ENDONUCLEOLYTIC CLEAVAGE OF MURINE LEUKEMIA VIRUS 35S RNA BY MICROSOME-ASSOCIATED NUCLEASE

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Summary: Moloney murine leukemia virus 35S RNA (molecular weight 3 to 3.4 x 10^6) is cleaved by nuclease activity present in microsomal fractions from MLV infected or uninfected mouse embryo cells to two RNA species of approximate molecular weights 1.8 x 10^6 and 1.5 x 10^6. Microsomal fractions from MLV infected and uninfected cells also contained nucleolytic activity that solubilized [^{3}H]poly(A)•poly(U) but not [^{3}H]poly(C) or [^{3}H]poly(U); the cleavage of poly(A)•poly(U) was inhibited by ethidium bromide. The cleavage of MLV RNA was also inhibited by ethidium bromide, suggesting double stranded regions in 35S RNA as the site of cleavage.

Two virus-specific RNA species that sediment at 35S and 20S have been found in transformed rat cells replicating murine sarcoma-leukemia viruses (1,2); 35S RNA was present in both free and membrane-bound polyribosomes while 20S RNA was detected only in membrane-bound polyribosomes derived from the microsomal fraction (3). It was suggested that the 20S RNA species present in the microsomal fraction may be a cleavage product of 35S RNA (3). Therefore, an attempt was made to detect endonuclease activity in the microsomal fraction of cells replicating MLV that would cleave viral 35S RNA. This communication presents evidence for the existence of microsome-associated ribonuclease that cleaves viral 35S RNA to products of discrete size. This activity appears to be specific for double-stranded regions of RNA since the cleavage of viral RNA was inhibited by ethidium bromide.

Materials and Methods

Cells and Virus. High passage Swiss mouse embryo (HPME) cells and Moloney murine leukemia virus (MLV) were obtained through the courtesy of Dr. A.J. Hackett. Cell monolayers were grown in Eagles minimum essential medium (MEM) with 10% fetal calf serum and infected with MLV as described (4,5).

Isolation of [^{32}P]MLV RNA. At 48 hours post infection, MLV-infected HPME monolayers (150 cm^2) were labeled for 1 hour with 5 mCi of [^{32}P]phosphate in 20 ml of phosphate-free MEM. Purification of labeled virus harvested at 1 hour intervals and extraction and sucrose density gradient centrifugation of viral RNA were done according to published procedures (2).

Abbreviations: MLV, murine leukemia virus; HPME, high passage Swiss mouse embryo

Sucrose gradient fractions containing viral 60-70S RNA were pooled and precipitated with ethanol; the precipitate was collected by centrifugation and dissolved in 1 to 2 ml of Tris buffer (50 mM Tris-HCl, pH 7.5 at 20°, 25 mM KCl and 5 mM $MgCl_2$).

Preparation of Microsomal Fraction. Cells were collected from infected and uninfected HPME monolayers using crushed frozen phosphate buffered saline (6) followed by low speed centrifugation (3). The cell pellet was suspended in 5 volumes of modified reticulocyte standard buffer (10 mM Tris-HCl, pH 7.5 at 4°, 10 mM KCl and 1.5 mM $MgCl_2$), homogenized and nuclei removed (7). The salt concentration in the post-nuclear supernatant was adjusted to that of Tris buffer and the supernatant was centrifuged at 27,000 x g for 5 minutes in order to sediment microsomes (3,7). The 27,000 x g pellet resuspended in Tris buffer by vortexing for 1 minute at maximum speed was termed as the microsomal fraction. The protein content of the microsomal fraction was determined by the method of Lowry et al. (8).

Incubation Conditions. The incubation mixture (1 ml) for MLV RNA cleavage studies contained 50 mM Tris-HCl, pH 7.5 at 20°, 25 mM KCl, 5 mM $MgCl_2$, heat dissociated [^{32}P]MLV RNA, microsomal fraction and 1.5 mM ethidium bromide (Sigma) when used. Samples were incubated at 37° for 10 minutes. After incubation, RNA was isolated by phenol extraction and dissolved in 15 mM NaCl-1.5 mM Na_3 citrate (7).

[^{3}H]poly(A)·poly(U), 15.1 μCi/μmole phosphate; [^{3}H]poly(C), 49 μCi/μmole phosphate; and [^{3}H]poly(U), 81.6 μCi/μmole phosphate were obtained from Miles Laboratories, Inc. These were incubated in 0.1 ml of high salt buffer (20 mM Tris-HCl, pH 7.8 at 25°, 10 mM $MgCl_2$, 200 mM NaCl, 5 mM 2-mercaptoethanol and 10% glycerol) with the desired amounts of microsomal fraction and 1.5 mM ethidium bromide, when used. At the end of incubation, 0.02 ml of bovine serum albumin (200 μg), 0.2 ml H_2O and 0.2 ml of cold 20% trichloroacetic acid were added and the mixture was centrifuged for 10 minutes at 12,000 x g. The radioactivity in 0.2 ml of supernatant was determined and multiplied by 2.6 in order to determine total acid soluble counts.

Polyacrylamide Gel Electrophoresis. RNA samples were electrophoresed for 3 to 4 hours in gels (0.6 x 10 cm) containing 2% acrylamide-0.1% bisacrylamide and 0.5% agarose as described (3). The gels were fractionated into 2 mm fractions and counted in 10 ml of Aquasol (9). The molecular weights of 35S RNA and its cleavage products were calculated by comparison of their mobilities with those of 28S and 18S rRNAs as previously described (3).

Results and Discussion

^{32}P-labeled 60-70S RNA was isolated from virus harvested at 1 hour intervals and heat-dissociated at 70° for 2 minutes in Tris buffer to obtain 35S RNA. Such RNA preparations contain over 85% intact 35S RNA (2) (see also Fig. 1A). The heated RNA was incubated at 37° for 10 minutes with different amounts of microsomal fraction from MLV-infected and uninfected HPME cells. At the end of incubation, RNA was extracted and electrophoresed in 2% acrylamide-0.5% agarose gels. [^{32}P]MLV RNA not treated with the microsomal fraction but incubated and analyzed under the same conditions showed a homogeneous peak with a molecular weight of 3 to 3.4 x 10^6 (species I, Fig. 1A). In contrast, [^{32}P]MLV RNA samples that were incubated with the microsomal fraction showed the presence of two species (II and III) that migrated faster than the MLV 35S RNA (Fig. 1B and 1C). Complete conversion of species I (35S, molecular weight 3.4 x 10^6) to

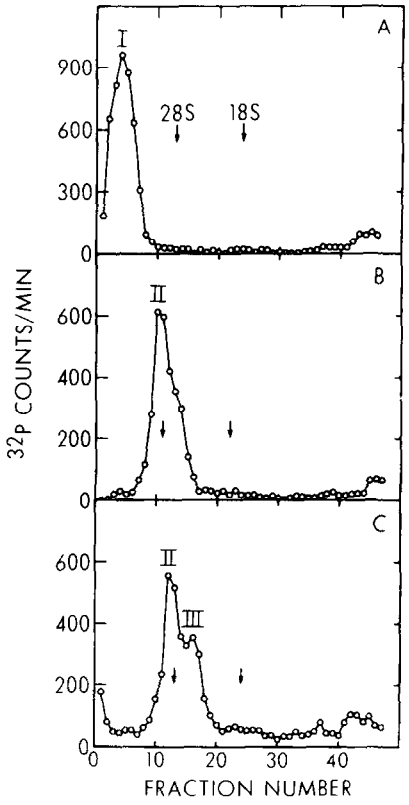
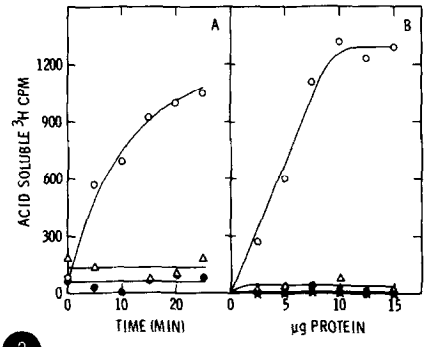


Fig. 1 Electrophoretic patterns of heat-dissociated [^{32}P]MLV RNA and its cleavage products on polyacrylamide-agarose gels. [^{32}P]MLV RNA extracted from virus harvested at hourly intervals was heated for two minutes at 70° in Tris buffer and quickly cooled in ice. Aliquots of heat-dissociated MLV RNA containing 35,000 cpm were incubated in 1 ml of Tris buffer at 37° for 10 minutes without (A) or with microsomes containing 8 µg (B) or 16 µg protein (C). RNA was extracted from the incubation mixture and electrophoresed in 2.5% polyacrylamide-0.5% agarose gels as described in Materials and Methods.

species II (molecular weight 1.7 to 1.9 x 10^6) was observed when MLV RNA was treated with the microsomal fraction from infected cells containing 8 µg protein (Fig. 1B). Increases in the concentration of microsomal fraction to 16 µg protein (Fig. 1C) or more led to the formation of species III (approximate molecular weight, 1.5 x 10^6). Over 70% of the radioactivity present in the 35S RNA peak of untreated controls was recovered in the cleavage products (Fig. 1B and 1C) suggesting endonucleolytic cleavage of 35S RNA into products approximately one-half its size. Microsomal fraction (5 µg) obtained from uninfected HPME cells also contained nuclease activity that cleaved MLV 35S RNA to species II (unpublished data).



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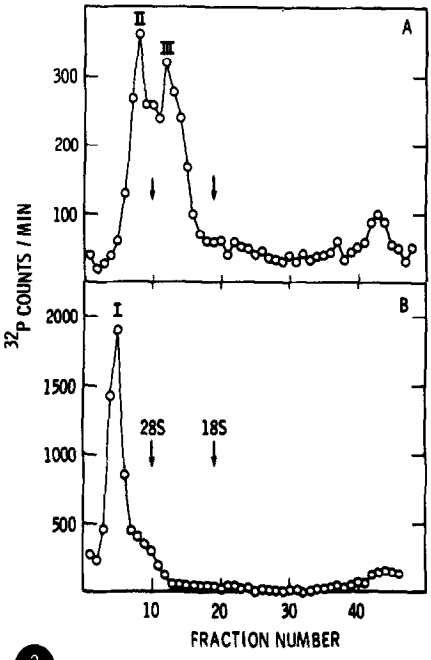

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Fig. 2. Cleavage of ^{3}H-labeled synthetic polynucleotides by microsomal fractions from MLV infected (A) and uninfected (B) HPME cells. The incubations were done in 0.1 ml of high salt buffer that contained 5,800 cpm [^{3}H] poly(A)·poly(U) without (o-o) or with 1.5 mM ethidium bromide (●-●), 10,750 cpm [^{3}H]poly(C) (▲), or 7,500 cpm [^{3}H]poly(U) (×). The acid soluble radioactivity present in samples incubated without microsomal fractions [7.8% for poly(A)·poly(U), 8.8% for poly(C), 12% for poly(U)] was considered as background and subtracted from all determinations. Conditions of incubation and determination of trichloroacetic acid soluble radioactivity are described in Materials and Methods.

Fig. 3. Gel electrophoretic analysis of cleavage of [^{32}P]MLV RNA by microsomes (A) and inhibition of cleavage by ethidium bromide (B). Heat dissociated [^{32}P]MLV RNA (9,600 cpm) was incubated in Tris buffer for 10 minutes at 37° with microsomes from infected cells containing 5 µg protein (A), or 16 µg protein and 1.5 mM ethidium bromide (B). RNA was extracted from the incubation mixture and electrophoresed on 2.5% acrylamide-0.5% agarose gels as described in Materials and Methods.

The RNA-cleavage activity associated with microsomal fraction was further studied using synthetic polynucleotides as substrates. Microsomal fractions from infected (Fig. 2A) and uninfected (Fig. 2B) cells solubilized [^{3}H]poly(A) ·poly(U). The solubilization of [^{3}H]poly(A)·poly(U) increased with time (Fig. 2A) and was proportional to microsomal protein concentration up to 10 µg (Fig. 2B). Under the conditions used, the microsomal fractions did not solubilize [^{3}H]poly(C) or [^{3}H]poly(U). Fig. 2A and 2B also show that the [^{3}H]poly(A)· poly(U) cleavage activity associated with either infected or uninfected microsomal fraction was inhibited by ethidium bromide which is known to inter-

calate with double stranded polynucleotides (10). The solubilization of [^{3}H]poly(A)·poly(U) by microsomal fraction and the inhibition of this reaction by ethidium bromide indicate the presence of a nuclease activity in the microsomal fraction that is specific for double stranded regions of RNA.

The cleavage of MLV 35S RNA by microsomal fractions that contain double-stranded RNA-specific nuclease activity suggests base paired regions in MLV RNA as possible sites of cleavage. In order to obtain further evidence regarding the nature of cleavage of MLV RNA, the effect of ethidium bromide was studied. The cleavage of [^{32}P]MLV RNA by microsomal fraction from infected cells and the inhibition of cleavage by ethidium bromide are shown in Fig. 3A and 3B, respectively. Only 5 µg of protein was required to produce species II and III with the microsomal fraction used in Fig. 3A, while 16 µg of another preparation was needed to produce these species (Fig. 1C). This difference could be due to inherent differences in enzyme levels and varying extents of solubilization of the enzyme in the two microsomal fractions. As shown in Fig. 3B, almost complete inhibition of cleavage of [^{32}P]MLV RNA was observed in the presence of 1.5 mM ethidium bromide. In the presence of lower amounts (0.45 to 0.9 mM) of the drug, 50-60% of the MLV RNA was not cleaved (data not shown). Recent studies demonstrate the presence of double stranded loop regions in feline C type viral RNA (11) and helical structures in heat dissociated murine leukemia viral RNA (12). Since ethidium bromide intercalates with double stranded polynucleotides (10) such as poly(A)·poly(U) and thereby inhibits the cleavage activity, the inhibition by this drug of cleavage of 35S MLV RNA by the microsomal fraction suggests double stranded region(s) as the site of action of the microsomal nuclease.

The specificity of cleavage activity for synthetic double stranded polynucleotides, the inhibition of cleavage by ethidium bromide and association of the enzyme activity with microsomal fractions parallels closely the properties of *E. coli* RNase III (13) which has been implicated in the processing of ribosomal RNA precursors and bacteriophage T7 messenger RNA transcripts (14-16). HeLa cell nuclei has been shown to contain a nuclease specific for double stranded regions of RNA (17). Similar endonuclease activity that was shown to be associated with microsomal fractions of HPME cells in the present work may be present in other subcellular fractions, including nuclei. Preliminary experiments show that the post-microsomal supernatant fraction of HPME cells also contains poly(A)·poly(U) and MLV RNA cleavage activity; in this fraction, over two-thirds of the cleavage activity is associated with ribosomes (unpublished observations). In *E. coli*, a ribosomal particulate fraction obtained from the RNase III^{+} strain was shown to cleave double-stranded RNA while ribosomes from the enzyme negative strain did not show significant double-stranded RNA specific cleavage activity (18).

The presence of endonucleolytic activity in the microsomal fraction is compatible with the presence of 20S virus-specific RNA species in microsomes of virus producing cells (3), but it is not clear at present whether similar enzyme activity plays a role in vivo in the cleavage of 35S MLV RNA. The microsome-ribosome associated endonuclease activity detected in this study may be useful for specific fragmentation of RNAs for structural studies and further work on its purification and characterization will be useful in understanding the biological role of this enzyme in RNA processing.

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