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Evaluation of pepsin treatment for electron microscopic RNA in situ hybridization on ultra-thin cryosections of cultured cells

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Abstract The in situ hybridization (ISH) technique, as applied to electron microscopic detection of RNAs, was evaluated for ultra-thin cryosections of cultured rat fibroblasts (rat 9G). Experimental variables to balance penetration of detection reagents and preservation of ultrastructural morphology included various strengths of aldehyde fixation and pepsin treatment. We performed ISH for 28S ribosomal RNA (rRNA) followed by ultra-small colloidal gold immunocytochemistry and silver enhancement. An acceptable balance for 28S rRNA ISH detection was obtained using mild cross-linking fixation followed by treatment with a relative high concentration of pepsin for a short time. The ISH method presented in this study was compatible with immunocytochemical detection of protein as demonstrated by double-labeling experiments.

Introduction

In situ hybridization (ISH) at the electron microscopic (EM) level provides the resolution necessary to determine the spatial relation between messenger RNA (mRNA) and cognate protein, factors involved in mRNA metabolism, as well as intracellular ultrastructure. The major concerns for EM ISH are to maintain cell morphology and to retain endogenous RNA, while gaining access for detection agents, i.e. nucleic acid probes and antibodies.

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Recently, we developed a pre-embedding EM ISH method in which saponin permeabilization afforded good ISH signals in ultrastructurally well-preserved cells (Macville et al. 1995), using peroxidase/diaminobenzidine (DAB) detection. Despite the high sensitivity of the DAB detection system, colloidal gold labeling is still preferred for transmission electron microscopy (TEM) because of its better localization properties and electron density. In addition, antibodies conjugated to different sizes of gold particles allow, in principle, double immunolabeling on the same specimen. However, to gain access for immunogold labels, cells often have to be permeabilized with proteolytic enzymes or detergents, and such treatments can compromise cell morphology to an unacceptable extent (Webster et al. 1987; Wolber et al. 1989; Lloyd et al. 1990), even if $F(ab)_2$ fragments adsorbed to ultra-small colloidal gold (Sibon et al. 1994, 1995; Macville et al. 1995) or covalently bound to gold-atom clusters (Huang et al. 1994) are used. Alternatively, post-embedding EM ISH methods allow direct access for nucleic acid probes and gold-conjugated antibodies at the sectioned surface. In methodological studies for post-embedding EM ISH, several embedding media such as Lowicryl K4 M, LR White, LR Gold, and also cryosections were evaluated with respect to detection efficiency and preservation of ultrastructural morphology. The highest detection efficiency was found on ultra-thin cryosections using 5-nm gold particles, whereas the ultrastructure was best maintained in Lowicryl K4M-embedded tissue (Wenderoth and Eisenberg 1991; Dirks et al. 1992; Le Guellec et al. 1992). Compared with pre-embedding EM ISH, in our opinion the detection efficiency on cryosections was low. The aim of this study was to improve the EM ISH detection efficiency for ultra-thin cryosections, while preserving the ultrastructural morphology. For this purpose, we performed ISH experiments in which aldehyde fixation and pepsin treatment were the main variables.

The application of proteolytic enzymes in electron microscopy poses some special problems because the integrity of ultrastructural components can be affected.

Previous studies employing proteolytic enzymes on ultra-thin sections of hydrophilic and epoxy resins have shown that the extraction of cell components is dependent on the type of embedding medium used (Leduc and Bernhard 1962; Leduc and Holt 1965; Lewis and Knight 1992). Although pepsin has not been used before in post-embedding EM ISH on ultra-thin sections of frozen material, it is supposed to be most effective since the material is free of embedding medium. Therefore, to obtain high detection efficiency while preserving the ultrastructure, the strength of proteolytic treatment (time and concentration) must be balanced carefully with the strength of fixation.

We used 28S rRNA as target RNA because it is present in very high abundancy in most cell types and its localization is well defined, i.e. over the ribosomes and the nucleoli. Moreover, it is a reliable reference for hybridizable RNA (Yoshii et al. 1995). The rat 9G cell line expresses 28S rRNA and, in addition, harbors an inducible transcription unit for human cytomegalovirus immediate early antigen (IEA; Boom et al. 1986), which is synthesized in the cytoplasm and then transported to the nucleus. We could, therefore, investigate the feasibility of simultaneous detection of RNA and protein on rat 9G cells. For detection of 28S rRNA-DNA hybrids, we used ultra-small (<0.8 nm) colloidal gold-conjugated $F(ab)_2$ fragments and silver enhancement. Post-embedding immunoelectron microscopic studies have shown that labeling efficiency increases with decreasing size of gold label (Humbel and Biegelmann 1992; Dulhunty et al. 1993). As a consequence, ultra-small colloidal gold conjugates should yield the highest labeling efficiency. For consecutive antigen detection we used 10-nm protein A-gold complexes (Slot and Geuze 1985).

Materials and methods

Cells and fixation

Rat 9G cells were cultured in Dulbecco's minimal essential medium supplemented with 5% fetal calf serum at 37°C in a 5% CO_2 atmosphere. Cell monolayers were washed at 37°C with Ringer's phosphate-buffered saline (PBS) (136 mM NaCl, 4 mM KCl, 2.2 mM $CaCl_2$, 1.4 mM Na_2HPO_4/NaH_2PO_4, pH 7.4), harvested by trypsinization and fixed in 1% (w/v) formaldehyde (FA), 1% or 2% (v/v) glutaraldehyde (GA, EM grade; Fluka Chemie, Buchs, Switzerland), or a mixture of 1% FA with 0.05% or 0.5% GA for 30 min at room temperature (RT). Stock solutions of 2% formaldehyde were prepared from paraformaldehyde shortly before use and diluted 1:1 in 300 mM $NaHCO_3$, pH 8.6 (Artvinli 1975).

Probes and labeling

A pGEM plasmid containing a 2.1-kb insert specific for human 28S rRNA (Bauman and Bentvelzen 1988; Erickson et al. 1981) was labeled with digoxigenin-11-deoxyuridine triphosphate (Boehringer-Mannheim, Mannheim, Germany) by nick translation and purified by Sephadex G50 (Pharmacia Biotech, Woerden, The Netherlands) gel filtration. The probe lengths were 100–400 bp as estimated by Southern blotting.

Ultra-thin cryosectioning

Cell processing for ultra-thin cryosectioning was performed essentially according to Tokuyasu (1984) and Van Bergen en Henegouwen (1989). In order to inhibit exogenous RNAse activity, all solutions used until the pepsin treatment were supplemented with 2 mM vanadyl ribonuleoside complex (VRC, Gibco BRL/Lifetechnologies, Breda, The Netherlands). After fixation, the cell suspension was spun down in a 10% gelatin matrix and post-fixed with 1% FA/150 mM $NaHCO_3$ for 16 h at 4°C. Gelatin-embedded cell pellets were cut into 1-mm^3 blocks, impregnated with 2.3 M sucrose for 30 min, placed on a roughened copper specimen holder, and plunged into liquid nitrogen. Ultra-thin cryosections (80–100 nm) were cut on a Reichert FCS cryo-ultramicrotome at –110°C, collected on a drop of 2.3 M sucrose, and mounted on nickel grids with a carbon-coated collodion film. Grids were placed inverted on a layer of 2% gelatin/PBS (136 mM NaCl, 2.7 mM KCl, 8.4 mM Na_2HPO_4, 0.9 mM KH_2PO_4, pH 7.4) in a petri dish on ice during the sectioning procedure. The sections were equilibrated to RT shortly before use.

Pepsin treatment and hybridization

Excessive gelatin was removed and fixation-induced free aldehydes were quenched by washing three times for 2 min in PBSG [PBS supplemented with 0.15% glycine, 0.1% bovine serum albumin (BSA), and 0.1% gelatin]. Proteolytic treatment was performed by placing the specimens inverted on pre-warmed (37°C) drops of 0.001%, 0.01%, or 0.1% pepsin (P-7000, Sigma; St. Louis, Mo., USA) in 10 mM HCl, pH 2.0, for 2, 5, or 10 min (Table 1). Specimens were then rinsed for 5 min with RNAse-free, diethylpyrocarbonate-treated PBS (PBSD), and post-fixed in 1% FA/PBSD for 10 min at RT to inactivate pepsin and prevent further morphological damage. Alternatively, specimens not treated with pepsin (0 min) were hybridized directly after PBSG washes (Dirks et al. 1992). Sections were hybridized in a humidified chamber, overnight at 37°C, floating on 5-µl drops of hybridization mixture containing 5 ng/µl denatured probe, 60% formamide, 2×SSC (1×SSC=150 mM NaCl, 15 mM sodium citrate), 0.1% polyvinyl pyrrolidone, 0.1% Ficoll type 400, 0.1% BSA, 5% dextran sulfate, 100 µg/ml yeast transfer RNA, and 100 µg/ml sonicated herring sperm DNA. Sections were rinsed three times for 10 min in 60% formamide, 2×SSC at 37°C, three times for 5 min in 0.1×SSC at 60°C, and two times for 5 min in 2×SSC at RT. Control hybridizations were performed on RNAse-A-treated cells (100 µg/ml in 2×SSC for 30 min at 37°C), and with hybridization mock solution containing no probe or 28S rRNA nonspecific probe.

Immunocytochemical procedures

Sections were incubated in TNB [0.5% blocking reagent (Boehringer-Mannheim) in 100 mM Tris-HCl, 150 mM NaCl, pH 7.5] for 30 min at 37°C. Digoxigenin-labeled probes were detected with sheep anti-digoxigenin $F(ab)_2$ fragments conjugated to ultra-small colloidal gold particles (Aurion, Wageningen, The Netherlands) diluted 1:50 in TNB for 2 h at RT.

Next, sections were rinsed six times for 5 min in PBS containing 0.1% BSA-C (Aurion) and then two times for 5 min in PBS, post-fixed for 10 min in 2% GA/PBS, and rinsed once for 5 min in PBS and six times for 5 min in double-distilled water. Silver enhancement was performed with EM-grade silver enhancement chemicals (Aurion) for 5 min according to the manufacturer's instructions. Double-labeling immunocytochemistry was performed

on rat 9G cells that were induced for IEA expression by adding 50 µg/ml cycloheximide for 5 h to exponentially growing cells (Boom et al. 1986). Cells were cultured for 16 h after release of cycloheximide and processed for ISH as described above. After completion of 28S rRNA ISH detection, the sections were rinsed in PBSG for 5 min and then incubated at RT for 2 h with mouse monoclonal anti-IEA (E13; Biosoft, Paris, France) diluted 1:1000 in PBSG, followed by two consecutive incubations at RT for 1 h each with unconjugated rabbit anti-mouse (Cappel-Organon Teknika, Boxtel, The Netherlands) diluted 1:500 in PBSG and 10 nm protein A-gold complex (Slot and Geuze 1985) diluted 1:400 in PBS/1% BSA. Between incubations, the sections were rinsed three times for 5 min with PBSG. Ultra-thin cryosections were post-stained with a mixture containing 3% uranyl acetate diluted 1:9 in 2% methylcellulose embedding medium. Sections were examined in a Philips EM 410LS transmission electron microscope operated at 80 kV.

Results

Influence of fixation on ISH signals and morphology

Hybridization experiments with 28S rRNA specific probes on ultra-thin cryosections of cultured rat 9G cells that were not pre-treated with pepsin revealed the strongest labeling when cells were fixed with 1% FA (Fig. 1). The ultrastructural morphology, however, was inferior to that of cells fixed in GA containing fixatives. In 1% FA-fixed cells, the cytoplasm and nucleus were less electron-dense and the membrane structures of mitochondria and endoplasmic reticulum were less distinct than those of 1% FA/0.05% GA-fixed cells. With increasing concentrations of GA, the labeling density decreased proportionally (compare Figs.1, 2a, 3a). The use of strong cross-linking fixatives such as 1% and 2% GA resulted in the complete absence of hybridization signals (Table 1).

Influence of pepsin treatment on ISH signals and morphology

In general, treatment of ultra-thin cryosections with pepsin increased the labeling density but decreased the ultrastructural morphology. Chessboard experiments in which degree of fixation and pepsin treatment were varied demonstrated that this effect is fixative dependent (Table 1). Cells fixed with 1% FA or 1% FA/0.05% GA showed damaged cell morphology after brief (2 min) treatment with pepsin at low (0.001%) or intermediate (0.01%) concentration, whereas in cells fixed with a stronger cross-linking fixative, such as 1% FA/0.5% GA, 1% GA, or 2% GA, such damage was only visible when pepsin was used for longer than 5 min at a high (0.1%) concentration (Table 1). We empirically determined dense labeling for 28S rRNA over the cytoplasm and nucleolus and good preservation of the nucleus and cytoplasmic membrane structures in 1% FA/0.5% GA-fixed cells that were treated with 0.1% pepsin for 2 min (Fig. 2B).

The effect of pepsin on ISH signal density and preservation of morphology appeared dependent on the section thickness. Thinner sections were more severely affected than thicker sections. Therefore, the results shown in this study are obtained on sections of equal thickness (80–100 nm).

Attempts to improve the reproducibility by performing ISH on cells fixed with stronger cross-linking strength (1% or 2% GA) were apparently unsuccessful. In addition, treatment with 0.1% saponin for 10–30 min, previously successful in pre-embedding EM ISH (Macville et al. 1995), appeared ineffective (data not shown).

Other RNA targets, such as immediate early (IE) messenger RNA (mRNA) in rat 9G cells or human elonga-

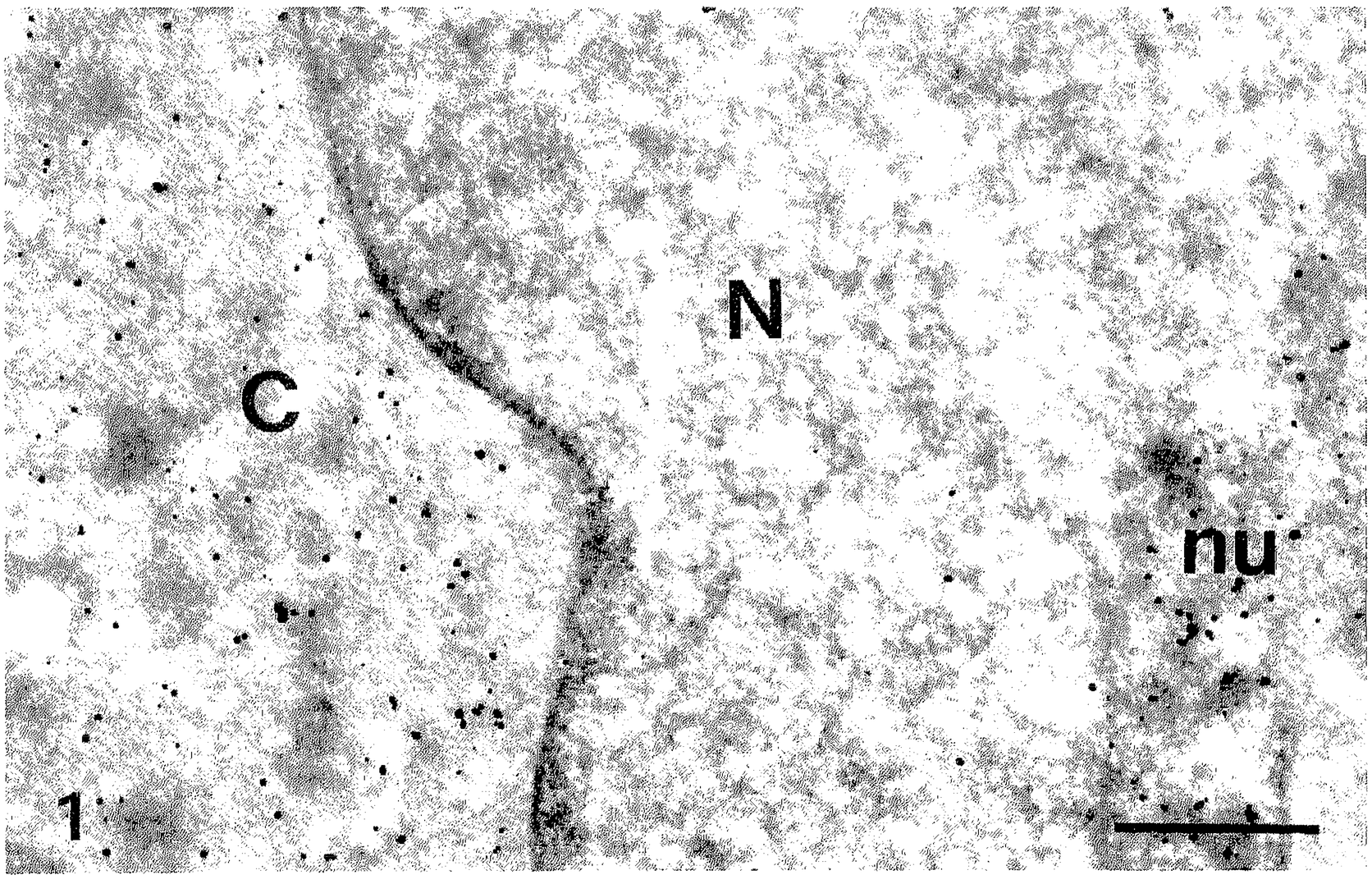

Fig. 1 TEM image of 28S rRNA ISH signals on ultra-thin cryosections of 1% FA-fixed rat 9G cells. Hybrid detection was performed by ultra-small colloidal gold $F(ab)_2$ fragments and silver enhancement. Gold/silver grains are present over the cytoplasm (*C*) and the nucleolus (*nu*). Very little label is present in the nucleoplasm (*N*) and mitochondria (*m*) are devoid of label. *Bar*: 1 µm

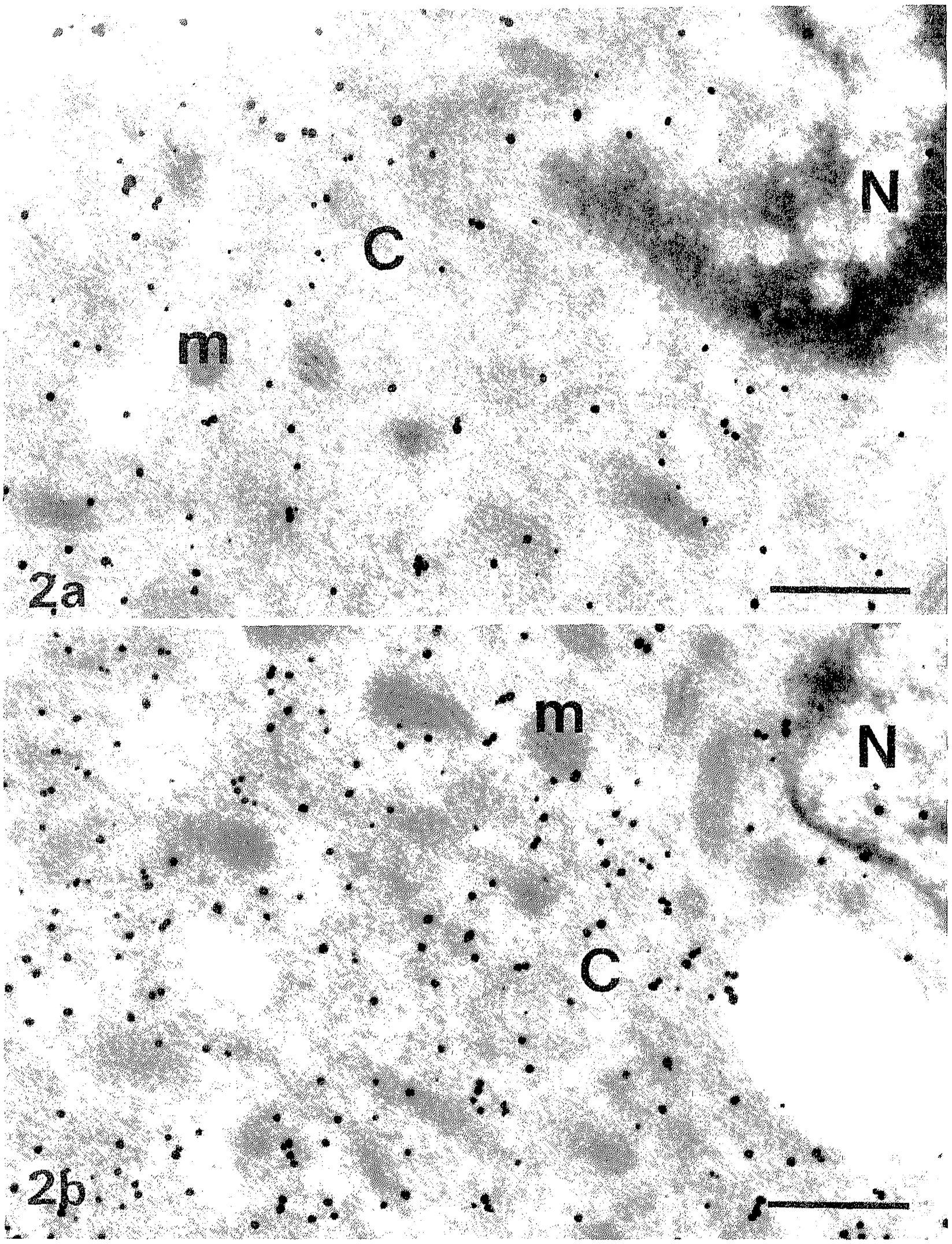

Fig. 2a, b TEM images of 28S rRNA ISH signals on ultra-thin cryosections of 1% FA/0.5% GA-fixed rat 9G cells that were (**a**) not treated with pepsin and (**b**) treated with 0.1% pepsin for 2 min. In the latter, the density of gold/silver grains has increased considerably, while the ultrastructural morphology has remained well preserved. *N* Nucleus, *C* cytoplasm, *m* mitochondria. *Bars*: 1 μm

tion factor (HEF) mRNA in HeLa cells, could not be detected with the ISH method as described in this article, even if pepsin was used (data not shown).

Simultaneous detection of 28S rRNA and IEA

Detection of IEA, performed after hybridization with 28S rRNA on the same specimen, was feasible, as illustrated in Fig. 3A. The 28S rRNA labeling over rat 9G cells did not differ visibly from that found with single detection. IEA was predominantly detected over the euchromatin in the nucleus, and nucleoli were devoid of label. Approximately 30% of the cells were labeled, and varied in labeling density because of the intrinsic variation in the IEA expression level (Boom et al. 1986). Some cells demonstrated 10-nm gold labeling in the cytoplasm, revealing newly synthesized protein. IEA detection was compatible with all fixatives tested. The highest labeling density, nevertheless, was observed in 1% FA-fixed cells (not shown).

Remarkably, pepsin pre-treatment enhanced the overall IEA labeling density. For example, in 1% FA/0.05%-fixed and 10 min 0.01% pepsin-treated cells the IEA labeling was more dense than in untreated cells (Fig. 3B). In this case, pepsin treatment apparently improved the accessibility to IEA but did not affect the antigenicity, al-

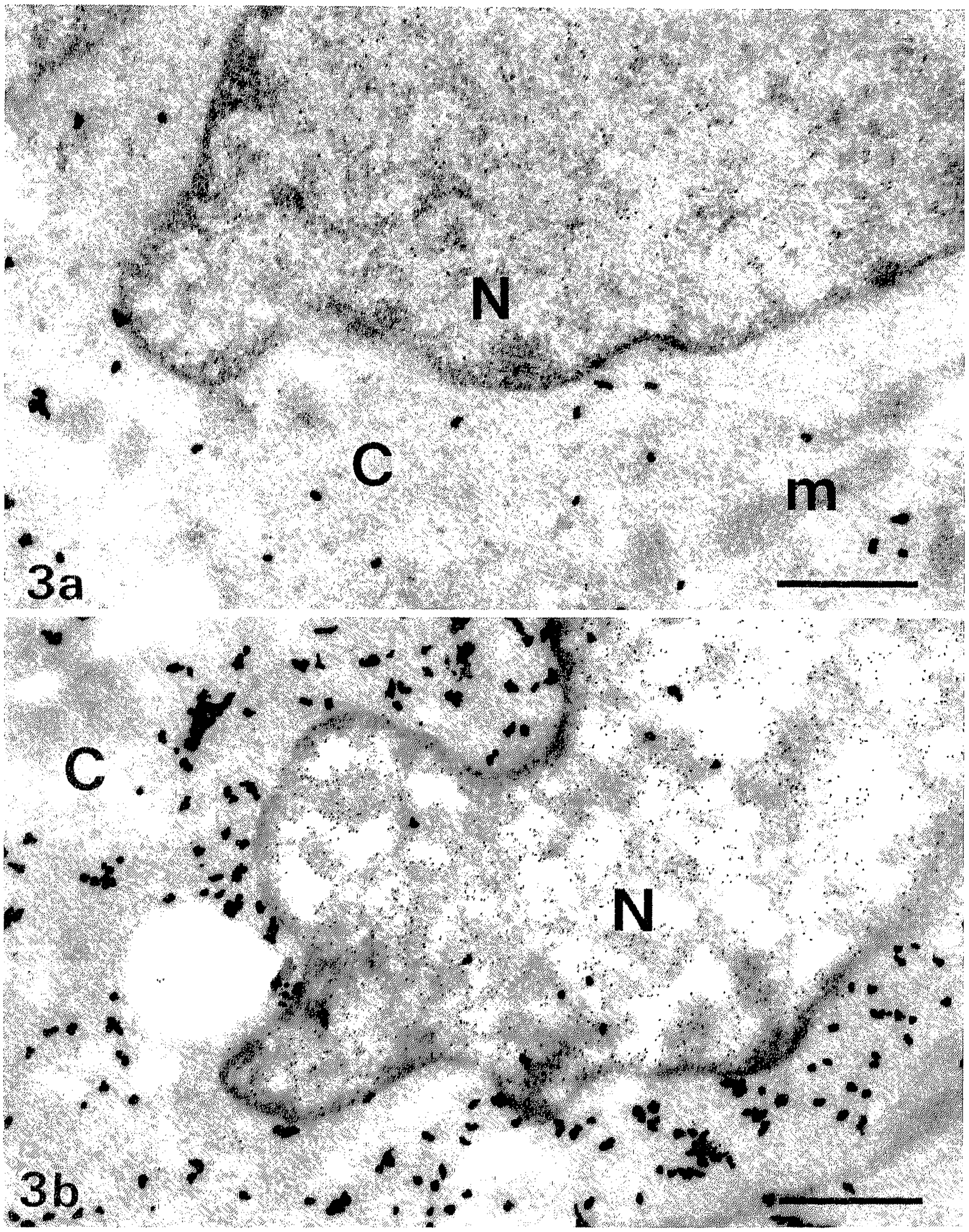

Fig. 3a, b TEM images of simultaneous 28S rRNA and IEA detection on ultra-thin cryosections of 1% FA/0.05% GA-fixed rat 9G cells. The large grains (30–50 nm) in the cytoplasm are silver-enhanced ultra-small gold particles representing 28S rRNA, and the small grains in the nucleoplasm are 10-nm gold particles representing IEA. (**a**) Cells that were not treated with pepsin show better preservation of ultrastructural morphology but weaker labeling than (**b**) cells treated with 0.01% pepsin for 10 min. *N* Nucleus, *C* cytoplasm, *m* mitochondria. *Bars*: 1 μm

though the ultrastructural morphology was already severely damaged. Conditions affording 28S rRNA ISH detection and preservation of morphology were compatible with simultaneous IEA detection.

Specificity of hybridization and immunocytochemistry

ISH signals for 28S rRNA were located over the cytoplasm and in the nucleolus, mainly localized at the granular component (Fig. 1). No labeling was observed over the Golgi apparatus, mitochondria, or vacuoles, but some labeling was found over the nucleoplasm. Cells were completely devoid of label when incubated in a mock solution containing no probe or 28S rRNA nonspecific probe, or when the antibody incubation was omitted. The ISH signals were obtained without in situ denaturation of DNA and were RNAse-A sensitive, proving that ISH signals were specific for RNA.

IEA expression is cell cycle dependent and, therefore, approximately 30% of a rat 9G cell population is IEA positive upon cycloheximide induction (Boom et al. 1986), thus providing an internal specificity control.

Discussion

In developmental studies for EM ISH the main challenge is to find a good balance between RNA detection effi-

Table 1 Influence of fixation and pepsin treatment on ultrastructural morphology (*morpho*) and 28S rRNA ISH signal (*signal*) on ultrathin cryosections of cultured rat 9G cells. *FA* Formaldehyde, *GA* glutaraldehyde; morphology: ++ well-preserved cytoplasmic and nuclear ultrastructure, + fairly well-preserved cytoplasmic and nuclear ultrastructure, ± cell damage, organelles distinguished, – cell damage, organelles not distinguished, – – severe cell damage, – – – very severe cell damage, *n.d.* not determined; 28S rRNA ISH signal: ++ cytoplasm and nucleolus very densely labeled, + cytoplasm and nucleolus densely labeled, ± cytoplasm and nucleolus weakly labeled, – cytoplasm weakly labeled, nucleolus not labeled, – – cytoplasm and nucleolus not labeled, *n.d.* not determined

Fixation		Pepsin pretreatment									
		0 min	2 min 0.001%	5 min 0.001%	10 min 0.001%	2 min 0.01%	5 min 0.01%	10 min 0.01%	2 min 0.1%	5 min 0.1%	10 min 0.1%
1% FA	morpho	+	±	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	signal	+	+	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
1% FA/0.05% GA	morpho	++	++	+	–	±	–	– –	n.d.	– –	n.d.
	signal	±	±	+	+	+	+	+	n.d.	++	n.d.
1% FA/0.5% GA	morpho	++	++	+	+	+	±	±	+	±	– –
	signal	–	–	±	±	±	±	+	++	++	++
1% GA	morpho	++	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	+	±	–
	signal	– –	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	–	+	+
2% GA	morpho	++	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	+	±	–
	signal	– –	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	–	±	+

ciency and preservation of ultrastructural morphology. In comparative post-embedding EM ISH studies, in which various embedding media and non-embedded frozen materials were tested, the most efficient ISH detection was found for ultra-thin cryosectioned tissue using 5-nm colloidal gold (Wenderoth and Eisenberg 1991; Dirks et al. 1992; Le Guellec et al. 1992). Consequently, the use of ultra-small colloidal gold particles with silver enhancement on ultra-thin cryosections should yield an even higher detection efficiency.

In ultra-thin sections of fixed cells, RNA molecules are present not only at the sectioned surface but also inside the section. Since there is no embedding medium present in cryosections, probes and antibodies may diffuse throughout the section and detect the internal RNA sequences. The accessibility of internal RNA molecules is, however, hindered by RNA-associated proteins that are cross-linked by fixation. Thus, by breaking the peptide bonds, e.g. by pepsin treatment, the accessibility can be increased. Indeed, decrease of cross-linking strength and increase of pepsin treatment resulted in higher RNA detection efficiency. In chessboard experiments, we empirically found good detection efficiency and preservation of morphology in cells fixed with 1% FA/0.5% GA, and then briefly (2 min) treated with a high concentration (0.1%) of pepsin. The balance, however, is fragile because these results were consistently reproduced only on sections of equal thickness.

In comparison with earlier post-embedding EM ISH studies for 28S rRNA, equally strong signal intensities were achieved (Thiry and Thiry-Blaise 1989; Puvion-Dutilleul et al. 1991; Wachtler et al. 1992), albeit at the cost of ultrastructural preservation. Post-embedding EM ISH on ultra-thin cryosections of cultured cells was less sensitive than pre-embedding EM ISH. RNA targets successfully detected by pre-embedding EM ISH on whole-mount cells, such as IE mRNA and HEF mRNA (Macville et al. 1995), were not detected. Nevertheless, for some applications post-embedding EM ISH will remain the method of choice, e.g. when the material has to be stored because immediate processing is not possible or when double labeling is desired. For elucidation of many cellular processes, simultaneous detection of protein and RNA at the ultrastructural level could provide useful information. Post-embedding EM ISH on ultra-thin sections of frozen tissue has proven compatible with simultaneous antigen detection (Dirks et al. 1992). We showed, by using two different sizes of immunogold labels, the simultaneous detection of 28S rRNA and IEA protein on ultra-thin cryosection of cultured cells. The majority of silver-enhanced ultra-small gold particles were easily distinguished from 10-nm gold by their larger size. However, interpretation problems may occur when targets are localized to the same cell compartment and ultra-small gold particles are enlarged to the size of the second label (Egger et al. 1994).

Higher sensitivity may be achieved (a) by taking stronger measures against RNAse activity or (b) by using more sensitive immunogold detection systems. The cells are susceptible to RNAse in many steps of the protocol. For instance, immediately after pepsin treatment RNA targets are exposed not only to probe hybridization but also to RNAse digestion. RNAse inhibition by VRC was sufficient for retention of 28S rRNA but not for mRNA species which are present in lower abundancies. Full access to internal RNA targets without pre-treatment might be readily gained by gold-atom clusters covalently bound to $F(ab)_2$ fragments (Takizawa and Robinson 1994). In addition, improvement of silver enhancement efficiency may contribute to enhance the labeling intensity (Stierhof et al. 1991; Gilerovitch et al. 1995). Alternatively, Sibon et al. (1994) used ultra-small colloidal gold and silver enhancement alternately for double labeling of mRNA and protein in pre-embedding EM ISH on nuclear matrices. Application of these three methods to ultrastructurally intact cells would combine the present advantages of both

post-embedding (double labeling) and pre-embedding (high detection efficiency) EM ISH techniques.

In conclusion, post-embedding EM ISH on ultra-thin cryosections of cultured cells while preserving morphology is feasible for high-abundance RNA. In addition, the ISH protocol described in this article is compatible with simultaneous protein detection. One should realize, however, that the detection efficiency is suboptimal when the cells are ultrastructurally intact. For improvement of detection efficiency, pepsin treatment was very effective but appeared too harsh for stably balancing labeling intensity and ultrastructural morphology. Further refinement of the protocol to detect mRNA species is currently under investigation.

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