110 Analyzing RNA Synthesis: Nonisotopic Labeling

INTRODUCTION

Although autoradiography can be used to detect sites of transcription after incubating cells with radiolabeled RNA precursors (e.g., [^{3}H] uridine), this approach has some disadvantages:

- It is rather specialized and technically demanding.
- Detection of transcription sites requires very short pulses because the transcription rate is so rapid (transcripts are extended by 1000 nucleotides/minute in vivo). Because pools of unlabeled triphosphates are relatively high (e.g., many cells contain ~0.5 μM UTP), little radio-label is incorporated during short pulses, necessitating lengthy autoradiographic exposures.
- The path length of the particles emitted by ^{3}H is so long that autoradiographic grains can lie hundreds of nanometers away from the incorporation site.
- Labeling additional markers is technically difficult.
- The resultant grains sit on top of the cell and only provide a two-dimensional localization of the sites of incorporation.

Therefore, the ability to label sites of transcription in permeabilized cells using nonisotopic RNA precursors simplifies the analysis of transcription sites and allows simultaneous three-dimensional immunodetection of proteins or sites where DNA is replicated or repaired. Sites of nascent transcription in permeabilized HeLa cells are shown in Figure 110.1.

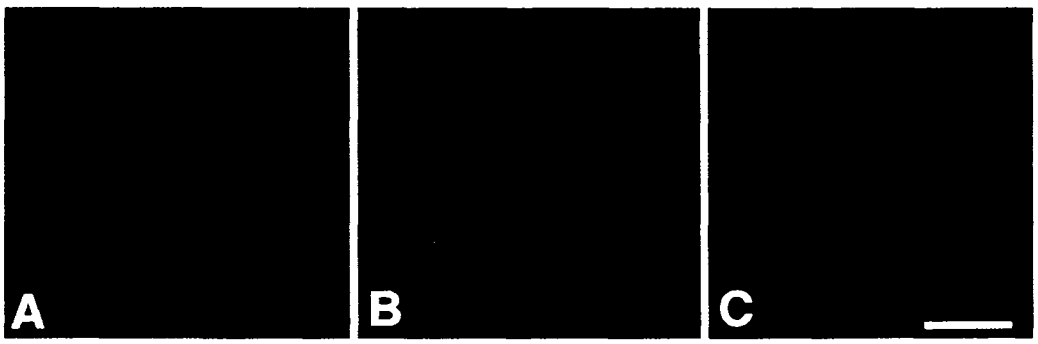

FIGURE 110.1
Sites of nascent transcription observed by fluorescence microscopy. Encapsulated HeLa cells were permeabilized with saponin and incubated with BrUTP for 10 minutes. Cells were fixed and stained using mouse anti-BrdU (Boehringer Mannheim) and a Texas Red-conjugated secondary antibody. (*A*) Transcription pattern showing strong nucleolar and weaker nucleoplasmic incorporation. (*B*) Inhibition of RNA polymerase II transcription with α-amanitin eliminates much of the nucleoplasmic signal. (*C*) DAPI staining of cell in *B*. Bar, 2.5 μm. (Photo provided by P.R. Cook, University of Oxford.)

Preparation of Cells

IMMOBILIZING CELLS

Procedures for labeling transcription sites require various manipulations and are applied with difficulty to cells free in suspension. These approaches can be more successfully applied to immobilized cells; this section describes the preparation of cells attached to coverslips or trapped in agarose microbeads.

Preparing Cells Grown on Coverslips

1. Prepare acid-washed coverslips as described in Chapter 98.

   For most lenses, coverslips having a thickness of 0.16–0.19 mm (no. 1–1/2) are best.

2. Remove an acid-washed coverslip from the ethanol storage solution, flame it to burn off the ethanol, and place it in a 35-mm diameter petri dish.

3. Seed cells (~20% confluency) in the dish and allow to adhere 1–2 days.

NOTES

- Best results are obtained with well-spread cells, covering 30–50% of the coverslip during labeling.
- Manipulation detaches some permeabilized cells; attachment can be improved by coating coverslips with gelatin or poly-L-lysine (0.5 mg/ml) prior to plating.

Preparing Cells Encapsulated in Agarose Microbeads

Encapsulation in agarose microbeads (Jackson and Cook 1985) provides a convenient way of protecting cells from damage during manipulation. Encapsulated cells can be permeabilized using a wide range of treatments, including buffers that cause nonencapsulated cells to aggregate into an unworkable mess. Both adherent and nonadherent cells can be permeabilized and labeled after encapsulation in agarose microbeads.

1. Warm 10 ml of liquid paraffin (BDH 29436; Merck 7162) to 37°C.

2. Heat 0.25 g of low-gelling agarose (e.g., Sigma type VII) in 10 ml of PBS at 95°C until dissolved, then cool to 37°C.

3. Resuspend 1×10^7 cells in 4 ml of PBS at 37°C in a 100-ml round-bottomed flask.

4. Add 1 ml of agarose solution at 37°C to 4 ml of cell suspension at 37°C and mix thoroughly.

5. Add 10 ml of paraffin at 37°C, seal flask with plastic film, and immediately shake

(by hand or at 800 cycles/minute using a flask shaker) until a creamy emulsion forms (about 15 seconds).

6. Cool flask by periodic rotation in ice-cold H_2O for 10 minutes; this allows spherical droplets of molten agarose suspended in the paraffin to gel.

7. Add 35 ml of ice-cold PBS, mix, and transfer to a 50-ml plastic centrifuge tube.

8. Pellet microbeads by spinning at 1000 rpm on a benchtop centrifuge at 20°C for 5 minutes.

9. Aspirate the supernatant and wash pelleted microbeads once in PBS. If some beads remain at the H_2O/paraffin interface, remove most paraffin, mix thoroughly, and respin.

10. Encapsulated cells can now be regrown in medium or permeabilized directly.

NOTES

- A cell density of 2×10^6/ml is convenient for most labeling procedures, but densities up to 10^8/ml can be used.
- Small volumes of microbeads can be prepared by homogenizing 50–500 μl of cell/agarose mixture with 1 ml of paraffin in a 50-ml round-bottomed tube.
- Adequate emulsions can be prepared by shaking manually (shake as fast as possible for 10–15 seconds). Microbead quality can be assessed microscopically; acceptable microbeads should be spherical, relatively uniform in size (r = 25–75 μm) with evenly dispersed cells.
- Microbead preparations should pass freely through tips used with automatic pipettes (10–200 μl). If not, large beads can be removed by filtration through monofilament nylon filters (R. Cadisch and Sons) using a Swinex filter (Millipore).
- To prevent losses during aspiration, samples should be transferred to 10-ml plastic centrifuge tubes at the earliest convenient point.
- Cells can be grown following encapsulation; simply resuspend beads in medium and incubate at 37°C. Encapsulated HeLa cells grow with normal cell-cycle kinetics for at least one generation.

CELL PERMEABILIZATION

After permeabilization, precursor pools of NTPs can be depleted by washing, and nucleotide levels can be adjusted to give the required rate of elongation. In the absence of the natural triphosphate, any modified triphosphate that is recognized as a substrate by the endogenous RNA polymerase is then incorporated relatively efficiently.

Permeabilization with Detergents

Permeabilization can be achieved by treatment with any of a variety of detergents. It is critical to control carefully both the concentration of detergent used and the timing of the treatment to permeabilize about 95% of the cells.

1. Prepare H_2O treated with DEPC. Add 0.5 ml of DEPC to 500 ml of distilled H_2O, mix, let stand at 37°C overnight, and autoclave.

   Caution: DEPC (See Appendix 5 for Caution)

   Alternatively, molecular biology grade H_2O can be obtained commercially (e.g., 5302–336550, 5 Prime→3 Prime).

2. Prepare fresh PB from stock solutions prepared in sterile purified H_2O and cool on ice.

   Chromatin structure is especially sensitive to changes in its ionic environment, and choice of the buffer to use with permeabilized cells is particularly important. Many buffers are in common use, but the following "physiological" buffer preserves considerable nuclear structure and function:

   Physiological Buffer (PB)
   100 mM KCH_3COOH
   30 mM KCl
   10 mM Na_2PO_4
   1 mM $MgCl_2$
   1 mM Na_2ATP
   1 mM DTT
   100 mM KH_2PO_4 is added, if required, to give pH 7.4.

   Protease and nuclease inhibitors are added as required.

   Caution: KCl; $MgCl_2$; DTT (See Appendix 5 for Caution)

3. Wash cells on coverslips, or cells encapsulated in agarose, twice with PBS.

   Coverslips: Place in convenient container (e.g., 24-well flat-bottomed tissue culture plate; Falcon 3047), add 2 ml of PBS to each well, wait for 2 minutes and aspirate; repeat once.

   Microbeads: Transfer 1 ml of cells in agarose beads and 9 ml of PBS into a 10-ml plastic centrifuge tube, centrifuge at 1000 rpm in a benchtop centrifuge for 30 seconds to pellet, aspirate supernatant, replace with appropriate buffer, and invert tube to mix.

4. Wash cells twice with ice-cold PB.

5. Transfer to ice-cold PB containing 0.01–0.1% detergent for 1–2 minutes (coverslips) or 2–3 minutes (microbeads). To define conditions, use a twofold dilution series of detergent in PB.

   The following (and related) detergents can be used; guideline concentrations are indicated:

   0.02–0.05% Triton X-100 (Pierce 28314)
   0.01–0.02% digitonin (Sigma D 1407)
   0.02–0.05% lysolecithin (Sigma L 4129)
   0.01–0.02% saponin (Sigma S 7900)

6. Assess the level of permeabilization using trypan blue exclusion (the extent of lysis is critical).

a. Add 50 μl of 1% trypan blue in PB to cells on a coverslip or to 50 μl of packed microbeads; wait 2 minutes.

b. Inspect by light microscopy; score % permeabilized (dark-blue) cells.

c. Choose the detergent concentration that permeabilizes >95% cells. If cells detach from coverslips during washing, use a lower concentration of detergent.

7. Wash detergent-treated cells three times with ice-cold PB. Cells are now ready for use in the transcription reaction.

NOTES

- It is sometimes useful to mark the backs of coverslips to ensure the correct orientation.
- If the buffer composition is to be varied, the following factors should be considered:
  (1) Various combinations of monovalent anion can be used. Different chloride/acetate/glutamate (or polyglutamate) combinations support almost identical rates of transcription or replication. However, acetate/glutamate is preferred to the smaller Cl^-, which is more damaging to tertiary protein structure.
  (2) The concentration of divalent cation must be carefully controlled. As little as 0.5 mM free Mg^{++} causes the visible (by EM) collapse or aggregation of chromatin. The equimolar Mg/ATP combination used here preserves chromatin structure and supports the action of Mg-dependent enzymes.
  (3) DTT, protease inhibitors, and ribonuclease inhibitors protect the sample and preserve cell morphology.

Permeabilization with Proteins

Streptolysin O and α-toxin assemble multimeric pore complexes in cell membranes, thereby permeabilizing them. Streptolysin generates 15-nm pores in cholesterol-containing membranes, sufficient to allow passage of large proteins like antibodies. Because excess protein can be removed prior to lysis, internal membranes should remain intact. α-Toxin pores are only 2 nm, allowing passage of small molecules, such as nucleic acid precursors, but few proteins.

1. Prepare PB, as described in steps 1–2 above.

2. Wash cells on coverslips or cells encapsulated in agarose with ice-cold PBS three times.

3. Lyse with streptolysin O or α-toxin:

Lyse with streptolysin O

a. Prepare streptolysin O (Gibco BRL 3493SA; Sigma S 5265) in PBS as instructed by supplier. Cool on ice.

b. Bind streptolysin O to cells on coverslips or in agarose beads.

Coverslips: Immerse in streptolysin O/PBS for 30 minutes on ice.

Microbeads: Mix 1 ml of beads with 9 ml of streptolysin O/PBS and incubate for 30 minutes on ice. Mix periodically.

c. Wash cells once in ice-cold PBS to remove unbound reagent.

d. Wash cells once in ice-cold PB.

e. Transfer cells to PB at 33°C for 3 minutes to lyse membranes.

f. Wash cells once in ice-cold PB.

Lyse with α-toxin

a. Dissolve α-toxin (Gibco BRL 3463SA) as instructed by supplier.

b. Add 5–10 volumes of PB and warm to 33°C.

c. Treat cells with α-toxin.

Coverslips: Immerse cells on coverslips in α-toxin/PB and incubate at 33°C for 10–30 minutes.

Microbeads: Mix 1 ml of cells in agarose beads with 9 ml of α-toxin/PB and incubate at 33°C for 10–30 minutes. Mix periodically.

d. Wash cells twice in ice-cold PB.

Cells are now ready for use in the transcription reaction.

NOTES

- Because cholesterol inhibits permeabilization by streptolysin O, it is important that samples contain no serum (which usually contains cholesterol) during binding.
- Concentrations of streptolysin O and α-toxin should be titrated to optimize permeabilization (monitored by trypan blue uptake, as described in preceding protocol).
- Permeabilization by α-toxin is relatively slow. Shorter lysis times are recommended and incubation longer than 30 minutes should be avoided.

TRANSCRIPTION IN VITRO

Cells permeabilized in PB and incubated at 33°C resume RNA synthesis once the necessary precursors are added. Unfortunately, the rate of RNA synthesis in vivo is not known, so the relative efficiency in vitro cannot be established. In the presence of optimal concentrations of triphosphates, however, permeabilized cells do synthesize DNA at the in vivo rate, and because (under optimal conditions) they synthesize tenfold more RNA than DNA, it is probable that most RNA polymerase complexes engaged prior to lysis survive permeabilization. Under these conditions, there is little or no initiation.

1. Prepare fresh PB supplemented with 0.5 mM PMSF.

   Caution: PMSF (See Appendix 5 for Caution)

2. Prepare 10× IM

   10× IM

   PB/PMSF
   500 μM CTP
   500 μM GTP
   10–500 μM modified UTP
   $MgCl_2$
   (molarity equal to molarity of added triphosphates)
   Warm to 33°C.

   Caution: $MgCl_2$ (See Appendix 5 for Caution)

3. Incubate permeabilized cells at 33°C for 5 minutes.

   Coverslips: Place in wells of 24-well tissue culture plate with 0.45 ml of PB.

   Microbeads: Mix 200 μl of beads with 250 μl of PB in a 1.5-ml microfuge tube.

4. Add 50 μl of prewarmed 10× IM and mix.

5. Incubate cells at 33°C for 1–30 minutes, as required.

6. Terminate transcription by washing the reaction mixture five times with ice-cold PB over a 30-minute period.

Samples can now be fixed and stained.

NOTES

- A range of precursors is available, but of those tested, only one, BrUTP (Sigma B 7166), is used efficiently by the endogenous RNA polymerase complex (Jackson

et al. 1993; Wansink et al. 1993). Biotin-14-CTP is also incorporated into RNA, but less efficiently (Iborra et al. 1996). Biotin-11-UTP, digoxigenin-11-UTP, fluorescein-12-UTP, coumarin-5-UTP, and lissamine-5-UTP are not incorporated (or incorporated very poorly) by permeabilized HeLa cells.

- Great care must be taken to ensure that only transcription sites are labeled. The concentration of modified precursor can be adjusted to control the rate of elongation. At 33°C in PB, 50 μM BrUTP supports an elongation rate of 50 nucleotides/minute, and then a 10-minute incubation probably ensures that >90% of the incorporated label remains at the site of polymerization (Jackson et al. 1993). If higher concentrations or longer times are used, a greater proportion of labeled RNA might move away from transcription sites.
- The elongation rate can also be reduced using a lower temperature.
- If inhibitors are to be used, incubate them for 15 minutes at 0°C and 5 minutes at 33°C prior to addition of 10× IM.

FIXATION AND ANTIBODY BINDING

Paraformaldehyde-based fixatives preserve nuclear structure well and are preferred for light microscopy, whereas glutaraldehyde-based fixatives provide more stable cross-linking and are therefore used for electron microscopy.

1. Prepare PB+ buffer by adding 0.5 mM PMSF and 2.5 units/ml HPRI (Amersham International E 2310Y) to PB (see p. 110.4).

   Caution: PMSF (See Appendix 5 for Caution)

2. Prepare 4% formaldehyde.

   Caution: Formaldehyde (See Appendix 5 for Caution)

   a. Dissolve 4 g of paraformaldehyde in 50 ml of distilled H_2O in a 50-ml tube.

   b. Heat at 60°C until dissolved; add a few drops of 2 M NaOH to help dissolve, if necessary.

   c. Cool to room temperature and add an equal volume of 2× concentrated PB+.

   d. Filter through nitrocellulose (0.22-μm pore size) and cool on ice.

3. To washed samples of cells from the preceding transcription protocol, add 1 ml of ice-cold 4% formaldehyde in PB+ to coverslips in 24-well tissue-culture plates or 200 μl microbeads in a 1.5-ml microfuge tube. Incubate on ice for 15 minutes.

4. Wash fixed cells twice in ice-cold PB+.

5. Replace PB+ with ice-cold PB+ containing 0.25% Triton X-100 and incubate on ice for 10 minutes.

6. Repeat step 3.

7. Add primary antibody to fixed cells:

   Coverslips: Cover samples with a few drops of PB+ containing 0.5% acetylated BSA (Sigma B 2518), 0.05% Tween 20, and 1/50 to 1/250 dilution anti-BrdU antibody, and incubate 1–2 hours on ice.

   Microbeads: Mix 50–100 µl of beads with an equal volume of PB+/acetylated BSA/Tween containing anti-BrdU antibody and incubate 1–2 hours on ice; mix periodically.

   Many antibodies to BrdU are commercially available. Cross-reactivities with BrUMP incorporated into RNA are indicated below (reactivity: poor [−] to good [+++]):

   Boehringer Mannheim (BMC 9318; 1170 376)+++
   Sera-Lab (clone BU1/75 MAS 250p) +−
   Becton Dickinson (clone B44; No. 7580) +/++
   Amersham RPN 202) +
   Sigma (clone BU-33; B 2531) +
   Caltag (clone BR-3) +/−
   Dako (clone Bu20a; M744)−

   These antibodies are all mouse monoclonals (IgG1) except for the product from Sera-Lab, which is a rat monoclonal. Products from other sources should be tested for binding.

8. Wash cells three times in ice-cold PB+/BSA/Tween over a 15-minute period.

9. Add secondary antibody:

   Coverslips: Cover samples with a few drops of PB+/BSA/Tween and 1/500 dilution of fluorochrome-labeled second antibody (e.g., Texas Red-donkey anti-mouse; Jackson Laboratories 715-075-137). Incubate 1–2 hours on ice.

   Microbeads: Mix 50–100 µl cells in agarose beads with 400 µl of PB+/BSA/Tween and 1/500 dilution of second antibody. Incubate 1–2 hours on ice.

10. Wash samples three times in ice-cold PB+/BSA/Tween, over a 15–30 minute period.

11. Wash samples three times in ice-cold PB+, for 5 minutes each time. To the second wash add 0.02 µg/ml **DAPI** (Boehringer Mannheim 236 276).

    Caution: DAPI (See Appendix 5 for Caution)

12. Mount samples and seal with nail polish.

    For recipe for mounting media, see Chapter 98.

Specimens are now ready for observation.

NOTES

- It is important to protect the labeled RNA from nuclease digestion. Always use DEPC-treated or molecular biology grade H_2O to prepare buffers. A ribonuclease inhibitor should also be added following transcription; adding HPRI (or equivalent) at 2.5 units/ml should be sufficient during most steps, but this can be increased to 25 units/ml during antibody binding.

- BSA *must* be essentially nuclease-free. The use of acetylated BSA in buffers is recommended; acetylation inactivates nucleases.
- Commercial reagents with cross-reactivity designated +++ or ++ should be used for routine staining. Antibodies with weaker binding will give signals close to the detection limits of unenhanced light microscopy. Weak cross-reactivities are sensitive to DTT (required by the ribonuclease inhibitor). Staining under nonreducing conditions can be beneficial (the antibody from Sera-Lab, for example, can give much improved staining in the absence of DTT).
- If antibodies with moderate cross-reactivity are used, it may be necessary to increase sensitivity using a biotin-conjugated secondary antibody followed by streptavidin coupled to a fluorochrome (Wansink et al. 1993). Texas Red-streptavidin usually gives less background staining than FITC-streptavidin.
- Standard techniques can be used to visualize transcription sites by EM (Hozak et al. 1994; Iborra et al. 1996).

LABELING IN VIVO

The distribution of transcription sites labeled in vitro can be confirmed if cells are first labeled in vivo. This can be achieved simply by growing cells in medium supplemented with bromouridine: 50 μM for 10 minutes for EM analysis (Hozak et al. 1994) or 100 μM for 15 minutes for flow cytometry (see see Chapter 16; Jensen et al. 1993). Because bromouridine added directly to cells will be incorporated into DNA as well as RNA, short labeling periods, preferably outside S-phase, should be used. However, sites of transcription can be labeled during S-phase if the DNA is not denatured.

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