CHAPTER 16

Analysis of Ribosome Loading onto mRNA Species: Implications for Translational Control

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Analysis of the association of an mRNA species with ribosomes can give great insight into, first, whether translational control of expression of the mRNA occurs and, second, possible mechanisms of regulation. Methods are described in detail for the preparation of cellular extracts, the display of ribonucleoprotein particles and polysomes by sucrose gradient centrifugation, and the detection of mRNA species of interest.

I. POLYSOME PROFILES AND TRANSLATIONAL CONTROL

Regulation of expression of the protein product of a gene can occur at several levels (for reviews, see Harford and Morris, 1997). Although the early observations of translational control in metazoic organisms were made in echinoderm eggs during activation by fertilization, it is now clear that messenger RNA (mRNA)-specific regulation of translation occurs in many somatic cells in response to a variety of stimuli (Morris, 1995, 1997; Mathews *et al.*, 1996). It is equally clear that there are many mechanisms for regulating the translational efficiency of a particular mRNA (reviewed in Hershey *et al.*, 1996; Morris, 1997).

Translation of an mRNA molecule occurs in a multimeric structure, the polysome, which consists of multiple ribosomes arrayed along the length of the mRNA (Warner *et al.*, 1963; Gierer, 1963; Wettstein *et al.*, 1963). Polysomes are readily separated according to the number of associated ribosomes ("polysome

mRNA Formation and Function
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size") by velocity sedimentation through a gradient of sucrose, which serves to stabilize the boundaries of the sedimenting zone. This experimental approach was first applied to the analysis of protein synthesis in bacteria (McQuillen *et al.*, 1959), and has been used for characterization of a variety of macromolecules and macromolecular assemblies (for example, see Martin and Ames, 1961).

Our current understanding of the state of an mRNA molecule in the cytosol is schematized in Figure 1. Molecules of mRNA can be "masked" or sequestered away from the translational apparatus through assembly into messenger ribonucleoprotein (mRNP) particles (Spirin, 1996). These particles generally sediment at 20S to 35S, somewhat more slowly than does the small ribosomal subunit. Those mRNAs that are not sequestered can be loaded with ribosomes to form polysomes. The rate of translational initiation relative to the rate of elongation determines the number of ribosomes loaded per mRNA and therefore polysome size.

Considering the scheme in Figure 1, one can readily see how the information gained from studying the location of individual mRNA species in sucrose gradients can provide considerable insight into the mechanism of translational control. To illustrate, the movement of an mRNA from mRNP particles into polysomes can readily be monitored; one example, studied in the authors' laboratory, is that of the mRNAs encoding the ribosomal proteins, which become translationally activated on mitogenic activation of vertebrate cells (Kaspar *et al.*, 1990, 1992). As a second example, if the rate of initiation is modulated, one can detect a

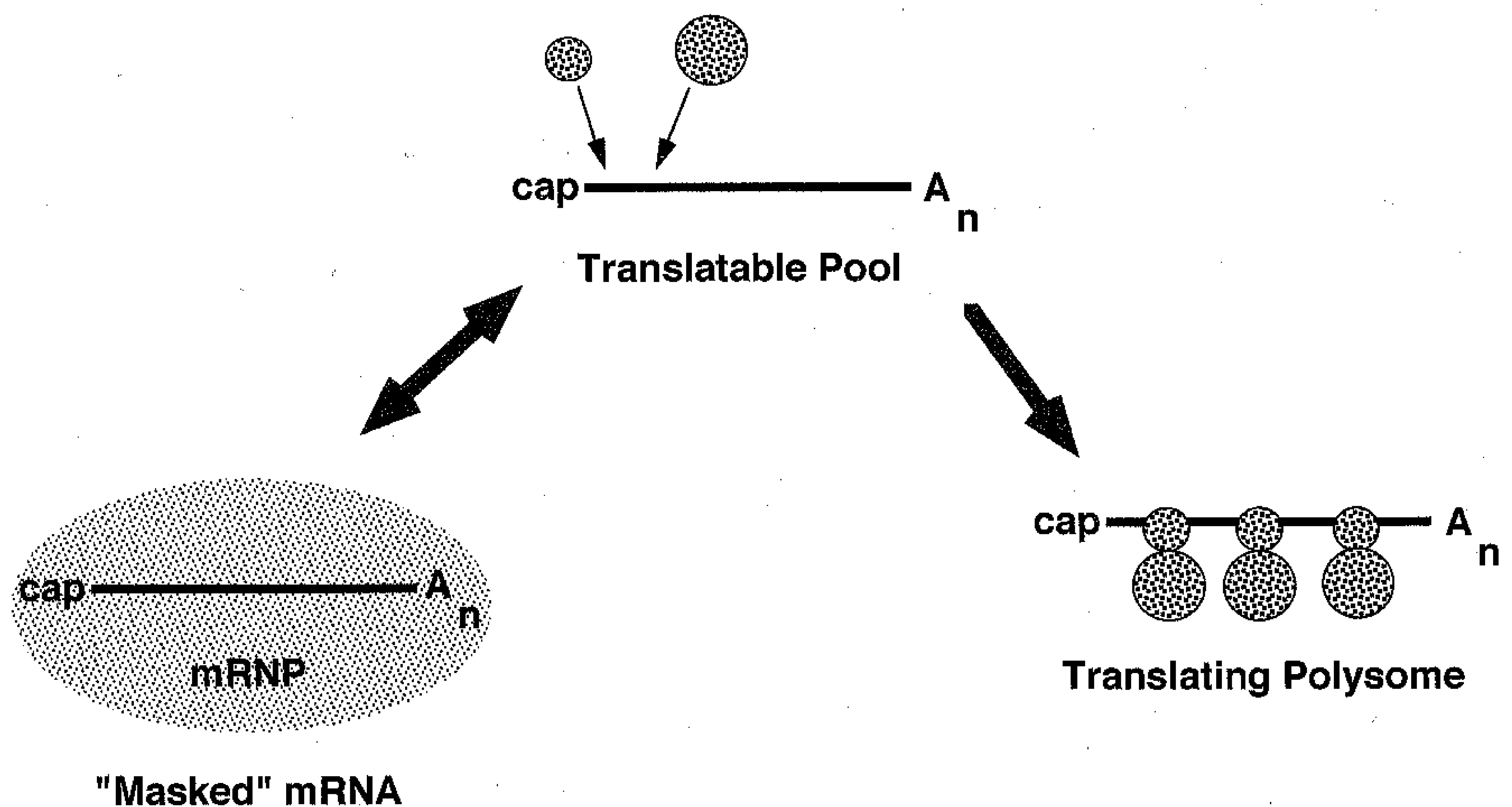

Figure 1 Functional localization of mRNA molecules in the cytosol. The pool of mRNP particles is represented by the large oval. The 40S and 60S ribosomal subunits are represented by the small and large stippled circles, respectively. mRNA molecules, with their capped 5′ termini and polyadenylated 3′ termini, are indicated by the bold horizontal lines.

change in polysome size. This situation was found with the mRNA encoding *S*-adenosylmethionine decarboxylase in response to polyamine starvation (White *et al.,* 1990; Ruan *et al.,* 1996) and also after mitogen activation of T lymphocytes (Mach *et al.,* 1986). In interpreting a change in polysome size, however, one must be sensitive to the fact that it can arise from a change in either initiation or elongation. For example, an increase in polysome size could result from increased initiation or decreased rate of elongation (discussed in Mathews *et al.,* 1996). However, when an increase in polysome size is accompanied by an increase in the rate of synthesis of the protein product, initiation must have been stimulated. In equivocal instances, or when one wants to measure the absolute rate of initiation, one should consider measuring the transit time, thus deriving the rate of elongation (Palmiter, 1975).

II. EXTRACTION AND DISPLAY OF POLYSOMES

A. Cell Harvest and Extraction

1. Materials

a. Phosphate-buffered saline (PBS): 0.14 *M* NaCl, 5 m*M* KCl, 8 m*M* Na_2HPO_4, and 1.5 m*M* KH_2PO_4, pH 7.2
b. Trypsin solution: 0.5% (w/v) trypsin and 5 m*M* disodium ethylenediaminetetraacetic acid (EDTA) in PBS
c. Stock cycloheximide (CH) solution (1000×): 100 mg/ml CH in ethanol
d. Stock phenylmethylsulfonylfluoride (PMSF) solution (100×): 100 m*M* PMSF in ethanol
e. Low-salt buffer (LSB) (10×): 200 m*M* Tris (pH 7.4–7.5), 100 m*M* NaCl, 30 m*M* $MgCl_2$
f. Detergent buffer: 1.2% (v/v) Triton N-101 in LSB

2. Methods

a. The following procedure (White *et al.,* 1990) is for a monolayer cell culture grown in a 150-mm tissue culture plate. The amount of reagents used to harvest cells can be scaled appropriately for plates of other sizes. Before harvesting, cells are replaced with fresh medium containing 100 μg CH per milliter (freshly prepared) and kept at 37°C for 10 to 15 min to immobilize the ribosomes on mRNAs. Cells are washed twice with 10 ml PBS containing 100 μg CH per milliliter (prewarmed at 37°C).

b. Add 2 ml trypsin solution and incubate at 37°C for 2–5 min. Gently tap the plate to disperse the cells. After cells are detached from the plate, add 10 ml PBS containing 1 m*M* PMSF and 100 μg CH per milliliter (prewarmed at 37°C), wash the cells off the plate, and transfer the solution to 5 ml crushed, frozen PBS containing 100 μg CH per milliter.
c. Centrifuge the cells at 4°C, 1000 *g* for 5 min, wash the cell pellets with 10 ml ice-cold PBS containing 100 μg CH per milliliter, and centrifuge again.
d. Drain the tube for 5 sec and put it on ice. Resuspend the cell pellet in 750 μl LSB at 4°C, gently dispersing the cells into the solution by pipetting. Swell the cells on ice for 3 min.
e. Add 250 μl detergent buffer and immediately transfer the solution to a 7-ml Dounce homogenizer on ice. Lyse the cells with eight strokes of the homogenizer. Transfer the solution to an ice-chilled 15-ml Falcon tube and centrifuge at 4°C, 10,000 *g*, for 1 min. Transfer the supernatant to a 1.5-ml microfuge tube containing 100 μl of 10 mg heparin per milliliter and 1.5 *M* NaCl in LSB. Cell lysates should immediately be layered onto sucrose gradients for centrifugation.
f. For cells in suspension culture, the procedure (Degen *et al.*, 1983) for cell harvesting is modified as follows. Cultures are poured into 50-ml conical tubes containing one fifth of a volume of frozen, crushed PBS and a final concentration of 100 μg CH per milliliter. The chilled cultures are centrifuged at 1000 *g* for 5 min at 4°C. Cell pellets are washed with 10-ml ice-cold PBS and centrifuged again at 1000 *g* for 5 min at 4°C. The cell pellets are extracted as in step d.

B. Sucrose Gradient Centrifugation and Polysome Fractionation

Two different sucrose gradients can be employed, depending on the region of interest. The 0.5- to 1.15-*M* sucrose gradient gives better separation in the region of the mRNP particles, 40S, 60S subunits, and 80S monosomes. However, there is loss of resolution in the large polysome region, which collects on a layer of dense sucrose at the bottom of the tube. The 0.5- to 1.5-*M* sucrose gradient is used to analyze the broad distribution of ribosomes from monosomes through large polysomes.

1. Materials

a. 0.5 *M* (15%) sucrose: 8 g sucrose in 42 ml LSB
b. 1.15 *M* (35%) sucrose: 17 g sucrose in 33 ml LSB

c. 1.5 *M* (50%) sucrose: 21.5 g sucrose in 28.5 ml LSB
d. 2 *M* (70%) sucrose: 28.5 g sucrose in 21.5 ml LSB
e. 10% (w/v) sodium dodecyl sulfate (SDS)
f. Protease K (20 mg/ml)

2. Methods

a. *Preparation of sucrose gradients.* To Beckman 14 × 95-mm tubes (Part No. 331374 from Beckman Instruments, Fullerton, CA), add 5.5 ml of 1.5 *M* (or 5 ml of 1.15 *M*) sucrose solution to the bottom of the tube. Then carefully layer 5.5 ml of 0.5 *M* (or 5 ml when preparing 0.5- to 1.15-*M*) sucrose solution onto the top, without disturbing the interface of the two gradients. Wrap the top of the tube tightly with parafilm. Carefully lay the tube **horizontally** in a 4°C walk-in cold room (not in a refrigerator since it vibrates). The gradient is ready for use after 6–24 hr of storage (R. Kaspar, personal communications). For a 0.5- to 1.15-*M* sucrose gradient, 1 ml of 2 *M* sucrose solution is layered at the bottom of the tube just before use. This is done by gently sliding the tip of a long Pasteur pipette along the tube wall to the bottom of the tube and slowly releasing 1 ml of 2 *M* sucrose solution.

b. *Sucrose gradient centrifugation.* Carefully layer cell extracts (prepared as described above) onto the top of the sucrose gradient without disturbing the interface and centrifuge in a Beckman SW 40 rotor at 4°C. For the 0.5- to 1.15-*M* gradient, centrifuge at 36,000 rpm for 220 min; for the 0.5- to 1.5-*M* sucrose gradient, centrifuge at 36,000 rpm for 110 min.

c. *Gradient fractionation.* Centrifuged gradients are fractionated into 12 1-ml fractions using an ISCO Density Gradient Fractionator (Model 185, ISCO, Inc., Lincoln, NE) at a flow rate of 3 ml/min. The fractionator is equipped with an ultraviolet (UV) monitor, and the polysome profile of the gradient is monitored via UV absorbance at 254 nm (shown in Fig. 2). The fractions are collected into 1.5-ml microfuge tubes containing 100 μl 10% SDS. Each sample is digested with 11 μl protease K solution at 37°C for 30 min. Samples can be stored at −70°C for several months prior to analysis.

3. Troubleshooting

Unsatisfactory polysome patterns can arise either from "runoff" of ribosomes during harvesting and extraction of the cells or from degradation by RNase activity present in cell extracts or reagents. Both of these results are manifested in a decrease in the ratio between polysomes and monosomes/subunits (Fig. 3) relative to a normal polysome profile (compare with Fig. 2). Large polysomes disappear and absorbance in the regions of small polysomes, monosomes, and ribosome subunits increases. This problem can often occur during analysis of monolayer cells, where rapid harvesting in the absence of runoff becomes a challenge, or in cell preparations

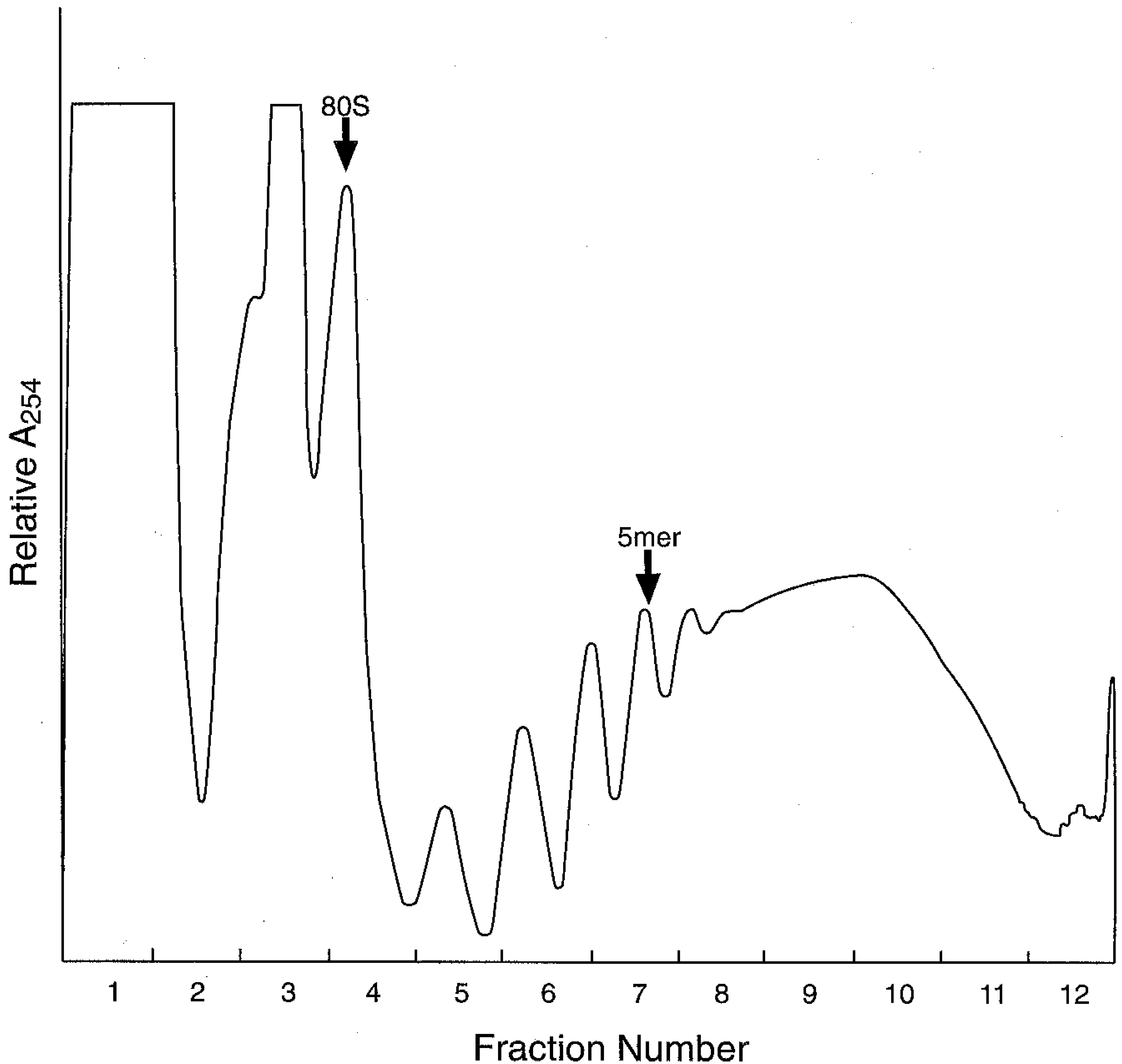

Figure 2 The UV absorbance profile at 254 nm of material sedimented through the 0.5- to 1.5-*M* sucrose gradient. Arrows indicate the positions of monosomes (80S) and pentasomes (5-mer).

like normal lymphocytes with high endogenous RNase activity. In attempting to remedy this situation, there are two possible causes to explore: (1) ribosomes must be immobilized by CH at the time of cell harvest or (2) cells must be cooled as quickly as possible after the harvest. If analysis of mRNA (below) suggests degradation by RNase, the inhibitory activity of the heparin in the polysome buffer can be supplemented by addition of diethylpyrocarbonate (0.2 μl/ml).

C. RNA Isolation from Fractionated Samples

1. Materials

a. Acidic phenol (buffered with 0.25 *M* sodium acetate, pH 5.0)
b. Chloroform : isoamyl alcohol mix (24 : 1)

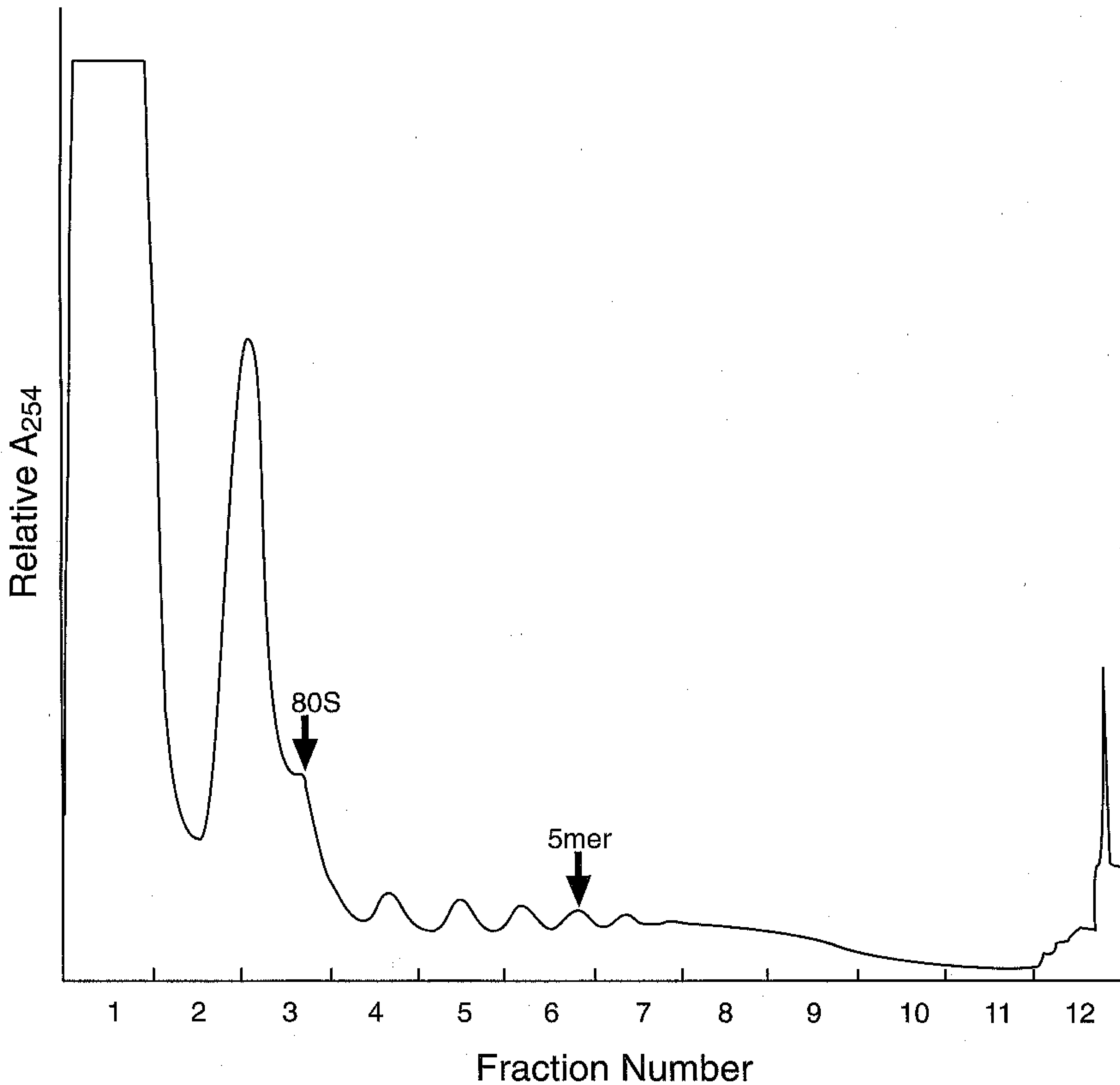

Figure 3 The UV absorbance profile of a 0.5- to 1.5-*M* sucrose gradient at 254 nm of an extract showing either runoff or degradation. Indicated by arrows are the peaks of the monosome (80S) and the pentasome (5-mer) region.

c. 3 *M* sodium acetate (pH 5.0)
d. Glycogen (2 mg/ml)
e. Ethanol and 75% ethanol

2. Methods

a. Add 0.50 volume of acidic phenol to the RNA sample and vortex twice for 30 sec. Add another 0.50 original volume of chloroform : isoamyl alcohol mix, vortex again, and centrifuge in a microcentrifuge at 4°C for 5 min.
b. Transfer the aqueous (upper) phase to a clean tube, avoiding precipitated material from the interface. Repeat step a.

c. Transfer the aqueous phase to a clean tube. A final concentration of 20 μg glycogen per milliliter is added as a carrier, and the RNA is precipitated at $-20°C$ for 30 min with 0.1 volume of 3 *M* sodium acetate (pH 5.0) and 2.5 volumes of ethanol.
d. The RNA pellet is collected by centrifugation at 10,000 *g* for 30 min at 4°C and washed with 70% ethanol.
e. The RNA pellet is dried for 5 min in a fume hood and resuspended in RNase-free H_2O.

III. RNA ANALYSIS BY NORTHERN BLOTS

A. Materials

1. Stock running buffer (20×): 0.2 *M* 3-(*N*-morpholino)propanesulfonic acid (MOPS), 80 m*M* sodium acetate, 10 m*M* EDTA (pH 8.0).
2. 1.2% Agarose/formaldehyde gel (total volume 100 ml): 1.2 g agarose, 5 ml stock running buffer, 77 ml H_2O and autoclave or microwave to melt. Add 18 ml 37% formaldehyde.
3. Gel electrophoresis buffer (total volume 1.5 liters): 75 ml stock running buffer, 270 ml 37% formaldehyde, 1155 ml H_2O.
4. Stock loading buffer (10×): 60% glycerol, 50 m*M* EDTA, 0.05% bromophenol blue, 0.05% xylene green.
5. Formamide mix (total volume 266 μl): 180 μl formamide, 54 μl 37% formaldehyde, 18 μl stock running buffer, 4 μl ethidium bromide solution (2.4 mg/ml, 10 μl stock loading buffer.
6. Northern hybridization solution: QuikHyb™ solution (Stratagene, La Jolla, CA).
7. Stock standard saline citrate (SSC) buffer (20×): 175.3 g NaCl and 88.2 g sodium citrate in 1 liter H_2O (pH 7.0).

B. Agarose/Formaldehyde Gel Electrophoresis

1. The volumes of solutions are given for a 16 × 14-cm gel. The volumes should be scaled according to gel size. Pour 1.2% agarose/formaldehyde gel and allow it to set for 30 min.
2. Add an RNA sample to equal the volume of the formamide mix. Mix the samples by vortex, spin briefly in a microcentrifuge, and incubate for 5 min at 60°C.
3. Spin samples briefly and load them onto the gel. Run the gel at ~6 V/cm for about 3 hr or until the bromphenol blue band has migrated near the bottom of the gel.

C. Transfer to Membranes

1. Cut out the portion of the gel to be transferred. Soak it in water for 20 min to remove the formaldehyde. In the meantime, cut a piece of Duralose™ membrane (Stratagene) to the same size as the trimmed gel. Soak the membrane in water for 5 min and then in 20× SSC for 5 min.
2. Set up the transfer apparatus by placing a sponge in a tray filled with 20× SSC. Cover the sponge with two pieces of filter paper. Layer the gel upside down on top of the paper. Remove air bubbles by rolling a glass pipette across the gel. Place the Duralose membrane on the gel, avoiding any air bubble between them. Put strips of parafilm around the edges of the gel to prevent evaporation of the 20× SSC.
3. Wet three sheets of the same size filter paper in 20× SSC and place them on the Duralose membrane. Place a stack of paper towels (~5 cm thick) on the filter paper and, finally, stack your favorite book to add enough pressure (for details, see Sambrook *et al.*, 1989).
4. After a minimum 24-hr transfer period, remove the membrane, place it on a piece of water-dampened filter paper, and UV cross-link the membrane (UV Stratalinker, Stratagene). Rinse the membrane in water for 20 min.

D. Hybridization

1. Place the membrane into a "Seal-a-Meal" bag. Add ~10–15 ml of well mixed, prewarmed Quikhyb solution to the bag and seal the bag (usually a minimum of 40 μl of QuikHyb solution per square centimeter of membrane is needed). Prehybridize the membrane at 68°C for 20–30 min in a slowly shaking water bath.
2. Label the DNA fragment with [α-^{32}P]deoxycytosine triphosphate (dCTP) using a random-primed DNA-labeling kit according to the manufacturer's instructions (e.g., Boehringer Mannheim, Indianapolis, IN). The radiolabeled probe is purified by either (a) phenol/chloroform extraction and ethanol precipitation or (b) Elutip-D column purification according to the manufacturer's instructions (Schleicher & Schuell, Keene, NH). Take an aliquot of probe and measure the radioactivity. Calculate the total incorporation of [α-^{32}P]dCTP and the specific radioactivity of the probe. The specific radioactivity for a good probe should be around 1×10^9 cpm per microgram of template.
3. Take an appropriate amount of probe and mix it with 100 μl of sonicated salmon sperm DNA solution (10 mg/ml). The suggested

probe concentration for hybridization is 1.0–1.5 $\times$ 10^6 cpm per milliliter of Quikhyb solution. Denature the probe by placing it in a boiling water bath for 5 min. Immediately inject the probe into the hybridization bag using a needle and syringe. Seal the bag again and hybridize at 68°C for 1.5–2 hr in a slowly shaking water bath.

4. After hybridization, the blots are washed three times for 15 min each at room temperature with 2$\times$ SSC and 0.1% (w/v) SDS, followed by two washes for 30 min each at 60°C with 0.1$\times$ SSC buffer and 0.1% (w/v) SDS.
5. Place the membrane on a piece of filter paper and wrap it in plastic wrap. Expose the membrane on X-ray film with an intensifying screen at −80°C for an appropriate period (e.g., see Fig. 4).

E. Troubleshooting

1. *Poor quality of RNA samples.* RNA can be easily degraded by RNase contamination. RNA samples should be processed promptly whenever possible. SDS at a concentration of 0.1% can be included as an RNase

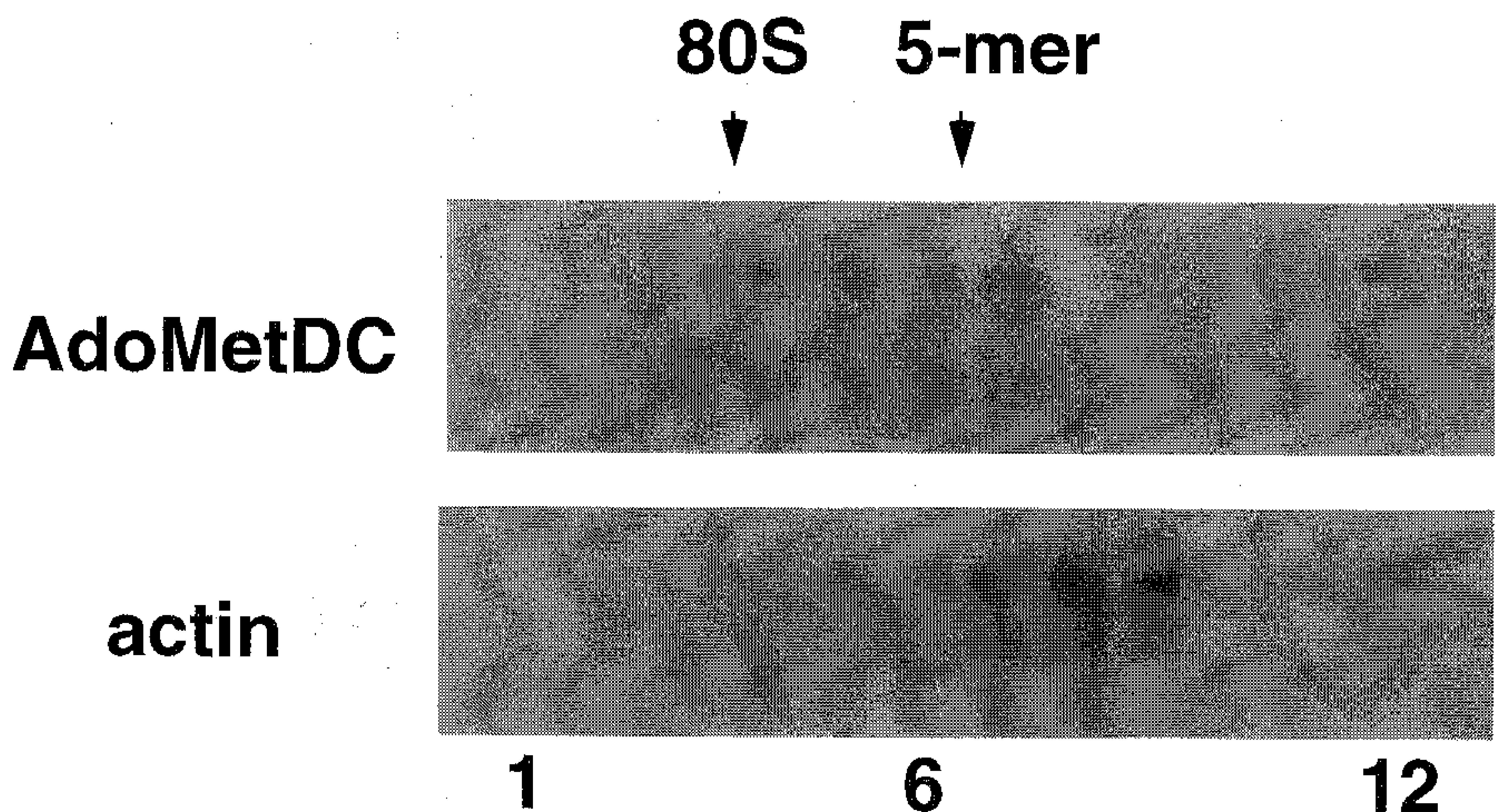

Figure 4 Distribution of mRNAs encoding *S*-adenosylmethionine decarboxylase (AdoMetDC) and actin in polysomes from Jurkat cells using Northern blot analysis. Cytoplasmic extracts from Jurkat cells were prepared and centrifuged through a 0.5- to 1.5-*M* sucrose gradient. The gradient was fractionated from top (fraction 1) to bottom (fraction 12). The positions of monosome (80S) and pentasome (5-mer) peaks are determined from the UV absorbance profile of the sucrose gradient at 254 nm (see Fig. 2) and are indicated by the arrows.

inhibitor when the RNA is resuspended in water (step e in Section II,C).

2. *Poor RNA transfer from gel to blot.* The most common problem is air bubbles trapped between the gel and the blot. Also, adequate time (at least 24 hr) for transfer is important for efficient transfer.
3. *Poor quality or impure DNA probe.* This is manifested by bad background radioactivity on the membranes after hybridization. ^{32}P-Labeled probe should be purified from free ^{32}P-dCTP and other contaminations, and the specific activity of a probe should be around 10^9cpm per microgram of template.
4. *Concentration of the probe during hybridization.* The concentration of a DNA probe should be around $1.0–1.5 \times 10^6$cpm per milliliter of hybridization solution.
5. *Insufficient mixing during hybridization.* The hybridization buffer should be constantly mixed with the blot. This can be done by incubating the hybridization bag in a slowly shaking water bath.
6. *Insufficient washing of the blot.* After hybridization the blot should be washed extensively, as specified in the manufacturer's instructions, to remove most of the background radioactivity.

IV. ANALYSIS BY RNase PROTECTION

A. *In Vitro* Transcription Reaction and Preparation of Probe

1. Materials

a. 5× T7 polymersase buffer: 200 m*M* Tris-HCl (pH 7.5), 50 m*M* $MgCl_2$, 10 m*M* spermidine
b. 5× T3 polymersase buffer: 200 m*M* Tris-HCl (pH 7.5), 250 m*M* NaCl, 40 m*M* $MgCl_2$
c. 5× SP6 polymerase buffer: 200 m*M* Tris-HCl (pH 7.5), 30 m*M* $MgCl_2$, 10 m*M* spermidine
d. TE: 10 m*M* Tris-HCl (pH 8.0), 1.0 m*M* EDTA, (pH 8.0)
e. Elution buffer: 0.5 *M* ammonium acetate, 0.1 m*M* EDTA, 0.1% SDS
f. DNA template: Digest to completion (check by agarose gel electrophoresis) 2–4 μg plasmid DNA harboring the desired sequence with a restriction enzyme that will result in DNA bearing 5′ protruding termini and at a site located 80–500 nucleotides from the bacteriophage promoter (T7, SP6, or T3). Extract with TE-saturated phenol/chloroform (1 : 0.8) and precipitate using RNase-free ethanol.

Resuspend in 30–40 μl RNase-free TE (volumes are empirical based on preliminary experiments).

2. Methods

a. For a 20-μl reaction, pipette the following components (all should be RNase-free) into the bottom of a microfuge tube and mix gently with the tip, always adding the template last (Goodall and Filipowicz, 1989). Incubate at 37°C for 1 hr.

5× T7, SP6, or T3 buffer	4 μl
500 m*M* dithiothreitol (DTT)	1 μl
100 μ*M* UTP	1 μl
2.5 m*M* ribonucleotides (minus UTP)	4 μl
RNase inhibitor (40 U/ml)	1 μl
[α-^{32}P]UTP (3000 Ci/mmol)	4 μl
Bovine serum albumin (2.55 mg/ml)	1 μl
T7, T3, or SP6 RNA polymerase	1 μl (10–20 units)
DNA template	3 μl (approx. 400 ng)

b. Digest the DNA template by adding 1 μl DNase (RNase free) and incubating at 37°C for 15 min. Add 200 μl TE, 1 μl transfer RNA (tRNA), 134 μl 5 *M* ammonium acetate, and 840 μl ethanol and precipitate the probe at −20°C for 20 min (or overnight).
c. Centrifuge at 12,000 rpm (15,850 *g*) for 25 min, wash the pellet with 70% ethanol, and dry it very briefly. All solutions should be RNase free.
d. The probe may then be phenol-extracted for use, but to ensure that background is minimal and to check the integrity of the probe, it is advisable to purify the probe by gel electrophoresis.
e. Resuspend the barely dry probe in 5 μl formamide loading buffer, vortex, denature at 95°C for 5 min, and resolve on a 6% denaturing polyacrylamide gel.
f. Remove the upper glass plate, place Saran Wrap over the gel, label it with fluorescent markers, and expose it to X-ray film for 90 sec.
g. Align the film and gel by the markers and excise the band. Place the acrylamide fragment in a microfuge tube, pellet the fragment by centrifuging for a few seconds, and then crush the acrylamide by repeatedly macerating with a round-ended yellow pipette tip, which enhances elution of the probe from the acrylamide. (Tips can be round-ended by briefly applying them to a flame and allowing the end to round over to create a smooth, round end of a size that will fit

snugly into the bottom of a microfuge tube.) Elute the probe from the gel at room temperature for 1.5–2 hr in 300 μl of elution buffer.

h. Centrifuge at 12,000 rpm (15,850 *g*) for 5 min to pellet the acrylamide. Decant 200 μl of the probe into a new microfuge tube. Add 84 μl 5 *M* ammonium acetate, 1 μl tRNA (10 mg/ml), and 696 μl ethanol, incubate at −20°C for 15 min, and microfuge at 4°C for 15 min. Wash the pellet with 70% ethanol, dry it very briefly, and resuspend it in 100 μl RNase-free H_2O.
i. Cerenkov count 1–5 μl and add approximately 20,000–100,000 cpm to each RNA sample (10–20 μg).
j. Dry the probe and RNA, add 10 μl of hybridizing solution (see step c in Section IV,A,2), denature at 80°C for 5 min, and hybridize for at least 16 hr at approximately 45°C (may vary depending on the probe).

3. Hints

a. Always make sure that the components of the transcription mix are added to the bottom of the tube and do not vortex; just mix with the tip.
b. Multiple probes may be simultaneously hybridized with RNA samples after preparation of templates that will protect RNAs of different sizes.
c. To save time, the probe may be resuspended in 100 μl hybridizing solution and, after counting, a reaction mix made with the hybridizing solution so that the appropriate radioactivity of probe per RNA sample is present in 10 μl. This is then added to the appropriate quantity of dry RNA.
d. For low-abundance RNAs or where RNA is present in very limited quantities, rather than adding more counts of probe, hybridize for 2 nights. As little as 2 μg of total RNA has been used to detect low-abundance mRNAs by using this approach.

4. Troubleshooting

a. Low *in vitro* transcription probe yield. (i) Transcription components not added to the bottom of the tube; (ii) probe sticking to the sides of the tube and not redissolving; do not overdry the probe at any time, but instead use a different brand of microfuge tube or siliconize the tubes if the problem persists; (iii) one or more components of the reaction mix has lost activity; (iv) radionuclide was incorporated inefficiently into the probe; check the activity date and quality; (v) The probe was not efficiently eluted from the acrylamide gel slice;

always check for the presence of probe in the elution buffer after elution.

b. No discrete band on the purification gel. Check the quality of the *in vitro* transcription components.

c. Probe of incorrect length synthesized. This may occur if restriction enzymes generating 3′ protruding termini are used to generate the template. Occasionally, aberrant initiation occurs with other enzymes, so use another restriction enzyme.

B. RNase Protection Assay

This assay uses Promega (Madison, WI) RNase ONE enzyme (refer to the manufacturer's technical bulletin). Several other RNase enzymes can be used, but this one obviates the need for a phenol/chloroform extraction and thus saves a considerable amount of time. This is particularly advantageous when dealing with a large number of samples.

1. Materials

a. Hybridizing solution: 40 m*M* 1,4-piperazinebis(ethanesulfonic acid) (PIPES) (pH 6.7), 0.4 *M* sodium chloride, 1 m*M* EDTA, 80% formamide

b. RNase buffer: 10 m*M* Tris-HCl (pH 7.5), 5 m*M* EDTA, 200 m*M* sodium acetate

2. Methods

a. Add 100 μl of RNase ONE solution (containing 1–10 units of enzyme; must be optimized by initial titration experiments) and incubate for 40 min at 37°C (optimize as necessary).

b. Add 1.6 μl 10% SDS and 1 μl tRNA (10 mg/ml), vortex, and then add 275 μl 100% ethanol. Precipitate at −20°C for 30 min and centrifuge at 12,000 rpm (15,850 *g*) at 4°C for 15 min.

c. Carefully remove the supernatant (pellets tend to float) and wash with 70% ethanol.

d. Dry, resuspend, in 4 μl formamide loading solution, vortex, denature at 95°C for 5 min, and resolve on a 6% denaturing acrylamide gel. Fix and dry the gel. Detect RNA by autoradiography or phosphorimaging (an example of an RNase protection assay is shown in Fig. 5).

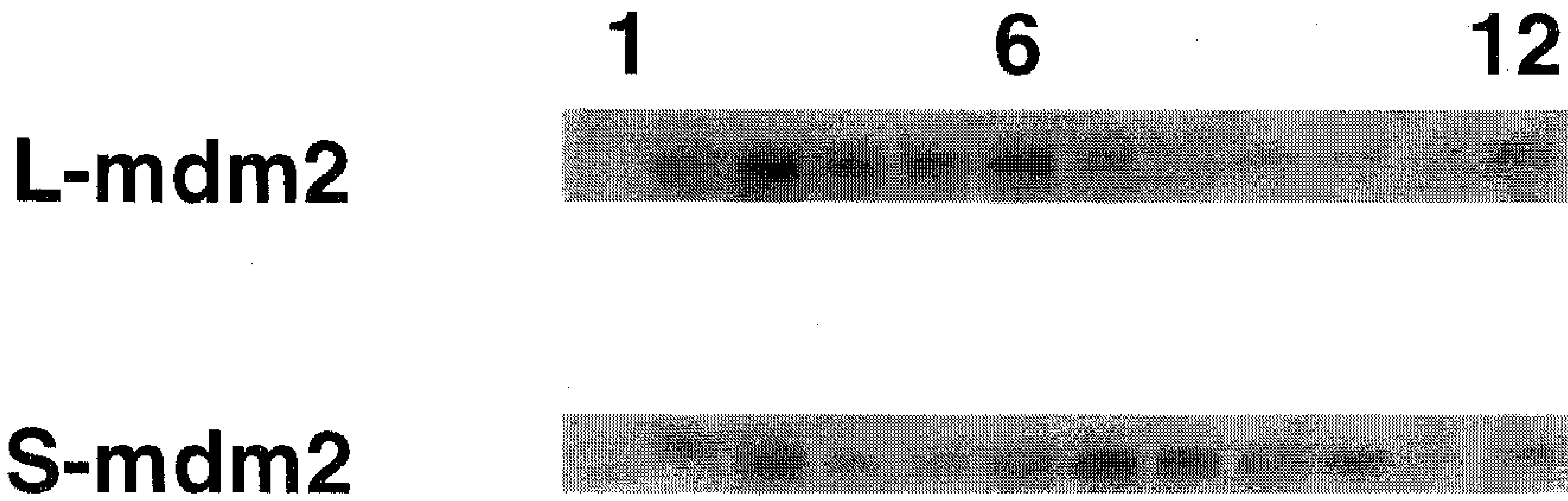

Figure 5 RNase protection assay indicating the presence of two forms of *mdm-2* RNA. RNA was isolated from cytoplasmic extracts of JAR choriocarcinoma cells, loaded onto 0.5- to 1.5-*M* sucrose gradients, and, after centrifugation, fractionated from top (1) to bottom (12) into 12 fractions. Protected bands from the long form (L-mdm2, poorly translated) and the short form (S-mdm2, well translated) are shown.

3. Hints

a. Always carry out initial titration experiments of the RNase enzyme with each new probe. Keep the hybridizing conditions consistent for each RNase protection assay. This eliminates a great deal of troubleshooting later on.
b. Always run a lane on the gel containing full-length probe to check the quality of the probe and to indicate the difference between full-length and RNase-digested products.
c. Always run a negative control containing tRNA to ensure the absence of contaminating bands.
d. If possible, run a positive control to check that the assay was successful.

4. Troubleshooting

a. *Extensive background or smear on electrophoretic gel.* (i) Degradation of the probe: check the quality of the probe by gel electrophoresis; (ii) degradation of the RNA: check the quality of the RNA by agarose gel electrophoresis and staining with ethidium bromide.
b. *Presence of additional bands on RNase protection assay gel.* (i) Sequence mismatch between the probe and the target RNA; check the sequences and use a lower concentration of RNase; alternatively, remake the probe, using a region of perfect base pairing with the target RNA; (ii) probe template DNA present; repeat DNase treatment of the probe; (iii) other complementary sequences present in the RNA sample; try making a probe from another region;

(iv) noncomplementary sequences not digested; titrate the RNase enzyme to optimize digestion conditions and check the tRNA control lane for complete absence of bands; (v) self-complementarity of the probe; try a different probe or increase the annealing and reaction temperatures; (vi) contamination of the reaction mix; run a tRNA control to check for the presence of contamination.

c. *Expected band absent on RNase protection assay gel.* (i) Poor probe quality; gel-purify the probe sample; (ii) overdigestion of the sample; titrate RNase enzyme in the initial experiments, and reduce the time/temperature of RNase digestion; (iii) mismatches between probe and target; check the template and probe for sequence mismatches; (iv) probe sensitivity too low; make sure that the probe is freshly prepared; if the RNA of interest is of low abundance, try increasing the cpm of the probe to be hybridized with the sample RNA or hybridizing for a longer time; (v) lost RNA pellet.

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