In situ hybridization of DIG-labeled rRNA probes to mouse liver ultrathin sections

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Thin section electron microscopy of nucleoli from mammalian cells reveals three nucleolar substructures. These are the fibrillar centers (FC), the dense fibrillar component, which forms a closely apposed layer upon the FCs, and the granular component, which constitutes the bulk of the nucleolar mass. Although there is no doubt that this ultrastructural organization somehow reflects the vectorial process of ribosome formation, the exact structure-function relationships have been a matter of much discussion and are still largely unclear. (For a review, see Scheer and Benavente, 1990.)

Here we describe an approach to localize specific intermediates for the pre-rRNA maturation process using *in situ* hybridization at the ultrastructural level. This approach will allow the correlation of each pre-rRNA processing step with the defined nucleolar substructures. The procedure described can also be applied to the localization of other RNA species involved in ribosome biogenesis, such as 5S rRNA or U3 snRNA (Fischer et al., 1991).

I. Embedding in Lowicryl K4M

(according to Carlemalm and Villiger, 1989, with alterations)

IA. Fixation of material

1. Prepare the following fixation solutions and keep them on ice:
   4% paraformaldehyde (PFA) in PBS (137 mM NaCl, 2.7 mM KCl, 7 mM Na_2HPO_4, 1.5 mM KH_2PO_4).
   0.5% glutaraldehyde (GA) plus 4% PFA in PBS.
2. Take a piece of freshly prepared mouse liver and submerge it in a petri dish filled with 4% PFA in PBS on ice.
3. Cut the liver into small cubes with a maximal side length of 1 mm.
   Note: The smaller the pieces, the better reagent penetrates them in the procedure below.
4. Transfer the liver cubes to other petri dishes on ice, containing the fixatives (i) 4% PFA and (ii) 0.5% GA plus 4% PFA.
5. Incubate the cubes in the fixatives for about 2 h.
6. To remove the fixative, wash the cubes 3 x 5 min with ice cold PBS.

IB. Dehydration of material

Transfer the object cubes to preparative glasses and dehydrate them in a series of ethanol solutions according to the following scheme:

- 2 x 15 min in 30% ethanol at 4° C.
- 1 x 30 min in 50% ethanol at 4° C.
- 1 x 30 min in 50% ethanol at –20° C.
- 2 x 30 min in 70% ethanol at –20° C.
- 2 x 30 min in 90% ethanol at –20° C.
- 2 x 30 min in 96% ethanol at –20° C.
- 2 x 30 min in 100% ethanol at –20° C.

Caution: *Leave the objects in a small amount of liquid during changes from one ethanol solution to another. This prevents them from drying out, especially at higher ethanol concentrations.*

IC. Infiltration

After dehydration, ethanol must be infiltrated with the embedding medium Lowicryl K4M (Chemische Werke Lowi, Waldkraiburg, FRG). This resin tolerates a little residual water in the object and produces good results in immunocytochemistry.

Caution: *When working with Lowicryl K4M, perform all steps:*
- At low temperatures
- Under the fume hood
- While wearing gloves
- While excluding air (oxygen) from K4M as much as possible.

To exclude air from the embedding medium, do one of the following:

- Fill all vessels which contain K4M with nitrogen.
- Perform all steps in a closed chamber cooled to constant temperature by liquid nitrogen, thus providing a nitrogen-saturated atmosphere in the closed preparation chamber (e.g., "CS-auto", Reichert & Jung, Cambridge Instr.).

- Prepare Lowicryl K4M as follows (to produce a medium hard plastic):
  Mix 2 g Crosslinker A, 13 g Monomer B, and 0.075 g Initiator C.
  Dissolve Initiator C by gentle stirring or by bubbling dry nitrogen through the mixture.
  Cool the mixture to -20°C in the freezer.
- Perform infiltration at about -20°C with suitably precooled material according to the following scheme:
  1 h with 100% ethanol:K4M (1:1, v/v).
  2 x 1 h with K4M.
  Overnight with K4M.
  2 x 3 h with K4M.

D. Embedding

Gelatin capsules (e.g. Capsulae Operculatae No. 2) are very suitable as molds for embedding because they are transparent to UV-light and can afterwards easily be cut away.

- Cool the gelatin capsules to the appropriate temperature.
- Add a few drops of Lowicryl K4M to each capsule.
- Drop one object cube into each capsule.
  Caution: Do this transfer very carefully. Do not mechanically damage the cube, allow it to dry out, or allow it to warm up.
- Fill the capsules with the embedding medium.
- Let the capsules stand for about half an hour to allow the air bubbles to leave.

E. Polymerization

- Before switching on the UV light, turn the temperature a bit lower to compensate for the heat from the light.
- Induce polymerization of Lowicryl K4M by 360 nm long-wave UV radiation ("CS-UV", Reichert & Jung, Cambridge Instr.) over a five day period. During the polymerization, do the following:
  For about three days, keep the polymerization temperature constant at any temperature between 0°C and -40°C.
  After three days, allow the temperature to rise to room temperature with about 8 K/h.

Note: For troubleshooting, see Carlemalm and Villiger (1989).

II. Sectioning

The embedded mouse liver cubes can easily be recognized in the transparent resin Lowicryl K4M. Carlemalm and Villiger (1989) recommend trimming with the ultramicrotome and a glass knife to get smooth surfaces.

- Obtain semithin sections with a glass knife and ultrathin sections with a diamond knife, according to standard protocols.
  Caution: Because of the hydrophilicity of Lowicryl K4M, be careful not to wet the surface of the block.
- Transfer sections to 200 mesh nickel grids which have been coated with 0.5% parlodion.
- Air dry the sections.
  Note: Now the sections are ready for hybridization.

III. Probe preparation

- Clone restriction fragments from mouse rDNA.
- Prepare *in vitro* transcripts from the rDNA clones and label them with DIG-UTP according to the protocols in Chapter 4 of this manual.
- Since probe length might influence the efficiency of *in situ* hybridization experiments, monitor transcript length by electrophoresis (in formaldehyde-containing gels), blotting onto nitrocellulose, and detection with the anti-DIG-alkaline phosphatase conjugate.
- If necessary, reduce probe length to approximately 150 nucleotides by limited alkaline hydrolysis of the transcripts according to Cox et al. (1984). Briefly, the procedure is:
  Incubate the transcripts with a solution of 40 mM $NaHCO_3$ and 60 mM Na_2CO_3 at 60°C.
  Calculate the Incubation time as follows:

$$t = \frac{L_0 - L_f}{k \cdot L_0 \cdot L_f}$$

  L_0 = initial length of transcript (in kb)
  L_f = desired probe length (in kb)
  k = constant = 0.11 kb/min

  Stop the hydrolysis by adding 3 M sodium acetate and acetic acid.
  Precipitate the hydrolyzate with ethanol.
  Dissolve the precipitate in water.

IV. Hybridization

Note: Perform all the following steps (including the hybridization) in a moist chamber.

- Prepare hybridization mixture [5 x SSC; 0.1 mg/ml tRNA; DIG-labeled antisense transcript at a final concentration of 10 ng/µl].
  Note: 1 x SSC contains 0.15 M NaCl, 0.015 M sodium citrate.
- To monitor the specificity of binding, prepare a "negative" probe by substituting the sense transcript for the antisense transcript in the hybridization mixture above.
- Place a droplet of the appropriate hybridization mixture onto Parafilm®.
- Incubate the ultrathin sections by putting the grid (section-side down) on top of the droplet.
- Perform hybridization for at least 3 h at 65° C.
- Wash the sections as follows, all at room temperature:
  3 x 5 min with 2 x SSC.
  2 x 10 min with PBST (PBS containing 0.1% Tween® 20).

V. Detection

- To block nonspecific sites, incubate each section for 15 min with PBST plus BG (PBST containing 1% bovine serum albumin, 0.1% CWFS-gelatin).
- Dilute 1 nm gold-conjugated anti-DIG antibody 1:30 in PBST plus BG.
- Apply diluted antibody to section and incubate for 1 h.
- Wash the sections as follows:
  3 x 5 min with PBST.
  6 x 5 min with redistilled water.
- Perform silver enhancement by incubating sections with developer and enhancer (from the set of silver enhancement reagents) at a ratio of 1:1 for 4–20 min (depending on the desired silver grain size).
- Wash the sections 6 x 5 min with redistilled water.
- Stain with 2% aqueous uranyl acetate (4 min) and Reynolds' lead citrate for 1 min (Reynolds, 1963) according to standard procedures.
- Evaluate the specimen in the electron microscope.

Results

In Figure 1, the hybridized probe was detected with monoclonal anti-DIG antibody coupled to 1 nm gold particles. The signal was enhanced with silver staining for 6 min at room temperature. Silver intensification facilitates visualization of the bound hybridization probe at low magnification. The silver grains scattered throughout the nucleoplasm might indicate transport of preribosomal particles from the nucleolus to the cytoplasm.

In Figure 2, a digoxigenin-labeled riboprobe complementary to the first 3 kb of the 5' region of the ETS1 was hybridized to a Lowicryl section of mouse liver and detected as described in Figure 1.

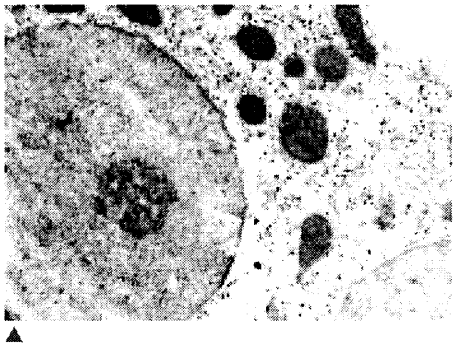

Figure 1: *In situ* hybridization of a digoxigenin-labeled RNA probe complementary to most of the 28 S rRNA region to an ultrathin section of mouse liver fixed with 3% formaldehyde and embedded in Lowicryl K4M. The nucleolus as well as the cytoplasm are clearly marked. Label is essentially absent from the mitochondria, demonstrating the specificity of the method. Cytoplasmic labeling is due to labeling of the ribosomes, especially the endoplasmatic reticulum-associated ones (Magnification: 25,200 x).

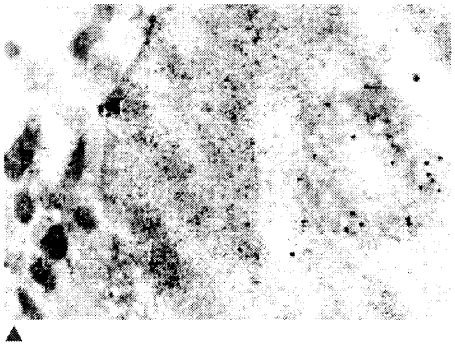

Figure 2: Localization of pre-rRNA molecules containing the 5' region of the ETS1. For details of the probe, see the text. The survey view shows selective labeling of the fibrillar components of the nucleolus
(Magnification: 27,000x).

References

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Reagents available from Boehringer Mannheim for this procedure

Reagent	Description	Cat. No.	Pack size
DIG RNA Labeling Kit*, **, ***	For RNA labeling with digoxigenin-UTP by *in vitro* transcription with SP6 and T7 RNA polymerase.	1175 025	1 Kit (2x10 labeling reactions)
Tween® 20		1 332 465	5x10 ml
BSA	Highest quality, lyophilizate	238 031 238 040	1 g 10 g
Anti-Digoxigenin-Gold	Affinity purified sheep IgG to digoxigenin conjugated with ultra small gold particles (average diameter ≤ 0.8 nm).	1 450 590	1 ml
Silver Enhancement Reagents	Detection of colloidal gold particles (anti-DIG-gold) by silver deposition on the particle surface	1 465 350	1 set

*Sold under the tradename of Genius in the US.

**EP Patent 0 324 474 granted to Boehringer Mannheim GmbH.

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