

## Invited Review

# Electron microscopic observation of intracellular expression of mRNA and its protein product: Technical review on ultrastructural *in situ* hybridization and its combination with immunohistochemistry

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**Summary.** *In situ* hybridization (ISH) at the electron microscopic (EM) level is essential for elucidating the intracellular distribution and role of mRNA in protein synthesis. Three different approaches have been applied by the investigators in this EM-ISH study: pre-embedding method; non-embedding method using ultrathin frozen sections; and postembedding method. In order to obtain satisfactory morphological preservation and retain the messages, we routinely utilized 6  $\mu\text{m}$ -thick frozen sections fixed in 4% paraformaldehyde for the preembedding method and tissues embedded in LR White resin for the postembedding method. The hybridization signal intensity by the postembedding method was lower, and non-specific signals were relatively frequent, in comparison with the pre-embedding method. The preembedding method thus appears to be easier and better than the postembedding method from the viewpoint of applicability and preservation of mRNA, although quantitative analysis of the expression of mRNA is rather difficult in the preembedding method. EM-ISH is considered to be an important tool for clarifying the intracellular localization of mRNA and the exact site of specific hormone synthesis on the rough endoplasmic reticulum. The simultaneous visualization of mRNA and encoded protein in the same cells using preembedding EM-ISH and subsequent postembedding immunoreaction with protein A colloidal gold complex is also described. This ultrastructural double-staining method for mRNA and encoded protein can be expected to provide an important

clue for elucidating the intracellular correlation of mRNA translation and secretion of translated protein.

**Key words:** *in situ* hybridization, mRNA, Immunohistochemistry, Electron microscopy, Pituitary cell

## Introduction

In 1969, the *in situ* hybridization (ISH) technique was introduced for the detection of ribosomal RNA genes (Gall and Pardue, 1969; John et al., 1969). Since then, many investigators have improved this technique to identify specific genes or gene products in cells. Radioactive probes, which were employed in earlier experiments, were sensitive enough for the detection of specific genes or transcripts. However, histological resolution of ISH studies using radioactive probes was unsatisfactory. The development of synthesized oligonucleotide probes contributed greatly to the refinement of ISH (Lewis et al., 1985). Synthesized oligonucleotide probes have merits in the readiness of availability and the arbitrariness of design. Moreover, non-radioactive synthesized oligonucleotide probes labeled with biotin or digoxigenin were introduced for the detection of ISH signals (Guitteny et al., 1988; Hankin and Lloyd, 1989; Larsson, 1989; Farquharson et al., 1990; Pringle et al., 1990; Schmitz et al., 1991; Shorrock et al., 1991). Thereafter, ISH at the light microscopic (LM) level (LM-ISH) has become a widely used method for examining the tissue distribution and expression of mRNA. This LM-ISH is lacking in the resolution required for the studies on the spatial relationship between mRNA and the protein product.

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This type of information can be provided by ultrastructural observation.

Immunoelectron microscopy has been developed for the observation of protein product and is now a sophisticated technique in the field of histopathology. ISH at the electron microscopic (EM) level (EM-ISH) is a recently developed technique and is essential for the intracellular identification of mRNA and the study of the role of mRNA in protein synthesis. Ultrastructural ISH for RNA was first described by Jacob et al. (1971). This method has further been developed by several investigators (Webster et al., 1987; Guitteny and Bloch, 1989; Morel et al., 1989a,b; Singer et al., 1989; Wolber et al., 1989; Jirikowski et al., 1990; Le Guellec et al., 1990, 1991, 1992; Trembleau et al., 1990; Pomeroy et al., 1991; Matsuno et al., 1994a,b, 1995). Three different approaches have been applied by the investigators to this EM-ISH study: preembedding method (Guitteny and Bloch, 1989; Wolber et al., 1989; Trembleau et al., 1990; Pomeroy et al., 1991; Le Guellec et al., 1992; Matsuno et al., 1994a,b, 1995), non-embedding method using ultrathin frozen sections (Morel et al., 1989a,b); and postembedding method (Jirikowski et al., 1990; Le Guellec et al., 1990, 1991; Matsuno et al., 1994a, 1995). Recently, we developed a non-radioisotopic EM-ISH method using biotinylated synthesized oligonucleotide probes, and applied this method to the ultrastructural visualization of growth hormone (GH) and prolactin (PRL) mRNAs and the pathophysiological studies in rat pituitary cells (Matsuno et al., 1994a,b, 1995). In addition, we developed a combined EM-ISH and immunohistochemistry method for the purpose of the simultaneous identification of pituitary hormone and its message in the same cell (Matsuno et al., 1996, 1998a,b). In this paper, the previously reported EM-ISH techniques including our non-radioisotopic EM-ISH method are reviewed, and, moreover, our combined immunohistochemistry and non-radioisotopic pre-embedding ISH method is described together with an overview on the reported method in the literature.

#### Electron microscopic *in situ* hybridization (EM-ISH)

As stated above, three different approaches have been applied by the investigators to this EM-ISH study: preembedding method, non-embedding method using ultrathin frozen sections; and postembedding method. The major concerns of EM-ISH are to maintain tissue morphology and to retain the messages. As Le Guellec et al. (1992) stated, the ultrastructural preservation in EM-ISH using ultrathin frozen sections is poor, and specimens embedded in Lowicryl K4M exhibit poorer ultrastructural preservation than those embedded in Epon resin. In order to obtain satisfactory morphological preservation, we routinely utilized 6  $\mu\text{m}$ -thick frozen sections fixed in 4% paraformaldehyde for the preembedding method and tissues embedded in LR White resin for the postembedding method.

#### 1) Preembedding EM-ISH method

The following is the protocol we employed for the preembedding EM-ISH studies.

Tissues were fixed at 4 °C overnight in 4% paraformaldehyde dissolved in 0.01 M phosphate-buffered saline, pH 7.4 (PBS). After immersion in graded concentrations of sucrose dissolved in PBS at 4 °C (10% for 1 hr, 15% for 2 hr, 20% for 4 hr), tissues were embedded in Optimal Cutting Temperature (OCT) compound (Tissue-Tek; Miles Laboratories Inc., Elkhart, IN, USA). 6  $\mu\text{m}$ -thick tissue specimens were mounted on 3-aminopropylmethoxysilane-coated slides. After being dried in air for 1 hr, tissue sections were washed in PBS for 15 min. Subsequently, they were treated with 0.1  $\mu\text{g}/\text{ml}$  proteinase K at 37 °C for 30 min, followed by treatment for 10 min with 0.25% acetic anhydride in 0.1M triethanolamine. The slides were washed in 2x sodium chloride sodium citrate (SSC) at room temperature for 3 min and then prehybridized at 37 °C for 30 min. The prehybridization solution consisted of 10% dextran sulfate, 3xSSC, 1x Denhardt's solution (0.02% Ficoll/0.02% bovine serum albumin (BSA)/0.02% polyvinylpyrrolidone), 100  $\mu\text{g}/\text{ml}$  salmon sperm DNA, 125 mg/ml yeast tRNA, 10  $\mu\text{g}/\text{ml}$  polyadenylic-cytidylic acid, 1 mg/ml sodium pyrophosphate pH 7.4 and 50% formamide. The biotinylated probe with the concentration of 0.1 ng/ $\mu\text{l}$  was diluted with this solution and hybridization was carried out at 37 °C overnight. After hybridization, the slides were washed at room temperature with 2xSSC, 1xSSC and then 0.5xSSC for 15 min, respectively. The hybridization signals were detected with streptavidin-biotin-horseradish peroxidase (ABC-HRP), using Vectastain's ABC kit (Vector Laboratories Inc., Burlingame, CA, USA). Tissue sections were dipped in diaminobenzidine (DAB) solution (incomplete Graham-Karnovsky's solution) for 30 min, and then hybridization signals were developed with DAB and H<sub>2</sub>O<sub>2</sub> (complete Graham-Karnovsky's solution). At this stage, hybridization signals were confirmed light microscopically. After osmification with 2% osmium tetroxide for 1 hr and dehydration with a graded concentration of ethanol, tissue sections were embedded in Epon resin, which was polymerized at 60 °C for 2 days. Ultrathin sections were inspected under electron microscopy. The negative control experiments should include hybridization with probes of sense or scramble sequence and without probes, and pretreatment with ribonuclease A (100 mg/ml) at 37 °C for 45 min before hybridization.

This preembedding EM-ISH study with an antisense biotinylated oligonucleotide probe localized rat GH mRNA diffusely on the polysomes of the entire rough endoplasmic reticulum (RER) (Fig. 1). The pre-embedding EM-ISH study with a sense probe for rat GH mRNA showed negative signals (Fig. 2). The sequence of antisense oligonucleotide probe for rat GH mRNA was described in our previous reports (Matsuno et al., 1994a,b, 1995, 1996, 1998a,b).

In the preembedding method, both ultrastructure and mRNA were sufficiently preserved. The preembedding method had a benefit in that hybridization signals could be confirmed light microscopically. The only demerit of this preembedding method was the failure of quantification of hybridization signals that were detected enzymatically.

## 2) Postembedding EM-ISH method

The following is the protocol we employed for the postembedding EM-ISH studies.

Tissues were embedded in LR White resin (Polyscience, Warrington, PA, USA) after fixation at 4 °C overnight in 4% paraformaldehyde dissolved in PBS. They were carefully placed at the bottom of 00 gelatin capsules (Lilly Pharmaceuticals, Indianapolis, IN, USA), which were filled with LR White resin and sealed. After polymerization at 50 °C for 24 hr in a vacuum oven, ultrathin sections were retrieved on Nickel grids. Hybridization was carried out on the grids at 37 °C overnight after prehybridization at the same temperature for 30 min. The hybridization solution was the same as that used for the preembedding method. The concentration of the antisense probe was 1 ng/ml. After hybridization, the grids were dipped in 2xSSC, 1xSSC and then 0.5xSSC for 5 min, respectively. The hybridization signals were developed for 30 min with 20 nm streptavidin gold (British Biocell International, Cardiff, UK) diluted 1:50 in 1% BSA-PBS. After having been dipped in PBS and distilled water (DW) and dried at room temperature, the grids were inspected under electron microscopy. The control experiments carried out were hybridization with probes of sense or scramble sequence and without probes.

This postembedding EM-ISH study localized hybridization signals for rat GH mRNA on the poly-

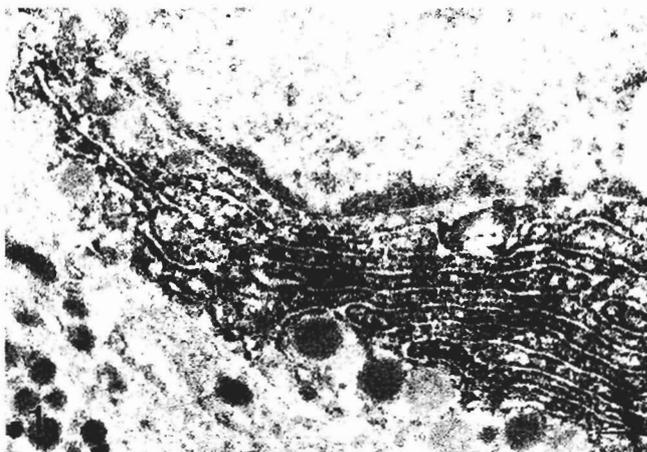
somes of the RER using 20 nm streptavidin gold (Fig. 3). EM-ISH study with a sense probe for rat GH mRNA showed no hybridization signals (Fig. 4). The hybridization signal intensity was lower than that for the preembedding method.

In the postembedding method using tissues embedded in LR White resin, ultrastructure was also sufficiently preserved. Compared with the preembedding method, the postembedding method has several difficulties: 1) difficulty of message preservation during polymerization of LR White resin at relatively high temperature for an extended period of time, which leads to mRNA degradation; and 2) possible non-specific signals due to the non-specific affinity of gold particles. Quantification of hybridization signals can be obtained through counting the number of gold particles. Nevertheless, we should note that the signals may be decreased in the postembedding method.

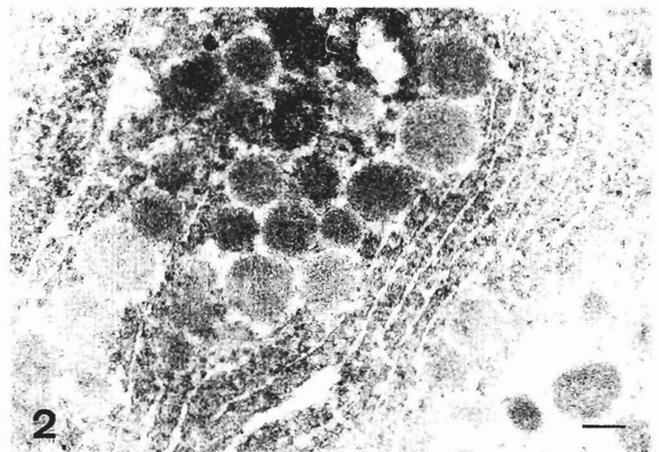
## 3) Non-embedding EM-ISH method using ultrathin frozen sections

Morel et al. (1989a) applied this non-embedding method for the detection of atrial natriuretic peptide synthesis in pituitary gonadotroph cells. They utilized ultrathin frozen sections of rat pituitary glands, which were cut at -120 °C and mounted on collodion-coated Nickel grids. Ultrathin frozen sections were incubated for 3 hr at 40 °C with 2.5  $\mu$ mol antisense biotinylated oligonucleotide probe. Grids were then washed twice in 2xSSC at room temperature. Hybridization signals were detected with rabbit anti-biotin serum and anti-rabbit immunoglobulin G-colloidal gold.

In general, as Le Guellec et al. (1992) stated, non-embedding EM-ISH method using ultrathin frozen sections may be highly sensitive, but that this method has a demerit in the poor morphological preservation.



**Fig. 1.** Photograph of preembedding EM-ISH for rat GH mRNA. Rat GH mRNA is localized diffusely on the polysomes of the entire rough endoplasmic reticula (RER). Cited from Matsuno et al., 1998b. Bar: 200nm.



**Fig. 2.** Control study with a sense probe for rat GH mRNA. Rat GH mRNA is not localized on the RER. Cited from Matsuno et al., 1998b. Bar: 200nm.

4) *Pathophysiological studies on changes in ultrastructural expression of rat PRL mRNA induced by estrogen and bromocriptine: preembedding method*

Female Wistar-Imamichi rats treated intramuscularly with 5 mg estradiol dipropionate (E2 depot: Ovahormon Depot; Teikoku Zoki Co. Ltd., Tokyo, Japan) every 4 weeks were sacrificed 3, 5 and 7 weeks after injection with or without a subcutaneous injection of 1 mg bromocriptine (Sandoz Pharmaceutical Co. Ltd., Basel, Switzerland) for 4 days. The anterior lobes removed from their pituitary glands were immediately fixed at 4 °C overnight in 4% paraformaldehyde dissolved in PBS, which served for ultrastructural ISH studies. The sequence of antisense oligonucleotide probe for rat PRL mRNA has been described in our previous reports (Matsuno et al., 1994b, 1995, 1998b).

Preembedding EM-ISH studies revealed the whirling changes of the RER in the specimens of female rats given estrogen for 7 weeks and also frequent but focally localized hybridization signals of rat PRL mRNA on the polysomes of the whirling RER (Fig. 5). After bromocriptine administration, rat PRL mRNA expression at the light microscopic level decreased markedly, and electron microscopic examination revealed diffuse localization of rat PRL mRNA hybridization signals on the distorted, vesiculated, and partly dilated RER (Fig. 6). There was also an increased number of secretory granules, which resulted in the increased PRL immunoreactivity induced by bromocriptine. For the quantitative analyses of PRL mRNA expression, other experiments including Northern blot hybridization and LM-ISH studies are required. Using the <sup>32</sup>P-labeled oligonucleotide probe for rat PRL mRNA, a 1.0-kb transcript was detected on the nitrocellulose membrane, and the PRL mRNA and  $\beta$ -actin mRNA hybridization signal density ratios for the pituitary glands of untreated, control female rats, those

treated with estrogen alone for 3 and 7 weeks, and those with estrogen plus bromocriptine treatment, were evaluated densitometrically. As shown in our previous report, the hybridization signal density of PRL mRNA was enhanced as the duration of estrogen treatment increased, and decreased markedly after bromocriptine administration (Matsuno et al., 1995). Our previous LM-ISH studies revealed that the hybridization signals of PRL mRNA from estrogen-treated rats were more intense than those of that from normal rats without estrogen administration, and the hybridization signal frequencies increased as the duration of estrogen treatment increased. After bromocriptine treatment, PRL mRNA expression decreased considerably (Matsuno et al., 1995).

In this study we found the difference in localization of GH and PRL synthesis on the polysomes of the RER. Hybridization signals of GH mRNA were distributed diffusely on the entire RER, whereas those of PRL mRNA were scattered and distributed focally on the RER even after having been stimulated by estrogen. After bromocriptine treatment, hybridization signals of PRL mRNA were shown to be localized diffusely on the RER. These differences and alteration of mRNA distribution on the RER may be difficult to explain. However, these phenomena are considered to be evoked possibly by the dynamics of signal recognition particle and its receptors on the RER. EM-ISH is an important technique for clarifying the intracellular localization of mRNA and the exact site of specific hormone synthesis on the surface of the RER. As shown in the studies on changes in ultrastructural expression of PRL mRNA induced by estrogen and bromocriptine, EM-ISH can serve the morphological and pathophysiological investigation on mRNA expression induced by some stimulatory factors.

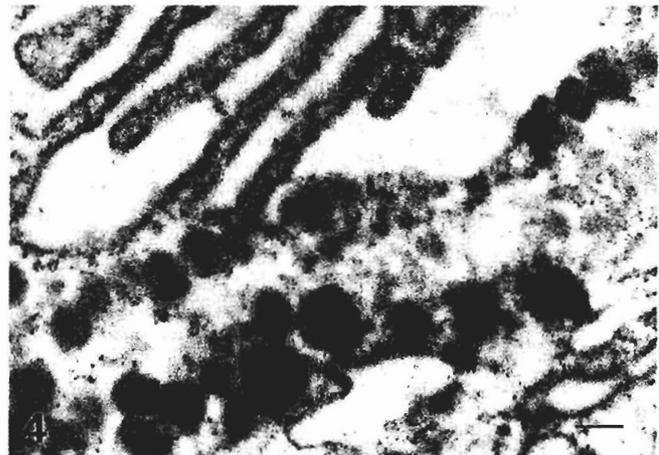


Fig. 3. Hybridization signals for rat GH mRNA are localized on the polysomes of the RER using 20 nm streptavidin gold. The hybridization signal intensity is lower than that for the preembedding method. Cited from Matsuno et al., 1998b. Bar: 200nm.

Fig. 4. EM-ISH with a sense probe for rat GH mRNA shows no hybridization signals. Cited from Matsuno et al., 1998b. Bar: 200nm.

### Combined immunohistochemistry and pre-embedding ISH at an electron microscopic level

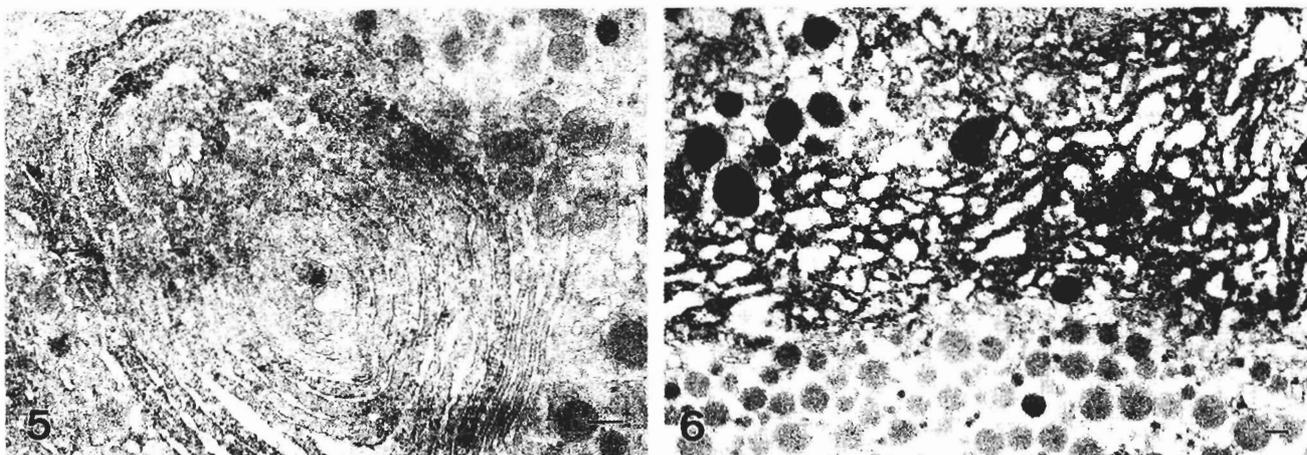
As for mRNA preservation, the preembedding EM-ISH method using frozen sections fixed in 4% paraformaldehyde has more advantages over the postembedding EM-ISH method using tissues embedded in LR White resin. Frozen sections fixed in 4% paraformaldehyde have better morphological preservation than immediately frozen sections. Based on this assessment, apropos of maintaining tissue morphology and retaining the messages, we utilized the pre-embedding EM-ISH method using 6  $\mu\text{m}$ -thick frozen sections fixed in 4% paraformaldehyde for the simultaneous detection of mRNA and encoded protein. The following is the protocol of the combined immunohistochemistry and preembedding EM-ISH we employed.

The hybridization procedure on 6  $\mu\text{m}$ -thick tissue specimens mounted on 3-aminopropylmethoxysilane-coated slides was the same as described as above. The hybridization signals were detected with ABC-HRP. Tissue sections were dipped in DAB solution (incomplete Graham-Karnovsky's solution) for 30 min, and then hybridization signals were developed with DAB and  $\text{H}_2\text{O}_2$  (complete Graham-Karnovsky's solution). After osmification with 2% osmium tetroxide for 1 hr and dehydration with a graded concentration of ethanol, tissue sections were embedded in Epon resin, which was polymerized at 60  $^\circ\text{C}$  for 2 days. After polymerization, ultrathin sections were attached to Nickel grids. Subsequently, in order to retrieve the immunoreactivity of the targeted protein, the ultrathin sections embedded in Epon resin were etched with 10%  $\text{H}_2\text{O}_2$  for 30 min or with 4% sodium periodate for 10

min, followed by washing with DW. Immunohistochemical staining with primary antibody was carried out at room temperature for 1 hr. The grids were washed with 0.1M phosphate buffer (PB), and the immuno-reaction was visualized at room temperature for 1 hr with 20 nm protein A colloidal gold (E.Y. Laboratories Inc., San Mateo, CA, USA) diluted 1:40 in 0.1M PB. After washing with 0.1M PB and DW, the grids were dried at room temperature, and observed under electron microscopy. The immunohistochemical control experiments included substitution of normal serum for primary antibody.

As shown in the above section, EM-ISH with an antisense probe for rat GH mRNA revealed its diffuse localization on the polysomes of the entire RER. Subsequent immunohistochemical staining using anti-rat GH antibody (monkey, diluted 1:100 with PBS), supplied by the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) (Bethesda, MD, USA), and 20 nm protein A colloidal gold identified rat GH on the secretory granules (Fig. 7). Colloidal gold signals for GH were distributed mainly on the secretory granules, and were also identified in the cisternae of the RER (Fig. 7). The immunoreactivity retrieved after etching process with 10%  $\text{H}_2\text{O}_2$  was similar and comparable to that with 4% sodium periodate. Immunohistochemical control experiments substituting normal monkey serum for anti-GH antibody combined with preembedding EM-ISH method revealed no reactions of protein A colloidal gold particles on the secretory granules (Fig. 8).

The only flaw of this combined method is the deosmification and degradation of the signals of mRNA caused by etching process using  $\text{H}_2\text{O}_2$  or sodium periodate. In order to solve this problem, we have



**Fig. 5.** Photograph of pituitary cells from female rats given estrogen for 7 weeks subjected to preembedding electron microscopic ISH. Rat PRL mRNA is frequently localized on the polysomes of the whirling RER. Bar: 200nm.

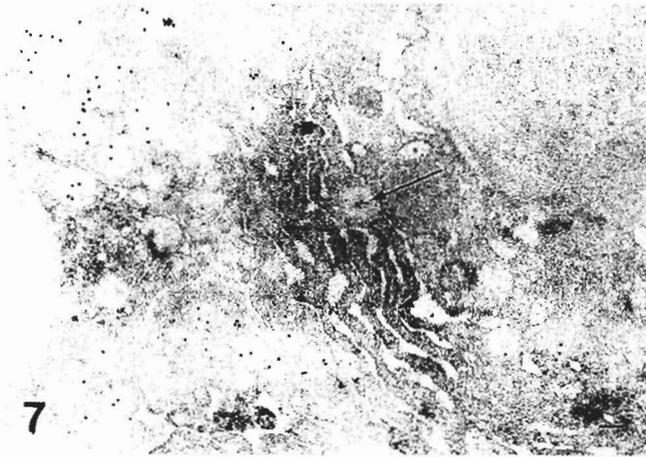
**Fig. 6.** Electron microscopic observation of pituitary cells after bromocriptine administration reveals rat PRL mRNA hybridization signals localized on the distorted, vesiculated, and partly dilated RER, and also increased number of accumulated secretory granules. Cited from Matsuno et al., 1998b. Bar: 200nm.

recently used LR White resin for tissue embedment (Matsuno et al., 1998a). EM-ISH using LR White resin for tissue embedment also localized rat GH mRNA on the polysomes of the entire RER (Fig. 9). Subsequent immunohistochemical staining identified rat GH protein both on the secretory granules and in the cisternae of the RER (Fig. 9).

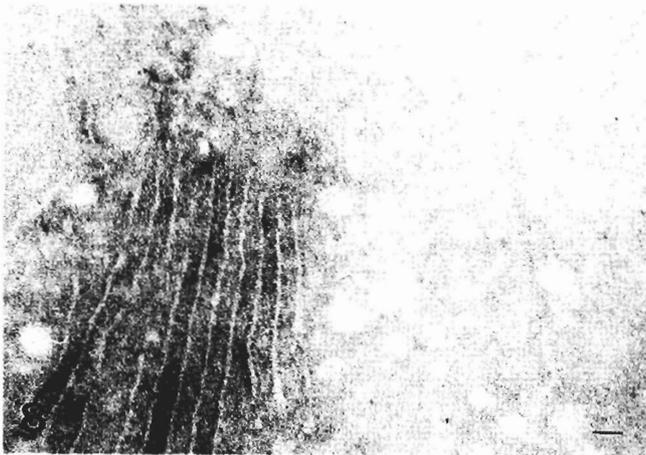
As shown in our previous reports (Matsuno et al., 1994a,b, 1996), GH mRNA is distributed diffusely on the RER. In these reports using tissues embedded in Epon resin, somewhat heterogeneous electron density was observed in GH mRNA expression. In LR White resin-embedded tissues, GH mRNA was also distributed

diffusely on the RER, its electron density being homogeneous. The cause of this difference in electron density of heterogeneity and homogeneity cannot be determined, but may be attributed to the characteristic difference of both resins. As the manufacturer stated, the hydrophilic and low lipid solvent character of LR White resin is known to serve for the excellent visualization of membrane and cytosol structures, and thus may be helpful for the preservation of the reaction products in LR White resin-embedded tissues.

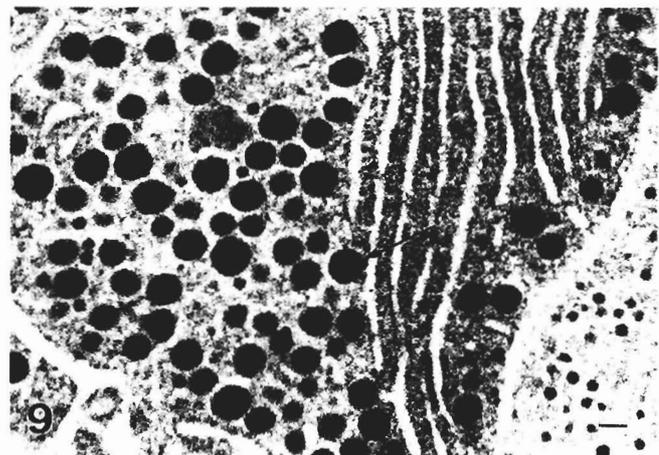
There seem to be two major problems to be resolved in this ultrastructural double staining method to visualize mRNA and encoded protein simultaneously in the same cell. One is to retain the messages and the other is to maintain the immunoreactivity of the encoded protein in the same cell. Based on the above-mentioned assessment, apropos of maintaining tissue morphology and retaining the messages, we utilized the pre-embedding EM-ISH method using 6  $\mu\text{m}$ -thick frozen sections fixed in 4% paraformaldehyde for the simultaneous detection of mRNA and encoded protein. The immunoreactivity can be retrieved by the etching process with  $\text{H}_2\text{O}_2$  or sodium periodate even after modification, such as osmification and embedment in Epon resin, and those tissues embedded in Epon resin can serve for the ultrastructural simultaneous detection of messages and encoded proteins. The only problem is the deosmification and degradation of the signals of mRNA, which are caused by etching process with  $\text{H}_2\text{O}_2$  or sodium periodate. In order to solve this problem, we have recently used LR White resin for tissue embedment (Matsuno et al., 1998a). In LR White resin-embedded tissues, retrieval of immunoreactivity with  $\text{H}_2\text{O}_2$  or sodium periodate is not required, and therefore the degradation of the signals of mRNA can be avoided. To our knowledge, only five reports, except for our previous ones (Matsuno et al., 1996, 1998a,b), describing the



**Fig. 7.** Combined immunohistochemistry and preembedding EM-ISH at the ultrastructural level. Positive signals for rat GH mRNA are localized as osmium black on the polysomes of the RER using biotinylated antisense oligonucleotide probe. Rat GH protein is identified mainly on the secretory granules with 20 nm protein A colloidal gold particles (arrow), and is also identified in the cisternae of the RER (arrow head). Cited from Matsuno et al., 1998b. Bar: 200nm.



**Fig. 8.** Immunohistochemical control experiments at the ultrastructural level with the substitution of normal monkey serum for anti-rat GH antibody. No positive reactions of protein A colloidal gold particles are observed on the secretory granules, whereas positive signals for rat GH mRNA are identified as osmium black on the polysomes of the RER. Cited from Matsuno et al., 1998b. Bar: 200nm.



**Fig. 9.** Using LR White resin for tissue embedment, rat GH mRNA is also localized on the polysomes of the entire RER. Subsequent immunohistochemical staining identifies rat GH protein both on the secretory granules (arrow) and in the cisternae of the RER (arrow head). Bar: 200nm.

ultrastructural simultaneous detection of mRNA and encoded protein, have been published, in each of which postembedding EM-ISH method using colloidal gold particle was utilized (Singer et al., 1989; Escaig-Haye et al., 1992; Egger et al., 1994; Gingras and Bendayan, 1995; Morey et al., 1995). However, in these postembedding EM-ISH studies, the relatively frequent non-specific reactions of colloidal gold particles used for the detection of mRNA were observed in the cisternae of the RER. EM-ISH method for Lowicryl K4M-embedded tissues is generally supposed to have some difficulty in morphological preservation. The decreased message preservation was observed in the postembedding EM-ISH studies using LR White-embedded tissues (Matsuno et al., 1994a; Morey et al., 1995). From the viewpoint of specific hybridization signal detection and morphological preservation, the combined immunohistochemistry and preembedding EM-ISH method is supposed to be superior to the previously reported postembedding EM-ISH method, and is successful and satisfactory for ultrastructural simultaneous identification of mRNA and encoded protein. Translation of mRNA for secreted proteins is initiated on free ribosomes and then translocated to the polysomes on the RER with the aid of signal recognition particle once the signal peptide is produced. Synthesized proteins are secreted into the luminal space of the RER and subsequently transported to secretory granules via the Golgi apparatus. This ultrastructural double-staining method for mRNA and encoded protein can provide an important clue for elucidating the intracellular correlation of mRNA translation and secretion of translated protein.

## Conclusion

Non-radioisotopic EM-ISH study, especially the preembedding method, has favorable characteristics of high resolution and well-preserved messages, and the combination of non-radioisotopic EM-ISH with immunohistochemistry is a useful tool for the identification of the intracellular correlation of mRNA translation and secretion of translated protein.

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