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Biochemistry

Biochemical studies on capped RNA primers identify a class of oligonucleotide inhibitors of the influenza virus RNA polymerase

(RNA chain length requirements/capped oligonucleotide inhibitors)

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ABSTRACT **A synthetic 67-nt RNA substrate, containing a ^{32}P-labeled cap-1 structure ($m^7G^{32}pppGm$) was specifically cleaved by the influenza virus RNA polymerase (EC 2.7.7.48) to yield a single capped 11-nt fragment capable of directly priming transcription. An analysis of systematic truncations of this RNA substrate demonstrated that an additional nucleotide beyond this cleavage site was required for cleavage. The minimal RNA chain length required for priming activity was found to be 9 nt, while in contrast an RNA chain length of at least 4 nt was required for efficient binding to the viral polymerase. On the basis of these chain length requirements we show that a pool of capped oligonucleotides too short to prime transcription, but long enough to bind with high affinity to the viral polymerase, are potent inhibitors of cap-dependent transcription *in vitro*.**

Influenza A virus is a segmented, negative-strand RNA virus which encodes its own RNA-dependent RNA polymerase (EC 2.7.7.48). The polymerase exists as a complex of three proteins (PB1, PB2, and PA) at the 3′ termini of nucleoprotein (NP)-encapsidated viral genome segments. The first step of viral replication, transcription of mRNA from genome virion RNA (vRNA), is initiated by a "scavenging" of nascent host cell mRNA transcripts by the influenza virus RNA polymerase (VPC). These cellular transcripts possess a "cap-1" structure ($m^7GpppNm$) at their 5′ ends and are bound and then cleaved by the VPC 9–15 nt from their 5′ ends, preferentially after purine residues, to yield primers for viral transcription (1, 2). This overall process produces chimeric viral mRNAs that contain host-derived heterogenous sequences at their 5′ ends (3, 4).

The endonuclease reaction has been studied *in vitro* by incubating virus or viral cores with eukaryotic mRNAs (5–9). Endonuclease activity of these mRNAs was strictly dependent upon the presence of the 7-methyl group on the guanyl cap (m^7G) but could be further stimulated by additional *O*-methylation of the ribosyl 2′-hydroxyl group (2, 10). In the absence of added rNTPs or Mg^{2+}, the binding step can be studied independently from other steps of transcription *in vitro*. A cosedimentation analysis (2) of cap-1 alfalfa mosaic virus (AlMV) RNA with viral cores through glycerol gradients demonstrated that at least the 5′ cap and 7 additional nucleotides ($m^7GpppGmUUUUUAp$) were required for high-affinity binding. Neither a cap-1 nucleotide ($m^7GpppGm$) nor a dinucleotide ($m^7GpppGmUp$) cosedimented efficiently.

The minimal chain length requirements for the binding, cleavage, and elongation of mRNA substrates by the VPC were further analyzed through the use of a systematic set of 3′ truncations of a capped 67-nt synthetic RNA substrate. Minimal RNA chain lengths were determined for each of the above functions of the VPC. Interestingly, a window exists between the minimal chain length required for efficient binding to the VPC and that needed for use as a primer. Those capped RNAs that are too short to prime transcription, but are long enough for high-affinity binding to the VPC, are shown to be potent inhibitors of cap-dependent transcription *in vitro*.

MATERIALS AND METHODS

Purification of Viral Cores. Influenza A/PR/8 virus (H1N1) was grown in the allantoic sacs of 10-day-old embryonated eggs and isolated by differential centrifugation (11). Viral cores were prepared from 2–3 mg of whole virus by standard disruption methods as described (9, 12, 13).

Generation of Endonuclease Substrates. Full-length RNA transcripts (67 nt) were prepared by *in vitro* run-off transcription (13) from *Sma* I-digested pGEM-7Zf(+) plasmid DNA (Promega), using SP6 RNA polymerase. The 5′-triphosphorylated ends of the RNA transcripts were converted to ^{32}P-radiolabeled cap-1 ($m^7G^{32}pppGm$) structures by using vaccinia virus capping and methylating enzymes (10, 14–17), *S*-adenosylmethionine, and [α-^{32}P]GTP. Capped transcripts were further purified by electrophoresis in 20% polyacrylamide gels containing 7 M urea (9), eluted by soaking overnight at 37°C in 0.75 M ammonium acetate containing 0.1 mM EDTA, 0.1% SDS, and RNase-free *Escherichia coli* tRNA (18) at 10 μg/ml, extracted with phenol/chloroform, and precipitated with ethanol. A panel of 3′ truncations was generated by partial digestion of the 67-nt capped substrate with PhyM, T1, or CL3 RNases (United States Biochemical), followed by gel purification as described above. These 3′-phosphate-terminated fragments were partially converted to their 3′-hydroxyl (3′-OH)-terminated counterparts by the reverse reaction of T4 polynucleotide kinase (Promega). The 5′ caps were removed from some RNAs (19) by β-elimination or with tobacco acid pyrophosphatase (TAP; Epicentre Technologies, Madison, WI).

Viral Endonuclease Reaction Conditions. The capped [^{32}P]RNAs were incubated with purified viral cores for 45 min at 31°C, in a reaction volume of 5 μl containing 200 ng of viral cores as described (9), and analyzed by electrophoresis on 7 M urea/20% polyacrylamide gels. For reactions that elongate the primer fragments, one or more of the rNTPs were included during the cleavage reactions or added as a chase after the initial cleavage.

***In Vitro* RNA Transcription and Capped RNA Binding Assays.** *In vitro* transcription reactions were done as described (9). Binding of capped RNAs was analyzed by a centrifugation method similar to that previously described (2). After binding, the reaction mix was centrifuged in a Beckman Airfuge for 90 min at 90,000 rpm through a 150-μl

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Abbreviations: VPC, influenza virus RNA polymerase; vRNA, virion RNA; AlMV, alfalfa mosaic virus; TAP, tobacco acid pyrophosphatase.

cushion of 10% (vol/vol) glycerol/10 mM Tris·HCl, pH 7.8/100 mM NaCl/1 mM EDTA. The complexes in the pellet were resuspended in appropriate buffers for liquid scintillation counting or electrophoretic analysis.

RESULTS

Cleavage and Elongation of Capped Synthetic Substrate. In the absence of nucleoside triphosphates (NTPs), a 5′-^{32}P-radiolabeled capped 67-nt RNA was specifically cleaved by viral cores to a single 3′-OH-terminated capped 11-nt fragment (Fig. 1, lane 2; see Table 1 legend). Cleavage was dependent on Mg^{2+} and was linear for about 1 hr. Occasionally, additional minor, nonspecific, cleavage products were detected. Their formation is not Mg^{2+} dependent, and they are found in the absence of viral cores (Fig. 1, lane 1). They are attributed to general RNA hydrolysis of labile sites of this transcript (20) or to the presence of contaminating host-derived nucleases.

Incubation of the capped 67-nt transcript with viral cores in the presence of one or several NTPs (Fig. 1, lanes 3–8) resulted in more slowly migrating products, depending on the NTP added. A single CMP was quantitatively added to the G11-OH product (lane 3), whereas 1–3 GMPs were efficiently added (lane 4). Neither AMP nor UMP alone could be incorporated by the VPC (lanes 5 and 6). These data are consistent with previous results involving nucleotide additions to G-terminated primer fragments which prime off the second or third base from the 3′ end of the vRNA (3′-UCG; Table 1 legend), whereas A-terminated primers can prime off only the penultimate base (9, 21). The additional G residues may be added nonspecifically (lane 4) and could be related to the proofreading property associated with the VPC (21). Addition of only ATP, CTP, and GTP (3NTPs) blocks transcription from each of the eight viral genome segments at the first virally encoded UMP incorporation, resulting in elongation of the G11-OH cleavage product by 10 (genes *PB2*, *PA*, and *M*), 11 (genes *NP* and *NS*), 12 (gene *NA*), 16 (gene *HA*), or 17 (gene *PB1*) nucleotides if priming occurred off the third G residue of the viral template (3, 22, 23). In the presence of all four nucleotides these limited elongation products chase into a band trailing from the gel origin (not shown) consistent with elongation to longer transcripts. Elongation was not quantitative, as significant amounts of a cleavage band elongated by one nucleotide remained even in the presence of 3 or 4 NTPs (lanes 7 and 8). This may result from the partitioning between premature termination ("abortive initiation") and productive elongation, as has been noted for influenza (24) and other (25) RNA polymerases.

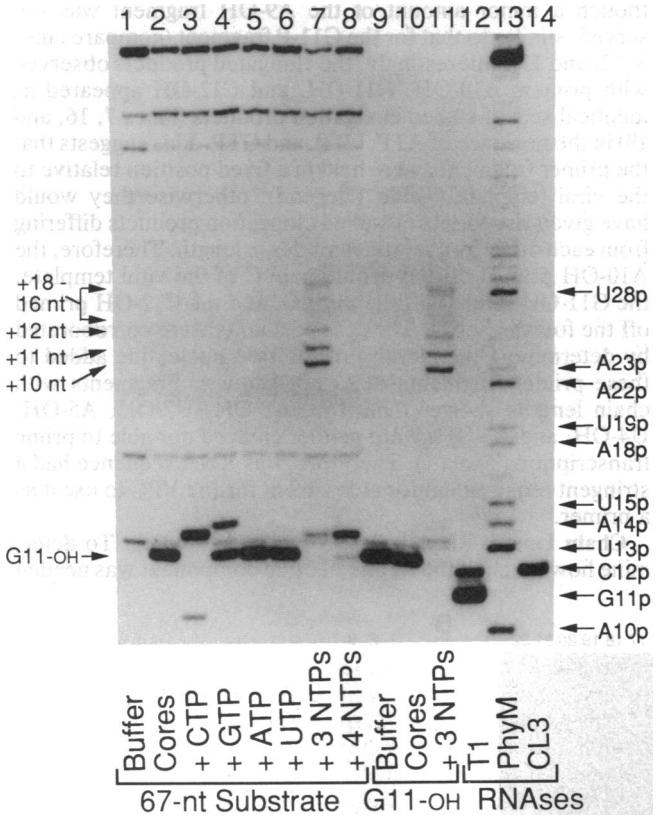


FIG. 1. Priming by the capped 67-nt substrate and its cleavage product. Viral cores were incubated with the 67-nt substrate (m^7G^{32}pppGmAAUACUCAAGCUAU$(N)_{52}$-OH) without (lane 2) or with one or more nucleoside triphosphates (NTPs) (lanes 3–8). The gel-purified G11-OH product (m^7G^{32}pppGmAAUACUCAAG-OH) was similarly incubated (lanes 9–11). Elongation of the G11-OH product in the presence of ATP, CTP, and GTP (3 NTPs, lanes 7 and 11) by 10–18 nt is indicated. Digestion products of the capped 67-nt substrate by RNase T1, PhyM, or CL3 (lanes 12–14) are used as markers. The additional negative charge conferred by the 3′-phosphate causes the 3′-phosphorylated fragments to migrate faster than their corresponding 3′-OH fragments. The G11-OH fragment migrates only slightly ahead of the 3′-phosphorylated U13 fragment marker (U13p) and almost comigrates with the C12p fragment marker. The cleavage product was identified by comparison of its migration with specific dephosphorylated fragments generated from the capped 67-nt substrate.

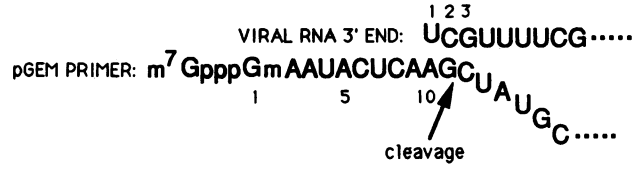
The gel-purified G11-OH cleavage product was not cleaved further (Fig. 1, lane 10); however, it primed transcription with an efficiency similar to that obtained with the full-length substrate (compare lanes 7 and 11). Thus, cleavage and elongation of this synthetic substrate could be uncoupled, as observed previously for other substrates *in vitro* (5, 26). These results indicated that the G11-OH cleavage product functioned as an authentic primer for viral transcription.

Chain Length Requirements for Endonucleolytic Cleavage of RNA Substrates. 5′-Radiolabeled cap-1-containing fragments terminating in 3′-phosphates were isolated by gel purification following limited RNase digestions of the radiolabeled capped 67-nt substrate. Portions of these fragments were partially dephosphorylated and further gel purified. These capped fragments were used to determine the shortest RNA fragment that could be cleaved by the VPC. All of the capped substrates containing RNA chains of more than 13 nt were

Table 1. Activities of substrates in cleavage and elongation reactions

Substrate	Cleavage	Elongation
m^7GpppGmAAUACUCAAGCUAUGp	+	+
m^7GpppGmAAUACUCAAGCUAUp	+	+
m^7GpppGmAAUACUCAAGCUAp	+	+
m^7GpppGmAAUACUCAAGCUp	+	+
m^7GpppGmAAUACUCAAGCp	−	−
m^7GpppGmAAUACUCAAGC	−	+
m^7GpppGmAAUACUCAAGp	−	−
m^7GpppGmAAUACUCAAG	−	+
m^7GpppGmAAUACUCAAp	−	−
m^7GpppGmAAUACUCAA	−	+
m^7GpppGmAAUACUCA	−	±
m^7GpppGmAAUACUC	−	−
m^7GpppGmAAUACU	−	−
m^7GpppGmAAUACUCAAGGp	+	+

± denotes inefficient elongation. The structure is



cleaved to the G11-OH primer fragment (data not shown). Cleavage results for capped fragments with chains of 13 or fewer nucleotides are presented in Fig. 2. Capped fragments with an RNA chain of less than 13 nt did not yield a significant amount of additional cleavage products (lanes 2–15, compare ± core lanes), although a minor amount of G11-P fragment was cleaved to the A9-OH fragment. Since little if any dephosphorylation of the A10-P, G11-P, and C12-P is observed (Fig. 2*A*, lanes 9 and 12; Fig. 2*B*, lane 2), the VPC does not appear to have an active 3′-phosphatase, but it must directly cleave on the 5′ side of a phosphodiester bond to yield a 3′-OH. This is similar to nuclease P1 but different from RNases T1, T2, PhyM, and CL3. Fragments with chain lengths shorter than 9 nucleotides (C8-OH, U7-OH, A5-OH, U4-OH, and A3-OH) were not cleaved (data not shown). However, the 13-base chain U13-P fragment (containing a 3′-phosphate) was cleaved to the G11-OH primer as observed for longer substrates (lane 17 and 18). Whether the purified C12-P fragment was cleaved to the G11-OH product could not be determined, since these two species nearly comigrate. However, minor amounts of cleavage products could be observed which correspond to (see below) A10-OH and A9-OH (Fig. 2*B*, lane 2). When the 3′-phosphate is removed (C12-OH), this fragment is not cleaved (Fig. 2*A*, lanes 14 and 15), but it acts directly as a primer (see below). Therefore, the presence of 2 nt past the G11 cleavage site was apparently required to yield a cleavable substrate.

To verify that this 2-nt minimum required for a cleavable substrate was a general feature, a site-specific C → G mutation was created at position 12 (Table 1). This gel-purified, capped G12-OH fragment was specifically cleaved to the G11-OH primer (not shown). Therefore the VPC cannot cleave a 3′-phosphate but requires a minimum of 1 nt past the G11 cleavage site for cleavage to occur (summarized in Table 1).

Chain Length Requirements for Priming by RNA Substrates. All of the capped substrates with RNA chains of 13 nt or more primed transcription, since they generate the G11-OH primer fragment (data shown only for U13-P fragment in Fig. 2*A*, lane 19). The A10-P, G11-P, or C12-P fragments were poorly elongated by three NTPs (Fig. 2*A*, lanes 10 and 13; Fig. 2*B*, lane 3). The lack of elongation of these fragments, which are not cleaved by the VPC and terminate in a 3′-phosphate, is consistent with the general requirement of RNA polymerases for 3′-OH termini on the growing ends of polynucleotide transcripts or primers. As these fragments could not be utilized through dephosphorylation by the VPC, they were dephosphorylated as described above to ascertain the chain length requirements for priming activity. The A10-OH segment was not cleaved, but it directly primed elongation (Fig. 2*A*, lanes 5–7) off the penultimate C residue of the viral template, since the first nucleotide added to this primer was a G (data not shown). The A9-OH fragment promoted synthesis of an abortive elongation product or primed transcription very inefficiently, as most of the product is only 2 nt longer (Fig. 2*A*, lanes 2–4).

A small amount of elongation was observed with the C12-P fragment with three NTPs (Fig. 2*B*, lane 3). This elongation appears to originate from the minor amounts of A10-OH fragment generated, rather than the A9-OH fragment which is inefficiently elongated as noted above. This suggests that little or no cleavage of the C12-P fragment to the G11-OH fragment occurred, since this latter oligonucleotide should efficiently prime transcription.

When the 3′-phosphate was removed to generate a C12-OH fragment, this capped oligonucleotide was able to prime transcription without cleavage (Fig. 2*A*, lanes 14–16). No G11-OH or A10-OH accumulated in the absence of NTPs, though a minor amount of the A9-OH fragment was observed, similar to that for the G11-P fragment (compare lanes 3, 12, and 15). Interestingly, the elongated products observed with primers A10-OH, G11-OH, and C12-OH appeared as identical sets of stalled elongation products (lanes 7, 16, and 19) in the presence of ATP, CTP, and GTP. This suggests that the primer fragments were held in a fixed position relative to the viral template (Table 1 legend), otherwise they would have given rise to sets of stalled elongation products differing from each other by a few nucleotides in length. Therefore, the A10-OH primed off the penultimate C of the viral template, the G11-OH primed off the third G, and the C12-OH primed off the fourth C or U. These assignments were corroborated by determining the identity of the first nucleotide added to these primer fragments (data not shown). Fragments with chain lengths shorter than 9 nt (C8-OH, U7-OH, A5-OH, U4-OH, and A3-OH) were neither cleaved nor able to prime transcription (Table 1). Therefore, this RNA sequence had a stringent requirement for at least 9 nt for the VPC to use it as a primer.

Chain Length Requirement for mRNA Binding. To determine how much of the ribonucleotide component was needed

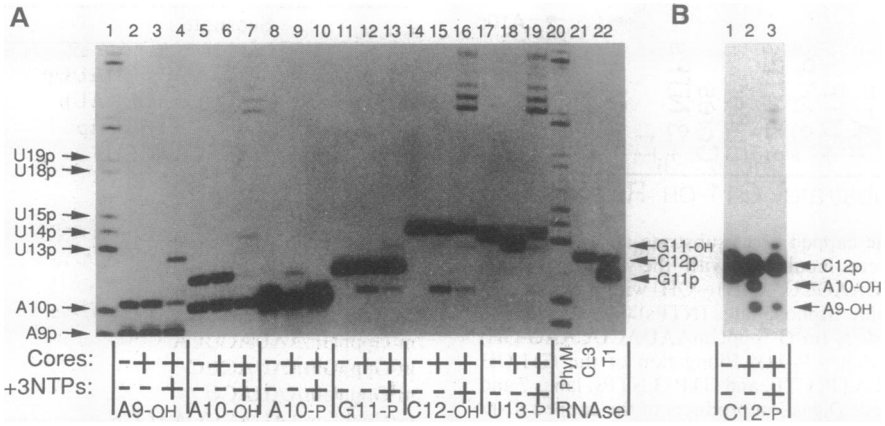


FIG. 2. Chain length requirements for cleavage and priming activity of the RNA substrate. (*A*) 5′-Radiolabeled and capped fragments were incubated with or without viral cores in the absence or presence of ATP, CTP, and GTP (3NTPs) as indicated. Since recovery from gels and dephosphorylation of capped oligonucleotides with RNA chains of less than 11 nt was inefficient, these fragments contained mixtures of phosphorylated (faster-migrating) and dephosphorylated (slower-migrating) oligonucleotides (lanes 2–7). For the A9-OH and A10-OH fragments (lanes 2–7), the partial dephosphorylation reaction products were used directly in elongation reactions. The C12-OH fragment (gel purified and recovered in sufficient quantity) appears as a single band (lanes 14–16). Sequencing ladders from partial RNase digests of the capped 67-nt substrate with PhyM (lanes 1 and 20) are indicated. (*B*) Similar incubation of the 5′-radiolabeled capped C12-P fragment. Positions of secondary cleavage fragments are indicated.

for capped RNA binding, a ladder of capped oligomers (Fig. 3, lane 1) was obtained from partial PhyM RNase digestion of the 5′-radiolabeled cap-1 67-nt transcript. The smallest species that bound to the VPC was the m^7G cap plus 4 nt (lane 2). Capped RNA species larger than this also bind, though there appears to be less binding of RNA chains longer than 14 nt (compare lanes 1 and 2). The same chain length limit for binding was obtained in both the absence (lane 2) and the presence (lane 3) of 5 mM Mg^{2+}, so binding does not require Mg^{2+}. All of the larger species were cleaved to the capped G11 product in the presence of Mg^{2+} (arrow in Fig. 3), while shorter species were not affected. Binding of all capped species was effectively blocked by competition with nonradiolabeled globin mRNA (lane 4). Taken together with the cleavage/elongation data above, these results show that short capped oligomers with RNA chains of more than 4 but less than 9 nt attached to a capping m^7G can bind to the VPC but cannot be further cleaved or be elongated.

Inhibitory Activity of Short Capped Oligomers. Since short capped oligomers with RNA chains of 4–9 nt bind well to the VPC but cannot be elongated, they should be potent inhibitors of primary influenza transcription. A complete T1 RNase (Gp↓N) digest of commercially available rabbit globin mRNA (BRL; α and β chains) yields a mixture of uncapped short oligonucleotide fragments and two short capped oligomers (Fig. 4*A*, lane 2) with RNA chain lengths of 9 nt (m^7Gpppm^{6}AmCACUUCUGp) and 7 nt (m^7Gpppm^{6}AmCACUUGp), respectively (27). These major capped species are both predicted to bind well to the VPC but not be cleaved, dephosphorylated, or elongated by virtue of their short lengths and 3′-phosphates. Indeed, complete T1 digestion of globin mRNA destroys its ability to prime transcription *in vitro* (data not shown).

This oligonucleotide mixture was tested for its ability to inhibit cap-dependent transcription *in vitro* and was found to

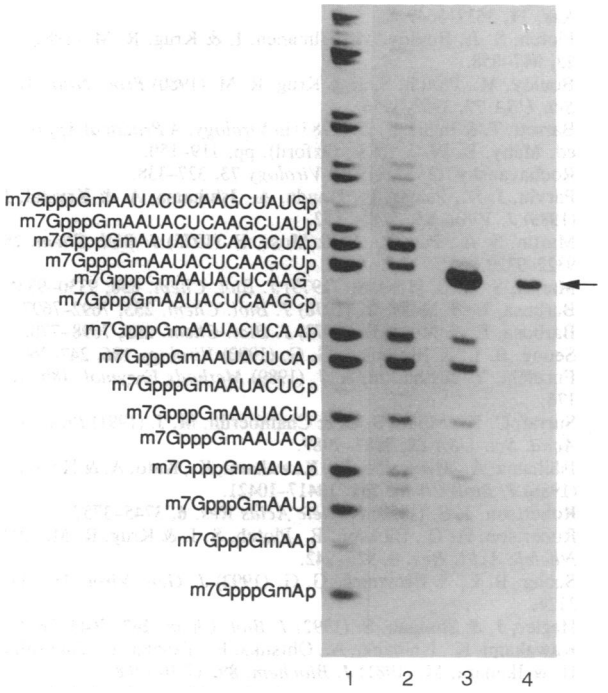


FIG. 3. Chain length requirements for capped RNA binding to VPC. The radiolabeled 67-nt RNA was partially digested with RNase PhyM (lane 1), incubated with viral cores in the presence of EDTA (lane 2), $MgCl_2$ (lane 3), or 96 nM globin mRNA (lane 4), pelleted, and analyzed. Identities of the various bands were determined by coelectrophoresis of sequencing ladders as indicated. The arrow at lane 4 indicates the G11-OH cleavage product from the capped 67-nt substrate in a standard viral core endonuclease reaction.

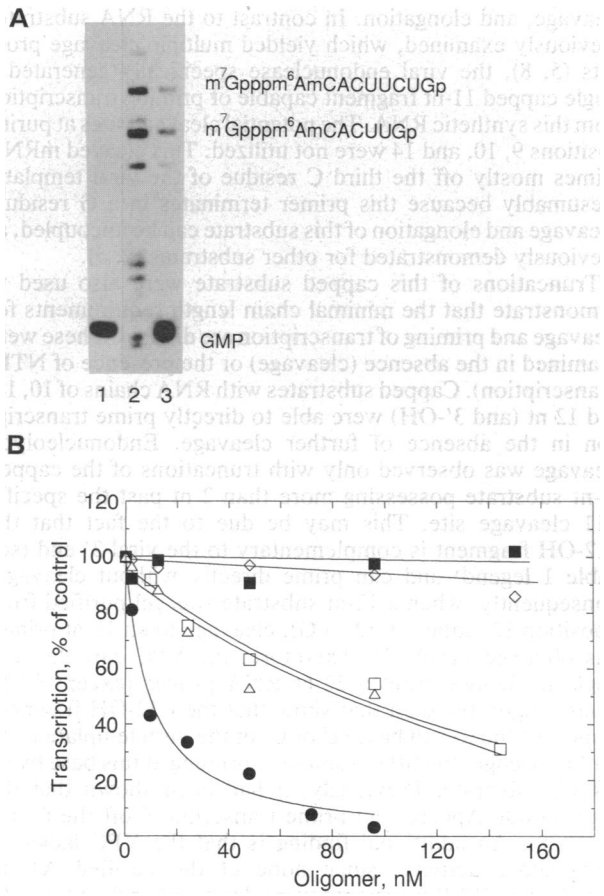


FIG. 4. (*A*) Electrophoretic analysis of T1 RNase-digested globin mRNA. Globin mRNA was decapped and recapped using [α-32P]GTP and added to monitor digestion. Lane 1, GMP standard; lane 2, T1 RNase-digested globin mRNA; lane 3, T1 RNase-treated sample after digestion with TAP. Lane 2 shows that the major products produced are the expected RNA bands with chain lengths of 7 and 9 (indicated on right). Minor products are also observed; they presumably correspond to impurities in the commercial preparations or could have resulted from chemical cleavage during β-elimination. Amount of decapping was calculated by quantitation of band radioactivity in a model 603 Betascanner (Betagen). (*B*) Inhibition of cap-dependent transcription by globin RNA fragments. Lysed virus was incubated in a standard AlMV-primed transcription mix containing rNTPs and uridine 5′-[α-[^{35}S]thio]triphosphate in the presence of T1 RNase-digested globin mRNA fragments that were fully capped (●), 65% uncapped by β-elimination (△), or 75% uncapped by treatment with TAP (□). Controls were 5S ribosomal RNA (◇), which contains no cap structure, and its T1 RNase digest (■).

be an extremely effective inhibitor of AlMV-primed influenza virus transcription (Fig. 4*B*) with an IC_{50} of 17 nM (Fig. 4*B*). When the 5′ caps were partially removed by either enzymatic (Fig. 4*A*, lane 3) or chemical methods, the potency decreased in proportion to the extent of decapping. Addition of either uncapped 5S rRNA or T1-digested 5S rRNA did not inhibit AlMV-primed transcription, showing that both full-length uncapped RNAs and a pool of shorter uncapped RNAs were not inhibitory at these concentrations. Both the 5′ cap and the attached short (4–9 nt) RNA component were required for effective inhibition of cap-dependent transcription.

DISCUSSION

Previous studies concerning the influenza virus endonuclease have centered on the use of natural mRNA substrates. In this study, 3′ truncations of a capped and fully methylated synthetic 67-nt RNA were used to systematically examine the effect of RNA chain length on the three steps of binding,

cleavage, and elongation. In contrast to the RNA substrates previously examined, which yielded multiple cleavage products (5, 8), the viral endonuclease specifically generated a single capped 11-nt fragment capable of priming transcription from this synthetic RNA. The potential cleavage sites at purine positions 9, 10, and 14 were not utilized. This cleaved mRNA primes mostly off the third C residue of the viral template, presumably because this primer terminates in a G residue. Cleavage and elongation of this substrate can be uncoupled, as previously demonstrated for other substrates (2, 5).

Truncations of this capped substrate were also used to demonstrate that the minimal chain length requirements for cleavage and priming of transcription are distinct. These were examined in the absence (cleavage) or the presence of NTPs (transcription). Capped substrates with RNA chains of 10, 11, and 12 nt (and 3′-OH) were able to directly prime transcription in the absence of further cleavage. Endonucleolytic cleavage was observed only with truncations of the capped 67-nt substrate possessing more than 2 nt past the specific G11 cleavage site. This may be due to the fact that the C12-OH fragment is complementary to the viral 3′ end (see Table 1 legend) and can prime directly without cleavage. Consequently, when a 12-nt substrate was gel purified from a position 12 mutant (C12 → G), cleavage to an 11-nt primer was obtained (Table 1). Therefore, the VPC can cleave a single nucleotide from a 12-nt RNA primer (except C12). Interestingly, the data that show that the C12-OH fragment primes off the fourth base (U or C) of the viral template is, to our knowledge, the first example of priming at this base by an mRNA substrate. Previously, it had been shown that the trinucleotide ApGpC can prime transcription off the fourth base (28). An additional finding is that the VPC lacks 3′-phosphatase activity, since none of the purified A10-P, G11-P, and C12-P fragments were dephosphorylated or efficiently elongated by the VPC.

Of the RNA substrates shorter than 11 nt, only the 9- and 10-nt substrates exhibited any priming activity, suggesting that for this primer sequence, 9 nt is the minimum length required for activity (Fig. 2). However, the 9-nt primer exhibited only weak priming ability and produced mostly abortive transcripts corresponding to addition of only 2 nt. The length requirement obtained for priming by this substrate is similar to the 9- to 15-nt heterogenous sequences transferred to viral mRNA previously observed *in vivo* (3, 4, 29–32) and *in vitro* (5, 7, 31). The inability of fragments shorter than 9 nt to prime transcription is presumably due to the lack of a sufficient length by which to span the distance between the cap-binding site and the 3′ end of the vRNA proximal to the cleavage or elongation sites.

The binding of capped RNA substrates to the VPC does not require Mg^{2+} and is therefore conveniently separated from transcription by omitting Mg^{2+} and rNTPs. To determine if the requirements for binding of capped RNA to the VPC mirrored the requirements for priming ability, a cosedimentation analysis with a panel of capped fragments was done. The VPC bound to capped oligomers with RNA chains of 4 nt or more. Capped oligomers with RNA chains of less than 4 nt do not bind with high affinity. Interestingly, binding to the shorter oligomers (<14 nt) seemed more efficient than binding to the capped RNAs with longer RNA chains (Fig. 3, compare lanes 1 and 2), perhaps due to the increased secondary structures of longer RNA substrates which may inhibit binding to the VPC. These data suggest that the VPC has a separate ribonucleotide-binding site aside from the cap-binding site and that it is upstream from the cleavage site.

The disparate RNA chain length requirements for binding to the VPC and for priming of transcription led us to postulate that a short capped oligomer could function as a competitive inhibitor of viral transcription. The RNA chain length of this prospective inhibitor should be somewhere between 4 and 9 nt. To readily generate such RNAs, a mixture of rabbit globin mRNAs (α and β chains) was digested with T1 RNase, resulting in two major species of capped oligomers of 7 and 9 nt in length (Fig. 4*A*). These oligomers also have 3′-phosphates, which could not be used by the VPC unless cleavage occurred. Indeed, this mixture is an extremely good inhibitor of viral transcription (Fig. 4*B*). The calculated IC_{50} for this mixture is approximately 17 nM, which is equimolar to the amount of AlMV primer added. The calculated IC_{90} of this mixture is 63 nM. If the 5′ cap component is removed from the RNA component, the level of inhibition is reduced in proportion to the amount of capped RNA still present in the mixture. This digested globin mixture also contains an excess of short uncapped oligomers. It is unlikely that these are inhibitory, since neither 5S RNA nor T1 RNase-digested 5S RNA exhibited much inhibition at equivalent or 1000-fold higher concentrations (Fig. 4*B* and unpublished observations). Free dinucleotide cap structures do not significantly inhibit viral transcription unless millimolar concentrations are used (10, 32), indicating that both the cap and the RNA moieties are critical for inhibition. These short capped RNAs therefore represent a class of highly efficient non-sequence-specific oligonucleotide inhibitors of viral transcription.

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