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Identification of the RNA region transferred from a representative primer, β-globin mRNA, to influenza mRNA during in vitro transcription

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ABSTRACT

Capped eukaryotic mRNAs strongly stimulate influenza viral RNA transcription in vitro and donate their cap and also additional nucleotides to the viral transcripts (1). To identify which bases of a given primer mRNA are transferred, we synthesized influenza viral mRNA using a primer rabbit globin mRNA (enriched in β-globin mRNA) which had been labeled in vitro to high specific activity with ^{125}I. We show that during transcription the same ^{125}I-labeled oligonucleotides were transferred to the 5' termini of each of the eight viral mRNA segments. The predominant sequence, representing 75 percent of the transferred oligonucleotides, was identical to the first 13 nucleotides at the 5' end of β-globin mRNA ($m^7G^5'ppp^5'm^6AmC(m)ACUUGCUUUUG$). Because only the C-residues are labeled with ^{125}I, these results indicate that either the first 12, 13 or 14 5' terminal bases of β-globin mRNA were transferred to the viral mRNAs. ^{125}I-labeled oligonucleotides recovered from the viral mRNA in minor yields indicated that shorter 5' terminal pieces of β-globin mRNA were sometimes transferred and that G was probably the first base inserted by transcription.

INTRODUCTION

Eukaryotic viral and cellular RNA transcription involves a number of steps including initiation, elongation and termination. These steps are followed in most cases by processing of the primary transcripts to produce the mature RNA molecules. Any of these steps are potential points for regulation. In one viral transcription system, that of influenza virus, the initiation step has recently been shown to require an RNA primer (2-4). The influenza virus genome consists of eight single-stranded RNA segments which serve as templates for the synthesis of eight polyadenylated mRNA segments catalyzed by the virion-associated transcriptase (1-4). Cell-free synthesis of these mRNA's is strongly stimulated by the dinucleotide AG (3) and, with greater efficiency, by certain eukaryotic mRNA's containing a 5' terminal methylated cap structure (1,4,5). By using rabbit globin mRNA containing ^{32}P label only in its cap, it was shown that this cap was transferred to the 5' end of newly synthesized viral mRNA (1). In addition, gel electrophoretic analysis of the size of the viral mRNA

segments primed by globin mRNA suggested that about 10-15 nucleotides were transferred from the globin mRNA primer to the viral mRNA (1). Similar results were obtained using a mixture of the ten reovirus mRNA's as primers (5).

None of these previous studies, however, established where the transferred nucleotides were located in the primer mRNA (aside from the cap), nor whether they were physically transferred as an intact unit. We therefore carried out sequence analysis of the bases transferred *in vitro* from ^{125}I-labeled rabbit β-globin mRNA to influenza viral mRNA. The results obtained directly demonstrate that the bloc of nucleotides transferred to the viral mRNA is the 5' terminal region of the primer RNA. Using this information, we propose a likely and testable mechanism for priming by globin mRNA and other capped RNAs.

MATERIALS AND METHODS

Preparation of ^{125}I-labeled Globin mRNA. Globin mRNA was purified from the total RNA of rabbit reticulocytes by oligo(dT) cellulose chromatography and sucrose density gradient centrifugation as described previously (1,4,6,7). Deadenylation of globin mRNA by the use of RNase H and poly(dT) and iodination of globin mRNA (before and after deadenylation) with ^{125}I in the Commerford reaction were carried out as previously described (1,4,8-11). For the large-scale iodination reaction, two 12-microgram aliquots of deadenylated globin mRNA were iodinated with carrier-free Na^{125}I from New England Nuclear (6,8-11). The yield from the two preparations was 10^9 and 7.75×10^8 dpm, as determined by counting in NaI-jacketed Bicron vials in a scintillation spectrometer at 20% efficiency. Since rabbit β-globin mRNA is at least three to four times more efficient than α-chain mRNA in stimulating cell-free influenza viral mRNA synthesis (1,4), and since β-chain mRNA constitutes about 80 percent of our preparations (see Results and Figure 1), at least 92 percent of any sequences transferred from globin mRNA to the influenza viral mRNAs should originate from the β-chain mRNA.

In vitro Synthesis of Influenza Viral mRNA's. For preparative-scale RNA synthesis, 24 micrograms of ^{125}I-labeled globin mRNA were added to an influenza virion transcriptase reaction (0.4 ml) containing 1 mM each of ATP, CTP, UTP and GTP. Reaction conditions were as described previously (1,4) except that *E. coli* tRNA was added to a concentration of 2 mg/ml to inhibit nuclease, and virus concentration was increased four-fold. After incubation for 60 min at 31° C, the reaction mixture was deproteinized by phenol-chloroform extrac-

tion, and poly(A)-containing influenza viral mRNA was purified by two successive chromatographic steps on oligo(dT) cellulose. Prior to polyacrylamide gel electrophoresis, the viral mRNA was deadenylated using RNase H in the presence of poly(dT)(4).

Polyacrylamide Gel Electrophoresis. Analysis of ^{125}I-labeled globin mRNA before and after deadenylation was carried out on 20 x 40 cm x 0.3 mm 5% polyacrylamide gels run in 8 M urea according to Sanger and Coulson (12). Influenza viral mRNA (after deadenylation) was separated on the 3% acrylamide 6 M urea system described in previous publications (1,4). Autoradiography was carried out at ambient temperature using Dupont Cronex-2 X-ray film.

RNA Fingerprinting Techniques. Samples were digested for 40 min at 37° C in two microliters of either 1 mg/ml RNase T1 (Sankyo) or 1 mg/ml pancreatic RNase. The concentration of total RNA was adjusted to 5 mg/ml with purified carrier tRNA from E. coli. Digests were spotted onto cellulose acetate strips wetted with pyridine acetate buffer, pH 3.7, and subjected to high-voltage electrophoresis at pH 3.5. Separated oligonucleotides were blotted onto thin layers of DEAE cellulose (Machery-Nagel) and subjected to ascending homochromatography using homomix c (13).

RNA Sequencing Techniques. Oligonucleotides were eluted from DEAE-cellulose thin layers as described by Barrell (13) and subjected to secondary enzymatic analysis as described by Dickson et al. (11,14). Treatment with RNases T1, T2, U2 or pancreatic RNase was carried out at 37° C in the buffers and for the times previously employed (11). The 5-microliter reactions were spotted onto Whatman DE81 paper, and electrophoresis was carried out at pH 1.9 as before (11). Comparison of the electrophoretic mobilities of the products with those of known standards allowed deduction of oligonucleotide sequences. Characterization of the cap-containing oligonucleotides from ^{125}I-labeled viral mRNA was done in a similar way. These species were directly compared with authentic Cap-AmCp^{S} obtained from globin mRNA itself (15). ^{125}I-labeled globin mRNA (10^{8} dpm) was digested with RNase T2, subjected to high-voltage electrophoresis at pH 1.9 on DEAE paper, and the 0.5% of the radioactivity migrating at about 10% of the mobility of the iodo-CMP spot recovered. This species, the major RNase T2-resistant cap structure of globin mRNA, has been previously identified as Cap-AmCp (15).

Cap-containing, ^{125}I-labeled oligonucleotides were characterized by their changes in electrophoretic mobility following treatment with three enzymes. The concentrations and times of incubation for these enzymatic digestions (in a final volume of 5 microliters) were as follows: Tobacco Acid Pyrophosphatase

(TAP) from Bethesda Research Laboratories, 100 units/ml in 0.01 M beta-mercaptoethanol, 0.05 M sodium acetate, pH 6.0, 45 min; Bacterial Alkaline Phosphatase (BAP) from Bethesda Research Laboratories, 200 units/ml, in 0.015 M sodium acetate, 0.003 M beta-mercaptoethanol, 0.03 M Tris-HCl, pH 8.3, 30 min; and RNase P1, 0.33 mg/ml, in 0.01 M sodium acetate, pH 6.0, 60 min. Electrophoresis was carried out at pH 1.9 on Whatman DE81 DEAE paper as before (11). Autoradiography of DEAE thin layers and papers was at -70°C in Halsey-Radelin X-ray exposure cassettes containing two Dupont Lightning-plus intensifying screens and Dupon Cronex-2 X-ray film.

RESULTS

The strategy employed here to determine which bases of β-globin mRNA are transferred to influenza viral mRNA during transcription *in vitro* was: (i) to remove the poly(A) from the 3' terminus of globin mRNA, iodinate this mRNA to high specific activity, and test it for purity and intactness; (ii) to carry out a mRNA synthesis reaction catalyzed by influenza virions in the presence of ^{125}I-labeled deadenylated globin mRNA and unlabeled ribonucleoside triphosphates; (iii) to separate the newly synthesized influenza viral mRNAs, which contain extensive poly(A) tracts (1), from the deadenylated globin mRNA by oligo(dT)-cellulose chromatography; (iv) to fractionate the eight influenza viral mRNA species by polyacrylamide gel electrophoresis; and (v) to subject the ^{125}I-labeled influenza viral mRNA's recovered from the gel to sequence analysis.

Characterization of ^{125}I-labeled Rabbit Globin mRNA. Purified rabbit globin mRNA, both before and after deadenylation, was iodinated in vitro with ^{125}I. As shown in Figure 1, panels a and b, this RNA remained intact despite its specific radioactivity of 10^8 dpm/microgram. The poly(A)-containing globin mRNA migrated as a diffuse band in the 10S region of 5% polyacrylamide gels (Figure 1a). Following deadenylation, two distinct bands of higher mobility appeared (Figure 1b). Previous work (4,7) has shown that the upper band in this gel system is β-globin mRNA, and the lower band, α-globin mRNA. The upper band contained 80 percent of the total radioactivity. Furthermore, comparison of the fingerprints of the deadenylated RNA (Figure 1c and 1d) with those published by Legon et al. (6,7) indicated that our mRNA preparations were significantly enriched in β-chain mRNA.

Cell-free Synthesis and Isolation of Influenza Viral mRNAs Primed by ^{125}I-labeled Rabbit Globin mRNA. Control experiments indicated that iodinated, dea-

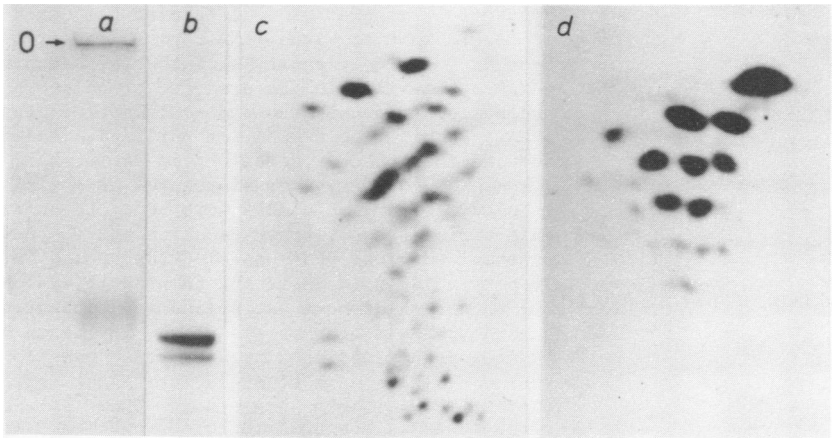

Figure 1. Characterization of ^{125}I-Labeled Rabbit Globin mRNA. Purified 10S rabbit globin mRNA was prepared and labeled with ^{125}I, as described in Materials and Methods. Electrophoresis of this mRNA on a 5% polyacrylamide gel was carried out before (panel a) and after (panel b) deadenylation. (Panel c): RNase T1 fingerprinting analysis of deadenylated ^{125}I-labeled globin mRNA. (Panel d): Pancreatic RNase fingerprint of deadenylated ^{125}I-labeled globin mRNA.

denylated globin mRNA effectively stimulated influenza viral mRNA synthesis in vitro (data not shown). A preparative-scale reaction was then carried out using 24 micrograms of deadenylated, ^{125}I-labeled globin mRNA (a total of 1.7×10^9 dpm) in the presence of unlabeled nucleoside triphosphates. Since only a small amount of total ^{125}I label would be expected to be transferred to the viral mRNA (1,4), the products of the reaction were subjected to two rounds of oligo(dT)-cellulose chromatography. The vast majority of the deadenylated globin mRNA flowed through the column, while the influenza viral mRNA was retained, due to its poly(A) tracts, along with about 0.01% of the ^{125}I radioactivity. Figure 2 depicts the autoradiogram of a 3% polyacrylamide gel on which the ^{125}I-labeled influenza viral mRNAs (after deadenylation) have been separated from each other, as well as a set of marker viral RNAs synthesized using unlabeled rabbit globin mRNA and α-^{32}P-labeled GTP. The electrophoretic mobilities of the six bands of ^{125}I-labeled RNA are indistinguishable from those of the 32 P-labeled RNA. Thus it appeared that iodo-CMP residues from within the rabbit β-globin mRNA were transferred into covalent linkage with all of the influenza viral mRNAs. In order to test whether these residues were still present in sequences characteristic of globin mRNA, each of the six RNA bands indicated in Figure 2 was eluted by standard techniques (14) and subjected to fingerprinting and se-

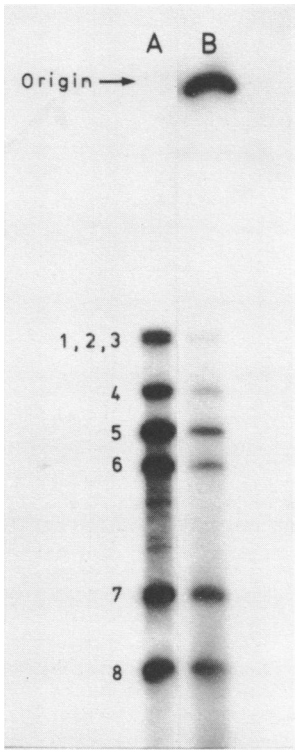

Figure 2. Transfer of ^{125}I-labeled Component(s) of Globin mRNA to Viral mRNA during Transcription. Lane B: Gel electrophoresis of the influenza viral mRNAs synthesized in the presence of unlabeled nucleoside triphosphates and ^{125}I-labeled globin mRNA. Rabbit globin mRNA was purified, deadenylated and iodinated as described in Materials and Methods. Twenty-four micrograms of ^{125}I-labeled rabbit globin mRNA were added to an influenza virion transcriptase reaction and the newly synthesized viral mRNA was electrophoresed on a 3% acrylamide gel containing 6M urea (1,4) following phenol-chloroform extraction and deadenylation. Lane A: Gel electrophoresis of the influenza viral mRNA's synthesized in a transcriptase reaction mixture containing unlabeled globin mRNA as primer and (α-^{32}P)GTP as labeled precursor. The ^{32}P-labeled viral mRNA was prepared for electrophoresis in a manner identical to the reaction analyzed in Lane B. The numbers refer to the eight viral mRNA segments.

quence analysis as described below.

Fingerprinting and Sequence Analysis of ^{125}I-labeled Influenza Viral mRNAs

The strategy for this part of these studies was (a) to carry out comparative fingerprinting analysis of each of the viral mRNA segments eluted from the 3% polyacrylamide gel depicted in Figure 2 in order to compare the ^{125}I-label transferred from globin mRNA to influenza viral mRNA with both the ^{125}I-labeled starting material and ^{32}P-labeled influenza viral mRNA's synthesized in the control reaction; (b) to carry out compositional analysis on ^{125}I-labeled oligonucleotides transferred to influenza viral mRNA in order to identify those containing 5' terminal caps (1); and (c) to carry out sequence analysis on both capped and uncapped oligonucleotides in order to determine the portion of globin mRNA transferred.

(a) Comparative Fingerprinting Analysis. RNase T1 fingerprints of ^{32}P-labeled mRNAs synthesized in the control reaction (Figure 2a) have complexities expected for RNA species between 800 and 3000 bases in length (Figure 3a-f). In contrast, all six fingerprints of the fractionated ^{125}I-labeled mRNAs were

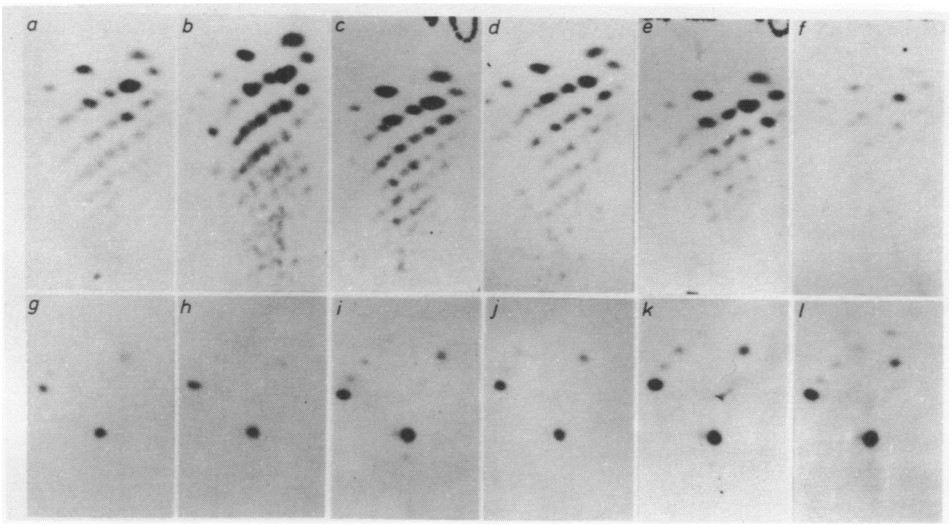

Figure 3. RNase T1 Fingerprints of ^{32}P- and ^{125}I-labeled Influenza Viral mRNA's Primed by Globin mRNA. Viral mRNA segments 1-3, 4,5,6,7, and 8 were eluted from the acrylamide gel depicted in Figure 2 and subjected to RNase T1 fingerprinting analysis as described in Materials and Methods. Panels a-f depict fingerprints of the ^{32}P-labeled influenza viral mRNA segments 1-3, 4, 5,6,7, and 8, respectively. Panels g-l depict fingerprints of ^{125}I-labeled influenza viral mRNA segments 1-3, 4,5,6,7, and 8, respectively. In each panel, the origin is at the lower right and the first dimension is depicted as running from right to left.

identical to each other and had strikingly simple patterns (Figure 3g-l). Thus, it is apparent that (i) the same region of globin mRNA appears to be transferred into covalent linkage with all eight of the influenza viral mRNAs; (ii) there is no similarity in pattern or complexity to the fingerprints of ^{32}P-labeled influenza viral mRNA synthesized *in vitro* (compare Figure 3a-f with Figure 3g-l), ruling out the possibility that the ^{125}I-labeled globin mRNA primer has been broken down into mononucleotides and randomly re-incorporated into influenza viral mRNA (2); and (iii) the fact that the fingerprints in Figures 3g-l are simple while the RNase T1 fingerprint of the ^{125}I-labeled globin mRNA starting material depicted in Figure 1c is complex indicates that only a small portion of the total globin mRNA sequence was transferred to influenza viral mRNA.

(b) A Search for 5' Terminal Cap Structures in the Transferred Region. Figure 4 depicts typical RNase T1 and pancreatic RNase fingerprints of the

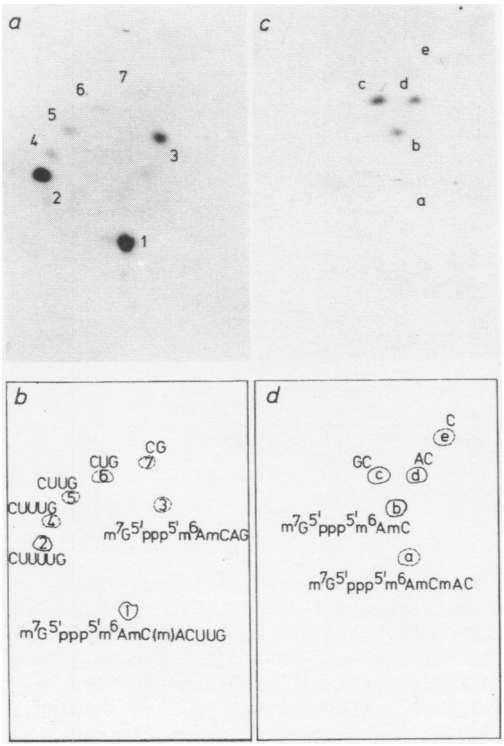

Figure 4. RNase T1 and Pancreatic RNase Fingerprinting Analysis of ^{125}I-labeled Influenza Viral mRNAs. (a) RNase T1 fingerprint of pooled bands 5 and 6 from the gel depicted in Figure 2. The numbered oligonucleotides are those which were eluted and subjected to further analysis. (b) Schematic drawing of panel (a) including proposed sequences (see Tables 2 and 3). (c) Pancreatic RNase fingerprint of pooled bands 3 and 4 from the gel depicted in Figure 2. The lettered oligonucleotides are those chosen for further analysis. (d) Schematic drawing of (c) including our proposed sequences. RNase T1 and pancreatic RNase fingerprinting analysis was carried out as described in Materials and Methods. In addition to those spots marked, there are also a few additional faint spots visible in Figure 4a and 4c. In panel (a), the faint spot immediately to the left of spot #1 is the result of a small percentage of spot 1 acquiring two, rather than one, iodo-CMP residues (11). The faint spot immediately below spot 1 could represent low levels of the 5' terminal RNase T1-resistant oligonucleotide from α-globin mRNA, which is a ten-nucleotide sequence that should migrate in approximately this location (15). In panel (c), the gray streak to the left of spot a is an artifact of X-ray film development, and did not contain any ^{125}I radioactivity.

^{125}I-labeled transferred region. Because of the demonstration by Plotch et al. (1) that the 5' terminal cap of globin mRNA is transferred to viral mRNA, we expect that at least one of the oligonucleotides in each of the fingerprints in Figure 4 contains a 5' terminal cap. Of the seven RNase T1- and five pancreatic RNase-resistant oligonucleotides, a total of four (two each from the RNase T1 and pancreatic RNase fingerprints) were found to have ^{125}I-labeled RNase T2-resistant products with lower electrophoretic mobility at pH 1.9 on DEAE paper than iodo-CMP. These four candidates for capped oligonucleotides were each isolated and compared with authentic Cap-AmCp , prepared from ^{125}I-labeled globin mRNA by RNase T2 digestion. For example, spot "b" from the pancreatic RNase fingerprint contained a major and a minor species, both of which were resistant to RNase T2 digestion (Figure 5a, lanes 1 and 4). The major spot was eluted and found to have a mobility identical to that of authentic Cap-AmCp when these two were run

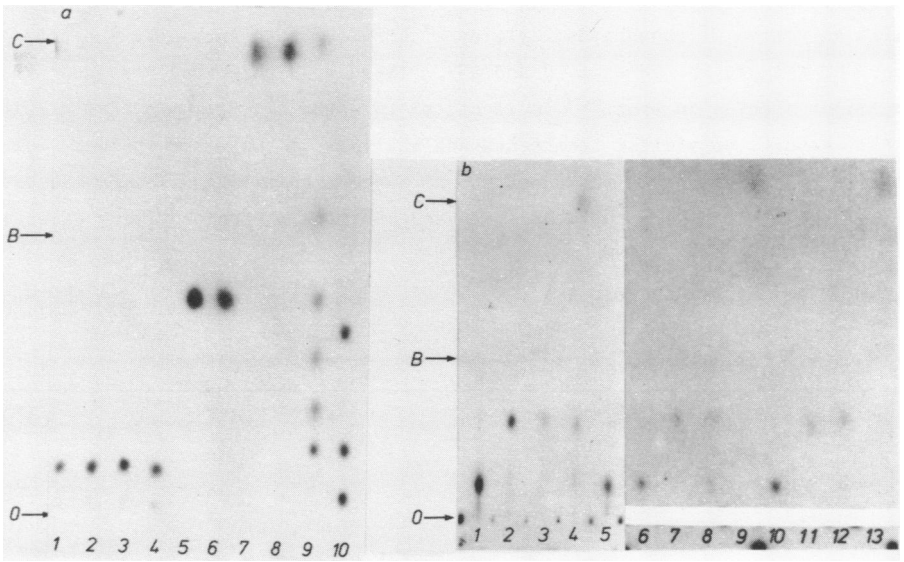

Figure 5. Identification and Characterization of the 5' Terminal Cap-containing Oligonucleotides Transferred from Globin mRNA to Influenza Viral mRNA. Panel (a): DEAE paper electrophoresis of two of the oligonucleotides eluted from the pancreatic RNase fingerprint shown in Figure 4c. Lanes 1-4: oligonucleotide "b"; Lanes 5-8: oligonucleotide "c"; Lanes 1 and 5: untreated; Lanes 2 and 6: after RNase U2 treatment; Lanes 3 and 7: after RNase T1 treatment; Lanes 4 and 8: after RNase T2 treatment. The concentrations and times of incubation for these RNase digestions are described in Materials and Methods. Lane 9: mixture of the following iodinated marker oligonucleotides (in order from the origin)--GGCp, (A,G)Cp, AACp, GCp, ACp and Cp. Lane 10: marker oligonucleotides (in order from the origin)--CUUGp, CUGp and CGp. Panel (b): DEAE paper electrophoresis of authentic Cap-AmCp from globin mRNA (Lanes 1-5), of the intense spot depicted in Lane 1 of panel (a) (Lanes 6-9), and of the predominant spot eluted from Lane 4 of panel (a) (Lanes 10-13) after various enzymatic treatments. Lanes 1, 6 and 10: untreated; Lanes 2, 7 and 11: after treatment with TAP; Lanes 3, 8 and 12: after treatment with BAP; Lanes 4, 9 and 13: after treatment with RNase P1 (in Lane 4, digestion is incomplete); Lane 5: after treatment with RNase T2. The concentrations and times of incubation for these enzymatic digestions were described in Materials and Methods. In the autoradiograph of panel (b), the region in the vicinity of the origin for Lanes 6-13 has been covered over prior to photography because reference marks between the lanes caused by radioactive ink were overly intense. No ^{125}I radioactivity migrated closer to the origin than those spots visible in Lanes 6-13 (see also panel (a), Lane 1, for the mobility of the starting material).

side-by-side (Figure 5b, lanes 1, 5, 6 and 10). Furthermore, mobility increases caused by treatment with tobacco acid pyrophosphatase (TAP); bacterial alkaline phosphatase (BAP), or RNase P1 were identical for these two samples: TAP removes $m^7G^{5'}pp$ to release pAmCp (Figure 5b, lanes 2, 7 and 11), BAP removes the 3'

phosphate group (Figure 5b, lanes 3, 8 and 12), and RNase P1 (which also contains an active 3' phosphatase) yields 5' CMP from both authentic Cap-AmCp and spot "b" (Figure 5b, lanes 4, 9 and 13). These results strongly suggest that the major species in spot "b" is identical to authentic Cap-AmCp from globin mRNA. Results of similar treatments and analyses of the minor species visible in spot "b" in Figure 5a (data not shown) suggest that it is ring-opened Cap-AmCp.

Table 1 summarizes the mobilities and mobility shifts occurring after the

TABLE 1

Analysis of putative 5'-terminal cap-containing oligonucleotides

Spot[a]		No Treatment	RNase T2	TAP	BAP	P1	Proposed Sequence
Control	R_{IC}[b]	0.097	0.099	0.300	0.288	1.0	
	Product[c]	Cap-AmCp	Cap-AmCp	pAmCp	Cap-AmC_{OH}	pC	$m^7G5'ppp5'm^6AmpCp$
1-T2	R_{IC}	0.100	0.099	0.303	0.290	1.0	
	Product	Cap-AmCp	Cap-AmCp	pAmCp	Cap-AmC_{OH}	pC	$m^7G5'ppp5'm^6AmpCp$
3-T2	R_{IC}	0.10	0.10	0.301	-	1.0	
	Product	Cap-AmCp	Cap-AmCp	pAmCp	-	pC	$m^7G5'ppp5'm^6AmpCp$
a-T2	R_{IC}	0.080	0.080; 0.9[d]	0.223	-	1.0	
	Product	Cap-AmCmAp	(Cap-AmCmAp); Cp	(pAmCmAp)	-	pC	$m^7G5'ppp5'm^6AmpCmpAp$
b-T2	R_{IC}	0.097	-	0.290	0.296	1.0	
	Product	Cap-AmCp	-	pAmCp	Cap-AmC_{OH}	pC	$m^7G5'ppp5'm^6AmpCp$

[a]The numbers and letters of the oligonucleotides analyzed are those from Figure 4. Oligonucleotides "a" and "b" from the pancreatic RNase fingerprint (Figure 4c) were analyzed directly. Oligonucleotides #1 and #3 from the RNase T1 fingerprint (Figure 4a) were first digested with RNase T2, and the products were separated by electrophoresis at pH 1.9 on DEAE paper. Oligonucleotide #3 yielded only a single slow-moving species, which was analyzed as described here. Oligonucleotide #1 yielded a major species representing about 80% of the radioactivity, and a minor species, representing 20%. Only the major species was further analyzed here. The minor species had a mobility intermediate between that of Cap-AmCp and Cap-AmCmACp and, on this basis, was concluded to be Cap-AmCmAp.

[b]The R_{IC} refers to the electrophoretic mobility at pH 1.9 on DEAE paper with respect to iodo-CMP (11).

[c]The product of each digestion is based on knowledge of the 5' terminal composition of globin mRNA (15) and the behavior of the authentic Cap-AmCp isolated from globin mRNA and analyzed in the top line of the table. Two of the products were put in parentheses because we had no comparable species to serve as marker.

[d]The 3' CMP (Cp) released by RNase T2 digestion migrates about 10% slower during high-voltage electrophoresis at pH 1.9 on DEAE paper than does the marker 5' CMP (pC).

above enzymatic treatments for each of the four putative cap-containing oligonucleotides and lists our proposed structures for each. Since the presence of methylated bases cannot be demonstrated directly in ^{125}I-labeled RNA, the methylated bases shown are based on the known composition of the 5' terminus of β-globin mRNA (15) and on the demonstrated resistance of the phosphodiester bonds adjacent to these residues to T1, T2, U2 and pancreatic RNases. As shown in Table 1, all RNase T2-resistant moieties were identified as either Cap-AmCp or Cap-AmCmAp, both of which were previously identified in rabbit β-globin mRNA (15). As in β-globin mRNA, we found Cap-AmCp to be the majority species in the viral mRNA molecules.

(c) Sequence Analysis of the Oligonucleotides Transferred from Globin mRNA to Influenza Viral mRNA. Conventional sequencing methods for small RNAs call for complete analysis of RNase T1- and pancreatic RNase-resistant oligonucleotides followed by overlapping of the two sets of data into a consistent sequence or sequences (13). The method described by Dickson et al. (11) for sequence analysis of ^{125}I-labeled oligonucleotides was used here. The results are summarized in Tables 2 and 3. Two major RNase T1-resistant oligonucleotides, #1 and #2, representing 75% of the total radioactivity, have the sequences Cap-AmC(m)ACUUGp and CUUUUGp, respectively, which together represent the first 13[¶] bases of the rabbit β-globin mRNA sequence, Cap-AmC(m)ACUUGCUUUUG... (15). GCp, the expected overlap between oligonucleotides #1 and #2, was identified as spot "b" of the pancreatic RNase fingerprint (Figure 4c; see Table 2 and Figure 5). The other two major pancreatic RNase-resistant oligonucleotides were found to correspond to CapAmCp and ACp (spots "c" and "d", respectively) as would be predicted from this sequence.

Analysis of oligonucleotides recovered in minor yield is summarized in Table 3. Minor RNase T1 spots #4-#7 have the sequences CUUUGp, CUUGp, CUGp and CGp, respectively. Cap-AmCAGp is the most likely sequence for spot #3, since (i) Cap-AmCp is released by RNase T2 digestion (Table 1); (ii) the fact that spot #3 is produced by RNase T1 digestion indicates it contains a 3' terminal G residue, and (iii) RNase U2 digestion released a product intermediate in mobility between spot #3 itself and Cap-AmCp, suggesting an A residue adjacent to the Cap-AmCp. However, our data do not rigorously rule out the possibility that the sequence of T1 spot #3 is Cap-AmCGp. Finally, only very low levels of an RNase T1-resistant oligonucleotide which could correspond to the capped 5' terminus of α-globin mRNA (15) were observed (see legend to Figure 4).

TABLE 2

Secondary analysis of major ^{125}I-labeled oligonucleotides found in influenza mRNA's

Spot[a]	Percent Yield[b]	R_{IC}[c]	Products of digestion with nucleases:[d] RNase T2	Pancreatic RNase	RNase U2	RNase T1	CMCT and CMCT/Pancreatic RNase	Proposed Sequence[e]
(a) RNase T1-resistant oligonucleotides								
1	39.4	0	Cp Cap-AmCp Cap-AmCmAp	ACp Cap-AmCp Cap-AmCmACp	Cap-AmCmAp[f] CUUGp	-	-	Cap-AmC(m)ACUUGp
2	33.1	0.002	Cp	Cp	CUUUUGp	-	CU̇U̇U̇U̇Gp[g] → Cp	CUUUUGp
(b) Pancreatic RNase-resistant oligonucleotides								
b	18.9	0.105	Cap-AmCp	-	Cap-AmCp	Cap-AmCp	-	Cap-AmCp
c	34.4	0.45	Cp	-	GCp	Cp	-	GCp
d	29.7	0.59	Cp	-	Cp	ACp	-	ACp

[a]The numbers and letters of the oligonucleotides analyzed are those from Figure 4.

[b]The percentage yield was calculated by counting each eluted oligonucleotide using a "mini-Assay" gamma scintillation counter.

[c]R_{IC} refers to the electrophoretic mobility of the untreated oligonucleotide with respect to iodo-CMP (11).

[d]RNase T2, pancreatic RNase, RNase U2, RNase T1, and CMCT derivitization and pancreatic digestion of CMCT-oligonucleotides was carried out, and the digests were separated by high-voltage electrophoresis at pH 1.9 on DEAE paper. Oligonucleotide identifications were made by comparison to parallel analyses of known standards as described (11). Under the conditions used here involving high amounts of unlabeled RNA from homochromatographic separations, the RNase U2 sometimes failed to digest to completion. 5' terminal cap-containing oligonucleotides generated during these analyses were characterized using the methods demonstrated in Figure 5 and Table 1.

[e]The sequences proposed here were derived by combining the data obtained here with that from Table 1.

[f]The conclusion that RNase U2 gave Cap-AmCmAp here is based on the fact that this enzyme created a product intermediate in electrophoretic mobility (R_{IC} = 0.085) between that of spot #3 (R_{IC} = 0.075, Table 3) and that of authentic Cap-AmCp (R_{IC} = 0.10-0.11).

[g]These sequences were assigned using the indicated data and by electrophoresing spot #2 on Whatman 3MM paper with and without CMCT treatment. These were run alongside a known standard of identical composition eluted from an RNase T1 fingerprint of ^{125}I-labeled rabbit globin mRNA. Furthermore, pancreatic RNase treatment of the CMCT-modified oligonucleotide #2 from the transferred region showed that one hundred percent of the radioactivity was released as iodo-CMP, demonstrating that C was at the 5' end.

TABLE 3

Secondary analysis of minor ^{125}I-labeled oligonucleotides found in influenza mRNA's

Spot[a]	Percent Yield[b]	RIC[c]	Products of digestion with nucleases:[d] RNase T2	Pancreatic RNase	RNase U2	RNase T1	CMCT and CMCT/Pancreatic RNase	Proposed Sequence[e]
(a) RNase T1-resistant oligonucleotides								
3	11.4	0.075	Cap-AmCp	Cap-AmCp	Cap-AmCAp[f]	-	-	Cap-AmCAGp
4	4.6	0.033	Cp	Cp	CUUUGp	-	CÙÙÚGp[g] → Cp	CUUUGp
5	6.0	0.066	Cp	Cp	CUUGp	-	CÙÚGp[g] → Cp	CUUGp
6	2.4	0.170	Cp	Cp	CUGp	-	CÙG[g] → Cp	CUGp
7	3.1	0.40	Cp	Cp	CGp	-	CG[g] → Cp	CGp
(b) Pancreatic RNase-resistant oligonucleotides								
a	8.2	0.076	Cp Cap-AmCmAp	-	Cap-AmCmAp[f]	Cap-AmCmACp	-	Cap-AmCmACp
e	8.8	1.0	Cp	-	Cp	Cp	-	Cp

[a-f]Please refer to footnotes a-f of Table 2.

[g]These sequences were assigned using the indicated data and by electrophoresing spots #4-#7 on Whatman 3MM paper with and without prior CMCT treatment. These were run alongside known standards of identical composition eluted from an RNase T1 fingerprint of ^{125}I-labeled rabbit globin mRNA. Furthermore, pancreatic RNase treatment of the CMCT-modified oligonucleotides #4-#7 from the transferred region showed that in each case, 100% of the radioactivity was released as iodo-CMP, demonstrating that C was at the 5' end.

DISCUSSION

The Region(s) Transferred from β-globin mRNA to Influenza Viral mRNAs.

We have demonstrated that during the synthesis of influenza viral mRNA *in vitro* in the presence of ^{125}I-labeled rabbit globin mRNA, a 5' terminal capped oligonucleotide is transferred from β-globin mRNA onto the 5' terminus of all eight viral mRNAs. The 5' terminus of β-globin mRNA (15) has the sequence

$$m^7G^{5'}ppp^{5'}m^6AmC(m)A\ C\ U\ U\ G\ C\ U\ U\ U\ U\ G\ A\ C\ A\ C\ A\ A\ C\ \ldots$$
$$1\qquad 5\quad 8\quad 10\ 12\ 13\ 14\ 15\qquad 20$$

and our analysis indicates that the predominant sequence at the 5' terminus of the viral mRNA molecules is identical to the first 13 residues (plus the cap) of this sequence. Because only the C-residues are labeled with ^{125}I (8-11), we cannot conclude with certainty that all 13 5' terminal nucleotides are

transferred from β-globin mRNA. The data, however, certainly indicate that the β-globin mRNA donates at least the first eight, but no more than the first 14, 5' terminal bases to the viral mRNAs. We know that the C at position 15 is not transferred because none of the RNase T1-resistant oligonucleotides has the proper sequence to include this residue. We can also reasonably conclude that the ..UUUU.. sequence (residues 9-12) originates from the globin mRNA molecules since there is no complementary ..AAAA.. sequence in the 12-base 3' terminal common sequence of the influenza viral RNA templates (3' UCGUUUUCGUCC ...5'; ref. 16-19). On the other hand, the G-residue at position 13 of the viral mRNA (identified at the 3' end of the RNase T1-resistant oligonucleotide CUUUUGp) could originate either from the globin mRNA, or as the first influenza viral-specific base transcribed. Our data do not establish whether or not the A-residue at position 14 of the β-globin mRNA is transferred to the viral mRNA.

Analysis of the minor oligonucleotides transferred sheds light on the probable identity of the first viral-specific base transcribed. CUUUGp, CUUGp, CUGp and CGp were recovered in a total yield of 14% of the ^{125}I-label transferred (see Table 3). The only straightforward way to account for these RNase T1 products (which occur neither in the globin mRNA 5' terminal regions (15,20,21) nor as complements to the 3' terminal regions of the influenza virus genomic RNAs) is to suggest that the C- and U- residues are derived from globin mRNA while the G-residue comes from *in vitro* transcription of the influenza viral RNA. Thus, these minor products provide the first strong evidence of "hybrid" oligonucleotides (i.e. those containing bases from both globin and influenza viral mRNAs). Likewise Cap-AmCAGp (RNase T1-resistant spot #3) containing 11% of the ^{125}I-label, clearly must derive its Cap-AmCp from globin mRNA (since the C-residue is ^{125}I-labeled), could derive its next A-residue either from the β-globin mRNA or as a result of transcription, and must obtain its G-residue as a result of transcription.

Thus, the simplest interpretation of our results is that (i) about 75% of the time the cap and the first 12, 13 or 14 5' terminal bases of β-globin mRNA are transferred to the 5' end of influenza viral mRNA; (ii) about 14% of the time shorter 5' terminal cap-containing pieces 8-11 bases in length are transferred; and (iii) the remaining 11% of the time only the cap and the adjacent three bases are transferred. These findings indicate that β-globin mRNA is almost certainly cleaved to release major and minor cap-containing fragments prior to their incorporation into the 5' termini of influenza viral mRNAs, strongly suggesting that influenza virions contain both a specific endonucleo-

lytic RNase (i.e. an RNA processing enzyme) which cleaves specifically after base 12, 13 or 14 of β-globin mRNA, along with an additional activity (e.g. an exonuclease) which creates the shorter fragments. Furthermore, our data indicate that the transferred sequence is linked to G as the first base transcribed. As a consequence, the viral RNA transcripts would not necessarily contain an A complementary to the 3' terminal U of the template.

Mechanism of Capped Oligonucleotide Transfer during Initiation of Transcription. How do capped RNA fragments from β-globin mRNA become linked to influenza viral mRNA transcripts? One straightforward way to explain this finding would be a priming mechanism. We define RNA priming to be all events in which transcription of the new RNA proceeds by stepwise addition of single nucleotides onto the 3' end of a pre-existing RNA segment. Another possibility would be a splicing mechanism, which would involve joining of one RNA segment to another (two or more bases in length) by the action of an RNA ligase. For a splicing mechanism to operate here, a small piece of influenza viral mRNA would need to be present. To distinguish between priming and splicing, it is necessary to study events accompanying the initiation of influenza viral mRNA synthesis. Experiments using globin mRNA as primer and α-^{32}P-labeled GTP as the only ribonucleoside triphosphate have yielded short 3' terminally labeled fragments of β-globin mRNA, the size and other properties of which strongly favor the priming mechanism (S.J.P, M. Bouloy and R.M.K, in preparation).

In this system, where viral RNA templates are copied, it might be expected that priming involves some hydrogen bonding between primer and template. Earlier studies showing that influenza viral RNA transcription is stimulated 110-, 90- and 19-fold over background by the dinucleotides AG, GG, and GC, respectively, while all 13 other possible dinucleotides caused little or no stimulation, indicated that base pairing of a dimer to the 3' end of the template strand (whose sequence is 3' UCGU...) is one way to stimulate influenza viral RNA transcription (2,3). However, in the case of β-globin mRNA, hydrogen bonding does not appear to be involved since sequence analysis of the major transferred region reveals no complementarity at its 3' end to the 3' terminal common sequence of the viral RNA templates. Furthermore, recent data would seem to eliminate the previous suggestion (1) that splicing within the β-globin mRNA itself could create a fragment about 15 bases in length which begins with the cap and ends with an AG derived from the interior of the globin mRNA molecule. These new data indicate that (i) capped fragments of globin mRNA too short to include an AG sequence are effective primers for

influenza viral mRNA transcription in vitro (M. Bouloy, S.J.P. and R.M.K., unpublished experiments; and (ii) synthetic capped heteropolymers not containing AG also stimulate transcription (A. Shatkin, A. LaFiandra, B. Broni and R.M.K., unpublished observations).

In the absence of hydrogen bonding between primer and template, one can reasonably propose a specific interaction between the primer and the transcriptase complex which stimulates the initiation of transcription. The most likely recognition signal for this specific interaction is the 5' terminal methylated cap, modification or removal of which inhibits priming activity (1,4,5). In addition, the eight-base 5'-UGCUUUUG-3' present both in the transferred region of β-globin mRNA and in the 12-base 3' terminal common region of influenza viral RNA templates could be involved in recognition. However, since other capped mRNAs with little 5' terminal sequence homology to rabbit β-globin mRNA can also stimulate influenza viral mRNA synthesis in vitro (1,4), it seems unlikely that recognition of such an extensive homology is needed. Nonetheless, some feature of this sequence, for example a U-rich region, may be important.

Implications for Viral and Cellular Transcription. The studies reported here have utilized a representative primer of known sequence -- rabbit globin mRNA -- in order to understand general features of RNA-primed influenza viral mRNA synthesis. Previous data indicated that the influenza viral mRNAs primed by different mRNAs in vitro or synthesized in the infected cell contain about 10-15 extra nucleotides at their 5' termini, including the cap, which are not viral-coded (1,4,22). The present results reveal the source of these additional bases in globin mRNA, and it is reasonable to propose that in all cases nonviral nucleotides come from the 5' terminal regions of capped RNAs which serve as primers. Consequently, the virion-associated RNA processing enzyme which cleaves β-globin mRNA and other capped RNAs to produce the short 5' terminal fragments which prime influenza viral mRNA synthesis in vitro, would also have to be present and operating in the infected cell. This enzyme, which may be encoded in either the viral or the cellular genome, may function in a manner similar to the cellular enzymes that are thought to cleave heterogeneous nuclear RNA near its 5' end during the early stages of the processing of this RNA (23-25). A nuclear location for the synthesis of influenza viral mRNAs has been suggested because of the presence of m^6A in influenza viral transcripts (22) and because of the sensitivity of their synthesis to α-amanitin (26-28). It will thus be of great interest to identify and characterize the cleavage enzyme(s) in influenza virions and to compare this activity to RNA processing activities found in the nuclei of infected and uninfected cells.

Robertson and Dickson (25,29) have proposed that RNA-primed transcription utilizing segments of RNA cleaved from nuclear precursors during normal processing might occur in eukaryotic cells and could operate in the control of gene expression. Two aspects of this model -- (i) specific cleavage of one RNA molecule to form a primer and (ii) use of this RNA fragment during initiation of transcription of a second RNA molecule -- have now been shown to operate in the influenza viral transcriptase system. Whether such a process is unique to influenza viral mRNA synthesis, or whether (as in the case of adenovirus RNA splicing (30)) it reflects a property of cellular RNA synthesis, is a question of fundamental importance.

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FOOTNOTES

§Abbreviation for nucleotide sequences used here have been adapted to depict 5' terminal cap structures, 2'O-methylated bases, and to indicate 3' phosphate termini. Thus, the abbreviation "Cap" appearing within an oligonucleotide sequence means "$m^7G^{5'}ppp^{5'}m^6$-"; the abbreviation "m" means "2' O-methyl"; and "p" means "3' phosphate end". The internal nucleotide residues of the RNA chain are indicated by the conventional abbreviations C, A, G, and U for cytidine, adenosine, guanosine and uridine, respectively.

¶The numbering system of the β-globin mRNA sequence adopted here assigns the number 0 to the m^7G cap residue and the number 1 to the m^6A, the first residue encoded by the genome. Thus, the region containing bases #1-#13 is really 14 bases in length.

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