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Polyadenylation-dependent screening assay for respiratory syncytial virus RNA transcriptase activity and identification of an inhibitor

Stephen W. Mason*, Carol Lawetz, Yvon Gaudette, Florence Dô, Erika Scouten, Lisette Lagacé, Bruno Simoneau[1] and Michel Liuzzi

Department of Biological Sciences and [1]Department of Chemistry, Boehringer Ingelheim (Canada) Ltd, Laval, Québec H7S 2G5, Canada

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ABSTRACT

RNA-dependent RNA polymerase from respiratory syncytial virus (RSV) is a multi-subunit ribonucleoprotein (RNP) complex that, in addition to synthesizing the full 15 222 nt viral genomic RNA, is able to synthesize all 10 viral mRNAs. We have prepared crude RNP from RSV-infected HEp-2 cells, based on a method previously used for Newcastle disease virus, and established a novel polyadenylation-dependent capture [poly(A) capture] assay to screen for potential inhibitors of RSV transcriptase activity. In this homogeneous assay, radiolabeled full-length polyadenylated mRNAs produced by the viral RNP are detected through capture on immobilized biotinylated oligo(dT) in a 96-well streptavidin-coated FlashPlate™. Possible inhibitors identified with this assay could interfere at any step required for the production of complete RSV mRNAs, including transcription, polyadenylation and, potentially, co-transcriptional guanylylation. A specific inhibitor of RSV transcriptase with antiviral activity was identified through screening of this assay.

INTRODUCTION

Respiratory syncytial virus (RSV), from the Paramyxoviridae family and genus Pneumovirus, is a major cause of hospitalization of infants <2 years of age, particularly those born prematurely or having underlying cardiac or pulmonary disease (1). Treatment of hospitalized infants infected with RSV has been limited to ribavirin, which elicits variable responses (2,3). Prophylaxis with humanized monoclonal antibody, Synagis™ (4,5), although very expensive, has become a reasonably effective way of combating the disease in infants at high risk. Nevertheless, it would be desirable to obtain small molecule inhibitors of an essential activity of RSV that would provide a less-expensive therapy for treatment or prevention of RSV disease.

In order to find potential therapeutics against RSV, it would be advantageous to screen for inhibitors that specifically target an enzymatic activity that is unique to the virus. The viral RNA-dependent RNA polymerase (RdRp) fulfills this criterion since such an activity is not present in the host cell. The RSV RdRp comprises at least five viral components (1), namely the genomic RNA, which is encapsidated by the nucleocapsid (N) protein, the catalytic subunit L and at least two transcription factors, phosphoprotein (P), and the M2-1 protein, an antitermination/elongation factor for the viral polymerase (6,7). Together these components form a ribonucleoprotein (RNP) complex that is essential for the synthesis of both viral genomic RNA ('replicase' activity) and sub-genomic mRNAs ('transcriptase' activity). The P protein seems to be a central player in the RNP by its interaction with N (8–17), L (16) and M2-1 (18). Crude, transcriptionally active RNP can be prepared from virus-infected cells (19–21) and RSV mRNAs produced by viral RNP *in vitro* are co-transcriptionally capped (i.e. guanylylated and methylated) (22) at their 5′ ends and polyadenylated at their 3′ ends (19). Although disassembly and reassembly of viral RNP is feasible (23), reconstitution of the RSV RNP from recombinant subunits has not been reported in the literature. In addition, inhibitor screening of such polymerases has been problematic since most methods, such as TCA precipitation or DE81 filter binding (19,24), require washing or filtering of radioactivity, techniques which are not amenable to today's demanding ultra-high-throughput screening technologies. Thus, the lack of an efficient means for obtaining and measuring transcriptionally active RNP has hindered the thorough screening of this activity for potential inhibitors.

Here, we show both a means for preparing highly active RSV-RNP-containing extracts and a new method for assessing RSV 'transcriptase' activity. As expected, RSV RNP complexes contain viral proteins that are known to be important for RdRp activity and produce transcripts that are the sizes expected for RSV mRNAs. RSV transcriptase activity present in RNP complexes can be assessed by a method that utilizes capture of polyadenylated mRNA and detection of incorporated radiolabel via FlashPlate™ technology. Moreover, this

*To whom correspondence should be addressed. Tel: +450 682 4640; Fax: +450 682 4642; Email: smason@lav.boehringer-ingelheim.com
Present address:
Michel Liuzzi, Cooperative Laboratory Idenix—Universita di Cagliari, Sesta Strada Ovest, Zona Industriale Macchiareddu, 09010 UTA (CA), Italia

assay format can be extended to any viral RNA polymerase that produces polyadenylated transcripts, such as that of influenza virus (25,26). High-throughput screening of this assay has yielded an interesting and unique inhibitor of the RSV transcriptase activity.

MATERIALS AND METHODS

Cells and viruses

Cell culture reagents and media were obtained from Invitrogen. The HEp-2 cells and the Long strain of RSV were from American Type Culture Collection (ATCC). The HEp-2 cells were grown in DMEM supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin sulfate and 100 μg/ml kanamycin sulfate at 37°C in an atmosphere of 5% CO_2. Virus stocks were grown in HEp-2 cells and virus titers were determined by a standard plaque assay.

Inhibitors

Foscarnet was purchased from Sigma. Compound A was synthesized according to a method described previously (27).

RSV RNP preparation

Crude RSV RNP complex was extracted from virus-infected cells by a method modified from Hamaguchi *et al* (28). Adherent HEp-2 cells at $\sim 6.4 \times 10^4/cm^2$ to $9.6 \times 10^4/cm^2$ were infected at 5 MOI (multiplicity of infection) with RSV Long strain by allowing 1 h of adsorption at 37°C (at 5% CO_2) in DMEM containing 2% inactivated FBS followed by replacement of the innoculum with fresh media and continuation of the incubation. At 19 h post-infection, the medium was replaced with DMEM + 2% FBS supplemented with 2 μg/ml actinomycin D and incubation was continued for 1 h, at which time, the medium was removed and the cells were washed with ice-cold phosphate-buffered saline (PBS). All further steps were performed on ice or at 4°C and all subsequent volumes of buffer are for $\sim 5 \times 10^7$ cells (as determined prior to infection). The cells were treated for ~1 min with 75 ml of PBS + 250 μg/ml of lyso-lecithin (29), and the solution was decanted out of the plate and the excess liquid aspirated. The cells were scraped into 2 ml of buffer A [50 mM Tris-acetate (pH 8), 100 mM potassium acetate, 1 mM DTT, 2 μg/ml actinomycin D and 50 mM ATP]. The cells were disrupted by repeated passage through an 18 gauge needle (10 strokes), left on ice for 5 min and centrifuged for 10 min at 2400 *g*. The supernatant (S1) was removed and kept aside for analysis. The pellet fraction (P1) was disrupted in 1 ml of buffer B [10 mM Tris-acetate (pH 8), 10 mM K-acetate and 1.5 mM $MgCl_2$] supplemented with 1% Triton X-100, by repeated passage through an 18 gauge needle (10 strokes), left on ice for 10 min and centrifuged for 10 min at 2400 *g*. The supernatant (S2) was removed and kept aside for analysis. The pellet fraction (P2) was disrupted in 1.5 ml of buffer B supplemented with 0.5% deoxycholate and 1% Tween-40 (polyoxyethylenesorbitan monopalmitate) as above, left on ice for 10 min and centrifuged for 10 min at 2000 *g*. The RNP fractionated into the supernatant (S3), and the pellet (P3) was disrupted in the same buffer and volume as the previous step and retained for analysis.

RSV polymerase assays

Transcription reactions (60 μl) contained 1–2 μl RSV RNP and 1 μCi [^{3}H]CTP (~20 Ci/mmol; ~1 μM; Amersham) in reaction buffer [50 mM Tris-acetate (pH 7.5), 120 mM K-acetate, 4.5 mM $MgCl_2$, 5% glycerol, 2 mM ethyleneglycol-bis(aminoethylether)-tetraacetic acid (EGTA), 3 mM DTT, 50 μg/ml BSA, 0.4 mM each of ATP, GTP and UTP, and 4% dimethyl sulfoxide (DMSO)]. Reactions were assembled in 96-well streptavidin-coated FlashPlates™ (PerkinElmer) to which 3.5 pmol of biotinylated oligo$(dT)_{30}$ was immobilized. Following 2 h incubation at 30°C, reactions were stopped with hybridization buffer [17 mM Tris–HCl (pH 7.5), 1.7 mM EDTA, 0.85 M LiCl, 0.85% SDS, 120 mM NaCl and 3.75 mM KCl]. Incorporated radionucleotide was detected by using a TopCount™ (PerkinElmer). For DE81 filter binding assays, transcription reactions as above containing 1 μCi [α-^{33}P]CTP (3000 Ci/mmol) and 1 μM CTP were spotted (5 μl) on DE81 filter paper (Whatman), washed with 0.35 M Na_2HPO_4, 0.01 M $Na_2P_2O_7$, exposed to a Storm™ phosphoimager screen (Molecular Dynamics) and quantified using the ImageQuant™ software.

For analysis of RNA products by gel electrophoresis, transcription reactions were performed with 15 μCi [α-^{33}P]CTP (3000 Ci/mmol) and 1 or 2 μM unlabeled CTP. Following 2 h incubation at 30°C, reactions were incubated with 5 μg oligo$(dT)_{15}$, and then with 7 U of RNaseH to remove poly(A) tails (19). Reactions were stopped with 80 μg tRNA and 20 mM EDTA, and the transcripts were extracted with Trizol® LS (Invitrogen) as per the manufacturer's protocol, and precipitated with isopropanol. Dried pellets were resuspended in 10 μl of water. Prior to electrophoresis, the RNA was treated with glyoxal: for each 3.7 μl of RNA, 12.3 μl of a mixture of 22% deionized glyoxal, 65% DMSO, 13% 10× MOPS (200 mM MOPS (pH 7), 50 mM sodium acetate and 10 mM EDTA) were added and heated to 55°C for 1 h. The glyoxylated RNA was run on a 1.5% agarose/1× MOPS (pH 7) gel for 14 h at 30 V. The gel was dried and exposed to Kodak MR film and Storm™ Phosphorimager screen.

Testing of inhibitors

Non-linear regression analysis using the SAS software (SAS Institute, Cary, NC) was employed to determine the inhibitor concentration needed to give 50% reduction of enzyme activity (IC_{50}). All IC_{50}s were determined by testing of 2- or 3-fold serial dilutions of the inhibitor. Preparation and testing of RNA polymerase from hepatitis C virus (HCV) and polioviruses RNA polymerase II from calf thymus was as described previously (30). RNA polymerase from influenza virus was as described previously (31,32).

Antiviral assays

Antiviral activity was assessed in an enzyme-linked immunosorbent assay (ELISA) using HEp-2 cells (obtained from ATCC) in DMEM containing 2% FBS at 37°C in an atmosphere of 5% CO_2. Virus stocks were grown in HEp-2 cells and virus titers were determined by a standard plaque assay. The cells in 96-well plates were infected with RSV Long strain at an MOI of 0.1 syncytia-forming unit (s.f.u.) and incubated for 48 h in the presence or absence of 3-fold serial dilutions of inhibitors. Compound solutions in 1% final

DMSO were filtered through 0.22 μm μStar LB™ filters (Costar) and concentrations verified by high-performance liquid chromatography analysis. The monolayers were fixed with 0.063% glutaraldehyde and the plates blocked with 1% BSA in PBS for 1 h. Virus replication was detected with a monoclonal antibody directed against RSV F (Serotec MCA 490 clone B016) in PBS/1% BSA. After 1 h incubation, the plates were washed and the bound monoclonal antibody was detected using sheep anti-mouse immunoglobulin G horse radish peroxidase (Amersham) for 1 h. The plates were washed and developed with *O*-phenylendiamine dihydrochloride for 30 min, and the absorbance was read at 450 nm. EC_{50} values (i.e. the compound concentration to inhibit virus replication by 50%) were calculated using SAS program for non-linear regression analysis. Compound cellular cytotoxicity was determined under identical assay conditions using a modified tetrazolium MTT assay and results expressed as 50% cytotoxic concentrations (CC_{50}).

Analysis of proteins

Protein concentrations were determined with Coomassie® Plus Protein Assay Reagent (Pierce) using BSA as standard. Analysis of proteins by SDS–PAGE and western blotting was as described previously (33). Blots were probed with anti-N (K126; 1:10 000 dilution), anti-P (K109; 1:10 000 dilution) or anti-M2-1 (K115; 1:5000 dilution) rabbit polyclonal antisera produced using purified recombinant proteins [(18); Mason *et al*., manuscript in preparation] as antigen. Monoclonal anti-actin (C4; 1:200 dilution) was purchased from Roche. Alkaline-phosphatase-conjugated goat anti-rabbit secondary antibody or goat anti-mouse antibody (both were purchased from Sigma and used at 1:50 000 dilution) was used. Alkaline phosphatase activity from the secondary antibody was detected using SuperSignal® WestFemto reagent (Pierce) followed by exposure to Kodak MR X-ray film. Western blots were stripped between each application of antibody by incubation of the membrane in 62.5 mM Tris–HCl (pH 6.8), 2% SDS, 100 mM 2-mercaptoethanol at 65°C.

RESULTS AND DISCUSSION

Preparation and characterization of RSV polymerase complex

To facilitate development and screening of an RSV RdRp activity assay, it was important to find an easily obtainable source of soluble and active RSV RNP. Previous studies have shown that simple cytosolic lysates of RSV-infected cells should provide significant amounts of RSV RdRp activity (19–21). In these previous studies, RdRp activity was detected using methods such as DE81 anion exchange filter binding assay (shown schematically in Figure 3B). Such assays allow the quantification of all labeled RNA produced in a transcription reaction, but are not selectively based on RNA size or sequence. When RSV-infected HEp-2 cells were extracted as described previously (19–21) and a DE81 assay was utilized, it was found that significant RdRp activity was indeed present in cytosolic lysates (Figure 1A, fraction labeled S1). However, when using a method that quantifies only full-length mRNAs such as Oligotex (Qiagen; data not shown) or a multi-well plate method that will be described herein ('poly(A) capture' assay schematically shown in Figure 3A), relatively little RdRp activity was present in the S1 fraction, with the majority (>90%) of the activity partitioning into the pellet (P1) fraction (Figure 1A). Thus, an alternative strategy was necessary to obtain a source of soluble RSV RNP.

A method utilizing a combination of detergents that disrupts the interaction of proteins with the cellular cytoskeleton was previously used to extract active RNP from MDBK cells infected with Newcastle disease virus (NDV), a Paramyxovirus related to RSV (28,34). As outlined in Figure 1B, a similar method was applied to RSV-infected HEp-2 cells that were treated with actinomycin D to inhibit DNA-dependent RNA polymerases (DdRp). Briefly, RSV-infected cells were permeabilized with lyso-lecithin (29), scraped into an isotonic buffer (T = total lysate) followed by low-speed centrifugation. The pellet fraction (P1) was resuspended and washed with hypotonic buffer containing 1% Triton X-100 and, following low-speed centrifugation, the pellet (P2) was resuspended in hypotonic buffer containing 0.5% deoxycholate and 1% Tween-40. This suspension was centrifuged for a third time to produce S3 and P3 fractions. The relative RSV RdRp activity within each fraction derived from the scheme shown in Figure 1B was determined in transcription reactions containing [^{33}P]CTP and actinomycin D. Synthesis of RNA was detected in the poly(A) capture assay (Figure 1C). Significantly, the final supernatant fraction (S3) contained most of the RdRp activity. Only a minor amount of RdRp activity was observed in the first two supernatant fractions (S1 and S2). In addition, based on the increase in specific activity observed in the S3 fraction (Figure 1D), a small but noticeable enrichment in RdRp activity was obtained (~4-fold). Subsequently, it was found that this extraction procedure was scalable for obtaining quantities of RSV RdRp activity containing extract sufficient for high-throughput screening.

The majority of RSV RdRp activity monitored by poly(A) capture was found in the insoluble material following extraction of RSV-infected cells. Interestingly, RSV RdRp could be further separated from insoluble material upon treatment with deoxycholate and Tween-40, an approach developed for the extraction of RNP from cells infected with the distantly related Rubulavirus NDV (28,34). It had been suggested that this double detergent extraction procedure dissociated the NDV RNP from the cytoskeletal framework of the cells (34). Since both RSV and NDV are from the family Paramyoviridae, it may not be surprising that the same procedure was efficient at releasing RNP from the insoluble material (cytoskeleton) for both of these viruses. Furthermore, it has been shown that cytoskeletal proteins such as actin, profilin and tubulin are involved in RdRp activity from RSV and other Mononegaviruses (35–39).

In order to characterize the proteins present in the RNP fractions, S3 derived from mock- or RSV-infected cells were analyzed on SDS–polyacrylamide gels followed by coomassie staining (Figure 2A) or western blotting (Figure 2B–E) to detect RSV and host proteins known to be important for RSV RdRp activity. The coomassie stained gel revealed that protein bands corresponding to the size expected for N and P were present in the S3 fraction from infected cells, but not from mock-infected cells. Western blotting with polyclonal

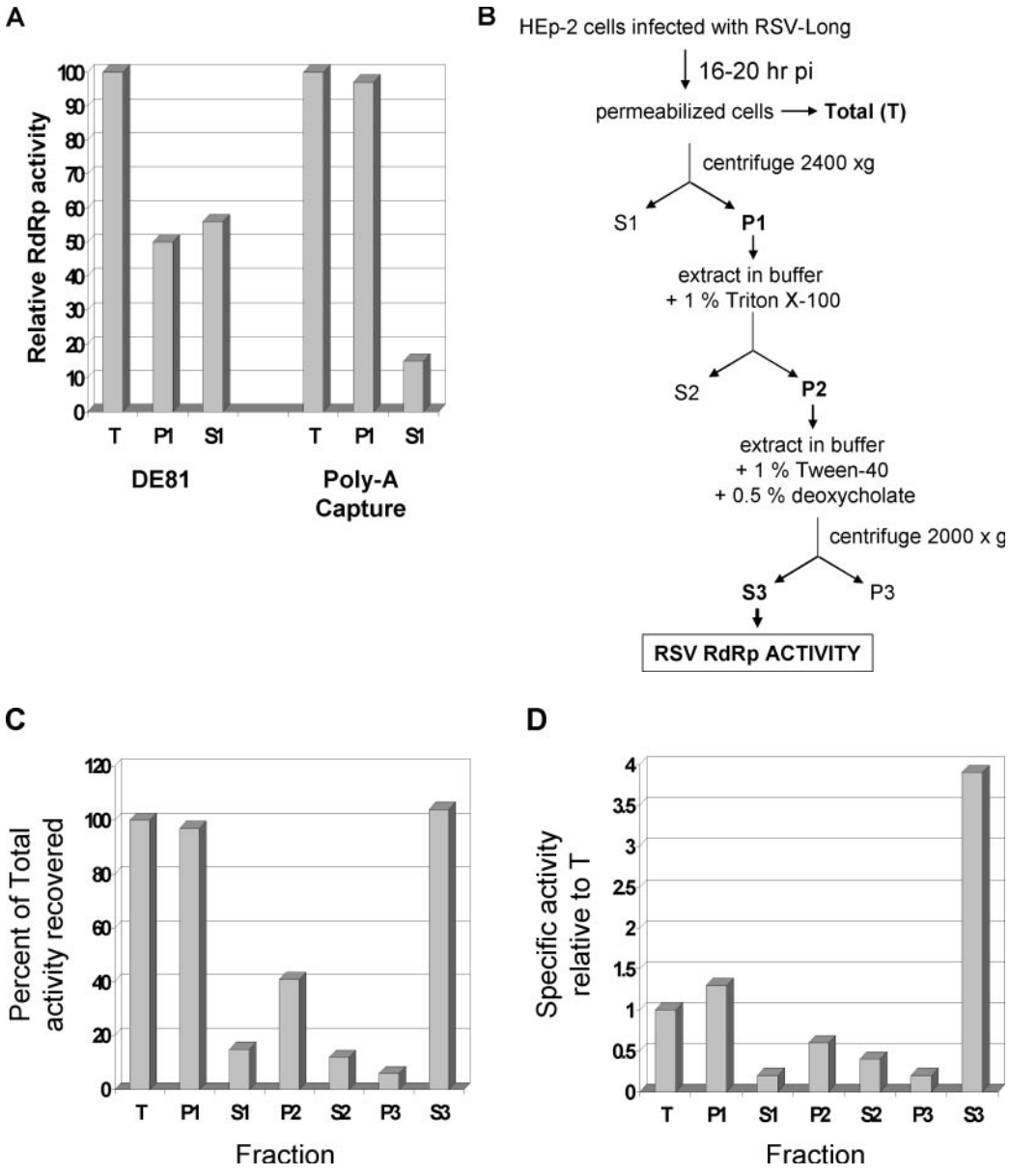

Figure 1. Extraction and activity of RSV RNP. (**A**) Measurement of RSV RdRp activity present in Total (T), cytosolic (S1) and pellet (P1) fractions derived from the extraction of RSV-infected HEp-2 cells. Incorporation of radiolabeled CTP was monitored by either DE81 filter binding assay (left) or poly(A) capture assay (right). (**B**) Schematic diagram of extraction procedure for RSV RdRp. Each centrifugation step in the procedure resulted in a supernatant (S) and pellet (P) fraction. T = Total lyso-lecithin permeabilized lysate fraction prior to the first centrifugation. (**C**) RSV RdRp activity of each fraction is represented relative to the T fraction. Each fraction was added to transcription reactions containing [^{33}P]CTP and actinomycin D. The mRNA products from each reaction were detected in the poly(A) capture assay. (**D**) Taking into account the amount of protein recovered at each step, the specific activities of each fraction is represented relative to the T fraction.

antisera raised against recombinant N, P or M2-1 proteins (Figure 2B–D) showed that only S3 from infected cells contained each of these viral proteins. Previously, cellular actin has been shown to be important for RSV transcription *in vitro* (38,39). When the blot was probed with anti-actin monoclonal antibody, it was found that actin was indeed present in the S3 fraction from RSV-infected cells, although a similar amount of actin was also present in S3 derived from mock-infected cells (Figure 2E). Even though this result suggests that actin is not enriched by the presence of RSV RNP, actin may still be functionally important for RdRp activity by providing stability to the RNP complex. Thus, the S3 RNP fraction contains at least three viral and one host protein known to be important for RdRp activity. Though the catalytic subunit L is not apparent in the S3 fraction shown in Figure 2A, possibly because of its very low abundance in RSV-infected cells, it is presumably present based on the RdRp activity of this fraction shown in Figure 1B and C. As described below in Figure 2F, synthesis of RSV specific transcripts by the S3 fraction indirectly support the presence of the catalytic subunit for RSV RdRp activity.

To confirm whether the S3 RNP-containing fraction is capable of synthesis of authentic RSV mRNAs (1,19), labeled transcripts produced by this fraction were analyzed by agarose gel electrophoresis. Transcription reactions containing

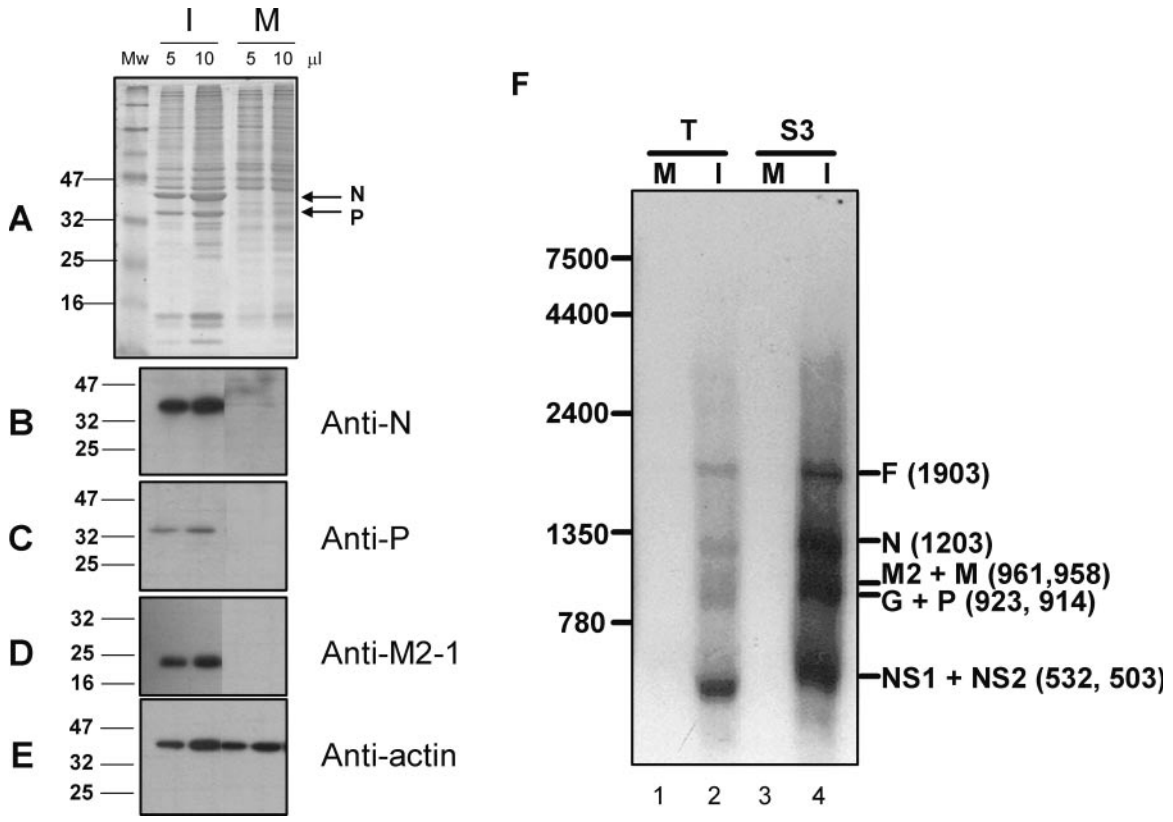

Figure 2. Analysis of RSV RNP. (**A**) Two amounts (5 and 10 µl) of S3 fraction from either RSV-infected (I) or mock (M) cells were analysed on 8% polyacrylamide/SDS gel and stained with coomassie (GelCode Blue; Pierce). For western blot analysis, one-tenth the amount of each fraction in (A) was run on the same type of gel and transferred to polyvinyl difluoride (PVDF) membrane for sequential probing with different antisera in the following western blots probed with (**B**) polyclonal anti-N (K-126); (**C**) polyclonal anti-P (K-109); (**D**) polyclonal anti-M2-1 (K-115); (**E**) monoclonal anti-actin (C4; Roche). The same blot was sequentially stripped and reprobed. (**F**) Analysis of transcripts by gel electrophoresis. Transcription reactions contained either total T (lanes 1 and 2) or S3 fractions (lane 3 and 4). The fractions were obtained either from mock-infected cells (M; lanes 1 and 3) or RSV-infected cells (I; lanes 2 and 4). Transcripts produced in the presence of 15 µCi [α-^{33}P]CTP and 2 µM unlabeled CTP were treated with oligo(dT)/RNaseH prior to denaturation in the presence of glyoxal and run on a 1.5% agarose gel. Labeled transcripts were detected by phosphorimaging. The relative mobility of labeled RNA markers (Gibco) is indicated on the left of the panel. The identity of RSV transcripts, based on expected size (nucleotide length shown in parentheses), is indicated on the right.

unfractionated cell lysates (T) or S3 RNP fraction from either mock- or RSV-infected cells were incubated in the presence of [^{33}P]CTP and actinomycin D. Prior to electrophoresis on agarose gels, the RNA was treated with oligo(dT)$_{15}$ and RNaseH to remove the heterogeneous poly(A) tails (19), allowing each transcript to migrate at the size expected for RSV mRNAs. When the poly(A) tails were not removed, distinct transcripts were not observed (data not shown). As shown in Figure 2F, labeled transcripts were produced from both T and S3 fractions but only when derived from virus-infected cells. These transcripts were of the size expected for many of the RSV mRNAs (Figure 2F; lanes 2 and 4). In contrast, no labeled RNA was produced in reactions containing fractions obtained from mock-infected cells (lanes 1 and 3). Furthermore, the S3 fraction produced more of each RSV transcript than the unfractionated cell lysate, consistent with the increase in specific activity for the S3 fraction observed in Figure 1D. Slot-blot hybridization was performed to confirm that RNA produced in these reactions was complementary to the viral genome as expected (data not shown). Thus, the RSV RNP fraction prepared by the method outlined here are highly active for the production of viral mRNAs. Albeit, this preparation is clearly crude (Figure 2A), it contains all the RSV proteins and displays the activities expected for RSV RdRp.

Development of RSV transcriptase assay

RSV mRNAs produced *in vitro* are polyadenylated and can be captured by oligo(dT) resin (19). Based on this observation, a sensitive, high-throughput multi-well plate assay for the detection of RSV RdRp activity was developed. In this assay, schematically shown in Figure 3A, the wells of a streptavidin-coated FlashPlate™ (PerkinElmer/NEN) were pre-bound with biotinylated oligonucleotides composed of the homopolymeric sequence dT$_{30}$. Polyadenylated transcripts that were synthesized and labeled with [^{33}P]CTP or [^{3}H]CTP by the RSV polymerase are 'captured' by hybridization to the immobilized oligo(dT)$_{30}$. The radioactivity incorporated into the transcripts is detected on a TopCount™ (PerkinElmer/Packard) plate reader by proximity of the captured transcripts to a scintillant embedded in each well of the FlashPlate™ (40,41). Thus, this assay specifically detects polyadenylated transcripts that are synthesized by the RSV transcriptase only when all of the steps in the RSV transcription cycle (initiation, elongation and polyadenylation) have been completed. Reactions that

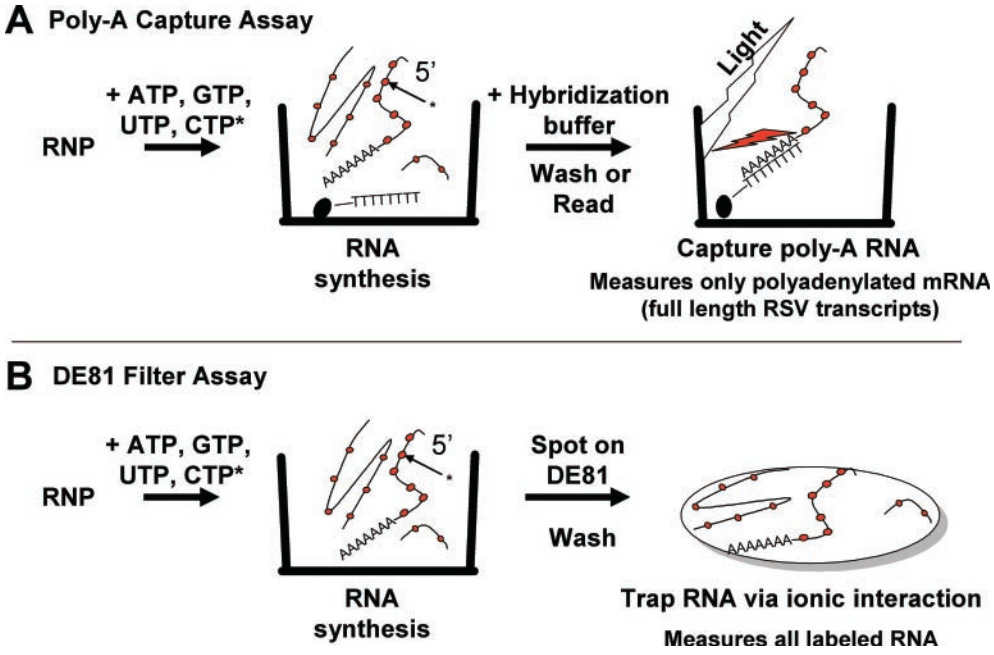

Figure 3. Comparison of poly(A) capture and DE81 filter binding assays. (**A**) Schematic diagram of poly(A) capture assay. *In vitro* transcription reactions are assembled by adding RSV RNP, unlabeled nucleoside triphosphates (ATP, GTP and UTP), radiolabeled CTP (asterisk) and buffer to the wells of a streptavidin-coated FlashPlate™ that has been pre-coated with biotinylated oligo(dT)$_{30}$ (black oval with multiple Ts). Nascent transcripts (curved lines) are synthesized resulting in the incorporation of radiolabeled CTP (red circles). Transcription reactions are stopped with buffer that favors hybridization of RNA to DNA bringing the labeled RNA into proximity of the scintillant that is embedded in the plastic well of the plate. Decay of the radiolabel (red lightening bolt) causes the scintillation signal (white lightening bolt) that is detected on a TopCount™ (Beckman) plate reader. In this assay, only RNA that is polyadenylated is captured. Therefore, only full-length RSV mRNA transcripts are detected. (**B**) Schematic diagram of DE81 filter binding assay. The transcription reactions are essentially the same as in (A). Following synthesis of the RNA, a part of the reaction mixture is spotted onto DE81 filter paper, washed with a phosphate-containing solution, dried and read on a phosphoimager. Since the DE81 filter paper is a weak anion exchanger, all RNA produced in the reaction is trapped and subsequently detected.

contain [^{3}H]CTP have an additional advantage that the assay plate could be read directly without prior washing of the wells, thus providing a solid-phase homogeneous read-out that is highly amenable to high-throughput screening. A compound that inhibits any step that is essential for the completion of the transcription cycle would cause a decrease in the signal produced in this poly(A) capture assay. Thus, this assay could be used in a high-throughput screen to detect RSV 'transcriptase' inhibitors.

In optimizing the assay for various parameters such as pH, divalent cation/counterion pair and concentration, ionic constitution and strength, we found that the conditions originally found by Barik (19) for RSV RdRp activity detected using DE81 assay (Figure 3B) were quite optimal. This suggests that the RNP extracted by these two distinct procedures was indeed similar. In testing the effect of various detergents and additives, we found the addition of 2 mM EGTA was stimulatory for the transcriptase activity (data not shown). It is possible that chelation of divalent cations may increase the yield of full-length mRNAs produced in the reaction by inhibiting any endogenous Ca^{2+}-dependent nucleases that may be present in the S3 fraction. In addition, we found that radiolabeled CTP produced a better signal compared to other radiolabeled ribonucleotides (Figure 4A). Additional parameters specific to the poly(A) capture format, such as the concentration of the pre-coated oligo(dT)$_{30}$, time and temperature of incubation for synthesis and hybridization phases of the reaction were also optimized. For example, the co-optimization of the time and temperature during RNA synthesis established that incubation at 30°C provided linear incorporation of radiolabel for 2 h (Figure 4B) or as long as 6 h (data not shown). The assay was also linear for the addition of increasing amounts of RNP-containing S3 fraction (data not shown). Once diluted into assay buffer, the S3 fraction was found to be stable on ice for ~1 h with only minimal losses to the transcriptase activity (data not shown).

The assay was found to be very stable and reproducible as determined by statistical analyses. Table 1 shows the signal obtained in negative (blank) and positive control wells and well-to-well variation measured using the Z'-statistic (42), for which $Z' > 0.5$ is acceptable with decreasing variation as the Z'-value approaches 1.0. Thus, the assay had excellent reproducibility either in homogeneous mode ($Z' = 0.70$) or when the assay wells were washed with buffer prior to reading of the scintillation proximity signal ($Z' = 0.73$). As shown in Table 1, the optimized homogeneous assay produced a signal-to-background (blank) of ~7, whereas the same parameter for the washed assay was 80. The background signal was obtained in wells that contained no immobilized oligo(dT)$_{30}$. When the transcripts were treated with oligo(dT) and RNaseH prior to hybridization in the FlashPlate wells, no signal was obtained (data not shown) further validating the capture through poly(A) tails. Although the homogeneous version of the assay is preferable for initial compound screening, the washed version of the assay provides mechanistic information during characterization of potential inhibitors. For example, some compounds may appear to inhibit the assay if they quench the scintillation signal. Such false positives can be ruled out

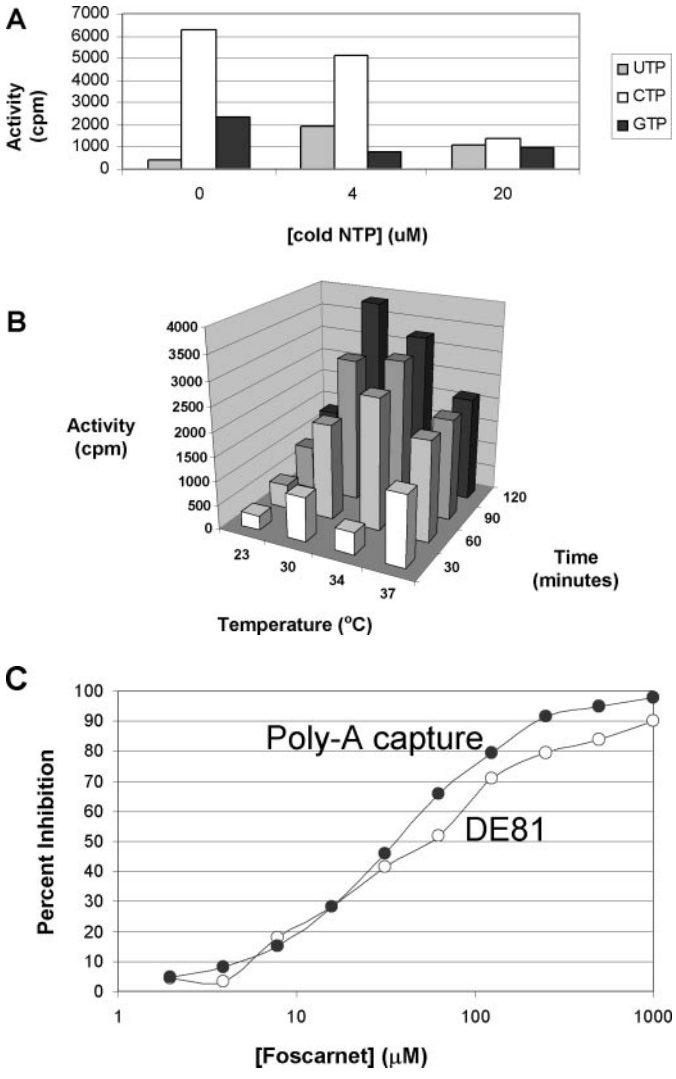

Figure 4. Optimization and characterization of poly(A) capture assay. (**A**) Testing of various ^{33}P-labeled NTP (UTP, CTP or GTP) with 0, 4 or 20 μM cold NTP. (**B**) Poly(A) capture assay was co-optimized for time and temperature. (**C**) Inhibition curves for foscarnet on RSV polymerase activity in DE81 (open circles) and poly(A) capture (closed circles) assays. The percentage inhibition (ordinate) versus the concentration of inhibitor (abscissa) is presented.

Table 1. Reproducibility of Poly(A) capture assay

Assay mode[a]	Blank[b] Avg ± SD (n = 8)	Control[c] Avg ± SD (n = 38)	Z'[d]
Homogenous	420 ± 22	3093 ± 248	0.70
Washed	38 ± 7	3044 ± 261	0.73

[a]Assay mode is explained in the text.
[b]Blank refers to assay wells that contained no biotin-dT30, but contained all other assay components including RNP (negative control).
[c]Control refers to assay wells containing all assay components, but no inhibitors (Positive control).
[d]Z' was calculated according to Zhang *et al*. (42):

$$Z' = 1 - \frac{[3 \times (\text{SD control}) + 3 \times (\text{SD blank})]}{(\text{Avg control} - \text{Avg blank})},$$

where SD is the standard deviation and Avg is the average.

by washing compounds out of the well prior to reading the scintillation signal.

The optimized reaction conditions were used to measure the K_m for nucleotides in both the poly(A) capture and DE81 assay formats. In the poly(A) capture format, it was found that the K_m values for ATP, GTP and UTP were 27, 38 and 24 μM, respectively. However, the K_m for CTP was much lower (1.7 μM), providing a possible reason that radiolabeled CTP gave the best results for RdRp activity in this assay (Figure 4A). A similar K_m for CTP was found in the DE81 assay (1.3 μM). Interestingly, a difference between the two assays was apparent in the K_m for GTP which was 38 μM in the poly(A) capture format compared to 1.8 μM in the DE81 assay. Such a difference in the K_m for GTP could reflect a divergence in the RNA being measured in the two assays such that higher GTP concentration seems to be necessary for the synthesis of full-length polyadenylated RSV transcripts. This suggests that a major contributor to the signal measured in the DE81 assay may arise from abortive or non-processive mRNA synthesis.

The pyrophosphate mimic foscarnet (phosphonoformic acid) has previously been shown to inhibit a variety of RNA- and DNA-dependent RNA polymerases (43). We tested foscarnet in both the DE81 and poly(A) capture assay formats as a potential inhibitor of RSV polymerase (Figures 4C). We found that the IC_{50} values obtained for foscarnet were in good agreement between the two assays [39 μM in poly(A) capture versus 41 μM in DE81]. Thus, it is possible to inhibit the RdRp activity of the RSV RNP with a known, general polymerase inhibitor.

Identification of a novel RSV transcriptase inhibitor

We have implemented the homogeneous format of the RSV poly(A) capture assay to screen several hundred thousand compounds from our corporate collection. A confirmed hit rate of ~0.2% was obtained. Out of several hundred confirmed hits that were profiled, only three compounds appeared to be specific and selective inhibitors of RSV transcriptase activity. One compound in particular, Compound A, shown in Figure 5A, was characterized further. Compound A had an IC_{50} of 4.5 μM (± 1.3 μM, $N > 200$) in the poly(A) capture assay, but was inactive against several other viral RdRp activities including influenza virus (either poly(A) capture or DE81 assay formats), HCV and poliovirus polymerases, as well as DdRp II isolated from calf thymus (Table 2). Compound A was also active in both washed and unwashed versions of the poly(A) capture assay (Figure 5B). Interestingly, this compound was only weakly active in DE81 filter binding assay attaining <50% inhibition at the maximal concentration (Figure 5B). Thus, this inhibitor would not have been found using an assay that simply measures radiolabel incorporation since the upper limit for selection of a hit is typically set at 40–50% inhibition for the compound concentrations used in most screening campaigns (~5–40 μM). Next, to elucidate the mechanism of action of Compound A, kinetic analysis of RSV polymerase inhibition was performed with a range of concentrations of both nucleotide and inhibitor. Double-reciprocal plots of the data indicated that inhibition by Compound A was non-competitive versus CTP (Figure 5C) or other nucleotides (data not shown). These kinetic experiments

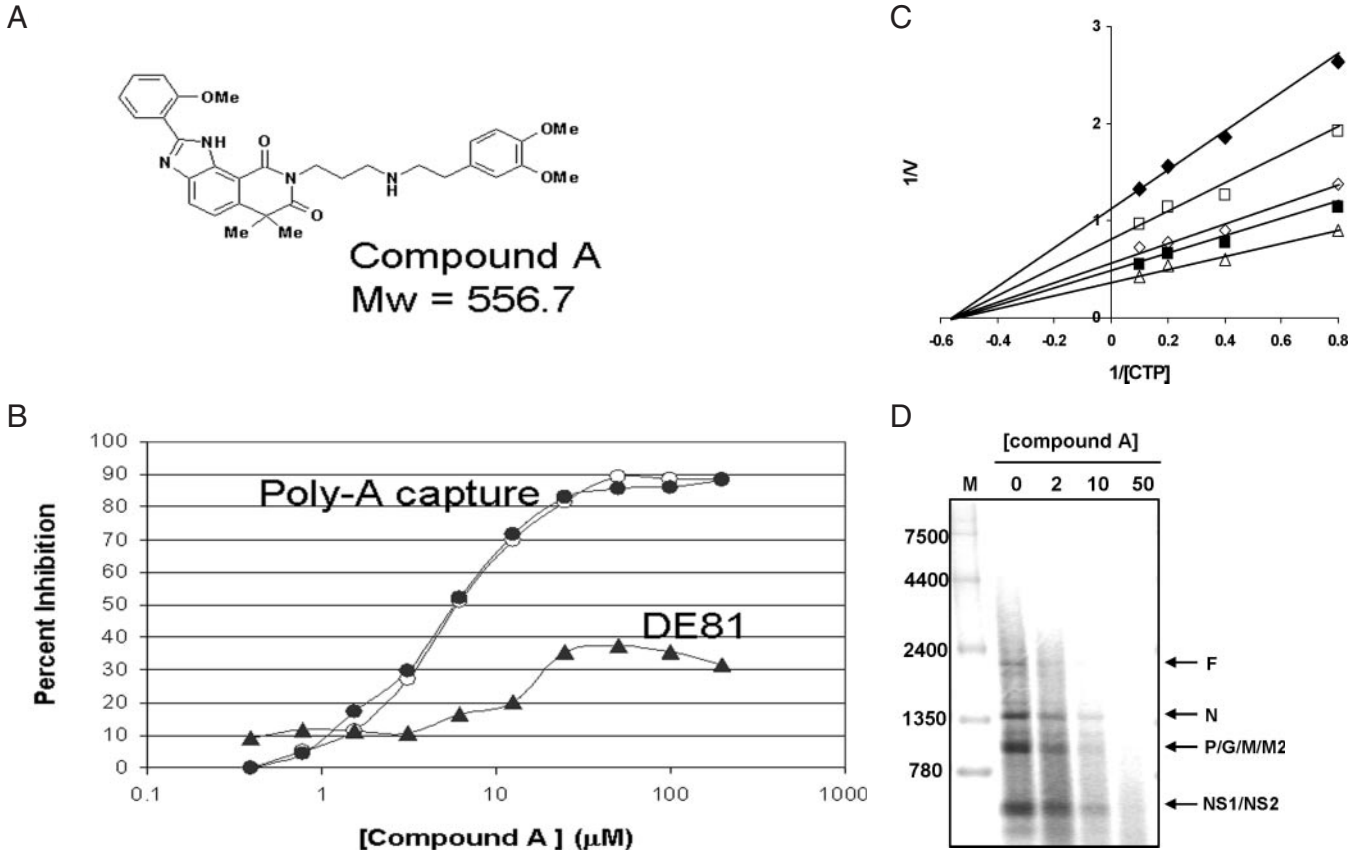

Figure 5. Characterization of novel RSV transcriptase inhibitor. (**A**) Chemical structure of RSV transcriptase inhibitor Compound A (dihydrochloride salt form). *In vitro* activity (IC_{50} in μM) of compound in various assays as indicated. (**B**) Inhibition curves for Compound A on RSV polymerase activity in DE81 assay (closed triangles) and poly(A) capture assay in either washed (closed circles) or unwashed (open circles) assays. The percent inhibition (ordinate) versus the concentration of inhibitor (abscissa) is presented. (**C**) Mode of inhibition of Compound A and effect on RSV transcripts. Transcription reactions were performed as described in Materials and Methods using the poly(A) capture assay with various CTP concentrations (1.25–10 μM) in the absence (open triangles) or in the presence of 6.25 (closed squares), 12.5 (open diamonds), 25 (open squares) and 50 (closed diamonds) μM Compound A. The *y*-axis represents the inverse velocity of the reaction expressed in picomoles CTP incorporated per second. (**D**) Transcription reactions were performed with 15 μCi [α-^{33}P]CTP and 2 μM unlabeled CTP in the presence of the indicated concentrations (0–50 μM) of Compound A and RSV transcripts were treated with oligo(dT)/RNaseH prior to denaturation in the presence of glyoxal and run on a 1.5% agarose gel. The relative mobility of labeled RNA markers (Gibco) is indicated on the left and the identity of RSV transcripts (based on size) is indicated on the right.

Table 2. Selectivity of inhibition by Compound A

Assay	IC_{50} (μM)
RSV	4.5
Influenza	>200[a]
HCV	183
Polio	>125[a]
Calf thymus	>125[a]

IC_{50} of Compound A was determined in RSV Poly(A) capture assay and compared to IC_{50} obtained in assays with other polymerases, including Influenza virus polymerase (31,32); HCV and poliovirus polymerase (30); and calf thymus RNA polymerase II (30).

[a]When Compound A gave <50% inhibition at the highest concentration tested the IC_{50} was designated as greater than that concentration (e.g > 125 or 200 μM).

also demonstrate the versatility of the poly(A) capture format for analyses of RSV transcription. We also investigated the effect of inhibitors on the synthesis of the major mRNA transcripts produced by the RSV RNP complex *in vitro* (Figure 5D). Analysis on 1.5% agarose gels showed that the synthesis of all major RSV mRNA species was equally inhibited. Thus, inhibition by Compound A results in a general impairment of mRNA synthesis by RSV RNP.

Importantly, we have also found that Compound A inhibits the replication of RSV (Long strain) in HEp-2 cells: in anti-RSV F ELISA assays, Compound A had an EC_{50} of 1.3 μM which was 6-fold lower than the CC_{50} for this compound on the same cells (7.7 μM). In further testing, we have found that Compound A does not interfere with the hybridization of mRNA to immobilized oligo(dT) nor does it seem to inhibit polyadenylation of the mRNAs by RSV polymerase (data not shown). Rather, coupled with the selectivity profile that this compound has for RSV polymerase over other RdRp or DdRp activities (Table 2), it appears that Compound A specifically inhibits a component of the RSV RNP which is essential for the synthesis of RSV mRNAs. We have evidence that the mode of action for this inhibitor is unique and involves the inhibition of 5′-cap formation by the viral RNA polymerase (M. Liuzzi *et al.*, manuscript in preparation). Moreover, since three different chemical or structural classes of selective RSV

RdRp inhibitors were obtained in this screen, it suggests that the RSV RNP is a highly drugable target for potential anti-RSV therapeutic intervention.

Although our poly(A) capture assay was originally developed for screening of compounds as potential inhibitors of RSV RdRp, it was obvious that this assay has potential for broader application. We have found that the poly(A) capture assay format can be used to assay other viral RNA polymerases. For example (data not shown), this assay format has been applied to influenza virus polymerase that catalyzes the polyadenylation of its own nascent transcripts (31,44,45). In addition, the poly(A) capture assay could also be applied to any cellular *in vitro* transcription reactions that include endogenous nuclear polyadenylation machinery or even human poly(A) polymerase (46). Recently, a FlashPlate assay that did not rely on the capture of RNA was developed for screening of human poly(ADP–ribose) polymerase-1 (PARP-1) activity (40). In order to expand the general applicability of our assay, we also have successfully tested this assay format with sequence-specific probes to capture different RSV transcripts (data not shown). Therefore, this assay opens the door for screening a variety of nucleic acid synthesizing and modifying activities.

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