Ribonuclease Activity Is Associated with Subviral Particles Isolated from Interferon-Treated Vesicular Stomatitis Virus-Infected Cells

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Previously we have shown that inhibition of replication of vesicular stomatitis virus in interferon-treated JLSV-11 cells is at least partly caused by impaired viral primary transcription. Here we report that subviral particles isolated from interferon-treated infected cells were deficient in mRNA synthesis in vitro compared with the particles isolated from untreated cells. This was due to the presence of an associated ribonuclease activity which hydrolyzed not only newly synthesized viral mRNAs but also exogenously added viral transcripts.

The detailed mechanism by which interferon (IFN) inhibits the replication of a virus remains unclear despite extensive studies on the antiviral actions of IFN (6, 9). We have been studying the underlying mechanisms by which IFN inhibits vesicular stomatitis virus (VSV) replication. Using a VSV temperature-sensitive mutant and two pairs of IFN-sensitive and -resistant cell lines, we demonstrated that accumulation of VSV primary transcripts is inhibited by IFN-treatment of GM2767 and JLSV-11, two sensitive cell lines (2). Since virus entry and uncoating are not affected under these conditions, it appears that viral primary transcription itself is impaired in IFN-treated cells. Here we report that VSV subviral particles isolated from IFN-treated JLSV-11 cells, which had been restricted to primary transcription conditions, synthesized less VSV mRNA in vitro than did similar particles isolated from untreated cells. Results are presented which suggest that this was caused by the presence of a ribonuclease activity associated with the subviral particles isolated from IFN-treated cells.

We have previously described procedures for culturing JLSV-11 cells and infecting them with VSV (2), methods for VSV mRNA transcription in vitro, and analyses of the products (4) and methods for the preparation of mRNA of the phosphoprotein (NS) of VSV in vitro by using a plasmid vector containing NS cDNA inserted behind a bacteriophage SP6 transcriptional promoter (3). IFN treatment was done for 18 h with 500 U of a partially purified mouse beta IFN preparation per ml (specific activity, 2×10^8 U of protein per mg) purchased from Lee BioMolecular, San Diego, Calif. VSV subviral particles (RNPs) were isolated from IFN-treated or untreated infected JLSV-11 cells (10). For this purpose, cells were grown in roller bottles; at 50% confluency the cells were treated with 500 U of mouse IFN per ml for 18 h, if desired, and infected with VSV at a multiplicity of infection of 10 in the presence of 100 μ M anisomycin, as described before (2). After 1 h of virus adsorption and after an additional 2.5 h of virus infection in the presence of anisomycin, cells were harvested by scraping, after washing them three times in cold phosphate-buffered saline. Cell pellets were suspended in a cold buffer containing 10 mM Tris hydrochloride (pH 7.5)-1.5 mM MgCl₂-10 mM KCl-1 mM dithiothreitol, left on ice for 30 min, and then homoge-

nized in a Dounce homogenizer with 60 strokes. KCl was added to a final concentration of 150 mM, and the extract was centrifuged at $16,000 \times g$ for 20 min. To the supernatant, 2 mM EDTA was added to dissociate the polysomes, and the mixture was centrifuged in a Ti50 rotor at 40,000 rpm for 2 h. The resulting pellet was suspended in TE buffer (10 mM Tris chloride [pH 7.5], 1 mM EDTA). Then, 150 μ l of TE buffer was used to suspend the pellet obtained from 2×10^8 cells from one roller bottle. The protein concentration of this suspension, which was used as the source of the RNPs, was about 3.5 mg/ml.

Since we sought to reproduce in vitro the observed inhibition of VSV primary transcription in vivo in IFN-treated cells, we first examined the transcriptional ability of VSV RNPs isolated from cells that had been infected with virus in the absence of ongoing protein synthesis. The electrophoretic migration patterns and the relative quantities of VSV transcripts synthesized in vitro were very similar irrespective of whether detergent-disrupted virions (Fig. 1, lane 1) or viral RNPs isolated from BHK cells with (lane 2) or without (lane 3) ongoing protein synthesis were used. N, NS, and M mRNAs were the predominant species, whereas G mRNA was less abundant, with little L mRNA being synthesized. A similar pattern of synthesis was observed when we used VSV RNP isolated from infected JLSV-11 cells that had been treated with anisomycin (lane 4). The qualitative pattern of synthesis of different VSV mRNAs did not change appreciably even when the JLSV-11 cells were pretreated with IFN. However, the relative amount of RNA synthesis was reduced appreciably (lane 5). The extent of this inhibition varied from lot to lot of RNP preparations, which will be apparent from the other experiments described here. To guard against this variability, pairs of RNPs from IFN-treated (RNP_I) and untreated (RNP_C) cells were always prepared at the same time and a different pair of RNP_C and RNP_I was used in each experiment described here.

To quantitate the extent of inhibition more rigorously, the experiment in Fig. 2 was done with one pair of RNP_C and RNP_I cells. In vitro transcription was carried out with various concentrations of RNP_C and RNP_I, the products were analyzed by gel electrophoresis, and the amount of N mRNA synthesized in each reaction was quantitated by excising the N mRNA band from the gel and counting it in a scintillation counter. At every concentration tested, less N

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FIG. 1. Transcription in vitro by different VSV RNP complexes. In vitro transcription reactions were carried out for 2 h at 30°C by using 0.35 mg of RNP protein per ml of reaction mixture. Products were analyzed as described before (4). Positions of different VSV mRNAs are shown. (A) Transcription by detergent-disrupted virion RNPs (lane 1), RNPs isolated from infected BHK cells (lane 2), and RNPs isolated from BHK cells infected in the presence of 100 µg of anisomycin per ml of culture medium (lane 3). (B) Transcription by RNPs isolated from anisomycin-treated infected JLSV-11 cells (RNP_C) (lane 4) and anisomycin-treated infected JLSV-11 cells that were pretreated for 18 h before infection with 500 U of mouse IFN per ml of culture medium (RNP_I) (lane 5). mRNA species are shown at the left and right.

mRNA was synthesized by RNP_I compared with that synthesized by RNP_C, but the extent of inhibition varied from 3- to 10-fold, depending on the actual concentration of RNPs in the reaction mixture.

When a high concentration of RNPs of another lot was used for in vitro transcription, the amount of VSV mRNAs synthesized decreased with increasing amounts of RNP_I (Fig. 3A, lanes 3 and 4), suggesting that the RNP_I preparation might contain an inhibitor of VSV mRNA synthesis. Indeed, when RNP_I was mixed with RNP_C, about 50% less mRNA was synthesized than that synthesized by RNP_C

FIG. 2. Quantitation of VSV N mRNA synthesis by RNP_C and RNP_I. In vitro transcription reactions were carried out with different concentrations of RNP_C and RNP_I, and the products were analyzed by gel electrophoresis. The bands representing N mRNA were excised from the gel, and the associated radioactivities were quantitated by being counted in a scintillation counter. RNP_C and RNP_I were isolated from JLSV-11 cells infected in the presence of anisomycin.

alone (lanes 1 and 2). At this concentration range, synthesis of VSV mRNA increased almost linearly with increasing concentrations of RNP_C (data not shown). To distinguish whether RNP_I contained an inhibitor of VSV mRNA synthesis or whether the newly synthesized mRNA was rapidly hydrolyzed by an activity associated with it, a pulse-chase experiment (Fig. 3B) was done. VSV mRNA was synthesized by RNP_C (lane 7) or RNP_I (lane 5) in the presence of radiolabeled CTP and then a 20-fold excess of unlabeled CTP was added to the reaction mixture, and the incubation was continued. The product of RNP_I was hydrolyzed at a much faster rate (lane 6) than was the product of RNP_C (lane 8), suggesting that a ribonuclease activity was associated with RNP_I. The amounts of N mRNA in each lane were 563 cpm (lane 5), 54 cpm (lane 6), 2,571 cpm (lane 7), and 2,586 cpm (lane 8). During the chase period, therefore, 90% of mRNA synthesized by RNP_I had been hydrolyzed, whereas the mRNA synthesized by RNP_C remained essentially intact. In a separate experiment in which labeled VSV mRNA was synthesized by detergent-disrupted virions and incubated further with RNP_I in the presence of excess of unlabeled ribonucleotide triphosphates, 95% of the mRNA was degraded in 1 h (data not shown).

Direct demonstration of RNP_I-associated RNase activity is documented (Fig. 4). In these experiments, we used VSV NS mRNA transcripts synthesized by SP6 RNA polymerase from an NS cDNA-containing plasmid vector (3). RNA was extensively hydrolyzed (83%) in the presence of RNP_I compared with that in the presence of RNP_C (19%) (Fig. 4A). RNase reaction in the presence or in the absence of ribonucleoside triphosphates did not alter the extent of mRNA degradation (data not shown). The RNase activity was found to be tightly associated with RNP_I. This was demonstrated when RNP_I pellets were suspended in cell homogenization buffer and resedimentated; the RNase activity still remained associated with the pellet, and the supernatant was virtually free of RNase activity (Fig. 4B). No such activity was

FIG. 3. Presence of an inhibitor of transcription in RNP_I. Transcription reactions were carried out for 1 h. Lanes: 1, 0.35 mg of RNP_C per ml of reaction mixture; 2, 0.35 mg of RNP_C and 0.35 mg of RNP_I per ml; 3, 0.35 mg of RNP_I per ml; lane 4, 0.70 mg of RNP_I per ml. (B) Pulse-chase reactions. Standard transcription reactions with 0.35 mg of RNPs per ml were carried out for 1 h. One set of aliquots was saved for analyses; to another set of equal aliquots 20-fold excess of unlabeled CTP was added, and the incubation reactions were continued for another 1 h. Lanes: 5, RNP_I transcription products before chase; 6, RNP_I transcription products after chase; 7, RNP_C transcription products before chase; 8, RNP_C transcription products after chase. mRNA species are shown.

FIG. 4. Degradation of SP6 transcripts of VSV NS mRNA upon incubation with RNP₁. (A) Radiolabeled NS mRNA was synthesized from pGEMNS and purified (3). Equal amounts of this mRNA were incubated with TE buffer, RNP_c, or RNP₁ in a standard *in vitro* transcription reaction mixture containing unlabeled CTP, and the products were analyzed by gel electrophoresis. Amounts of NS mRNA left undegraded were quantitated by excision of the gel bands. Results are presented as percent of input RNA left undegraded. (B) RNP_c and RNP₁ were suspended in cell homogenization buffer and resedimented by ultracentrifugation. The pellets were suspended in the same volume of TE buffer as the homogenization buffer, and equal portions of supernatants (SUP_c, SUP₁) and resedimented RNPs were assayed for RNase activity. The substrate, reaction conditions, and methods of analysis were the same as those in panel A.

associated with similar pellets obtained from IFN-treated uninfected cells, suggesting that the presence of virion RNP was necessary for sedimentation of this activity (data not shown).

In the experiments described here, we have demonstrated that VSV RNPs isolated from IFN-treated JLSV-11 cells synthesized significantly less VSV mRNA *in vitro* than did RNPs isolated from the same number of untreated cells. This result is consistent with earlier observations of impaired VSV primary transcription *in vivo* (2, 8). Moreover, our data clearly indicated that this reduced RNA synthesis was caused by the action of an RNase activity associated with RNP₁. Purification and further characterization of the RNase activity are needed for understanding the basis of its association with VSP RNP. It remains to be seen whether this activity is related to the IFN-inducible RNase L activity which has been implicated to be responsible for impaired mRNA transcription by reoviral cores isolated from IFN-treated cells (5, 7) and by reovirions to which extract of IFN-treated cells has been added (1). Wallach and Revel (11)

have reported trapping 2',5'-oligoadenylate synthetase in VSV and murine leukemia virus particles released from IFN-treated cells (11). Activation of the RNase activity associated with RNP₁ by 2',5'-oligoadenylate synthesis *in situ* is however unlikely, since this RNase could hydrolyze mRNA in the absence of any added ATP. Irrespective of whether this RNase activity is related to RNase L activity, the important mechanistic question is whether it can account for the inhibition of VSV primary transcription observed in IFN-treated cells *in vivo*. Experiments to clarify these issues are in progress.

The secretarial assistance of Jaklin Levi is gratefully acknowledged.

This study was supported in part by Public Health Service grant AI-22510 and by core grant CA-08748 from the National Institutes of Health.

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