JOURNAL OF VIROLOGY, May 1974, p. 1055-1060
Vol. 13, No. 5

Polyadenylation of Vesicular Stomatitis Virus mRNA

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Received for publication 2 January 1974

Vesicular stomatitis virus (VSV) mRNA isolated from infected cell polysomes contains polyadenylic acid [poly(A)] sequences. Detergent-activated purified virions in vitro can transcribe complementary RNA, which has sedimentation properties similar to mRNA, and this RNA also contains poly(A) sequences. Digestion of virion RNA with U2 RNase under conditions where hydrolysis is specific for purine linkages leaves no sequences of polyuridylic acid corresponding in length to the poly(A) on the transcripts. Growth of infectious virus is not inhibited by 3-deoxyadenosine (cordycepin) under conditions in which it inhibits polyadenylation of cellular mRNA. The virus-specific mRNA produced in the presence of cordycepin has poly(A) sequences of the same size distribution as that synthesized in the absence of cordycepin.

Vesicular stomatitis virus (VSV) contains a single-stranded RNA genome with a molecular weight of approximately 3.6×10^6 (13, 20). During the replication cycle, virus-specific mRNA, which is smaller than and complementary to the RNA contained in the virion, is synthesized in the cytoplasm (12, 19, 25, 31). At least part of this mRNA is synthesized by an RNA transcriptase which is present in purified virions and which catalyzes the synthesis of RNA species in vitro (3). The product RNA species contain sequences which are complementary to the entire VSV genome (5) and which have sedimentation properties similar to the 13-15*S* VSV mRNA obtained from polysomes in vivo (6).

Previous work has demonstrated that mRNA isolated from VSV-infected HeLa cells contains polyadenylate [poly(A)] sequences (9). These sequences are present even when infection is carried out in the presence of cycloheximide (27). Under these conditions, virus-specific protein synthesis and virion RNA replication are inhibited, but primary transcription of the infecting genome by the virion-associated transcriptase apparently occurs, and the RNA synthesized in the inhibited cells is polyadenylated. Recently, Villarreal and Holland (30) and Bannerjee and Rhodes (4) examined the RNA product synthesized in vitro by detergent-activated purified virions or by nucleocapsids purified after removal of the virus envelope (28). Their results demonstrate that poly(A) sequences are present covalently attached to the in vitro product RNA and that they comprise the same size distribution as those sequences synthesized in vivo.

Similar poly(A) sequences are present in animal cell mRNA and heterogeneous nuclear RNA. The cellular polyadenylation reaction occurs post-transcriptionally in the nucleus (7), perhaps catalyzed by an enzyme present in nuclear ribonucleoprotein particles containing and processing mRNA precursor molecules (21). Since VSV replicates in the cytoplasm of its host cell, and since RNA synthesized in vitro as well as in vivo by purified virions already contains poly(A) sequences, it is important to determine the origin of the poly(A) polymerizing activity. In this report, we demonstrate that the virion RNA does not contain polyuridylic acid [poly(U)] sequences corresponding in length to the poly(A) in the transcription product, and show that the activity responsible for poly(A) addition to VSV mRNA is apparently different from the corresponding cellular activity that adds poly(A) to HnRNA.

MATERIALS AND METHODS

Cells and virus. Suspension cultures of HeLa S_3 cells were grown at a density of 4×10^5 to 8×10^5 cells per ml in Eagle minimal essential medium supplemented with 7% calf serum. Growth, purification, and plaque assay of the Indiana serotype of VSV have been described (20).

Preparation of radioactive VSV RNA. HeLa cells (5×10^8) were concentrated to a density of 5×10^6 cells per ml and infected with VSV at a multiplicity of 10 PFU/cell. Actinomycin D (gift of Merck, Sharp and Dohme) was added to 5 μg/ml at 1 h p.i., and 1 mCi of [^{3}H]adenosine (5 Ci/mmol) or [^{3}H]uridine (20 Ci/mmol; New England Nuclear Corp.) was added at 1.5 h postinfection (p.i.). Cultures were incubated overnight at 32 C, and virus was purified and stored frozen in 10% dimethylsulfoxide. For isolation of the RNA,

samples were thawed, made 1% in sodium dodecyl sulfate (SDS), and sedimented through sucrose gradients. Gradient fractions were collected through a Gilford automatic recording spectrophotometer, samples of each fraction were transferred directly into scintillation vials and counted in a Beckman scintillation spectrometer, and the appropriate fractions were pooled, precipitated with 2 volumes of ethyl alcohol in the presence of 100 μg of yeast RNA, and stored frozen.

Preparation of intracellular VSV RNA. HeLa cells were concentrated to a density of 5×10^6 cells/ml and infected with VSV at a multiplicity of 10 PFU/cell. Actinomycin D was added to 5 μg/ml at 1 h p.i., and the cultures were diluted with medium to a density of 10^6 cells/ml to reduce readsorption of released virus. [^{3}H]uridine (2 μCi/ml) was added at 1.5 h p.i., and the cells were incubated at 37 C for a further 4.5 h. The cells were sedimented, washed twice with Earle salts solution, suspended in hypotonic buffer (0.01 M Tris, pH 7.4, 0.01 M NaCl, 0.0015 M $MgCl_2$) and allowed to swell for 5 min before disruption in a Dounce homogenizer. Cell breakage was monitored by phase microscopy and was 95% complete. Nuclei were removed by centrifugation, and the cytoplasmic extract was made 1% in SDS and layered over sucrose gradients. Cellular RNA served as absorbance markers. Samples of each gradient fraction were analyzed for radioactivity, and the appropriate samples were pooled, precipitated with ethyl alcohol, and stored at −20 C.

Preparation of virion-associated transcriptase product RNA. The reaction conditions for VSV-associated transcriptase activity were essentially those described by Bishop and Roy (6). Reaction mixtures were incubated at 28 C and terminated by adjusting to 1% SDS. Samples were diluted with 0.05 M sodium acetate, pH 5.0, containing 0.01 M EDTA and extracted twice at 65 C with phenol:chloroform (1:1). The aqueous phases were pooled, and 25 μg of yeast RNA was added. Samples were adjusted to 0.2 M NaCl, and RNA was precipitated with 2 volumes of ethyl alcohol at −20 C.

RNase digestion. U2 RNase was received lyophilized as a kind gift from Tsuneko Uchida at the Mitsubishi-Kasei Institute of Life Sciences, Tokyo, Japan (2). The enzyme was dissolved in distilled water and stored frozen at 0.1 A_{280}, equivalent to 75 U/ml. One enzyme unit is defined as the amount of enzyme that produces an increase in acid-soluble absorbance at 260 nm of 1,000 after 15 min of incubation at 37 C with yeast RNA (29).

Pancreatic RNase was purchased from Worthington Biochemical Corp.; T1 RNase was purchased from Calbiochem. Substrates poly(U), polycytidylic acid [poly(C)], and poly(A) were obtained from Miles Laboratories, Inc. RNA samples were diluted to a final volume of 0.5 ml with sodium acetate buffer, pH 4.5, or Tris-chloride buffer, pH 7.5, for RNase U2 or pancreatic and T1 RNase digestion, respectively. RNA and enzyme concentrations and times of digestion are indicated in the figure legends.

Polyacrylamide gel electrophoresis of poly(A) sequences. Precipitated RNA was dissolved in 0.15 ml of a solution containing 0.01 M sodium phosphate (pH 6.8), 0.02 M EDTA, 0.1% SDS, 1% glycerol, and bromophenol blue dye marker and was layered over an 18-cm column of 10% polyacrylamide gel. Electrophoresis buffer was the same as above except that the sodium phosphate was 0.1 M. Electrophoresis was performed for 18 h, 10 mA/gel at room temperature. Gels were fractionated in a Savant autogel divider and analyzed for radioactivity.

RESULTS

RNase U2, purified from *Ustilago sphaerogena*, has been shown to cleave polyribonucleotides preferentially at purine linkages (23) and can be utilized as a purine-specific reagent when reacted under specific conditions of enzyme and substrate concentrations. At an enzyme concentration of 0.75 U/ml and an enzyme:substrate ratio of 0.75 U/mg of RNA, there was no loss of acid-insoluble radioactivity in the pyrimidine ribopolymer poly(U) (Fig. 1). The pyrimidine-specific pancreatic RNase, on the other hand, hydrolyzed the sample to less than 1% of the remaining acid-insoluble radioactivity. Similar experiments with [^{3}H]poly(C) as substrate gave the same results, whereas digestion of RNAs containing purine nucleotides proceeded rapidly (see below, for example, Fig. 4).

Previous experiments have demonstrated that digestion of VSV mRNA, complementary to virion RNA, with pancreatic and T1 RNases

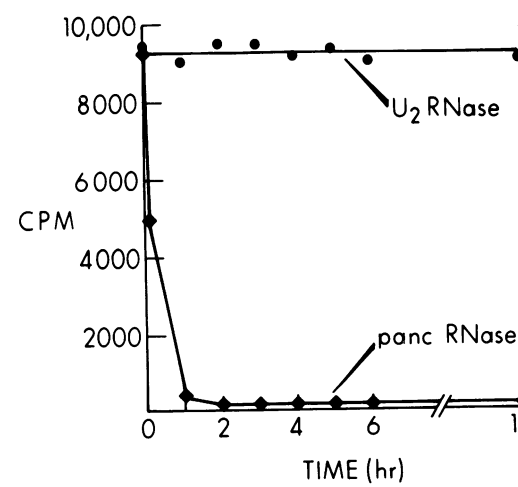

FIG. 1. *Digestion of [^{3}H]poly(U) by U2 and pancreatic RNases. [^{3}H]poly(U) (0.2 μCi; 20 μCi/μmol P) was incubated at 37 C in the presence of 500 μg of yeast RNA with either U2 or pancreatic RNase. Reaction mixtures contained 0.375 U of U2 RNase, 0.05 M sodium acetate buffer, pH 4.5, or 50 pg of pancreatic RNase, 0.05 M Tris-chloride buffer, pH 7.5, in final volumes of 0.5 ml. At the indicated times, 50-μliter samples were removed and assayed for trichloroacetic acid-insoluble radioactivity.*

resulted in an RNase-resistant fraction amounting to approximately 20% of the incorporated adenosine nucleotide (9). This fraction contained a heterogeneous population of molecules composed of approximately 95% AMP which migrated in polyacrylamide gels as polymer of between 70 and 250 nucleotides in length. RNA molecules transcribed in vitro from virion RNA by the virus-associated transcriptase also contained sequences labeled with [^{3}H]ATP but not [^{3}H]GTP which were resistant to digestion by pancreatic and T1 RNases (Fig. 2). The finding that purified virions could synthesize poly(A) sequences (see also references 4 and 30) prompted us to look for poly(U) sequences of corresponding length in the virion RNA.

VSV RNA labeled with either [^{3}H]uridine or [^{3}H]adenosine was prepared as described in Materials and Methods. Figure 3 shows a sucrose gradient sedimentation analysis of the viral RNA. The appropriate fractions (indicated by bar, Fig. 3) were pooled, precipitated with ethyl alcohol, dissolved, and digested with RNase U2 under conditions where pyrimidine linkages are not hydrolyzed. For comparison, similar samples of VSV RNA were digested with pancreatic RNase which would cleave any poly(U) sequence present. Figure 4 shows that (i) there was no difference in the RNase-resistant radioactivity of VSV RNA labeled with adenosine or uridine, and (ii) there was no more resid-

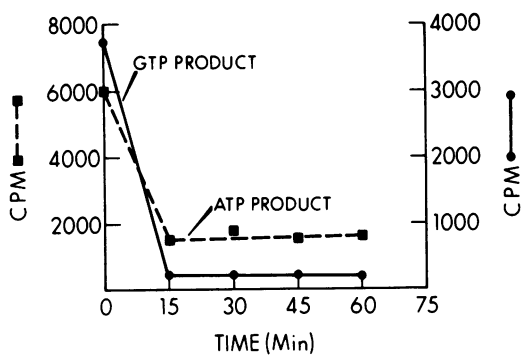

FIG. 2. *Digestion of VSV transcription product RNA with pancreatic and T1 RNases. 10" PFU of purified VSV was incubated for 4 h as described in Materials and Methods for the assay of virion-associated transcriptase. Product RNAs labeled with [^{3}H]ATP and with [^{3}H]GTP were extracted, precipitated with ethyl alcohol, and dissolved in 1 ml of 0.01 M Tris buffer, pH 7.4, containing 0.2 M NaCl and 0.01 M EDTA. Duplicate 20-μliter samples were removed and precipitated with 5% trichloroacetic acid for analysis of acid-insoluble radioactivity. Pancreatic (10 μg) and T1 ribonucleases (50 U) were added, and the samples were incubated at 37 C. At the indicated times, duplicate samples were removed and analyzed for acid-insoluble radioactivity.*

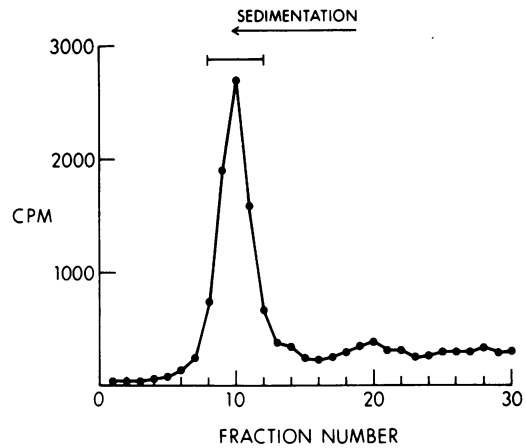

FIG. 3. *Sucrose gradient sedimentation analysis of [^{3}H]uridine-labeled VSV RNA. Radioactive VSV RNA was prepared as described in Materials and Methods. Sedimentation was through linear 15 to 30% sucrose gradients in 0.01 M Tris, pH 7.4, 0.1 M NaCl, 0.002 M EDTA, 0.1% SDS for 17 h at 20,000 rpm at 23 C in a Spinco SW27 rotor. Gradients were collected, and 50-μliter samples of each fraction were analyzed for radioactivity. The sucrose gradient profiles for [^{3}H]uridine- and [^{3}H]adenosine-labeled VSV RNA were virtually identical.*

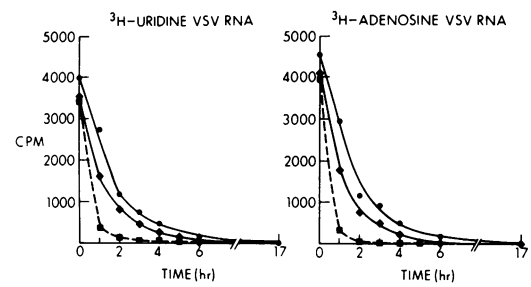

FIG. 4. *Digestion of [^{3}H]adenosine- and [^{3}H]uridine-labeled VSV RNA with U2 and pancreatic RNases. Radioactive VSV RNA was prepared as shown in Fig. 3 and described in Materials and Methods. Samples were incubated at 37 C in the presence of 100 (■---■) or 500 μg (■——■) of yeast RNA with 0.375 U of U2 RNase or 5 pg pancreatic RNase (●) under conditions described in Materials and Methods. At the indicated times, 50-μliter samples were removed and analyzed for acid-insoluble radioactivity.*

ual uridine radioactivity resulting from purine-specific RNase U2 digestion than results from pyrimidine-specific pancreatic RNase digestion. No oligonucleotides which migrated more slowly than bromophenol blue dye could be detected on polyacrylamide gels (not shown).

The polyadenylation of cellular heterogeneous nuclear RNA is effectively inhibited by 3-deoxyadenosine (cordycepin) at a concentration of 50 μg/ml (1), and the appearance of

cytoplasmic mRNA stops. Uridine or adenosine incorporation into VSV-specific RNA, however, was not reduced by cordycepin at concentrations between 10 and 50 μg/ml. At concentrations of 50 μg/ml or greater, a slight reduction occurred, most probably due to interference with nucleoside transport. Figure 5 shows a sucrose gradient analysis of virus-specific intracellular RNA made in the presence or in the absence of 50 μg of cordycepin per ml. Although this experiment was performed in the presence of actinomycin D to aid in the detection of viral RNA, parallel experiments performed in the absence of actinomycin showed that cellular mRNA synthesis was completely inhibited by cordycepin. All three sedimentation classes of virus-specific RNA (13), 42S virion RNA, 26S mRNA, and 13–15S mRNA were synthesized in the presence of cordycepin. In addition, the 18-h yields of infectious virus from HeLa cells infected in the presence or absence of cordycepin were identical (Table 1). When the 13–15S mRNA, labeled with [^{3}H]adenosine in the presence of cordycepin, was collected from sucrose gradients similar to those shown in Fig. 5, a distribution of RNase-resistant poly(A) sequences could be demonstrated on polyacrylamide gels which was indistinguishable from those obtained after labeling in the absence of drug (Fig. 6). These data show that cordycepin does not inhibit either the production of infectious virions or the polyadenylation of VSV mRNA (27).

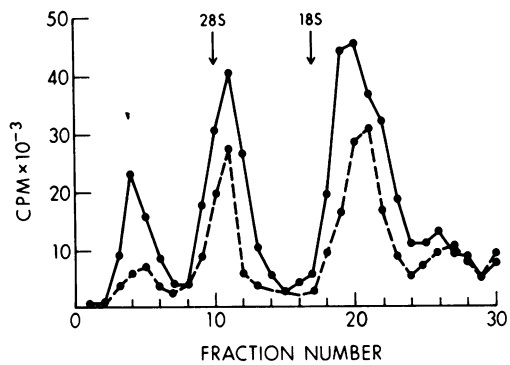

FIG. 5. *Sucrose gradient sedimentation analysis of [^{3}H]uridine-labeled VSV-specific intracellular RNA synthesized in the presence and in the absence of cordycepin. 5 × 10^7 HeLa cells were infected with 10 PFU of VSV per cell, and the culture was divided; one-half received 50 μg of cordycepin per ml (●- -●), the other remained untreated (●——●). Both cultures were treated with actinomycin D and [^{3}H]uridine, diluted, and incubated at 37 C for 5 h. Cytoplasmic extracts were prepared as described in Materials and Methods, treated with SDS, and sedimented in 15 to 30% sucrose gradients prepared in 0.1 M NaCl, 0.01 M Tris-chloride, pH 7.4, 0.002 M EDTA, 0.1% SDS for 18 h at 22,000 rpm at 23 C in the SW27 rotor. Gradients were collected and analyzed for trichloroacetic acid-insoluble radioactivity.*

DISCUSSION

Several investigators have shown that VSV mRNA contains poly(A) sequences, even when synthesized by purified virions in vitro (4, 30). A similar result was found for vaccinia virus mRNA synthesized in vitro (15), and a poly(A) polymerase has been solubilized and purified from vaccinia virions (18). Despite the high uridylic acid content of VSV virion RNA (20), previous attempts in this laboratory and others (11, 17) to find poly(U) sequences in virion RNA by hybridization to synthetic poly(A) have failed, and in this report, we show that no such sequences of poly(U) corresponding in length to mRNA poly(A) occur. We conclude, therefore, that the poly(A) sequences are not transcribed, but are added post-transcriptionally, as is cellular poly(A). To date, no poly(A) polymerase activity independent of all four nucleoside triphosphates has been detected in VSV virions. This suggests that polyadenylation of VSV RNA transcripts is accomplished either by a cellular enzyme routinely incorporated into VSV nucleocapsids, or by one or a combination

TABLE 1. *Incorporation of adenosine and production of infectious VSV from HeLa cells infected in the presence and in the absence of cordycepin*

Treatment	Adenosine incorporation at 5 h[a] (% of control)	Virus yields at 18 h[b] (PFU/ml)
Untreated	100	6.3×10^8
Cordycepin (6, 12.5, 25 μg/ml)	100	
Cordycepin (50 μg/ml)	82	6.9×10^8
Cordycepin, (75 μg/ml)	73	

[a] HeLa cells (5×10^6/ml) were infected with 10 PFU of VSV per cell in the presence or absence of the indicated concentrations of cordycepin. [^{3}H]adenosine (2 μCi/ml) was added at 1 h p.i., and duplicate samples were removed at 0.5-h intervals for analysis of acid-precipitable radioactivity.

[b] Two milliliters of HeLa cells (5×10^5/ml) were infected with 1 PFU of VSV per cell in the presence or absence of 50 μg of cordycepin per ml. A control culture was infected in the presence of 100 μg of cycloheximide per ml for the determination of residual input virus. The cultures were incubated at 32 C for 18 h and examined by phase microscopy, and the cell debris was removed by centrifugation. Virus in the supernatant was measured by plaque formation on L-cell monolayers.

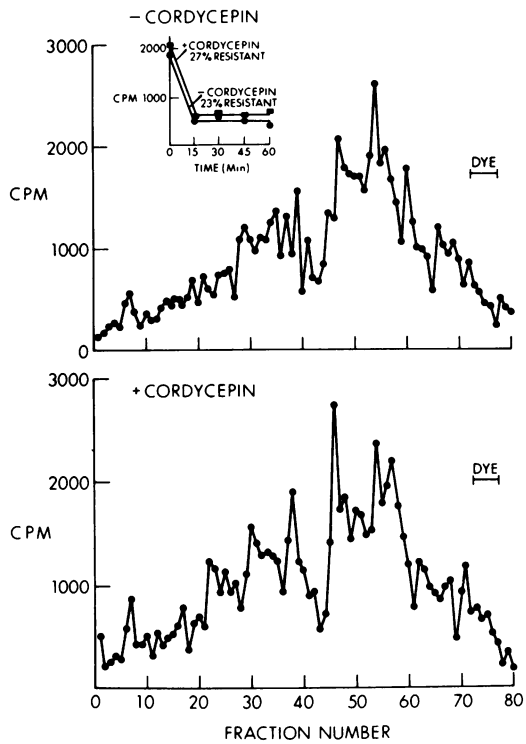

FIG. 6. *Polyacrylamide gel electrophoresis of RNase-resistant sequences in VSV 13–15S RNA synthesized in the presence and in the absence of cordycepin. VSV-infected HeLa cells were incubated in the presence and in the absence of cordycepin, and cytoplasmic extracts were analyzed on sucrose gradients as described in the legend to Fig. 5, except both cultures were labeled with 250 μCi of [^{3}H]adenosine. Samples (50 μliter) of each gradient fraction were analyzed for radioactivity, and samples containing 13–15S mRNA were pooled and digested with pancreatic and T1 RNase as described (8). The inset shows the kinetics and extent of RNase digestion. RNase-resistant material was precipitated with ethyl alcohol in the presence of 50 μg of carrier yeast RNA and analyzed on 10% polyacrylamide gels as described in Materials and Methods. Marker 4S RNA from HeLa cells migrated as a peak with maximal radioactivity in fraction 65.*

of the three viral proteins found in viral nucleocapsids. Emerson has presented evidence that the L protein (molecular weight, 150,000) is involved in transcription of VSV RNA in vitro (10). It is possible that the poly(A) synthesizing activity is an integral part of the transcriptase, but no such data are yet available. The virus-associated activity appears to be different from the corresponding cellular enzyme, since the former is resistant to inhibition by cordycepin whereas the latter is sensitive. However, other cellular, perhaps cytoplasmic, poly(A) synthesizing activities which may be cordycepin insensitive have not been ruled out. In fact, the results of R. Perry (personal communication) suggest the presence of a cytoplasmic poly(A) polymerase in L cells, and experiments with sea urchin embryos strongly implicate such a cytoplasmic activity operating before activation of stored mRNA particles in the fertilized egg (26, 32). The cordycepin sensitivity of these cytoplasmic enzymes has not been reported. An alternative interpretation of the cordycepin insensitivity of the VSV polyadenylation reaction is that cytoplasmic cordycepin triphosphate is inaccessible to the nucleocapsid synthesizing site, although ATP and other nucleoside triphosphates are available. Thus, the differential sensitivities of cellular and viral polyadenylation might be due to selective permeability rather than differential enzyme specificity. The effect of cordycepin triphosphate on the in vitro polyadenylation reaction has not been measured.

Growth of two parainfluenza viruses, Newcastle disease and Sendai, has been shown to be markedly inhibited by cordycepin (16); under the same conditions, however, influenza virus replication was not inhibited. Presumably, the mRNA's for all of these viruses contain poly(A) sequences (22; S. R. Weiss and M. A. Bratt, Abst. Amer. Soc. Microbiol., p. 202, 1973) but the enzymatic activities responsible for AMP polymerization or the nucleocapsid permeabilities show different sensitivities to 3-deoxyadenosine triphosphate.

The mechanism of poly(A) addition to VSV mRNA is not clear. No data are yet available to distinguish between sequential addition of AMP residues by an enzyme similar to that reported by Edmonds and Abrams (8), as has been suggested for the nuclear cellular enzyme (14), or a repeated transcription by "slippage" of short tracts of uridylic acid, as has been described for T3 polymerase (24). The presence of short tracts of UMP in VSV RNA has not been looked for.

ACKNOWLEDGMENTS

I thank Ethel Hurston for skillful technical assistance and Amiya Bannerjee for sending his manuscript prior to publication.

This work was supported by National Science Foundation grant GB 18026 and American Cancer Society grant VC-33. I am supported by Public Health Service career development award IK04 A170020.

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