THE VIRUS-SPECIFIC RNA SPECIES IN FREE AND MEMBRANE-BOUND POLYRIBOSOMES OF TRANSFORMED CELLS REPLICATING MURINE SARCOMA-LEUKEMIA VIRUSES

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Summary: Ribonucleic acids extracted from polyribosomes of cells replicating murine sarcoma-leukemia viruses (M-MSV(MLV)) were resolved by electrophoresis on 2.5% polyacrylamide gels. Virus-specific RNA was detected by hybridization of RNA in the gel fractions with the ^{3}H-DNA product of the viral RNA-directed DNA polymerase. The postmicrosomal supernatant and the free polyribosomes contained one peak of virus-specific RNA with a molecular weight of about 2.9 x 10^6 (35S). In contrast, the microsomes and the membrane-bound polyribosomes contained two peaks of virus-specific RNA in approximately equal amounts with molecular weights of 2.9 x 10^6 (35S) and 1.5 x 10^6 (approximately 20S). The high molecular weight viral RNA species might serve as polycistronic mRNA for the synthesis of large polypeptides that are cleaved to form the smaller viral proteins.

Virus-specific RNA has been detected in the polyribosomes of the transformed rat cell line (78A1) replicating murine sarcoma-leukemia viruses (MSV(MLV)) (1). The same cell line was found to contain two viral RNA species sedimenting at 35S and 20S (2) and to synthesize several virus-specific proteins (3). The size of viral mRNA species present in polyribosomes that code for viral proteins has not been determined. In this report we describe the analysis of several polyribosome-containing cell fractions for the size of virus-specific RNA molecules utilizing polyacrylamide gel electrophoresis and molecular hybridization. Our data show that 35S RNA is present in both free and membrane-bound polyribosomes, while 20S RNA is unique to membrane-bound polyribosomes.

Materials and Methods

Viral ^{3}H-DNA Product. Viral DNA was prepared by the endogenous RNA-directed DNA polymerase reaction (4) of detergent disrupted M-MSV(MLV) utilizing ^{3}H-TTP as the labeled substrate in the presence of 20 µg/ml of actinomycin D.

Abbreviations: M-MSV(MLV), Moloney strain of murine sarcoma-leukemia virus; PBS, phosphate-buffered saline (137 mM NaCl-2.7 mM KCl-8.1 mM Na_2HPO_4-1.47 mM KH_2PO_4).

S-1 Nuclease. S-1 nuclease was prepared from Aspergillus oryzae α-amylase (Sigma Chemical Company) by a modification of the methods of Ando (5) and Sutton (6). α-Amylase was dissolved in column buffer (0.01 M potassium phosphate (pH 6.9) containing 5% glycerol), clarified by brief centrifugation, absorbed onto a Whatman DEAE cellulose (DE-52) column (1.8 x 50 cm), washed with 200-250 ml of column buffer, and eluted with a linear gradient of 400 ml of 0 to 0.4 M NaCl in column buffer. Fractions with S-1 nuclease activity were pooled, diluted with an equal volume of 50% glycerol, and stored at -20°C.

Isolation of Polyribosomes. Free and membrane-bound polyribosomes were obtained from transformed rat cells (78A1) replicating M-MSV(MLV), essentially as described by Vecchio et al (1). Cells grown in suspension culture were incubated for 10 minutes with 100 μg/ml of cycloheximide, and harvested by the addition of an equal volume of frozen phosphate-buffered saline (PBS) containing 200 μg/ml of cycloheximide and 0.01% diethylpyrocarbonate. Cells were washed with PBS containing 200 μg/ml of cycloheximide and resuspended in 5 volumes of reticulocyte standard buffer containing cycloheximide and $CaCl_2$ (1). The cells were homogenized and the nuclei were isolated (1). Heparin (final concentration of 500 μg/ml) was added to the postnuclear supernatant (7) which was then centrifuged at 27,000 x g for 5 minutes to sediment microsomes. Free and membrane-bound polyribosomes were obtained from the postmicrosomal supernatant and detergent treated microsomes, respectively, by centrifugation through 2 ml of 1.28 M sucrose over 2 ml of 2 M sucrose, both containing 100 μg/ml of heparin, for 16-18 hours at 36,000 rpm in a Spinco SW41 rotor (1,7).

Extraction of RNA. The polyribosome pellets were suspended in Na dodecyl SO_4 buffer and RNA was isolated (1). RNA from the postmicrosomal supernatant and microsome fractions was isolated by the hot phenol method (8).

Polyacrylamide Gel Electrophoresis. RNA samples, after addition of bromophenol blue (0.003%) and sucrose (25%), were electrophoresed in 2.5% polyacrylamide-0.12% bisacrylamide gels (0.6 x 9.5 cm) for 3 to 6 hours with constant current (5 ma/gel) as described (8). Cytoplasmic RNA labeled with ^{3}H-uridine was isolated from KB cells and electrophoresed in parallel gels. The molecular weights of virus-specific RNA peaks were calculated by comparison with 28S and 18S ribosomal RNA markers, assumed to have molecular weights of 1.65×10^6 and 0.65×10^6 daltons respectively (9,10).

Hybridization and S-1 Nuclease Assay for Duplex Formation. Gel slices (2.3 mm) were placed in 0.3 ml of 0.45 M NaCl-0.045 M Na_3citrate (3 X SSC) containing 5 μl of ^{3}H-DNA product (1400 cpm) and annealed for 20 hours at 66°C. To each tube, 4.4 ml of 3 X SSC was added and two 2 ml

aliquots were taken. To one aliquot was added 30 µg of denatured calf thymus DNA, 300 µl of 10 X S-1 buffer (S-1 buffer: 0.03 M Na acetate (pH 4.5), 1.8 x 10^{-3} M $ZnCl_2$), and a predetermined amount of S-1 nuclease sufficient to digest 97% of the MSV(MLV) single-stranded ^{3}H-DNA product. The mixture (total volume 3 ml) was incubated at 45°C for 2 hours. The second aliquot was treated by similar conditions but without enzyme. After incubation, 100 µg of calf thymus DNA and 0.75 ml of 50% trichloroacetic acid were added and the mixture kept in ice for 20 minutes. The nuclease-resistant hybrid was collected on nitrocellulose membrane filters, and the acid-precipitable radioactivity was determined using 10 ml of toluene containing 0.4% Omnifluor (New England Nuclear Corp.). Control hybridizations with 70S RNA and with E. coli tRNA was performed with each experiment. The background from the latter (2-4%) was subtracted from each experimental value.

Results and Discussion

Previous studies with MSV(MLV) producing 78A1 cells showed that virus-specific mRNA was present in polyribosome fractions that synthesized virus-specific polypeptides (1). In the present study, the size of virus-specific RNA species in the postmicrosomal supernatant, microsomes, and free and membrane-bound polyribosomes was analyzed. Polyribosomes were isolated by sedimenting the postmicrosomal supernatant (free polyribosomes) and detergent-treated microsomes (membrane-bound polyribosomes) through sucrose layers (1,11). RNA extracted from polyribosome-containing fractions, or from purified polyribosomes, was electrophoresed on 2.5% polyacrylamide gels. Two or three of each RNA preparation were analyzed. The RNA present in the gel slices was annealed with MSV(MLV) ^{3}H-DNA product, and treated with S-1 nuclease to digest unhybridized single-stranded DNA. The acid-precipitable radioactivity represents virus-specific RNA in hybrid form.

The virus-specific RNA in the postmicrosomal supernatant migrated as a major peak with a molecular weight of 2.9 x 10^6, as calculated from the 28S and 18S RNA markers (Fig. 1A). This peak has a molecular weight similar to the 35S virus-specific RNA detected in the total RNA extracted from 78A1 cells (12). In contrast, two peaks of virus-specific RNA in nearly equal amounts were detected in microsomes (Fig. 1B). The slower migrating peak has a molecular weight similar to the 35S virus-specific RNA of the post-microsomal supernatant. The fast migrating peak has a molecular weight of about 1.5 x 10^6, similar to that of the 20S virus-specific RNA species of 78A1 cells (12). The small amount of virus-specific RNA in the postmicrosomal supernatant with a lower mobility than the 28S

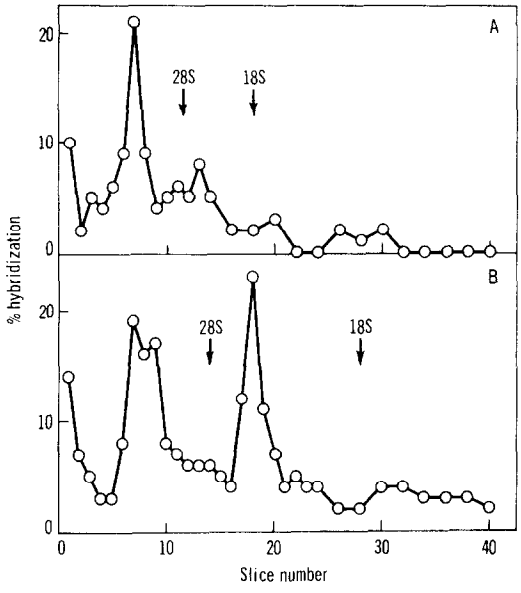

Fig. 1. Size of virus-specific RNA in postmicrosomal supernatant (A) and microsomes (B) of MSV(MLV) producing rat cells transformed by MSV (78A1 cells). RNA extracted from the postmicrosomal supernatant (102 μg) and from microsomes (35 μg) was electrophoresed on 2.5% polyacrylamide gels for 3 and 6 hours respectively. RNA in gel slices were hybridized with MSV(MLV) ^{3}H-DNA product. Details of the isolation of the cell fractions, the extraction of RNA, polyacrylamide gel electrophoresis, and DNA-RNA hybridization are given in Materials and Methods.

ribosomal RNA may represent breakdown products of 35S virus-specific RNA or contaminating 20S RNA from microsomes.

Free and membrane-bound polyribosomes were isolated by sedimentation through sucrose layers and the size distribution of virus-specific RNA was determined (Fig. 2). Essentially the same pattern of virus-specific RNA was obtained in free polyribosomes as in the postmicrosomal supernatant, i.e., one major peak of 35S virus-specific RNA (Fig. 2A). Electrophoresis for 6 hours to provide better resolution also gave a single 35S RNA peak (unpublished data).

The virus-specific RNA in the membrane-bound polyribosomes migrated as two peaks (Fig. 2B), 35S and 20S, similar to the two RNA peaks in microsomes from which they were derived. Better resolution and sharper peaks of virus-specific RNA were obtained with RNA from the postmicrosomal supernatant and microsome fraction than from the purified free and membrane-bound polyribosomes. This probably represents some degradation of RNA during the long periods that were required to isolate polyribosomes.

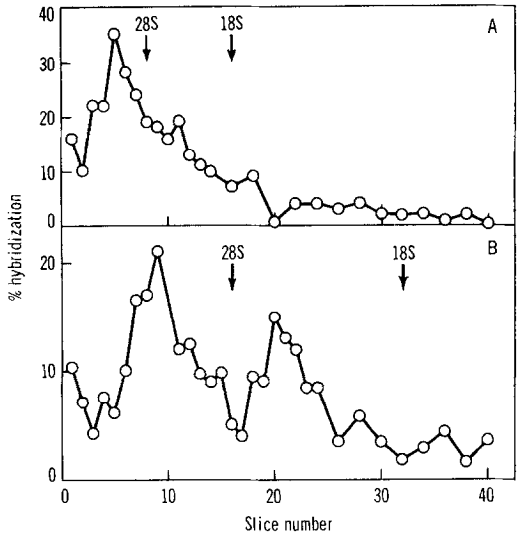

Fig. 2. Size of virus-specific RNA in free polyribosomes (A) and membrane-bound polyribosomes (B) of 78A1 cells. RNA extracted from free polyribosomes (104 µg) or membrane-bound polyribosomes (30 µg) was electrophoresed on 2.5% polyacrylamide gels for 3 and 6 hours respectively. RNA in gel slices were hybridized with the MSV(MLV) ^{3}H-DNA product.

The 35S and 20S virus-specific RNA species in polyribosome fractions probably serve as mRNA for the synthesis of virus-specific proteins since (i) virus-specific RNA in polyribosomes that synthesize virus-specific proteins could be dissociated with EDTA (1), and (ii) 35S and 20S RNA species were present in the RNA released from polyribosomes by EDTA (unpublished data). The occurrence of poly(A) tracts in 35S and 20S RNA species obtained from 78A1 cells (13) further strengthens the view that these RNA species could function as mRNA.

Our observations that polyribosomes of 78A1 cells contain two large species of virus-specific RNA, 35S and 20S, and that the major polypeptides made by these cells have molecular weights of 31,000 and lower (3), suggest that these high molecular weight RNAs might code for the synthesis of large polypeptides that are subsequently cleaved. The recent identification of precursor polypeptides of avian myeloblastosis virus (14), and the *in vitro* synthesis of large polypeptides by 78A1 polyribosomes (Bhaduri, Shanmugam, Vecchio and Green, unpublished data) support this possibility.

The second species of virus-specific RNA, 20S RNA, is present mainly in microsomes and membrane-bound polyribosomes. It is possible that (i)

both species of RNA serve as messenger, or that (ii) the smaller species (20S) is a cleavage product. Further experiments are needed to resolve this question.

Acknowledgments

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