The broad spectrum antiviral nucleoside ribavirin displays activity against a variety of RNA and DNA viruses. A number of possible mechanisms have been proposed during the past 30 years to account for the antiviral activity of ribavirin, including the possibility that ribavirin might have a negative effect on the synthesis of the RNA cap structure of viral RNA transcripts. In the present study, we investigated the possibility that ribavirin can directly serve as a substrate for the vaccinia virus RNA capping enzyme. We demonstrate that ribavirin triphosphate can be used as a substrate by the capping enzyme and can form a covalent ribavirin monophosphate-enzyme intermediate reminiscent of the classical GMP-enzyme intermediate. Furthermore, our data indicate that ribavirin monophosphate can be transferred to the diphosphate end of an RNA transcript to form the unusual RpppN structure. Finally, we provide evidence that RNA transcripts that possess ribavirin as the blocking nucleoside are more stable than unblocked transcripts. However, in vitro translation assays indicate that RNA transcripts blocked with ribavirin are not translated efficiently. Our study provides the first biochemical evidences that ribavirin can directly interact with a viral capping enzyme. The ability of a purified RNA capping enzyme to utilize ribavirin as a substrate has not been previously documented and has implications for our understanding of the catalytic mechanisms of RNA capping enzymes. The biological implications of these findings for the proposed ribavirin-mediated inhibition of capping are discussed.

Ribavirin is a broad spectrum antiviral nucleoside that displays activity against a variety of RNA and DNA viruses (1, 2). Ribavirin is a synthetic nucleoside analogue with a structure closely related to guanosine (3). Once inside the cells, ribavirin is phosphorylated by cellular kinases, with ribavirin triphosphate (RTP) as the major intracellular metabolite (4, 5). A number of possible mechanisms have been proposed during the past 30 years to account for the antiviral activity of ribavirin. For instance, ribavirin monophosphate (RMP) has been shown to inhibit the host cell inosine monophosphate dehydrogenase, an enzyme involved in the de novo synthesis of GTP (6, 7). Because GTP is required for the transcription of viral genomes and replication of RNA viruses, it has been assumed that the decrease in the cytosolic concentration of GTP could affect the multiplication of viruses. Ribavirin also modulates the host immune system by engendering a bias toward helper T-cell type 1 cytokine response (8, 9). This would ultimately lead to an enhanced immune response against viral infections. Ribavirin has also been shown to have an inhibitory effect on viral polymerases by competitively inhibiting the binding of the nucleotides (10–12). More recently, elegant studies demonstrated that ribavirin can actually be used by viral polymerases and incorporated into viral RNA with the potential to base pair with UMP and CMP, leading to ribavirin-mediated mutagenesis of viral genomes (13–15). This would ultimately drive the viruses beyond a critical mutation rate and lead to an overall reduced fitness of the viral populations.

Other mechanisms of action have been proposed for ribavirin but have not been fully explored, including the possibility that ribavirin may have a negative effect on the capping of viral RNA transcripts (12). The 5′-end of most eukaryotic mRNAs and many viral mRNAs harbors a 5′GpppN cap structure that plays a critical role in the translation and stability of mRNAs (16, 17). The first step in the synthesis of the cap structure involves the hydrolysis of the RNA 5′-triphosphate end of the nascent RNA by an RNA triphosphatase to form a diphosphate end. An RNA guanylyltransferase enzyme then catalyzes a two-step reaction in which it initially utilizes GTP as a substrate to form a covalent GMP-enzyme intermediate. The GMP moiety is then transferred to the diphosphate end of the RNA transcript in the second step of the reaction to form the GpppN structure (18). The guanosine residue is finally methylated by an RNA methyltransferase to form the typical 5′GpppN cap structure. Support for the possibility that ribavirin might inhibit the capping of viral RNA transcripts comes from the finding that mutations in the Sindbis virus genome that confer resistance to ribavirin map to the RNA guanylyltransferase coding region of the genome (19, 20). Furthermore, the inhibition of vaccinia virus RNA cap synthesis by ribavirin has been noted previously, although the mechanism of action has not been explored (21).

In the present study, we investigated the possibility that ribavirin can directly serve as a substrate for the vaccinia virus RNA capping enzyme. We demonstrate that RTP can be used as a substrate by the capping enzyme and can form a covalent RMP-enzyme intermediate reminiscent of the classical GMP-enzyme intermediate. Furthermore, our data indicate that RMP can be transferred to the diphosphate end of an RNA transcript to form the unusual RpppN structure. Finally, we provide evidence that RNA transcripts, which possess ribavirin as the blocking nucleoside, are more stable than unblocked transcripts. However, in vitro translation assays indicate that RNA transcripts blocked with ribavirin are not translated efficiently. Our study provides the first biochemical evidences that ribavirin can directly interact with a viral capping enzyme. The ability of a purified RNA capping enzyme to utilize ribavirin as a substrate has not been previously documented and has implications for our understanding of the catalytic mechanisms of RNA capping enzymes. The biological implications of these findings for the proposed ribavirin-mediated inhibition of capping are discussed.
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**MATERIALS AND METHODS**

**Expression and Purification of the Vaccinia Virus D1 Protein (1–545)—** A plasmid for the expression of the N-terminal segment of the vaccinia virus D1 protein was generated by PCR amplification of the sequence corresponding to residues 1–545 of the D1 coding region. The 5'- and 3'-primers harbored restriction sites for NheI and XhoI, respectively. Note that the 3'-primer was designed to introduce a translation stop codon 546. The PCR-generated recombinant protein was inserted on the NheI and XhoI sites of the pET28a expression plasmid (Novagen). In this context, the resulting D1-(1–545) protein is fused in-frame with an N-terminal peptide containing six tandem histidine residues, and expression of the His-tagged protein is driven by a T7 RNA polymerase promoter. The resulting recombinant plasmid, pET-D1-(1–545), was transformed into *Escherichia coli* BL21(DE3). A 1-liter culture of *E. coli* BL21(DE3)pET-D1-(1–545) was grown at 37 °C in Luria-Bertani medium containing 50 μg/ml kanamycin until the A600 reached 0.5. The culture was adjusted to 2% ethanol, and the incubation continued at 18 °C for 48 h. The cells were then harvested by centrifugation, and the pellet was stored at −80 °C. All subsequent procedures were performed at 4 °C. The pellets were resuspended in water in lysis buffer A (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 10% sucrose), and cell lysis was achieved by the addition of lysozyme and Triton X-100 to final concentrations of 50 μg/ml and 0.1%, respectively. The lysates were sonicated to reduce viscosity, and any insoluble material was removed by centrifugation at 15,000 rpm for 45 min. The soluble extract was applied to a 5-ml column of nickel-nitrilotriacetic acid-agarose (Qiagen) that had been equilibrated with buffer B (50 mM Tris-HCl, pH 8, 0.1 M NaCl, and 10 mM DTT) containing 50, 100, 200, 500, and 1000 mM imidazole. The polypeptide composition of the column fractions was monitored by SDS-PAGE. The recombinant D1-(1–545) protein was retained on the column and recovered in the 100 mM imidazole eluate. This fraction was applied to a 5-ml column of phosphocellulose that had been equilibrated with buffer A containing 0.1 M NaCl, 10% glycerol, and 0.05% Triton X-100. The column was washed with the same buffer and then eluted stepwise with buffer B containing 50 mM Tris-HCl, pH 8, 5 mM DTT, and 2 mM dithiothreitol (10% glycerol, and 0.05% Triton X-100). The column was washed with the same buffer and then eluted stepwise with buffer C containing 0.1, 0.2, 0.3, 0.4, 0.5, and 1 M NaCl. The recombinant protein was retained on the column and recovered predominantly in the 0.2 M NaCl fraction. The fraction was then dialyzed against buffer C that was supplemented with potassium pyrophosphate (1 mM) to ensure a homogenous nonguanyllylated enzyme. The fraction was finally stored at −80 °C. Protein concentration was determined by the Bio-Rad dye binding method using bovine serum albumin as the standard.

**Assay for Enzyme-GMP Complex Formation—** The assay was performed by incubating the enzyme with 10 μM [α-32P]GTP in a buffer containing 50 mM Tris-HCl, pH 8, 5 mM DTT, and 5 mM MgCl2 for 5 min at 37 °C. The reactions were stopped by the addition of EDTA to 10 mM and SDS to 1%. The reactions were analyzed by electrophoresis through a 12.5% polyacrylamide gel containing 1% SDS. The radiolabeled proteins were visualized by autoradiography of the gel. The extent of covalent complex formation was quantitated by scanning the gel with a PhosphoImager (Amersham Biosciences).

**Assay for Enzyme-RMP Complex Formation—** An assay similar to the one performed for the evaluation of the enzyme-GMP complex formation was used. The reaction mixture was incubated with [3H]RTP, and the reaction was monitored by the covalent binding of [3H]RTP to the enzyme. A single SDS-stable GMP-enzyme complex that migrated as a 61-kDa species was detected following SDS-PAGE (Fig. 1B). Labeling of the enzyme was not detected in the absence of divalent cations (data not shown). We conclude that the D1-(1–545) protein is active in the formation of a protein-GMP covalent complex.

**Formation of an RMP-Enzyme Covalent Intermediate—** A His-tagged version of the vaccinia virus D1 (1–545) protein was expressed in bacteria and purified by sequential nickel-agarose and phosphocellulose chromatography steps (Fig. 1A). This N-terminal fragment of the D1 protein has been shown previously to functionally be equivalent to the full-sized capping enzyme with respect to triphosphatase and guanylyltransferase activities (22–25). The first step of the RNA guanylyltransferase reaction entails the nucleophilic attack of the α-phosphate of GTP by the enzyme and the subsequent formation of a covalent enzyme-GMP intermediate. The ability of the purified D1-(1–545) protein to form a covalent GMP-enzyme intermediate was detected by label transfer from [α-32P]GTP to the enzyme. A single SDS-stable GMP-enzyme complex that migrated as a 61-kDa species was detected following SDS-PAGE (Fig. 1B). Labeling of the enzyme was not detected in the absence of divalent cations (data not shown). We conclude that the D1-(1–545) protein is active in the formation of a protein-GMP covalent complex.

Because the nucleoside analogue ribavirin is structurally related to guanosine (Fig. 2A), we intended to monitor the activity of RTP to serve as a substrate for the D1-(1–545) protein by measuring the covalent binding of [α-32P]GTP to the enzyme. Note that the tritium label is located on the triazole ring of RTP. The D1-(1–545) protein was incubated in the presence of magnesium and increasing concentrations of radiolabeled RTP,
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Fig. 1. Expression, purification, and RNA guanylyltransferase activity of the vaccinia virus D1-(1–545) protein. A, an aliquot (2 μg) of the purified preparation of the D1-(1–545) protein was analyzed by electrophoresis through a 12.5% polyacrylamide gel containing 0.1% SDS and visualized by staining with Coomassie Blue dye. The positions and sizes (in kDa) of the size markers are indicated on the left. B, RNA guanylyltransferase activity of the D1-(1–545) protein. The enzyme (2 μg) was incubated for 5 min at 37 °C with 10 μM [α-32P]GTP in a buffer containing 50 mM Tris-HCl, pH 8, 5 mM DTT, and 5 mM MgCl2. The reactions were stopped by the addition of EDTA to 10 mM and SDS to 1%. The reactions were analyzed by electrophoresis through a 12.5% polyacrylamide gel containing 0.1% SDS. An autoradiogram of the gel is shown. The positions and sizes (in kDa) of the size markers are indicated on the left.

and the reaction products were submitted to SDS-PAGE. The ability of the D1-(1–545) protein to catalyze the formation of a covalent protein-RMP complex was demonstrated by label transfer from [3H]RTP. The proteins were stained with Coomassie Blue dye, and the corresponding bands were excised from the gel and crushed, and the radioactivity was evaluated by liquid scintillation counting. The yield of protein-RMP formation by the D1-(1–545) protein increased with RTP concentrations up to 300 μM and leveled off thereafter (Fig. 2B). Note that the amount of the protein-RMP complex formed during a 10-min incubation at 37 °C in the presence of 1 mM [3H]RTP was proportional to the amount of the added D1-(1–545) protein and that formation of the enzyme-RMP complex was dependent on the presence of a divalent ion (data not shown). These data demonstrate that RTP can serve as a substrate for the D1-(1–545) protein and form a stable covalent RMP-enzyme complex.

A second assay was used to demonstrate the covalent transfer of RMP to the D1-(1–545) protein. In this assay, the enzyme was incubated in the presence of RTP and magnesium, and the polypeptide was analyzed by capillary electrophoresis. The appearance of a slower migrating protein species was observed repeatedly when the protein was incubated with RTP (Fig. 2C). We hypothesized that the slower migrating protein species corresponds to the enzyme with covalently bound ribavirin (RMP-enzyme). The addition of pyrophosphate to the typical RNA guanylyltransferase reaction has been shown to reverse the reaction, i.e., promote the release of GTP by reversal of the reaction (18, 26, 28). We therefore added pyrophosphate to the reaction to evaluate if the formation of the RMP-D1-(1–545) complex was reversible. Our results demonstrate that the slower migrating protein species was converted into the faster migrating species following incubation with pyrophosphate. These data clearly demonstrate that the D1-(1–545) protein can form a covalent RMP-enzyme complex and that the reaction is reversible.

In the typical RNA guanylyltransferase reaction, the nucleophilic attack on the α-phosphate of GTP by the enzyme results in the formation of a covalent intermediate in which GMP is linked via a phosphoamide bond to a lysine residue of the enzyme (18, 29, 30). To determine the nature of the enzyme-RMP linkage, the RMP-D1-(1–545) labeled complex was isolated by gel filtration and submitted to chemical treatment, and the products were analyzed by thin layer chromatography (Fig. 3B). The RMP-D1-(1–545) intermediate was resistant to NaOH treatment, but treatments with HCl or NH4OH resulted in the release of RMP, which is indicative of a phosphoamide linkage. As a control, the D1-(1–545) protein was labeled with GMP and analyzed in parallel to confirm the nature of the phosphoamide bond (Fig. 3A).

Transfer of RMP to RNA—The D1-(1–545) protein clearly has the ability to form a covalent RMP-enzyme intermediate, but can it transfer RMP to an RNA substrate containing a 5′-diphosphate end? Because the D1-(1–545) protein harbors both the triphosphatase and guanylyltransferase active sites, the enzyme can hydrolyze the 5′-triphosphate end of RNAs to generate a 5′-diphosphate end that can subsequently serve as a substrate for capping. The ability of the enzyme to transfer RMP to an acceptor RNA molecule was tested by incubating the D1-(1–545) protein with [3H]RTP, an RNA substrate (84 nucleotides) containing a triphosphate 5′-end and magnesium. The products of the reaction were extracted with phenol/chloroform to remove the radiolabeled protein, and the RNA acceptor molecules were recovered by ethanol precipitation. Aliquots of the RNA samples were then digested with nuclease P1 and alkaline phosphatase and analyzed by polyethyleneimine-cellulose thin layer chromatography (Fig. 4B). The transfer of radiolabeled RMP to RNA was confirmed by demonstrating the release of a RpppG structure following digestion of the RNA samples with nuclease P1, with a simultaneous resistance to alkaline phosphatase treatment. Similar patterns were obtained when radiolabeled GTP was used in these experiments (Fig. 4A). We conclude that the D1-(1–545) protein can transfer the RMP moiety to an RNA acceptor molecule.

A second assay was used to demonstrate the transfer of ribavirin to RNA. A 32P-radiolabeled RNA transcript (84 nucleotides) was synthesized by in vitro transcription and gel-purified after electrophoresis on a denaturing 20% polyacrylamide gel. The radiolabeled RNA was then incubated in the presence of the D1-(1–545) protein, ribavirin, and magnesium, and the reaction products were analyzed on a denaturing polyacrylamide gel. As can be seen in Fig. 4B, the addition of ribavirin, magnesium, and the D1-(1–545) protein to the radiolabeled RNA resulted in a slower migrating RNA species. We hypothesized that the slower migrating RNA species results from the addition of the ribavirin moiety to the RNA transcript. As observed in the typical RNA capping reaction, incubation of the reaction product with pyrophosphate drove the reaction in the opposite direction (18, 26, 28). This demonstrates that the second step of the reaction, i.e., the D1-(1–545)-mediated transfer of RMP to RNA, is readily reversible.

Stability of RNAs Blocked with Ribavirin—The presence of the cap structure found at the 5′-end of eukaryotic mRNAs has been shown to be involved in the stabilization of mRNAs by protecting the 5′-ends from exonucleolytic degradation (17). The presence of a blocking, unmethylated capping guanosine residue at the 5′-end is sufficient for protection against degradation (31). In an effort to evaluate the stability of RNAs capped with ribavirin, in vitro kinetic analyses were performed in the presence of total protein extracts isolated from human HEK293 cells. A radiolabeled RNA transcript (84 nucleotides) was synthesized by in vitro transcription and gel-purified after electrophoresis on a denaturing 20% polyacrylamide gel. The radiolabeled RNA was then blocked with either guanosine, ribavirin, or the classical methylated guanosine by incubating the transcript with the D1-(1–545) protein and magnesium. The various RNA species were then recovered by multiple
rounds of ethanol precipitation. Note that the presence of a blocking nucleoside (guanosine or ribavirin) was confirmed by thin layer chromatography (data not shown). Radiolabeled RNAs were incubated at 37°C in the presence of HEK293 total protein extracts, and aliquots were removed at various time points. As observed previously, our data indicate that RNA transcripts with an unblocked 5′-end are degraded more rapidly than RNAs blocked with GTP. The informative finding is that RNAs blocked with ribavirin are significantly more stable than unblocked RNAs (Fig. 5). In fact, 65% of the unblocked RNAs are degraded after 30 min in comparison to 20% for RNAs blocked with ribavirin. As shown in Fig. 5, the stability of RNAs harboring a RpppN 5′-end is similar to the stability of RNAs possessing a GpppN 5′-end. We conclude that the presence of a blocking ribavirin residue at the 5′-end of RNAs can protect them from exonucleolytic degradation.

Translation of RNAs Blocked with Ribavirin—The presence of the 7-methyl group of the classical methylated guanosine 5′-
cap has been shown previously to be critical for cap-dependent translation (32). Various studies have demonstrated that the 7-methylguanosine interacts with the eukaryotic initiation factor 4E (eIF4E), a phylogenetically conserved subunit of the heterotrimeric eIF4F initiation complex, and establishes the foundation for the assembly of a functional translational initiation complex (33–35). In an effort to investigate the relationship between translation and stability of RNA transcripts blocked with ribavirin, in vitro translation studies were performed using wheat germ cell-free extracts (Fig. 5B). It is worth emphasizing that all attempts to methylate RNAs blocked with ribavirin by the vaccinia virus capping machinery were unsuccessful (data not shown). Our in vitro translation data indicate that only the RNA transcripts blocked with the classical $^{m}GpppG$ cap structure were efficiently translated (Fig. 5B). Unblocked RNA transcripts, or RNAs blocked with either guanosine or ribavirin, were not efficiently translated. Overall, these results indicate that RNAs blocked with ribavirin are not efficiently recognized by the translational machinery and do not serve as substrates for protein synthesis.

**Specificity for GTP—** In an effort to evaluate the efficiency of RTP as a substrate for the capping reaction, we compared the respective efficiency of RTP and GTP in nucleotidyl-enzyme formation. The D1R-(1–545) protein was incubated in the presence of magnesium and [$\alpha^{-32}$P]GTP or [$^{3}$H]RTP. The radiolabeled SDS-stable nucleotidyl-enzyme complex was quantified as described under “Materials and Methods.” The yield of enzyme-GMP formation increased as a function of GTP concentration and reached saturation at 200 $\mu$m. The titration assay indicated that half-saturation was reached at a concentration of 20 $\mu$m GTP. Formation of the enzyme-RMP complex also saturated at 200 $\mu$m RTP. However, the extent of enzyme-RMP formation at that concentration was ~15% of that of enzyme-GMP formation. Furthermore, a concentration of RTP of 120 $\mu$m was required to reach half-saturation, indicating that GTP is more effective than RTP in the formation of the covalent intermediate. This was also confirmed by competition experiments in which the ability of RTP to inhibit the RNA guanylyltransferase activity mediated by the purified D1-(1–545) protein was investigated (Fig. 6B). Increasing concentrations of RTP were added to the standard RNA guanylyltransferase assay containing 10 $\mu$m [$^{32}$P]GTP and 5 $\mu$m MgCl$_2$, and the effect on the formation of the covalent enzyme-GMP complex was monitored. Using this assay, RTP was shown to inhibit the RNA guanylyltransferase activity mediated by the D1-(1–545) protein by 50% at 2 $\mu$m and by 80% at 3 $\mu$m. The findings that RTP elicited 50% inhibition when present at concentrations 200-fold more than input GTP raises the possibility that RTP might inhibit the guanylyltransferase by acting as a chelator to compete with GTP for the essential magnesium cofactor.

**DISCUSSION**

Renewed interest in the broad spectrum antiviral ribavirin stems from recent studies reporting the use of the nucleoside analogue in the treatment of severe acute respiratory syndrome (SARS) and its ability to inhibit the replication of variola major virus, a major potential threat as a biological weapon (36, 37). Although its mechanism of action is still debated, ribavirin has been shown to prevent the replication of a large number of DNA and RNA viruses (1, 2). It has previously been suggested that ribavirin could have a negative effect on the capping of viral RNA transcripts, although this possibility has not been fully explored (16, 19–21).

Our study provides the first biochemical evidences that ribavirin can directly interact with a viral capping enzyme. The ability of a purified RNA capping enzyme to utilize ribavirin as a substrate has not been previously documented and has implications for our understanding of the catalytic mechanisms of RNA capping enzymes. The use of radiolabeled RTP allowed us to monitor precisely the use of ribavirin as a substrate for the vaccinia virus RNA capping enzyme. Our results indicate that the vaccinia virus enzyme has the ability to form a covalent protein-RMP intermediate and subsequently transfer the RMP moiety to an RNA acceptor. These two nucleotidyl transfer reactions are identical to the typical RNA capping reaction in which GTP is used as the capping nucleotide. RNA guanylyltransferases are members of the covalent nucleotidyl transferase superfamily, which also includes DNA and RNA ligases (38). Interestingly, the crystal structures of five family members revealed a common tertiary structure consisting of an N-terminal nucleotide binding domain and a C-terminal OB-fold domain (39–43). Analysis of the Chlorella virus PBCV-1 RNA capping enzyme revealed numerous contacts between the protein and the GTP substrate (41). Furthermore, important conformational changes have been shown to occur between both domains during substrate binding and reaction chemistry (41, 44). The carbonyl oxygen 07 and the amino nitrogen N8 of ribavirin occupy positions stereochemically similar to those of the carbonyl oxygen O6 and the ring nitrogen N1 of guanosine. However, an important structural property of ribavirin is the absence of both the ring nitrogen N3 and the 2-amino group normally found in guanosine. A simple explanation for the weaker affinity of the vaccinia virus RNA capping enzyme for...
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RTP versus GTP is that the latter has an extra 2-amino group, not available in RTP, that enables additional electrostatic or hydrogen-bonding contacts with the enzyme. Interestingly, the 2-amino group of GTP has been shown to be involved in a hydrogen bond with a proline (Pro-59) of the N-terminal nucleotide binding domain of the Chlorella enzyme (41). Furthermore, the 2-amino group of GTP also forms van der Waals interactions with two additional residues (Leu-232 and Pro-61) of the protein (41). The importance of these interactions in reaction chemistry has never been assessed directly, but our study clearly shows that GTP, which contains the additional 2-amino and nitrogen N3 of guanosine, is a more potent substrate than RTP for capping chemistry. Interestingly, recent studies indicate that ribavirin can be used as a substrate by the RNA polymerases of both the poliovirus and the hepatitis C virus (13–15). Both enzymes can use RTP as a nucleotide substrate in a template-dependent manner, incorporating it opposite pyrimidine residues. However, the efficiency of incorporation was estimated to be much lower than that of natural nucleotides (1,000–7,000-fold less) (13–15). Nonetheless, this low catalytic incorporation efficiency is sufficient to induce both mutagenic and inhibitory effects on RNA synthesis. The mechanistic differences responsible for this lower efficiency of incorporation have not been investigated but are reminiscent of the situation that is observed in our study using RTP as a substrate for the vaccinia virus RNA capping enzyme.

Two mechanisms by which ribavirin could potentially inhibit the capping of viral RNA transcripts have been suggested previously (16). Ribavirin has been shown to cause a reduction of the cellular GTP pools through the inhibition of inosine monophosphate dehydrogenase (IMPDH), an enzyme required for the de novo biosynthesis of GTP (6, 7). A decrease in GTP concentrations could potentially have a negative effect on the capping of viral RNAs. Evidence for this mechanism comes from studies performed with both the Sindbis virus and the Borna disease virus that showed that the viruses cannot replicate in cultured cells treated with ribavirin because the level of GTP falls too low to permit the capping of viral RNAs (19, 20, 45). However, mounting evidence indicates that the antiviral effect of ribavirin is not mediated entirely through the reduction of the intracellular GTP pool. For instance, other inosine monophosphate dehydrogenase inhibitors, such as mycophenolic acid, are not necessarily potent viral inhibitors (13). The second mechanism by which ribavirin could potentially inhibit the capping of viral RNAs is by binding directly to the active site of viral capping enzymes, thereby preventing the binding of GTP. Ribavirin possesses a five-membered heterocyclic base linked to a β-D-ribofuranose moiety. X-ray diffraction studies revealed that the structure of ribavirin closely resembles that of guanosine (3). The geometry of the carbamoyl function is of...
Ribavirin and GTP appear a much more potent substrate for the enzyme. Nonetheless, we do not exclude the possibility that RTP may act as a substrate for the vaccinia virus capping enzyme and transferred to an acceptor RNA molecule. Various adenosine analogues have been used previously to indirectly inhibit the in vivo formation of the mRNA cap structure by raising the cellular S-adenosylhomocysteine (SAH) levels through the inhibition of S-adenosylhomocysteine hydrolase (27). In contrast, our study demonstrates for the first time that a nucleoside analogue can be recognized directly by a viral enzyme and transferred to an acceptor RNA molecule. However, biochemical assays showed that ribavirin could not be methylated efficiently by the vaccinia virus RNA capping machinery (data not shown). The absence of methylation on the blocking ribavirin residue appears to be critical, as reflected by our in vitro translation assays that indicate that unmethylated RNA transcripts blocked with ribavirin are not efficiently recognized by the cellular translational apparatus, providing yet another mechanism of action for this pleiotropic antiviral agent. Because of the crucial importance of the cap structure for mRNA translation and stability, viral enzymes involved in the synthesis of the cap structure are attractive targets for the development of antiviral drugs. Ribavirin, with its ability to interact with the RNA capping machinery, has the potential to serve as a template for the development of more potent inhibitors. In fact, a number of ribavirin derivatives have been developed in recent years. Analysis of the interaction between these derivatives and RNA capping enzymes will also undoubtedly shed light on the chemistry of the RNA capping reaction.

What is the biological relevance of the present findings? Numerous studies suggest that the potency of ribavirin is determined in part by virus-host interactions, i.e. ribavirin sensitivity can vary considerably depending on the virus and cell line used (37, 45). However, support for the hypothesis that ribavirin acts at least in part through interference with GTP biosynthesis has now been found in numerous viral systems. For example, the antiviral effect of ribavirin can be abrogated by the addition of guanosine, but not by other nucleotides in cells infected with the measles virus and the Borna disease virus (6, 45). The extent by which the pleiotropic antiviral ribavirin mediates its in vivo activity through a negative effect on capping is difficult to assess. However, our study clearly demonstrates that the vaccinia virus capping enzyme can interact with the antiviral ribavirin and use it as a substrate. Subsequent in vitro studies performed in the absence of ribavirin should indicate whether the antiviral ribavirin serves as a substrate when cellular GTP concentrations are reduced.

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