THE 5' SEQUENCE OF VSV VIRAL RNA AND ITS IN VITRO TRANSCRIPTION PRODUCT RNA.

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Received June 30,1975

SUMMARY: The 5' terminal sequence of vesicular stomatitis virus (VSV) RNA (pppApCpGp...) is not capped, however in the viral nucleocapsid it is protected from pancreatic ribonuclease digestion, as are most or all of the internal sequences of the viral RNA. Some of the 5' in vitro transcription product sequences synthesized in the absence of S-adenosyl-L-methionine, SAM, are capped. In the presence of SAM those sequences which are capped, become methylated.

INTRODUCTION

Vesicular stomatitis virus, Indiana serotype, possesses the 5' nucleotide sequence pppApCpGp.. (1). In the virus particle the RNA is associated with a 10-to 20-fold greater mass of N protein as well as with two minor virion proteins, NS and L (2). We show here that the 5' sequence is protected against nuclease removal by the N protein. VSV possesses its own RNA-dependent RNA polymerase enzymes which are capable of completely transcribing the virion genome (either in vitro or in vivo) into viral complementary RNA species (3,4,5). At least some of the viral complementary RNA functions as mRNA in in vitro translation systems (6, 7). In previous publications, we reported that the direction of product RNA synthesis occurs in a 5' to 3' mode (6), and we characterized (8, 9) four distinct 5' initiation sequences (pppApCpGp.., pppApApPypXpGp.., pppGpCp.., and pppGpGpCp.., where Py is a pyrimidine and X is either A, C, or U). Recent analyses

(10, 11) have indicated that some VSV product RNA species are capped and can be methylated by S-adenosyl-L-methionine (SAM). We report here that only certain product RNA species are capped and that capping is rate-limiting to subsequent methylation when SAM is present.

MATERIALS AND METHODS

Both [^{3}H]uridine (20 Ci/mmole) and ^{32}P phosphate were obtained from I. C. N., Irvine, California. [α-^{32}P]purine ribonucleoside triphosphates were obtained from N. E. N. Boston, Mass. [γ-^{32}P]ATP or [γ-^{32}P]GTP were synthesized as described previously (12). S-adenosyl-L-methionine was purchased from Boehringer Mannheim, New York. The production and purification of ^{32}P or [^{3}H]uridine labeled VSV, Indiana serotype, and extraction of RNA have been described (1). VSV transcription assays were incubated and their reaction products purified and analyzed as reported previously (8, 9). Alkaline phosphatase treatment of nucleotides involved incubating a sample in 1 ml of 0.01 M Tris-HCl buffer, pH 8.0, 0.001 M $MgCl_2$ with 3.5 units of alkaline phosphatase (BAPF, Worthington Biochem. Corp., Freehold, N. J.) at 37° C for 30 min.

RESULTS AND DISCUSSION

The viral 5' nucleotide sequence is protected by N protein in the nucleocapsid. A sample of purified ^{32}P labeled VSV Indiana was dissociated with 1% Triton N101 and 1 M NaCl. The viral RNA-N protein complex was freed from the viral G, M, NS and L proteins by centrifugation (13). The RNA-N complex was then suspended in 0.001 M Tris-HCl buffer, pH 8.0, verified by protein gel electrophoresis to be free of other viral proteins (2), and aliquots mixed with a sample of 2 x 10^4 cpm of ^{3}H-labeled viral RNA and either centrifuged directly on a gradient of sucrose or treated with ribonuclease and then centrifuged (Fig. 1). It was found that although the ^{3}H RNA was rendered completely acid-soluble, the profile of the ^{32}P-RNA-N protein complex remained essentially unchanged. The rest of the original RNA-N protein complexes (7.5 x 10^5 cpm) was mixed with 2 x 10^4 cpm of ^{3}H-viral RNA, digested with ribonuclease and centrifuged as before. The slowly-sedimenting material recovered at the top of the gradient (pool 2) and

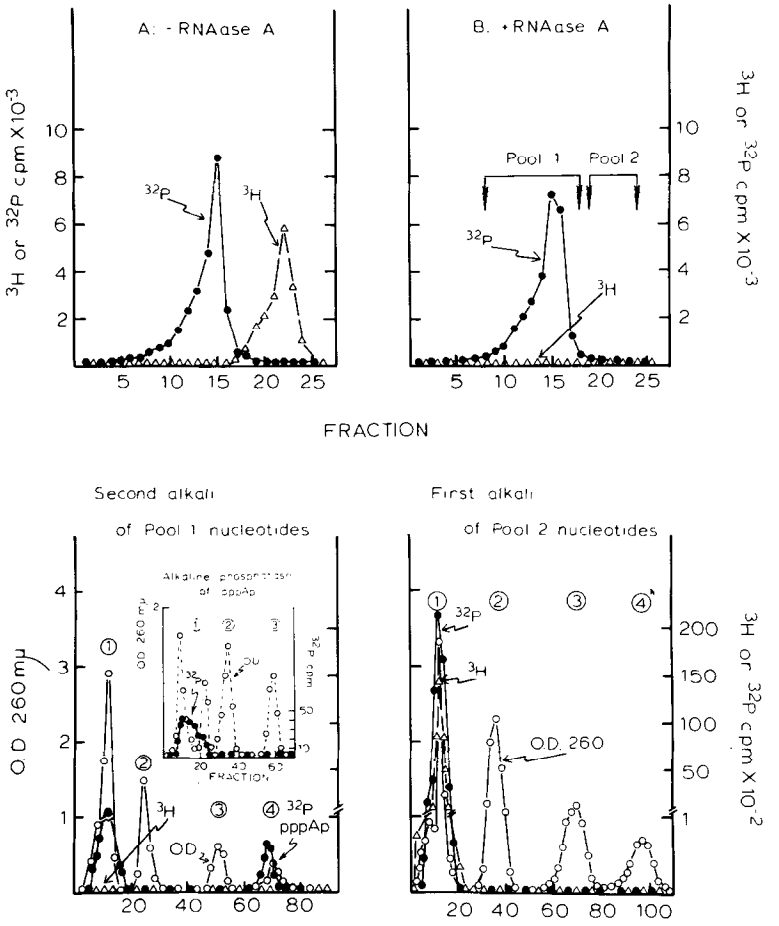


FIG. 1. The effect of pancreatic ribonuclease on VSV nucleocapsids. ^{32}P-labeled VSV nucleocapsids (RNA + N protein) were mixed with purified ^{3}H VSV RNA (see text) and either (A), centrifuged on a 70% - 15% gradient of sucrose containing 1 M NaCl, 0.01 N Tris, pH 7.4, or (B) incubated with 10 μg ribonuclease per ml of 0.001 M Tris-HCl buffer pH 7.4 (containing 100 μg chick embryo RNA) for 10 min at 20° C, then centrifuged. In each case the recovery of acid-insoluble material was determined. From a similar experiment involving ribonuclease treatment of 7.5 x 10^5 cpm of ^{32}P nucleocapsids, material was recovered and treated as described in the text. The proceeds were resolved by DEAE cellulose column chromatography with marker nucleotides derived from a pancreatic ribonuclease digest of chick embryo RNA (1). A 5' terminal nucleotide (pppAp) from ^{32}P labelled VSV RNA was obtained by alkali digestion as described previously (1), subjected to alkaline phosphatase treatment and the proceeds likewise resolved on DEAE cellulose (insert, bottom left panel).

that in the nucleocapsid region (pool 1), were extracted by phenol and quantitatively precipitated by addition of saturated $BaCl_2$ and alcohol (1). Following alkali digestions and DEAE cellulose chromatography, only the pool 1 material was found to possess the alkali resistant pppAp sequence (Fig. 1). Note that the pool 2 nucleotides contained ^3H mononucleotides, as well as some ^{32}P mononucleotides, the amount of which corresponded closely to the amount of acid-insoluble ^{32}P cpm in the pool 2 fraction. When a 5' terminal pppAp sequence was isolated by alkali digestion of ^{32}P labeled viral RNA (1) and treated with alkaline phosphatase, all of the label was converted into inorganic phosphate (Fig. 1, insert). From these results it was confirmed that the viral RNA pppAp sequence is uncapped (1) but, in the nucleocapsid complex (RNA-N), it is protected from pancreatic ribonuclease release by the viral N protein.

Heterogeneity of transcript initiation, capping and methylation of certain VSV product RNA species. VSV transcription product RNA labeled by [γ-^{32}P] ATP or [γ-^{32}P] GTP, was purified as described previously (6) and, for the ATP labeled product, digested with alkali or, for the GTP labeled product, ribonuclease T_1 (Figure 2). As shown previously, both ATP and GTP labeled termini were obtained (8). After phosphatase treatment, in each case the label was recovered as inorganic phosphate (Fig. 2 inserts). We conclude therefore that these sequences are uncapped product termini which represent the initial stage of RNA synthesis. Since evidence has been presented (11) which indicates that 11-18S VSV transcription product RNA have capped and methylated sequences (viz. m^7GpppAmpAp...), we have conducted product analyses to determine how much of the product we synthesize is capped and how many of those sequences subsequently become methylated.

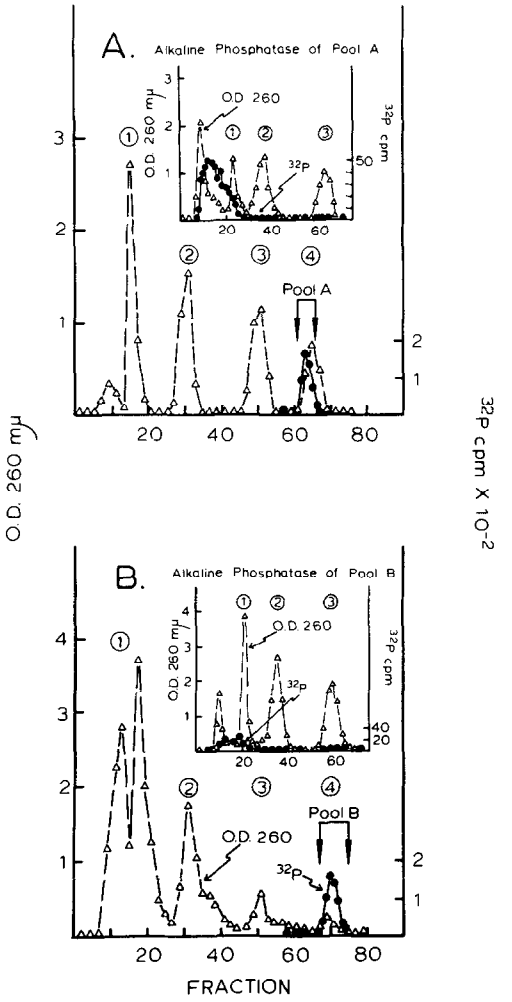
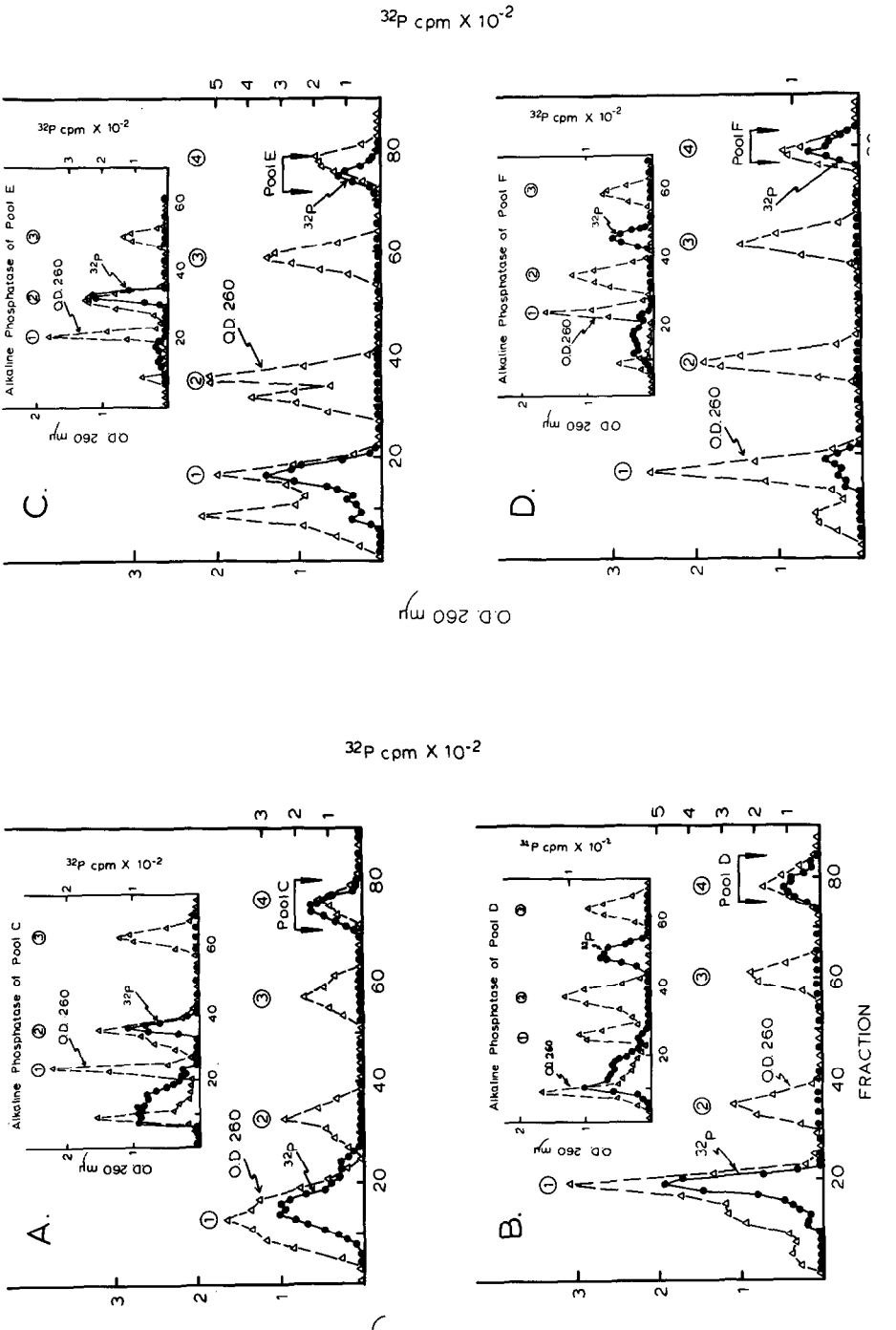


FIG. 2. Analysis of (A) [γ-^{32}P]ATP or (B) [γ-^{32}P]GTP labeled VSV transcription product RNA. Purified samples of 8 x 10^2 cpm of transcription product (8) were digested with alkali (A) or ribonuclease T_1 (B) and the nucleotides resolved by DEAE cellulose chromatography (1) with suitable marker nucleotides (Fig. 1).

VSV transcription product RNA labeled by [α-^{32}P]ATP or [α-^{32}P]GTP in the presence or absence of SAM, was treated with two cycles of alkali digestion and chromatographed on DEAE cellulose at pH 8.0 (Fig. 3). The labeled termini were recovered from the tetranucleotide isoplith, treated with alkaline phosphatase and rechromatographed (Fig. 3, inserts). For reaction


32P cpm X 10-2
O.D. 260 mμ
A.
B.
C.
D.
Alkaline Phosphatase of Pool C
Alkaline Phosphatase of Pool D
Alkaline Phosphatase of Pool E
Alkaline Phosphatase of Pool F
Pool C
Pool D
Pool E
Pool F
O.D. 260
32P
FRACTION

mixtures lacking SAM, both inorganic phosphate and a phosphatase resistant nucleotide was obtained which eluted with the dinucleotide marker. For reaction mixtures containing SAM, both inorganic phosphate and a phosphatase resistant residue was obtained which eluted from the column after the dinucleotides. In the latter case no nucleotide sequences were detected which eluted with the dinucleotides. It was concluded therefore that some of the 5' sequences were capped, and that in the presence of SAM, all of the capped sequences were subsequently methylated. In the labeled ATP experiments (± SAM), in each case there was more ^{32}P released by phosphatase than protected from removal; therefore it was concluded that there was a heterogeneous mixture of product termini with more uncapped sequences by comparison to capped sequences. Although less phosphate was removed from the GTP labeled sequences, it was concluded that both capped and uncapped sequences were present, a conclusion consistent with the pppGp initiations described above, as well as the guanosine nucleotide capping (11) identified on some product species (m^7GpppAmpAp...).

DISCUSSION

The results obtained are compatible with a scheme of RNA synthesis involving initiation with purine nucleoside triphosphates, subsequent capping and methylation at the 5' end. The capping appears to be rate-limiting to subsequent methylation. Which sequences are capped and which are not is under investigation.

FIG. 3. Analysis of VSV product labeled (A, B) by [α-^{32}P]ATP or (C, D) [α-^{32}P]GTP. VSV transcription product was obtained from reaction mixtures with (B, D) or without (A, C) SAM. About 8 x 10^5 cpm of ATP or 6 x 10^5 cpm of GTP-labeled product were used for each analysis.

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