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# Effect of ribavirin triphosphate on primer generation and elongation during influenza virus transcription in vitro

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## Summary

These studies examine the effect of ribavirin triphosphate (RTP) on two replicative functions associated with influenza virus nucleocapsids, primer generation and its subsequent elongation. To study primer generation influenza virus cores were added to β-globin mRNA in the presence of only [$^{32}$P]GTP. To examine elongation, ATP and CTP were added to the reaction mixture to permit limited elongation, and products from both reactions were separated on polyacrylamide gels and quantified. Under these conditions, the 50% inhibitory concentration of RTP for primer generation was 3.0 mM, and the 50% inhibitory concentration for elongation was 0.6 mM. RNA polymerase activity associated with cores isolated from clinical strains of influenza A and B viruses reacted as did the laboratory strain of influenza virus and was equally susceptible to inhibition by RTP.

ribavirin triphosphate; influenza virus transcription

## Introduction

The antiviral agent ribavirin is a purine analogue known to inhibit influenza virus replication as measured by viral RNA synthesis [14] and viral protein synthesis [10]. A mechanism by which ribavirin inhibits viral replication has been postulated to involve reduction of the intracellular GTP pool size [3,16]. However, we have separated ribavirin's antiviral effects from its effects on GTP pool sizes by simultaneously measuring influenza virus ribonucleoprotein production and GTP pool sizes in

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infected cells treated with ribavirin and/or guanosine [20]. The results showed that the GTP pool was restored to normal values in the presence of both ribavirin and guanosine; however, under these conditions, viral ribonucleoprotein synthesis was only 37% of that in cells treated with guanosine alone. Thus we have shown that ribavirin retains substantial antiviral activity when the GTP pool size is restored to normal values by the addition of exogenous guanosine. Therefore, a mechanism of ribavirin antiviral activity may exist that is independent of the GTP pool size.

This action may depend upon direct interactions of phosphorylated metabolites of ribavirin with viral replicative enzymes. Eriksson et al. [4] showed one such interaction when they demonstrated that ribavirin triphosphate (RTP) specifically inhibits the activity of the influenza virus RNA-dependent RNA polymerase. With the elucidation of the in vivo pathway for influenza virus transcription by Robert Krug and others [1,12] the mechanism of RTP inhibition of the influenza polymerase complex may now be studied in greater detail.

In this pathway, viral P proteins (the polymerase complex) generate capped oligonucleotides from cellular mRNAs which act as efficient primers for the transcription of the viral genome [12]. Initiation of elongation occurs on this primer by the addition of GMP, and subsequently elongation proceeds. These enzymatic reactions are dependent upon protein interactions with purine nucleotides. For example, the synthesis of primers requires recognition of a cap 1 structure on host mRNAs [12] and the subsequent cleavage of these mRNAs has been reported to occur on the 3′ side of purine nucleotides [12]. Thus, either or both of these activities associated with the viral polymerase complex may be inhibited by a purine analogue such as ribavirin.

Our studies have examined the effect of RTP on the early events of influenza virus transcription using two assays. The first assay tested initiation of transcription (primer generation); specifically, cap recognition of host mRNAs, cleavage, and initiation by GMP addition. The second assay tested elongation of the host-derived primer fragments. The results demonstrated that RTP interfered with both primer generation and elongation catalyzed by the cores from both influenza A and B viruses, though elongation was at least 5-fold more sensitive to inhibition by RTP.

## Materials and Methods

### *Chemicals*

RTP tetrasodium salt was a gift of Dr. Roland K. Robins (Brigham Young University) and was 95% pure as determined by high performance liquid chromatography (personal communication). Guanosine 5′-[$\alpha$-$^{32}$P]triphosphate (410 Ci/mmol) and adenosine 5′-[$\gamma$-$^{32}$P]triphosphate (3000 Ci/mmol) were purchased from Amersham Corp. Rabbit $\beta$-globin mRNA, $T_4$ polynucleotide kinase and oligo-dT size standards were purchased from Bethesda Research Laboratories, Inc. Nucleotide triphosphates and other chemicals were obtained from Sigma Chemical Corporation.

### *Virus*

The X-31 strain of influenza A [5] was obtained from the Influenza Research

Center, Baylor College of Medicine. A/WSN virus was present in the laboratory. Two viral isolates were selected from samples obtained from patients during ribavirin aerosol studies conducted at Texas A & M University [9]. The influenza A virus was isolated in January 1983 and most closely resembled A/England/333/80 (H1N1). The influenza B virus was isolated in January 1982 and most closely resembled B/Singapore/222/79. These clinical strains were passaged once in eggs before use.

*Preparation of viral cores*

Viral cores were used as a source of RNA-dependent RNA polymerase and were prepared by the method of Rochovansky [13]. Egg allantoic fluid was harvested from influenza virus-infected eggs and the virus was pelleted by centrifugation in a Beckman 30 rotor for 75 min at 27 000 rpm. The pellet was resuspended in lysing buffer (0.1 M Tris, pH 8.1; 0.1 M KCl; 0.005 M $MgCl_2$; 0.0015 M dithiothreitol; 5% glycerol; 1.5% Triton N101; 1% lysolecithin) and incubated at room temperature for 1 h. Virus cores were isolated by centrifugation on glycerol gradients prepared by layering 70, 60, 50, 40 and 30% glycerol in glycerol gradient buffer (0.05 M Tris, pH 7.8; 0.15 M NaCl). After centrifugation at 39 000 rpm for 4 h at 4°C, the 50% layer was removed and frozen at −70°C. Purified core protein (50–260 μg/ml) contained in the 50% layer was dialyzed against transcription buffer containing 50% glycerol immediately before use.

*Assays for initiation of transcription and elongation of primers*

The standard initiation assay was that of Plotch et al. [12]. Gradient purified influenza cores (2–13 μg of protein, 50 μl volume) were incubated with 0.5 μg of rabbit β-globin mRNA, 30 μCi guanosine 5′-[α-$^{32}$P]triphosphate and 0.2 nmol of GTP in transcription buffer (0.05 M Tris, pH 7.8; 0.1 M KCl; 0.01 M NaCl; 0.001 M dithiothreitol; 0.2% Triton N101; 0.005 M $MgCl_2$) with or without RTP. The reaction volume was 100 μl. Incubation was for 1 h at 31°C. Phenol was equilibrated with an equal volume of 0.1 M Tris, pH 8.8, mixed 1 : 1 with chloroform and used to extract the samples. The aqueous fraction was made 0.3 M sodium acetate, precipitated with ethanol and electrophoresed on polyacrylamide gels.

The elongation assay was identical to the initiation reaction except that after the initial incubation period 2.0 nmol of ATP and CTP was added to the reaction. The samples were then reincubated for 30 min with or without RTP before extraction. For the ApG primed elongation reaction, 500 μM ApG was substituted for β-globin mRNA [11].

*End labeling of oligo-dT size standards*

Oligo-dT size standards (4–22 nucleotides in length) were incubated with 150 μCi of adenosine 5′[γ-$^{32}$P]triphosphate and 7 units of $T_4$ polynucleotide kinase in kinase buffer (0.1 M Tris, pH 7.6; 0.02 M $MgCl_2$; 0.01 M dithiothreitol; 0.0002 M spermidine; 0.0002 M EDTA) according to the procedure of Maniatis [8]. The samples were extracted with a 1 : 1 equilibrated phenol–chloroform mixture and the aqueous fraction was precipitated twice with ethanol and electrophoresed on 20% polyacrylamide gels.

*Gel electrophoresis*

Samples were resuspended in 20 μl of 10 mM Tris, pH 8.0; 2 mM EDTA and electrophoresed at 450 V for 16 h on 20% polyacrylamide–0.75% bisacrylamide gels containing 7 M urea in 50 mM Tris–borate, pH 8.3, 1 mM EDTA. Following electrophoresis, the gels were autoradiographed for 3–24 h, depending upon the core preparation used. The autoradiographs were analyzed by a densitometer and quantified by comparing peak areas.

## Results

*Initiation of transcription by influenza virus RNA-dependent RNA polymerase*

Initiation of influenza virus transcription was measured by the assay of Plotch et al. [12]. Purified viral cores generated labeled primers through endonucleolytic cleavage of β-globin mRNA and addition of [$^{32}$P]GMP to the 3′ end. The primers were visualized by gel electrophoresis followed by autoradiography (lane 6, Fig. 1A). The labeled primers comigrated with 15 and 16 nucleotide length end-labeled oligo-dT standards (Fig. 1B) and have been shown to be a 14 nucleotide length cleavage product of the extreme 5′ end of β-globin mRNA with the addition of 1 or 2 GMPs at the 3′ end of the fragment [12]. The intensity of the bands was dependent upon the amount of β-globin mRNA and viral core protein present in the assay and upon the time of incubation (data not shown). Bands were not observed when proteins from mock-infected eggs (lane 7, Fig. 1A) replaced influenza cores or when β-globin mRNA was omitted from the reaction mixture (lane 8, Fig. 1A).

Radiolabeled material retained at the top of the gels was observed when reaction mixtures containing RTP were phenol extracted (Figs. 1 and 2). Since this material was not degraded by various enzyme treatments (RNase $T_1$, proteinase K), the exact nature of the precipitate is not known.

*RTP inhibition of initiation of transcription*

The initiation activity of purified influenza cores from the type A virus X-31 was tested in the presence of various concentrations of RTP. Fig. 1A, lane 6 shows the labeled primers made in the absence of RTP. Lanes 1–5 show the effects of increasing concentrations of RTP (1.25–10 mM) on initiation of transcription as quantitated by the intensity of bands corresponding to the two primer fragments. In lane 2, 2.5 mM RTP inhibited primer production by 45%.

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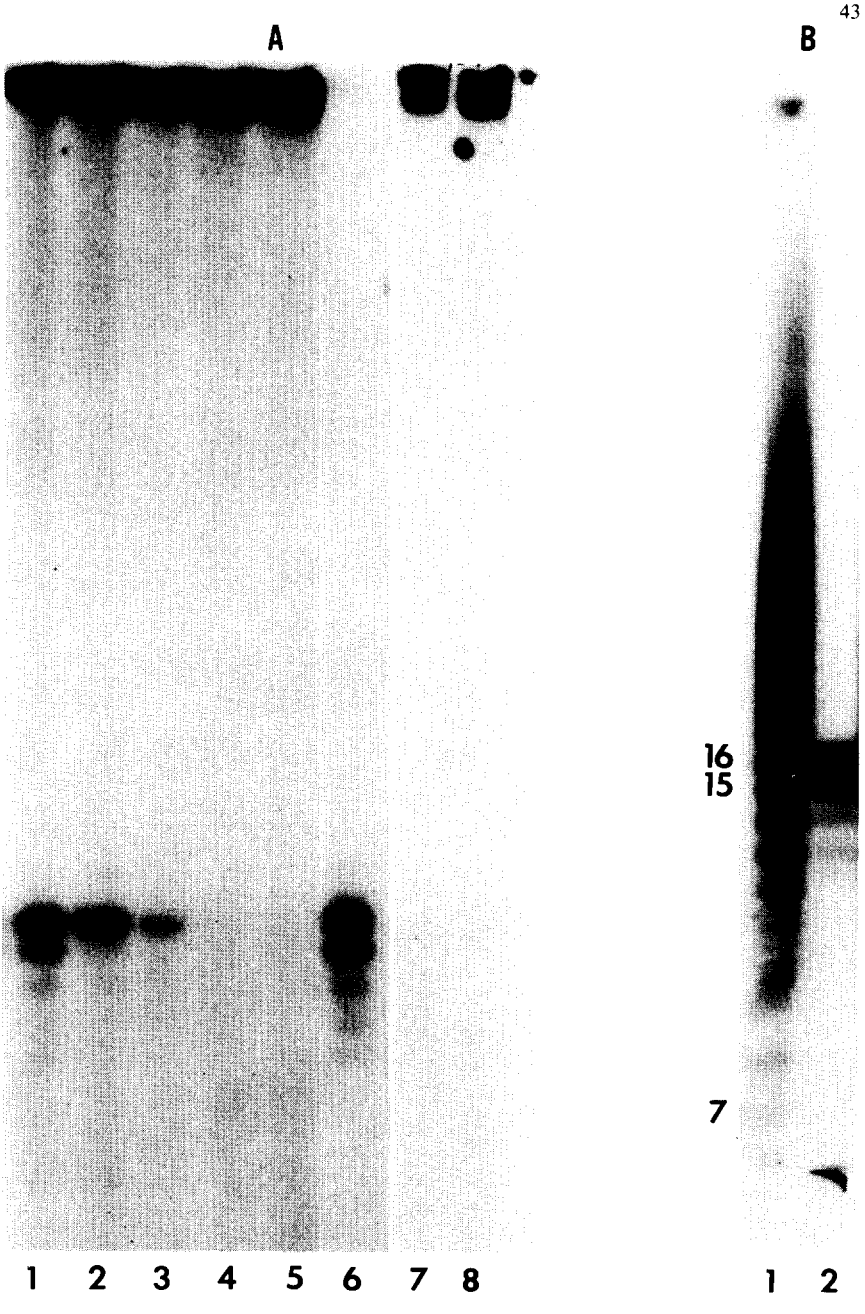
Fig. 1. (A) Effect of RTP on initiation of transcription. Purified cores from the type A virus X-31 containing polymerase activity were mixed with 0.2 nmol of GTP, 30 μCi guanosine 5′-[α-$^{32}$P]triphosphate, 0.5 μg rabbit β-globin mRNA in transcription buffer without RTP (lane 6), 1.25 mM RTP (lane 1), 2.5 mM RTP (lane 2), 5.0 mM RTP (lane 3), 7.5 mM RTP (lane 4), or 10.0 mM RTP (lane 5) and incubated for 1 h at 31°C. Samples containing the generated primer fragments were phenol extracted, ethanol precipitated, electrophoresed on polyacrylamide gels and autoradiographed. In lane 7, proteins from mock-infected eggs were substituted for viral core proteins. In lane 8, β-globin mRNA was omitted from the reaction mixture. (B) Sizing of primer fragments. Oligo-dT size standards of lengths 4–22 were end-labeled using polynucleotide kinase and adenosine 5′-[γ-$^{32}$P]triphosphate, phenol extracted, ethanol precipitated and electrophoresed on polyacrylamide gels (lane 1). Primer fragments were co-electrophoresed (lane 2).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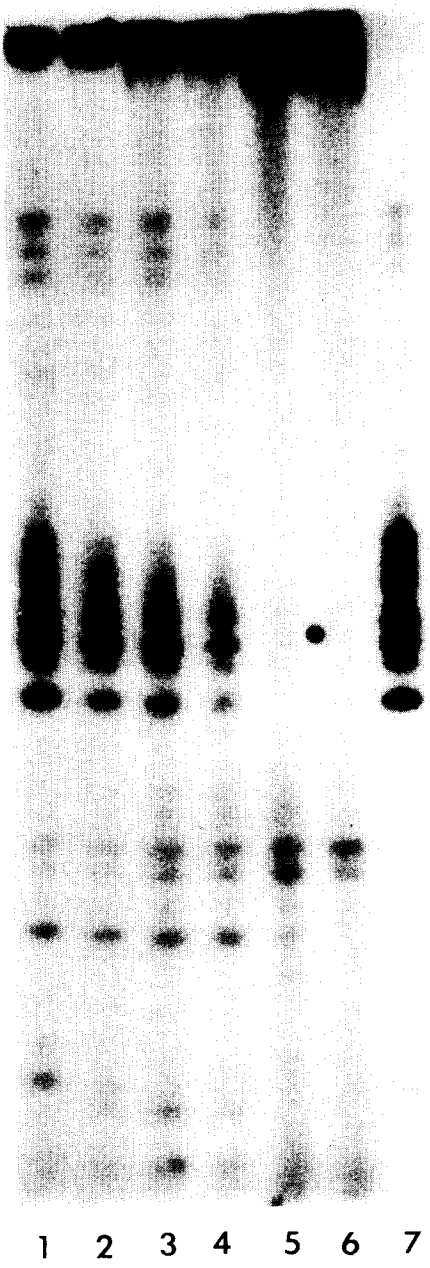


Fig. 2. Effect of RTP on elongation of primer fragments. Purified cores from the type A virus X-31 containing polymerase activity were mixed with 0.2 nmol of GTP, 30 μCi guanosine 5'-[α-$^{32}$P]triphosphate, and 0.5 μg rabbit β-globin mRNA in transcription buffer and incubated for 1 h at 31°C. After the addition of 2.0 nmol of ATP and CTP only (lane 7) or with 0.20 mM RTP (lane 1), 0.4 mM RTP (lane 2), 0.6 mM RTP (lane 3), 0.8 mM (lane 4), 1.6 mM RTP (lane 5), 3.2 mM RTP (lane 6) incubation was continued for 30 min. Samples containing the elongated products were phenol extracted, ethanol precipitated, electrophoresed on polyacrylamide gels and autoradiographed.

*RTP inhibition of elongation of primer fragments*

In Fig. 2, the effect of RTP on elongation of primer fragments was tested. Lane 7 demonstrates the elongated primers produced in the presence of only ATP, CTP and GTP. These elongated primers are 25–32 nucleotides in length (size markers not shown) since the primers are elongated only to the initial uridine nucleotide [1]. Lanes 1–6 show the inhibitory effects of increasing concentrations of RTP (0.2–3.2 mM) on this limited elongation. In lane 3, densitometric tracings showed that 0.60 mM RTP inhibited elongation of primers by 46%. In lanes 5 and 6, primer fragments reappear when elongation has been inhibited.

In order to completely differentiate the elongation reaction from the priming reaction, an elongation assay stimulated by the dinucleotide ApG, rather than by β-globin mRNA was utilized. The sensitivity of this assay to RTP was measured and the 50% inhibitory concentration was found to be approximately 0.07 mM (data not shown).

In Fig. 3, the sensitivities of the two β-globin mRNA stimulated reactions to RTP are compared. The elongation assay was 5-fold more sensitive to inhibition by RTP than the initiation assay. The 50% inhibitory concentrations for elongation and initiation were 0.6 and 3.0 mM, respectively.

*Effect of RTP on the polymerase activity of other strains of influenza virus*

We compared the sensitivity of the polymerase complex isolated from various strains of influenza virus to RTP. RNA polymerase activity of cores prepared from two laboratory strains (X-31 and A/WSN) and one clinical isolate (A/England/333/80) showed similar sensitivities to RTP when tested by both the initiation and elongation assays (Table 1). Polymerase activity isolated from an influenza B

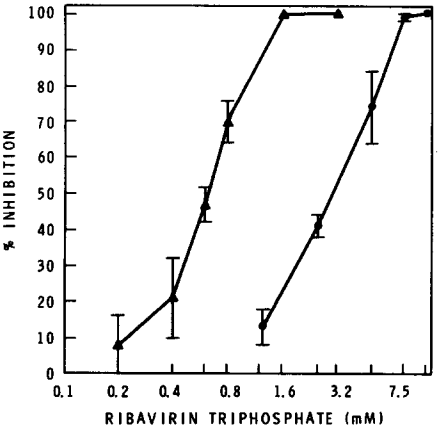


Fig. 3. Relative susceptibility of initiation of transcription and elongation of primer fragments to RTP. Autoradiography following gel electrophoresis was used to detect product formation in the primer generation and elongation reactions. Autoradiographs were analyzed by densitometric analysis and peak areas were used to determine the percent inhibition of initiation of transcription (●) and elongation of primer fragments (▲) at various concentrations of RTP. Vertical lines represent ± S.E.M. of 3 experiments.

TABLE 1

Effect of RTP on the polymerase activity of various strains of influenza virus

| Strain | Initiation (% inhibition at 3.00 mM RTP) | Elongation (% inhibition at 0.75 mM RTP) |
|---|---|---|
| X-31 | 48 | 62 |
| A/WSN | 34 | 70 |
| A/England/333/80 | 68 | 33 |
| B/Singapore/222/79 | 100 | 54 |

For the initiation or primer generation reaction, purified influenza virus core preparations from various strains containing polymerase activity (2–13 μg protein) were mixed with 0.5 μg rabbit β-globin mRNA, 30 μCi of guanosine 5'-[α-$^{32}$P]triphosphate, and 0.2 nmol of GTP in transcription buffer with or without 3.00 mM RTP. After incubation at 31°C for 1 h, samples were phenol extracted, ethanol precipitated, electrophoresed on polyacrylamide gels and autoradiographed. Percent inhibition was calculated from peak areas after densitometric analysis. For the elongation reaction, the assay was identical except that following the initial incubation, 2 nmol of ATP and CTP was added with or without 0.75 mM RTP, and incubation was continued for an additional 30 min. The 50% inhibitory concentration for influenza B was 1.45 mM RTP.

virus (B/Singapore/222/79) produced a single primer fragment 15 nucleotides long (data not shown). Production of this primer was more susceptible to RTP (50% inhibitory dose = 1.45 mM) than polymerase activity from influenza A viruses. Elongated products made by B/Singapore in the presence of ATP, CTP and GTP had similar mobilities on polyacrylamide gels as those of influenza A viruses and their length corresponded to the positions of the initial uridine residues in the published sequences of B/Lee cRNA [7,2,15]. Elongation of primers by cores of B/Singapore was susceptible to RTP at concentrations similar to those inhibitory for type A viruses.

## Discussion

Our studies have demonstrated that RTP inhibits the early events of influenza virus transcription. The initiation assay, sensitive to RTP at a concentration of 1–3 mM, measured the parameters involved in primer generation: (1) recognition of the cap-1 structure of host mRNA, (2) endonucleolytic cleavage of host mRNAs, and (3) initiation by GMP addition. Thus, one or more of these steps are inhibited by RTP. Further purification of the polymerase complex proteins will be necessary to identify the specific site of action.

The β-globin mRNA stimulated elongation assay, catalyzed by the core protein $PB_2$ [18], measured transcription to the first uridine residue and was inhibited at approximately one-fifth the concentration needed to inhibit the initiation reaction. Stimulation of elongation by the dinucleotide ApG did not involve the cap recognition and cleavage steps necessary in β-globin mRNA priming, and thereby simplified the measurement of elongation. This simplified reaction was 9-fold more sensitive to RTP than the β-globin stimulated reaction. The greater susceptibility of these elongation

assays to RTP suggests that elongation is an important site of action for the drug, possibly due to an affinity for $PB_2$. This does not exclude RTP inhibitory effects on the initiation of transcription, as catalyzed by $PB_1$ [18] during virus replication, since this reaction was also inhibited by RTP, albeit at higher concentrations. An additional site of action, such as primer generation, would enhance the antiviral effect of RTP over that of a single site of action.

The concentration of RTP required to inhibit the elongation reaction is greater than that reported for the in vitro inhibition of RNP synthesis by ribavirin in Madin Darby Canine Kidney (MDCK) cells [20]. This may reflect the artificial nature of the in vitro assay which employs a partially purified preparation of polymerase (i.e. viral cores) or the ability of MDCK cells to efficiently utilize ribavirin to inhibit influenza replication. In any event, clinical studies of respiratory syncytial virus [17] and influenza A and B virus infections [6,9] show that ribavirin has a significant therapeutic effect when applied to the site of infection, such as by small-particle aerosol. Endotracheal aspirates from such patients contained ribavirin concentrations in the range of 200–800 μM [19]. Although the intracellular concentration of RTP in respiratory epithelium following aerosol therapy is unknown, Zimmerman et al. [21] found that after a short period of incubation, the intracellular concentration of RTP approximates the extracellular concentration of ribavirin in L51784 cells. Thus, RTP may, in the virus-infected respiratory epithelium, reach concentrations similar to those inhibitory in the present in vitro studies. However, further studies will be needed to determine dose–effect relationships in infected tissues.

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