Virus-Specific Messenger RNA and Nascent Polypeptides in Polyribosomes of Cells Replicating Murine Sarcoma-Leukemia Viruses

(hybridization/RNA-directed DNA polymerase/membrane-bound polysomes/immunoprecipitation)

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Communicated by Rita Levi-Montalcini, April 26, 1973

ABSTRACT We present evidence that virus-specific RNA is present in polyribosomes of transformed cells replicating the murine sarcoma-leukemia virus complex and that it serves as messenger RNA for the synthesis of viral-coded proteins. Both virus-specific RNA (detected by hybridization with the [³H]DNA product of the viral RNA-directed DNA polymerase) and nascent viral polypeptides (measured by precipitation with antiserum to purified virus) were found in membrane-bound and free polyribosomes. Membrane-bound polyribosomes contained a higher content of both virus-specific RNA and nascent viral polypeptides. From 60 to 70% of viral RNA sequences were released from polyribosomes with EDTA, consistent with a function as messenger RNA. Maximum amounts of both virus-specific RNA and nascent viral polypeptides were found in the polyribosome region sedimenting at about 350 S.

Cells transformed by RNA tumor viruses possess virus-specific RNA sequences (1-5), but direct proof for their function as mRNA in translation is lacking. To understand the role of virus-specific RNA in the synthesis of viral structural proteins, we have studied different size classes of free and membrane bound polyribosomes in rat cells transformed by murine sarcoma virus that produce the murine sarcoma-leukemia virus complex [MSV(MLV)]. Virus-specific RNA was detected by molecular hybridization with the [³H]DNA product of the viral RNA-directed DNA polymerase. Nascent viral polypeptides were detected by immunological precipitation with antiserum directed against purified virus (6). Our results suggest that virus-specific RNA functions as mRNA for the synthesis of virion polypeptides in free and membrane-bound polyribosomes.

MATERIALS AND METHODS

Buffers. The following buffers were used: Tris buffer [20 mM Tris·HCl (pH 7.5) at 4°-50 mM KCl-5 mM MgCl₂]; RSB-CC [10 mM Tris·HCl (pH 7.5) at 4°-10 mM NaCl-1.5 mM MgCl₂-3.6 mM CaCl₂-200 μg/ml of cycloheximide]; phosphate-buffered saline (137 mM NaCl-2.7 mM KCl-8.1 mM Na₂HPO₄-1.47 mM KH₂PO₄); TKM [50 mM Tris·HCl (pH 7.5) at 20°-25 mM KCl-5 mM MgCl₂]; puromycin buffer [1 mM puromycin, 1 M KCl, 50 mM Tris·HCl (pH 7.5) at 4°, 5 mM MgCl₂, 100 μg/ml of bovine-serum albumin]; Na

dodecyl sulfate buffer [10 mM Tris·HCl (pH 7.0) at 25°-100 mM NaCl-1 mM EDTA-0.5% Na dodecyl sulfate]. All solutions were sterilized by autoclaving except bovine-serum albumin.

Cells and Viruses. Transformed rat cells (78A1) producing M-MSV(MLV), uninfected Wistar rat cells, and rat cells transformed by adenovirus 2 (8617), were grown as described (6, 7). Labeled M-MSV(MLV) was isolated from the culture medium of 78A1 cells grown for 48 hr in phosphate-free Eagle's medium containing 100 μCi/ml of carrier-free ³²P or in Eagle's medium with 10 μCi/ml of [³H]uridine (40-50 Ci/mmol, NEN Chemicals) (6, 8).

Isolation of Free and Membrane-Bound Polyribosomes. Unlabeled polyribosomes were isolated from confluent monolayers of 78A1 cells, and labeled polyribosomes from 78A1 or 8617 cells grown in suspension. To obtain maximum yields of undegraded polyribosomes and to avoid "runoff" (9-13), we took the following precautions: cultures were fed 4 hr before harvesting (9, 10), 200 μg/ml of cycloheximide was added during harvesting (13) and homogenization of cells, and a 150,000 × *g* rat-liver supernatant (S-3) was added throughout the isolation (14).

Polyribosomes were isolated by two procedures (at 2-4°), *direct layering* and *pelleting*. Direct layering was used when quantitative recovery was desired, especially for isolation of small quantities of nascent polypeptides. Pelleting was used in experiments requiring high resolution and a larger proportion of heavier polysomes. (a) *Direct layering*: Cells were washed with phosphate-buffered saline containing cycloheximide (200 μg/ml), swollen for 15 min in five volumes of RSB-CC plus S-3, and homogenized with 10-20 strokes of a Kontes Dounce homogenizer (B pestle). Nuclei and cell debris were removed by centrifugation at 750 × *g* for 10 min. The post-nuclear supernatant was centrifuged at 27,000 × *g* for 5 min to separate the microsome fraction containing membrane-bound polyribosomes (15, 16) and the supernatant fluid containing free polyribosomes. The microsome fraction was solubilized with 1% sodium deoxycholate and 1% Nonidet P40 to liberate the polyribosomes. (b) *Pelleting*: Free and membrane-bound polyribosome fractions, obtained as described above, were layered onto a discontinuous sucrose gradient (14) containing S-3 and centrifuged at 36,000 rpm in an SW41 rotor or at 45,000 rpm in a titanium 50 rotor of a Spinco ultracentrifuge for 12-18 hr at 4°. The pellet was suspended in Tris buffer containing 0.1 volumes of S-3, and clarified by centrifugation at 750 × *g* for 5 min.

Abbreviations: M-MSV(MLV), Moloney strain of murine sarcoma-leukemia virus; RSB-CC, reticulocyte standard buffer with CaCl₂ and cycloheximide.

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FIG. 1. Virus-specific RNA in free and membrane-bound polyribosomes. Free (A) and membrane-bound (B) polyribosomes were isolated by pelleting. About 4 $A_{260\text{ nm}}$ units of free and 1.2 $A_{260\text{ nm}}$ units of membrane-bound polyribosomes were layered onto 15–45% (w/v) sucrose gradients in Tris buffer and centrifuged for 75 min at 36,000 rpm in a SW41 rotor. Fractions (0.5 ml) were collected with continuous UV monitoring with a Gilford recording spectrophotometer. RNA was extracted from each fraction and hybridized with MSV-(MLV) [^3H]DNA product (2000 dpm) (2). Sedimentation is from right to left.

Release of mRNA from Polyribosomes. Messenger RNA was released from free and membrane-bound polyribosomes (prepared by “pelleting”) by suspension in Tris buffer containing 25 mM EDTA and 0.1 volumes of S-3.

Extraction of RNA. Pooled or individual fractions of polyribosomes resolved by zonal centrifugation were precipitated with two volumes of ethanol in the presence of 30 μg of yeast RNA and dissolved in Na dodecyl sulfate buffer. The solution was stirred with a Vortex mixer for 1 min with an equal volume of water-saturated phenol, and for 30 sec with an equal volume of chloroform-isoamyl alcohol 24:1. After a centrifugation at low speed, RNA was precipitated from the aqueous phase with two volumes of ethanol at -20° overnight and dissolved in 15 mM NaCl–1.5 mM Na_3 citrate.

[^3H]DNA Product was prepared by the endogenous, RNA-directed DNA polymerase reaction (1) of detergent-disrupted M-MSV(MLV) with [^3H]TTP (11–12 Ci/mmol, New England Nuclear Corp.), using conditions that transcribe most (80–90%) of the viral genome (Green, unpublished data).

Hybridization. Polyribosomal RNA was annealed with [^3H]DNA (2000 dpm) in 300 mM NaCl–30 mM Na_3 citrate (pH 5.5) at 66° for 18 hr. The formation of DNA–RNA hybrids was measured by batch elution from hydroxyapatite (2).

Labeling of Polyribosomes. 3 Liters of 78A1 cells were centrifuged and incubated in 500 ml of leucine-free Eagle’s medium plus 5 $\mu\text{Ci/ml}$ of [^3H]leucine (30–50 Ci/mmol, New England Nuclear Corp.) for 30 sec. Incorporation was stopped with $1/5$ volume of crushed frozen phosphate-buffered saline containing cycloheximide (200 $\mu\text{g/ml}$). Polyribosomes were isolated as described above.

Release of Nascent Polypeptides. Polyribosome fractions from sucrose gradients were centrifuged at 45,000 rpm in a Spinco Ti50 rotor for 12–18 hr and suspended in 0.2 ml of puromycin buffer (17). After incubation at 0° for 15 min, the preparation was layered over 4.0 ml of 1.0 M sucrose and centrifuged for 1 hr at 40,000 rpm in a Spinco Ti50 rotor. The upper layer containing released polypeptides was used for immunoprecipitation.

Immunoprecipitation. Preparations of nascent polypeptides from free or membrane-bound polyribosomes were incubated with antiserum against MSV(MLV) in the presence of detergent-disrupted MSV(MLV) (15–20 μg of protein) and 0.05% Na dodecyl sulfate (6). The antiserum against MSV(MLV) was obtained from rabbits inoculated with purified MSV(MLV) after disruption with Na dodecyl sulfate and was absorbed with an extract of normal rat-embryo fibroblasts. Details of specificity and characterization of antiserum against MSV(MLV) have been described (6). As controls, corresponding fractions of nascent polypeptides from labeled 8617 cells were incubated with the same antiserum. Immunoprecipitates were dissolved in 10% Na dodecyl sulfate and counted. The radioactivity of immunoprecipitates from 78A1 cell polypeptides were corrected for nonspecific precipitation by subtraction of the corresponding values from 8617 cells.

Measurement of Radioactivity. Nitrocellulose membrane filters containing radioactive hybrids were dried and counted in 10 ml of toluene containing 0.4% 2,5-bis(2-5-tert-butylbenzoxazolyl-thiophene) (BBOT). Aliquots of sucrose gradient fractions and immunoprecipitates (100 μl) in 10% Na dodecyl sulfate were counted in 10 ml of Aquasol (New England Nuclear Corp.).

FIG. 2. Dissociation of free polyribosomes and release of virus-specific RNA by EDTA. Polyribosomes isolated by pelleting were suspended in 1.0 ml of Tris buffer. Half ($5 A_{260 \text{ nm}}$ units) served as a control (A) and 0.5 ml was treated with 25 mM EDTA (B). Each sample was layered on a 15–45% (w/v) sucrose density gradient in Tris buffer (MgCl_2 deleted for EDTA-treated samples) and sedimented as described in Fig. 1. The recovery of virus-specific RNA was about 50% in EDTA-treated samples, perhaps due to the lower stability of viral mRNA after release from polyribosomes.

RESULTS

Detection of Virus-Specific RNA in Polyribosomes of Virus-Producing Cells Transformed by M-MSV. Free (Fig. 1A) and membrane-bound (Fig. 1B) polyribosomes from 78A1 cells were fractionated by zonal centrifugation in sucrose density gradients. RNA was extracted from each polyribosome fraction and annealed to viral [^3H]DNA to detect virus-specific RNA. Virus-specific RNA was detected in all size classes of polyribosomes (Fig. 1). A major peak of virus-specific RNA was found at about 350 S in both free and membrane-bound polyribosomes [size estimated as described (18)]. To provide evidence that viral RNA in polyribosomes has the properties of mRNA, duplicate aliquots of free and membrane-bound polyribosomes were treated with 25 mM EDTA, a procedure that dissociates polyribosomes and releases mRNA (19). As shown in Figs. 2 and 3, most of the hybridizable RNA (76% for free polyribosomes and 62% for membrane-bound polyribosomes) was released from polyribosomes and displaced towards the top of the gradient.

To eliminate the possibility that contamination of polyribosomes with virus particles was responsible for viral RNA

detected in polyribosomes, [^{32}P]- or [^3H]uridine-labeled virus was mixed with 78A1 cells before homogenization to prepare free and membrane-bound polyribosomes (microsome fraction). The radioactivity was distributed 60–80% in free polyribosome fraction and 20–40% in the microsomes. The radioactivity sedimented in the central region of the free polyribosome gradient (7.5–45% sucrose, SW27 rotor, 85 min at 25,000 rpm); for microsomes freed from membranes, the bulk of the radioactivity sedimented in a region close to the 80S ribosome peak and at the top of the gradient. However, no shift of the radioactivity peak was found when free or membrane-bound polyribosomes containing radioactivity derived from added virions was treated with EDTA. Thus, virus-specific RNA identified on polyribosomes by hybridization cannot be due to virion particles cosedimenting with polyribosomes.

Relative Amount of Virus-Specific RNA in Free and Membrane-Bound Polyribosomes. From the $A_{260 \text{ nm}}$ units of RNA and the extent of hybridization (Fig. 1), it may be estimated that 4.3-times as much virus-specific RNA is present in membrane-bound as in free polyribosomes. The hybridization-saturation experiment presented in Fig. 4 confirms this estimate. RNA was isolated from pooled regions in sucrose gradients of free or membrane-bound polyribosomes, and increasing amounts were annealed with a constant amount of viral [^3H]DNA. Comparison of the RNA concentrations that give half-maximal hybridization (see Fig. 4) shows that membrane-bound polyribosomes contain about 4-times as much virus-specific RNA as do free polyribosomes.

Nascent Viral Polypeptides in Polyribosomes of 78A1 Cells. To determine whether polyribosomes containing virus-specific RNA synthesize viral proteins, 78A1 cells were incubated with [^3H]leucine for 30 sec to label nascent polypeptide chains. Free and membrane-bound polyribosomes were isolated by the direct layering procedure, and 30% of the polypeptide radioactivity was released by treatment with puromycin. The degree of immunoprecipitation of released polypeptides from free fraction and membrane-bound polyribosomes with completely absorbed antiserum against MSV (MLV) prepared in rabbits are shown in Fig. 5. The immunoprecipitation of released nascent polypeptides in the

FIG. 3. Dissociation of membrane-bound polyribosomes and release of virus-specific RNA by EDTA. 1.3 $A_{260 \text{ nm}}$ units of polyribosomes were layered on each gradient. See Fig. 2 for further details. (A) Untreated; (B) treated with EDTA.

peak fraction from membrane-bound polyribosomes was 10%, while that from free polyribosomes was 1%, as obtained from the peak fraction of free polyribosomes. The highest concentration of viral polypeptide chains was found in the heavy regions of the gradient (about 350 S in several experiments) (Fig. 5B), which correspond to the region containing the highest concentration of virus-specific RNA (Fig. 1B).

DISCUSSION

Virus-specific RNA molecules in cells transformed by and producing RNA tumor viruses may serve two functions: (i) precursor to the viral 70S RNA genome, and (ii) mRNA for synthesis of viral-coded proteins. The presence of virus-specific RNA in a polyribosome fraction of a mouse-mammary tumor has been reported (4), but neither its origin nor messenger function has been established. We have described two lines of evidence that virus-specific RNA in polyribosomes of 78A1 cells function as mRNA: (i) virus-specific RNA associated with polyribosomes can be released by treatment with EDTA, and (ii) immunologically reactive virion polypeptides are synthesized by the same size polyribosomes. Furthermore, 78A1 polyribosomes synthesize polypeptides *in vitro* that resemble virion polypeptides by immunological and electrophoretic criteria (20).

From the data used in Fig. 4 and from the calibration curve showing that half-saturation of [³H]DNA occurs with 0.012 μ g of viral 70S RNA, we calculate that 0.69% of total cellular RNA and 0.58% of polyribosomal RNA is virus specific. Since polyribosomal RNA represents about 80% of total cellular RNA and 70% of virus-specific RNA in polyribosomes is mRNA as judged by EDTA release, we estimate that 0.32% of the total cellular RNA is viral mRNA. Thus, about half of the total virus-specific RNA in 78A1 cells may function as mRNA.

A peak of virus-specific RNA and immunologically reactive polypeptides was found in polyribosomes sedimenting at about 350 S. Similar sedimentation rates were reported for polyribosomes of ascites cells infected with encephalomyocarditis virus (21) whose RNA has a molecular weight of 2.6×10^6 (22). The size of the MSV(MLV) major RNA subunits formed by denaturation of the viral 70S RNA genome

FIG. 4. Hybridization-saturation of RNA from whole cells (□) and from free (○) and membrane-bound (●), polyribosomes with MSV(MLV) [³H]DNA. Polyribosomes were obtained by direct layering, and fractions 4–14 of sucrose gradients of free and membrane-bound polyribosomes (see Fig. 5A and B) were pooled for RNA extraction. Increasing amounts of RNA were annealed with a constant amount (2000 dpm) of [³H]DNA product.

FIG. 5. Immunoprecipitation of labeled nascent polypeptides from free and membrane-bound polyribosomes. Cells were labeled with [³H]leucine. Polyribosomes were isolated by direct layering and centrifuged in 7.5–45% (w/v) sucrose density gradients in Tris buffer for 85 min at 25,000 rpm in a Spinco SW27 rotor. Nascent polypeptides were released from each gradient fraction with puromycin and were immunoprecipitated with antiserum against MSV(MLV). Nonspecific precipitation with the same serum and the corresponding fractions from 8617 cell polyribosomes was subtracted from each fraction of 78A1 polyribosomes. (A) Free polyribosomes; (B) membrane-bound polyribosomes.

is 35 S, corresponding to 3×10^6 daltons (Tsuchida and Green, manuscript in preparation). This value is close to that of encephalomyocarditis virus and is compatible with a polyribosome of 350 S. Virus-specific RNA in smaller polyribosomes could be intact viral mRNA loaded with fewer ribosomes or breakdown products of viral RNA still bound to polyribosomes.

Membrane-bound polyribosomes contain a much higher content of virus-specific RNA and nascent virion polypeptides than did free polyribosomes per unit of polyribosomal RNA. However, the absolute content in each polyribosome class is about the same since 78A1 cells contain 4-times as much free as membrane-bound polyribosomes (unpublished data). Although membrane-bound polyribosomes have been thought to function generally in the synthesis of exportable proteins (23), recent findings suggest other possibilities. For example, immunoglobulin that is exported from cells is synthesized on both free and membrane-bound polyribosomes (24). In addition, the attachment of all newly synthesized RNA to ribosomes appears to occur free in the cytoplasm, and subsequently a portion of the mRNA ribosome complex attaches to membranes (25). Furthermore, a recent report suggests

that free polyribosomes become membrane-bound only when the growing polypeptide chain has reached a certain length (26). It is interesting that in our experiments (unpublished data) and those reported by Vassart (16), nascent polypeptide chains released from free polyribosomes are smaller on the average than those released from membrane bound polyribosomes. This finding could represent an additional explanation for the low immunological reactivity of the nascent chains derived from free polysomes.

Thus, it is possible that the synthesis of RNA tumor virus proteins is initiated on free polyribosomes, as may be the case for most cellular proteins, and that virus-specific complexes attach to membranes to a higher degree than do cellular polyribosomes. Perhaps the attachment to membranes of virus-specific polyribosomes is necessitated by maturation of virion polypeptides, a process that may involve proteolytic cleavage and glycosylation and may require enzymes present on microsomes.

We thank Dr. David Schlessinger for a critical reading of the manuscript and Susan Loewenstein for technical assistance. This work was supported by Contract PH43-67-692 within the Special Virus-Cancer Program of the National Cancer Institute, National Institutes of Health, Public Health Service, Bethesda, Md. M.G. is a Research Career Awardee of the National Institutes of Health (5K6-AI-4739).

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