Influenza virus utilizes a unique mechanism for initiating the transcription of viral mRNA. The viral transcriptase ribonucleoprotein complex hydrolyzes host cell transcripts containing the cap 1 structure (m7GpppG(2′-OMe)-) to generate a capped primer for viral mRNA transcription. Basic aspects of this viral endonuclease reaction are elucidated in this study through the use of synthetic, radiolabeled RNA substrates and substrate analogs containing the cap 1 structure. Unlike most ribonucleases, this viral endonuclease is shown to catalyze the hydrolysis of the scissile phosphodiester, resulting in 5′-phosphate- and 3′-hydroxyl-containing fragments. Nevertheless, the 2′-OH adjacent to the released ribosyl 3′-OH is shown to be important for catalysis. In addition, while the endonuclease steady-state turnover rate is measured to be 2 h−1, phosphodiester bond hydrolysis is not rate-limiting. The direct generation of a free 3′-OH and the subsequent slow release of this product are consistent with the viral need for efficient use of the capped primer in subsequent reactions of the influenza transcriptase complex.

Most viral and cellular mRNA molecules contain a methylated cap structure at the 5′-end (for reviews see Refs. 1 and 2). The presence of a cap is important for mRNA maturation, initiation of translation, and protection against degradation by RNases present in the cell. The general structure of a capped RNA can be designated as m7G(5′)ppp(5′)Puo- (Puo, the penultimate base, is typically a purine), and the penultimate base of cap 1-containing RNAs is 2′-O-methylated and can be designated m7G(5′)ppp(5′)Puo(2′-OMe).

The first step in the synthesis of influenza virus mRNA is the binding of host cell nuclear mRNA having a cap 1 structure (3–5) by the PB2 protein of the viral transcriptase complex (6–8). Subsequent cleavage of the capped RNA 10–15 bases from the penultimate nucleoside generates capped oligoribonucleotides that serve as primers in mRNA synthesis by the viral transcriptase (9–12).

Heretofore, the RNA endonuclease reaction of the influenza transcriptase complex has not been well defined, either in terms of the chemical structure of substrates and products or in the quantification of reaction kinetics. The difficulty has been the unavailability of homogeneous preparations of short substrates containing the cap 1 structure. Initial characterizations of the endonuclease have employed mRNAs isolated from natural sources (10) or prepared by in vitro “run-off” transcription, so that the preparations were heterogeneous in length or in cap structure. In the present study, the basic mechanism of the endonuclease reaction is defined through the use of chemically synthesized, short capped RNAs.

EXPERIMENTAL PROCEDURES

Materials—The phosphitylating reagent 2-chloro-4H-1,3,2-benzodiazepin-4-one, tetra-n-butylammonium fluoride, and N,N,N′,N′-dimethylformamide were obtained from Fluka AG (Neu-Ulm, Germany). Bis[tri-n-butylammonium] pyrophosphate was prepared as described (13). Ribonucleoside phosphoramidates and synthesis columns were obtained from Milligen/Biodesign (Eschborn, Germany). The 2′-O-methyl guanosine ribonucleotide phosphoramidite and 3′-deoxyadenosine-controlled pore glass (CPG) were purchased from GlenResearch (Sterling, VA). Preparation of 2′-fluoro-deoxyadenosine was according to Olsén et al. (14). Guanyllytransferase and poly(A) polymerase were obtained from Life Technologies, Inc., 3′-adenosyl-1-methionine from U.S. Biochemical Corp., RNasin from Promega (Madison, WI), and radiolaabeled nucleoside triphosphates from DuPont NEN. Multiscreen-NC 0.22-μm nitrocellulose filtration plates were purchased from Millipore (Bedford, MA), and nitrocellulose membranes (0.45-μm pore size) were from Schleicher and Schuell. Oligoribonucleotides were purchased from Life Technologies, Inc.

Synthesis and 5′-Triphosphorylation of Oligoribonucleotides—Oligoribonucleotides were synthesized on an Applied Biosystems 380B DNA/RNA synthesizer (1-μmol scale) using standard phosphoramidite chemistry. The method of Ludwig and Eckstein (13) for the solution phase synthesis of the nucleoside 5′-O-(1-thiotriphosphates) was modified for the conversion of the CPG-bound oligoribonucleotides into the corresponding oligoribonucleotide triphosphates. After oligonucleotide synthesis the column was dried in a vacuum oven for 15 min. A 0.2-μmol aliquot was then removed, depurinated, and purified as described below. The remaining CPG-bound RNA was transferred into a 2-ml vial and dried for 2 h at 35 °C. The CPG was covered with pyridine (50 μl) and dioxane (150 μl) under argon. A 0.5 M solution of 2-chloro-4H-1,3,2-benzodiazepin-4-one in dioxane (20 μl, 20 μl, 10 μl, 10 μl) was added to the suspension followed by (after 15 min) 150 μl (75 μmol) of 0.5 M bis[tri-n-butylammonium] pyrophosphate in N,N,N′,N′-dimethylformamide and tri-n-butylamine (50 μl). The solution was removed after 15 min, and 500 μl (47 μmol) of 3% iodine in tetrahydrofuran/pyridine/water (80:10:10, v/v/v) was added. After 20 min, the CPG was washed three times each with tetrahydrofuran (2 ml) and ethanol (2 ml). The oligoribonucleotides were depurinated and polyacrylamide gel-purified as described (16, 17).

Purification of the Oligoribonucleotides—Crude products of the oligoribonucleotides and their corresponding 5′-triphosphates were purified on the same gel for comparative analysis, revealing, in general, a...
TABLE I

Sequences of triphosphorylated synthetic RNAs

| Code | Primary structure | Sequence based on | Feature |
|------|------------------|-------------------|---------|
| I    | ppGGmUUUUUUUAUUUUAAUUUC-3' | AIVm<sup>5b</sup> | Unmodified substrate |
| II   | ppGGmUUUUUUUAUUUUU(da)AUUUUC-3' | AIVm<sup>5b</sup> | 2'-da at the cleavage site |
| III  | ppGGmUUUUUUUAUUUUU(2'-FA)AUUUUC-3' | AIVm<sup>5b</sup> | 2'-Fluoro-da at the cleavage site |
| IV   | ppGGmUUUUUAUUUUU(3'-da)-3' | BMV<sup>5</sup> | "Product-analog" nonextendible 3'-end |

<sup>a</sup>The 5'-triphosphorylated nucleoside of each RNA contains a 2'-O-methyl modification.

<sup>b</sup>AIVm, alfalfa mosaic virus.

<sup>c</sup>BMV, brome mosaic virus.

The homogeneity of the oligoribonucleotides was analyzed by reverse phase high performance liquid chromatography. The oligoribonucleotides were purified by reverse phase high performance liquid chromatography (RP-HPLC) using a Vydac C18 column. The RNA was eluted with a gradient of acetonitrile (1.4–14% in 20 min, flow rate = 1.5 ml/min) in 50 mM aqueous triethylammonium acetate buffer (pH 7.0). Coinjection of oligoribonucleotides with their corresponding oligoribonucleotide 5'-triphosphates revealed two peaks. The <sup>31</sup>P NMR of the gel purified fully unprotected oligoribonucleotide III showed the characteristic signals of a triphosphate (δJ = 8.84 (d, J = 18.9 Hz); δP = 18.2 Hz, δP = 21.99 (dd, J = 18.5 Hz)) in addition to the phosphodiesters signals, 0.05 to −0.22 (m). <sup>31</sup>P NMR spectra were recorded at 145.79 mHz on a Bruker WH 360 spectrometer with 1H decoupling and 85% H<sub>3</sub>PO<sub>4</sub> as the external standard. Samples contained 50% D<sub>2</sub>O and 10 mM EDTA, pH 8.

Preparation of Cap 1 RNAs—High specific activity RNA was prepared by adding 16 units of guanylyltransferase to a 100-μl reaction containing 50 mM Tris-HCl, pH 8, 1.25 mM MgCl<sub>2</sub>, 6 mM KCl, 2.5 mM dithiothreitol, 5.0 μM triphosphorylated oligoribonucleotide (Table I), 100 μM 5'-adenosyl-<sup>33</sup>P-methionine, 0.35 μCi of [α-<sup>32</sup>P]GTP, 3.5 μg GTP, and 120 units of RNasin. The reaction was incubated for 5 h at 37°C before being chloroform-extracted (18). Control reactions using the nontriphosphorylated RNAs were not capped. The capped 19-base RNAs were purified by polyacrylamide gel electrophoresis using 20% gel containing 8M urea followed by Elutrap electroelution (Schleicher and Schuell) and ethanol precipitation. Alternatively, the capped RNAs were purified by reverse phase high performance liquid chromatography using a Spectra Physics 8800 instrument coupled with a SP8450 UV/VIS detector and a Vydac C18 column. The RNA was eluted with a gradient as described above. Low specific activity RNA was prepared using 50 μM GTP and 10 μg of [α-<sup>32</sup>P]GTP.

Enzyme Kinetics—Enzymic reactions were carried out at 25 °C in the presence of 100 mM Tris-HCl, pH 7.8, 50 mM KCl, 5 mM dithiothreitol, 0.25 mM MgCl<sub>2</sub> (buffer A); 2, 1.6, or 1.2 μg of ribonuclease/ml (50, 40, and 30 μM endonuclease, respectively) and 400 pm <sup>32</sup>P-capped I. Reactions were initiated by the addition of I or MgCl<sub>2</sub>. Aliquots (4.3 μl) were quenched after 0.5–90 min by mixing with 3 μl of glycerol-tolerant gel buffer (U.S. Biochemical Corp.) stop mix (GTGB-SM; 30 mM Tris-HCl, pH 9, 30 mM Taurine and 0.5 mM EDTA, 90% formamide, 1% (w/v) PEG-8000, 0.1% (w/v) sodium dodecyl sulfate, 0.1% (w/v) sodium dodecyl sulfate, 0.1% (w/v) SDS, and 0.005% (w/v) bromophenol blue). Alternatively, the reaction was stopped by filtering aliquots through a Multiscan-NC nitrocellulose membrane and diluting with 4 μl of GTGB-SM. Reaction products were separated from the substrate by running the samples on a 20% acrylamide denaturing sequencing gel and quantified with a Phosphorimager (Molecular Dynamics, Sunnyvale, CA). Biphasic time course data were fit to the equation,

\[
p(t) = a(1 - \exp(-k_1t)) + (k_2t) \quad \text{(Eq. 1)}
\]

where \(p(t)\) is the amount of product formed at time \(t\), \(a\) is the burst amplitude, \(k_1\) is the first order rate constant, and \(k_2\) is the steady-state turnover rate constant. The concentration of endonuclease active sites was determined from the burst amplitude, correcting for the difference between the burst and steady-state rates by multiplying the burst amplitude by \((k_0 + k_1)/k_2\).

IC<sub>50</sub> Determination for Capped 10-Base Oligoribonucleotide—Reactions were performed at 25 °C in buffer A containing 3.3 μg protein/ml, 0–2.0 nm capped I and were initiated after a 30-min preincubation by the addition of 10 μl of 1.2 nm 32P-capped I. Aliquots (4.5 μl) of the reaction mixture were quenched after 0.5, 1.0, 1.5, and 2.0 min by mixing with 3 μl of GTGB-SM. The amount of cleavage of the substrate was quantified using a Phosphorimager. Capped 32P-labeled IV was used to determine lethality by incubation with purified ribonuclease. Inhibitor saturation data were curve-fit using a nonlinear least-squares algorithm to the Hill equation (19).

Structure of the 3'-Uncapped RNA Cleavage Product—Capped I and 5'‐AUUUUC-3' were 3'-end labeled using the enzyme poly(A) polymerase and [γ-<sup>32</sup>P]ATP according to the manufacturer's instructions. The other control oligoribonucleotide, 5'-AUUUUC(3'-da)-3', was 5'-radio labeled using [γ-<sup>32</sup>P]ATP and polynucleotide kinase. Cleaved I was incubated with the endonuclease to yield the capped labeled 13- and 6-base cordycepin-labeled products, which were analyzed on a 20% acrylamide sequencing gel and compared with the 5'-phosphorylated and nonphosphorylated 6-base cordycepin-labeled control RNAs.

Equilibrium Binding—Ribonucleoprotein (2 μg of protein/ml) was incubated at 25 °C for 30 min with <sup>32</sup>P-capped IV in 50 μl of buffer A. Uncapped IV (50 nm) was included in all samples to displace nonspecific binding of the labeled capped IV. Enzyme-bound oligoribonucleotides were captured by nitrocellulose filtration in a 96-well manifold and were washed three times with 200 μl of buffer. Enzyme-bound capped IV was quantified using a Phosphorimager.

RESULTS

Substrate Requirement and Product Determination—Fig. 1 demonstrates the specificity of the influenza endonuclease toward the cleavage of capped RNA substrate I. In the absence of added ribonucleoprotein, capped I was stable against hydrolysis. In the presence of the enzyme there was a time-dependent decrease in the amount of capped 19-base RNA with a concomitant increase in the amount of a specifically cleaved product. The position of hydrolysis was previously determined to be between nucleotides A<sub>13</sub> and A<sub>14</sub> (20). When GTP was added to the reaction, one or more GMP residues was added by the endonuclease-associated transcriptase activity to the cleavage product, as directed by the viral RNA template. This indicates that the product is specifically derived from influenza endonuclease and was not due to nonspecific hydrolysis of the RNA (Fig. 1). In order to determine the fate of the phosphate at the site of cleavage, doubly labeled (5'-capped and 3'-cordycepin) capped I was incubated in the presence of the endonuclease. As shown in Fig. 2, the 6-base cordycepin-labeled product from the cleavage reaction co-migrated with the control 5'-32P-labeled 6-base cordycepin synthetic oligoribonucleotide, 5'-<sup>32</sup>P-AUUUUUC(3'-da)-3', and not with the nonphosphorylated RNA, 5'-HO-AUUUUC(3'-da)-3', indicating cleavage of the 3'-O-P bond.

The ability of the endonuclease to cleave modified capped RNAs, where the 2'-OH at the preferred cleavage site was replaced by a hydrogen or a fluorine atom, was investigated. Oligoribonucleotides I and III, having either an unmodified adenine or a 2'-deoxyadenosine located at A<sub>13</sub>, respectively, were incubated with ribonucleaseprotein in the presence or absence of one of the four nucleoside triphosphates. The results are shown in Fig. 3. First, there was no detectable cleavage at A<sub>13</sub> for the 2'-da containing RNA, and the site of cleavage was shifted from 3' to A<sub>13</sub> to the 3' side of A<sub>14</sub>. In addition, the hydrolysis rate was approximately 10 times slower than that for capped I, and similar results were obtained for the 2'-fluoro-containing oligoribonucleotide III (data not shown). The data presented in Fig. 3 also show that the specific cleavage products for both substrates (capped I and II) are extended when incubated in the presence of GTP and not with any of the other three nucleoside triphosphates.
Fig. 1. Sequencing gel (8% polyacrylamide) electrophoretic analysis of the products from an endonuclease cleavage reaction of 32P-labeled RNA-capped I. Incubation was from 3 to 60 min in the absence and presence of 40 μM GTP. The major product results from specific hydrolysis at position 13. When GTP was included, the cleavage product was extended by the endonuclease-associated transcriptase activity by one or more GMP residues. A small amount of nonspecific cleavage was obtained that was not extended in the presence of GTP.

Fig. 2. Sequencing gel analysis of the endonuclease cleavage products of the doubly labeled 20-base m7G32pppGmUUUU-AUUUUUAUUUUC-32P-(3-dA)-3 analog of capped I. Lane 1 contains the 13-base product (c) of the endonuclease reaction with labeled capped I (b). Lane 2 is a control of doubly labeled analog of capped I (a) with no reaction, whereas lane 4 contains the 13-base (c) and 6-base cordycepin-labeled RNA cleavage products after incubation with the endonuclease. Lanes 3 and 5 contain control synthetic oligoribonucleotides 5'-AUUUUC-32P-(3'-dA)-3' (d) and 5'-32P-AUUUUUC(3'-dA)-3' (e), respectively.

Endonuclease Reaction Kinetics—Typical biphasic reaction progress curves for the cleavage of I to the 13-base product are shown in Fig. 4A. Similar curves were obtained regardless of whether the reaction was initiated with capped I or with 0.25 mM Mg2+, the concentration of Mg2+ that produces the maximal rate of reaction in this system (data not shown). Chung et al. (23) have shown that the endonuclease was able to bind but was unable to cleave capped RNA when the divalent metal ion was absent from the reaction. The amplitude of the burst phase was approximately proportional to the amount of endonuclease added. This observation suggests that the early phase of the reaction signifies a reaction that occurs prior to the establishment of steady-state turnover, which was likely due to a slow capped product off-rate. To confirm this interpretation, the same reaction was performed wherein the endonuclease-bound product (E-cap-13-mer) was removed from the substrate (cap-19-mer) and free cleaved product (cap-13-mer) by filtering the reaction mixture at various times through a nitrocellulose membrane. The results are shown in Fig. 4B (open symbols) and compared with those obtained from the same reaction but without the removal of enzyme-bound product (closed symbols). With the removal of the E-cap-13-mer, the burst phase of the reaction was absent and only the steady-state phase was observed. The rate was identical to the steady-state rate obtained for the unfiltered reaction. Using the burst amplitude to determine the endonuclease active site concentration we calculate that 0.5 mol of substrate was converted to product in the burst per mol of enzyme (concentration based on our equilibrium binding data below). In addition, burst and steady-state rates of 21 ± 3 h−1 and 2.1 ± 0.1 h−1, respectively, were determined for the endonuclease reaction under these conditions.

Equilibrium binding experiments were performed by separation of free from bound capped IV using a nitrocellulose filter retention assay. The equilibrium titration results are shown in Fig. 5B. The data were consistent with a single class of noninteracting binding sites. Nonlinear least-squares fitting returned values of KD = 170 pM and a saturation amplitude of 52 pm. Only 10% of the bound 32P-capped IV was competed by up to 150 nM uncapped IV. In contrast, 95% was displaced by competition with unlabeled capped IV. Thus, the binding to the endonuclease was dependent on the presence of a 5'-cap and reflected direct binding to the endonuclease active site. The effective endonuclease active site concentration was derived from the value of the saturation amplitude.
nases catalyze the cleavage of the phosphodiester bond between $-\text{OH}$ at the cleavage site is important for either incorporation of nucleoside analogs.

1-containing RNA molecules, which allowed for the site-specific development for the synthesis of short, sequence-specific cap.

influenza endonuclease. To carry out this study, a method was

FIG. 4. Reaction progress curve for the cleavage of capped I by influenza endonuclease. Reactions were carried out as described under "Experimental Procedures." A, protein concentrations were 2.0 (●), 1.6 (□), and 1.2 (▲) μg/ml. B, the reaction was quenched using the standard protocol before being loaded on the gel (●). Alternatively, aliquots were filtered through a nitrocellulose membrane to remove enzyme and RNA-associated enzyme before being loaded (□). The data were fit to Equation 1.

DISCUSSION

The aim of this work was to elucidate the mechanism of influenza endonuclease. To carry out this study, a method was developed for the synthesis of short, sequence-specific cap 1-containing RNA molecules, which allowed for the site-specific incorporation of nucleoside analogs.

The data presented for the cleavage of capped II indicate that the $2^\prime$-OH at the cleavage site is important for either recognition or catalysis (Fig. 3). A large number of ribonucleases catalyze the cleavage of the phosphodiester bond between the $3^\prime$ and $5^\prime$ riboses of adjacent nucleosides by using the ribose $2^\prime$-OH as the nucleophile. This results in the formation of a $2^\prime,3^\prime$-cyclic phosphate-terminated RNA, which slowly hydrolyzes to the $3^\prime$-phosphate (21). In contrast, the final product of the influenza endonuclease reaction is an RNA with a free $3^\prime$-OH. This species is the required end product because it is utilized as a primer for the subsequent transcriptase reaction (10). In addition, the data presented in Fig. 2 indicate that the phosphate remains with the six-nucleotide $3^\prime$-cleavage fragment of I. Thus, the influenza endonuclease carries out the nucleophilic attack of the internucleotidic linkage with a group other than the $2^\prime$-OH, with the concomitant cleavage of the $3^\prime$-O-phosphorous bond. This is analogous to the mechanism proposed for the cleavage of RNA in DNA-RNA duplexes by Escherichia coli ribonuclease H (22). When the endonuclease was incubated with another analog of capped I, where the nucleoside at the site of cleavage was replaced with a $2^\prime$-fluoro-modified ribose, no cleavage at this position was observed. In contrast, RNaseH is able to hydrolyze $2^\prime$-fluoro-substituted RNA, albeit at a reduced rate (22). This suggests that the $2^\prime$-OH is required for influenza endonuclease, whereas it is not likely to be utilized as the nucleophile at the site of cleavage.

The cleavage of capped 19-base I yielded a biphasic reaction progress curve. Fig. 4A illustrates that there was a burst in the conversion of substrate to product at a rate of 21 h$^{-1}$, followed by a much slower steady-state rate of 2 h$^{-1}$. The presence of a burst was consistent with the rapid accumulation of enzyme-bound product (E-cap-13-mer) followed by a slow breakdown of E-cap-13-mer to yield free enzyme. The less than stoichiometric conversion of substrate to product per enzyme determined from the burst amplitude was consistent with the presence of some ribonucleoprotein that was able to bind capped IV but was inactive catalytically.

Previous studies have shown that influenza endonuclease was able to bind but was unable to cleave capped RNA when divalent metal ion was absent from the reaction (23). The progress curves for reactions initiated with capped I or Mg$^{2+}$ were compared to determine whether productive binding of the capped RNA I was rate-determining for the burst part of the reaction. No significant difference in the reaction progress curves was observed, indicating that the rate of substrate binding does not significantly affect the rate of the burst phase. Thus, the rate-limiting step during the burst phase might be due to a catalytic event or a rate-determining conformational change.

The linear steady-state rate observed in the biphasic reaction progress curve (Fig. 4A) was consistent with the slow release of E-cap-13-base to yield free enzyme. In order to con-
firm this, a method was developed that removed all the E-capped 13-base at various time points during a reaction by filtering an aliquot through a nitrocellulose membrane. If release of the enzyme-bound product was rate-limiting, then the data would originate near the origin and linearly parallel the data obtained for the reaction with the burst. The results from this experiment (Fig. 4B) clearly show that the data from the two methods parallel each other. The slow phase of the reaction with the rate of 2 h⁻¹ was consistent with the dissociation of the capped 13-base product from the enzyme. It is reasonable that the product dissociates slowly from the enzyme since it is the substrate for the ensuing transcriptase reaction. Also, it would be deleterious to the virus if the endonuclease efficiently catalyzed the cleavage of all the host cellular mRNA molecules, thus killing the cell.

The slow release of the capped RNA product is consistent with the tight binding and potent inhibition by the product-analog, capped IV (Fig. 5). In fact, a recent report suggested that a mixture of short length capped RNAs inhibited the RNA polymerase activity of influenza ribonucleaseprotein (23). The capped 10-base IV that was selected for the current study was reported by Plotch and co-workers (10) to be the shortest influenza endonuclease-derived fragment from the cleavage of brome mosaic virus RNA 4. The tight binding of this product-analog also allowed for the determination of enzyme concentration and turnover number. It will be interesting to determine if shorter or linker-substituted capped RNA analogs also exhibit tight binding characteristics. Such compounds might prove useful as specific antiviral agents.

In summary, basic aspects of the influenza endonuclease reaction have now been elucidated with the use of chemically defined capped RNAs. In addition to establishing the bond hydrolyzed at the site of cleavage, endonuclease-catalyzed turnover was quantitatively determined.

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Elucidation of Basic Mechanistic and Kinetic Properties of Influenza Endonuclease Using Chemically Synthesized RNAs
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