Rifampin-Susceptible Mutant of Vesicular Stomatitis Virus: Protein and RNA Synthesis

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Received for publication 14 January 1976

A rifampin-susceptible strain (VSV Rif+) was selected from the wild vesicular stomatitis virus (VSV) population unsusceptible to rifampin. The VSV Rif+ was blocked in its intracellular replication in the presence of rifampin. In cells, rifampin affected primarily VSV Rif+ transcription, but to a different extent than in a cell-free system. In addition, a decrease in the amount of VSV Rif+ protein M was detected, linked to a stimulation of protein NS. In the absence of rifampin, protein M, although synthesized, was not immediately incorporated into the cell membrane. An interpretation of these observations is proposed.

Rifampin, a semisynthetic derivative of the antibiotic rifamycin, is well known to inhibit the growth of bacteria (6, 22) and poxviruses (7, 23). In contrast, the majority of RNA viruses, except RNA tumor viruses, appear to be resistant to rifampin and its derivatives. In particular, the wild type of vesicular stomatitis virus (VSV), a membrane-coated and RNA-containing virus, is not susceptible to this drug.

We have previously described the intracellular replication of a spontaneous VSV Indiana strain mutant (VSV Rif+) susceptible to rifampin and the selection of a revertant variant (VSV Rif-) resistant to the antibiotic (16, 17). When the mechanism of the inhibitory effect of rifampin was explored in a cell-free system, total inhibition of the transcriptase activity at an early stage of transcription was observed. However, in cells the rate of total VSV Rif+ RNA synthesis was only decreased about 50%. This partial defectiveness in VSV Rif+ RNA synthesis observed in the presence of rifampin could result from a selective inhibition of one viral mRNA. Consequently, an analysis of viral RNA and protein synthesis was carried out during a single replicative cycle.

Data are presented in favor of the hypothesis that the mutation affected VSV transcription and involved both structural and functional changes in the transcription complex.

MATERIALS AND METHODS

Cells and virus. The Indiana strain of VSV was used in this study. VSV Rif+ and VSV Rif- mutants were concentrated and purified as described by McSharry and Wagner (14). The selection of these mutants was previously described (17).

L-cells were propagated in monolayers and incubated with Eagle medium (minimal essential medium 0111), supplemented with tryptose phosphate broth and 10% heat-inactivated calf serum.

Chemicals. [3H]uridine (21 Ci/mmol) and a 14C-labeled amino acid mixture (473 µCi/mg) were purchased from C.E.A. (Saclay, France); cycloheximide from Sigma Chemical Co. (St. Louis, Mo.). Actinomycin D was a gift from Merck, Sharp and Dohme Research Laboratories (Rahway, N.J.). Rifampin was supplied by Gruppo Lepetit Spa (Milan, Italy).

Extraction of viral RNA. L-cell monolayers in Roux bottles were infected with VSV at a multiplicity of infection of 20 PFU/cell in the presence of actinomycin D (5 µg/ml) for 1 h at 37°C. One hour postinfection (p.i.), nonadsorbed virus was eliminated and rifampin was added at selected concentrations until the end of the experiment. To study primary transcription, the same procedure was used, except that cycloheximide (100 µg/ml) was added together with rifampin.

A [3H]uridine labeling (10 µCi/ml) was performed from 2.5 to 3.5 h p.i. Cells were then washed three times with TNE buffer (0.01 M Tris, 0.1 M NaCl, 0.001 M EDTA), pH 8.4, at 4°C. Cell sheets were incubated for 10 min at room temperature with TNE buffer, pH 8.4, adjusted to 1% sodium dodecyl sulfate (SDS) and 1% β-mercaptoethanol. RNA was then extracted twice with phenol buffered in NTE, pH 8.4, and 0.5% β-mercaptoethanol, according to a previously described technique (2, 9). A final extraction was performed with phenol buffered in NTE, pH 7.4. The RNA was precipitated from the aqueous phase overnight at −20°C with 2 volumes of ethanol in the presence of sodium acetate 20% (0.1 volume). The precipitate was resuspended after centrifugation in NTE buffer, pH 7.4, containing 0.5% SDS and analyzed on polyacrylamide agarose gel. The production of cellular 14C-labeled RNA markers was performed by the same procedure.

Affinity chromatography on poly(U)-Sepharose. The following procedure, described by Lindberg and Personn (12), was used. Polyuridylic acid [poly(U)]-
Sepharose 4B, obtained from Pharmacia Fine Chemicals (Uppsala, Sweden), was swollen in NaCl (1 M, pH 7.5) for 10 min at room temperature at a concentration of 10 ml/0.2 g of dry powder. The gel was then washed with 10 ml of 0.1 M NaCl and packed in a 1-ml syringe column. A second wash was performed with eluting buffer (PO₄/NaCl, 0.1 M; EDTA, 0.01 M; SDS, 0.2%; in 90% formamide, pH 7.5). The poly(U)-Sepharose was equilibrated with a concentrated salt buffer: NaCl, 0.7 M; Tris-hydrochloride, 0.05 M; EDTA, 0.01 M; in 25% formamide (pH 7.5).

The viral RNA, after a phenol extraction performed as described above, was prepared in detergent solution (1% SDS and 0.03 M EDTA) and diluted five times with the salt buffer before fractionation on a poly(U) column. The viral mRNA binding was carried out at pH 7.5 in concentrated salt buffer for 18 h at room temperature. At that time, nonadenylated RNA was washed through with concen-

Fig. 1. Polyacrylamide gel profiles of ³H-labeled RNA extracted from L-cells infected with VSV Rif⁻. Monolayers containing 10⁸ cells were infected with VSV Rif⁻ (multiplicity of infection = 20 PFU/cell) in the presence of actinomycin D (5 μg/ml). Rifampin (200 μg/ml) was added 1 h p.i., and labeling was performed with [³H]uridine (10 μCi/ml) from 2.5 to 3.5 h p.i. After SDS-phenol extraction, RNA was subjected to electrophoresis for 2.5 h at 10 mA/gel on 2% acrylamide and 0.5% agarose gels. Arrows indicate position of ¹⁴C-labeled L-cell rRNA markers. VSV Rif⁻-infected cells untreated (●) and treated (○) with rifampin (200 μg/ml); RNA extracted from uninfected cells treated (●) with actinomycin D.
trated salt buffer until the radioactivity of the eluted fractions was insignificant. The mRNA's retained by the poly(U) column were then eluted with the formamide eluting buffer in 1-ml fractions and precipitated with ethanol. The precipitate was re-suspended for analysis on polyacrylamide agarose gels.

RNA polyacrylamide gel electrophoresis. The method of Loening (13), modified by Schincariol and Howatson (20), was chosen to perform polyacrylamide gel electrophoresis. The 2% acrylamide and 0.5% agarose gels (23 by 1 cm) were prerun for 1.5 h at 10 mA/gel. Samples of 100 µl containing 20 µg of RNA were then layered onto the gels, and electrophoresis was carried out for 2.5 h at room temperature. Gels were sliced into 1.5-mm fractions. The fractions were dissolved with 200 µl of 2 N NH₄OH and counted in Scintix scintillation fluid (Isotec, France).

Analysis of viral proteins. L-cell monolayers were infected with VSV at a multiplicity of infection of 20 PFU/cell. After 1 h of viral adsorption, L-cells were incubated in Earle serum-free saline solution. Rifampin (100 µg/ml) and a 14C-labeled amino acid mixture (5 µCi/ml) were added at the indicated times. Cells were then harvested by scraping in Earle saline solution and frozen at -80°C. Thawed extracts were treated with cold acetic acid diluted 10

![Image]

Fig. 2. Polyacrylamide gel profiles of [3H]RNA extracted from L-cells infected with VSV Rif+. Cytoplasmic extracts were analyzed as described in the legend to Fig. 1. (A) VSV Rif+ -infected cells (●) untreated with rifampin; (B) VSV Rif+ -infected cells treated with (○) 100 µg, (□) 200 µg, and (△) 400 µg of rifampin per ml.
times, urea (0.5 M), and SDS (1% final concentration) using the technique of Wagner et al. (27).

After incubation for 1 h at 37°C, the protein extracts were dialyzed at room temperature for 18 h against phosphate-buffered saline with 0.1% SDS, urea (0.5 M), and 0.1% β-mercaptoethanol. Samples of 100 μl containing about 1 mg of protein were layered on 7.5% acrylamide gel (8 by 1 cm), and electrophoresis was carried out at 10 mA/gel for 7 h. Gels were then sliced into lengths of 1 mm. After one night at room temperature in 200 μl of NH₄OH, the ¹⁴C-labeled protein content of each slice was measured by scintillation spectrometry.

The cellular distribution of viral proteins was analyzed after high-speed centrifugation of the cell extract to separate soluble and insoluble components (28, 26). Electrophoresis of cell extracts was then performed in the same manner.

RESULTS

Analysis of viral RNA synthesis. It has been shown that de novo synthesis is necessary for replication and secondary transcription of VSV. Consequently, primary transcription (i.e., synthesis of viral mRNA's from the genome of input virions) can be artificially separated from secondary transcription whose activity is asso-

![Diagram of gel electrophoresis](http://jvi.asm.org/Downloaded from)
associated with newly synthesized progeny virus genome (8, 15). These two events were studied with VSV Rif+ and its revertant variant, VSV Rif−.

Total transcription was performed as described in Materials and Methods. L-cells were infected in the presence of rifampin, and at 3.5 h p.i. intracellular RNA species were analyzed on polyacrylamide gels after phenol-SDS extraction. Control cells without the antibiotic were infected with both viruses in parallel. The three major size classes of viral RNA previously identified by Schincariol and Howatson (20) and by others (11, 25) were also observed with VSV Rif− in the presence of the drug (Fig. 1). In spite of a poor separation of the RNAs sedimenting in the area of 18 to 13S, a pronounced inhibition of their synthesis was observed with VSV Rif+ in the presence of rifampin (Fig. 2). As could be expected, this inhibition appeared to be proportional to the concentration of rifampin used. In contrast, no change in replicative 42S RNA or 28S mRNA was detectable. These experiments seem to indicate that rifampin affects the smallest mRNA species.

Primary transcription was analyzed when L-cells were infected in the presence of cycloheximide to block de novo protein synthesis, and thus viral replication and secondary transcription. As primary transcription accounts for only 10% of the total RNA synthesis (8, 25), a predictable reduction of RNA synthesis was observed with both viruses independently of rifampin action. In addition, no 42S RNA synthesis was detected (Fig. 3 and 4). The same results were found as in the case of total transcription: rifampin seems to act selectively on the VSV Rif+ 18-13S RNA fraction synthesis (Fig. 4). Thus, rifampin affects the transcription of VSV Rif+ but not that of VSV Rif−.

Analysis of VSV Rif+ mRNA's after fractionation on a poly(U)-Sepharose column. It has been previously reported that VSV mRNA's, but not virion RNA, contain adenylate-rich sequences (4, 21). To obtain a better resolution of VSV Rif+ mRNA's, an affinity chromatography on poly(U)-Sepharose was performed. When L-cells were infected under permissive conditions with the VSV Rif+ mutant,
Fig. 5. Polyacrylamide gel electrophoresis of VSV Rif+ RNA fractionated on a poly(U)-Sepharose column. L-cells (4 x 10⁸ cells) were infected with VSV Rif+ (multiplicity of infection = 20 PFU/cell) in the presence of actinomycin D (5 μg/ml). Rifampin (200 μg/ml) was added 1 h p.i. and a pulse-label was performed with [³H]uridine (50 μCi/ml) at 3.5 to 4 h p.i. After an SDS-phenol extraction, viral RNA was fractionated on a poly(U)-Sepharose column and subjected to electrophoresis for 2.5 h at 10 mA/gel on 2% acrylamide and 0.5% agarose gels along with marker ⁴C-labeled rRNA (arrows). VSV Rif+-infected cells untreated (●) and treated (Δ) with rifampin. (A) Electropherogram profiles of VSV Rif+ polyadenylated mRNA's eluted with the 90% formamide buffer. (B) Electropherogram profiles of VSV Rif+ nonadenylated RNAs eluted with the concentrated salt buffer.
polyadenylated 28S and 13-18S mRNA's were detected after affinity chromatography. In the presence of rifampin, 28S mRNA was also synthesized, but in a somewhat lesser amount. In contrast, no 13-18S mRNA species were detectable (Fig. 5A).

Since only 13-18S mRNA synthesis seems to be affected by the drug, these data could indicate a defect in polyadenylation of 28S mRNA. To investigate this hypothesis, an analysis of the RNA fraction washed through the column with concentrated salt buffer was performed in a similar manner and compared with the poly(A)-RNA fraction eluted with formamide. In the absence of rifampin, a peak of 40S RNA, which could represent the viral genome, was detected. In addition, a very low level of 28S RNA was observed and could be related to the binding efficiency of this RNA species. In the presence of the drug, 28S RNA was clearly found in the nonadenylated RNA fraction. A low level of 13-18S mRNA's was also detected, perhaps in relation with the heterogeneity of this messenger category (Fig. 5B).

Analysis at the end of the replicative cycle (7 h p.i.) of viral proteins synthesized in the presence of rifampin. Cytoplasmic extracts and polyacrylamide gel electrophoresis were performed as described in Materials and Methods. Rifampin was added 1 h after cell infection and was maintained during a complete cycle. The labeling period with 14C-amino acids was 4

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**Fig. 6.** Electropherograms of viral proteins extracted from L-cells. Cells were infected with VSV Rif⁺ or Rif⁻ (multiplicity of infection = 20 PFU/cell). Rifampin (100 μg/ml) diluted in Earle salt solution was added after a 1-h adsorption period until the end of the experiment. Labeling was performed with a 14C-amino acid mixture (5 μCi/ml) from 4 to 7 h p.i. Cytoplasmic extracts were prepared at 7 h p.i. and analyzed on SDS-7.5% polyacrylamide gels for 7 h at 10 mA/gel. The arrows mark the positions of the viral proteins L, G, N, NS, and M. VSV Rif⁺-infected cells: (A) treated (●) with rifampin and (B) untreated (○). VSV Rif⁻-infected cells: (C) treated (●) with rifampin and (D) untreated (○).
to 7 h p.i. In the absence of the antibiotic, the electropherogram profiles observed with both VSV Rif+ and Rif− (Fig. 6B, C) were identical to those previously described for the wild strain (10, 28). However, in our experiments, it was often difficult to detect protein L in significant amounts. In the presence of rifampin, no modification was observed with VSV Rif− (Fig. 6C). For VSV Rif+, the total amount of protein labeling increased by an average of 20% (Fig. 6A). In agreement with the data described above, it is more likely that this increase is due to an accumulation of the five viral proteins than to a stimulation of their synthesis.

To support this view, VSV Rif+ protein synthesis was analyzed, adding rifampin at two different time periods during the first replicative cycle. When rifampin was present only during the first 4 h p.i., an accumulation of viral proteins was observed (Fig. 7A). In contrast, when rifampin was added at the end of the cycle (4 to 7 h), no such modification was found (Fig. 7B). In parallel, extracellular radioactivity was measured. When an increase in the amount of viral proteins was noted in the electropherogram, the corresponding extracellular accumulation of viral label was reduced by about 30%.

Analysis at 4 h p.i. of viral proteins synthesized in the presence of rifampin. During the 2- to 4-h p.i. period, protein synthesis was observed to be maximal for the wild strain of VSV (28). As the growth cycles of VSV Rif+ and Rif− were identical in the absence of rifampin and presented no significant difference with the standard virus, cell extracts were performed in the following experiments at 4 h p.i., before the complete maturation of the virus. When protein content of L-cells infected with VSV Rif+ was analyzed under these conditions and in the presence of rifampin, the intracellular level of protein M accumulation decreased about 40% as compared to the other viral proteins. In addition, protein NS labeling was in-
Fig. 7. Viral proteins synthesized when rifampin was added at two different time intervals. L-cells were infected with VSV Rif+ (multiplicity of infection = 20 PFU/cell). The 14C-labeled amino acid mixture (5 μCi/ml) was added from 2 to 4 h p.i. Cell extracts were performed at 7 h p.i. and analyzed as described in Materials and Methods. (A) Rifampin (100 μg/ml) was added at the beginning of the replicative cycle from 1 to 4 h p.i. After rifampin removal, L-cells were incubated with minimum essential medium (MEM) 0111 without calf serum from 4 to 7 h p.i. (B) MEM 0111 without calf serum was added at the beginning of the replicative cycle from 1 to 4 h p.i. After its removal, L-cells were incubated in the presence of rifampin (100 μg/ml) from 4 to 7 h p.i.
increased by about 50% (Fig. 8A). A slight reduction (never more than 20%) of protein M, synthesized in the presence of rifampin, was sometimes found with VSV Rif− and might relate to the reversion rate of the viral population used. Nevertheless, there was no change in the amount of VSV Rif− protein NS labeling (Fig. 8B), and the stimulation observed with VSV Rif+ seemed to be specific for this virus. No change was induced by rifampin in the behavior of either protein M or protein NS in the case of the wild strain (not shown).

**Cellular distribution of VSV Rif+ proteins in the presence of rifampin.** It was interesting to investigate whether VSV Rif+ protein G or M, synthesized in the presence of rifampin, was correctly inserted in the cell membrane. Sedimentable and non-sedimentable viral protein fractions were analyzed 4 h p.i. using a technique described by Wagner and others (19, 26, 28).

As previously shown for the wild strain of VSV, the majority of protein G and about two-thirds of protein N were recovered in the soluble fraction (Fig. 9). However, in the presence of rifampin, the peak of protein NS was increased by 40% (as described above). Normally, protein M should be inserted in the cell membrane immediately after its synthesis (3). In the case of VSV Rif+, when cell extracts were performed at 4 h p.i., protein M was not yet incorporated into the cell membrane, since 80 to 90% of this protein was found in free form. This event was observed even in the absence of rifampin. The drug seemed to act only on the intracellular accumulation of the protein, the amount of which was reduced about 30%. Protein M of VSV Rif− was normally found inserted in the cell membrane and was not affected by the drug (Fig. 10).

**DISCUSSION**

Our experimental data show that rifampin could inhibit the multiplication of VSV Rif+, affecting two different metabolic steps of intracellular viral synthesis. Viral RNA synthesis is decreased and followed by more complex changes in the synthesis of viral proteins. The first site of rifampin action seems to be primary transcription, which is in agreement with in vitro studies. Inhibition of transcriptase activity is complete in a cell-free system, whereas in cells only the 18-13S mRNA synthesis appears to be affected. These discrepancies could be due to...
Fig. 9. Cellular distribution of VSV Rif<sup>+</sup> proteins. L-cells were infected and labeled as described in the legend to Fig. 7. Cytoplasmic extracts prepared at 4 h p.i. without detergent were centrifuged at 4°C (33,000 rpm) for 2 h in a no. 40 rotor. Viral proteins of the sedimentable and non-sedimentable fractions were then analyzed on SDS-polyacrylamide gels using the technique described in Materials and Methods. (A) VSV Rif<sup>+</sup>-infected cells treated with rifampin (100 µg/ml) from 1 to 4 h p.i.: (○) soluble material; (○) sedimentable material. (B) VSV Rif<sup>+</sup>-infected cells, untreated: (●) soluble material; (○) sedimentable material.
to the fact that in vitro transcription of VS virions is greatly dependent on temperature (24). In addition, in cells a partial defect in polyadenylation of 28S mRNA is also detected.

The main site of mutation of VSV Rifₚ is probably the transcriptase itself. The observation that VSV Rifₚ transcriptase can polymerize deoxyribonucleotides in a cell-free system, in opposition to Rif⁻ and the wild strain, supports this view (18). The changes involved in the protein structure could be considered a consequence of this mutation. Indeed, in addition to RNA synthesis, protein synthesis is also modified when rifampin is added early during the replicative cycle, regardless of whether the antibiotic is kept 1 to 4 h after the onset of the replicative cycle or during the whole period. An intracellular accumulation of all viral proteins is observed at the end of the replicative cycle, but no similar increase is detectable in the extracellular compartment. Thus, a relationship could exist between the inhibition of transcription and the modification of protein structure or synthesis. When rifampin is added from 1 to 4 h p.i. and cell extracts are performed at that time, a decrease of protein M labeling is apparently linked to a stimulation of protein NS. In contrast, for the Rif⁻ variant, protein NS labeling is normal and that of protein M is only slightly diminished, or not at all.

It has been shown that the smallest viral mRNA's (12S) are responsible for the translation of proteins M and NS (1). Since protein NS is directly involved in viral transcription (5), its apparent stimulation could reflect a direct effect of the drug on the transcriptase. Moreover, the cytoplasmic accumulation of VSV Rifₚ protein M at 4 h p.i., even in the absence of rifampin, could be a consequence of a modified transcription. This matrix protein is later incorporated into the cell membrane since it is found in the Rif⁺ virions. Thus, nucleocapsids are produced, but the process of envelopment could be defective in the presence of the drug.

In conclusion, rifampin inhibits VSV Rif⁺ transcription. Its direct action on the maturation process is not fully evident, and this problem is currently under investigation using different rifampin-susceptible mutants.

ACKNOWLEDGMENTS

We thank Charles Chany and Carole Girard for many helpful discussions and contributions in the preparation of the manuscript. The help and advice on affinity chromatography by N. Hanania is gratefully acknowledged. This study was supported in part by research grant 65.75 R from U.E.R. Cochin Port-Royal, Paris, France.

LITERATURE CITED


Fig. 10. Cellular distribution of VSV Rif⁺ proteins. Cytoplasmic extracts were analyzed in the absence of rifampin as described in the legend to Fig. 8. Symbols: (●) soluble material; (○) sedimentable material.