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In Vitro Synthesis of a Possible Precursor RNA by Purified Vesicular Stomatitis Virus

Douglas Testa and Amiya K. Banerjee

Roche Institute of Molecular Biology, Nutley, New Jersey 07110

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SUMMARY

The RNA products synthesized in vitro by the virion-associated RNA polymerase of purified vesicular stomatitis virus have previously been shown to contain two distinct 5'-terminal sequences. The mRNA species contain the blocked 5'-terminal G(5')ppp(5')A-A-C-A-G sequence and the initiated lead-in RNA segment (approximately 50 bases) contains the unblocked 5' ppA-C-G sequence. In the present studies, using inosine 5'-triphosphate in place of GTP it is shown that RNA species as large as 14.5S contain an unblocked 5'-ppA-C-(I) sequence indicating that the GTP analogue permits synthesis of a possible precursor of viral mRNA in vitro.

INTRODUCTION

Vesicular stomatitis virus (VSV) is a rhabdovirus containing a single stranded genome RNA of negative polarity (1). Purified VSV contains a virion-associated RNA polymerase that synthesizes five mRNA species in vitro that are capped and contain identical sequences at their 5' termini as G(5')ppp(5')A-A-C-A-G (2). Recent in vitro studies have shown that transcription on the viral genome begins directly at the 3' end, with the synthesis of a short leader RNA molecule, followed by the sequential synthesis of the remaining five mRNA species (3). Thus, the gene order of VSV is established as 3'N-NS-M-G-L 5' (4,5), the letters denoting the five structural proteins of the virus (1). In contrast to the mRNA species, the leader RNA is uncapped and contains the 5'-terminal sequence ppA-C-G (6).

The precise mechanism for the generation of these mRNAs is still unclear and two models have been proposed (7). The five mRNA species are synthesized either by (a) independent sequential initiations as the virion RNA polymerase moves along the genome RNA, or (b) a single initiation at the

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3' end of the genome followed by subsequent cleavages, generating mRNAs sequentially from a larger precursor RNA molecule. The capping of the 5' termini and polyadenylation of the 3' termini are linked with this cleavage process. So far, in our laboratory, VSV mRNAs containing uncapped, polyphosphorylated 5'-termini have not been found and RNA molecules containing the leader RNA covalently linked to mRNAs (precursor mRNA) has not been detected (7). Consequently, it is still an open question as to which of the above mechanisms is operative in the VSV system.

The formation of the capped 5' termini of VSV mRNAs presumably involves reaction with GTP and the 5' termini of either the initiated or cleaved mRNA species. In the present studies we have used an analogue of 5'-GTP, inosine 5'-triphosphate (ITP), in an attempt to understand the capping reaction and identify the nature of the 5' terminus of the mRNA species. The high molecular weight RNA products synthesized in vitro in the presence of ITP contain a 5'ppA-C-(I) sequence which is in contrast to that found with 5'-GTP. This indicates that the leader RNA may not have been cleaved during synthesis of some of the mRNA species.

MATERIALS AND METHODS

Purification of Virus: The procedures for the growth and purification of VSV (Indiana serotype) in baby hamster kidney (BHK 21/13) spinner cells, have been described previously (8).

Synthesis and Purification of RNA In Vitro: Standard in vitro RNA polymerase reactions (2 ml) contained 50 mM TRIS-HCl (pH 8.0), 100 mM NaCl, 5 mM $MgCl_2$, 4 mM dithiothretol, 0.05% Triton N101 and the following: 25 μM ITP, 50 μM UTP and 25 μM CTP and 500 μM ATP when labeling with [α^{32}P]-CTP or, [α^{32}P]-ATP. Reaction mixtures were incubated with 1 mCi of the appropriate radioisotope and 150 μg of purified VSV at 30° for 6 hours and terminated by the addition of sodium dodecyl sulfate (SDS) to 0.5%. The RNA was extracted with an equal volume of phenol saturated with acetate buffer (pH 5.0) and the soluble nucleotides were separated from the product RNA by Sephadex G-50 chromatography as described previously (9). mRNAs containing poly(A) were separated from poly(A)$^-$ RNAs by oligo(dT)-cellulose chromatography and sedimented through a linear SDS-sucrose gradient as described previously (5). VSV leader RNA was identified by polyacrylamide gel electrophoresis as described previously (6).

Enzyme Digestions: The conditions used for the enzymatic digestions of VSV RNA with RNAse T_2, nuclease P1, and bacterial alkaline phosphatase (BAP) are described in detail elsewhere (10).

Electrophoretic and Chromatographic Analyses: DEAE-cellulose chromatography in 7M urea and high voltage paper electrophoresis in a pyridine-acetic acid-water (1:10:89) system at pH 3.5 have been described previously (10).

Chemicals and Enzymes: ITP was purchased from Miles Laboratories, Ind.; RNAse T2 was from Sanyko Co., Japan, Nuclease P1 was from Yamasa Shoya Co., Japan and BAP was from Worthington, Mass. Oligo (dT)-cellulose was purchased from Collaborative Research, Mass.

RESULTS

RNA was synthesized with [α^{32}P]-ATP in a standard reaction mixture containing ITP in place of GTP. The product RNAs were purified and separated into poly(A)$^+$ and poly(A)$^-$ RNA species and co-sedimented through linear SDS-sucrose gradients with [^{3}H]-UMP labeled marker VSV mRNAs synthesized *in vitro* in the presence of GTP to determine their sizes. The base composition analysis of the RNA products showed that the RNA was totally substituted with IMP (data not shown). Figure 1 demonstrates that while 80-85% of RNA synthesized in the presence of ITP lacks poly(A) (Figure 1B), the poly(A)$^+$ IMP substituted RNA is similar in its sedimentation profile to GMP containing RNA (Figure 1A). The lack of coincidence of the two preparations of poly(A)$^+$ RNA in the same gradient (Figure 1A) suggests that conformational differences may exist between the IMP- and GMP- substituted RNA.

Before analyses of the 5'-terminal sequences of the poly(A)$^+$ or Poly(A)$^-$ RNA species, it was important to test for possible contamination of leader RNA in IMP-substituted high molecular weight RNA species. The RNA from the sucrose gradients was pooled into poly(A)$^+$ RNA (Figure 1A, fractions 10-20), poly(A)$^-$ high molecular weight RNA (Figure 1B, pool A) and poly(A)$^-$ low molecular weight RNA (Fig. 1B, pool B). Each pool of RNA was precipitated with ethanol and the RNA was analyzed by electrophoresis in 20% polyacrylamide gels with [^{3}H]-labeled leader RNA marker. Figure 2 panels A and B show that the RNA in both high molecular weight regions of a sucrose gradient, i.e. poly(A)$^+$ and poly(A)$^-$ pool A, do not contain free leader RNA species comigrating with the [^{3}H]-labeled leader RNA marker. RNA isolated from the slower sedimenting region of the poly(A)$^-$ sucrose gradient in Figure 1B however, demonstrates the

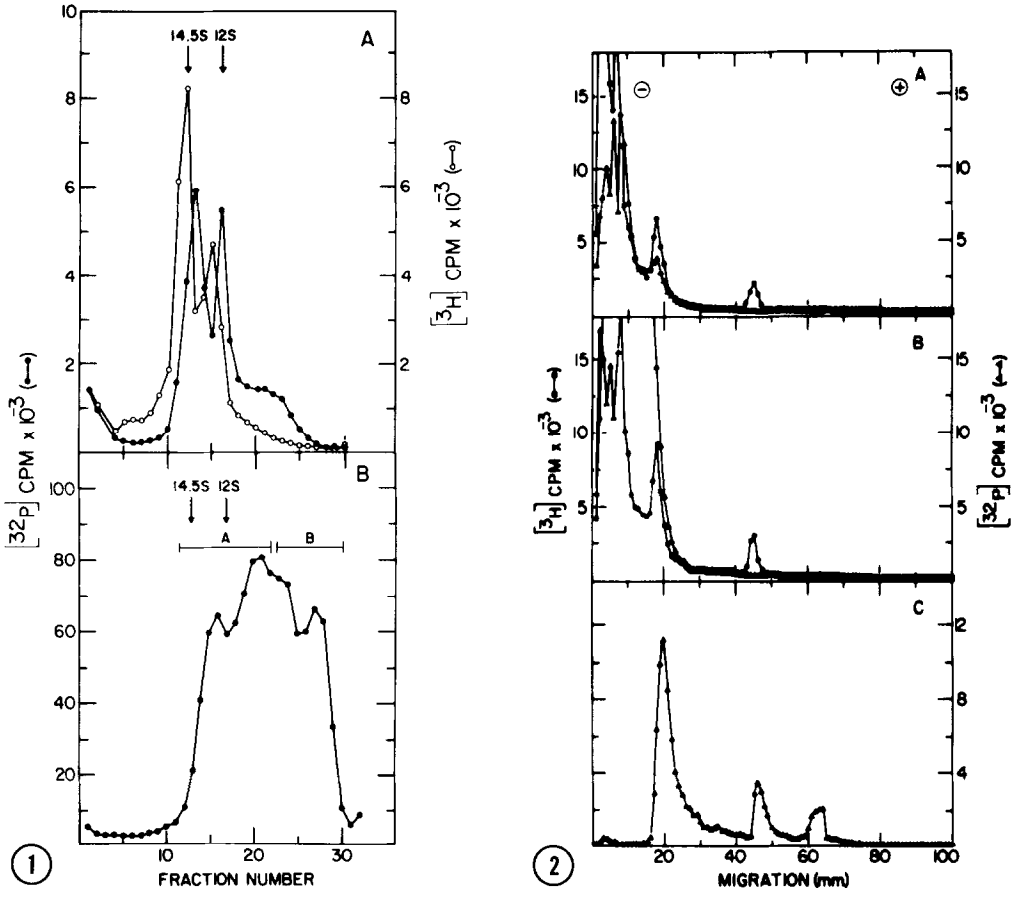


Figure 1. Velocity sedimentation analysis of IMP-substituted RNA products synthesized in the presence of [α^{32}P]-ATP.

The reaction was labeled with 1.0 mCi [α^{32}P]-ATP in the presence of 25 μM ITP as described. The purified product RNA was separated into poly(A)$^+$ and poly(A)$^-$ species by binding to and subsequent elution from an oligo(dt)-cellulose column. An aliquot of the bound and unbound RNAs were mixed with [^{3}H]-UMP labeled VSV-marker poly(A)$^+$ RNA and cosedimented through linear 15-30% sucrose gradients at 33,000 rpm. The gradients were fractionated and the total radioactivity in each fraction was determined. IMP-substituted RNA (A) poly (A)$^+$, (B) poly(A)$^-$. -●—●- Represents the [^{32}P] and -0--0 the [^{3}H] radioactivities.

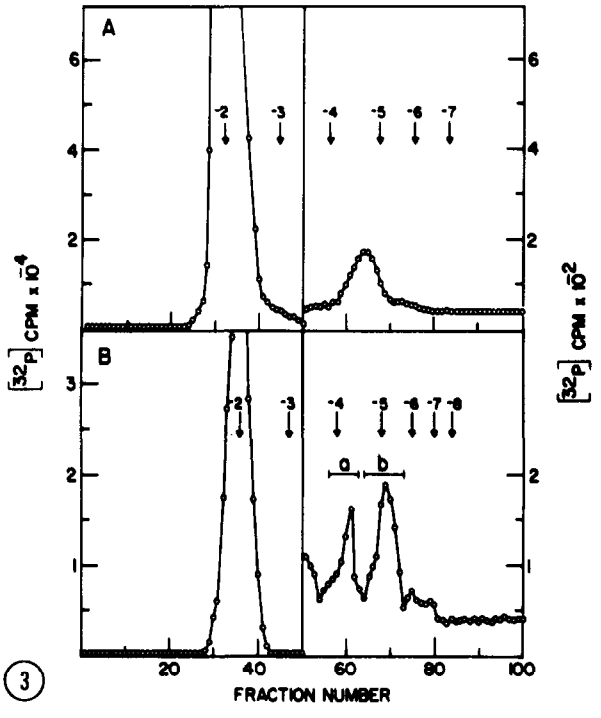
Figure 2. Polyacrylamide gel electrophoresis of [^{32}P]-labeled IMP-substituted RNA.

[^{32}P]-labeled IMP-substituted RNA, pooled from SDS-sucrose gradients was precipitated and electrophoresed in a 20% polyacrylamide gel with [^{3}H]-UMP labeled marker RNA. (A) High molecular weight IMP-substituted poly(A)$^+$RNA, (B) High molecular weight IMP-substituted poly(A)-RNA, (C) Low molecular weight IMP-substituted poly(A)-RNA. The symbol -Δ--Δ- represents the [^{32}P]-labeled IMP-substituted RNA and -●—● the [^{3}H]-labeled UMP marker RNA preparation.

presence of low molecular RNAs (Fig. 2C). The middle peak was confirmed to be the leader RNA by the presence of 5'-terminal ppA-C-(I) sequence. The first and the last peak are mRNA and leader RNA fragments, respectively (data not shown). These analyses eliminate the possibility of a leader RNA nonspecifically associated with and co-sedimenting with a high molecular weight RNA species.

In order to determine the 5'-terminal sequence of the high molecular weight species (12-18S, Fig. 1A), IMP-substituted poly(A)$^+$ RNA was labeled with either [α^{32}P]-ATP or [α^{32}P]-CTP and digested to completion with RNase T2. The digests were applied to a DEAE-cellulose column and eluted with a linear salt gradient in 7M urea. As shown in Figure 3A the [α^{32}P] CMP-labeled RNA displayed a major peak of radioactivity eluting at -2, in addition to a minor peak of radioactivity eluting between charges -4 to -4.5. In contrast, the [α^{32}P] AMP-labeled product released, in addition to the major peak eluting at -2 charge, two minor peaks eluting between charges -4 to -4.5 (Peak a) and -5 to -5.5 (Peak b). Similar profiles were also obtained when [α^{32}P]-AMP or [α^{32}P] CMP-labeled poly(A)$^-$ high molecular weight RNAs were digested with RNase T2 and analyzed by DEAE-cellulose chromatography as described above (data not shown). The radioactivity eluting between -4 and -5 charges (Fig. 3A) was recovered and a portion treated with bacterial alkaline phosphatase (BAP) and analyzed by paper electrophoresis. As shown in Fig. 4A, the untreated material migrated slightly ahead of the ATP marker, however following digestion with BAP all the radioactivity was rendered as Pi. These results indicate that the 5'-terminal sequence of the [α-^{32}P] CMP-labeled IMP product is pp$\overset{*}{A}$pC. In separate experiments not shown, [α-^{32}P] CMP-labeled IMP product was treated with RNase T1 and the oligonucleotides separated by DEAE-cellulose chromatography. The radioactivity eluting between -6 and -7 released pp$\overset{*}{A}$p after RNase T2 digestion indicating that the third base is inosine which is consistent with the sequence ppA-C-(I).

In order to determine the 5'-terminal sequences of the [α-^{32}P] AMP-labeled IMP product, both peaks a and b (Fig. 3B) were pooled separately, dialyzed, lyophylyzed, and digested with BAP prior to electrophoretic analyses. It can



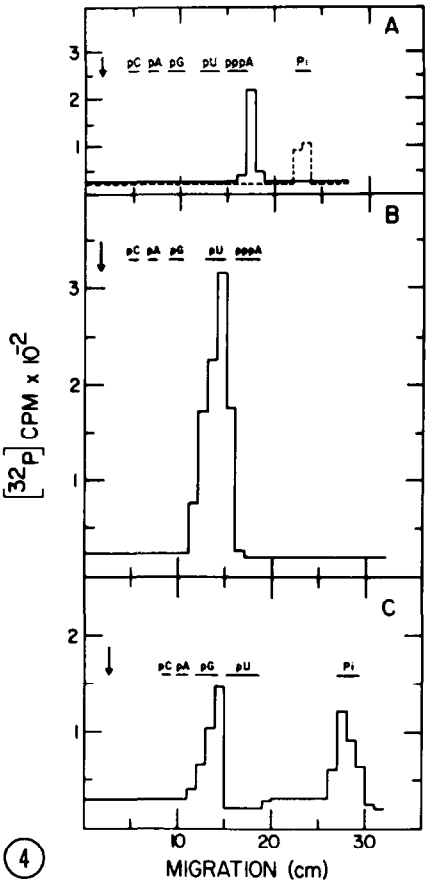


Figure 3. DEAE cellulose chromatography analyses of poly(A)+ RNA.

[^{32}P] labeled poly(A)+ IMP-substituted RNA species purified as described in Materials and Methods was digested to completion with RNase T2. The digested RNA was bound to and eluted from a DEAE cellulose column with a linear gradient of NaCl, 0 to 0.3M, containing 50mM Tris-HCl, pH 8 and 7M Urea. An unlabeled pancreatic RNase digest of yeast RNA was included as the marker. IMP-substituted RNA labeled with [α^{32}P]-CMP (A) and [α^{32}P]-AMP (B).

Figure 4. Paper electrophoresis analyses of enzymatically digested peak fractions from the DEAE cellulose column.

Fractions eluting from a DEAE cellulose column at a high net negative charge, (i.e. -4, -5), were pooled, dialyzed and concentrated as described and analyzed electrophoretically in a pyridine-acetate buffer pH 3.5 system with marker nucleotides. The radioactivity eluting between -4 and -5 in figure 3A (see text page 4) before (solid line) and after (dotted line) treatment with alkaline phosphatase. Peak b of Figure 3B: (B) before and (C) after treatment with alkaline phosphatase. The positions of the origin (arrow) and marker nucleotides are as indicated.

be seen in Figure 4B that the undigested peak B material migrated with the marker p while the same material, when digested with BAP (Fig. 4C) released two peaks

of equal radioactivity, one migrating with Pi and the other between pG and pU markers. By a series of experiments not shown using nucleotide pyrophosphatase and BAP, the latter material was found to contain an inverted 5'-5' pyrophosphate linkage in the oligomer with the structure (Fig. 3B) $(I)^{5'}ppp^{5'}A\overset{*}{p}$. The radioactivity eluting in peak a (Fig. 3B) was treated similarly and the results show that all of the radioactivity was rendered as Pi when digested with BAP and furthermore, nuclease P1 digestion of the sample followed by electrophoresis shows that the radioactivity comigrates with a 5' ADP marker (data not shown). Hence the structure of 5' terminus eluting in peak a (Fig. 3B) is $5'p\overset{*}{p}Ap...$. Table 1 summarizes the data obtained from the 5'-terminal analyses of both poly(A)$^+$ and poly(A)$^-$ IMP-substituted high molecular weight RNAs labeled with [α^{32}P]-ATP or CTP. Only one type of terminus, 5'ppAp, is observed with [α^{32}P]CMP-labeled RNA indicating that the 5'-sequence is $pp\overset{*}{A}pC$. However [α^{32}P]-AMP labeled RNA permits the identification of both capped and uncapped termini, i.e. $(I)^{5'}ppp^{*5'}A\overset{*}{p}A$ and $5'\ p\overset{*}{p}Ap$ sequences in total poly(A)$^+$ RNA (Fig. 1A). The results were similar when two individual peaks of radioactivity (Fig. 1A) were analyzed separately for those 5'-termini. These results indicate that the use of ITP in place of GTP during *in vitro* transcription by VSV results in the synthesis of high molecular weight RNAs, some of which contain the 5'-terminal sequence ppA-C-(I). Previously this sequence was found to be present only in the small leader RNA molecule (5).

DISCUSSION

In this communication we have demonstrated that 5' ITP--an analogue of 5' GTP can be used as substrate for RNA synthesis by the virion-associated RNA polymerase of VSV. The overall incorporation of radioactive nucleotides into RNA in the presence of ITP is approximately 15% of that synthesized in the presence of 5' GTP. The RNA products synthesized *in vitro* contain poly(A) (15% of the product) and the poly(A)$^+$ and poly(A)$^-$ products sediment around 14.5S and between 4S to 14.5S, respectively (Fig. 1). The 5'-terminal analyses of the high molecular weight poly(A)$^+$ and poly(A)$^-$ RNA species (>12S) revealed two types

Table I

5' Termini of IMP Substituted Products

[α-^{32}P] Nucleotide	RNA Species	Proportion of Total Radioactivity[a]	Relative Proportion of Radioactivity in Each Terminus[b]	5'-Terminus[c]
ATP	poly(A)$^+$	0.10	40%	5'p$\overset{*}{p}$Ap
		0.30	60%	(I)$^{5'}$pp$\overset{*}{p}^{5'}$A$\overset{*}{p}$
	poly(A)$^-$	0.08	30%	5' p$\overset{*}{p}$Ap
		0.37	70%	(I)$^{5'}$pp$\overset{*}{p}^{5'}$A$\overset{*}{p}$
CTP	poly(A)$^+$	0.06	100%	5'ppA$\overset{*}{p}$
	poly(A)$^-$	0.08	100%	5'ppA$\overset{*}{p}$

[a]Determined from the distribution of radioactivity eluted from a DEAE column.
[b]Relative quantities of each terminus with the appropriate label.
[c]Asterisks denote radioactive phosphates.

of termini: capped 5'-termini with the structure (I)$^{5'}$ppp$^{5'}$ A-A and uncapped, polyphosphorylated structure ppA-C-(I). The RNA products that contain the blocked 5'-termini presumably represent the mRNA species since the nucleotide sequence of the RNA chain begins with A-A similar to that found in the VSV mRNA species containing GMP (2).

It should be noted that due to the unavailability of [α-^{32}P] ITP, it is difficult to demonstrate conclusively that inosine is indeed the blocking base. In this respect we have put I within parentheses throughout this paper. However, the faster migration of the T2-cap (Fig. 3B) with respect to G$^{5'}$ppp$^{5'}$Ap or A$^{5'}$ppp$^{5'}$Ap indicates that the material must contain inosine. We have also attempted unsuccessfully to methylate (12) the 5'-blocking base *in vitro* using S-adenosyl methionine since only 2'-O methyladenosine was recovered as the methylated residue (data not shown). These results indicate that the methyltransferases present in the purified virion may be specific only for G and A residues (12) and furthermore inosine can be used as a blocking base if replaced by guanosine. So far, only guanosine has been found to be the blocking base at the 5'-terminus of eukaryotic RNAs (11).

The interesting observation is that some of the IMP-containing RNA products (>12 S) contain the 5'-terminal sequence ppA-C-(I). This sequence has previously been shown to be present only in the low molecular weight leader RNA (6). These results taken together indicate that in the presence of ITP, the VSV RNA polymerase synthesizes in vitro RNA products which contain leader RNA covalently associated with mRNA. Since the gene order of VSV is established as 3' leader RNA-N-NS-M-G-L 5' (4,5), the above results indicate the leader RNA is most probably associated with the adjacent mRNA, namely the N protein mRNA. In fact, hybridization-competition experiments with the poly(A)$^+$ RNA products (Fig. 1A) show that the most predominant RNA species is the 14.5 S RNA or N protein mRNA (data not shown). Thus, it seems that some of the virion-associated RNA polymerases in the presence of ITP, read through the putative "processing" site and fail to further modify the synthesized RNA generating a larger unprocessed RNA, e.g. leader RNA covalently linked to the 14.5S mRNA. Read-through of transcription termination site in the presence of ITP has been documented in prokaryotic system (13) and occurs very efficiently in IMP-substituted RNAs. The ability to isolate both processed and unprocessed high molecular weight VSV RNA, may be due to a slow down in the cleavage and the capping processes caused by the presence of an IMP residue. It therefore, appears that biosynthesis of the VSV mRNA species may involve cleavage at specific sites on a growing precursor RNA chain.

REFERENCES

1. Wagner, R. R. (1975). In Comprehensive Virology, ed. by Fraenkel-Conrat, H. and Wagner, R. R. Vol. 4, 1-161, Plenum Press, New York.
2. Rhodes, D. P., and Banerjee, A. K. (1976). J. Virol. 17, 33-42.
3. Abraham, G., Colonno, R. J., and Banerjee, A. K. (1976). In Animal Virology, ed. by Baltimore D., Huang, A. S. and Fox, F, pp. 439-455, Academic Press, New York.
4. Ball, L. A., and White, C. N. (1976). Proc. Nat. Acad. Sci. U.S.A. 73, 442-446.
5. Abraham, G., and Banerjee, A. K. (1976). Proc. Nat. Acad. Sci. U.S.A. 73, 1504-1508.
6. Colonno, R. J., and Banerjee, A. K. (1976). Cell, 8, 197-204.
7. Banerjee, A. K., Abraham, G., and Colonno, R. J. (1977). J. Gen. Virol. 34, 1-8.
8. Banerjee, A. K., Moyer, S. A., and Rhodes, D. P. (1974). Virology 61, 547-558.

9. Colonno, R. J., and Banerjee, A. K. (1977). Virology 77, 260-268.
10. Abraham, A., Rhodes, D. P., and Banerjee, A. K. (1975). Cell 5, 51-58.
11. Shatkin, A. J. (1976). Cell 9, 645-653.
12. Testa, D., and Banerjee, A. K. (1977). J. Virol. 24, 786-793.
13. Lee, F., and Yanofsky, C. (1977). Proc. Nat. Acad. Sci. U.S.A. 74, 4365-4369.