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RNA primers and the role of host nuclear RNA polymerase II in influenza viral RNA transcription

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Influenza viral RNA transcription in the infected cell is inhibited by α-amanitin, a specific inhibitor of the host nuclear RNA polymerase II. Because viral RNA transcription *in vitro* catalysed by the virion-associated transcriptase is greatly enhanced by the addition of a primer dinucleotide, ApG or GpG, we have proposed that viral RNA transcription *in vivo* requires initiation by primer RNAs synthesized by RNA polymerase II. In addition, because we did not detect any capping and methylating enzymes in virions, we have proposed that the 5′ terminal methylated cap found on in-vivo viral messenger RNA (mRNA) is derived from the putative primer RNAs. Our recent experiments have proved these two hypotheses. Purified globin mRNAs were shown to stimulate viral RNA transcription *in vitro* very effectively. The resulting transcripts directed the synthesis of all the non-glycosylated virus-specific proteins in cell-free systems. Other eukaryotic mRNAs were also active as primers. The presence of a 5′ terminal methylated cap structure in the priming mRNA was required for its priming activity. Thus, with globin mRNA, removal of the cap eliminated essentially all of its priming activity, and much of this activity could be restored by enzymically recapping the globin mRNA. Using globin mRNA containing ^{32}P only in its cap, we demonstrated that the 5′ cap of the globin mRNA primer was physically transferred to the viral RNA transcripts during transcription. Gel electrophoretic analysis suggested that, in addition to the cap, about 10–15 other nucleotides were also transferred from the globin mRNA to the viral RNA transcripts. A mechanism for the priming of influenza viral RNA transcription by globin mRNA is proposed. Initial experiments strongly suggest that priming by capped host mRNAs also occurs during the synthesis of viral mRNA *in vivo*.

1. Background: influenza viral RNA transcription requires RNA synthesis by the host nuclear DNA-dependent RNA polymerase II

A feature distinguishing influenza virus from other non-oncogenic RNA viruses is that a host nuclear function is required for virus replication. Virus replication is inhibited by actinomycin D and α-amanitin (Barry *et al.* 1962; Barry 1964; Rott *et al.* 1965; Mahy *et al.* 1972; Rott & Scholtissek 1970), and the α-amanitin sensitivity has been shown to be due to inhibition of the host nuclear DNA-dependent RNA polymerase II (Lamb & Choppin 1977; Spooner & Barry 1977), the enzyme concerned with the synthesis of cellular heterogenous nuclear RNA, the precursor to messenger RNA (mRNA).

The RNA polymerase II function has been shown to be required for viral RNA transcription. When α-amanitin is added at the beginning of infection, no detectable virus-specific proteins are synthesized (Lamb & Choppin 1977; Spooner & Barry 1977). This drug was reported to inhibit early RNA synthesis in infected cells (Mahy *et al.* 1972; Spooner & Barry 1977), and one experiment suggested that α-amanitin inhibited transcription (Rott & Scholtissek 1970). To document the effect of α-amanitin on viral RNA transcription, we used

^{125}I-virion RNA (vRNA) and ^{32}P-complementary DNA (synthesized with oncornavirus reverse transcriptase) as hybridization probes to measure the amount of complementary RNA (cRNA, the product of transcription) and vRNA, respectively, synthesized in the absence and presence of this drug (Mark *et al.* 1979). When α-amanitin was added at the beginning of virus infection, no detectable vRNA and only extremely small amounts of cRNA (about 0.5% of normal) were synthesized. Thus, even primary transcription, the transcription catalysed by the transcriptase associated with the inoculum virus, was almost totally inhibited. The sensitivity of viral cRNA synthesis to α-amanitin disappeared rapidly, and by 1.5 h after infection addition of this drug had no effect on subsequent viral cRNA synthesis. These results strongly suggest that RNA synthesized by RNA polymerase II during the first 1.5 h of infection is required for viral RNA transcription.

Actinomycin D also inhibits viral RNA transcription, but not as effectively as α-amanitin (Taylor *et al.* 1977; Mark *et al.* 1979). When actinomycin D was added at the beginning of infection, about 10–15% of the normal amount of viral RNA transcripts continued to be synthesized. Almost all (about 90%) of this residual cRNA was found in the nucleus. These nuclear cRNA molecules were shown to be composed of polyadenylated transcripts of all of the vRNA segments for which we assayed, consistent with these transcripts being complete viral mRNA molecules. These results suggest that actinomycin D does not completely inhibit host RNA polymerase II and consequently some viral cRNA is synthesized. As actinomycin D has been shown to block the migration of newly synthesized cellular mRNA sequences from the nucleus to the cytoplasm (Levis & Penman 1977), the fact that the residual viral cRNA synthesized in the presence of actinomycin D was restricted to the nucleus points to the nucleus as the site of primary transcription.

Also consistent with primary transcription occurring in the nucleus are the results that we obtained when cycloheximide was added at the beginning of infection (Taylor *et al.* 1977; Mark *et al.* 1979). Because this drug inhibits vRNA replication (Scholtissek & Rott 1970; Pons 1973; Taylor *et al.* 1977; Mark *et al.* 1979), viral RNA transcription in the presence of cycloheximide can be presumed to be confined to primary transcription. Under these conditions, about 50% of the cRNA synthesized was found in the nucleus during the first 2 h of infection, and the nucleus always contained a larger fraction of the cRNA than in infected cells not treated with cycloheximide (Mark *et al.* 1979). In addition, our demonstration that influenza viral mRNA contains internal methylated adenosine residues (Krug *et al.* 1976) suggests that at least one step in viral mRNA synthesis occurs in the nucleus, as internal methylated adenosine residues have been found in only those mRNAs, both cellular and viral, that are synthesized in the nucleus.

In contrast to their effect *in vivo*, actinomycin D and α-amanitin do not inhibit viral RNA transcription *in vitro* catalysed by the virion-associated transcriptase (Chow & Simpson 1971; Penhoet *et al.* 1971). Thus, in an apparent paradox, these drugs inhibit the virion transcriptase when it is introduced into the cell to carry out primary transcription, but not when it is assayed *in vitro*.

Our recent experiments have resolved this apparent paradox and have identified the host nuclear RNA polymerase II function required for influenza viral RNA transcription in the infected cell. We shall first summarize the experiments that led us to a hypothesis for the role of RNA polymerase II, and shall then present the experiments which essentially proved our hypothesis.

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2. Experimental basis for our hypothesis for the role of RNA polymerase II in influenza viral RNA transcription

In order to gain an insight into the role of host RNA polymerase II in viral RNA transcription, we determined whether the virion transcriptase has the capability of synthesizing *in vitro* viral cRNA that is identical to the viral mRNA synthesized in the infected cell. We demonstrated that the virion transcriptase, in the presence of Mg^{2+}, transcribes *in vitro* each of the eight vRNA segments into cRNA segments containing polyadenylate (poly(A)), as also occurs *in vivo* (Plotch & Krug 1977, 1978). This in-vitro synthesis, however, is almost totally dependent on the addition of a specific dinucleotide primer, ApG or GpG, which is incorporated into the 5′ end of the transcripts and stimulates viral cRNA synthesis about 100-fold. Synthesis with the ApG primer was shown to initiate exactly at the 3′ end of the vRNA segments (Plotch *et al.* 1978; Skehel & Hay 1978). After enzymic removal of poly(A), the cRNA segments synthesized *in vitro* are slightly smaller than the corresponding vRNA segments because transcription terminates (and poly(A) addition occurs) at a point about 30 nucleotides before the 5′ end of the vRNA template is reached (Plotch & Krug 1978). This termination is a specific event which also occurs *in vivo* during the synthesis of viral mRNA (Hay *et al.* 1977*a*, *b*; Plotch & Krug 1978; Skehel & Hay 1978). Because the in-vitro synthesis of cRNA is almost totally dependent on the addition of a primer, we postulated that viral RNA transcription *in vivo* also requires a primer and that the in-vivo primer is an RNA synthesized by host RNA polymerase II (Plotch & Krug 1977, 1978). This would explain why α-amanitin inhibits viral RNA transcription *in vivo* but not *in vitro*.

We have also postulated that capping and methylation of viral cRNA is dependent on primer RNA. Using 5′ phosphorylated derivatives of ApG or GpG as primers, we have not detected in influenza virions any capping and methylating enzymes active on the 5′ initiated termini of cRNA synthesized *in vitro*, whether these termini possess one, two or three phosphates (Plotch & Krug 1977; Plotch *et al.* 1978). In contrast, we have shown that in-vivo viral mRNA contains 5′ terminal cap structures (Krug *et al.* 1976). We proposed several hypotheses to explain these results, one of which was that the putative primer RNA itself contains a 5′ cap structure which is transferred to the viral cRNA (Plotch *et al.* 1978).

3. Proof of our hypothesis for the role of RNA polymerase II

The identification of primer RNAs (Bouloy *et al.* 1978) arose from our experiments to determine whether cRNA synthesized *in vitro* and primed by ApG could be translated in cell-free systems. We tested a number of different cell extracts for their effect on viral RNA transcription and translation, and we observed that reticulocyte extracts were unique in being able to stimulate viral RNA transcription in the absence of ApG and that the resulting cRNA was functional as viral mRNA. These results suggested that reticulocyte extracts contain a factor, possibly an RNA primer, that stimulates the synthesis of functional viral mRNA by the virion transcriptase. To determine whether this factor was indeed an RNA primer, RNA was extracted from a reticulocyte extract and was added to a transcriptase reaction (figure 1). This RNA very effectively stimulated cRNA synthesis. Stimulation was proportional to the amount of RNA added, and at the highest level of RNA tested, the stimulation was 80-fold. Most (about 75%) of the RNA with priming activity bound to oligo(dT)-cellulose and thus

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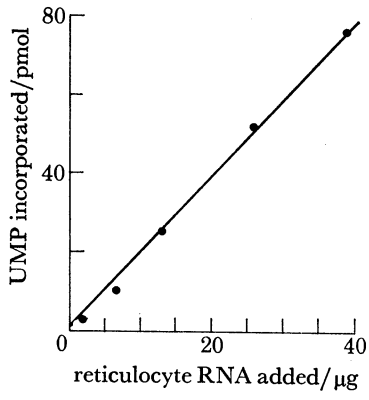

FIGURE 1

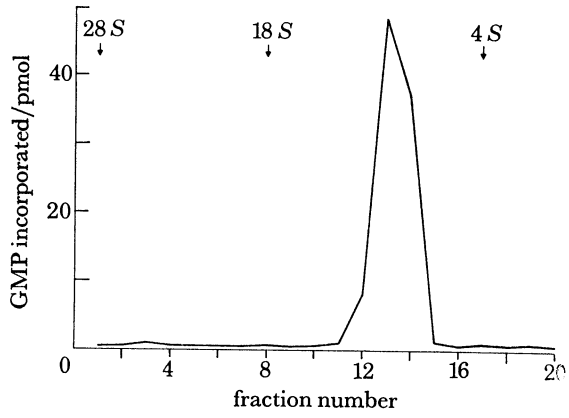

FIGURE 2

FIGURE 1. Stimulation of the influenza virion transcriptase by unfractionated reticulocyte RNA. The RNA was assayed for 1 h at 31 °C in a transcriptase reaction with [α-^{32}P]GTP as labelled precursor.

FIGURE 2. The sedimentation value of the poly(A)+ RNA from reticulocytes that stimulates viral RNA transcription *in vitro*. An aliquot of each sucrose gradient fraction was assayed for 1 h at 31 °C in a transcriptase reaction with [α-^{32}P]GTP as labelled precursor. From Bouloy *et al.* (1978).

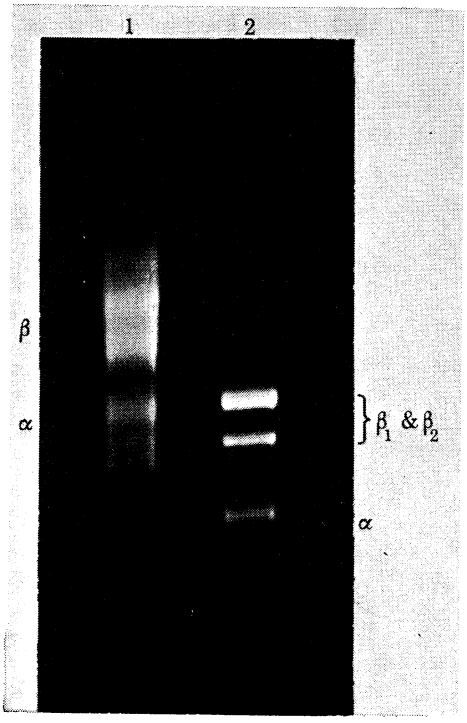

FIGURE 3

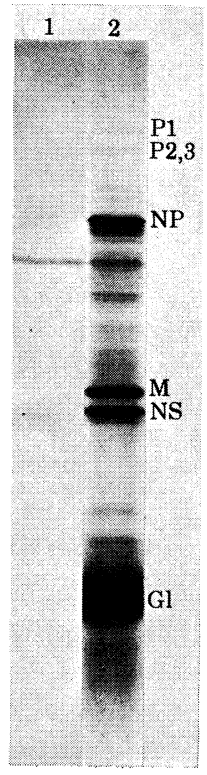

FIGURE 4

FIGURE 3. Polyacrylamide gel electrophoresis of the 10*S* poly(A)+ RNA from reticulocytes. Only the bottom 10 cm of a 40 cm gel is shown; no RNA bands were visible in the top 30 cm. From Bouloy *et al.* (1978).

FIGURE 4. Polyacrylamide gel electrophoresis of the [^{35}S]methionine labelled proteins synthesized in a micrococcal nuclease-treated cell extract with no added RNA (lane 1) and with the addition of poly(A)+ RNA isolated from a transcriptase reaction primed by globin mRNA (lane 2). The mobilities of virus-specific proteins P1, P2+3, NP (nucleocapsid protein), M (membrane protein) and NS (non-structural protein) are indicated. Gl, globin. From Bouloy *et al.* (1978).

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contained poly(A). Sucrose density gradient analysis of the poly(A) + RNA revealed that the priming RNA sedimented as a sharp peak at 10*S* (figure 2), the known sedimentation of globin mRNA. We confirmed that this 10*S* RNA was globin mRNA by translation experiments.

TABLE 1. STIMULATION OF INFLUENZA VIRAL RNA TRANSCRIPTION *IN VITRO* BY GEL-PURIFIED GLOBIN mRNAs

primer added	GMP incorporated/pmol†
none	0.6
ApG (0.4 mM)	55.7
β-globin mRNA (1.5 μg)	45.1
α-globin mRNA (1.0 μg)	9.7

† Transcriptase assays (50 μl) were carried out for 1 h at 31 °C with [α-^{32}P]GTP as labelled precursor. From Bouloy *et al.* (1978).

To verify that the priming RNA was globin mRNA, the 10*S* poly(A) + RNA was further purified by electrophoresis on polyacrylamide gels (30g/l) containing 6 M urea (Bouloy *et al.* 1978) (figure 3, lane 1). The RNA was resolved into two relatively broad bands, which were identified as β-globin mRNA and α-globin mRNA by translation experiments. After extraction from the gel, the β-globin mRNA, at a level of 1.5 μg (in a 50 μl reaction volume), stimulated viral cRNA synthesis *in vitro* about 75-fold (table 1). It should be noted that, on a molar basis, β-globin mRNA is about 1000–2000 times more effective as a primer than ApG. The eluted α-globin mRNA was about 30% as effective per microgram as β-globin mRNA. The broadness of the gel bands shown in lane 1 of figure 3 is due to the poly(A) sequences in the globin mRNAs. When the 10*S* poly(A) + RNA was enzymically deadenylylated, the β (β_1 and β_2)-globin and α-globin mRNAs were resolved into three sharp bands by gel electrophoresis (figure 3, lane 2). Each of the deadenylylated globin mRNAs was approximately as active per microgram in stimulating cRNA synthesis as their adenylylated counterparts.

A critical question arising from these results was whether the cRNA primed by globin mRNA was a functional viral mRNA. To determine this, the globin mRNA-primed cRNA was extracted and translated in a micrococcal nuclease-treated L-cell extract (Bouloy *et al.* 1978). As shown in figure 4, all of the non-glycosylated virus-specific proteins were synthesized. In addition, globin was synthesized in response to the excess poly(A) + globin mRNA that co-purified with the poly(A) + viral cRNA. Per microgram of cRNA, the globin mRNA-primed viral cRNA was translated into virus-specific proteins about three times more efficiently than the ApG-primed cRNA.

To investigate whether the globin mRNA-primed cRNA contains a 5′ terminal methylated cap structure, we determined whether the translation of this viral cRNA was inhibited by 7-methylguanosine-5′-phosphate (pm^{7}G) (Bouloy *et al.* 1978), an inhibitor of the translation of mRNAs containing 5′ methylated caps (Hickey *et al.* 1976; Weber *et al.* 1976). *S*-adenosyl-homocysteine was added to prevent de-novo methylated cap formation during translation (Both *et al.* 1975). First, we established that the translation of uncapped, ApG-primed viral cRNA was not inhibited by pm^{7}G (at 0.5 mM) (figure 5*a*). Under the same conditions, the translation of globin mRNA-primed viral cRNA into virus-specific proteins was inhibited by 75% (figure 5*b*). This level of inhibition is equal to, or greater than, that observed with

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in-vivo viral mRNA and globin mRNA, both of which contain 5′ terminal caps. These results suggest that the viral cRNA primed by globin mRNA contains a 5′ terminal methylated cap structure.

To determine whether a 5′ cap structure was synthesized *de novo* during transcription by virion-associated enzymes, viral cRNA was synthesized *in vitro* with globin mRNA as primer and [α-^{32}P]GTP and S-adenosyl[methyl-^{3}H]methionine (AdoMet) as labelled precursors (Bouloy *et al.* 1978). DEAE-Sephadex chromatography of the ribonuclease T_2 (RNase T_2)

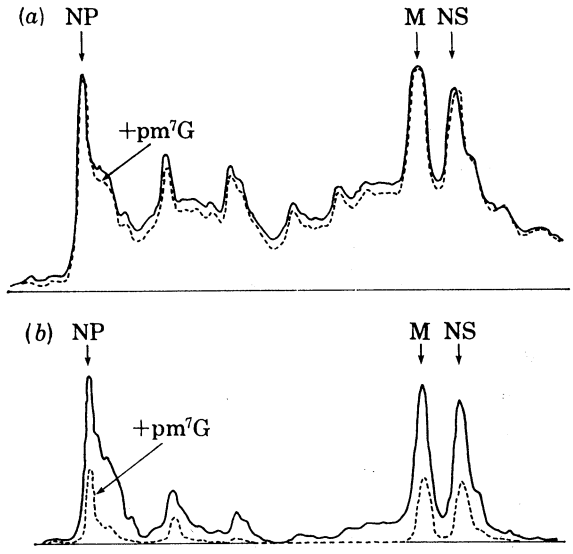

FIGURE 5. Effect of pm^{7}G on the translation of (*a*) ApG-primed cRNA and (*b*) globin mRNA-primed cRNA (bottom panel) in micrococcal nuclease-treated L-cell extracts. This is a densitometer tracing of the polyacrylamide gel of the [^{35}S]methionine labelled proteins synthesized *in vitro* (see figure 4).

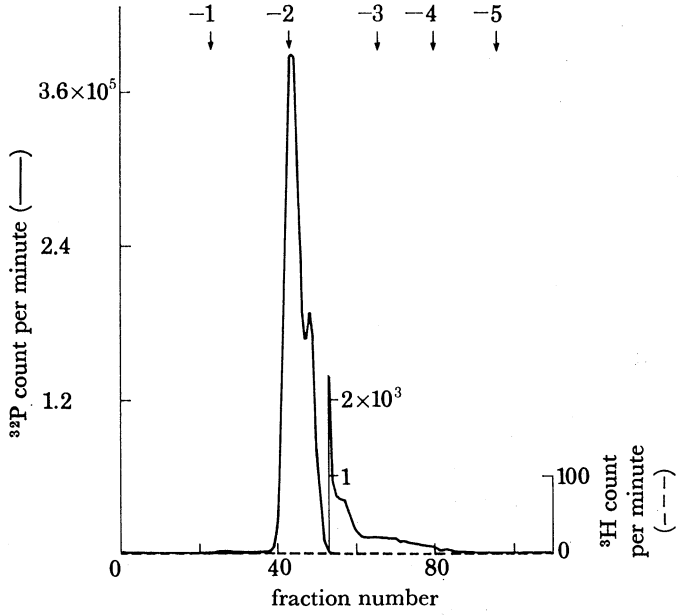

FIGURE 6. DEAE-Sephadex chromatography of the RNase T_2 digest of the poly(A)+ cRNA synthesized in the presence of globin mRNA, [α-^{32}P]GTP and Ado[methyl-^{3}H]Met. The elution position of the charge markers is indicated. The change of scale for ^{32}P at fraction 52 should be noted.

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digest of the poly(A)+ cRNA product demonstrated no ^{3}H incorporation at all and the absence of a ^{32}P labelled cap structure at a charge of -4 to -6 (figure 6). Based on this result, we proposed that the cap of the globin mRNA primer was transferred to the viral cRNA during transcription (Bouloy *et al.* 1978).

TABLE 2. REQUIREMENTS OF A 5′ TERMINAL METHYLATED CAP STRUCTURE FOR THE PRIMING ACTIVITY OF GLOBIN mRNA

globin mRNA added (2.5 μg)	GMP incorporated/pmol†
none	0.2
untreated	37.2
β-eliminated	0.5
TAP decapped	0.6
β-eliminated and enzymically recapped	22.1

† Transcriptase assays (50 μl) were carried out for 1 h at 31 °C with [α-^{32}P]GTP as labelled precursor. From Plotch *et al.* (1979). TAP, tobacco acid pyrophosphatase.

Consistent with this proposal, the 5′ terminal methylated cap of globin mRNA was shown to be required for its priming activity (Plotch *et al.* 1979). As shown in table 2, removal of the cap by either chemical or enzymic treatment eliminated essentially all of the priming activity of the globin mRNA. This loss of activity was not due to non-specific degradation of the globin mRNA, because after the decapping procedure, the 10*S* 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 with the use of vaccinia virus guanylyl and methyl transferases (Moss 1977; Muthukrishnan *et al.* 1978), and then tested for priming activity. By using Ado[methyl-^{3}H]Met to monitor the recapping reaction, about 40% of the mRNA was recapped, and the resulting 10*S* 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 only in its cap (Plotch *et al.* 1979). After β-elimination, the globin mRNA was recapped with the vaccinia virus enzymes in the presence of [α-^{32}P]GTP and unlabelled AdoMet, and the recapped globin mRNA was purified by sucrose density gradient centrifugation (figure 7*a*). The recapped globin mRNA sedimented as a sharp peak at about 10*S*. When analysed by gel electrophoresis (figure 7*b*), the ^{32}P-labelled 10*S* RNA migrated as a diffuse band at the same position as unlabelled marker 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 T_2 digest of the recapped globin mRNA (figure 7*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 (Lockard 1978).

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 unlabelled nucleoside triphosphates (Plotch *et al.* 1979). The poly(A)+ cRNA synthesized was isolated, deadenylylated and examined by gel electrophoresis. As shown in figure 8*a* (lane 1), each of the viral cRNA

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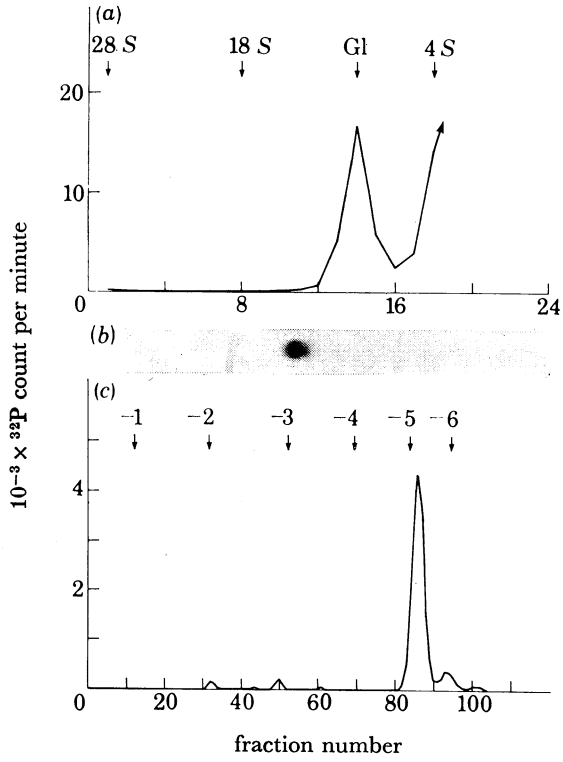

FIGURE 7. Preparation and characterization of globin mRNA containing ^{32}P only in its cap. Globin mRNA was first decapped by β-elimination and then recapped with vaccinia virus guanylyl and methyl transferases in the presence of AdoMet and [α-^{32}P]GTP. The recapped globin mRNA was subjected to sucrose density gradient centrifugation (*a*). The 10*S* RNA from the sucrose gradient was analysed by electrophoresis on a acrylamide gel (30 g/l) containing 6 M urea (*b*). Electrophoresis was from left to right. An aliquot of the 10*S* RNA was hydrolysed with RNase T_2, and the digest analysed by DEAE-Sephadex chromatography in 7 M urea (*c*). From Plotch *et al.* (1979).

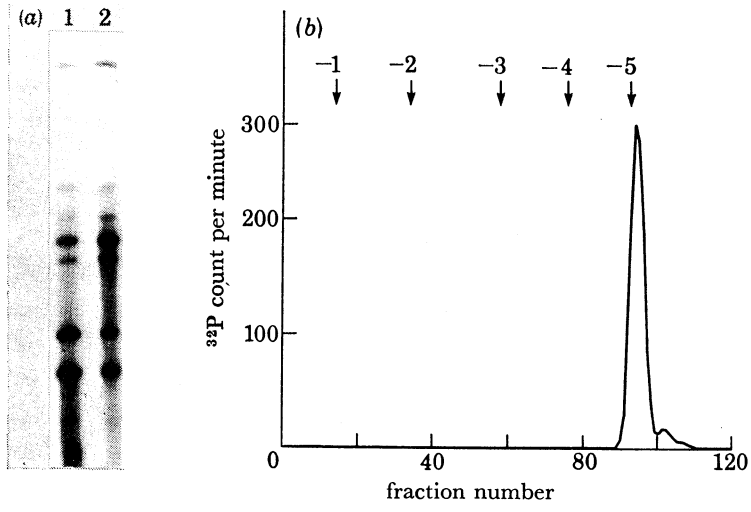

FIGURE 8. Transfer of the ^{32}P-labelled cap of globin mRNA to viral cRNA during transcription. (*a*) Electrophoresis of in-vitro cRNA, after deadenylylation, on an acrylamide gel (30 g/l) containing 6 M urea. Under the electrophoresis conditions employed, globin mRNA migrates off the gel. The cRNA was synthesized in transcriptase reaction mixtures containing: lane 1, the ^{32}P-labelled globin mRNA from figure 7*a* and unlabelled nucleoside triphosphates; lane 2, unlabelled globin mRNA with [α-^{32}P]GTP as labelled precursor. (*b*) DEAE-Sephadex chromatography of the RNase T_2 digest of the RNA eluted from the bands in lane 1 of (*a*). From Plotch *et al.* (1979).

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bands was labelled 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 unlabelled globin mRNA was used to prime the synthesis of cRNA in the presence of [α-^{32}P]GTP as labelled precursor. To demonstrate conclusively that the ^{32}P-labelled cap of globin mRNA was transferred whole to the viral cRNA, the cRNA bands in lane 1 were eluted, digested with RNase T_2 and analysed by DEAE-Sephadex chromatography (figure 8*b*). All of the ^{32}P label eluted at a charge of -5 to -6, indicating that this viral cRNA contained radiolabelled cap 1 and cap 2 structures and no radiolabel in internal residues.

FIGURE 9. Difference in size between ApG-primed and globin mRNA-primed cRNA. Gel electrophoresis of cRNA, after deadenylylation, synthesized in reaction mixtures containing [α-^{32}P]GTP as labelled precursor, and as primer either ApG (lane 1) or globin mRNA (lane 3). Lane 2 contains a mixture of the cRNA of lanes 1 and 3. In (*a*) the deadenylylated cRNA was electrophoresed directly; in (*b*) the deadenylylated cRNA was first treated with glyoxal in the presence of dimethylsulphoxide (McMaster & Carmichael 1977). From Plotch *et al.* (1979).

If sequences in addition to the cap were transferred from the globin mRNA primer to the 5′ end of viral cRNA, we should expect that the globin mRNA-primed cRNA segments would be larger than the ApG-primed cRNA segments, which are initiated at exactly the 3′ end of the vRNA templates (Plotch *et al.* 1978; Skehel & Hay 1978). To compare the size of the globin mRNA-primed and ApG-primed cRNAs, these two cRNAs were deadenylylated enzymically and analysed by gel electrophoresis, either directly (figure 9*a*) or after treatment with glyoxal in the presence of dimethyl sulphoxide (figure 9*b*) (Plotch *et al.* 1979). The latter treatment has been shown to eliminate all secondary structure in RNA (McMaster & Carmichael 1977). 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 can be seen, which are 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. Other experiments have established that these additional nucleotides are at the 5′ end of the globin mRNA primed cRNA segments (Plotch *et al.* 1979). Thus, in addition to the cap 10–15 other nucleotides are most probably transferred from the globin mRNA primer to the 5′ end of viral cRNA.

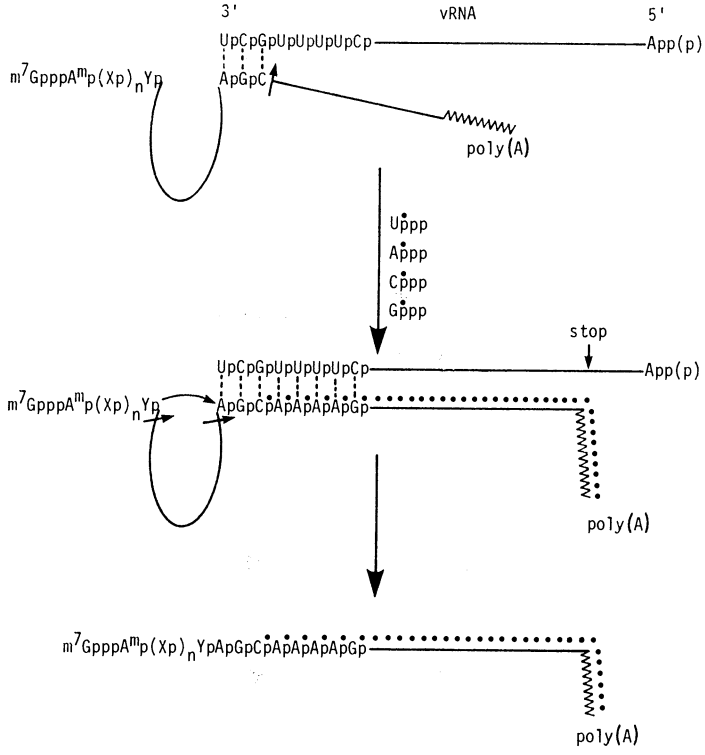

FIGURE 10. Postulated mechanism for the priming of influenza viral cRNA synthesis by β-globin mRNA. From Plotch *et al.* (1979). The 3′ terminal sequence of vRNA is from Skehel & Hay (1978).

The priming activity observed with our globin mRNA preparation can be presumed to be due primarily, if not totally, to the β-globin mRNA. Per microgram, β-globin mRNA is at least 3–4 times more effective as primer than α-globin mRNA (table 2), and β-globin mRNA is the major species present in our preparation of 10*S* poly(A)+ RNA from reticulocytes (figure 3). Based on our results, we can propose a mechanism for the priming of influenza viral cRNA synthesis by β-globin mRNA, as shown in figure 10. We assume that an AG, AGC or AGCA 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 (Plotch & Krug 1977, 1978; Skehel & Hay 1978; Plotch *et al.* 1978). 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

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in β-globin mRNA more effective as a primer, on a molar basis, than the dinucleotide ApG. If priming does occur at an AG-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 figure 10. In β-globin mRNA, the distance from the 5′ end to the first AG sequence is 46 nucleotides, to the first AGC is 316 nucleotides, and to the first and only AGCA is 547 nucleotides (Efstratiadis *et al.* 1977). No sequences longer than AGCA which are complementary to the 3′ end of vRNA exists in β-globin mRNA. Thus, to end up with a cRNA molecule which 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* (Bouloy *et al.* 1978, and unpublished experiments). With these mRNAs, priming activity also requires a 5′ terminal methylated cap structure. Thus, growth hormone mRNA, kappa chain mRNA, and 38*S* 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 which are synthesized *in vitro* (Bouloy *et al.* 1979). Reovirus 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. Experiments have been carried out that demonstrate that the 5′ cap of reovirus mRNAs is also transferred to influenza viral cRNA during transcription and that these cRNA segments also 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 (Krug *et al.* 1979).

These results thus strongly support our hypothesis that in the infected cell, product(s) of the host RNA polymerase II, i.e. mRNA(s) and/or precursor(s), serve as primers for viral RNA transcription and that the synthesis of these host mRNA(s) constitutes the α-amanitin-sensitive step required for viral RNA transcription. If it is verified that the nucleus is the site of primary transcription, then the requirement for new synthesis of host mRNAs would be explained at least partly. In the nucleus, the pool of available host mRNAs and/or their precursors, the potential primers for viral RNA transcription, is probably limited, and consequently new and continuous synthesis of these host mRNA precursors would be required.

Note added in proof (12 *November* 1979). Recent experiments with the use of ^{125}I-labelled globin mRNA to stimulate influenza viral cRNA synthesis have demonstrated that the first 12–14 nucleotides from the 5′ end of β-globin mRNA are transferred to the viral cRNA (Robertson, Dickson, Plotch & Krug, unpublished experiments). The fact that this transferred sequence is

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not complementary to the 3′ end of the vRNA suggested that priming does not require the hydrogen-bonding between an AG sequence in the primer mRNA and the 3′ end of the vRNA indicated in figure 10. This conclusion was verified by our recent finding that capped ribopolymers lacking AG are effective primers for viral RNA transcription. Therefore, we now favour a mechanism in which a capped 12–14 nucleotide fragment is cleaved from the 5′ end of the primer mRNA and then undergoes a specific interaction with the transcriptase which causes stimulation of transcription concomitant with the linking of the primer to the first base transcribed.

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References (Krug *et al.*)

Barry, R. D. 1964 *Virology* **24**, 563–569.
Barry, R. D., Ives, D. R. & Cruickshank, J. G. 1962 *Nature, Lond.* **194**, 1139–1140.
Both, G. W., Banerjee, A. K. & Shatkin, A. J. 1975 *Proc. natn. Acad. Sci. U.S.A.* **72**, 1189–1193.
Bouloy, M., Morgan, M. A., Shatkin, A. J. & Krug, R. M. 1979 *J. Virol.* (In the press.)
Bouloy, M., Plotch, S. J. & Krug, R. M. 1978 *Proc. natn. Acad. Sci. U.S.A.* **75**, 4886–4890.
Chow, N. L. & Simpson, R. W. 1971 *Proc. natn. Acad. Sci. U.S.A.* **68**, 752–756.
Efstratiadis, A., Kafatos, F. C. & Maniatis, T. 1977 *Cell* **10**, 571–585.
Hay, A. J., Abraham, G., Skehel, J. J., Smith, J. C. & Fellner, P. 1977*a* *Nucl. Acids Res.* **4**, 4197–4209.
Hay, A. J., Lomniczi, B., Bellamy, A. R. & Skehel, J. J. 1977*b* *Virology* **83**, 337–355.
Hickey, E. D., Weber, L. A. & Baglioni, C. 1976 *Proc. natn. Acad. Sci. U.S.A.* **73**, 19–23.
Krug, R. M., Broni, B. A. & Bouloy, M. 1979 *Cell* **18**, 329–334.
Krug, R. M., Morgan, M. M. & Shatkin, A. J. 1976 *J. Virol.* **20**, 45–53.
Lamb, R. A. & Choppin, P. W. 1977 *J. Virol.* **23**, 816–819.
Levis, R. & Penman, S. 1977 *Cell* **11**, 105–113.
Lockard, R. E. 1978 *Nature, Lond.* **275**, 153–154.
Mahy, B. W. J., Hastie, N. D. & Armstrong, S. J. 1972 *Proc. natn. Acad. Sci. U.S.A.* **69**, 1421–1424.
Mark, G. E., Taylor, J. B., Broni, B. & Krug, R. M. 1979 *J. Virol.* **29**, 744–752.
McMaster, G. K. & Carmichael, G. G. 1977 *Proc. natn. Acad. Sci. U.S.A.* **74**, 4835–4838.
Moss, B. 1977 *Biochem. biophys. Res. Commun.* **74**, 374–383.
Muthukrishnan, S., Moss, B., Cooper, J. A. & Maxwell, E. S. 1978 *J. Biol. Chem.* **253**, 1710–1715.
Penhoet, E., Miller, H., Doyle, M. & Blatti, S. 1971 *Proc. natn. Acad. Sci. U.S.A.* **68**, 1369–1371.
Plotch, S. J. & Krug, R. M. 1977 *J. Virol.* **21**, 24–34.
Plotch, S. J. & Krug, R. M. 1978 *J. Virol.* **25**, 579–586.
Plotch, S. J., Bouloy, M. & Krug, R. M. 1979 *Proc. natn. Acad. Sci. U.S.A.* **76**, 1618–1622.
Plotch, S. J., Tomasz, J. & Krug, R. M. 1978 *J. Virol.* **28**, 75–83.
Pons, M. W. 1973 *Virology* **51**, 120–128.
Rott, R., Saber, S. & Scholtissek, C. 1965 *Nature, Lond.* **205**, 1187–1190.
Rott, R. & Scholtissek, C. 1970 *Nature, Lond.* **228**, 56.
Scholtissek, C. & Rott, R. 1970 *Virology* **40**, 989–996.
Skehel, J. J. & Hay, A. J. 1978 *Nucl. Acids Res.* **4**, 1207–1219.
Spooner, L. L. R. & Barry, R. D. 1977 *Nature, Lond.* **268**, 650–652.
Taylor, J. M., Illmensee, R., Litwin, S., Herring, L., Broni, B. & Krug, R. M. 1977 *J. Virol.* **21**, 530–540.
Weber, L. A., Feman, E. R., Hickey, E. D., Williams, M. C. & Baglioni, C. 1976 *J. biol. Chem.* **251**, 5657–5662.

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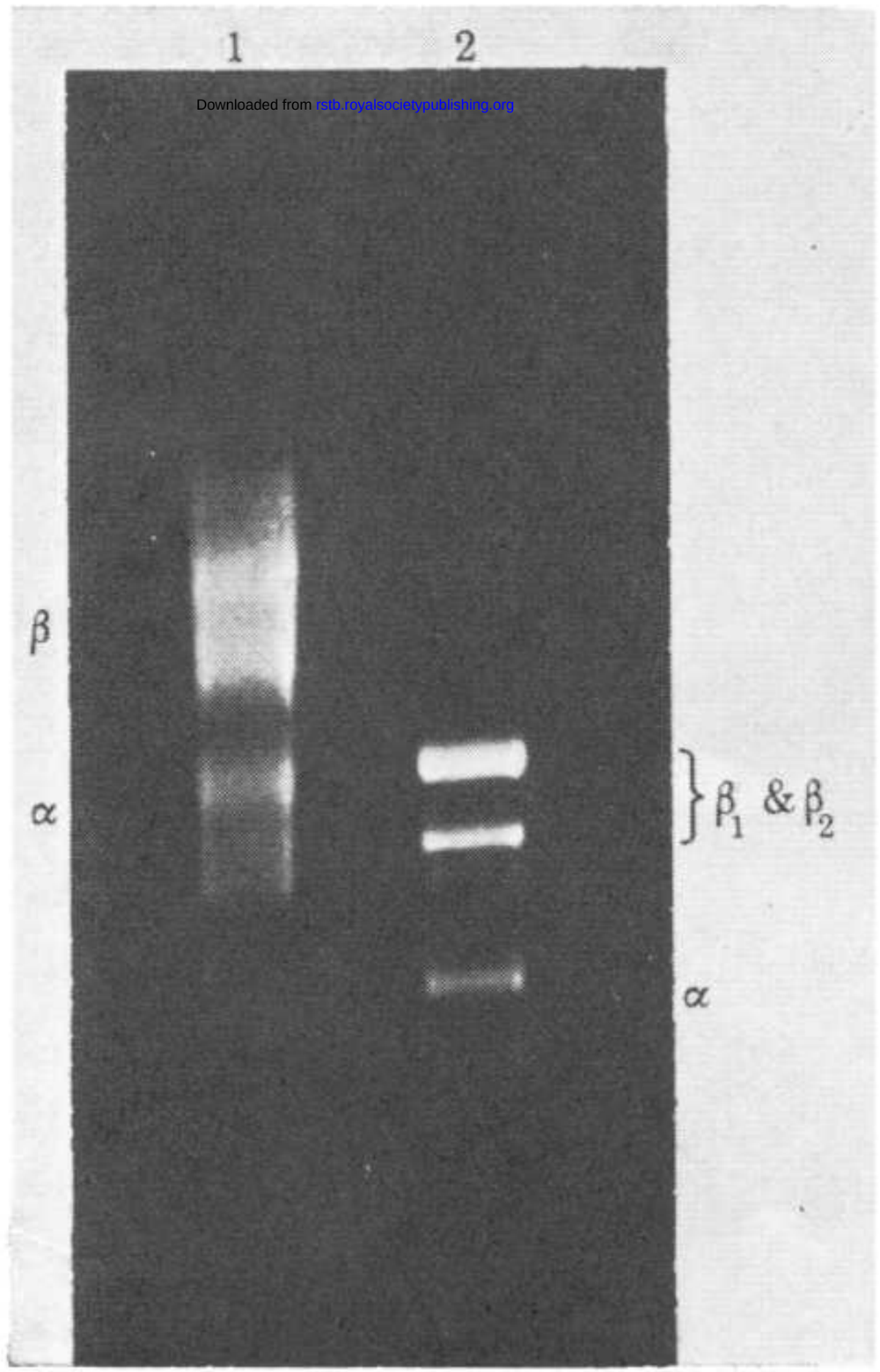

FIGURE 3. Polyacrylamide gel electrophoresis of the 10*S* poly(A)+ RNA from reticulocytes. Only the bottom 10 cm of a 40 cm gel is shown; no RNA bands were visible in the top 30 cm. From Bouloy *et al.* (1978).

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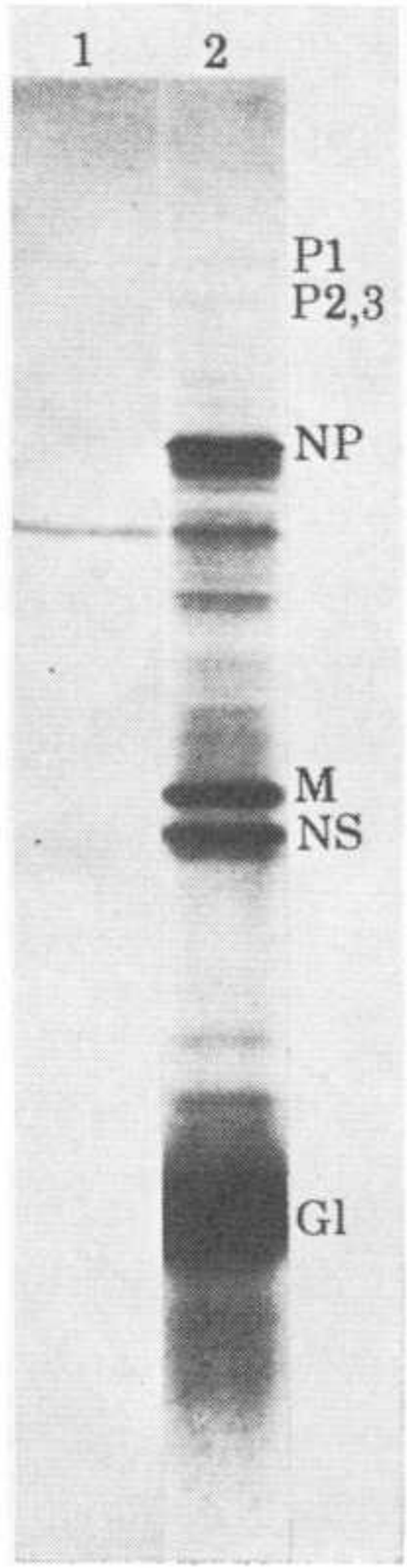

IGURE 4. Polyacrylamide gel electrophoresis of the [^{35}S]methionine labelled proteins synthesized in a micrococcal nuclease-treated cell extract with no added RNA (lane 1) and with the addition of poly(A)+ RNA isolated from a transcriptase reaction primed by globin mRNA (lane 2). The mobilities of virus-specific proteins P1, P2+3, NP (nucleocapsid protein), M (membrane protein) and NS (non-structural protein) are indicated. Gl, globin. From Bouloy *et al.* (1978).

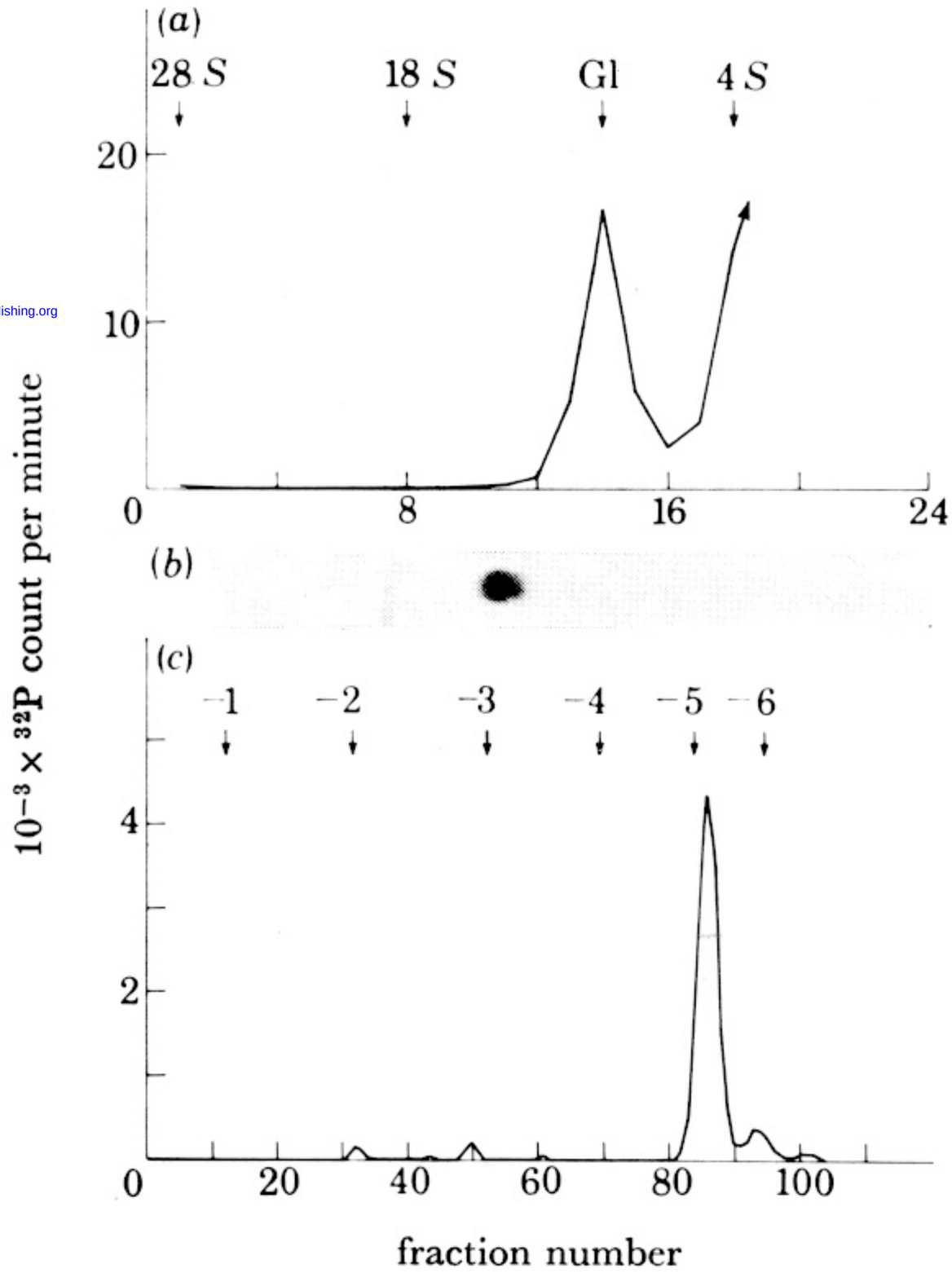

FIGURE 7. Preparation and characterization of globin mRNA containing ^{32}P only in its cap. Globin mRNA was first decapped by β-elimination and then recapped with vaccinia virus guanylyl and methyl transferases in the presence of AdoMet and [α-^{32}P]GTP. The recapped globin mRNA was subjected to sucrose density gradient centrifugation (*a*). The 10*S* RNA from the sucrose gradient was analysed by electrophoresis on a acrylamide gel (30 g/l) containing 6 M urea (*b*). Electrophoresis was from left to right. An aliquot of the 10*S* RNA was hydrolysed with RNase T_2, and the digest analysed by DEAE-Sephadex chromatography in 7 M urea (*c*). From Plotch *et al.* (1979).

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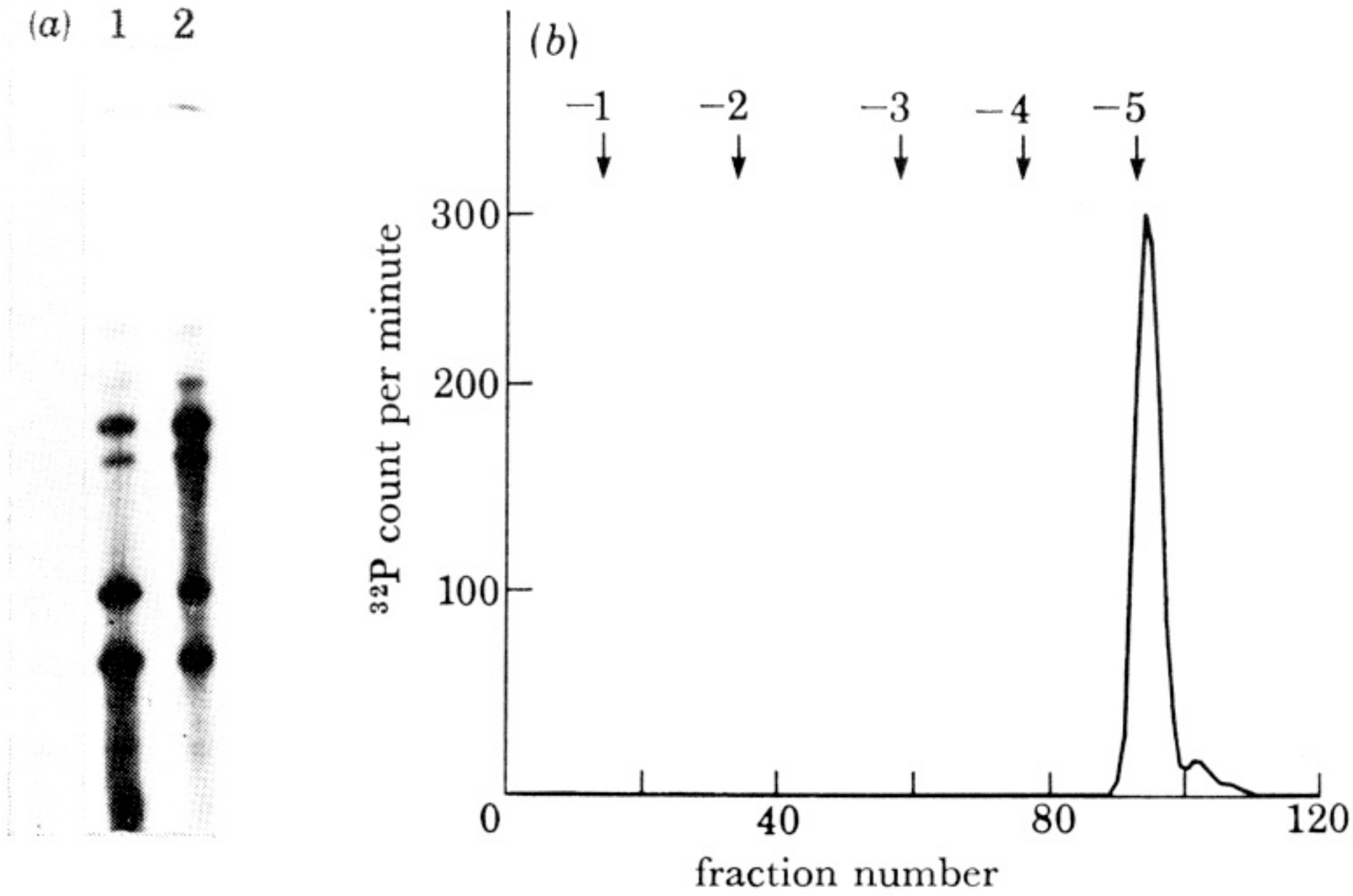

FIGURE 8. Transfer of the ^{32}P-labelled cap of globin mRNA to viral cRNA during transcription. (*a*) Electrophoresis of in-vitro cRNA, after deadenylylation, on an acrylamide gel (30 g/l) containing 6 M urea. Under the electrophoresis conditions employed, globin mRNA migrates off the gel. The cRNA was synthesized in transcriptase reaction mixtures containing: lane 1, the ^{32}P-labelled globin mRNA from figure 7*a* and unlabelled nucleoside triphosphates; lane 2, unlabelled globin mRNA with [α-^{32}P]GTP as labelled precursor. (*b*) DEAE-Sephadex chromatography of the RNase T_2 digest of the RNA eluted from the bands in lane 1 of (*a*). From Plotch *et al.* (1979).

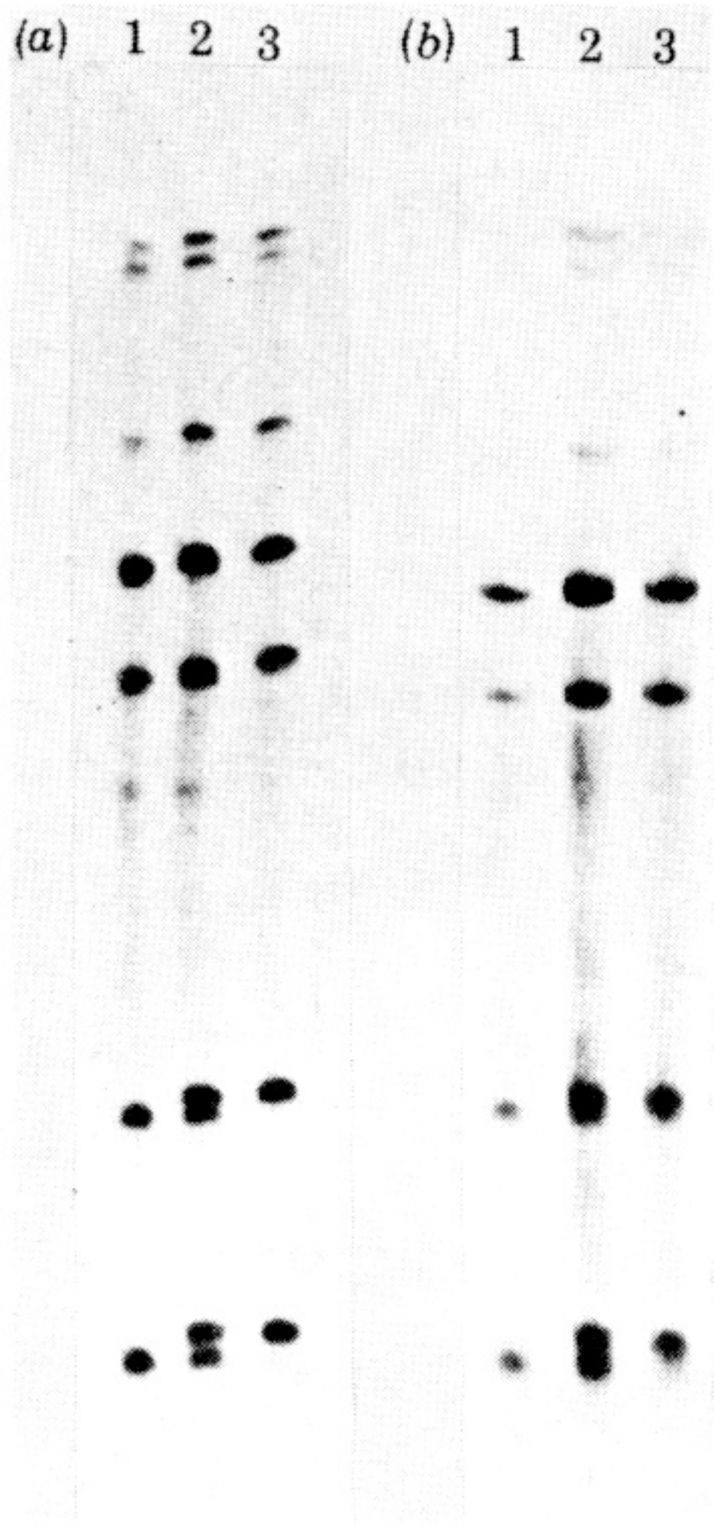

FIGURE 9. Difference in size between ApG-primed and globin mRNA-primed cRNA. Gel electrophoresis of cRNA, after deadenylylation, synthesized in reaction mixtures containing [α-^{32}P]GTP as labelled precursor, and as primer either ApG (lane 1) or globin mRNA (lane 3). Lane 2 contains a mixture of the cRNA of lanes 1 and 3. In (*a*) the deadenylylated cRNA was electrophoresed directly; in (*b*) the deadenylylated cRNA was first treated with glyoxal in the presence of dimethylsulphoxide (McMaster & Carmichael 1977). From Plotch *et al.* (1979).

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