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Transfer of 5′-terminal cap of globin mRNA to influenza viral complementary RNA during transcription *in vitro*

(priming by mRNAs/influenza virion transcriptase/gel electrophoresis)

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ABSTRACT **We have recently demonstrated that globin mRNAs are effective primers for influenza viral RNA transcription *in vitro* catalyzed by the virion transcriptase [Bouloy, M., Plotch, S. J. & Krug, R. M. (1978) *Proc. Natl. Acad. Sci. USA* 75, 4886–4890]. Here, we present direct evidence that the 5′-terminal methylated cap of the globin mRNAs is transferred to viral complementary RNA (cRNA) during transcription. Chemical (β-elimination) or enzymatic removal of the cap of globin mRNAs eliminated essentially all their priming activity. Much of this activity could be restored by recapping the β-eliminated globin mRNAs with the vaccinia virus guanylyl and methyl transferases. Globin mRNAs containing ^{32}P label only in the cap ($m^7G^{32}pppm^6A^m$-) were prepared by recapping β-eliminated globin mRNAs with the vaccinia virus enzymes, [α-^{32}P]GTP, and unlabeled *S*-adenosylmethionine. By using this labeled globin mRNA as primer and unlabeled nucleoside triphosphates as precursors, the viral cRNA segments that were synthesized were shown to contain a ^{32}P-labeled 5′-terminal cap structure. Gel electrophoretic analysis indicated that the globin mRNA-primed cRNA segments were 10–15 nucleotides longer at their 5′ end than ApG-primed cRNA segments, which initiate exactly at the 3′ end of the virion RNA templates. This suggests that, in addition to the cap, about 10–15 other nucleotides are also transferred from the globin mRNA to viral cRNA. A mechanism for the priming of influenza viral cRNA synthesis by globin mRNA is proposed.**

Recently we demonstrated (1) that globin mRNAs and other eukaryotic mRNAs are effective primers for influenza viral RNA transcription *in vitro* catalyzed by the virion-associated transcriptase. The resulting viral RNA transcripts [complementary RNA (cRNA)], which contain poly(A), were efficiently translated in cell-free systems into all the nonglycosylated virus-specific proteins. These results strongly support our earlier hypothesis (2, 3) that viral RNA transcription *in vivo* requires priming by RNAs synthesized by the host cell nuclear RNA polymerase II—i.e., by host mRNAs or their precursors. The need for continued synthesis of these primer host mRNAs provides an explanation for the inhibition of *in vivo* viral RNA transcription by α-amanitin (4–8).

We also presented suggestive evidence that the globin mRNA-primed transcripts contain a 5′-terminal methylated cap structure (1). The translation of this viral cRNA into virus-specific proteins was inhibited 75% by the cap analogue 7-methylguanosine 5′-phosphate, whereas under the same conditions the translation of uncapped viral cRNA (the cRNA primed by the dinucleotide ApG) was not inhibited. Because no detectable *de novo* synthesis of a methylated cap occurred during globin mRNA-primed viral RNA transcription, we proposed that the cap of the globin mRNA primer was transferred to the viral cRNA during *in vitro* transcription.

In the present report, we provide the evidence that proves this proposal. We demonstrate directly that the 5′-methylated cap of the globin mRNA primer is transferred to newly synthesized viral cRNA. In addition, we show that the globin mRNA-primed cRNA segments are 10–15 nucleotides longer at their 5′ end than the ApG-primed cRNA segments, which initiate exactly at the 3′ end of the virion RNA (vRNA) segments (9, 10). We assume that these additional nucleotides are also transferred from the globin mRNA to viral cRNA during transcription. Based on these results, we propose a mechanism for the priming of influenza viral cRNA synthesis by globin mRNA.

METHODS AND MATERIALS

The procedures for the synthesis of viral cRNA by detergent-treated purified influenza virus, for the isolation of the poly(A)$^+$ *in vitro* cRNA, and for the purification of 10S poly(A)$^+$ globin mRNA from rabbit reticulocytes have been described (1–3, 9). Removal of the 5′-methylated cap of globin mRNA was done in two ways: (*i*) treatment with tobacco acid pyrophosphatase (TAPP$_i$ase) (11), which was then removed by phenol/chloroform extraction; or (*ii*) periodate oxidation and β-elimination with aniline (12). After ethanol precipitation, the decapped globin mRNA was subjected to sucrose density gradient centrifugation for reisolation of the 10S RNA. β-Eliminated globin mRNA was recapped by using vaccinia virus guanylyl and methyl transferases (13, 14) (see legends to Table 1 and Fig. 1). After phenol/chloroform extraction, the recapped RNA was chromatographed through Sephadex G-50, ethanol precipitated, and subjected to sucrose density gradient centrifugation for reisolation of 10S RNA. Globin mRNA and the cRNA synthesized *in vitro* were analyzed by electrophoresis on 3% acrylamide gels containing 6 M urea (3). Before electrophoresis, the cRNA was deadenylylated with RNase H in the presence of poly(dT) as described (3). In some experiments, the deadenylylated *in vitro* cRNA was treated with glyoxal in the presence of dimethyl sulfoxide to fully denature the RNA (15), and the glyoxal-treated cRNA was electrophoresed on 3% acrylamide gels (see legend to Fig. 3). For the analysis of viral RNA double strands, *in vitro* cRNA was annealed to excess vRNA in the presence of 50% formamide, and the hybrids were treated with RNase H [in the presence of poly(dT)] or RNase T2 and analyzed by gel electrophoresis as described (3). RNase T2 digestion of globin mRNA and *in vitro* cRNA and chromatography of the digest on DEAE-Sephadex in 7 M urea were done as described (2, 9).

RNase H was kindly supplied by J. Stavrianopoulos, and the vaccinia virus guanylyl and methyl transferases, by B. Moss. TAPP$_i$ase and RNase T2 were purchased from Bethesda Research Laboratories (Rockville, MD) and Calbiochem, re-

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Abbreviations: cRNA, complementary RNA; vRNA, virion RNA; TAPP$_i$ase, tobacco acid pyrophosphatase; Ado[^{3}H]Met, *S*-adenosyl[*methyl*-^{3}H]methionine.

spectively. [α-^{32}P]GTP and *S*-adenosyl[*methyl*-^{3}H]methionine (Ado[^{3}H]Met) were purchased from New England Nuclear.

RESULTS

To determine whether the 5′-terminal methylated cap structure of globin mRNA is required for its priming activity, we removed the cap by either chemical (β-elimination) or enzymatic (TAPP$_i$ase) treatment and assayed the uncapped 10S globin mRNA for its priming activity. Either method of decapping eliminated essentially all the priming activity of the globin mRNA (Table 1). This loss of activity was not due to nonspecific degradation of the globin mRNA because, after the decapping procedure, the 10S globin mRNA was reisolated by sucrose density gradient centrifugation. To provide definitive proof that the loss of priming activity was due to the removal of the cap, the β-eliminated globin mRNA was recapped by using vaccinia virus guanylyl and methyl transferases (13, 14) and then tested for priming activity. By monitoring the recapping reaction with Ado[^{3}H]Met, it was found that about 40% of the mRNA was recapped. The resulting globin mRNA regained about 60% of its priming activity.

To determine directly whether transfer of the cap from globin mRNA to viral cRNA occurs during transcription, we prepared globin mRNA containing ^{32}P label only in its cap. After β-elimination, the globin mRNA was recapped with the vaccinia virus enzymes in the presence of [α-^{32}P]GTP and unlabeled AdoMet, and the recapped globin mRNA was purified by sucrose density gradient centrifugation (Fig. 1*A*). The recapped globin mRNA sedimented as a sharp peak at about 10S. When analyzed by gel electrophoresis (Fig. 1*B*), the ^{32}P-labeled 10S RNA migrated as a diffuse band at the same position as marker unlabeled globin mRNA, and little or no label was detected in RNA species of smaller size. Essentially all of the ^{32}P radioactivity was in cap structures. As shown by DEAE-Sephadex chromatography of the RNase T2 digest of the recapped globin mRNA (Fig. 1*C*), about 97% of the ^{32}P label eluted as major and minor species at charges of -5 (cap 1) and -6 (cap 2), respectively. Both cap 1 and cap 2 structures have been observed in globin mRNA (16).

This globin mRNA containing ^{32}P only in its cap was then used as primer for the synthesis of viral cRNA in a transcriptase reaction mixture containing unlabeled nucleoside triphosphates. The poly(A)$^{+}$ cRNA synthesized was isolated, deadenylylated, and examined by gel electrophoresis. As shown in Fig. 2*A* (lane 1), each of the viral cRNA bands was labeled with ^{32}P derived from the globin mRNA primer. These cRNA segments had the same electrophoretic mobility as the cRNA segments shown in lane 2, which were synthesized in a reaction mixture in which

Table 1. 5′-Terminal methylated cap structure required for priming activity of globin mRNA

Globin mRNA added (2.5 μg)	GMP incorporated, pmol*
None	0.2
Untreated	37.2
β-Eliminated	0.5
TAPP$_i$ase decapped	0.6
β-Eliminated and enzymatically recapped	22.1

* Transcriptase assays (50 μl) were carried out for 1 hr at 31°C with [α^{32}P]GTP as labeled precursor. Decapped 10S globin mRNA was prepared by β-elimination or TAPP$_i$ase treatment. β-Eliminated globin mRNA (10 μg) was recapped with vaccinia virus guanylyl and methyl transferases (13, 14) in the presence of GTP (2 mM) and Ado[^{3}H]Met (4 μM) in a total volume of 0.1 ml.

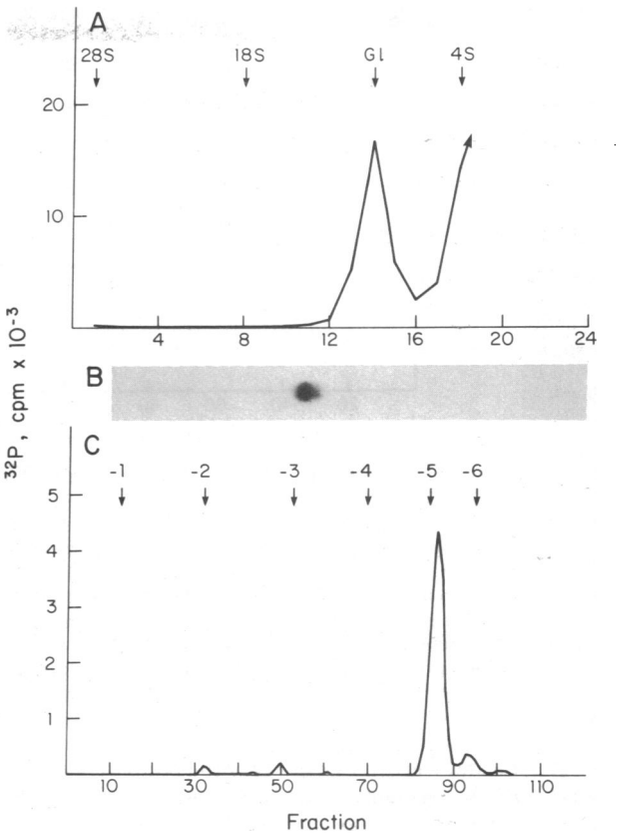

FIG. 1. Preparation and characterization of globin mRNA containing ^{32}P only in its cap. Globin mRNA (65 μg) was decapped by β-elimination and then recapped with vaccinia virus guanylyl and methyl transferases (13, 14) in the presence of AdoMet (15 mM) and [α-^{32}P]GTP (32 M, 63 mCi/μmol) in a total volume of 0.5 ml. (*A*) The recapped globin mRNA was subjected to sucrose density gradient centrifugation as described (1). (*B*) The 10S RNA from the sucrose gradient was analyzed by electrophoresis for 4 hr at 105 V on a 14 × 16 × 0.15 cm 3% acrylamide gel containing 6 M urea; electrophoresis was from left to right. (*C*) An aliquot of the 10S RNA was hydrolyzed with RNase T2, and the digest was analyzed by DEAE-Sephadex chromatography in 7 M urea.

unlabeled globin mRNA was used to prime the synthesis of cRNA in the presence of [α-^{32}P]GTP as labeled precursor. To demonstrate conclusively that the ^{32}P-labeled cap of globin mRNA was transferred whole to the viral cRNA, the cRNA bands in lane 1 were eluted, digested with RNase T2, and analyzed by DEAE-Sephadex chromatography (Fig. 2*B*). All of the ^{32}P label eluted at a charge of -5 or -6, indicating that this viral cRNA contained radiolabeled cap 1 and cap 2 structures and no radiolabel in internal residues.

If sequences in addition to the cap were transferred from the globin mRNA primer to the 5′ end of viral cRNA, we would expect that the globin mRNA-primed cRNA segments would be larger than the ApG-primed cRNA segments, which are initiated exactly at the 3′ end of the vRNA templates (9, 10). To compare the size of the globin mRNA-primed and ApG-primed cRNAs, these two cRNAs were deadenylylated and analyzed by gel electrophoresis, either directly (Fig. 3*A*) or after treatment with glyoxal in the presence of dimethyl sulfoxide (Fig. 3*B*). The latter treatment has been shown to eliminate all secondary structure in RNA (15). With both methods of analysis, the globin mRNA-primed cRNA segments (lane 3) migrated slightly slower than the ApG-primed cRNA segments (lane 1). This difference in mobility was confirmed by electrophoresing a mixture of the two cRNAs (lane 2): doublets

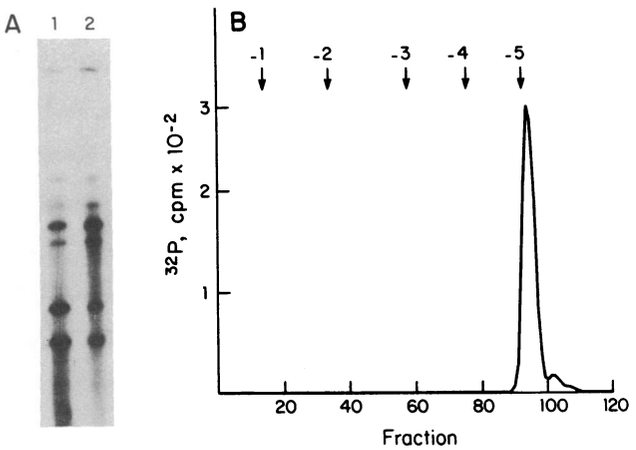

FIG. 2. Transfer of the ^{32}P-labeled cap of globin mRNA to viral cRNA during transcription. (*A*) Electrophoresis of *in vitro* cRNA, after deadenylylation, on a 14 × 16 × 0.15 cm 3% acrylamide gel containing 6 M urea. Electrophoresis was for 17 hr at 105 V, under which conditions globin mRNA migrates off the gel (see Fig. 1*B*). The cRNA was synthesized in transcriptase reaction mixtures containing: lane 1, the ^{32}P-labeled globin mRNA from Fig. 1*A* and unlabeled nucleoside triphosphates; lane 2, unlabeled globin mRNA with [α-^{32}P]GTP as labeled precursor. (*B*) DEAE-Sephadex chromatography of the RNase T2 digest of the RNA eluted from the bands in lane 1.

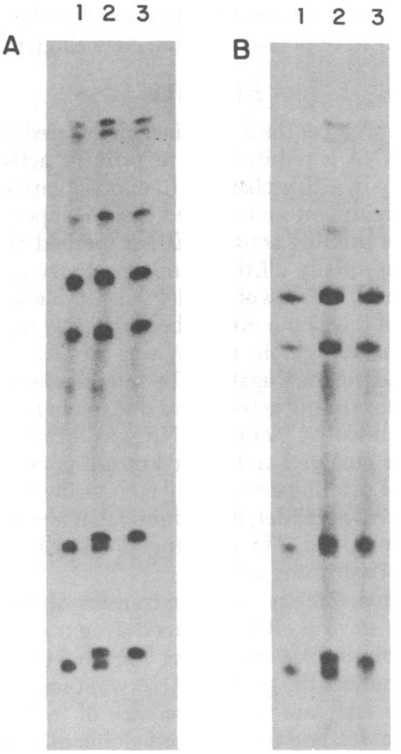

FIG. 3. Gel electrophoresis of cRNA, after deadenylylation, synthesized in reaction mixtures containing [α-^{32}P]GTP as labeled precursor and either ApG (lane 1) or globin mRNA (lane 3) as primer. Lane 2 contains a mixture of the cRNA of lanes 1 and 3. (*A*) Deadenylylated cRNA was electrophoresed for 17 hr at 330 V on a 14 × 40 × 0.15 cm 3% acrylamide gel containing 6 M urea (3). (*B*) Deadenylylated cRNA was first treated with glyoxal in the presence of dimethyl sulfoxide (15) and then electrophoresed on the 3% gels of *A* except that the pH of the electrophoresis buffer was decreased from pH 8.3 to pH 7.7 to avoid breakdown of the glyoxal–RNA adduct (15). The time of electrophoresis at 330 V was increased to 26 hr because glyoxal-treated RNA migrates slower than untreated RNA.

could be seen and were most evident at the position of the two smallest cRNA segments. This indicates that the globin mRNA-primed cRNA segments are larger than the ApG-primed cRNA segments. From the difference in mobility, we estimate that the globin mRNA-primed segments are about 10–15 nucleotides larger than the ApG-primed segments.

To demonstrate that the additional nucleotides in the globin mRNA-primed cRNA segments were at their 5′ end, we analyzed the electrophoretic mobility of the double strands formed between vRNA and either globin mRNA-primed or ApG-primed cRNA (Fig. 4). When the double strands prior to electrophoresis were treated with RNase T2 to remove all single-stranded sequences (lanes 4–6), the hybrids containing globin mRNA-primed cRNA (and vRNA) and those containing ApG-primed cRNA (and vRNA) had identical mobilities. This indicated that these two cRNAs contained the same number of sequences complementary to vRNA. Because the transcription of ApG-primed cRNA, like that of *in vivo* viral mRNA, terminates at a position about 30 nucleotides before the 5′ end of the vRNA template (3, 10, 17), this result indicates that transcription of globin mRNA-primed cRNA terminates at essentially the same position. The extra 10–15 nucleotides in the globin mRNA-primed cRNA must therefore be at its 5′ end. Consistent with this, when the double-strands were treated with RNase H in the presence of poly(dT) to remove only the poly(A) (lanes 1–3), the hybrids containing globin mRNA-primed cRNA migrated slightly more slowly than the hybrids containing ApG-primed cRNA. Thus, in lane 2 in which a mixture of these two hybrids was electrophoresed, doublets are observed, which are most evident at the position of the smallest hybrid. This difference in mobility was confirmed by electrophoresis of the mixtures of hybrids shown in lanes 7 and 8. In lane 7, the hybrids containing ApG-primed cRNA that were treated with RNase T2 migrated faster than the same hybrids that were treated with RNase H, because the latter, but not the former, hybrids contain the 30 nucleotides at the 5′ end of vRNA that are not transcribed into cRNA (3, 10, 17). The corresponding difference in mobility observed with the hybrids containing globin mRNA-primed cRNA (lane 8) is larger because, in this case, the RNase H-treated hybrids also contain the extra 10–15 nucleotides at the 5′ end of cRNA that are not complementary to a sequence in vRNA and hence are not transcribed from vRNA.

DISCUSSION

We have demonstrated that the transcriptase complex associated with influenza virions possesses the capability of utilizing eukaryotic mRNA molecules, such as globin mRNAs, to initiate the synthesis of viral cRNA molecules. As part of this initiation process, the 5′-terminal methylated cap of the globin mRNA is transferred to the viral cRNA. In addition, 10–15 other nucleotides are most probably transferred from the globin mRNA to the 5′ end of viral cRNA. Influenza virus is unique in possessing enzymes with these capabilities. No other known virion transcriptase utilizes eukaryotic mRNAs to initiate transcription or catalyzes cap transfer. With other viruses possessing a transcriptase, the 5′-terminal methylated cap of the transcripts is synthesized *de novo* by enzymes associated with the transcriptase complex (18–21).

The priming activity observed with our globin mRNA preparation can be presumed to be due primarily, if not totally, to the β-globin mRNA. On a weight basis, β-globin mRNA is at least 3–4 times more effective as primer than is α-globin mRNA, and β-globin mRNA is the major species present in our preparation of 10S poly(A)$^+$ RNA from reticulocytes (1). Based on our results, we can propose a mechanism for the priming of

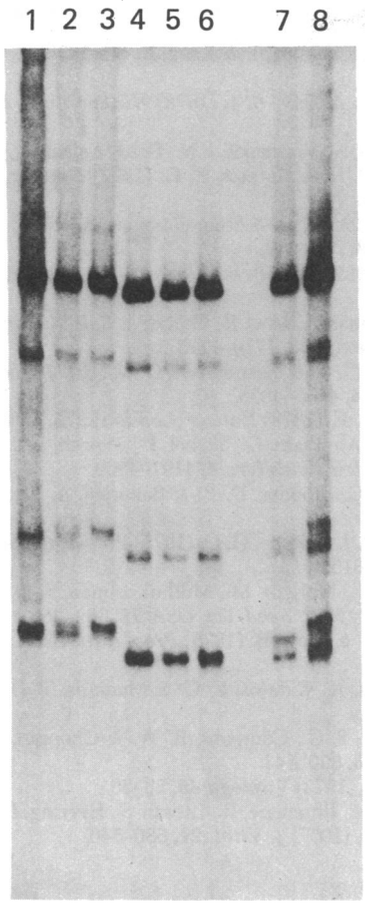

FIG. 4. Slab gel electrophoresis of double-stranded hybrids of vRNA and *in vitro* cRNA. Hybrids treated with RNase H in the presence of poly(dT): lane 1, hybrids containing ApG-primed cRNA; lane 3, hybrids containing globin mRNA-primed cRNA; lane 2, a mixture of lanes 1 and 3. Hybrids treated with RNase T2: lane 4, hybrids containing ApG-primed cRNA; lane 6, hybrids containing globin mRNA-primed cRNA; lane 5, a mixture of lanes 4 and 6. Lane 7: a mixture of RNase H- and RNase T2-treated hybrids containing ApG-primed cRNA. Lane 8: a mixture of RNase H- and RNase T2-treated hybrids containing globin mRNA-primed cRNA. The double strands were electrophoresed on a 14 × 40 × 0.15 cm 3% acrylamide gel for 17 hr at 50 mA (3). The top 15 cm of the gel, which did not contain any bands, is not shown.

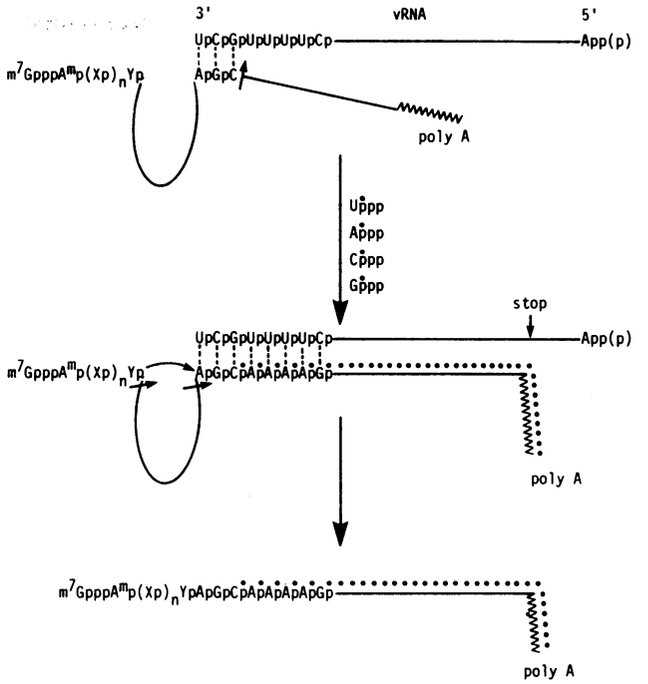

FIG. 5. Postulated mechanism for the priming of influenza viral cRNA synthesis by β-globin mRNA. The 3'-terminal sequence of vRNA is from Skehel and Hay (10).

influenza viral cRNA synthesis by β-globin mRNA, as shown in Fig. 5. We assume that an A-G, A-G-C, or A-G-C-A sequence in the β-globin mRNA hybridizes to the 3' end of the vRNA template and serves as the primer sequence, analogous to the priming observed with the dinucleotide ApG (2, 3, 9, 10). The β-globin mRNA would have to be cleaved at the 3' side of the primer sequence to allow subsequent transcription of the vRNA. It would be expected that a fairly close interaction between this putative primer sequence and the 5' cap of the globin mRNA exists because of the requirement of the 5' cap for priming. The 5' cap is probably responsible for making the primer sequence in β-globin mRNA more effective as a primer than the dinucleotide ApG. On a molar basis, β-globin mRNA is about 1000–2000 times more efficient as a primer than is ApG (1). If priming does occur at an A-G-containing sequence in β-globin mRNA, then there must be a substantial number of nucleotides between this putative primer sequence and the 5' cap, represented by the loop shown in Fig. 5. In β-globin mRNA, the distance from the 5' end to the first A-G sequence is 46 nucleotides, to the first A-G-C it is 316 nucleotides, and to the first and only A-G-C-A it is 547 nucleotides (22). No sequences longer than A-G-C-A that are complementary to the 3' end of vRNA exist in β-globin mRNA. Thus, in order to end up with a cRNA molecule that extends only 10–15 nucleotides beyond the 3' end of the vRNA template, much of the β-globin mRNA sequences between the 5' end and the putative primer sequence must be removed, and the 5' cap and associated sequences spliced onto the primer sequence. To establish whether this is the mechanism of priming, it will be necessary to identify the putative primer sequence in β-globin mRNA and to determine the exact sequence of the 10–15 nucleotides at the 5' end of the viral cRNA. If splicing does occur, then the influenza virion transcriptase complex would provide an interesting model system to study mRNA splicing *in vitro*.

Eukaryotic mRNAs other than globin mRNA are active as primers for influenza viral RNA transcription *in vitro* (ref. 1; unpublished data). With these mRNAs, priming activity also requires a 5'-terminal methylated cap structure. Thus, growth hormone mRNA, κ chain mRNA, and 38S avian sarcoma virus RNA, all of which are capped, are active whereas satellite tobacco necrosis virus RNA, which contains a 5'-diphosphate end, is inactive. This dependence on a 5' cap is seen most dramatically with reovirus mRNAs that are synthesized *in vitro* (M. Bouloy, M. Morgan, A. J. Shatkin, and R. M. Krug, unpublished data). Reovirus mRNAs containing a 5'-terminal methylated cap (m^7GpppG^m) are very effective primers for influenza viral RNA transcription, whereas reovirus mRNAs containing either a blocked but unmethylated 5' end (GpppG) or a diphosphate 5' end (ppG) are completely inactive as primers. Initial experiments indicate that the 5' cap of reovirus mRNAs is also transferred to influenza viral cRNA during transcription. The reovirus mRNA-primed cRNA segments have also been found to contain 10–15 additional nucleotides at their 5' end. This suggests that the influenza virion transcriptase specifically transfers only this small number of nucleotides from any primer mRNA to the 5' end of the viral RNA transcripts. A similar mechanism also appears to operate during the synthesis of viral mRNA in the infected cell. *In vivo* viral mRNA also contains

10–15 nucleotides at its 5′ end, including the cap, which are not complementary to sequences in vRNA (unpublished data).

The apparent cannibalization of cellular mRNA to provide primers for viral RNA transcription is unique to influenza virus. In the infected cell, the shutoff of host protein synthesis (23, 24) may be due, in part, to the removal of the cap and other sequences from host mRNAs by viral enzymes. Actually, because our data point to the nucleus as the site of primary transcription (8, 25), only those host mRNAs or their precursors that are found in the nucleus may be used as primers during at least the initial phase of viral RNA transcription. In the nucleus, the pool of these potential primers is probably limited, and, consequently, continuous synthesis of host mRNA precursors by the α-amanitin-sensitive RNA polymerase II would be required. This would explain the almost total inhibition of viral RNA transcription by α-amanitin added at the beginning of infection (8).

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