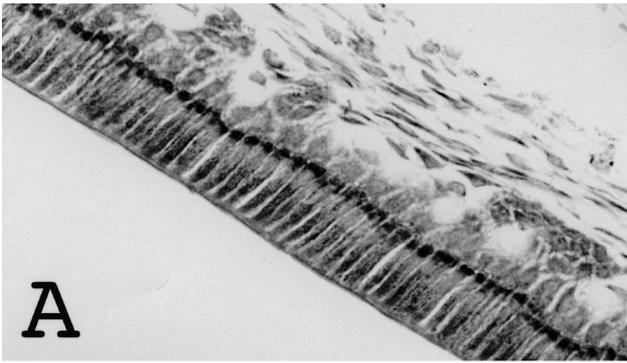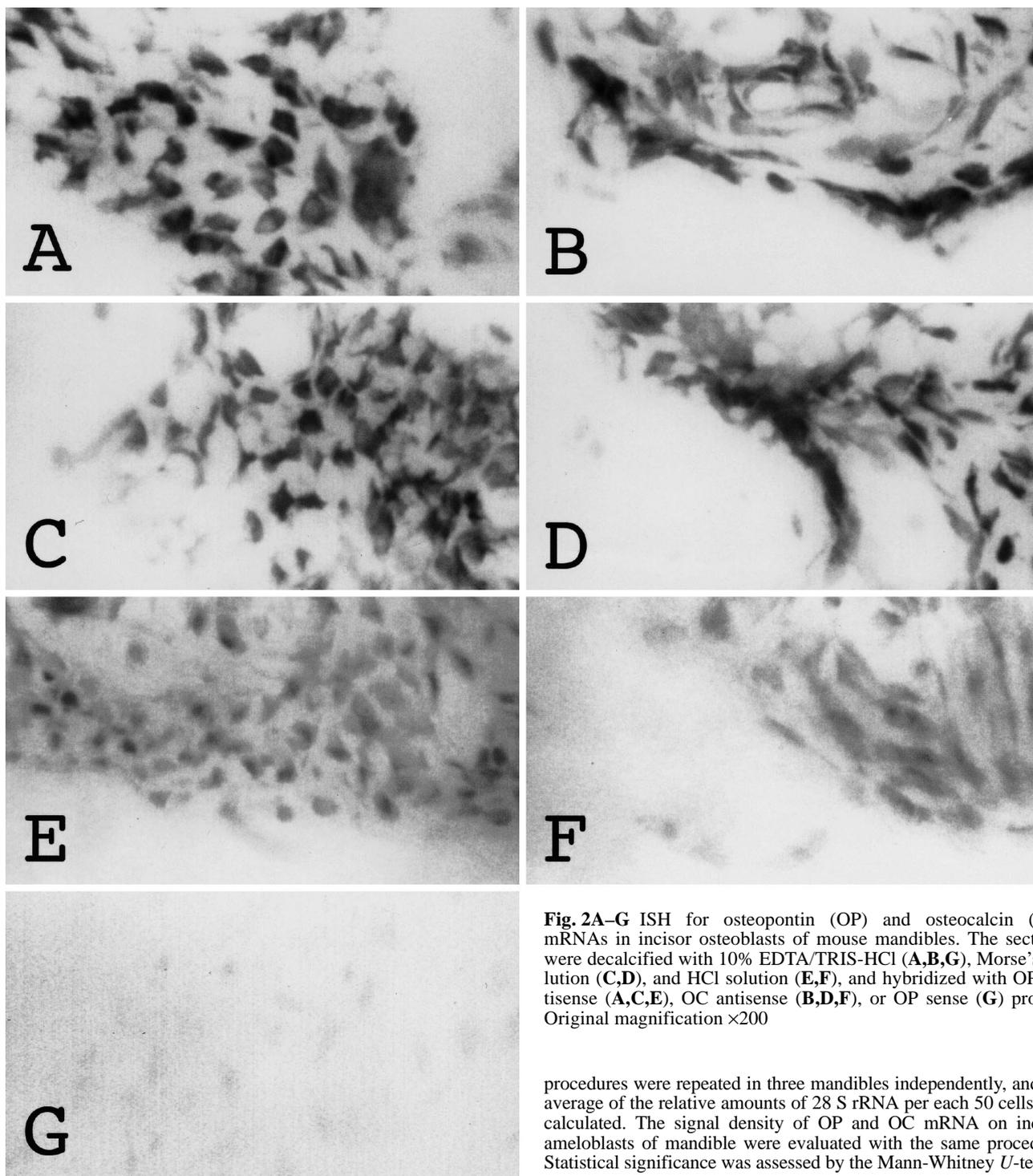
Fig. 2A–G ISH for osteopontin (OP) and osteocalcin (OC) mRNAs in incisor osteoblasts of mouse mandibles. The sections were decalcified with 10% EDTA/TRIS-HCl (**A,B,G**), Morse's solution (**C,D**), and HCl solution (**E,F**), and hybridized with OP antisense (**A,C,E**), OC antisense (**B,D,F**), or OP sense (**G**) probes. Original magnification ×200

procedures were repeated in three mandibles independently, and the average of the relative amounts of 28 S rRNA per each 50 cells was calculated. The signal density of OP and OC mRNA on incisor ameloblasts of mandible were evaluated with the same procedure. Statistical significance was assessed by the Mann-Whitney *U*-test.

Quantitation

Stained sections were exchanged to digital image and signal density was quantified using an Olympus microscope BH2 connected to an Olympus image analyzer (SP-200). To compare the degree of RNA retention in mandibles decalcified with each solution, three areas each containing over 50 incisor ameloblasts or 50 osteoblasts were selected randomly and the density of signals in each area was measured using the image analyzer. Relative amounts of 28 S rRNA were obtained by subtracting the sum of arbitrary units of 50 cells on sections with sense probe from one with antisense probe. These

Results

Decalcification duration and optimal concentration of proteinase K

The times required for decalcification and the optimal concentration of proteinase K were determined by the X-ray examination and 28 S rRNA staining, respectively. As shown in Table 1, decalcification took 24 h with acid

solution, i.e., 10% nitric acid, 10% HCl, 5% formic acid, 5% TCA, Morse's solution, Plank-Rychlo's solution, and K-CX solution, 7 days with 10% EDTA and 10% EDTA/TRIS-HCl, and 8 days with 10% EDTA with glycerol. The intensity of 28 S rRNA detected reached a maximum at 5 µg/ml proteinase K in mandible decalcified with acid solutions and 10 µg/ml in EDTA-based solutions (10% EDTA, 10% EDTA/TRIS-HCl, and 10% EDTA with glycerol).

Morphology

The arrangement and architecture of ameloblasts were well preserved in mandibles decalcified with Morse's solution (Fig. 1E), 10% HCl (Fig. 1F), Plank-Rychlo's solution (Fig. 1G), and K-CX solution (Fig. 1H), as well as the three EDTA-based solutions (Fig. 1A–C). The treatment with 5% formic acid (Fig. 1D) resulted in a slight vacuolation and shrinkage of ameloblasts. Ameloblasts in the sections decalcified with 5% TCA were severely shrunken and detached from each other (Fig. 1I), while with 10% nitric acid they were markedly swollen (Fig. 1J).

Retention of RNA assessed by 28 S rRNA staining

The average of the sum of pixel intensity and the standard deviation of 28 S rRNA in ameloblasts and osteoblasts decalcified in each solution are shown in Table 1. The treatment with 5% formic acid solution and Morse's solution as well as EDTA-based solutions resulted in retention of large amounts of rRNA (Table 1; Fig. 1A–E). In contrast, other acid-based solutions reduce the level of 28 S rRNA signal to approximately half (Plank-Rychlo's solution and 5% TCA) or one-third (10% HCl, K-CX solution, 10% nitric acid) of that with EDTA-based solutions (Table 1; Fig. 1F–J). There was no significant difference between the 5% formic acid, Morse's solution, and EDTA-based solutions for retention of rRNA.

Retention of specific mRNAs decalcified with Morse's solution and 10% HCl

ISH for OP and OC mRNAs was performed in the tissue sections decalcified with 10% EDTA/TRIS-HCl, Morse's solution, and 10% HCl (Fig. 2), and the signal intensities of these mRNAs in osteoblasts were measured by an image-analyzer (Table 2). These mandibles treated with these three solutions showed heterogeneous staining of OP (Fig. 2A,C,E) and OC (Fig. 2B,D,F) mRNAs. Osteoblasts treated with EDTA/TRIS-HCl (OP: Fig. 2A; OC: Fig. 2B) and Morse's solution (OP: Fig. 2C; OC: Fig. 2D) stained strongly, while those treated 10% HCl (OP: Fig. 2E; OC: Fig. 2F) stained weakly. Relative amounts of each mRNA per 50 osteoblasts are indicated in Table 2.

Table 2 Average pixels of osteopontin and osteocalcin mRNAs on 50 osteoblasts, in sections processed with 10% EDTA/TRIS-HCl, Morse's solution, and 10% HCl

	Relative amounts of mRNA (pixels per 50 osteoblasts)	
	Osteopontin	Osteocalcin
10% EDTA/TRIS-HCl	1592±132	1137±98
Morse's solution	1896±263	1311±187
10% HCl	574±62	312±28

On mandibles treated with each of the three decalcifying solutions, averages of the sum of arbitrary units of OC on osteoblasts were approximately two-thirds of that of OP, and there were significant differences between total pixels of these mRNAs ($P<0.05$). There were no significant differences between the retention of each OP and OC on osteoblasts treated with EDTA/TRIS-HCl and those of Morse's solution, however, 10% HCl solutions reduced the level of OP and OC to approximately one-third of that with EDTA/TRIS-HCl and Morse's solutions. λDNA was not detectable in mandibles treated with EDTA/TRIS-HCl (Fig. 2G), Morse's solution, or HCl (data not shown) even when an image analyzer was used.

Discussion

In the present study, we investigated the effects of various acid-based decalcifying solutions on the preservation of morphology and RNA, which was the prerequisite for successful demonstration of specific RNA expression in calcified tissues by ISH. We first estimated the retention of hybridizable RNA by non-radioactive ISH using the oligo-DNA probe complementary to 28 S rRNA and subsequent image analysis of the signal density in mouse mandibles. These experiments indicated that Morse's solution was an appropriate decalcifying solution. We used T-T dimer as a tag to detect RNA, since the procedures used in the present study already turned out to be equally applicable for the case with digoxigenin-labeled probe (Koji and Brenner 1993). Indeed, we detected ferritin both heavy chain and light chain mRNA in ameloblasts using digoxigenin-labeled cRNA probe in the sections decalcified with Morse's solution (unpublished data). Taken together, Morse's solution would be a favorable choice regardless of probes and labelings.

The results of 28 S rRNA staining were further confirmed by detection of specific mRNAs for OP and OC, which had been reported to be expressed in osteoblasts close to the bone surface (Weinreb et al. 1990), depending upon the differentiation stages of these cells. In the mandible treated with 10% EDTA/TRIS-HCl, the rimming osteoblasts expressed each mRNA at various levels of signal intensity, reflecting heterogeneous differentiation of osteoblasts. When Weinreb et al. (1990) per-

formed ISH for OP and OC using neonatal rat bone as a target tissue without decalcifying preparation, their quantitative analysis of signal density revealed a higher level of OP mRNA expression than that of OC mRNA. The results seem to be consistent with our results, suggesting the reliability of 28 S rRNA as a standard probe for evaluating the levels of hybridizable RNA even in decalcified tissue sections.

Walsh et al. (1993) reported that 20% EDTA as a decalcifying reagent retained good morphology and a large amount of RNA, while buffered formic acid and 6% nitric acid deteriorated morphology and reduced the amount of RNA to nearly one-half compared to EDTA by using human femoral heads as a model system. The present results confirmed the usefulness of EDTA-based solutions. In our hands, however, the amount of RNA in tissues treated with 5% formic acid was not significantly different from those with EDTA-based solutions. This discrepancy may be explained by the longer decalcification duration in their study, compared to our work. They decalcified the femoral heads for 2 days, while we decalcified the mandibles for only 24 h. In addition, the difference in GC contents of probes or the difference in cell type evaluated for morphology and RNA retention may have affected the results. We chose ameloblasts as a target cell type to assess the effects of decalcification on morphology and RNA retention. These cells have abundant cytoplasm and are arranged in a monolayer, providing a superior system to compare the morphological preservation and retention of RNA by the decalcifying solutions than the use of other cells such as osteocytes or osteoblasts which were used by Walsh et al. (1993).

Judging from morphology and quantitative analysis, Morse's solution had superior effects on retention of RNA equivalent to EDTA-based solution. Actually, the EDTA-based solutions have been used for the purpose of decalcification of tissues for ISH in many recent studies (Fujii et al. 1999; Iwasaki et al. 1998), while Morse's solution was utilized by Nomura et al. (1993) for detection of OC mRNA in odontoblasts. In our case, the latter solution completed decalcification within 24 h, while the former solution needed 7–8 days to decalcify similar samples. This merit is of great advantage in preparing surgical specimens. Very recently, decalcification by ascorbic acid useful for immunohistochemistry for calcified tissue has been reported (Merchán-Pérez et al. 1999). Since the authors discussed that the result obtained with ascorbic acid decalcification was, at least, comparable to those obtained with an EDTA-based decalcification technique (Merch·n-Pérez et al. 1999), the solution also could be considered as an alternative choice of decalcification solution for ISH.

On the other hand, mandibles decalcified with HCl-based solutions, such as Plank-Rychlo's solution and K-CX solution, which are routinely used for decalcifying biopsy and surgical specimens in many laboratories, resulted in only low levels of hybridizable RNA. In some retrospective studies, it is required to compare the signal intensities of mRNAs among sections treated with different decalcifying solutions. To minimize the influence of the decalcification-dependent variation in RNA retention, amounts of 28 S rRNA in various decalcifying solutions (Table 1) could serve as a standard to calibrate the level of such a signal. In fact, the ratio of signal intensities of specific mRNA (Table 2) correlated well with that of 28 S rRNA between 10% HCl-treated sections and Morse's solution (or 10% EDTA/TRIS-HCl)-treated sections. In other words, the result strongly indicates that the increasing or decreasing ratio of 28 S rRNA retention reflects that of specific mRNA between two arbitrary solutions. Therefore, our quantitative analysis may be useful for calibration of the intensities of specific mRNAs in sections decalcified with an inappropriate decalcifying solution, in comparison with that of Morse's solution.

In conclusion, we recommend the use of Morse's solution as a routine decalcifying solution, especially so in the field of surgical pathology, because of its minimal incubation duration and good preservation of morphology as well as good retention of RNA integrity.

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