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Segments of Influenza Virus Complementary RNA Synthesized In Vitro

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In the presence of Mg^{2+} and a specific primer, ApG or GpG, the influenza WSN virion transcriptase synthesizes large, polyadenylic acid-containing complementary RNA (cRNA) (Plotch and Krug, J. Virol., 21:24-34, 1977). After removal of its polyadenylic acid with RNase H in the presence of polydeoxythymidylic acid, the in vitro cRNA distributed into seven discrete bands during electrophoresis in acrylamide gels containing 6 M urea. The eight known segments of virion RNA (vRNA) also distributed into seven bands under these conditions as two, rather than the expected three, large-sized segments were resolved. Each of the in vitro cRNA segments migrated slightly faster than the corresponding vRNA segment. To determine whether this difference in mobility reflects a difference in size between cRNA and vRNA, the double-stranded RNA formed by annealing labeled in vitro cRNA to unlabeled vRNA was subjected to various nuclease treatments and was analyzed by gel electrophoresis. Hybrids treated with RNase T2 or a combination of RNase T2 and RNase H migrated slightly faster than those treated only with RNase H, indicating that RNase T2 removed an RNA sequence other than polyadenylic acid, most probably a short sequence of vRNA not hydrogen bonded to cRNA. These results suggest that the in vitro cRNA segments are shorter than, and thus incomplete transcripts of, the corresponding vRNA segments. All eight hybrids were resolved by gel electrophoresis, indicating that all eight vRNA segments are transcribed into cRNA in vitro. We also present evidence suggesting that the ApG primer initiates in vitro transcription exactly at the 3′ end of vRNA.

The genome of influenza A virus is comprised of eight single-stranded RNA segments ranging in molecular weight from 2.5×10^5 to 1.0×10^6 (2, 15-17, 20, 23). As we have shown previously, the viral mRNA isolated from infected cells, which is complementary to the virion RNA (vRNA), is also segmented (7). The complementary RNA (cRNA) segments migrate more slowly than the corresponding vRNA segments during gel electrophoresis. This is due to the polyadenylic acid [poly(A)] sequences in cRNA not found in vRNA. After enzymatic removal of the poly(A) sequences, the cRNA and the vRNA segments appear to comigrate when analyzed on cylindrical gels, indicating that they are approximately the same size (7).

Previously we have shown that the virion transcriptase is capable of synthesizing in vitro cRNA that is large and contains covalently linked poly(A) (11, 18). The synthesis of cRNA with these characteristics occurs in the presence of the proper divalent cation, Mg^{2+}, and a specific primer, ApG or GpG. Although the in vitro cRNA migrates in the same molecular weight range as vRNA during gel electrophoresis, it is much more heterogeneous, so that discrete in vitro cRNA segments corresponding to the vRNA segments cannot be discerned. We postulated that the heterogeneity of in vitro cRNA is due to its poly(A) sequences which are longer and more heterogeneous than the poly(A) sequences of in vivo cRNA (18).

In the present paper, we establish that the heterogeneity of this in vitro cRNA is, indeed, due to its poly(A) sequences. After enzymatic removal of the poly(A), in vitro cRNA distributed into the same number of discrete segments as is found in vRNA. Each of the in vitro cRNA segments migrated slightly faster than the corresponding vRNA segment, and our data strongly suggest that the cRNA segments are actually slightly smaller than the vRNA segments from which they are transcribed. It will also be shown that the very small amount of RNA made in the presence of Mg^{2+}, but in the absence of a primer, distributes into segments similar in size to the primed segments.

MATERIALS AND METHODS

Cells, virus, and vRNA. The procedures for cul-

ture of the MDCK and MDBK cell lines and for the growth of WSN (influenza A) virus in MDBK cells have been described previously (8, 9). Virus was purified by two sequential bandings in sucrose-D_2O gradients (8, 9). ^{32}P-labeled vRNA was obtained as described previously (8, 9).

RNA synthesis in vitro. The synthesis of cRNA in vitro by detergent-treated virus was carried out as described previously (18). The divalent cation was Mg^{2+} at 5 mM (added as $MgCl_2$), and the labeled ribonucleoside triphosphate was [α-^{32}P]GTP at 0.2 mM and, except where indicated, at 0.3 mCi/μmol. Where indicated, ApG or GpG at 0.4 mM or a trinucleoside diphosphate at 0.22 mM was added.

Removal of poly(A) from viral cRNA. The procedures for isolating the poly(A)-containing in vitro cRNA product from reaction mixtures and for purifying ^3H-labeled viral cRNA from infected MDCK cells (in vivo cRNA) have been described previously (6, 18). The in vitro or in vivo cRNA (or a mixture of the two) was hybridized to polydeoxythymidylic acid [poly(dT)], and the poly(A) was removed by digestion with RNase H as described previously (5, 25, 26). After extraction with phenol-chloroform (1:1) at pH 9.0, the RNA was ethanol precipitated.

Gel electrophoresis of single-stranded RNA. The RNA (vRNA, deadenylated in vivo cRNA, or deadenylated in vitro cRNA) was heated, fast-cooled, and subjected to gel electrophoresis on acrylamide gels containing 6 M urea, using Peacock-Dingman buffer (17, 18). Either cylindrical (12 cm; 2.1% acrylamide–0.7% agarose) or slab (14 by 16 by 0.15 cm; 2.8% acrylamide) gels were used. Electrophoresis at 140 V was for 5 to 6 h with the cylindrical gels and 13 to 15 h with the slab gels. After electrophoresis, the cylindrical gels were sliced, and the slices were processed for counting as described previously (10). Slab gels containing only ^{32}P-labeled RNA were transferred onto filter paper, dried, and exposed to Dupont Cronex 2DC X-ray film. Slab gels containing ^3H-labeled as well as ^{32}P-labeled RNA were treated with 2,5-diphenyloxazole–dimethyl sulfoxide as described by Bonner and Laskey (3) before transfer to the filter paper.

RNA-RNA annealing and nuclease treatment of hybrids. ^{32}P-labeled in vitro cRNA was heat denatured in 90% formamide and annealed to 20 μg of vRNA for 24 h at 37°C in 55% formamide, using the procedures described previously (11). To obtain good resolution of double strands by gel electrophoresis, it was necessary to remove poly(A) (7, 21). Two alternative methods for removing poly(A) were employed: (i) in vitro cRNA was treated with RNase H in the presence of poly(dT) before, or after, its annealing to vRNA; or (ii) after annealing, the double strands, in 0.02 M sodium acetate (pH 5)–0.2 M NaCl, were digested with 20 U of RNase T2 per ml for 1 h at 37°C. Nuclease was removed by phenol extraction, and the RNA was precipitated by ethanol.

Gel electrophoresis of double-stranded RNA. Nuclease-treated, double-stranded RNA was dissolved in 0.25 M Tris (pH 8.4)–0.001 M EDTA–10% sucrose and subjected to electrophoresis in slab gels (14 by 16 by 0.3 cm) containing 3% acrylamide. The electrophoresis buffer was 0.036 M Tris–0.030 M phosphate (pH 7.8)–0.001 M EDTA. Electrophoresis was carried out for 19 to 22 h at 50 mA with constant recirculation of the electrophoresis buffer.

Materials. [α-^{32}P]GTP, [2,8-^3H]adenosine, and carrier-free $^{32}P_i$ were purchased from New England Nuclear Corp. Purified RNase H free of detectable RNase activity of the pancreatic type (25, 26) was generously supplied by Janus Stavrianopoulos. RNase T2 was purchased from Calbiochem. Trinucleoside diphosphates were obtained as follows: UpApG (O. Uhlenbeck and Boehringer Mannheim Corp.); CpApG (O. Uhlenbeck); ApApG (C-W. Wei and B. Moss).

RESULTS

Gel pattern of deadenylated in vitro cRNA. In the presence of Mg^{2+} and a primer, ApG or GpG, the influenza virion transcriptase synthesizes large, poly(A)-containing cRNA (11, 18). The in vitro product is heterogeneous, and discrete segments cannot be discerned. To determine whether this heterogeneity is due to the poly(A) sequences of in vitro cRNA, the poly(A) was removed enzymatically with RNase H in the presence of poly(dT) as described above. The effectiveness of this treatment was tested by digesting RNase H-treated, [^{32}P]AMP-labeled in vitro cRNA with pancreatic and T1 RNase. The digest was analyzed by electrophoresis on a 20% acrylamide gel (18) for the presence of residual poly(A) or oligoadenylic acid [oligo(A)] sequences. More than 95% of the heterogeneous, large-sized (60 to 350 nucleotide) poly(A) of the in vitro cRNA (18) was no longer present, and no oligo(A) sequences longer than 6 nucleotides were found.

The deadenylated in vitro cRNA was subjected to coelectrophoresis with similarly deadenylated in vivo cRNA on cylindrical gels containing 6 M urea (Fig. 1). After removal of their poly(A) sequences, in vitro and in vivo cRNA's were resolved into six peaks, which were designated L, M1, M2, M3, S1, and S2. We will show later that the L peak is actually composed of three closely spaced segments. As analyzed on cylindrical gels, the in vitro and in vivo cRNA segments comigrated, indicating that they are approximately the same size. Both the ApG- and GpG-primed products contained all the cRNA segments, indicating that each segment can initiate with either A or G. In addition, similar ratios of the different segments were found whether ApG or GpG was used as primer.

The in vitro cRNA differs from the in vivo cRNA preparation in the relative proportion of the different segments. In Table 1, the amounts of the cRNA segments are expressed relative to the M2 segments. In both the in vitro and in vivo cRNA preparations, the smallest segment, S2, is synthesized in the largest amount, about three times the amount of the M2 segment. The

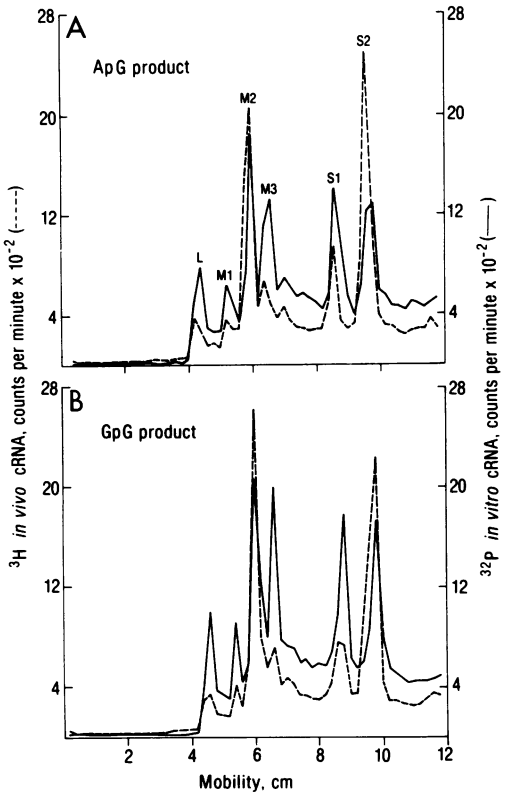

FIG. 1. *Gel electrophoresis of [^{32}P]GMP-labeled, deadenylated in vitro cRNA and [^{3}H]adenosine-labeled, deadenylated in vivo cRNA. The in vitro cRNA was synthesized with either ApG (A) or GpG (B) as primer. Cylindrical gels were used. Experimental details were as described in the text.*

two cRNA's, however, differ in the representation of the L, M1, M3, and S1 segments: in in vitro cRNA, these segments are found in about 2.5 times the relative amount found in the in vivo cRNA preparation. These differences are not unexpected, because the in vivo cRNA was obtained from cells under conditions of amplified transcription (5, 6). As the virion-associated transcriptase is responsible for primary transcription in the infected cell, it is possible that the ratio of the cRNA segments synthesized in vivo as a result of primary transcription may more closely approximate the ratio observed during in vitro transcription.

Unprimed in vitro cRNA. With Mn^{2+} as the divalent cation, only small RNA lacking poly(A) is synthesized whether or not the primer ApG or GpG is present (11, 18). With Mg^{2+}, the characteristics of the product cRNA made in the absence of primer have not been determined previously. In the absence of a primer, the amount of cRNA synthesized in the presence of Mg^{2+} is only 1 to 2% of that made in the presence of a primer. For this reason, it was necessary to employ a radioactive nucleoside triphosphate precursor with a high specific activity (10 mCi/μmol). About half of this unprimed product was found to bind to oligodeoxythymidylic acid-cellulose and to contain poly(A). The poly(A)-containing product was treated with RNase H and subjected to electrophoresis on a cylindrical gel (Fig. 2). Six peaks were resolved by slicing the gels, each peak comigrating with a peak of in vivo cRNA. The only significant difference between this unprimed product and the primed product (Fig. 1) is that about 100-fold less of each peak is made in the absence of the primer. These results indicate that the virion transcriptase in the pres-

TABLE 1. *Relative molar amounts of the different segments of in vitro and in vivo cRNA*[a]

cRNA	Molar ratio of segments					
	L	M1	M2	M3	S1	S2
In vitro	0.30	0.37	1.0	1.05	2.2	3.0
In vivo	0.11	0.13	1.0	0.40	0.95	3.25

[a] Deadenylated, ApG-primed in vitro cRNA ([^{32}P]-GMP labeled) and deadenylated in vivo cRNA ([^{3}H]-adenosine labeled) were subjected to electrophoresis on cylindrical gels as described in the legend to Fig. 1. The number of counts per minute under each peak in the gels was divided by the estimated molecular weight of the RNA in that peak to yield the molar amounts of each segment. The molecular weight estimates are those of Pons (20) for the vRNA segments. The molar ratios shown represent the average of four preparations of ApG-primed in vitro cRNA and of three preparations of in vivo cRNA.

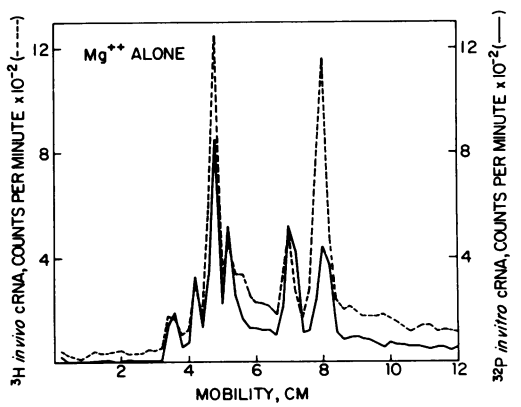

FIG. 2. *Gel electrophoresis of [^{32}P]GMP-labeled, deadenylated in vitro cRNA synthesized without a primer. [^{3}H]adenosine-labeled, deadenylated in vivo cRNA was added to the in vitro cRNA, and the mixture was subjected to electrophoresis on cylindrical gels.*

ence of Mg^{2+} has a real, but very limited, capacity to initiate cRNA chains in the absence of a primer. Once initiated, elongation and poly(A) addition proceed normally.

Comparison of the segments of vRNA, in vitro cRNA, and in vivo cRNA by slab gel electrophoresis. To critically compare the mobilities of the vRNA and cRNA segments, gel electrophoresis was carried out in slabs rather than in cylinders, and the gels were analyzed by autoradiography (Fig. 3). The eight known segments of vRNA were resolved into seven bands (lane 1), as two, rather than the expected three, large-sized (L) RNA segments were resolved. Deadenylated in vitro cRNA (lane 2) was also resolved into seven bands, including two L bands. Each of the in vitro cRNA segments migrated slightly faster than the corresponding segment of vRNA. To confirm this difference in mobility, deadenylated in vitro cRNA and vRNA were mixed, heated and fast-cooled to obviate formation of double strands, and subjected to electrophoresis in the same lane (lane 4). As compared with the pattern of vRNA or in vitro cRNA, a doublet at every position was observed, thus verifying the migration difference between the vRNA and in vitro cRNA segments. This mobility difference could simply reflect a difference in secondary structure between vRNA and in vitro cRNA, because the gel system employed is not totally denaturing. Alternatively, the cRNA segments could be slightly smaller than the corresponding vRNA segments. In vivo cRNA (lane 3) was also resolved into seven segments, and these segments migrated just between the vRNA and in vitro cRNA segments.

Slab gel electrophoresis of double-stranded RNA. To determine whether the difference in mobility between in vitro cRNA and vRNA reflects a difference in size or in secondary structure, we employed a method of analysis in which only size differences are detected: gel electrophoresis of the double-stranded RNA formed by annealing labeled in vitro cRNA to unlabeled vRNA. Because good resolution of double strands requires the removal of poly(A) (7, 21), the in vitro cRNA was first deadenylated with RNase H and poly(dT) before its annealing to vRNA (Fig. 4, lane 1). The two small-sized and three medium-sized hybrids were resolved, but the large-sized hybrids were not clearly resolved into the expected three species. After treatment of the hybrids between deadenylated in vitro cRNA and vRNA with RNase T2 (lanes 2a and b), three distinct large-sized (L) hybrids were resolved. Thus, all three L segments, and hence all eight vRNA segments, were transcribed into cRNA in vitro. Furthermore, it is evident that the deadenylated in vitro cRNA:vRNA hybrids treated with RNase T2 migrate slightly faster than the untreated hybrids. This reduction in size indicates that RNase T2 has hydrolyzed an RNA sequence other than poly(A), because essentially all the latter had already been removed by RNase H

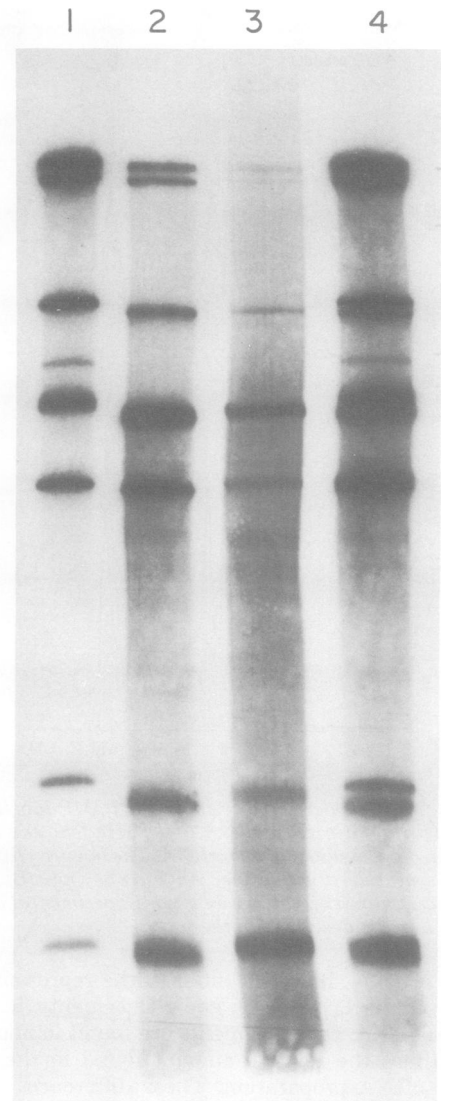

FIG. 3. *Fluorogram of a slab gel electrophoresis of vRNA, in vitro cRNA, and in vivo cRNA. (1) ^{32}P-labeled vRNA; (2) [^{32}P]GMP-labeled, deadenylated in vitro cRNA (ApG primed); (3) [^{3}H]adenosine-labeled, deadenylated in vivo cRNA; (4) mixture of vRNA and deadenylated in vitro cRNA. The extra band seen between two of the middle-sized vRNA segments in lanes (1) and (4) is 18S rRNA, which is found in variable amounts in purified preparations of influenza virus (4, 11, 12).*

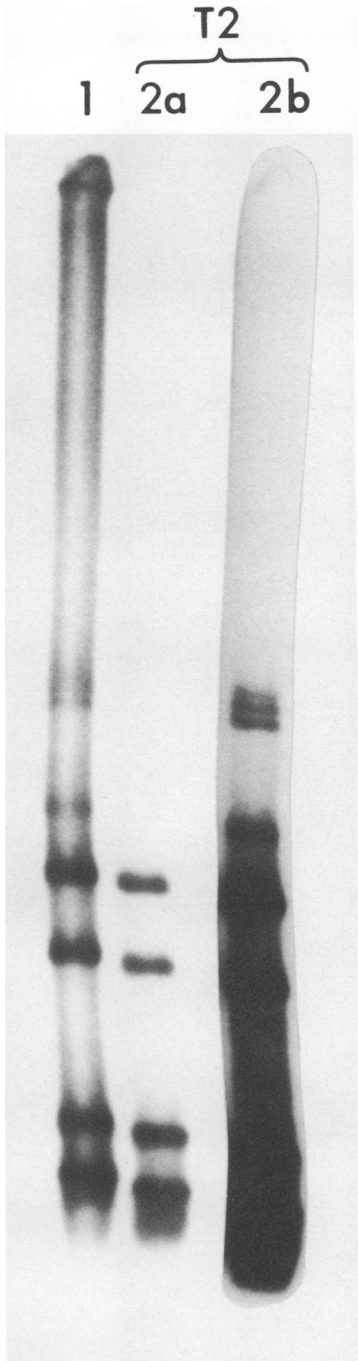

FIG. 4. *Slab gel electrophoresis of double-stranded RNA. (1) Double-stranded RNA formed by hybridizing [^{32}P]GMP-labeled, deadenylated in vitro cRNA (ApG primed) with excess unlabeled vRNA; (2a) as in (1), except that the hybrids were further treated with RNase T2 before electrophoresis; (2b) the material in (2a) after longer exposure of the gel to the X-ray film.*

(see above). This experiment thus provides good evidence that in vitro cRNA is actually smaller in size than vRNA. As a result of the smaller size of each cRNA segment, each hybrid would be expected to contain a relatively short sequence of vRNA that is not in double-stranded form because the complementary sequence is missing from the corresponding cRNA segment. This single-stranded vRNA sequence would be subject to hydrolysis by RNase T2.

The missing sequence in the cRNA segments could be a result of incomplete transcription in vitro or could be an artifact of the RNase H treatment employed in all of the above analyses. Thus, in addition to RNase H removing the poly(A) sequences at the end of the cRNA, it is possible that there is an RNase H-sensitive oligo(A) sequence near either end of the in vitro cRNA molecules. Alternatively, the shorter length of the in vitro cRNA could reflect the action of a contaminating nuclease in the purified RNase H preparation. To distinguish between these two possibilities and incomplete transcription, we compared the mobilities of the double-stranded hybrids treated with RNase H to those not treated with this enzyme. As shown in Fig. 5, the mobilities of the double strands were the same regardless of whether the RNase H treatment was carried out on the in vitro cRNA before (lane 1) or after (lane 2) its annealing to vRNA. After RNase T2 treatment of such hybrids, their mobility increased (lane 3), as already shown in Fig. 4. Figure 5 (lane 4) shows the mobility of hybrids not treated with RNase H. Here, poly(A)-containing in vitro cRNA was hybridized to vRNA, and the resulting double strands were treated with RNase T2 alone to remove the terminal poly(A) sequences of cRNA. If any vRNA sequences were not hydrogen bonded to cRNA, these sequences would also be hydrolyzed. These hybrids had the same mobility as the hybrids treated with both RNase H and RNase T2 (lane 3), demonstrating that the RNase H does not cause a reduction in size in addition to that caused by RNase T2 alone. Therefore, we conclude that the smaller size of in vitro cRNA results from incomplete transcription.

Priming by XpApG trinucleotides. The above observations suggest either that initiation does not occur precisely at the 3′ end of vRNA or that termination occurs before the transcriptase reaches the 5′ end of vRNA. As an initial approach to determine whether in vitro transcription initiates exactly at the 3′ end of vRNA, we examined the ability of trinucleotides of the form XpApG to serve as primers (Table 2). If the trinucleotide acted as primer by hydrogen bonding to the vRNA at some unique internal

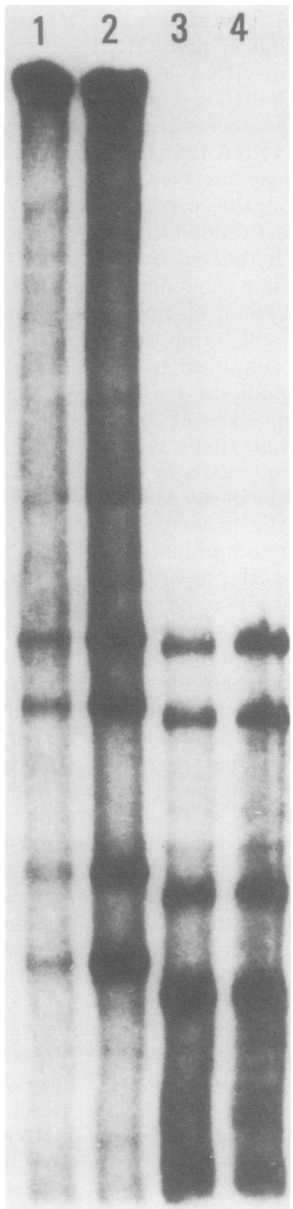

FIG. 5. *Electrophoretic mobility of the hybrids formed between [^{32}P]GMP-labeled in vitro cRNA (ApG primed) and unlabeled vRNA. (1) cRNA was annealed to vRNA, and the hybrids were then treated with RNase H and poly(dT) before electrophoresis. (2) cRNA was first treated with RNase H and poly(dT) and then annealed to vRNA. (3) RNase H-treated hybrids were prepared as in (1) and then further treated with RNase T2. (4) cRNA was annealed to vRNA, and the hybrids were treated with RNase T2 alone. Annealing conditions, enzymatic treatment, and gel electrophoresis of double-stranded RNA were as described in the text. In this exposure of the film, only the small-sized (S) and medium-sized (M) hybrids are seen. With longer exposure, the large-sized (L) hybrids are also seen.*

TABLE 2. *Stimulation of cRNA synthesis in vitro by XpApG trinucleotides*[a]

Oligonucleotide	GMP incorporated (pmol)
None	0.5
ApG	23.7
ApApG	25.6
CpApG	18.0
UpApG	22.0

[a] Reactions were for 1 h at 30°C. ApG was added at 0.22 mM rather than at 0.4 mM (which is optimal) because the trinucleoside diphosphates were added at 0.22 mM, since very limited quantities of the latter were available.

trinucleotide sequence, one of the XpApG trinucleotides would be expected to be a much more effective primer than the others. However, all three of the trinucleotides tested had similar priming activity, suggesting that the 5′-terminal base X is not involved in hydrogen bonding to the vRNA template. These results are consistent with ApG initiating transcription by hydrogen bonding to the 3′-terminal bases of vRNA.

DISCUSSION

Our results indicate that in the presence of Mg^{2+} the influenza virion transcriptase transcribes each of the vRNA segments into cRNA segments of almost the same size. Only a very small amount of these cRNA segments is made in the presence of Mg^{2+} alone, and a specific primer, ApG or GpG, causes about a 100-fold increase in the synthesis of the segments. This almost total dependence on the addition of a primer suggests that a primer RNA is also required for the effective transcription of vRNA in the infected cell. We have already postulated that the synthesis of such a primer RNA may be the actinomycin D- and α-amanitin-sensitive host function (18, 27), which has been shown to be required for in vivo viral RNA transcription (1, 19, 22, 24, 27).

Our gel electrophoretic analyses of single-stranded in vitro cRNA and vRNA and of the in vitro cRNA:vRNA hybrids strongly suggest that the in vitro cRNA segments are incomplete transcripts of the corresponding vRNA segments. One possible explanation for incomplete transcription is that initiation in vitro with ApG might occur internally and not at the 3′ end of vRNA. The results presented here, however, demonstrating the ability of trinucleotides of the form XpApG to prime cRNA synthesis sug-

gest that in vitro transcription with ApG as primer initiates exactly at the 3′ end of vRNA. However, definitive proof requires the demonstration by base sequence analysis that the 3′ end of vRNA and the 5′ end of in vitro cRNA are complementary. Initial experiments suggest that this is the case. We have recently established that the initial 5′ sequence of ApG-primed cRNA is ApGpC (Plotch and Krug, manuscript in preparation), which is complementary to the $GpCpU_{OH}$ 3′ terminus of vRNA (P. Fellner, *in* R. D. Barry and B. W. J. Mahy, ed., *Negative Strand Viruses and the Host Cell*, in press). Initiation of each cRNA segment in vitro by GpG would then be best explained by the ability of GpG to hydrogen bond to the penultimate GpC sequence at the 3′ end of vRNA. It has been shown that when a GpG dinucleotide is bound to a polynucleotide, G-to-G interactions between the dinucleotide and the polynucleotide are at least as strong as the Watson-Crick G-to-C interaction (14). If further evidence verifies that in vitro transcription with ApG initiates exactly at the 3′ end of vRNA, then the apparent smaller size of in vitro cRNA would result from termination of transcription before the 5′ end of vRNA is reached. It will be of considerable interest to identify and sequence the region of the vRNA that is not transcribed.

The in vivo cRNA segments migrate just between the vRNA and in vitro cRNA segments. This difference in mobility could indicate a size and/or a secondary structure difference between the in vivo and in vitro cRNA segments. A secondary structure difference could result from the 5′-terminal cap structure, which is present in in vivo cRNA (12) and absent from in vitro cRNA (11, 18). In fact, using in vitro cRNA primed with ppApG, we recently have found that the addition of a 5′-methylated cap structure, utilizing vaccinia virus capping and methylating enzymes, causes a slight decrease in the mobility of the capped cRNA segments compared with the uncapped segments during gel electrophoresis (Plotch and Krug, manuscript in preparation). It is important to determine whether the in vivo and in vitro cRNA segments are the same size to ascertain whether the incomplete transcription observed in vitro also occurs in vivo. If in vivo transcription is also terminated short of the 5′ end of the vRNA templates, then obvious questions arise as to the mechanism of synthesis of the complete cRNA chains required as templates for vRNA synthesis.

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ADDENDUM IN PROOF

Our recent results strongly suggest that the transcription of vRNA into mRNA in vivo also terminates short of the 5′ end of the vRNA templates. Thus, the electrophoretic mobility of the double strands formed by annealing vRNA to in vivo cRNA is greater after RNase T2 treatment than after RNase H treatment, in a manner exactly analogous to the effects of these nucleases on the vRNA:in vitro cRNA double strands shown in Fig. 5.

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