In Situ Hybridization at the Electron Microscope Level: Localization of Transcripts on Ultrathin Sections of Lowicryl K4M-embedded Tissue Using Biotinylated Probes and Protein A–Gold Complexes

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Abstract. A technique has been developed for localizing hybrids formed in situ on semi-thin and ultrathin sections of Lowicryl K4M-embedded tissue. Biotinylated dUTP (Bio-11-dUTP and/or Bio-16-dUTP) was incorporated into mitochondrial rDNA and small nuclear U1 probes by nick-translation. The probes were hybridized to sections of Drosophila ovaries and subsequently detected with an anti-biotin antibody and protein A-gold complex. On semi-thin sections, probe detection was achieved by amplification steps with anti-protein A antibody and protein A-gold with subsequent silver enhancement. At the electron microscope level, specific labeling was obtained over structures known to be the site of expression of the appropriate genes (i.e., either over mitochondria or over nuclei). The labeling pattern at the light microscope level (semi-thin sections) was consistent with that obtained at the electron microscope level. The described nonradioactive procedures for hybrid detection on Lowicryl K4M-embedded tissue sections offer several advantages: (a) rapid signal detection; (b) superior morphological preservation and spatial resolution; and (c) signal-to-noise ratios equivalent to radiolabeling.

The technique of in situ hybridization at the light microscope (LM) level has become a standard method for the localization of DNA and RNA sequences on tissue sections or cells (for review see Szabo and Ward, 1982). In particular, in the field of developmental biology this technique has recently shown to be the most direct way of answering questions of gene expression and its modulation at the individual cell level during developmental transitions (Angerer and Angerer, 1981; Venezky et al., 1981; Akam, 1983; Hafen et al., 1983; Ambrosio and Schedl, 1984; Cox et al., 1984; Hafen et al., 1984; Jamrich et al., 1984; and Akam and Carlson, 1985). Attempts have been undertaken to extend this technique to the electron microscope (EM) level (Jacob et al., 1971, 1974; Croissant et al., 1972; Rae and Franke, 1972; Geuskens and May, 1974; Geuskens et al., 1974; Manning et al., 1975; Steinert et al., 1976; Hutchison et al., 1982; and Steinert et al., 1984). Until recently, signal detection could be only accomplished using autoradiography. At the present time, however, alternative methods for signal detection are available (Rudkin and Stollar, 1977; Bauman et al., 1981; Langer-Safer et al., 1982a; and Forster et al., 1985); amongst them, the concept of Ward and his collaborators has already proved very effective (Langer-Safer et al., 1982a; Manuelidis et al., 1982; Singer and Ward, 1982; Brigati et al., 1983; Leary et al., 1983; and Manuelidis and Ward, 1984). This approach is based on the labeling of probes with a biotinylated nucleotide, and their detection with sensitive immuno- or affinity-cytochemical detection systems. It was successfully applied for in situ hybridization of mouse satellite DNA to whole mount chromosomes at the EM level (Hutchison et al., 1982). However, as yet no comparable method for in situ hybridization for thin sections has been described. Such a method would further extend the range of potential applications of in situ hybridization to questions requiring high precision correlation of molecular function with ultrastructural morphology.

In the present study, we (a) tested the suitability of the embedding resin Lowicryl K4M by checking RNA retention and accessibility by acridine orange staining on semi-thin sections; (b) tested the sensitivity of the detection system, namely anti-biotin antibody in consort with protein A–gold complexes with EM dot blots; and (c) developed hybridization and signal detection protocols for in situ hybridization to semi-thin and ultrathin sections of Lowicryl K4M-embedded tissue for radiolabeled and biotinylated probes. We chose mitochondrial large rRNA and U1 small nuclear RNA expression in Drosophila ovaries as our model systems because the transcripts are assumed to be confined within well-defined structures.
Materials and Methods

Tissue Processing

Drosophila melanogaster females (Canton-S strain) were dissected in a drop of Ringer solution containing either 4% formaldehyde (freshly made from paraformaldehyde) and 0.1% glutaraldehyde (pH 7.4), or only 4% formaldehyde, and fixed for 15 min. Tissue was then placed in Ringer solution and stored overnight at 4°C. Dehydration and infiltration at low temperature with Lowcrysyl K4M (Carlsmann et al., 1982) were performed as previously described (Roth et al., 1981) except that the individual steps were prolonged in order to achieve proper infiltration in late stage ovariian chambers. Semi-thin sections (1 µm) were cut and placed on polylysine-treated slides (Lucoq and Roth, 1984). Ultrathin sections were cut (50-60 nm) and picked up on carbon-parlodion-coated nickel grids (150 mesh).

Preparation of Protein A-Gold Complex

Monodisperse colloidal gold with a particle size of 14 nm was prepared by reducing a boiling solution of tetrachloroauric acid with trisodium citrate (Frens, 1973). Protein A was complexed to colloidal gold as previously described (Roth et al., 1978). After ultracentrifugation, the protein A-gold complex was resuspended with phosphate-buffered saline (PBS) and stored at 4°C.

Acridine Orange Staining and Pre-treatments of Semi-thin Sections

Semi-thin sections were stained with acridine orange according to the method described by Hafen et al. (1983). Several pre-treatments of semi-thin sections were carried out as follows: sections were incubated with (a) pancreatic RNAse (2 mg/m in 5 mM MgCl2, 50 mM Tris-HCl [pH 7.5]) (Boehringer Mannheim Biochemicals, Indianapolis, IN); (b) 0.2 N HCl for 20 min at room temperature followed by a wash in distilled water for 5 min. (c) pepsin (0.25 mg/ml in 50 mM Tris-HCl [pH 7.5], 3 mM EDTA) (Calbiochem-Behring Corp., La Jolla, CA), followed by a wash in glycine (2 mg/ml in PBS) for 30 s at room temperature and two washes in PBS for 30 s each; or (d) 0.5% aqueous solution of Triton X-100 followed by two washes with PBS for 5 min each. The above-listed treatments were also used in several combinations.

Probes and Nick-Translation with 3H-labeled and Biotin-labeled Nucleotides

Two different Drosophila DNA probes have been used in this study: a plasmid containing a 1.05-kb EcoRI fragment of the mitochondrial large rRNA gene cloned into pBR322 (Renaud, M., and F. Berthier, unpublished data), and a second plasmid containing 131 nucleotides from the U1 snRNA gene cloned at the Hin1 II site of pUC8 (Alonso et al., 1984). For nick-translation, the whole first plasmid was used, whereas for the second a 432-pb fragment encompassing the U1 region was excised with Pvu II, purified by agarose gel electrophoresis and electroelution. 3H-labeled hybridization probes were prepared by nick-translation according to a published protocol (Hafen et al., 1983). Biotinylated probes were prepared essentially according to the above protocol with the following modifications: instead of the 3H-labeled nucleotides, 3 µl of a mixture of dUTP, dCTP, and dGTP (0.5 mM in water), 2.5 µl biotinylated dUTP (Bio-11- or Bio-16-dUTP; 0.3 mM; Enzo Biochem, Inc., New York, NY; Cat. No. EBP-806) and 1 µl 32p-dCTP (2 µl of 1 mCi/ml stock solution diluted with 3 µl water) were added to the reaction mixture (50 µl final volume). Each preparation was checked for probe length by acrylamide gel electrophoresis (5%) and incorporation of label by trichloroacetic acid precipitation. The lengths of probes used in the experiments were within the range of 35-120 nucleotides.

EM Localization of Transcripts on Thin Sections

EM Dot Blots

Before hybridization with tritiated probes, semi-thin sections were subjected to acetylation in order to diminish background. The acetylation mixture consisted of 500 ml of 0.1 M triethanolamine-HCl adjusted to pH 8 with HCl including PBS = 3.4 M NaCl, 0.07 M KCl, 0.037 M KH2PO4, 0.19 M Na2HPO4, and fixed for 10 min. Tissue was then placed in Ringer solution and stored overnight at 4°C. Dehydration and infiltration at low temperature with Lowcrysyl K4M (Carlsmann et al., 1982) were performed as previously described (Roth et al., 1981) except that the individual steps were prolonged in order to achieve proper infiltration in late stage ovariian chambers. Semi-thin sections (1 µm) were cut and placed on polylysine-treated slides (Lucoq and Roth, 1984). Ultrathin sections were cut (50-60 nm) and picked up on carbon-parlodion-coated nickel grids (150 mesh).

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EM In Situ Hybridization on Thin Sections

EM In Situ Hybridization on Thin Sections

For denaturation the 3H-labeled rDNA probe was boiled for 2 min and immediately cooled to 0°C where it was kept until use. Hybridization was performed by floating the grids on drops (15 µl) of the hybridization solution (1 µg DNA in 500 µl hybridization buffer) in a moist chamber for 24 h at 37°C. After hybridization, the grids were placed on drops of formamide buffer at 37°C for ~20 h with four changes of buffer. The grids were then washed with PBS, distilled water, and air dried. A 1:40 dilution of anti-biotin antibody (Sigma Chemical Co., St. Louis, MO) (diluted 1:10 with PBS containing BSA, detergents, and NaN3 as above) for 2 h at room temperature. Incubation was followed by two washes in PBS for 2 min each. Then grids were incubated with protein A-gold (diluted to an optical density [OD] value of 0.44 at 525 nm with PBS containing BSA, detergents, and NaN3 as above) for 1 h at room temperature. The grids were then washed with PBS (twice for 2 min each) and again treated with protein A-gold as described above. Finally, after two washes with PBS for 2 min and one wash with distilled water for 5 min, the slides were air dried. For silver enhancement of the gold signal (Danscher and Ryter Norgaard, 1985) the grids were dipped in Kodak NTB II emulsion, placed on a pre-cooled metal plate (0°C) for 10 min, developed in Kodak developer D-19 for 15 min, rinsed in water for 30 s, fixed with 20% sodium thiosulfate (4 min), rinsed in water for 15 min, and air dried. After staining for 20 s with 0.1% toluidine blue in 0.1% aqueous borate solution the grids were washed with water for 15 min and air dried. Slides were then mounted in DPX and examined by light microscopy. The photomicrographs were obtained with a Agfa film 25 film.

EM Dot Blots

Slot grids were coated with a parlodion film. Various amounts of biotinylated rDNA probe (containing free 32P-dCTP as a tracer) of known DNA content (in a volume of 20-30 nl) in hybridization buffer were dispensed onto the parlodion film and air dried. After baking at 80°C for 1 h, the grids were subjected to Cerenkov counting (Gosling, 1976) for calculation of the exact applied volume. Grids were then incubated with anti-biotin antibody and protein A-gold as described under hybridization of biotinylated probes. After inspection of grids by electron microscopy and preparation of micrographs, particle density was determined as described above.
Results

RNA Retention and Accessibility on Semi-thin Sections

Retention and accessibility of cellular RNA are two important parameters to be checked at each stage during the development of a suitable protocol for in situ hybridization. The positive results obtained with acridine orange staining on semi-thin sections strongly favored the use of Lowicryl K4M. Additional aspects in favor of this resin are as follows. (a) Methacrylate embedding has already been successfully used for in situ hybridization techniques (Jamrich et al., 1984; Steinert et al., 1984). (b) This highly cross-linked acrylate- and methacrylate-based hydrophilic resin has been shown to provide superior preservation of structure in consort with significant lower background labeling compared to other resins (Roth et al., 1981). (c) The low temperature embedding protocol may prevent enzymatic degradation of RNA. Pre-treatment of semi-thin sections with a combination of detergent (Triton X-100) and pronase improved intensity of acridine orange staining (data not shown). However, as will be seen later, this finding did not bear closer examinations. RNase treatment of sections completely abolished acridine orange staining (data not shown).

LM In Situ Hybridization on Semi-thin Sections

Hybridization on semi-thin sections was checked at the LM level using 3H-labeled rDNA and U1 probes. In the autoradiographs of rDNA hybridization the silver grains were present only over the cytoplasm of cells; nuclei were nearly devoid of label (Fig. 1 a). Hybridization of rDNA probe to frozen sections gave the same results (data not shown). Controls performed at this stage were hybridization with nick-translated M13 vector without insert and RNase pre-treatment. In both controls, no significant hybridization signal over background could be detected (data not shown). Furthermore, the same results were obtained whether or not pre-treatments were performed before hybridization. This is in agreement with observations made by Steinert et al. (1984) with ribosomal cDNA probes on thin sections of glycolmethacrylate-embedded oocytes, embryos, and eggs of sea urchin.

In another series of experiments biotinylated rDNA and U1 probes were used for LM in situ hybridization to semi-thin sections. Fig. 1, b and c, clearly shows that it is possible to detect hybrids at the LM level if amplification steps are used. The labeling pattern thus obtained resembles that of LM autoradiography. The only difference appears to be the heterogeneous grain size compared to autoradiography. However, it must be emphasized that with this technique signal visualization is only possible after substantial amplification (i.e., anti–protein A antibody—protein A-gold and silver enhancement).

EM In Situ Hybridization on Thin Sections

A 3H-labeled rDNA probe was compared with the biotin-labeled probe with respect to performance of signal detection. As can be seen from Fig. 2 b, the labeling pattern obtained with the tritiated probe is similar compared to that of the biotinylated probe (Fig. 2 a). In addition, if an exposure time had been chosen at which both detection systems approximately equaled in background densities, the densities of specific label were similar. From Fig. 2 b it is also evident that the autoradiographic detection system is inferior to the biotin—anti-biotin—protein A-gold system in terms of spatial resolution and structural preservation. Controls of the same kind as described below were done.

Mitochondrial large rRNA as well as U1 genes, biotinylated by nick-translation, were hybridized to thin sections. After hybridization, the biotinylated hybrids were detected by antibiotin antibody in consort with protein A-gold. The gold particles could be observed either over mitochondria (Fig. 2 a) or over nuclei (Fig. 3); i.e., over structures known for the expression of the appropriate genes. In the case of hybridization with rDNA the labeling pattern of mitochondria was heterogeneous. Analysis of the labeling pattern (Table I) showed that approximately one-third of the mitochondria is unlabeled, nearly one-third is labeled with one gold particle, and the residual third is labeled with more than one gold particle with a number being reciprocal to the frequency of occurrence. With serial sectioning we found that the labeling pattern of individual mitochondria changed on consecutive sections.

Control Experiments

Table II represents the results of a series of controls of mitochondrial labeling (see also Figs. 1 d, and 2 d). In all the listed control experiments, labeling densities over mitochondria were determined. Background was estimated as the labeling density over the residual cell area (nucleus included). In none of the controls labeling density over mitochondria exceeded background levels, thus arguing in favor of the specificity of signal due to the interaction of anti-biotin and biotinylated hybrids.

To demonstrate the applicability of this technique for the localization of transcripts being present in a concentration above detection limit, we chose U1 small nuclear RNA transcripts as an other example. Fig. 3 shows that the labeling is restricted to the nucleus. It is interesting to note that the nucleolus is exempted from labeling. Control experiments of the same type as listed in Table II for rDNA probes were carried out confirming specificity of both hybridization and signal detection (data not shown).

EM Dot Blots

These experiments were done for two reasons—namely, to obtain some kind of measure for the sensitivity of the technique and to demonstrate that sensitivity is increased at the EM level. Dot blots on nitrocellulose strips with biotinylated probes and immunochemical signal detection (anti-biotin antibody—second antibody—protein A-gold and silver enhancement) resulted in a detection limit between 1–10 pg when inspected with the naked eye (data not shown). At the EM level, however, signal detection is more sensitive (detection limit is ~100 fg) because accumulation of a few gold particles relative to background can easily be recognized (Fig. 4).

Discussion

The work presented here improves and extends the technique of in situ hybridization at the EM and LM level. The procedures described for the nonradioactive hybrid detection on ultrathin and semi-thin sections of Lowicryl K4M-embedded
tissue display several advantages relative to radiolabeling: (a) rapid signal detection; (b) superior morphological preservation and spatial resolution; and (c) an equivalent signal-to-noise ratio. The data obtained by hybridization with tritiated and biotinylated rDNA and U1 probes—performed at the LM level using either autoradiography or immunochemical techniques with signal amplification as detection systems—are consistent with those obtained at the EM level. The rapidity of signal detection allowed us to carry out hybridization and signal detection in one day, although, for the sake of convenience, the hybridization step was usually done overnight. Thus it was possible to test several parameters in a reasonable time period in order to determine appropriate conditions for optimizing morphological preservation and specific labeling.

The best fixative for our system proved to be 4% formaldehyde with 0.1% glutaraldehyde. This is based on the observation that the autoradiographic signal on semi-thin sections of ovaries fixed with formaldehyde and glutaraldehyde was consistently stronger compared to formaldehyde-fixed ones. Although ethanol/acetic acid is most widely used as a fixative for in situ hybridization, a recent report (on quantitative
Figure 2. Comparison of the biotin detection system with EM autoradiography and amplification with anti–protein A and protein A–gold on K4M thin sections; rDNA probe. (a) Follicle cell hybridized with biotinylated rDNA. Signal detection with anti-biotin antibody in consort with protein A–gold. (b) Late stage ovarian chamber. Yolk. EM autoradiography, rDNA probe, exposure for 7 wk. (c) Follicle cell hybridized with biotinylated rDNA; signal detection with amplification. (d) Follicle cell. Control incubation with hybridization buffer only. Bars, 0.5 μm.

Analysis of in situ hybridization parameters has convincingly demonstrated the superior performance of short fixation with formaldehyde (Lawrence and Singer, 1985). Glutaraldehyde, however, when used in higher concentration (4%) reduces hybridization efficiency by ~60% relative to formaldehyde. The optimal length of hybridization time using biotinylated rDNA probes at the EM level was determined by using hybridization times ranging from 1 to 24 h. Specific label over mitochondria was already present after 1 h of hybridization. The labeling density increased with longer hybridization time, up to ~5 h. Thereafter, labeling remained constant. This is in agreement with the study of Lawrence and Singer (1985) who have shown that hybridization reaches a maximum within 3 to 4 h. Incubation time for the anti-biotin antibody and the
Figure 3. Nurse cell; hybridization with biotinylated U1 probe. Signal detection with anti-biotin antibody and protein A-gold. Bar, 0.5 µm.

Table I. Distribution and Density of Gold Particles on Mitochondria (n = 608) Labeled with rDNA Probe

<table>
<thead>
<tr>
<th>Number of gold particles per mitochondrion</th>
<th>Percentage of total number of mitochondria (number of mitochondria)</th>
<th>Mean density of specific label</th>
<th>Mean area per mitochondrion</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>gold particles/µm²</td>
<td>µm²-10²</td>
</tr>
<tr>
<td>0</td>
<td></td>
<td>35.55 (216)</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>28.29 (172)</td>
<td>10.98</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>14.64 (89)</td>
<td>15.24</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>9.37 (57)</td>
<td>19.56</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>6.41 (39)</td>
<td>20.08</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>1.48 (9)</td>
<td>30.61</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>1.97 (12)</td>
<td>27.27</td>
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<tr>
<td>7</td>
<td></td>
<td>0.33 (2)</td>
<td>29.12</td>
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<tr>
<td>8</td>
<td></td>
<td>0.49 (3)</td>
<td>54.54</td>
</tr>
<tr>
<td>9</td>
<td></td>
<td>0.66 (4)</td>
<td>20.69</td>
</tr>
<tr>
<td>10</td>
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<td>0.33 (2)</td>
<td>29.85</td>
</tr>
<tr>
<td>11</td>
<td></td>
<td>0.16 (1)</td>
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<tr>
<td>15</td>
<td></td>
<td>0.16 (1)</td>
<td>30.00</td>
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</table>

protein A-gold complex was found to be optimal at 1–2 h. In addition, we have initially tried to adapt LM pre-treatment procedures to the EM level prompted by the positive results obtained with a combined Triton-pronase treatment and subsequent acridine orange staining. While having no influence on RNA retention and accessibility at the LM level (semi-thin sections and autoradiographic signal detection) pre-treatments such as acetylation or incubation with pronase had a detrimental effect at the EM level. Lawrence and Singer (1985) investigating the effect of proteinase on hybridization and RNA retention also found on formaldehyde-fixed cells a loss of cellular RNA and lower hybridization resulting from this treatment.

We observed that post-hybridization washes, inserted in the EM protocol for biotinylated probes, represented the most critical step in the procedure. Here, in contrast to autoradiographic signal detection at the LM and EM levels, the formamide buffer had an adverse effect on labeling and morphology. Upon EM examination, sections treated with formamide buffer exhibited contamination with semi-electron dense specks unspecifically labeled with gold particles and an amorphous layer which obscured morphological de-

Table II. Controls of Specificity of Mitochondrial Labeling with Biotinylated rDNA Probes on Thin Sections of Lowicryl K4M-embedded Drosophila Ovaries*

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Mitochondria</th>
<th>Rest of the cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control 1</td>
<td>12.80 ± 0.51</td>
<td>1.87 ± 0.22</td>
</tr>
<tr>
<td>Control 2*</td>
<td>2.72 ± 0.33</td>
<td>1.55 ± 0.26</td>
</tr>
<tr>
<td>Control 3*</td>
<td>2.57 ± 0.42</td>
<td>2.87 ± 0.31</td>
</tr>
<tr>
<td>Control 4**</td>
<td>1.36 ± 0.38</td>
<td>1.67 ± 0.30</td>
</tr>
</tbody>
</table>

* Labeling densities over both mitochondria and the rest of the cell expressed as mean number of gold particles per µm² ± SEM.

1 In situ hybridization of rDNA probe to thin sections of Lowicryl K4M-embedded Drosophila ovaries.
1 rDNA probe was replaced by U1 probe.
1 rDNA probe was omitted; hybridization was carried out with hybridization buffer only.
1 Hybridization was omitted; incubation with anti-biotin antibody and protein A-gold complex.
1 Incubation with protein A-gold complex only.

Figure 4. EM dot blot. Part of a spot representing 1 pg rDNA on a parlodion film. Bar, 0.5 µm.
The ultimate use of an in situ hybridization technique depends on the sensitivity achievable with this method and the precision with which target molecules can be localized. It is obvious that the precision obtained with an EM technique is superior compared to an LM one, whereas sensitivity is not necessarily improved by adapting a technique to the EM level. The present EM technique using biotinylated probes depends on several parameters, some of which are different from those of an autoradiographic EM method: (a) cellular RNA retention during tissue processing for embedding; (b) accessibility of RNA species to be localized; (c) efficiency of hybridization; (d) the degree of biotinylation of the probe; (e) steric accessibility of the label; and (f) efficiency of signal detection. Since quantitative evaluation of some of these parameters is not feasible because of inherent uncertainties, an accurate overall estimate of sensitivity is also not possible. To obtain an approximate estimation of sensitivity, we compared grain particle densities and signal-to-noise ratios obtained with tritiated and biotinylated probes; we performed dot blots in order to evaluate sensitivity of the biotin-detection system; and we approximately calculated the number of RNA transcripts equivalent to one gold particle of specific label. Table III shows that signal-to-noise ratios do not vary considerably within the listed systems, presumably indicating that sensitivity is of the same order of magnitude. The signal-to-noise ratio of ~7 obtained with hybridization of biotinylated rDNA probes to ultrathin sections compares well with that obtained by Hutchison et al. (1982) with hybridization of a biotinylated probe to whole mount chromosomes at the EM and signal detection with colloidal gold (signal/noise [S/N] = -9). This is the only data available in the literature which is obtained with Lowicryl K4M-embedded tissue. Hence, we conclude that the use of this resin for in situ hybridization is highly advantageous because the results thus obtained are comparable to those obtained with unembedded cells.

On the other hand, Lawrence and Singer (1985) compared signal-to-noise ratios of three different detection systems (32P-labeled probe and Cerenkov counting) (Goulding, 1976); 3H-labeled probe and autoradiography; biotinylated and 35P-labeled probe, detection system not mentioned) obtained by hybridization of the correspondingly labeled actin probe to formaldehyde-fixed unembedded cells (skeletal myoblasts). They obtained an S/N = 70 with 3P-labeled probe, an S/N = 20 for 3H-labeled probe, and an S/N of at least 10 with the biotinylated probe. This again compares well with our result especially if we consider the thickness of tissue probably participating in signal generation. In contrast to signal-to-noise ratios, Table III shows that specific labeling densities vary over a broader range. In particular, we have no explanation for the considerably lower labeling density obtained with semi-thin sections and autoradiographical detection.

Dot blots performed with biotinylated rDNA probes, goat anti-biotin antibody, protein A–gold and silver enhancement on nitrocellulose resulted in a detection limit of ~1 pg of probe DNA, whereas ~10 times less than this amount could be recognized without amplification on the EM dot blots. This experiment was mainly devised to demonstrate the higher sensitivity of the protein A–gold system at the EM level simply by being capable to recognize gold particle densities relative to background noise under the EM. From the foregoing one might reason that the sensitivity of the colloidal gold technique at the EM level could be enhanced by adding some amplification steps to the protocol. While being beneficial for the detection of hybrids on semi-thin sections (LM), amplification at the EM level is rather disadvantageous. Concomitantly with the signal amplification nonspecific labeling is increased while spatial resolution is diminished due to the formation of clusters of gold particles (Fig. 2 c).

To obtain a rough estimate of the order of magnitude of sensitivity of hybridization plus signal detection in our system, we offer an extrapolation from the following set of data. (a) 0.11 μm3 represents the mean mitochondrial profile area on thin sections over which the average signal of 1.5 gold particles is observed (see Table I). From this the average volume of a 50-nm section of a mitochondrial profile calculates as 5.7 × 10−13 μm3. (b) From EM morphometry (P. Lcher, personal communication) we know that the long axis ranges from 2–4 μm and the short one from 0.3–0.5 μm. From this data we calculate the mitochondrial volume by approximation as cylinder (base area × length = 0.14–0.78 μm3). (c) The

<table>
<thead>
<tr>
<th>Technique</th>
<th>Probe</th>
<th>S/N</th>
<th>Specific label</th>
<th>Background</th>
<th>Number of fields inspected</th>
</tr>
</thead>
<tbody>
<tr>
<td>EM, thin sections protein A–gold</td>
<td>U1</td>
<td>5.82 ± 0.69</td>
<td>2.62 ± 0.20</td>
<td>0.45 ± 0.14</td>
<td>10</td>
</tr>
<tr>
<td>EM, thin sections protein A–gold</td>
<td>rDNA</td>
<td>6.82 ± 0.41</td>
<td>12.80 ± 0.51</td>
<td>1.87 ± 0.22</td>
<td>9</td>
</tr>
<tr>
<td>EM, thin sections autoradiography 4-wk exposure</td>
<td>rDNA</td>
<td>10.25 ± 2.76</td>
<td>2.46 ± 0.36</td>
<td>0.24 ± 0.14</td>
<td>6</td>
</tr>
<tr>
<td>7 wk exposure</td>
<td>rDNA</td>
<td>6.69 ± 0.73</td>
<td>6.56 ± 0.54</td>
<td>0.98 ± 0.21</td>
<td>8</td>
</tr>
<tr>
<td>LM, semi-thin sections autoradiography 4-wk exposure</td>
<td>rDNA</td>
<td>5.40 ± 0.53</td>
<td>0.14 ± 0.06</td>
<td>0.025 ± 0.02</td>
<td>10</td>
</tr>
<tr>
<td>LM, frozen sections autoradiography 2-wk exposure</td>
<td>rDNA</td>
<td>8.28 ± 0.46</td>
<td>3.13 ± 0.22</td>
<td>0.38 ± 0.12</td>
<td>14</td>
</tr>
</tbody>
</table>

* Mean values of particles per μm2 ± SEM.
In conclusion, the described technique for in situ hybridization on thin sections may be useful for investigations on the various cellular aspects of RNA processing.

We thank Dr. Bernard Jacq for critically reviewing the manuscript, Douglas J. Taatjes for correcting the English, Dr. Iain Mattaj for valuable suggestions, and Dr. Uwe Waldorf for his kind assistance.

We would like to express our gratitude to Dr. A. Alonso who kindly provided us with the monoclonal antibody anti-Jo-1. Much appreciation is due to Erica Oesch for her tireless work and to Daniel Wey for preparing the photographs.

During part of this research M. Binder was a European Molecular Biology Organization short-term fellow. S. Tourmente was the recipient of a Centre National de la Recherche Scientifique fellowship. The work was supported in part by a grant in aid from the "CIBA-Stiftung" Basel and the Swiss National Science Foundation grant no. 3.443-0.83.

Received for publication 24 October 1985, and in revised form 18 November 1985.

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