CORONAVIRUS CELL-ASSOCIATED

RNA-DEPENDENT RNA POLYMERASE

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INTRODUCTION

Infectious, single-stranded, nonsegmented, polyadenylated, genomic RNA has been demonstrated for the avian infectious bronchitis virus (Lomniczi and Kennedy, 1977; Schochetman et al., 1977), the mouse hepatitis virus (Lai and Stohlman, 1978; Wege et al., 1978), and the transmissible gastroenteritis virus (TGEV) of swine (Brian et al., 1980), three members of the coronavirus family. These properties alone would characterize these coronaviruses as positive-strand viruses and place them in category IV of the Baltimore Scheme (Baltimore, 1971). By analogy with the picornaviruses and togaviruses that have been characterized, also belonging to category IV of the Baltimore scheme, one would not expect to find an RNA-dependent RNA polymerase as part of the virion, but would expect to find such an enzyme in infected cells. In this paper we report that we are unable to detect an RNA-dependent RNA polymerase in purified virions, but we do find it associated with apparent membrane structures in infected cells between 4 and 6 h postinfection, a time when the rate of viral RNA synthesis is maximal. The [^{3}H]UMP-incorporating activity probably represents coronaviral- specified RNA-dependent RNA polymerase since (a) the activity was 10- to 100-fold greater than any similar activity in uninfected cells, (b) the activity was insensitive to actinomycin D, (c) the product was destroyed by RNase but not by DNase, (d) the activity was associated with an apparent replication complex that had sedimentation properties the same as cytoplasmic structures containing virus-specific RNA. In addition, the enzyme required the presence of all four ribonucleoside triphosphates, and Mg^{++} at approximately 5 mM concentration, for maximal activity. The enzyme was associated with cytoplasmic membrane-like structures. In an effort to determine the coronaviral specificity of the in vitro

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polymerase products, we examined the molecular weight, strandedness, (i.e., whether the RNA is single or double stranded), and polyadenylation of the in vitro polymerase products and compared these to the same properties for intracellular virus-specific RNA. Cells pulse-labeled with [^{3}H] uridine between 4 and 6 h post-infection contained five major species of single-stranded, polyadenylated RNA with apparent molecular weights of 6.8-, 3.15-, 1.40-, 0.94-, and 0.66 x 10^6 and seven minor species of single-stranded RNA with apparent molecular weights of 6.2-, 1.55-, 1.05-, 0.64-, 0.39-, 0.34- and 0.24 x 10^6 all of which were polyadenylated except for the 1.55 x 10^6 molecular weight species. When the membrane complex containing the viral RNA polymerase and endogenous template were used for the in vitro synthesis of RNA, three species of single-stranded, nonpolyadenylated RNA were made. These electrophoretically comigrated with three of the species made in vivo and they had molecular weights of approximately 3.15-, 1.40-, and 0.66 x 10^6. TGEV therefore apparently replicates as a positive-strand virus which employs a cell-associated, but not virion associated, RNA-dependent RNA polymerase for the synthesis of viral RNA. The virus also apparently replicates by synthesizing multiple mRNA species, and the synthesis of these species appears to be associated with membranous cytoplasmic structures.

MATERIALS AND METHODS

Cells, Virus, and Virus Purification

The epithelioid swine testicle cell line, (ST), developed by McClurkin (1966) was used as previously described (Brian et al., 1980). Clone 116 of the Purdue strain of swine TGEV was cloned again again by us and stocks were used between passages 3 and 6 as previously described (Brian et al., 1980).

TGEV was grown and purified as previously described (Brian et al., 1980). Essentially, supernatant fluids were collected at 18 to 20 h p.i, clarified by centrifugation at 10,000 x g for 10 min, layered onto an 18 ml continuous 20-60% (wt/wt) sucrose gradient, made up in, TNE (0.01 M Tris-hydrochloride [pH 7.5] - 0.1 M NaCl - 1.0 mM EDTA), and virus was isopycnically sedimented by centrifugation at 90,000 x g for 4 h at 4°C in a Sorvall AH-627 rotor. Virus was then pelleted at 150,000 x g for 2 h in a Sorvall AH-650 rotor.

RNA Polymerase Assay

The assay for virion-associated RNA-dependent RNA polymerase was one optimized by Huang et al.(1970), for the VSV-associated enzyme. In a standard reaction, 50 µl of purified virus, suspended to a concentration of 0.5 mg/ml in reticulocyte standard buffer (RSB) (0.01 M Tris hydrochloride (pH 7.4) - 0.01 M NaCl - 0.0015 M $MgCl^2$), was assayed in a 300 µl solution (total volume) containing 50 mM

Tris-hydrochloride (pH 7.3); 5.3 mM magnesium acetate; 100 mM sodium chloride; 3.3 mM 2-mercaptoethanol 830 µg Triton N-101 per ml; 0.83 µM [5-^{3}H] UTP (4.4 x 10^7 cpm/pmole) or 2.20 mM [8-^{3}H] GTP (1.65 x 10^7 cpm/pmole); 0.67 mM each of ATP, CTP, and GTP (or UTP when [^{3}H]GTP was used). The amount of labeled substrate incorporated into product in 30 min at 33°C was measured by collecting the product on membrane filters (Amicon, 0.45 µm) after precipitation with 5% trichloroacetic acid - 0.05 M sodium pyrophosphate at 0°C. Dried filters were counted by scintillation spectrophotometry.

The assay for cell-associated RNA-dependent RNA polymerase was a modification of that described by Polatnick and Arlinghaus (1967). In a standard reaction, 50 µl of sample, suspended to a protein concentration of 5 to 10 mg per ml in 0.25 M sucrose - 0.0015 M $MgCl_2$ - 40 µg dextran sulfate per ml - 0.28% (wt/wt) deoxycholate, was assayed in a 200 µl solution (total volume) containing: 50 mM Tris-hydrochloride (pH 8.0); 5 mM magnesium acetate; 10 mM phosphoenolpyruvic acid; 40 µg phosphoenol pyruvate kinase per ml; 17.5 mM 2-mercaptoethanol; 20 µg actinomycin D per ml; 1.25 M [5-^{3}H] UTP (4.4 x 10^7 cpm/pmole) or 3.30 µM [8 ^{3}H] GTP (1.65 x 10^7 cpm/pmole); 0.5 mM each of ATP, CTP, and GTP (or UTP when [^{3}H]GTP was used). The amount of labeled substrate incorporated into product in 60 min at 37°C was measured by collecting the product on membrane filters as described above.

Subcellular Fractionation

Batches of 2- to 8 x 10^8 cells (representing 1 to 4 confluent roller bottle cultures) were treated as described in individual experimental protocols with regard to infection and radioisotopic labeling. At the designated times, cells were drained and washed 5 times with ice cold TN buffer (0.01 M Tris-hydrochloride (pH 7.4)-0.14 M NaCl). All further steps were done at 0-4°C. Cells were scraped into TN buffer and pelleted by centrifugation at 500 x g for 5 min. Cells were resuspended in 5-10 ml of a 0.3 M sucrose solution, allowed to swell for 10 minutes, and then disrupted using 15 strokes in a tight fitting glass Dounce homogenizer. Cellular disruption and nuclear breakage were monitored by light microscopy. The suspension was centrifuged at 650 x g for 7 min and the pellet which contained nuclei and other large debris was designated the nuclear fraction. The resultant supernatant was then centrifuged at 13,000 x g for 20 minutes, conditions known to pellet mitochondria, endoplasmic reticulum, cytoplasmic membranes and other membranous organellar structures. This pellet was designated the post-nuclear fraction. The resultant supernatant was designated the soluble fraction and was either analyzed directly for measurement of trichloroacetic acid-precipitable radioactive material, or was further treated with 10% polyethylene glycol to form a precipitate which was then resuspended and analyzed for enzymatic activity.

Extraction of RNA

Total cytoplasmic RNA was extracted by the method of Erickson et al. (1973). RNA was extracted from the pelleted postnuclear fraction or from the in vitro reaction mixture by the SDS-proteinase K-phenol method that we have previously described (Brian et al., 1980).

Agarose Gel Electrophoresis and Fluorography

The method of Lerach et al. (1977) was used to analyze RNA by electrophoresis in agarose gels. RNA samples were heated in 2.2M formaldehyde-50% (wt/wt) formamide-0.018M Na_2HPO_4-0.002M NaH_2PO_4, for 5 min. at 60°C immediately prior to electrophoresis. Electrophoresis buffer was 2.2M formaldehyde-0.0018M Na_2HPO_4-0.002M NaH_2PO_4. Slab gels of 0.75% agarose were used with a horizontal apparatus of 11 x 13 cm dimensions. Gels were 6 mm thick. Electrophoresis was carried out using 50 volts of constant voltage with a current of approximately 50mA. Upon termination of electrophoresis, gels were dehydrated in two sequential baths of 10 volumes of methanol for 2.5 h each. Methanol was then blotted from the gels using filter paper until the gels were approximately 1.5 mm thick at which time they were treated with Enhance (New England Nuclear, Boston, Mass.) and dried in a slab gel drier under vacuum and low heat. Gels were fluorographed by the method of Laskey and Mills (1975).

Materials

[^{3}H] uridine (8 Ci/mole) was obtained from Schwartz/Mann. [5,6-^{3}H]UTP (40 Ci/mmole) and [8-^{3}H] GTP (15 Ci/mmole) obtained from ICN Chemical and Radioisotope Division were in a 50% ethanol solution. The ethanol was removed by lyophilization and the nucleoside triphosphate was dissolved in the reaction medium. Cellular and viral RNA marker species were prepared as previously described (Brian et al., 1980).

RESULTS

Absence of RNA-Dependent RNA Polymerase in Purified TGEV

To examine the possibility that TGEV does possess a viron-associated RNA-dependent RNA polymerase, semi-purified and purified viral preparations were examined using an RNA polymerase optimized for vesicular stomatitis virus-associated enzyme. Table 1 illustrates that no RNA polymerase activity could be found in purified TGEV. At no time could polymerase activity be detected when a variety of different protocols were used (data not shown). The assay as employed for the detection of cell associated enzyme was also used, but modified to include detergent, and no enzyme activity could be detected (data not shown).

Table 1. Absence of RNA polymerase in purified TGEV.

Experiment	[^{3}H]UMP incorporation (fmole/mg protein/hr) VSV	TGEV
1[a]	62	0.01
2[b]	49	0.01
3[c]	49	0.02

[a]Approximately 10^{10} pfu of virus was partially purified from 800 ml of tissue culture fluid and assayed directly. Tissue culture fluid harvested from cells at 24 h p.i. was clarified at 13,000 x g for 20 min and virus was pelleted from the supernatant at 90,000 x g for 2 h through an 8 ml barrier of 20% sucrose (wt/wt) made up in TNE.

[b]Approximately 10^{10} pfu of virus was purified from 800 ml of supernatant fluids as described in the text, except that virus was first concentrated by sedimentation onto a 60% (wt/wt) sucrose cushion before resuspension and isopycnic sedimentation. Purified virus was pelleted and stored at -80°C for 1 week prior to assay.

[c]Approximately 10^{10} pfu of virus was purified from 800 ml of supernatant fluids as described in the text, except that virus was first concentrated by polyethylene glycol precipitation before resuspension and isopycnic sedimentation. For polyethylene glycol precipitation, clarified supernatant was made 10% final concentration with polyethylene glycol (6000) - 0.1 M NaCl-0.001 M EDTA, held at 0°C for 0.5 h with constant stirring, and the precipitate was pelleted at 1500 x g for 40 min at 0°C. The pellet was resuspended by Dounce homogenization prior to isopycnic sedimentation. In each experiment, virus was resuspended and assayed as described in the text.

Kinetics and Site of In Vivo RNA Synthesis

For the purpose of optimizing chances of detecting an active cell-associated RNA-dependent RNA polymerase, studies were made to determine the interval, within the 20 h growth cycle of the virus (Brian et al., 1980), during which viral RNA was synthesized at a maximal rate, and to determine the site of RNA synthesis during this time. The interval was determined by pulse-labeling infected cells with [^{3}H]uridine in the presence of actinomycin D (Fig. 1). The rate of RNA synthesis was found to be maximal between 4 and 6 hours postinfection. Mock-infected cells showed no such increase throughout the 20 h period indicating that the observed RNA synthesis is induced by the viral infection and probably represents virus-specific RNA synthesis. To determine the site of viral RNA synthesis, infected cells were pulse-labeled at 5 h postinfection with [^{3}H]uridine in

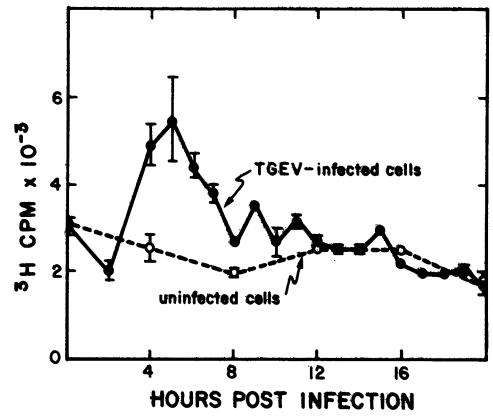

Fig. 1. Kinetics of viral RNA synthesis. Cells in 30 mm plates were infected simultaneously with TGEV at a multiplicity of 10 pfu/cell, or mock infected with balanced salt solution, and refed with medium containing 2% fetal calf serum. At 1 hour pior to labeling, actinomycin D was applied to the cells at a concentration of 0.1 µg/ml and kept there for the duration of the RNA labeling period. 4 µCi [^{3}H]uridine was added per plate at the indicated time postinfection for a 1 hr pulse. Periods were timed from the beginning of virus adsorption. Duplicate cultures were used for each time point. Acid precipitable radioactivity was determined for each culture.

the presence of actinomycin D, immediately fractionated into nuclear, postnuclear, and soluble fractions, and the fractions were analyzed for acid-precipitable radioactivity (data not shown). Infected cells incorporated 50 times more labeled uridine than did uninfected cells. The postnuclear fraction of the infected cells contained 80% of the total radioactivity. The postnuclear fraction of infected cells contained 50 times more radioactivity than the postnuclear fraction from uninfected cells. We concluded therefore that the viral RNA synthesizing structures were contained in the postnuclear fraction and this fraction was selected for further study of a viral RNA polymerase activity.

RNA Polymerase Activity in the Infected Cells

When we sought an RNA polymerase activity in the postnuclear fraction, 10 to 100-fold greater activity was found over any similar activity detectable in the postnuclear fraction from uninfected cells (Table 2).

Table 2. RNA polymerase activity in the sedimentable postnuclear fraction of TGEV-infected cells.[a]

Origin of postnuclear fraction	[^{3}H]UMP incorporation	
	cpm/assay/hr	fmole/mg protein/hr
Uninfected cells	122	2.5
Infected cells	6385	145

[a]Separate roller cultures, each containing 2 x 10^8 cells, were infected at a multiplicity of 10 pfu/cell or mock infected with a balanced salt solution, and refed with medium containing 2% fetal calf serum. At 5 h p.i., cells were scraped from the bottle and fractionated as described in the text. The postnuclear fraction was assayed for RNA polymerase as described in the text. Each assay was done in triplicate and the number shown represents the arithmetic mean of the three assays.

Characteristics of the Enzyme Activity

The [^{3}H]UMP incorporating activity was characterized with respect to the properties listed in Table 3. The polymerizing activity was reduced by 25% in the absence of Mg^{++}, and was reduced by 85% when Mn^{++} was substituted for Mg^{+}. The activity therefore apparently requires Mg^{++}. No other divalent cations were tested. The polymerizing activity was reduced by only 2% in the absence of 2-mercaptoethanol so a reducing agent is apparently not required for full activity. Enzymatic activity was not diminished when phosphoenol pyruvate and phosphoenol pyruvate kinase were omitted from the reaction mixture. An ATP generating system is evidently not required for activity. This may be evidence that ATPases which are normally present in crudely prepared postnuclear fractions were adequately removed by pelleting the postnuclear preparation through 20% sucrose. The presence of 20 µg actinomycin D per ml in the reaction failed to inhibit [^{3}H]UMP incorporation and therefore precludes the possibility that the product was made from a DNA template. Because there was decreased activity in the absence of all three supplementary nucleoside triphosphates when [^{3}H]UMP was used as the labeled compound, there is apparently a template requirement for the activity. It is therefore unlikely that we were merely detecting a polyuridylic acid synthetase activity. When dependence on individual nucleoside triphoshates was measured, we observed a complete dependence on ATP, but very little or no dependence on CTP and GTP. We are unable to explain these latter observations. When [^{3}H]GTP was used as the labeled nucleoside triphosphate a substantial dependence on each of the supplementary

Table 3. Characteristics of the RNA polymerase activity.[a]

Sample	[^{3}H]UMP or [^{3}H]GMP incorporation (fmole/mg protein/hr)	% of complete reaction
Complete reaction[b]	137	100
minus Mg^{++}[bd]	103	75
minus Mg^{++}, plus 2 mM Mn^{++}[bd]	20	15
minus 2-mercaptoethanol[b]	134	98
minus PEP and PEP kinase[b]	142	104
minus actinomycin D[b]	142	104
minus ATP[b]	13	9
minus CTP[b]	113	82
minus GTP[b]	123	90
minus ATP, CTP, and GTP[b]	3	2
minus dextran sulfate[b]	175	127
200 µg/ml dextran sulfate[be]	129	94
heated product plus DNase[bf]	137	100
heated product plus RNase[bg]	37	27
complete reaction[c]	286	100
minus ATP[c]	123	43
minus UTP[c]	87	31
minus CTP[c]	29	10
minus ATP, UTP, and CTP[c]	6	2
uninfected cell postnuclear fraction[c]	6	2

[a]The assay was performed as described in the text on a postnuclear fraction prepared in the following manner. Cells were lysed and and the nuclear fraction was removed as described. The supernant from the nuclear pellet was made 17 ml total volume by the addition of 0.3 M sucrose and layered onto a 20 ml cushion of 20% sucrose (wt/wt)-0.01 M Tris-hydrochloride (pH 7.4) in a 38 ml centrifuge tube. The pellet formed by centrifugation at 60,000 x g for 1.5 h at 4°C in a Sorvall AH-627 rotor was drained and resuspended in a solution of 0.01 M Tris-hydrochloride (pH 8.0) - 0.0015 M $MgCl_2$ - 40 µg dextran sulfate 500 (Sigma) per ml - 0.28% (wt/wt) sodium deoxycholate to a final protein concentration of 5-10 mg/ml.

[b][^{3}H]UTP was used.

[b]c[^{3}]HGTP was used.

[d]The postnuclear fraction was resuspended in 0.25 sucrose - 0.28% (wt/wt) - sodium deoxycholate.

[e]At the end of the 60 min reactions, the entire mixture was heated at 100° for 2 min, cooled to 37°C and treated with 50 µg DNase I (Worthington) per ml for 15 min at 37°C.

[f]At the end of the 60 min reaction, the entire mixture was heated at 100° for 2 min, cooled to 37°C and treated with 20 µg RNase A (Sigma) per ml and 20 units T1 RNase (Sigma) per ml for 15 min at 37°C nucleoside triphosphates was observed. These observations can most

readily be explained by the dependence of the activity on a template. Since the enzyme was not inhibited by actinomycin D, we conclude that the template was RNA. The heat denatured product was resistant to DNase but was 73% degraded by a mixture of pancreatic and T_1 RNases. We therefore conclude that the product was RNA.

When concentrations of Mg^{++} were used in 5 mM increments above 0 mM, a maximal activity of 325 fmole [^{3}H]UMP incorporation/mg protein/hour was observed at 5 mM concentration (data not shown). Increased concentrations above 5 mM caused an apparent aggregation within the reaction mixture which had the appearance of a precipitate. This may have been the cause of the inhibition at the higher concentrations. The rate of [^{3}H]UMP incorporation at 37°C increases rapidly for the first 10 min and then decreases by more than 50% to remain constant for at least 50 min. (data not shown). For studies in which the RNA product was analyzed reaction times were normally held to less than 15 minutes to minimize the liklihood of RNase degradation.

Apparent Membrane Association of the Polymerase

Viral RNA synthesizing complexes, of both positive-strand picornaviruses and togaviruses, and negtive-strand myxoviruses and rhabdoviruses, are found to have an intimate association with cytoplasmic membranous structures. These structures usually have buoyant densities of 1.20 g/cm^3 or less in sucrose gradients. We have found that the postnuclear fraction of TGEV-infected cells, pulse-labeled with [^{3}H]uridine in the presence of actinomycin D, can be resolved by isopycnic sedimentation on sucrose gradients into structures with buoyant densities of 1.15 and 1.19 g/cm^3 (data not shown). Material from each of these fractions contains virus-specific RNA, and therefore a membrane-association of the TGEV-RNA synthesizing apparatus is suggested. When the postnuclear fraction prepared in the same way was sedimented on a rate zonal sucrose gradient, the TGEV-specific RNA sedimented in one peak apparently as part of a complex having an average sedimentation coefficient of approximately 500S. This complex also contained the RNA-dependent RNA polymerase activity (data not shown). In an attempt to verify the membrane association of the polymerase, the postnuclear fraction of TGEV- infected cells prepared as described above, was treated with the nonionic detergent deoxycholate and sedimented on a rate zonal gradient in the presence of deoxycholate (Fig. 2). The TGEV-specific RNA now sediments as one peak with a sedimentation coefficient of approximately 230S. The detergent apparently destroyed a membrane association. When an unlabeled postnuclear fraction was sedimented on a rate zonal gradient in the presence of deoxycholate, two peaks of polymerase activity were resolved (Fig. 2). These peaks had sedimentation coefficients of approximately 600S and 160S, suggesting again that the detergent had destroyed a membrane association. As yet, we have no direct proof that the enzyme is associated with membranes.

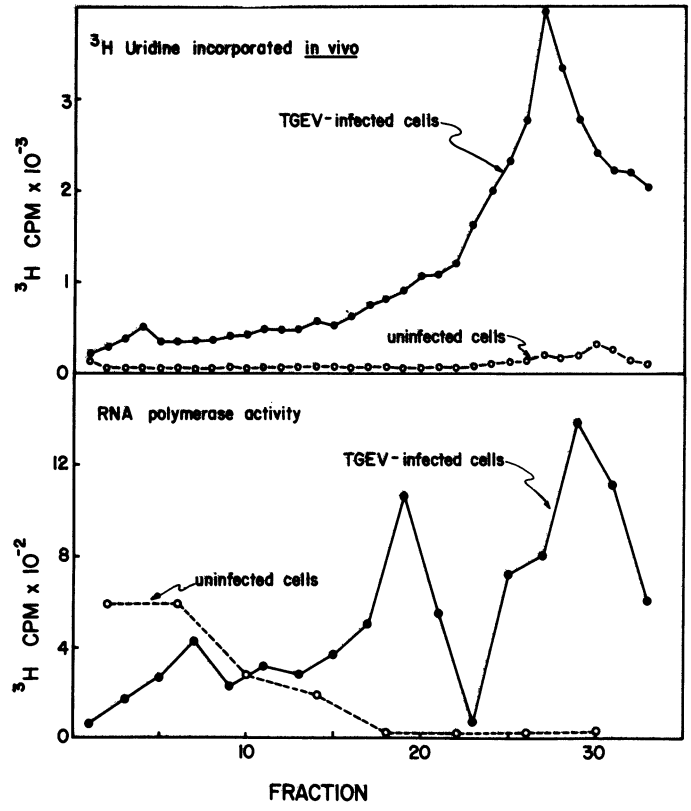

Fig. 2. Rate zonal sedimentation of deoxycholate-solubilized postnuclear fraction. a) 2 x 10^8 cells in roller culture were infected at a multiplicity of 10 pfu/cell and an identical culture was mock infected with a balanced salt solution. At 3 h p.i., timed from the beginning of the adsorption period, actinomycin D was added to make a final concentration of 1.0 μg/ml. At 5 h p.i., 100 μCi [^{3}H]uridine was added per culture and cells were incubated for 0.5 h at 37°C. Cells were then fractionated as described in the text except that the postnuclear fraction was resuspended in 0.25 M sucrose - 0.0015 M $MgCl_2$ and immediately made 1% (wt/wt) with sodium deoxycholate. The solubilized postnuclear fraction was then sedimented on a 15 to 40% (wt/wt) sucrose gradient which contained 1% (wt/wt) final concentration sodium deoxycholate. Sedimentation was at 23,500 x g for 1.5 h at 4°C in a Sorvall AH-627 rotor and the gradients were fractionated and analyzed for acid precipitable radioactivity. b) 2 x 10^8 each of infected and uninfected cells were treated and fractionated as described in part a) except that they were not isotopically labeled. The postnuclear fractions were sedimented on rate zonal gradients containing 1% (wt/wt) sodium deoxycholate in parallel with the gradients described in part a). Indicated fractions of the gradient were assayed for [^{3}H]UMP-incorporating activity. Sedimentation is from right to left.

Virus Specific RNA in Infected Cells.

To characterize the in vitro polymerase products and to determine their viral specificity, RNA extracts of infected cells, the postnuclear fraction of infected cells, and in vitro polymerase products, were examined on a comparative basis. All preparations were from cells between 4 and 6 h postinfection. To examine the RNA species made during this interval of time infected cells were labeled with [^{3}H] uridine in the presence of actinomycin D and the RNA was extracted from whole cells by the method of Erickson et al., 1973, and examined under denaturing conditions by electrophoresis on agarose gels (Fig. 3). Five major species having apparent molecular weights of 6.8-, 3.15-, 1.40-, 0.94-, and 0.66 x 10^6 were seen. In additon, 3 minor species having apparent molecular weights of 6.2-, 1.55-, and 1.05 x 10^6 were also seen. No RNA could be observed in extracts from uninfected cells even though the total rate of RNA synthesis under these conditions was 3% of that in infected cells. To determine whether the RNA species found in the postnuclear fraction were the same as those extracted from whole infected cells, infected cells were

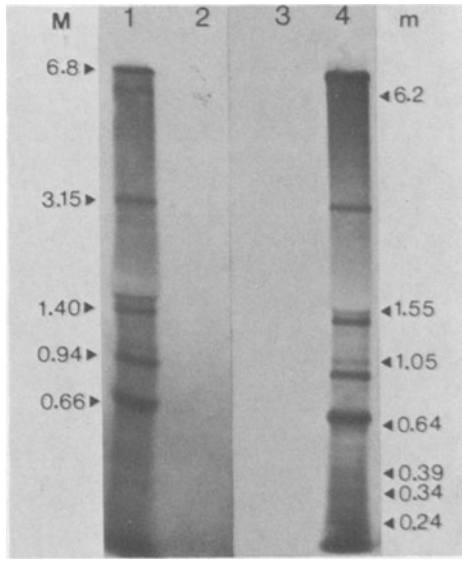

Fig. 3. Electrophoresis of intracellular RNA on denaturing agarose gel. Cells were infected with TGEV or mock infected and radiolabeled with [^{3}H]uridine using the conditions discribed in Fig. 2. (1) RNA extracted from infected whole cells. (2) RNA extracted from uninfected whole cells. (3) RNA extracted from the postnuclear fraction of uninfected cells. (4) RNA extracted from the postnuclear fraction of infected cells. M and m indicate the molecular weight (X10^{-6}) of the major and minor species respectively.

pulse-labeled with [^{3}H] uridine at 5 h postinfection in the presence of actinomycin D and the postnuclear fraction was prepared. RNA was extracted and analyzed by electrophoresis under denaturing conditions on agarose gels (Fig. 3). Five major RNA species were found which had the same electrophoretic mobilities as the five major species extracted from whole cells. In addition, there were seven minor species, three of which migrate the same as the three minor species in whole cell extracts. The minor species had apparent molecular weights of 6.2-, 1.55-, 1.05-, 0.64-, 0.39-, 0.34-, and 0.24 x 10^6 molecular weight. Molecular weights were estimated by extrapolation and interpolation from a plot of molecular weight vs relative mobility for known single-stranded RNA markers. The 6.8 x 10^6 molecular weight species comigrated with TGEV virion RNA. Approximately 5% of the total radioactivity was observed as unresolved species migrating with 4S RNA.

Single-Stranded Nature of Intracellular Virus-Specified RNA

All RNA species extracted from infected cells were single-stranded as judged by their sensitivity to digestion with RNase T_1 in 0.3M NaCl (Fig. 4). It is possible that double-stranded replicative intermediates were present but in too few numbers to be detected by this method.

Polyadenylation of Intracellular Virus-Specified RNA.

To determine whether virus-specified RNA is polyadenylated, infected cells were pulse-labeled with [^{3}H] uridine at 5 h postinfection in the presence of actinomycin D, and RNA was extracted from the postnuclear fraction and chromatographed on oligo (dT)-cellulose (Figures 4 and 5). All five major RNA species and six minor RNA species were polyadenylated by this criterion. The 1.55 x 10^6 molecular weight appeared not be be polyadenylated. In addition, some of the 6.8-, 1.40-, and 0.66 x 10^6 molecular weight species appeared not to be polyadenylated as well. The nonpolyadenylated species may be minus-strand RNA's, or they may reflect incomplete transcription of the 3' end of the molecule. Alternatively, they may represent molecules from which the 3' end was lost by breakage.

Viral RNA Species Synthesized During the In Vitro Polymerase Reaction

In order to make a direct comparison between RNA species made *in vitro* and those made *in vivo*, both were analyzed by electrophoresis on the same agarose slab gel following denaturation (Figures 4 and 5). By these analyses, two *in vitro* transcripts with apparent molecular weights of 1.40-, and 0.66 x 10^6 and a possible third transcript with an apparent molecular weight of 3.15 x 10^6 were observed. In addition a broad band of material with a molecular

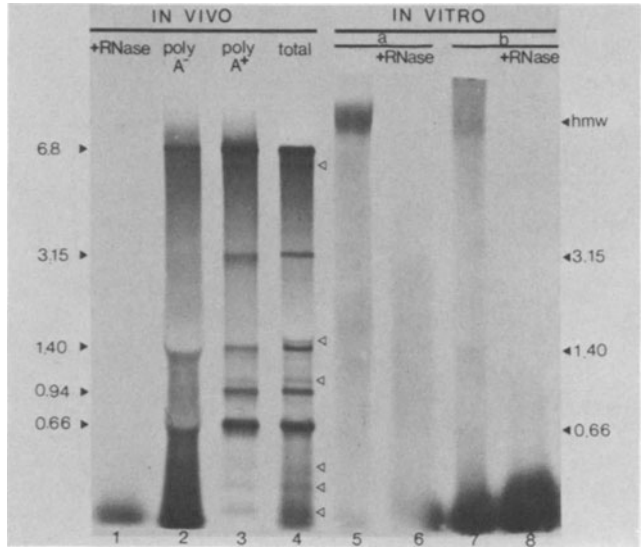

Fig. 4. RNase susceptibility of intracellular TGEV-specific RNA and *in vitro* polymerase products. The radiolabeled postnuclear fraction of infected cells was prepared as described in Fig. 2. The RNA was extracted and electrophoresed on denaturing agarose gels after the following treatments. (1) Digestion with 20 units T_1 RNase per ml - 0.3 M NaCl for Ih at 37°C. (2) and (3), oligo(dT)-cellulose chromatography using the procedure we have previously described (Brian et al., 1980). (4) No treatment. Radiolabeled *in vitro* reaction products from two separate experiments (a and b) were extracted as described in the text and electrophoresed after the following treatments. (5) No treatment. (6) RNase digestion as described in (1). (7) No treatment. (8) RNase digestion as described in (1).

weight of greater than 6.8×10^6 was observed. This is designated as high molecular weight (hmw) RNA. Digestion of the *in vitro* products with T_1 RNase in 0.3M NaCl demonstrates that all but the hmw RNA species are single stranded (Fig. 4). Oligo (dT)-cellulose chromatography of the *in vitro* products reveals that the 1.40- and 0.66×10^6 molecular weight species are apparently not polyadenylated (Fig. 5).

DISCUSSION

Our results indicate that the TGEV virion does not possess an RNA-dependent RNA polymerase but that the virus induces an enzyme of this type during intracellular replication. The absence of this enzyme(s) on the virion along with an infectious, single-stranded,

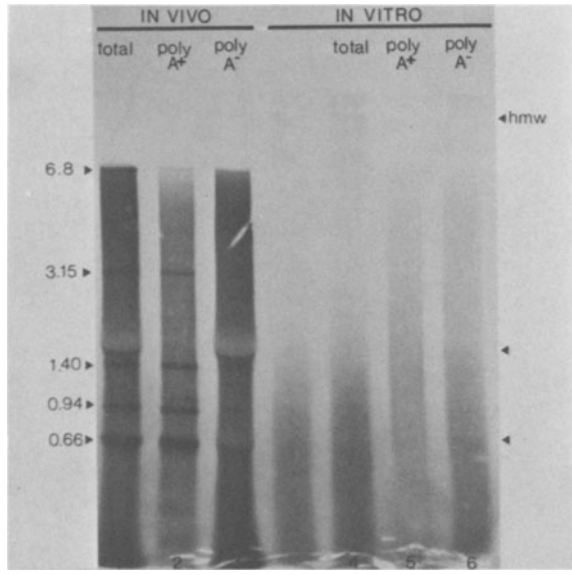

Fig. 5. Polyadenylation status of intracellular TGEV-specific RNA and in vitro polymerase products. The radiolabeled postnuclear fraction of infected cells was prepared as described in Fig. 2. The RNA was extracted and electrophoresed on denaturing agarose gels after the following treatments: (1) No treatment. (2) and (3), chromatography on oligo(dT)-cellulose using the previously described procedure (Brian et al., 1980). Radiolabeled in vitro reaction products were extracted as described in the text and electrophoresed after the following treatments: (4) No treatment. (5) and (6), chromatography on oligo(dT)-cellulose.

RNA genome are properties shared by picornaviruses and togaviruses. Many features of the coronavirus enzymatic activity are shared by the picornavirus and togavirus counterparts. Maximal activity requires Mg^{++} and the presence of all nucleoside triphosphates. The enzyme is apparently part of a membrane associated replication complex as judged by the bouyant density of the complex. Treatment of the complex with a nonionic detergent changes the sedimentation coefficient of the complex and further suggests a membrane-association.

One unique feature of coronavirus replication which contrasts sharply with the replicational schemes of picornaviruses and togaviruses is that apparent multiple mRNA species are employed during coronavirus replication (Stern and Kennedy, 1980). We demonstrate that a similar scheme is apparently true for the replication of TGEV as well.

While a cell-associated RNA-dependent RNA polymerase is undoubtedly used in the replication of the genomic RNA (i.e., synthesis of the minus-strand) as well as transcription of the genomic RNA and mRNA species (i.e., synthesis of the positive-strands), no effort was made in our studies to separate the two activities. Several attempts to demonstrate double-stranded forms of RNA from infected cells were not successful suggesting that perhaps negative-strand species were present in only very small numbers or possibly positive and minus strands were loosely associated in the replicative forms and were thus separated during RNA extraction. The only possible double-stranded candidates were the high molecular weight RNA species observed after nuclease treatment of in vitro transcripts suggesting that perhaps the in vitro polymerase reaction favors the production of minus-strand species which then anneal to readily present positive strand species during the incubated reaction. This interpretation is supported by the fact that the sizes of the in vitro transcripts are the same as nonpolyadenylated in vivo species found, namely 3.15-, 1.40- and 0.66 x 10^6, and at least two of the in vitro products appear nonpolyadenylated.

It is of interest to note that while every major and almost every minor virus-specific intracellular RNA species identified appears to be polyadenylated, three abundant species appear to be nonpolyadenylated as well. These have molecular weights of approximately 6.8-, 1.40- and 0.66 x 10^6. These may represent genomic and subgenomic minus-strand RNA species or alternatively they may be mRNA species with poly(A) tracts too short to anneal to the oligo (dT) cellulose.

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