JOURNAL OF VIROLOGY, Sept. 1979, p. 668-676
0022-538X/79/09-0668/09$02.00/0

Vol. 31, No. 3

Viral Genome RNA Serves as Messenger Early in the Infectious Cycle of Murine Leukemia Virus

REVITAL SHURTZ,[1] SARAH DOLEV,[1] MORDECHAI ABOUD,[2] AND SAMUEL SALZBERG[1]*

Department of Life Sciences, Bar-Ilan University, Ramat-Gan,[1] and Microbiology and Immunology Unit, Faculty of Health Sciences, Ben Gurion University of the Negev, Beersheva,[2] Israel

Received for publication 20 March 1979

When NIH/3T3 mouse fibroblasts were infected with the Moloney strain of murine leukemia virus, part of the viral genome RNA molecules were detected in polyribosomes of the infected cells early in the infectious cycle. The binding appears to be specific, since we could demonstrate the release of viral RNA from polyribosomes with EDTA. Moreover, when infection occurred in the presence of cycloheximide, most viral RNA molecules were detected in the free cytoplasm. Size analysis on polyribosomal viral RNA molecules indicated that two size class molecules, 38S and 23S, are present in polyribosomes at 3 h after infection. Analysis of the polyriboadenylate [poly(rA)] content of viral RNA extracted from infected polyribosomes demonstrated that such molecules bind with greatest abundance at 3 h after infection, as has been detected with total viral RNA. No molecules lacking poly(rA) stretches could be detected in polyribosomes. Furthermore, when a similar analysis was performed on unbound molecules present in the free cytoplasm, identical results were obtained. We conclude that no selection towards poly(rA)-containing viral molecules is evident on binding to polyribosomes. These findings suggest that the incoming viral genome of the Moloney strain of murine leukemia virus may serve as a messenger for the synthesis of one or more virus-specific proteins early after infection of mouse fibroblasts.

The viral genome of RNA tumor viruses (retroviruses) consists of two identical 35S RNA subunits, both forming a complex of 70S (9, 39). Retroviruses replicate via a DNA intermediate which is synthesized in the cytoplasm early in the infectious cycle of the virus (37). It is well established that the viral RNA serves as a template for viral DNA synthesis by the virion-associated reverse transcriptase occurring in the cytoplasm of exogenously infected cells. However, no particular attention has been paid to the possibility that this RNA might serve also as mRNA for early viral protein synthesis.

Viral RNA isolated from virus particles is characterized by its polyriboadenylate [poly(rA)] tail in the 3′ terminus (14, 27, 35), methylated "cap" structure in the 5′ terminus (12, 17), and internal methylations of adenosine residues (8). These features are typical for most mRNA molecules of eucaryotic cells and animal viruses. In addition, studies with cell-free systems have shown that the RNA isolated from viral particles can be translated to authentic viral proteins (23, 30). Knowing these properties, it could be speculated that the viral genome might be utilized as mRNA during the early stage of the infectious cycle of the virus. Indirect support for such a possibility has been provided by the finding that inhibition of protein synthesis by cycloheximide (CH) (33) and puromycin (our unpublished data) interferes with both viral RNA synthesis and virus production of murine sarcoma and leukemia viruses. These inhibitory effects were evident even when the drugs were present at the early stage of the infection. Furthermore, Gallis et al. (13) have shown that in the case of avian viruses, the precursor for the internal core proteins, Pr 76, is synthesized at about 3 h after infection, even in the presence of cytosine arabinoside.

In this study we present evidence that the viral genome serves as mRNA early in the infectious cycle of the Moloney strain of murine leukemia virus (MLV). The characteristics of this early message, including its size distribution, are described.

MATERIALS AND METHODS

Cells and viruses. NIH/3T3 mouse fibroblasts were used as control cells throughout this study. NIH/3T3 cells chronically infected with MLV [NIH/3T3(MLV)] were used as a source for infecting virus particles. Unless otherwise indicated, the cells were grown in 100-mm dishes (Nunc, Roskilde, Denmark)

in Dulbecco-modified Eagle medium (EM) containing 10% newborn calf serum.

Infection procedure. NIH/3T3(MLV) cells were seeded at 0.8×10^6 cells per plate. Two days later, the medium was replaced with 8 ml of EM supplemented with 10% calf serum for 16 to 18 h. The medium was then collected, centrifuged at $1,000 \times g$ for 10 min, and supplemented with 8 μg of Polybrene (Sigma Chemical Co., St. Louis, Mo.) per ml. Under such conditions the concentration of MLV was 3×10^6 to 5×10^6 PFU/ml.

For exogenous infections, NIH/3T3 cells were seeded at a density of 3×10^6 cells per plate in the presence of Polybrene (8 μg/ml). Eighteen hours later cells were infected with 2 to 3 PFU/cell for 1 h at 37°C, with occasional shaking. At the end of the adsorption period, cultures were washed with EM and, unless otherwise indicated, fresh medium containing 10% calf serum was added. Control cultures were mock infected with EM and treated under identical conditions.

Reverse transcriptase assay. Reverse transcriptase activity was measured in 50-μl samples of culture fluid, as described by Aboud et al. (3).

Virus purification and cDNA preparation. NIH/3T3(MLV) cells were seeded in Roux bottles at a density of 4×10^6 cells per bottle in 100 ml of EM supplemented with 10% calf serum. The medium was changed every 12 h for 2 to 3 days. Virus was collected from the medium and purified as described previously (32). The purified virus was used to prepare the viral ^{3}H-labeled complementary DNA (cDNA) by the endogenous reverse transcriptase reaction. The reaction mixture (3 to 5 ml) contained 50 mM Tris-hydrochloride (pH 8.2), 5 mM dithiothreitol, 100 mM NaCl, 0.5 mM $MnCl_2$, 0.1 mM each dATP, dGTP, dCTP, and dTTP (Sigma), 0.01 mM [^{3}H]TTP (specific activity, 50 Ci/mmol; The Radiochemical Center, Amersham, England), 0.03% Nonidet P-40 (Fluka AG, Buchs, Switzerland), and an amount of purified virus equivalent to 500 to 1,000 μg of protein per ml.

Incubation was at 37°C for 3 h. The reaction was stopped by adding 0.5% sodium dodecyl sulfate and 0.01 N EDTA. After an additional incubation of 10 min at 37°C, nucleic acids were twice extracted with 1 volume of phenol saturated with NTE (100 mM NaCl, 10 mM Tris-hydrochloride, pH 7.4, and 1 mM EDTA) and 0.5 volume of a mixture of chloroform-isoamyl alcohol (24:1). *Escherichia coli* tRNA (50 μg/ml; Sigma) was added as a carrier to the aqueous phase, and the nucleic acids were precipitated overnight with 2.5 volumes of ethanol at −20°C. The precipitate was dissolved in 1 ml of 0.1× SSC (SSC = 0.15 M NaCl plus 0.015 N sodium citrate, pH 5.5), and the RNA was hydrolyzed by treatment with 0.2 N NaOH for 60 min at 37°C. After neutralization with HCl, 50 μg of *E. coli* DNA (Sigma) was added as carrier and the DNA was precipitated with ethanol. The specific activity of the cDNA thus obtained was 1.8×10^6 cpm/μg. It was 90% sensitive to S_1 nuclease digestion and 88% resistant after hybridization to an excess of viral 70S RNA extracted from purified virions.

Isolation of polyribosomes and treatment with EDTA. A temperature of 2 to 4°C was maintained throughout the isolation. Cells were washed three times with phosphate-buffered saline (31) and resuspended in RSB buffer (10 mM Tris-hydrochloride, pH 7.6, 10 mM NaCl, 1.5 mM $MgCl_2$, containing 0.1% diethylpyrocarbonate). One-tenth volume of 10% Nonidet P-40 was added, and after 15 min the lysate was centrifuged at $800 \times g$ for 10 min. The supernatant fluid was collected, layered onto a 6-ml 1.5 M sucrose "cushion" in RSB, and centrifuged at $210,000 \times g$ for 2.5 h in a Spinco Ti-50 rotor. The pellet was gently suspended in 0.4 ml of gradient buffer (10 mM Tris-hydrochloride, pH 7.5, 100 mM KCl, and 5 mM $MgCl_2$), layered onto a 12-ml linear 17 to 40% sucrose gradient, and centrifuged at $109,000 \times g$ for 80 min in a Spinco SW41 rotor. The gradient was collected, and the absorbance at 260 nm (A_{260}) was recorded with a Gilford spectrophotometer.

Appropriate fractions were pooled, total material was precipitated with ethanol, and RNA was extracted. The amount of viral RNA at each fraction was measured by annealing with 750 cpm of MLV [^{3}H]cDNA.

The percentage of cDNA hybridized was converted to actual amounts of viral RNA, using a standard curve which was established by determining the percentage of input cDNA hybridized as a function of the amount of 70S viral RNA extracted from purified virions, and added to the annealing reaction.

For treatment of polyribosomes with EDTA, 1.5 mM $MgCl_2$ in RSB was replaced with 3 mM EDTA. Non-polyribosomal fraction (free cytoplasm) was obtained by collecting the layer above the sucrose cushion after ultracentrifugation.

Treatment of cultures with CH. Cultures were treated with 10 μg of CH (Sigma) per ml in EM supplemented with 10% calf serum. Cells were infected 30 min after treatment. The drug remained during and up to 3 h after infection. At the end of the treatment, cultures were washed three times with EM, either harvested or supplemented with fresh medium, and further incubated for 24 h.

RNA extraction. RNA extraction was adapted, with minor modifications, from procedures previously described by Fan and Baltimore (10). All steps were performed at room temperature, unless otherwise indicated.

Cell fractions (total cytoplasm, polyribosomes, and free cytoplasm) were suspended in 0.5 ml of DNase buffer (500 mM NaCl, 50 mM $MgCl_2$, and 50 mM Tris-hydrochloride, pH 7.2) and treated with 50 μg of DNase I (Sigma) per ml for 45 min at room temperature. A 1.5-ml amount of pronase buffer (0.1 M NaCl, 1 mM EDTA, and 5 mM Tris-hydrochloride, pH 7.4) was added, and the fractions were treated with Pronase P (Serva GMBH, Heidelberg, Germany) at a concentration of 100 μg/ml for 18 h at room temperature. RNA was extracted three times with water-saturated phenol and a mixture of chloroform-isoamyl alcohol (24:1) as described above for cDNA extraction. The aqueous phase was adjusted to 0.1 N NaCl, and RNA was precipitated with 2.5 volumes of ice-cold ethanol. After storage overnight at −20°C, the RNA was pelleted by centrifugation at $27,000 \times g$ for 30 min and resuspended in 0.1× SSC. The concentration of RNA was determined by measuring the A_{260} in a Gilford spectrophotometer. A_{280} was also determined.

Only RNA samples showing a ratio of at least 1.8:1 in the readings at A_{260}/A_{280}, respectively, were used for hybridization studies.

Since we used no internal standard for estimating the extraction efficiency during our extraction procedure, and assuming that the extraction efficiency is identical for all the cellular fractions used in our study, we expressed our results as nanograms of viral RNA per microgram of total RNA.

Nucleic acid hybridization. Viral RNA in cellular fractions was estimated by hybridizing RNA samples to about 600 cpm of [^{3}H]cDNA for 42 h at 68°C, as described by Salzberg et al. (31). The extent of hybridization was determined by the amount of [^{3}H]cDNA protected from S_1 nuclease digestion, as described previously (31). The amount of virus-specific RNA in each sample was computed from a standard curve, established as described above.

Size analysis of viral RNA in exogenously infected NIH/3T3 cells. Purified polyribosomal RNA (usually 1.5 A_{260} units) was dissolved in 0.1× SSC and heated for 2 min at 80°C to dissociate RNA aggregates. The sample was quickly cooled and applied to an exponential 15 to 30% (wt/wt) sucrose gradient in NTE. Centrifugation was performed at 4°C for 5 h at 115,000 × g in a Spinco SW41 rotor.

Fractions of 0.4 ml were collected from the bottom with a peristaltic pump, and the A_{260} was determined. *E. coli* tRNA (10 μg/ml) was added to each fraction, and RNA was precipitated with 2.5 volumes of ethanol, as described above. The amount of viral RNA was determined by annealing to viral [^{3}H]cDNA.

Sedimentation values were calculated from 18S and 28S rRNA and 4S RNA, obtained from the absorbance profile of each gradient.

Fractionation of RNA on oligo(dT)-cellulose. Oligodeoxythymidylate [oligo(dT)]-cellulose was obtained commercially (Collaborative Research, Waltham, Mass.). RNA was dissolved in 0.1× SSC at a concentration of 3 A_{260} units and was applied to a column containing 300 mg of oligo(dT)-cellulose. All steps were performed at room temperature, as described by Aviv and Leder (4).

The column was washed with adsorption buffer (0.5 M KCl, 10 mM Tris-hydrochloride, pH 7.4), and fractions of non-polyadenylated material were collected. Poly(rA)-containing RNA was eluted from the column with elution buffer (10 mM Tris-hydrochloride, pH 7.4).

The fractions were analyzed for the amount of RNA by measuring the A_{260}. Polyadenylated and non-polyadenylated RNAs were applied again to the column, and the same washing procedure was repeated. Fractions containing RNA were pooled, 10 μg of *E. coli* tRNA per ml was added, and RNA was precipitated with 2.5 volumes of ethanol.

Viral RNA was determined by hybridization to [^{3}H]cDNA, as described above.

Before applying the RNA samples, each column was tested for its validity by running 0.5 A_{260} unit of commercial poly(rA) (Collaborative Research).

RESULTS

Detection of virus-specific RNA in polyribosomes early after infection of NIH/3T3 cells with MLV.

Previous experiments (33) have indicated the possibility that the incoming virus-specific genomes of the murine sarcoma and leukemia virus complex, early after infection of a clonal line of mouse 3T6 cells, may function as mRNA for the synthesis of virus-specific proteins. These findings led us to investigate the possible function of the parental viral genome in our system. We used mouse NIH/3T3 fibroblasts that were infected with MLV. Polyribosomes were isolated from the cells and RNA was extracted. Virus-specific RNA was detected by annealing RNA samples with viral [^{3}H]cDNA, prepared in vitro from purified virions.

Figure 1A represents the binding pattern of

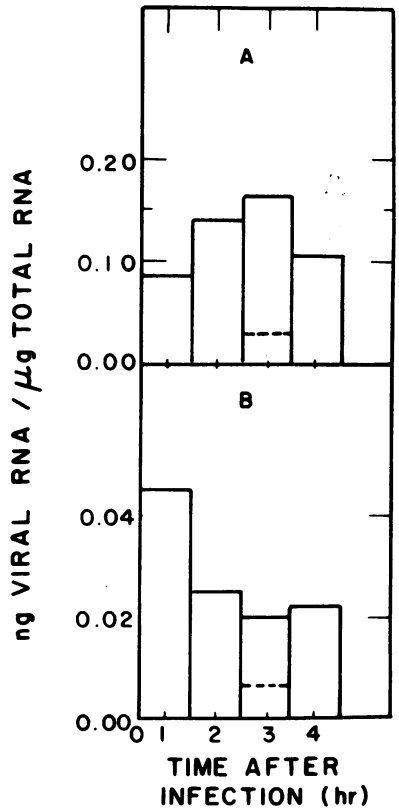

FIG. 1. *Detection of virus-specific RNA in cytoplasmic fractions of infected cells early after infection. NIH/3T3 cells were infected with 2 to 3 PFU of MLV per cell and collected at different times after infection, and the cytoplasm was separated from nuclei by sedimentation. Polyribosomes were prepared by pelleting through a 1.5 M sucrose cushion, and the free cytoplasmic fraction was simultaneously collected. RNA was extracted from both the polyribosomes (A) and the free cytoplasmic fraction (B), and the content of virus-specific RNA was determined by hybridization to viral [^{3}H]cDNA, prepared in vitro from purified virions. The dashed lines represent the values obtained in mock-infected cells.*

viral RNA found in the polyribosomes during the first 4 h after infection. Virus-specific RNA was detected 1 h after infection; its binding was maximal at 3 h and subsequently decreased. The amount of virus-specific RNA present in polyribosomes at 3 h after mock infection was no more than 0.05 ng/μg of total RNA.

The free cytoplasmic fraction which did not include the polyribosomes was simultaneously examined. This was performed assuming that the viral genome present in this fraction serves as a template for the synthesis of proviral DNA (15, 28, 36) by the RNA-dependent DNA polymerase (38).

Figure 1B demonstrates that the amount of viral RNA was maximal at 1 h after infection and then decreased, probably as a result of increased binding to polyribosomes, as shown above. In mock-infected cells the concentration of viral RNA was not higher than 0.005 ng/μg of total RNA. These data indicate a possible function of viral RNA as mRNA early in the infectious cycle of MLV.

Binding properties of virus-specific RNA to polyribosomes of infected cells. To provide evidence that the virus-specific RNA detected in polyribosomes is bound in a manner characteristic for mRNA, the polyribosomal pellet derived from cells at 3 h after infection was divided into two portions. One part was treated with EDTA to disaggregate polyribosomes and release bound mRNA molecules. The second part was suspended in RSB. Both samples were centrifuged in sucrose gradients, as described in Fig. 2. The profile thus obtained is shown in Fig. 2A. Each gradient was divided into the polyribosome region (fractions 1 to 21, region I) and the upper region (fractions 22 to 30, region II). The fractions from both regions were combined, and RNA was extracted and annealed to viral [^{3}H]cDNA.

Most of the virus-specific RNA was detected in polyribosomes (0.14 ng, I in inset to Fig. 2A), whereas the remainder (0.015 ng, II in inset to Fig. 2A) is present presumably as free viral RNA or viral RNA subunits at the top of the gradient.

Treatment with EDTA dissociated polyribosomes and released virus-specific RNA, which was displaced towards the top of the gradient (Fig. 2B), as expected for mRNA. Only 0.01 ng of viral RNA appeared in the lower region of the gradient (I in inset to Fig. 2B), whereas the majority (0.16 ng) was detected at the top (II in inset to Fig. 2B). Similar results were obtained in experiments with cells treated with cytosine arabinoside (33).

Effect of CH on virus production and the binding properties of viral RNA to polyribosomes of infected cells. It could be argued

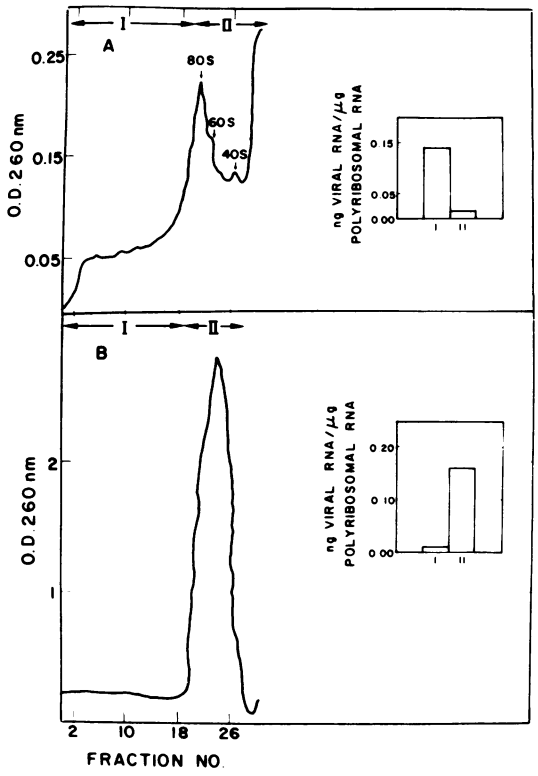

FIG. 2. *Effect of EDTA on the binding properties of viral RNA to polyribosomes of infected cells. Cells were collected 3 h after infection and divided into two portions. Polyribosomes were prepared from one portion by pelleting through a 1.5 M sucrose cushion (A). The other portion was similarly treated except that Mg^{2+} was replaced by EDTA in RSB (B). Both fractions were layered on 17 to 40% sucrose gradients, centrifuged for 80 min at 109,000 × g, fractionated, and pooled into two regions, I and II, as indicated by the arrows. The content of virus-specific RNA in each region was determined by hybridization to viral [^{3}H]cDNA (insets). O.D.$_{260\ nm}$, Optical density at 260 nm.*

that the viral genome of the penetrating virions is adsorbed non-specifically to the cellular polyribosome texture and that its displacement in a sucrose gradient after EDTA treatment resulted from destruction of this structure. It was previously shown that CH inhibits the translocation process in protein synthesis, thus preserving the preexisting cellular polyribosomes but preventing the formation of new polyribosomes (7, 19). Based on these findings, NIH/3T3 cells were treated with CH for 30 min before infection with MLV. The drug remained up to either 3 or 24 h after infection.

First, the effect of the drug on virus production at 24 h after infection was determined. As shown in Table 1, 95% inhibition in DNA

TABLE 1. *Effect of cycloheximide (CH) on virus production in NIH/3T3 cells infected with MLV*[a]

Plate no.	Treatment	[^{3}H]TMP incorporation (cpm)	% of control
1	Infected; no CH	15,275	100
2	Infected; CH, 0–3 h p.i.[b]	764	5
3	Infected; CH, 0–24 h p.i.	305	2
4	Uninfected; no CH	300	2
5	Uninfected; CH	458	3

[a] NIH/3T3 cells were treated with 10 μg of CH for 30 min before infection with MLV. The drug was present during infection and up to either 3 h (plate 2) or 24 h (plate 3) after infection. Plate 4 was mock infected, without treatment with CH, and plate 5 was mock infected and treated with CH (for 3 h). The culture medium was collected 24 h after infection and assayed for reverse transcriptase activity. Each value represents an average of three cultures.

[b] p.i., Postinfection.

polymerase activity is observed in the medium of the 3-h treated culture (plate 2), as compared with infected untreated cells. As expected, a strong inhibition in virus production is also evident in cultures treated for 24 h with CH.

No cytotoxic effects were found in cultures treated with CH, as was observed by cell number determination (unpublished data).

In a parallel experiment, untreated and CH-treated cells were harvested at 3 h after infection, and polyribosomes were prepared. RNA was extracted from the polyribosomal and the free cytoplasmic fractions, and 15 μg of RNA from each fraction was annealed with a constant amount of viral [^{3}H]cDNA.

Virus-specific RNA was readily detected in the polyribosomal fraction of infected untreated cells, whereas only a small amount was found in the free cytoplasmic fraction (Fig. 3). However, in CH-treated cells, most of the viral RNA was found in the free cytoplasmic fraction. In uninfected cultures, either treated or untreated with CH, almost the same low levels of viral RNA in both fractions were detected.

Size analysis of virus-specific mRNA early after infection. To further characterize the viral RNA molecules bound to polyribosomes early in the infectious cycle of MLV, cells were harvested 3 h after infection and layered on a sucrose gradient. Fractions were pooled and annealed to viral [^{3}H]cDNA.

Figure 4 demonstrates two major size classes of viral RNA present: the larger class of about 38S and an additional class of approximately 23S. These size classes are similar to those detected in polyribosomes of chronically infected

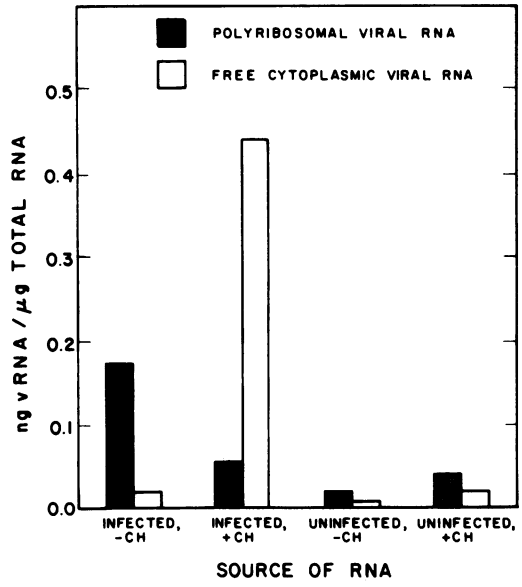

FIG. 3. *Effect of CH on the binding properties of viral RNA to polyribosomes in infected cells. Cells were treated with CH, infected, and collected 3 h after infection. Cytoplasm was separated from nuclei, and polyribosomes were prepared as described. RNA was extracted from the polyribosomes and free cytoplasm, and virus-specific RNA was measured by hybridization to viral [^{3}H]cDNA. Viral RNA (vRNA) is expressed as nanograms of virus-specific RNA per microgram of total RNA.*

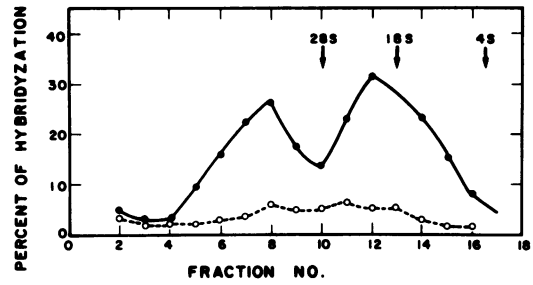

FIG. 4. *Size analysis of virus-specific mRNA early after infection. Cells were harvested 3 h after infection, and polyribosomes were prepared. RNA was extracted, and 1.5 A_{260} units were layered on a 15 to 30% sucrose gradient and centrifuged for 5 h at 115,000 × g. Fractions were collected and annealed with viral [^{3}H]cDNA (●). RNA from polyribosomes of mock-infected cells served as a control (○). The locations of 28S and 18S rRNA and 4S tRNA are indicated.*

cells (11). Small amounts of viral RNA with no definite size classes are detected in uninfected cells. These viral RNA molecules are heterogeneous in size, ranging from 35S to 18S (dashed line in Fig. 4).

Poly(rA)-containing, virus-specific RNA

molecules are present in the polyribosomes early after infection. Several investigators have reported that the 35S RNA molecules of retroviruses have several properties characteristic for mRNA, e.g., poly(rA) at the 3′ end (14, 27, 35) and a 5′-terminal methylated cap structure (12, 17). We investigated the possibility that the viral RNA molecules present in polyribosomes early after infection contain poly(rA) sequences.

Purified polyribosomes were prepared from infected cells at different times after infection. The extracted RNA was chromatographed through oligo(dT)-cellulose. Polyadenylated RNA was annealed to viral [^{3}H]cDNA. Figure 5 shows that polyadenylated viral RNA represents the same pattern of binding to polyribosomes as total viral RNA. The maximal amount of polyadenylated viral RNA is detected at 3 h after infection, followed by a decrease at 4 h. No more than 8% hybridization was obtained when polyadenylated RNA extracted from polyribosomes of uninfected cells at 3 h after mock infection was annealed with viral [^{3}H]cDNA (see Fig. 5).

Distribution of polyadenylated and non-polyadenylated viral RNA in the cytoplasm of MLV-infected NIH/3T3 cells. The finding that parental viral RNA may serve two separate functions early after infection of NIH/3T3 cells led us to investigate the distribution of polyadenylated viral RNA between polyribosomes and free cytoplasm in such cells. RNA was extracted from purified polyribosomes at 3 h after infection. The RNA was chromatographed twice through oligo(dT)-cellulose to separate the polyadenylated from the non-polyadenylated RNA. Each RNA fraction was annealed to viral [^{3}H]cDNA. RNA present in the free cytoplasm was similarly analyzed.

As can be seen in Fig. 6, the same distribution of viral RNA is evident in both fractions; namely, the majority of viral RNA present in either fraction was polyadenylated. Very few viral molecules were non-polyadenylated, and there was no preference of polyadenylated material towards either fraction. It is interesting to note that a significant amount of polyadenylated viral RNA is present in uninfected cells, both in polyribosomal and in free cytoplasmic fractions.

DISCUSSION

Our studies support some earlier reports on the role of the viral genome of retroviruses early in the infectious cycle of the virus. As has been previously demonstrated (33), our present data demonstrate that viral RNA binds to ribosomes during the first few hours after infection, before the onset of progeny viral RNA synthesis, which starts only at about 6 h after infection (32, 34). The binding appears to be specific, since treatment with EDTA causes the release of viral RNA from polyribosomes. Moreover, infection in the presence of CH results in the appearance of a large amount of viral RNA in the free cytoplasm, with a decreased amount present in

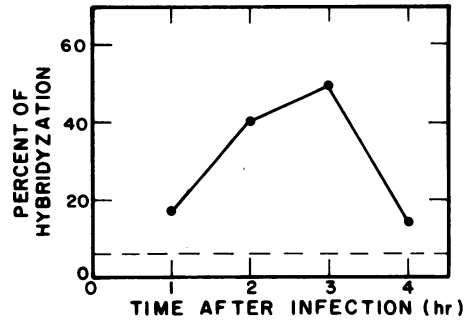

FIG. 5. *Poly(rA)-containing, virus-specific RNA molecules in polyribosomes early after infection. Purified polyribosomes were prepared from infected cells at the first 4 h after infection. Three A_{260} units of RNA extracted from the polyribosomes pellet were chromatographed through an oligo(dT)-cellulose column. The same elution profile was obtained with all RNA preparations. The polyadenylated RNA was precipitated with ethanol and resuspended in 200 μl of 0.1× SSC. A sample of 60 μl was annealed with viral [^{3}H]cDNA to determine virus specificity. The value obtained with RNA extracted from uninfected cells at 3 h after mock infection is shown by the dashed line.*

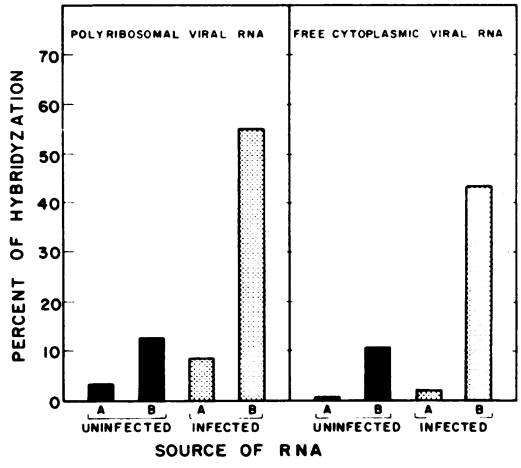

FIG. 6. *Distribution of polyadenylated and non-polyadenylated viral RNA in cytoplasmic fractions of infected cells. RNA was extracted from purified polyribosomes and free cytoplasmic fraction at 3 h after infection and chromatographed twice through an oligo(dT)-cellulose column. Non-polyadenylated (A) and polyadenylated (B) material from both fractions was annealed to [^{3}H]cDNA. Uninfected cells were mock infected and similarly treated.*

the polyribosomes. It is interesting to note that the total amount of viral RNA in the cytoplasm (polyribosomes and free cytoplasmic fraction) of infected cells after CH treatment is much higher than in untreated cells. It could be speculated that as a result of CH treatment, most of the incoming viral RNA molecules that do bind to polyribosomes are not functional. Hence, viral RNA is not degraded at a rate similar to that of untreated cells, resulting in the accumulation of viral RNA in the free cytoplasm.

Another possibility is based on the finding of Aaronson and Dunn (1), who demonstrated type C virus induction in mouse cells after treatment with protein synthesis inhibitors. It could thus be argued that at least part of the viral RNA detected in the free cytoplasmic fraction results from such an induction. However, this possibility is unlikely. First, we could not find an increased amount of viral RNA in uninfected cells treated with CH. Second, when the growth medium of such cultures was tested for the presence of virus particles, no such particles were observed (Table 1). Moreover, when the culture medium of infected cells after CH treatment was similarly tested, strong inhibition of virus production was observed, as compared with infected untreated cells (Table 1).

Based on these findings, we conclude that one of the roles of the viral genome early in the infectious cycle of MLV is to serve as messenger for the synthesis of an early viral protein(s), although the fact that some viral RNA molecules detected in CH-treated cells originate from endogenous viral information still cannot be ruled out.

The nature and biological significance of this protein is not clear. In the avian system, it has been demonstrated that Pr 76, the precursor for the core proteins, is synthesized early in infection (13). As indicated above, we could demonstrate that CH, as well as puromycin (unpublished data), even if present only in the first 4 h after infection of mouse cells with MLV, is sufficient to cause a significant inhibition in both progeny viral RNA synthesis (33) and virus production. Assuming that the major site of inhibition by these drugs is the protein synthesis machinery (95% inhibition as compared to 20 to 25% inhibition in DNA synthesis; unpublished data), these results mean that either cellular or viral protein(s) synthesized early in the infection is essential for the normal progression of the infectious cycle. In this respect, it is worth mentioning that Roa and Bose (25, 26) have demonstrated that treatment of mouse cells with ethidium bromide before infection with murine sarcoma virus results in inhibition of focus formation by the virus. Since the recovery from this effect is sensitive to protein synthesis inhibitors, these authors conclude that cellular proteins are essential early in the infectious cycle of murine sarcoma virus. Of special interest is the finding that interferon, which is known to be an inhibitor of viral protein synthesis, exerts its inhibitory effect on virus production early in the infectious cycle, before the onset of progeny viral RNA synthesis (2).

Since viral proteins are synthesized via specific viral mRNA molecules (11, 20–22, 40), we determined the size classes of viral RNA present in polyribosomes at 3 h after infection. Two major size classes of 38S and 23S are evident. Although the possibility that the smaller molecules are breakdown products cannot be excluded, this is unlikely, since distinct and reproducible size classes are obtained. Moreover, these classes are identical in size to those detected in mouse cells chronically infected with MLV (11). It has recently been demonstrated that 35S, as well as 23S, viral RNA is present in complete virions and can be translated in cell-free systems (5, 24; C. J. L. Saris, H. C. M. Eenbergen, and H. P. J. Blomers, Abstr. Fourth Int. Congr. Virol., The Hague, The Netherlands, p. 368, 1978). Thus, the distinct size class molecules that we detected in polyribosomes mostly represent authentic viral messages.

It has previously been demonstrated that the viral genome RNA contains poly(rA) sequences (14, 27, 35). Our studies on the kinetics of the appearance of poly(rA)-containing viral RNA in polyribosomes indicate a similarity to the kinetics observed with total viral RNA present in polyribosomes under the same conditions. These results mean that most, if not all, viral RNA present in polyribosomes is polyadenylated. Some reports indicate a difference in poly(rA) content of the viral RNA subunits present in mature virions (16), whereas other reports claim that all viral subunits contain poly(rA) (18). We could find no difference in the binding properties to oligo(dT)-cellulose between viral RNA molecules present in polyribosomes and in the free cytoplasm at 3 h after infection. Although the size of the poly(rA) stretch in each case was not determined, we assume from the similar elution profiles of both types of RNA that these molecules are identical in respect to their poly(rA) content. We conclude that no selection of poly(rA)-containing viral molecules binding to polyribosomes occurs in our system.

Our studies raise the question of the role of early viral proteins in the infectious cycle of retroviruses. Recent reports (6, 29) demonstrated that viral cDNA prepared in vitro is infectious. However, the amount used in these experiments was severalfold higher than that

anticipated to participate in a normal infection (about 100 equivalents of viral genome RNA, which equals about 5×10^{-4} pg [32]). It can be postulated that under normal conditions a regulatory protein which may bind to viral DNA and be essential for elongation, transport, supercoiling, or integration of such molecules is synthesized in infected cells.

Since progeny viral RNA synthesis was shown to be inhibited by CH in exogenously infected cells (33), it is possible that a virus-specific RNA polymerase subunit is essential for the normal progression of infection. Additional experiments are in progress to clarify these alternatives.

ACKNOWLEDGMENTS

This research was supported by a grant from the National Council for Research and Development, Israel, and by the GSF, Munich, Germany.

LITERATURE CITED

1. **Aaronson, S. A., and C. Y. Dunn.** 1974. High-frequency C-type virus induction by inhibitors of protein synthesis. Science **183**:422-423.
2. **Aboud, M., R. Shoor, and S. Salzberg.** 1978. Effect of interferon on exogenous murine leukemia virus infection. Virology **84**:134-141.
3. **Aboud, M., O. Weiss, and S. Salzberg.** 1976. Rapid quantitation of interferon with chronically oncornavirus-producing cells. Infect. Immun. **13**:1626-1632.
4. **Aviv, H., and P. Leder.** 1972. Purification of biochemically active globin mRNA by chromatography on oligothymidylic acid-cellulose. Proc. Natl. Acad. Sci. U.S.A. **69**:1408-1412.
5. **Beemon, K., and T. Hunter.** 1977. In vitro translation yields a possible Rous sarcoma virus SRC gene product. Proc. Natl. Acad. Sci. U.S.A. **74**:3302-3306.
6. **Clayman, C. H., E. Mosharrafa, and A. J. Faras.** 1979. In vitro synthesis of infectious transforming DNA by the avian sarcoma virus reverse transcriptase. J. Virol. **29**:242-249.
7. **Colombo, B., L. Felicetti, and C. Baglioni.** 1965. Inhibition of protein synthesis by cycloheximide in rabbit reticulocytes. Biochem. Biophys. Res. Commun. **18**: 389-395.
8. **Dimock, K., and C. M. Stolzfus.** 1977. Sequence specificity of internal methylation in B77 avian sarcoma virus RNA subunits. Biochemistry **16**:471-478.
9. **Fan, H.** 1978. Expression of RNA tumor viruses at translation and transcription levels. Curr. Top. Microbiol. Immunol. **79**:1-41.
10. **Fan, H., and D. Baltimore.** 1973. RNA metabolism of murine leukemia virus: detection of virus specific RNA sequences in infected and uninfected cells and identification of virus specific mRNA. J. Mol. Biol. **80**:93-117.
11. **Fan, H., and I. M. Verma.** 1978. Size analysis and relationship of murine leukemia virus-specific mRNA's: evidence for transposition of sequences during synthesis and processing of subgenomic mRNA. J. Virol. **28**:468-478.
12. **Furuichi, Y., A. J. Shatkin, E. Stavnezer, and J. M. Bishop.** 1975. Blocked, methylated 5'-terminal sequence in avian sarcoma virus RNA. Nature (London) **257**:618-620.
13. **Gallis, B. M., R. N. Eisenman, and H. Diggelman.** 1976. Synthesis of the precursor to avian RNA tumor virus internal structural protein early after infection. Virology **74**:302-313.
14. **Green, M., and M. Cartas.** 1972. The genome of RNA tumor viruses contains polyadenylic acid sequences. Proc. Natl. Acad. Sci. U.S.A. **69**:791-794.
15. **Hatanaka, M., T. Kakefuda, R. V. Gilden, and E. A. O. Callan.** 1971. Cytoplasmic DNA synthesis induced by RNA tumor viruses. Proc. Natl. Acad. Sci. U.S.A. **68**:1844-1847.
16. **Joseph, D. R., and J. N. Ihle.** 1977. Comparison of sequence homology of poly(A) and non-poly(A) containing 34S RNA of AKR murine leukemia virus. Biochem. Biophys. Res. Commun. **74**:499-505.
17. **Keith, S., and H. Fraenkel-Conrat.** 1975. Identification of the 5' end of Rous sarcoma virus RNA. Proc. Natl. Acad. Sci. U.S.A. **72**:3347-3350.
18. **King, A. M. Q., and R. D. J. Wells.** 1976. All intact subunit RNA's from Rous sarcoma virus contain poly(A). J. Biol. Chem. **251**:150-152.
19. **Lin, S. Y., R. D. Mosteller, and B. Hardesty.** 1966. The mechanism of sodium fluoride and cycloheximide inhibition of hemoglobin synthesis in the cell-free reticulocytes system. J. Mol. Biol. **21**:51-69.
20. **Mueller-Lantzch, N., and H. Fan.** 1976. Monospecific immunoprecipitation of murine leukemia virus polyribosomes: identification of P30 protein specific messenger RNA. Cell **9**:579-588.
21. **Panet, A., M. Gorecki, S. Bratosin, and Y. Aloni.** 1978. Electron microscopic evidence for splicing of Moloney murine leukemia virus RNAs. Nucleic Acids Res. **5**: 3219-3230.
22. **Parsons, J. T., P. Lewis, and P. Dierks.** 1978. Purification of virus-specific RNA from chicken cells infected with avian sarcoma virus: identification of genome-length and subgenome-length viral RNAs. J. Virol. **27**: 227-238.
23. **Pawson, T., G. S. Martin, and A. E. Smith.** 1976. Cell-free translation of virion RNA from nondefective and transformation-defective Rous sarcoma virus. J. Virol. **19**:950-967.
24. **Purchio, A. F., E. Erikson, and R. L. Erikson.** 1977. Translation of 35S and of subgenomic regions of avian sarcoma virus RNA. Proc. Natl. Acad. Sci. U.S.A. **74**: 4661-4665.
25. **Roa, R. C., and S. K. Bose.** 1974. Inhibition by ethidium bromide of the establishment of infection by murine sarcoma virus. J. Gen. Virol. **25**:197-205.
26. **Roa, R. C., and S. K. Bose.** 1975. Requirement for cellular protein synthesis in reversal of ethidium-bromide-induced inhibition of cell transformation by murine sarcoma virus. Proc. Natl. Acad. Sci. U.S.A. **72**: 4337-4340.
27. **Rho, H. M., and M. Green.** 1974. The homopolyadenylate and adjacent nucleotides at 3' terminus of 30-40S' subunits in the genome of murine sarcoma-leukemia virus. Proc. Natl. Acad. Sci. U.S.A. **71**:2386-2390.
28. **Robin, M. S., S. Salzberg, and M. Green.** 1975. Cytoplasmic synthesis of viral DNA early during infection and cell transformation by the murine sarcoma-leukemia virus. Intervirology **4**:268-278.
29. **Rothenberg, E., D. Smotkin, D. Baltimore, and R. A. Weinberg.** 1977. In vitro synthesis of infectious DNA of murine leukemia virus. Nature (London) **269**:122-126.
30. **Salden, M. H. L., and H. Bloemendal.** 1976. Translation of avian myeloblastosis virus RNA in a reticulocyte cell-free system. Biochem. Biophys. Res. Commun. **68**:249-255.
31. **Salzberg, S., Z. Levi, M. Aboud, and A. Goldberger.** 1977. Isolation and characterization of DNA-DNA and DNA-RNA hybrid molecules formed in solution. Biochemistry **16**:25-29.
32. **Salzberg, S., M. S. Robin, and M. Green.** 1973. Appearance of virus specific RNA, virus particles and cell surface changes in cells rapidly transformed by MSV. Virology **53**:186-195.

33. **Salzberg, S., M. S. Robin, and M. Green.** 1977. A possible requirement for protein synthesis early in the infectious cycle of the murine sarcoma-leukemia virus. Virology **76**:341-351.
34. **Schincariol, A. L., and W. K. Joklik.** 1973. Early synthesis of virus specific RNA and DNA in cells rapidly transformed with Rous sarcoma virus. Virology **56**:532-548.
35. **Stephenson, M., J. Scott, and P. Zemecnik.** 1973. Evidence that the polyadenylic acid segment of the 35S RNA of avian myeloblastosis virus is located at the 3'-OH terminus. Biochem. Biophys. Res. Commun. **55**:8-16.
36. **Varmus, H. E., R. V. Guntaka, W. J. Fan, S. Heasley, and J. M. Bishop.** 1974. Synthesis of viral DNA in cytoplasm of duck embryo fibroblasts and in enucleated cells after infection by avian sarcoma virus. Proc. Natl. Acad. Sci. U.S.A. **71**:3874-3878.
37. **Varmus, H. E., S. Heasley, H. J. Kung, H. Oppermann, V. C. Smith, J. M. Bishop, and P. R. Shank.** 1978. Kinetics of synthesis, structure and purification of avian sarcoma virus-specific DNA mode in the cytoplasm of acutely infected cells. J. Mol. Biol. **120**:55-82.
38. **Verma, I. M.** 1977. The reverse transcriptase. Biochim. Biophys. Acta **473**:1-38.
39. **Vogt, P.** 1977. The genetic structure of RNA tumor viruses. Annu. Rev. Genet. **11**:203-238.
40. **Weiss, S. R., H. E. Varmus, and J. M. Bishop.** 1977. The size and genetic composition of virus specific RNAs in the cytoplasm of cell producing avian sarcoma-leukosis viruses. Cell **12**:983-992.