

To the Graduate Council:

I am submitting herewith a dissertation written by Douglas Edward Dennis entitled "Coronavirus Replicational Strategy". I recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Microbiology.

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CORONAVIRUS REPLICATIONAL STRATEGY

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

Douglas Edward Dennis

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DEDICATION

This work is dedicated to Monica, Chris, and Megan for their love and support, and their willingness to participate in my existence. Together, we make a family.

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ABSTRACT

An enzymatic activity which incorporates [^3H] UMP into acid-precipitable material in the presence of endogenous template was found in the cytoplasm of porcine cells infected with the transmissible gastroenteritis virus of swine. This activity was not found in uninfected control cells, nor was it found in purified virus. The activity was associated with rapidly-sedimenting, particulate structures, suggesting that the enzyme is bound to a membrane-containing replication complex. The membrane association was confirmed by demonstrating detergent-dependent changes in sedimentation behavior. The enzymatic activity required the presence of all three ribonucleoside triphosphates in addition to [^3H] UTP and it was not inhibited by actinomycin D. The heated product was digested by ribonuclease but not by deoxyribonuclease. Mg^{++} was required for enzymatic activity and its optimal concentration was determined to be approximately 5 mM. These results support the classification of this coronavirus as a positive-stranded RNA virus. In an effort to determine the coronaviral specificity of the in vitro polymerase products, we examine 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 [^3H] uridine between 4 and 6 h postinfection, a time when the rate of viral RNA synthesis is maximal, 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 . Seven minor species of

single-stranded, polyadenylated RNA with apparent molecular weights of 6.2-, 1.55-, 1.05-, 0.64-, 0.39-, and 0.24×10^6 were also found. The 6.8×10^6 molecular weight species was indistinguishable from virion genomic RNA by denaturing gel electrophoresis. Intracellular virus-specified species were closely associated with cytoplasmic membrane structures and are probably part of a membrane-associated replication complex. When the membrane-associated complex containing the viral RNA polymerase and endogenous template were used for the in vitro synthesis of RNA, three species of single-stranded RNA were seen, of which the two smaller species appeared to be nonpolyadenylated. These species electrophoretically comigrated with three of the species made in vivo and they had molecular weights of approximately 3.15-, 0.94-, and 0.66×10^6 . This coronavirus, therefore, apparently replicates by the transcription and subsequent translation of multiple subgenomic mRNA molecules and the mechanism for regulating the length of at least some of the subgenomic transcripts resides within the replication complex.

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CORONAVIRUS REPLICATIONAL STRATEGY

I. REVIEW OF THE LITERATURE

CONCERNING POSITIVE-STRANDED

(CLASS IV) RNA VIRUSES

CHAPTER I

INTRODUCTION

Coronaviruses are one of the 10 families of animal RNA viruses. They have a helical nucleocapsid, are pleomorphic, and range from 80 to 120 nm in diameter. Their outer lipid bilayer is acquired by budding of the virus through reticular membranes into cytoplasmic vesicles (65). The genomic RNA of the coronaviruses is single-stranded, infectious, and polyadenylated (59, 60, 78). Although there is some controversy at this time, most researchers believe the genomic RNA to be nonsegmented (45, 55, 60, 62, 78, 88). The coronaviruses are characterized by large bulbous peplomers attached along the circumference of the virus, giving the virus the appearance of having a "corona" in electron micrographs. It was the description of these peplomers in the prototype coronavirus, infectious bronchitis virus, by Berry et al. in 1964 which first differentiated coronaviruses from myxoviruses, the family in which they were originally grouped (15). Subsequent studies on mouse hepatitis virus (76) and two human viruses (68) revealed the same distinct morphological features. This morphological difference was the reason for proposing coronaviruses as a separate family by an international committee in 1968. In 1975 the family was formally accepted by the International Committee on the Taxonomy of Viruses (91). The coronavirus family consists of 12 members (Table 1), and there is at least one more candidate for inclusion into the family (90).

Table 1. Coronavirus species with corresponding host animal and disease.

Coronavirus species	Host animal	Disease
avian infectious bronchitis virus	chicken	upper and lower respiratory tract infection
mouse hepatitis virus	mouse	hepatitis, encephalitis
transmissible gastro- enteritis virus	pig	gastroenteritis
hemagglutinating encephalomyelitis virus	pig	encephalomyelitis "wasting and vomit- ing disease"
human coronavirus (respiratory)	man	upper respiratory tract infection
rat coronavirus	rat	lower respiratory tract infection
sialodacryadenitis virus	rat	sialodacryadenitis
turkey bluecomb disease virus	turkey	gastroenteritis "bluecomb disease"
canine coronavirus	dog	gastroenteritis
bovine coronavirus	calf	gastroenteritis
feline infectious peritonitis virus	cat	infectious peritonitis
equine coronavirus	foal	gastroenteritis

Coronaviruses are responsible for a wide variety of diseases and they exhibit many different tissue tropisms. Their natural host range includes man, pigs, cattle, dogs, rats, mice, turkeys, chickens, cats, and horses. They appear to be responsible for about 20% of all human colds (49, 65) and are implicated in viral gastroenteritis (26, 27). In addition, they may cause such slowly developing diseases as endemic nephropathy (3) and myocarditis (5). The diseases they cause in food animals are of commercial importance because they result in a high incidence of mortality in young animals (1, 18, 34, 44, 66).

The inherent difficulties in cultivating host cells and growing coronaviruses to high titers have caused coronavirus research to lag behind that of other virus families. However, with the naming of coronaviruses as a separate family in 1975, the selection of high-titered strains, and the interest in coronaviruses as possible causes of chronic infections, many scientists entered the field of coronavirusology. By 1976 other biochemical features were known for at least the prototype coronavirus, avian infectious bronchitis virus as well as the mouse hepatitis virus, the porcine transmissible gastroenteritis virus (TGEV), and a human coronavirus. It had been shown that they possess single-stranded RNA as their genome, but there was disagreement with regard to the molecular weight and degree of segmentation of the RNA (55, 60, 78).

It was at this time that we proposed to answer a fundamental question concerning the molecular biology of coronavirus replication: Given that coronaviruses possess a single-stranded RNA genome, do they replicate by one of three known genetic strategies described for single-stranded RNA viruses as outlined by David Baltimore in 1971 (Fig. 1),

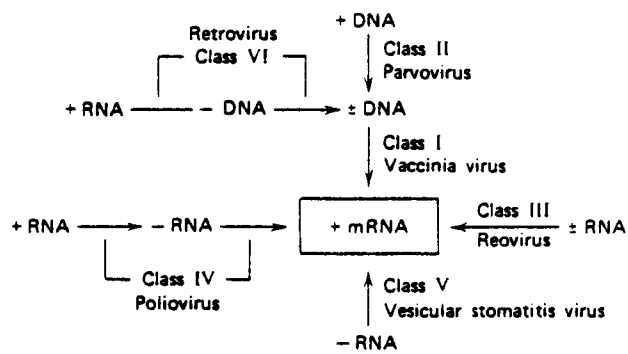


Fig. 1. Schematic diagram of RNA animal virus replication.

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or might they replicate by a heretofore undescribed strategy?

Using a rapidly replicating, tissue culture-adapted coronavirus, the transmissible gastroenteritis virus of swine (TGEV), we sought to answer the specific question of whether this virus replicates as a "positive-stranded" RNA virus (i.e., the single-stranded genomic RNA is of the same polarity as mRNA) or as a "negative-stranded" RNA virus (i.e., the single-stranded genomic RNA is of antimesage polarity). More specifically, could we find a nonsegmented, polyadenylated, infectious RNA genome which would be characteristic of a positive-stranded RNA virus, or, alternatively, could a virion-associated RNA-dependent RNA polymerase be detected which would be characteristic of a negative-stranded RNA virus?

Our initial work demonstrated that TGEV genomic RNA was in fact a nonsegmented, single-stranded, polyadenylated, infectious molecule (17). By this criterion, we classified TGEV as a positive-stranded RNA virus (i.e., a group IV virus) in the Baltimore scheme (12). Our findings were consistent with concurrent reports describing the RNA of the avian infectious bronchitis virus and the mouse hepatitis virus as also containing infectious RNA (55, 59, 60, 78). It was thus becoming clear that at least some coronaviruses are positive-stranded RNA viruses. Our initial work also allowed us to conclude that a virion-associated RNA-dependent RNA polymerase was not present, but that a cell-associated polymerase was present. This property was consistent with the behavior of the other two positive-stranded RNA virus families, the picornaviruses and togaviruses (13, 14, 52, 63, 71, 74, 85)

Because fundamental differences do exist between the picornaviruses and the togaviruses with respect to RNA structure and replicational and transcriptional strategies (Table 2), we sought to make careful comparisons with several features of coronavirus replication. Specifically, we asked whether there were fundamental differences between the cell-associated RNA-dependent RNA polymerase of the coronavirus and of the picornavirus and togavirus counterparts. Is the coronavirus enzyme(s) also part of a membrane-bound replication complex? Can intracellular replicative intermediates and replicative forms be found? How many mRNA species are made in the coronavirus-infected cell? Does actinomycin D influence coronaviral RNA synthesis? As will be seen in the following chapters, these questions were answered, at least in part, and led to still more specific questions which were investigated.

In essence, we found TGEV to behave as a unique positive-stranded RNA virus. If it is representative of other coronaviruses, then coronaviruses, as a family, have a fundamentally unique pattern of transcription. This raises the possibility that coronaviruses possess mechanisms of gene control that are not shared by other virus families. If this is true, then the study of coronavirus gene expression may serve as a unique approach for elucidating cellular mechanisms of gene control.

The following section is a detailed review of the picornavirus and togavirus literature that laid the groundwork in both methodology and comparative virology for my dissertation research.

Table 2. Features of positive-stranded virus RNA.

	picornavirus	togavirus	coronavirus
molecular weight	2×10^6	4×10^6	$5-8 \times 10^6$
3' poly (A)	60-80 bases	60-80 bases	?
5' end	protein	cap	?
internal methylation	none	m ⁵ C	?
form	linear	circular	?
number of mRNAs	1	2	?

CHAPTER II

PICORNAVIRAL RNA REPLICATION

Introduction

The picornavirus family is among the most extensively studied virus families. Because of their medical significance and their ease of growth, they have been instrumental in several significant discoveries in molecular biology over the last 20 years. Among these, are the discovery of a virus-specific RNA-dependent RNA polymerase, the identification of a base-paired, double-stranded replicative form of viral RNA, and the demonstration of the translation of a polycistronic gene product and its subsequent cleavage into functional polypeptide units (11, 57). There are in the literature several comprehensive reviews on the picornavirus family. The succeeding sections of this review draw from these, as well as current literature, in order that we may make a detailed study of the pertinent aspects of picornaviral RNA replication.

Overview of Picornavirus Replication

Picornaviruses begin their replication cycle by adsorption of the virus to specific receptor proteins on the cell surface. It is these cell-coded proteins which determine whether a cell will be susceptible to a particular virus, a restriction which may be bypassed by using viral RNA to infect cells (12). After adsorption, the virus penetrates the cell and is uncoated, releasing the positive-stranded RNA. The viral RNA then serves as a template for the translation of viral-specific proteins. Among the proteins

produced is a viral-specific RNA-dependent RNA polymerase which initiates primary transcription of the infecting viral RNA. The product of this transcription is a negative-stranded RNA complementary to genomic RNA, which serves as a template in secondary transcription to produce viral positive-stranded RNA. It has been suggested that secondary transcription is directed by a polymerase different than the polymerase which is responsible for primary transcription, but direct proof has not yet been obtained to support this hypothesis (79).

The replication complex, consisting of template RNA, nascent complementary RNA, and the RNA polymerase, is intimately associated with cellular cytoplasmic membranes (24). The role of this membrane association is not clear, but it is thought that it may play a part in sequestering the constituents necessary for the polymerase reaction.

The products of secondary transcription, viral positive-stranded RNA's are utilized as templates for further protein synthesis and for encapsidation into virions which are released by rupture of the host cell.

The Picornaviral RNA-Dependent RNA Polymerase

A picornavirus-specific RNA-dependent RNA polymerase was first discovered in 1962 by David Baltimore and Richard Franklin in lysates from Mengovirus-infected cells (13). Their discovery was followed soon afterwards by the discovery of such an enzyme for other picornaviruses (14, 52, 71, 74). Generally, the picornavirus polymerases have been found to be remarkably similar. They all require magnesium, all four nucleoside triphosphates, and an ATP-generating system, although polymerase isolates which are purified by centrifugation

through a dense sucrose solution lose the requirement for an ATP-generating system. This reflects the purification of the polymerase complex from ATPases present in the mitochondrial-microsomal fraction (13,000 x g for 20 min) which were shown to degrade ATP at a rate of 2 umoles/mg protein/hr at 37°C (72). The polymerases are inhibited by manganese, and, at least in one report, by calcium (56). The pH optimum of the enzyme is approximately 8. Treatment of the polymerase complex by heating at 100°C for 2 minutes abolishes all activity. The presence of actinomycin D or DNase in the reaction does not affect the activity, so it may be concluded that the template is not DNA. The product has been shown to be RNA by digestion with RNase and hydrolysis in 0.3 M NaOH. Also, the deoxyribonucleoside triphosphates can not substitute for the corresponding ribonucleoside triphosphates in the polymerase reaction. Kinetic experiments have revealed that the activity increases with time to about 15 minutes, whereupon the rate levels off and remains constant. This leveling off is probably a reflection of the fact that the in vitro reaction is restricted to the completion of previously initiated strands, because in more recent improved reaction mixes where initiation takes place in vitro, incorporation of the labeled ribonucleoside triphosphate increases linearly for up to 6 hours (54, 83).

The enzyme first appears intracellularly 3 hr postinfection and rises to its maximum activity at 5-6 hr postinfection. These kinetics generally parallel the course of viral RNA synthesis in the cell. In most cases where the in vitro detection of the virus-specific polymerase is the goal, actinomycin D is added to infected cells to decrease

production of cellular DNA-dependent RNA polymerase, without any adverse effect with regard to detection of the polymerase. However, in foot-and-mouth disease virus Arlinghaus and Polatnick have shown that pretreatment with actinomycin D can decrease measurable polymerase activity by as much as 80%. This inhibition apparently reflects the differences in actinomycin D sensitivity between picornavirus species in different cell lines (9, 75). Viral RNA synthesis is dependent upon concurrent protein synthesis because the addition of puromycin completely inhibits RNA synthesis. This and other data indicate that the polymerase is made de novo and its activity is unstable in the absence of protein synthesis (37, 77).

Because the polymerase activity can be pelleted from an enucleated cytoplasmic lysate at relatively low centrifugation speeds, the activity was thought to be associated with a large particle fraction of the cell. Subsequent experiments in which infected cells were separated into nuclei, large particle fraction (sometimes referred to as the microsomal fraction), and small particle fraction and then assayed for polymerase activity proved this hypothesis (14, 52, 71). Solubilization of the membranes in the large particle fraction with sodium deoxycholate renders the polymerase complex more susceptible to RNases present in the cytoplasm. This was demonstrated by the fact that the RNA products of sodium deoxycholate-solubilized polymerase complexes contained far less single-stranded RNA than nonsolubilized complex products (31, 39, 40). These findings suggest that the virus-specific RNA is produced in membranous structures which somehow protect the nascent RNA from degradation by endogenous RNases. Virus-infected cells incubated in growth

medium contain 3-3.5 fold more polymerase activity than virus-infected cells incubated in minimal medium (74). Therefore, it would appear that the physiological status of the cell is very important for polymerase production and detection.

Picornaviral Polymerase Products

Products of picornavirus polymerases fall into 3 classes, single-stranded genome-sized RNA (37S), double-stranded RNA which contains infectious viral RNA (20S), and a heterogeneous population of partially RNase-resistant RNA species having a sedimentation coefficient between 18S and 28S (6, 7, 8, 20, 32, 53, 73). These same RNA species are found within the infected cell. Dietzschold and Ahl (35) have shown that the foot-and-mouth disease virus polymerase product RNA species of the in vitro reaction are positive-stranded RNA, by virtue of the fact that the product RNA will not hybridize to viral RNA extracted from the intact virion, but it will hybridize to double-stranded RNA forms extracted from the infected cell (presumably replicative form RNA containing complementary negative strands). Therefore, the 37S product of the polymerase represents the intracellular form of viral RNA. Studies by McDonnell and Levintow revealed that the heterogeneous 18-28S partially RNase-resistant RNA species represented the functional intermediate in the synthesis of single-stranded viral RNA, and was therefore designated the replicative intermediate (64). The replicative intermediate was shown to be comprised of full-length template-strand RNA (positive or negative polarity), and 4-6 complementary nascent chains of varying length. RNA synthesis is assymmetric in that 5-10 times as much positive strand RNA is synthesized as

negative strand RNA (53). The 20S double-stranded RNA was subsequently found to be an artifact caused by annealing during extraction, and not a true intermediate of viral replication (70). These double-stranded RNA species were designated replicative forms. This data resulted in the model of picornavirus replication seen in Fig. 2. Support of this model was lent by the results of Koliais and Dimmock who found that the rhinovirus polymerase produces only double-stranded RNA in the first 1-1.5 minutes of the in vitro reaction, but thereafter produces single-stranded RNA (53). After the initial period of double-stranded RNA formation, the amount of double-stranded RNA in the reaction remains constant.

Unequivocal proof has not been gathered that there are two separate polymerases, but temperature-sensitive mutants for polymerase activity have been isolated which have their mutations mapped at different loci on the genome, suggesting that at least two proteins are responsible for RNA transcription (30).

Although it has been shown that 3' terminal poly (A) sequences are added in the in vitro reaction, it is not clear whether this is a transcriptive process, or is accomplished by template-independent posttranscriptional terminal addition. The demonstrated presence of 5' terminal poly (U) sequences on intracellular negative strand RNA suggests that poly (A) addition is a transcriptive process. However, nascent poly (A) sequences have never been detected on positive strand RNA obtained by denaturation of replicative intermediates isolated from infected cells or the in vitro reaction. This is true even in instances where product RNA species isolated as single strands (not as part of a replicative intermediate or replicative form) have been shown to contain 3' terminal poly (A) sequences (36, 83, 93, 94, 95).

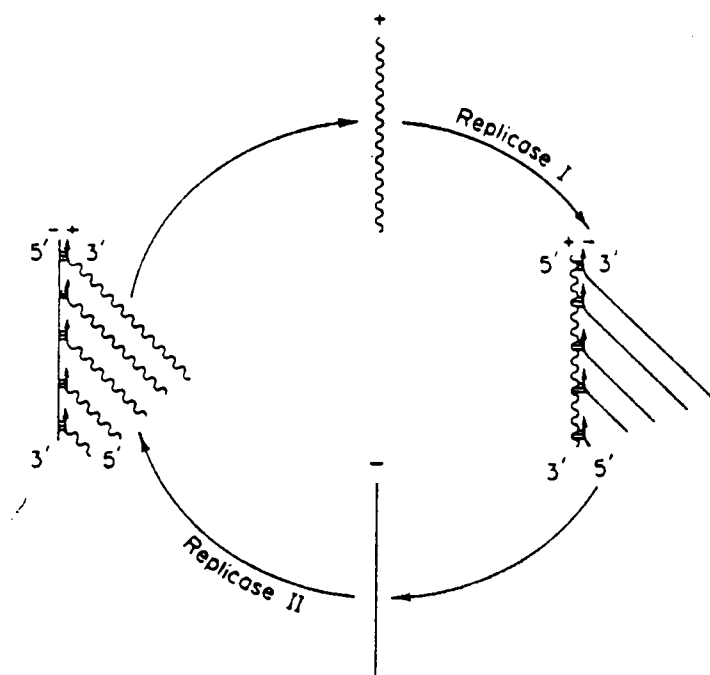


Fig. 2. Model of picornavirus RNA replication.

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Picornavirus Membrane-Associated Replication Complex

Alteration of lipid metabolism in Mengovirus-infected cells was first reported by Amako and Dales (2). Subsequently, Caliguiri and Tamm made a detailed study of the role of membranes in poliovirus replication (21, 22, 23, 24, 25, 69). In characterizing cellular alterations, they utilized the method of Bosman and Hagopian to separate cellular smooth membranes from cellular rough membranes (16). In this method, a cellular cytoplasmic lysate (minus the nuclei) is centrifuged isopycnically in a discontinuous sucrose gradient. Membranes of lighter density (smooth membranes) sediment in discrete bands near the top of the gradient, whereas rough membranes and particulate matter (rough endoplasmic reticulum, ribosomes) sediment in discrete bands near the bottom of the gradient. Isopycnic sedimentation of a poliovirus-infected cell lysate in a discontinuous sucrose gradient revealed 7 discrete bands of densities 1.08, 1.12, 1.18, 1.21, 1.25, 1.27, and 1.31 g/cm³. Analysis of these bands resulted in the discovery that the appearance and composition of the membrane bands fluctuates in relation to the course of the viral infection. More specifically, smooth membranes can be seen to proliferate starting 2.5-3.25 hours postinfection, and continue until late in the infection. At the same time, the rough endoplasmic reticulum decreases in quantity, but increases in buoyant density. The proliferating smooth membranes differ from their counterparts in uninfected cells by their 1) higher phospholipid:protein ratio, 2) altered phospholipid composition, and 3) reduced specific activity of NADH diaphorase. Detailed study of each membrane band gave the following results. Membrane band #2 at 1.12 g/cm³ (smooth membranes) is the site of poliovirus RNA replication. This was evidenced by the

location of the majority of the polymerase activity, and [^3H] uridine incorporation into viral RNA species. Membrane band #3 at 1.18 g/cm^3 contained a significant amount of polymerase activity and viral RNA species. Membrane band #5 at 1.25 g/cm^3 contained the rough endoplasmic reticulum and was the site of viral mRNA translation. Experiments in which cells were pulse-labeled for increasing periods of time revealed that for short pulse times, viral RNA was found predominantly in membrane band #2, but as the pulse times increased, viral RNA was found in fraction #5, presumably undergoing translation. This evidently represents the synthesis of viral RNA in association with smooth membranes and the subsequent translocation of the RNA to the rough endoplasmic reticulum, or, alternatively, the synthesis of viral RNA in association with smooth membranes and the subsequent alteration of the membrane to become rough endoplasmic reticulum. Membrane bands #2, #3, and #5 were also studied after sodium deoxycholate solubilization followed by rate zonal centrifugation of the solubilized structures through sucrose gradients. Band #2 contained a replication complex (i.e., template RNA, polymerase, and nascent complementary RNA) which sedimented at 130S and was composed of replicative intermediates, replicative forms, and ribosomal subunits. Band #3 contained a replication complex sedimenting at 230S which was composed of 74S ribosomes, single-stranded viral RNA, replicative intermediates, and replicative forms. Contained in band #5 were viral polyribosomes which sedimented at a coefficient of 320S. Taken together, this data indicates that poliovirus RNA replication takes place in association with the smooth membrane bands #2 and #3. In addition to viral RNA replication taking place in association with smooth membranes,

there is suggestive evidence which indicates that viral maturation may take place in association with smooth membranes, coupled to RNA replication. This evidence is the discovery of poliovirus procapsid particles late in infection in association with smooth cytoplasmic membranes which are harboring viral RNA synthesis (21).

Picornavirus Membrane-Free Replication Complex

In order to further study the picornavirus replication complex, several investigators have, using detergents, removed the membranes from the membrane-associated replication complex. This has been done most notably for poliovirus, Mengovirus, and foot-and-mouth disease virus, with results being similar for all three replication complexes (4, 10). When sedimented through rate zonal sucrose gradients, two size classes of soluble replication complexes are seen. These are designated "heavy" and "light". The light replication complex has a sedimentation coefficient of approximately 70S, contains 20S double-stranded RNA, and incorporates [^3H] UTP into 20S double-stranded RNA in vitro. The heavy complex, which has a sedimentation coefficient of 100-300S, contains largely single-stranded, viral-sized RNA, with some polydisperse RNase-resistant RNA. The heavy complex incorporates [^3H] UTP in vitro into viral-sized, positive-stranded, single-stranded RNA. The heavy complex can be reduced to a sedimentation coefficient of less than 60S using deproteinizing agents. In addition to these two complexes, Arlinghaus and Polatnick have discovered a third complex which sediments faster than 300S, and which synthesizes viral-sized RNA (37S), and 20S double-stranded RNA, leading to the speculation that this complex contains both heavy and light replication complex

components which are both necessary for effective viral RNA replication (5). In contrast to these findings, Ehrenfeld et al. have found only one soluble replication complex for poliovirus, having a sedimentation coefficient of 70S (38). This complex synthesizes both viral-size 37S RNA and double-stranded 20S RNA. The isolation of active complexes is inhibited by the addition of protein-synthesis inhibitors to the cell. The significance of this single soluble replication complex is not known, but the answer may lie in the different methods of solubilization and isolation used by the individual authors.

Purification of the Picornaviral Polymerase

In recent years several groups have achieved purification and characterization of the picornaviral polymerase, an accomplishment which has allowed a more detailed study of picornavirus transcription. This feat has been done most notably in polioviruses and encephalomyocarditis viruses. Dasgupta et al. isolated the poliovirus polymerase from the soluble phase of poliovirus-infected cells and purified it more than 60 fold by phosphocellulose chromatography and poly (U) sepharose chromatography (33). The purified enzyme has essentially the same cation and ribonucleoside triphosphate requirements as the membrane-associated crude polymerase. However, it does not require an ATP-generating system. The purified replicase preferentially copies poliovirus RNA, but it will also copy the homopolymer poly (A) : oligo (U). Other primed homopolymer templates are inactive. This suggests that a poly (U) polymerase is closely associated with the replicase. A major difference in the purified replicase and the crude membrane-associated replicase is that the purified replicase is able to

copy viral positive strands. That the product is full-length negative strand has been verified by hybridization. A nonstructural poliovirus-specific protein was found associated with the purified polymerase (61). The analogous protein has been found in the Mengovirus replication complex (58) and the encephalomyocarditis virus replication complex (89).

In contrast to the poliovirus replicase, the encephalomyocarditis virus replicase was extracted from smooth cytoplasmic membranes using 0.15% SDS-1,1,2 trichlorotrifluoroethane (89). The replicase was further purified by high salt dextran polyethylene glycol gradient sedimentation. The purified enzyme exhibits the same cation and nucleoside triphosphate requirements as the crude membrane-associated polymerase, but as with the purified poliovirus polymerase, it did not require an ATP-generating system. The replicase nonpreferentially transcribed EMC RNA, QB RNA, BHK ribosomal RNA, and the homopolymer poly (C). This suggests that the purified enzyme is a core replicase which lacks a protein factor responsible for the specific recognition of EMC RNA. As in the poliovirus and Mengovirus, the EMC replicase was found to contain a viral-specific protein associated with it. This protein, EMC viral protein E, is analogous to the poliovirus polymerase protein (NCVP2), and the Mengovirus polymerase protein. Therefore, this protein probably has an integral role in the functioning of picornaviral RNA replication.

CHAPTER III

TOGAVIRAL RNA REPLICATION

Introduction

Togaviruses, like picornaviruses, are grouped into the class IV viruses based on their infectious, polyadenylated RNA, the absence of a virion-associated RNA-dependent RNA polymerase, and the presence of a cell-associated RNA-dependent RNA polymerase. Togavirus transcriptional strategy differs markedly from the picornavirus strategy in that transcription includes a subgenomic message, as well as full-length genomic message. The subgenomic message serves to synthesize viral structural proteins, whereas the full-length message functions as message for nonstructural proteins. Contrasts between picornaviruses and togaviruses will be reviewed in the succeeding sections so that a focused comparison may be made among picornaviral, togaviral, and coronaviral RNA replicational and transcriptional schemes. Since the majority of the research has been conducted on alphaviruses, a serological subgroup of the togaviruses, the review will be necessarily limited to alphavirus RNA replication.

Overview of Alphavirus Replication

As in the picornaviruses, alphavirus adsorption to the cell is mediated by specific cellular receptor proteins. Following adsorption of the virus to a sensitive cell, the envelope of the virus apparently fuses with the cell plasmalemma to initiate the

infection cycle, although some researchers believe that pinocytosis is the initial event in virus penetration (11). After penetration, the viral RNA is uncoated and is translated into viral-specific proteins. One of these proteins is an RNA-dependent RNA polymerase which initiates primary transcription of the viral positive-stranded RNA. The resultant negative-stranded RNA is utilized as a template in secondary transcription to produce viral positive-stranded RNA. Alphaviral secondary transcription differs from picornaviral transcription in that in addition to a full-length positive strand being produced, a subgenomic messenger-polarity RNA is produced. This subgenomic message contains the genes coding for the viral structural proteins. Like the picornavirus replication complex, the alphavirus replication complex can be found in close association with cellular cytoplasmic membranes. After virion assembly and encapsidation of the viral RNA, newly synthesized viruses are released by budding through the cell plasma membrane.

Alphavirus RNA-dependent RNA Polymerase

Studies on alphavirus-specific RNA-dependent RNA polymerases have shown that they are remarkably similar to the picornaviral RNA-dependent RNA polymerases. They are isolated from the cytoplasm in association with particulate structures which are assumed to be membranous because they are solubilized by treatment with sodium deoxycholate (41). The polymerase activity is first seen 3 hours

postinfection and increases at a rate parallel with in vivo RNA synthesis to a maximum at 5-6 hours postinfection. Although early investigators were only able to synthesize 20S replicative form RNA using the crudely prepared polymerase, more recent isolation protocols employing rate zonal sedimentation in sucrose have allowed synthesis of not only double-stranded 20S RNA, but also heterogeneous replicative intermediates and single-stranded viral RNA (85). The rate zonal purification step apparently reduces the amount of endogenous RNases sufficiently to allow newly synthesized single-stranded RNA to retain its structural integrity. At least one investigator has reported the need for mercaptoethanol as a stabilizing agent during isolation of the polymerase and in the polymerase reaction (63). This phenomena is not seen for the picornaviruses or other alphaviruses. Characteristically, the pH optimum of the alphavirus polymerase reaction is 8.0. The optimal concentration of magnesium for polymerase activity ranges from 3mM to 8mM, and all four ribonucleoside triphosphates are required for maximal activity (63, 85). By nearest neighbor frequency analysis, the ribonucleoside triphosphates are incorporated into viral RNA in approximately equimolar amounts. However, the deletion of single ribonucleoside triphosphates from the reaction mixtures causes markedly different reductions in polymerase activity. For example, the deletion of UTP from a reaction assaying SFV polymerase causes only a 30% reduction in activity, whereas the deletion of CTP causes a greater than 90% reduction in activity. A satisfactory explanation for this phenomenon has not yet been put forth (63). The polymerase reaction increases linearly for the first 15-20 minutes of incubation and thereafter levels off (63, 85). The substitution of manganese for magnesium in the

reaction causes a severe inhibition of activity. Actinomycin D does not inhibit the reaction and the reaction is insensitive to DNase added before incubation. Therefore, the template must be RNA. Finally, no equivalent activity can be detected in uninfected cells.

Products of the Alphavirus polymerase

Michel and Gomatos have shown that the products of the Semliki Forest virus-specific polymerase comprise 4 species; three single-stranded RNA species, 49S, 26S, and 22S, and one double-stranded species, 18S (67). Each species is produced in a particulate structure which is separable from the others by sucrose gradient centrifugation. This result suggests a unique replication complex for each RNA species, perhaps with different polymerases. All four RNA species produced in vitro have been detected and characterized in vivo (81, 82). The 49S and 26S RNA species are transcribed from a full-length viral negative strand. Therefore, 49S RNA is the intracellular form of viral RNA. The 26S RNA species contains one third of the base sequences of the 49S RNA. These sequences represent the 3' end of the 49S RNA. The 22S RNA species is a minor RNA species which may be a configurational variant of 49S or 26S RNA. Hybridization experiments have revealed that the 18S double-stranded RNA species represents the replicative form of the single-stranded 26S RNA. Replicative intermediates seen in vivo sedimenting between 23S and 30S can be treated with RNase to remove single-stranded tails which results in the formation of 3 unique replicative forms. The largest replicative form, RF I, has a sedimentation coefficient of 23S and a molecular weight of 9×10^6 . It is a double-stranded form of genomic 49S RNA.

The smallest replicative form, RF III, has a sedimentation coefficient of 18S, a molecular weight of 3×10^6 , and represents a double-stranded form of the 26S RNA. The middle-sized replicative form, RF II, sediments at 20S, has a molecular weight of 5.6×10^6 , and represents the double-stranded form of the remaining two thirds of the genome sequences not found in 26S RNA. Because all replicative intermediates are as large or larger than the replicative form containing full-length double-stranded RNA, and because RF II and RF III are found in equimolar amounts, it was concluded that RF II and RF III are generated by digestion of a replicative intermediate containing full-length template RNA. Short pulses with [^3H] uridine given in the middle of the infectious cycle label only RF I and RF III in their nascent positive strands, indicating that 49S and 26S RNA species are synthesized with similar kinetics. These findings are summarized in a model for transcription of alphavirus-specific positive-stranded RNA seen in Fig. 3. Briefly, the model states the following: Both 49S and 26S RNA are transcribed from a full-length negative strand. Transcription of 49S RNA is initiated terminally at the 3' end of the template, whereas 26S RNA is transcribed by internal initiation two thirds of the way from the 3' end of the negative strand template, with transcription continuing to the end of the template. Regulation of this initiation is achieved via a virus-specified interconversion protein. Hypothetically, the interconversion protein binds near the 26S RNA initiation site, jamming polymerase molecules which are transcribing 49S RNA, thereby preventing readthrough of the initiation site.

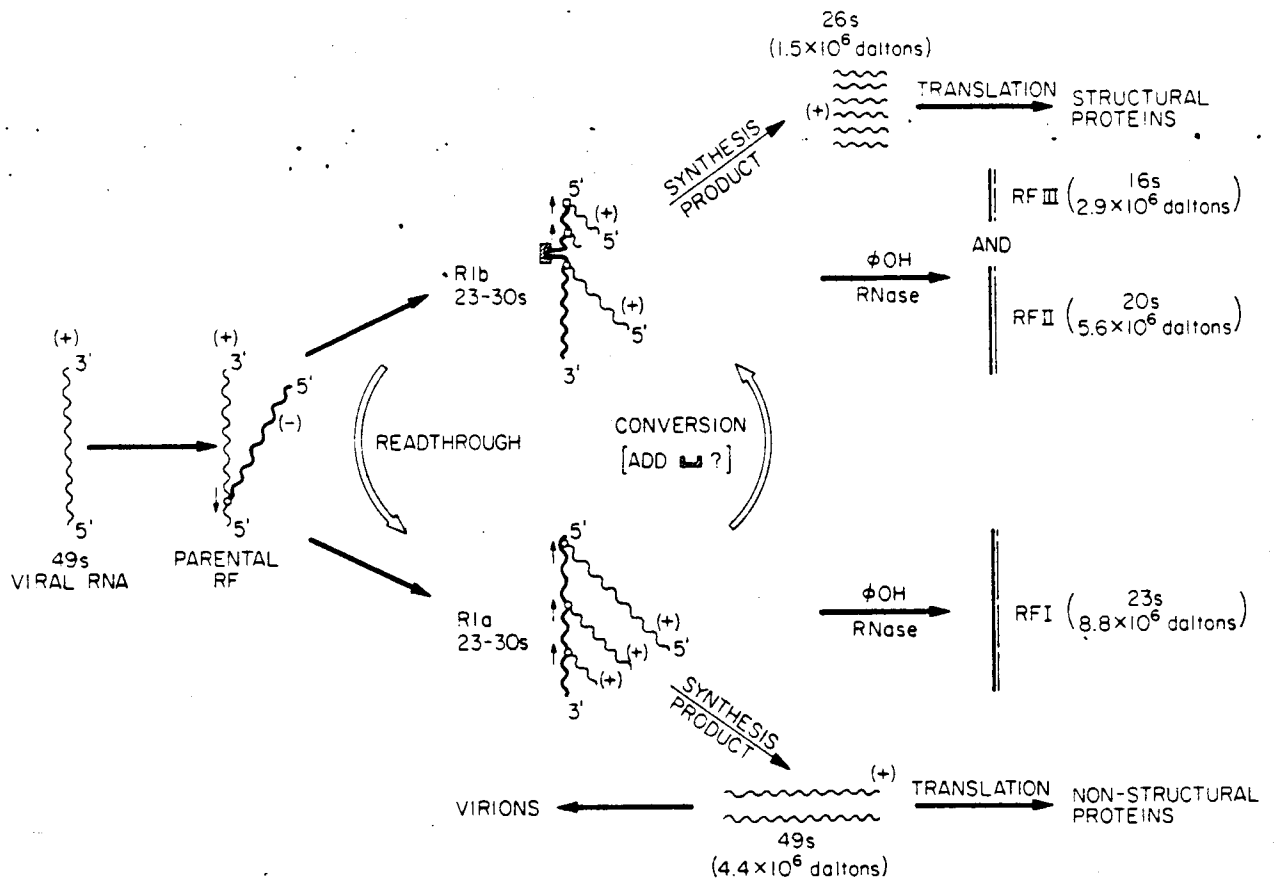


Fig. 3. Model for the replication of alphavirus RNA.

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Initiation is then allowed at the 26S initiation site, possibly by alterations in the secondary structure of the RNA template mediated by the interconversion protein (81, 82).

Intracellular kinetic experiments reveal that 49S negative-stranded RNA is transcribed first and reaches a maximum at 2.5 hours postinfection. Positive strand synthesis increases until it reaches a constant level at 3 hours postinfection, whereupon it remains constant for up to 6-7 hours postinfection (19).

The poly (A) tracts of 49S and 26S RNA are transcribed from a poly (U) sequence of about 60 residues at the 5' end of the negative strand template (28). Therefore, it would appear that the mechanism for polyadenylation is contained within the replication complex, by virtue of complementary transcription, and no additional enzymes are necessary, cellular or viral, to add poly (A) sequences posttranscriptionally.

Membrane-Associated Alphavirus Polymerase

The association of viral replication with cellular smooth cytoplasmic membranes has been studied in great detail by R. M. Friedman et al. working with Semliki Forest virus (41, 42, 43, 47, 48, 84). The predominant portion of this section of the review will deal with his studies. Friedman's initial efforts were kinetic studies in which pulse-labeled RNA was located in cellular fractions at various times postinfection. After a 5 minute pulse of [^3H] uridine, label was detected in large cytoplasmic vacuoles, designated CPV-1, which could be seen in electron micrographs of infected cells, but not in uninfected cells. The structures were isolated in sucrose gradients and were found to contain 42S, 26S, and 20S viral-specific RNA. Furthermore, high

resolution autoradiography revealed that the CPV-1 are loci of [^3H] uridine incorporation. In addition, the distribution of the silver grains suggested that the CPV-1 limiting membranes, rather than the vacuole interior, are the actual site of specific [^3H] uridine uptake. This data strongly suggested that CPV-1's were the site of membrane-associated viral RNA synthesis. Characterization of the CPV-1 showed that the vacuoles were approximately 50 nm in diameter, membrane-limited, and had a fine central density. They were detected by electron microscopy in cells 3-6 hrs postinfection, coinciding with the time of rapid viral growth, but preceeding the time of nucleocapsid formation. The number of CPV-1 per cell remained constant through multiplicities of infection from 0.1 to 10 PFU/cell. Actinomycin D, which affects cellular transcription and translation, did not affect biogenesis of the CPV-1. However, factors which affected viral RNA replication (guanidine) and viral protein synthesis (cycloheximide) caused a decrease in the amount of CPV-1 produced. Subsequent studies revealed that at 1 hr postinfection up to 36% of the input SFV RNA which was cell-associated, was found bound to CPV-1. Interestingly, the addition of cycloheximide to the cells almost totally inhibited this binding, but the addition of interferon did not. Therefore, it would appear that the binding is mediated by a protein.

Finally, Friedman conducted studies on membrane fractions isolated using the method of Bosman and Hagopian (16). This is the same method utilized for picornaviruses in which cellular smooth cytoplasmic membranes are separated from cellular rough membranes and particulate matter by centrifugation through a discontinuous gradient

of aqueous sucrose. Seven discrete bands resulted from the centrifugation of the SFV-infected chick embryo cytoplasmic fraction. These fractions were the following densities, 1.04, 1.07, 1.10, 1.12, 1.16, 1.19, and 1.22 g/cm³. The top four fractions contained predominantly smooth membranes, whereas the bottom fraction was enriched in rough endoplasmic reticulum. Membrane band #5 (1.16 g/cm³) was enriched in CPV-1 of the type described previously. In addition, membrane band #5 was heavily enriched in pulse-labeled RNA, viral polymerase activity, and RNA species associated with RNA replication (42S, 26S, 33S, replicative intermediates, and replicative forms). Thus, ultrastructural, biochemical, and biological evidence are consistent with the interpretation that CPV-1 membranes represent a significant element of the viral replication complex.

Using a slightly different approach, Sreevalsan found generally the same result for Sindbis virus-infected cells (84). Membranes with attached nucleic acids were isolated using the "M band" technique, which consists of lysing cytoplasmic extracts with the detergent sodium lauryl sarcosinate. In the presence of magnesium the detergent crystallizes and the crystals are banded by centrifugation through a sucrose gradient. The band of magnesium sarcosinate (M band) contains the cell membranes and any attached DNA or RNA. Soluble nucleic acids are not isolated within this band. Isolated membranes from cells infected with Sindbis virus had associated with them viral RNA, viral RNA polymerase, and about 60-70% of the viral RNA labeled when short pulses of [³H]uridine were used. This RNA contained most of the replicative intermediates and replicative forms of viral RNA found in infected cells.

Isolation of Alphavirus-specific Polymerase

At the present time the single report describing the isolation and purification of an alphavirus-specific replicase is that by Clewley and Kennedy done for Semliki Forest virus (29). The purification was accomplished by preparation of a crude lysate of infected cells by Dounce homogenization, differential centrifugation to obtain a mitochondrial pellet (13,000 x g for 20 minutes), isopycnic centrifugation on a discontinuous sucrose gradient to give a membranous band at 1.16 g/cm³ (Friedman's band #5), solubilization of this band followed by rate zonal centrifugation to obtain a 25S solubilized polymerase complex, and, finally, affinity chromatography through an oligo (dT)-cellulose matrix bearing immobilized 42S virion RNA. The isolation resulted in a 360 fold purification with a 5% recovery of activity. The membrane band at 1.16 g/cm³ was shown to have a sedimentation coefficient of 260S prior to solubilization. Therefore, the reduction in sedimentation coefficient from 260S to 25S argues for a membrane association of the replication complex. The purified polymerase was shown to be active by the synthesis in vitro of viral replicative intermediates and replicative forms (80% RF's). However, no single-stranded RNA species were produced, and no in vitro reinitiation took place. The reasons for this are not clear, but they may have to do with RNases present during the extraction, or, alternatively, a functional protein lost during purification. The addition of 42S viral RNA failed to stimulate the enzymatic activity. Three polypeptides of molecular weight 90,000, 63,000, and 40,000 were detected in the purified replicase complex. Of these, two were viral-specified, 90,000 and 63,000, and the

other protein was cellular in origin. It is not known whether the two viral proteins represent two separate polymerase activities as has been suggested by Segal and Sreevalsan (79). It is also not clear whether or not the 40,000 molecular weight cellular protein is necessary for the functioning of the polymerase.

CHAPTER IV

RATIONALE

At the present time coronaviruses are tentatively classified as class IV viruses in the replicational scheme of Baltimore, based on their single-stranded, polyadenylated, nonsegmented, infectious genome RNA. However, no viral-specific, cell-associated, RNA-dependent RNA polymerase has been detected for any of the coronaviruses. In addition, recent reports suggest a unique replicational strategy for the coronaviruses (80, 86). Therefore, coronaviruses may represent a totally new classification group of viruses. In view of this, the intent of this dissertation is to clarify the relationship between coronaviruses and the class IV viruses by reporting the discovery and characterization of a coronavirus-induced RNA-dependent RNA polymerase, to present preliminary data on the in vivo and in vitro transcription of coronavirus RNA, and, finally, to use this data to speculate on possible coronavirus transcriptional strategies.

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CORONAVIRUS REPLICATIONAL STRATEGY

II. A MEMBRANE-ASSOCIATED RNA-DEPENDENT RNA POLYMERASE ACTIVITY IN CORONAVIRUS-INFECTED CELLS

CHAPTER I

INTRODUCTION

Infectious RNA has been demonstrated in the avian infectious bronchitis virus (29, 38), mouse hepatitis virus (28), and transmissible gastroenteritis virus (TGEV) of swine (9), three members of the coronavirus family. This property alone would characterize these coronaviruses as positive-stranded viruses and place them in category IV of the Baltimore scheme (4). 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 do find it associated with apparent membrane structures in infected cells. The enzyme required the presence of all three ribonucleoside triphosphates in addition to the labeled UTP, and it was not inhibited by actinomycin D, indicating that the template was RNA. The heated product was digested by ribonuclease, and not digested by deoxyribonuclease, indicating that the product was RNA. Mg^{++} was required as a divalent cation for enzymatic activity and its optimal concentration was determined to be approximately 5 mM.

CHAPTER II

MATERIALS AND METHODS

Cells

The epithelioid swine testicle cell line, (ST), developed by McClurkin (32) was obtained from R. Woods, National Animal Disease Center, Ames, Iowa. Cells were grown as monolayers in growth medium consisting of Dulbecco modified Eagle medium, 4500 mg of glucose per liter, 0.35% NaHCO_3 , 50 ug of gentamicin per ml, 5% fetal calf serum (Grand Island Biologicals, Grand Island, N.Y.), and 3% calf serum (Kansas City Biologicals, Inc., Lenexa, Kansas). When grown in 850 cm^2 roller bottles, serum concentrations were changed to 9% fetal calf serum and 3% calf serum. In all studies, cells were infected within 48 hours of reaching confluency. Cells at this age were found to be the most productive of intracellular viral components as well as mature progeny virus.

Viruses

Clone 116 of the Purdue strain of swine TGEV was obtained from E. Bohl, Ohio Agricultural Research and Development Center, Wooster, Ohio. The virus was cloned twice again by us. Cloned virus was passaged twice at a multiplicity of infection of greater than 0.1 PFU/cell, and then viral stocks were prepared from passage 3 through 6 by infecting cells at a multiplicity of approximately 10 PFU/cell. Viral titers ranging from 10^8 to 10^9 PFU/ml were obtained in stock virus preparations. The Indiana strain of vesicular stomatitis virus was obtained from A. Brown, University of Tennessee, Knoxville.

Virus Titration

Virus was titered on cells in 24-well tissue culture plates (Falcon). Serial 10-fold dilutions of virus were made in serumless medium, and 0.1-ml amounts of each dilution were applied to cells that had been rinsed with Earle balanced salt solution. After a 1-h incubation, inoculum was replaced with medium containing 1% immunoglobulin G-free fetal calf serum (Grand Island Biologicals, Grand Island, N.Y.) and 0.4% methyl cellulose, and cells were incubated at 37°C for 4 days in a humidified incubator. Overlay medium was then removed, and cells were stained with a 1% solution of crystal violet. Alternatively, 0.1% agarose replaced methyl cellulose in the overlay medium and plaques were observed with the inverted microscope.

Virus Growth and Purification

Confluent monolayers of ST cells grown in 850 cm² plastic roller bottles (Falcon) were drained and rinsed three times with Earle balanced salt solution. Cells were then infected at a multiplicity of infection of approximately 10 PFU/cell. After a 1-h adsorption at 37°C, the inoculum was removed and 25 ml of medium containing 2% fetal calf serum was added per bottle. Incubation was continued for 15 to 17 h, at which time virus was harvested. Supernatant fluids were clarified by centrifugation at 10,000 x g for 10 min and layered onto a 24-ml continuous 20-60% (wt/wt) sucrose gradient. Virus was isopycnically sedimented by centrifugation at 90,000 x g for 4 h at 4°C in a Sorvall AH-627 rotor. The position of virus in the gradient was established by measuring the infectious titer of each gradient fraction and by electron microscopy. Routinely, virus was located spectrophotometrically

by measuring the absorbance at 254 nm wavelength or by measuring the radioactivity in cases where viral components were radioisotopically labeled. Fractions containing virus were pooled, diluted with an equal volume of TNE (0.01 M Tris-hydrochloride (pH 7.5)-0.1 M NaCl-1.0 mM EDTA) and pelleted at 150,000 x g for 2 h in a Sorvall AH-650 rotor. The pellet was either used immediately or stored at -80°C. Gradients were harvested from the top with an Isco gradient fractionator. Sucrose solutions were made in TNE.

Vesicular stomatitis virus was obtained by infecting roller bottles of Maden-Darby Bovine Kidney cells at a multiplicity of infection of 0.1 PFU/cell and incubating cells for 20 h. Purification of vesicular stomatitis virus was as described for TGEV.

RNA Polymerase Assay

The assay for virion-associated RNA-dependent RNA polymerase was one optimized by Huang, et al., for the VSV-associated enzyme (26). In a standard reaction, 50 ul 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 ul 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 ug/ml Triton N-101; 0.83 mM $[5-^3\text{H}]$ UTP (4.4×10^7 cpm/pmole) or 2.20 mM $[8-^3\text{H}]$ GTP (1.65×10^7 cpm/pmole; 0.67 mM each of ATP, CTP, and GTP (or UTP when $[^3\text{H}]$ 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 μ m) after precipitation with 5% trichloroacetic acid-0.05 M sodium pyrophosphate at 0°C. Dried filters were counted in toluene containing 4 mg/ml of 2,5-bis (5'-tert-butyl-benz-oxazolyl-(2')) thiophene (BBOT).

The assay for cell-associated RNA-dependent RNA polymerase was a modification of that described by Arlinghaus and Polatnick (1). In a standard reaction, 50 μ l of sample, suspended to a protein concentration of 10 to 20 mg per ml in 0.25 M sucrose-0.0015 M MgCl_2 -40 μ g/ml dextran sulfate-0.28% (wt/wt) deoxycholate, was assayed in a 200 μ l solution (total volume) containing: 50 mM Tris-hydrochloride (pH 8.0); 5 mM magnesium acetate; 10 mM phosphoenolpyruvic acid; 40 μ g/ml phosphoenolpyruvate kinase; 17.5 mM 2-mercaptoethanol; 20 μ g/ml actinomycin D; 1.25 mM 5- ^3H UTP (4.4×10^7 cpm/pmole) or 3.3 mM 8- ^3H GTP (1.65×10^7 cpm/pmole); 0.5 mM each of ATP, CTP, and GTP (or UTP when ^3H GTP was used). The amount of labeled substrate incorporated into product on 60 min at 37°C was measured by collecting the product on membrane filters after TCA precipitation.

Subcellular Fractionation

Batches of $2-8 \times 10^8$ cells (representing 1 to 4 confluent roller bottle cultures) were treated as described in 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 (8). This pellet was designated the postnuclear 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.

Materials

[^3H] uridine (8 Ci/mmol) was obtained from Schwartz/Mann. ^{32}P as orthophosphate (144 Ci/mg) was obtained from Amersham. [5,6- ^3H] UTP (40 Ci/mmol) and [8- ^3H] GTP (15 Ci/mmol) obtained from ICN Chemical and Radioisotope Division, were in 50% ethanol solution. The ethanol was removed by lyophilization and the nucleoside triphosphate was dissolved in the reaction medium.

CHAPTER III

RESULTS

Growth Characteristics

When confluent monolayers of ST cells were infected with TGEV at a multiplicity of 10 PFU/cell, there was total destruction of the monolayer by 22 to 26 h postinfection. Affected cells became rounded and detached from the plastic. Peak titers under these conditions were obtained by 10 to 20 h postinfection (Fig. 1), a time preceding the development of maximal cytopathic effect. Virus to be used for inoculum or to be purified for further study was collected at 18 to 22 h postinfection.

Virus titers were extremely dependent on the type of serum and lot of serum used in the growth medium. Titers ranged from a low of 1×10^6 PFU/ml for virus grown in cells on medium containing 10% calf serum, to a high of 8×10^8 PFU/ml for virus grown in cells on medium containing 5% fetal calf serum and 3% calf serum. Titers of virus grown in medium containing 10% newborn calf serum varied from 2×10^6 PFU/ml for a poor lot to 2×10^8 for a good lot. This variability in titer evidently stems from the effect of the serum on the physiological status of the cells (37).

Virus Purification

The results of isopycnic sedimentation of TGEV are shown in Fig. 2. From a 15 to 17 h postinfection harvest, a major peak of [^3H] uridine activity with a buoyant density of 1.20 g/cm^3 and a minor peak (or sometimes only a shoulder) with buoyant density of

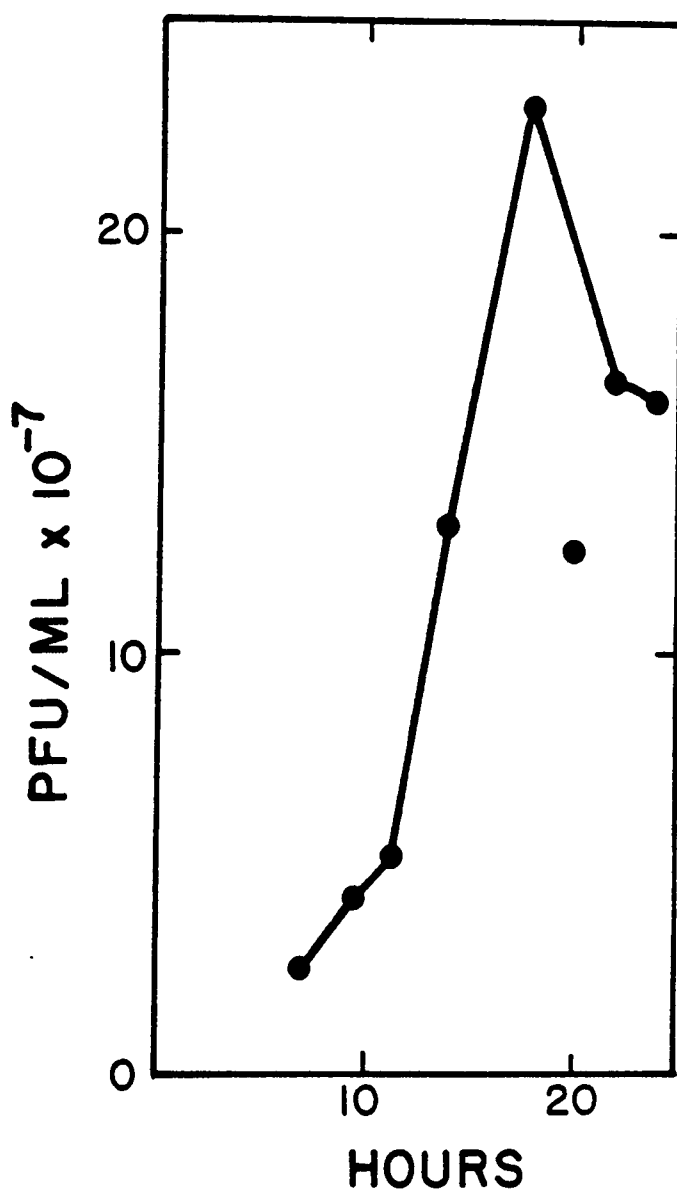


Fig. 1. Growth curve of TGEV. A confluent roller bottle of ST cells was infected at a multiplicity of infection of 10 pfu/cell. Samples of the medium were taken at 2-h intervals and immediately frozen at -80°C . Virus was titrated as described in the text.

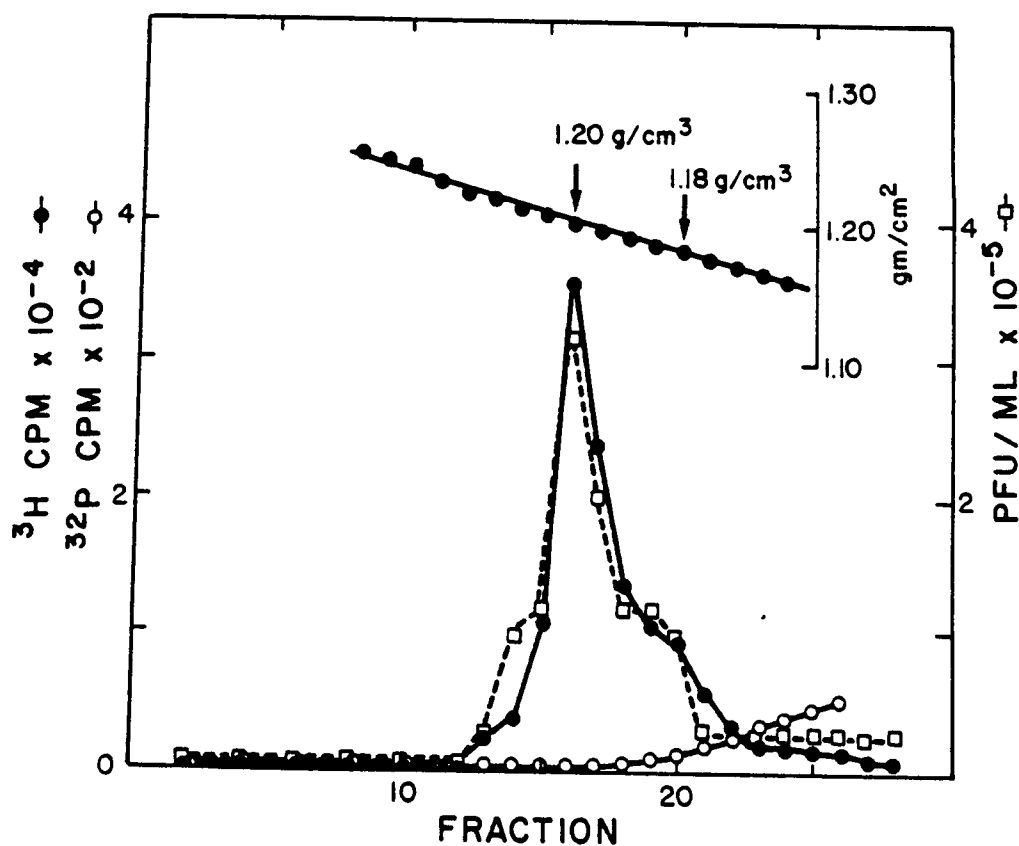


Fig. 2. Isopycnic sedimentation of TGEV. Virus labeled with [^3H] uridine was purified as described in the text. Infectivity and trichloroacetic acid-precipitable radioactivity was measured in 10 lambda portions. The buoyant density of each fraction was determined by reading the refractive index. ^{32}P -labeled material from uninfected cells was sedimented in parallel as described in the text.

1.18 g/cm³ were seen. The results of infectivity assays (Fig. 2) and electron-microscopic studies (Fig. 3 and data not shown) indicate that TGEV is found at both densities, but that more are found at a buoyant density of 1.20 g/cm³. Our impression from preliminary studies is that particles of buoyant density of 1.18 g/cm³ have fewer surface projections and many appear to be ruptured. RNA extracted from particles of both densities was indistinguishable in our analyses, however (9), and particles with buoyant densities ranging from 1.18 to 1.20 g/cm³ were routinely collected for further study.

To determine the degree of purity of virus prepared in this manner, uninfected ST cells were labeled with [³²P]orthophosphate to label cellular nucleic acids and other phosphorous-containing components and the supernatant fluids were centrifuged in parallel (Fig. 2). No [³²P]-labeled material was found in the isopycnic gradient at the density region of 1.18 to 1.20 g/cm³, indicating that the [³H]-labeled material is probably all viral RNA and that there is very little, if any, uninfected cellular material contaminating the virus preparation. In other experiments (data not shown) in which differentially labeled infected and uninfected cells were mixed, ruptured by freezing, and copurified, there was also no evidence of cellular RNA contamination of purified TGEV. These tests, however, do not rule out the possibility that RNA-containing vesicles are being produced by infected cells which would copurify with TGEV.

To examine the possibility that TGEV does possess a virion-associated RNA-dependent RNA polymerase, semi-purified and purified viral preparations were examined using an RNA polymerase assay optimized for vesicular stomatitis virus-associated enzyme. Table 1 demonstrates

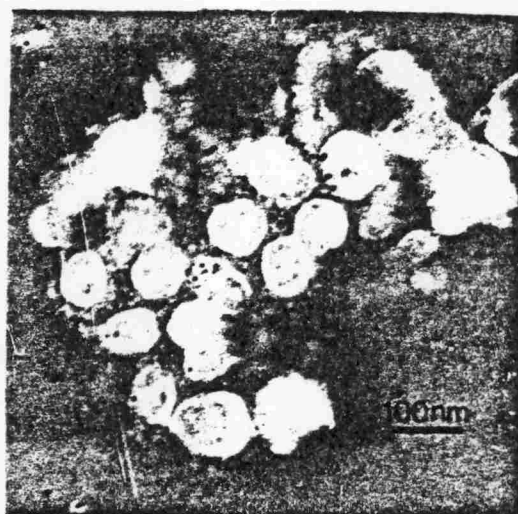


Fig. 3. Electron micrograph of purified TGEV. Virus was purified by isopycnic sedimentation as described in the text. An aliquot from the fraction having a buoyant density of 1.20 g/cm^3 and showing the peak infectivity titer (Fraction 16 in Fig. 2) was applied to a carbon-coated formvar-filmed copper grid, and stained with a 0.5% solution of uranyl acetate. Electron microscopy was done on an RCA EMU-4. The bar represents 100 nm.

Table 1. Absence of RNA polymerase in purified TGEV.

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

^aApproximately 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.

^bApproximately 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.

^cApproximately 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, chilled 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 all experiments, virus was resuspended and assayed as described in the text.

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 noenzyme activity could be detected (data not shown).

Actinomycin D Inhibition of Cellular RNA Synthesis

Early experimentation designed to detect an RNA-dependent RNA polymerase activity in whole infected-cell lysates yielded equivocal results. An RNA-dependent RNA polymerase activity could be detected, but at best, it was only double that found in uninfected cells. A systematic effort was therefore made to define the time period in which viral RNA was synthesized at a maximum rate and to define the intracellular location of this synthesis to enhance our chances of detecting an active polymerase in vitro. In our measurements of RNA synthesis, no effort was made to distinguish between transcription (i.e., synthesis of viral mRNA species) or replication (i.e., synthesis of viral genome RNA species). Rather, only total virus-specific RNA synthesis was measured. To measure this, we needed first to establish the conditions under which all (or most) cellular RNA synthesis would be inhibited while at the same time allowing viral RNA synthesis to progress normally. Our approach was to use actinomycin D which has often been used to inhibit cellular RNA synthesis in studies of RNA viruses known to replicate solely in the cytoplasm (6, 25, 35). Only in the case of myxoviruses is actinomycin D shown to completely inhibit RNA synthesis (7). Reports regarding the effects of this antibiotic

on coronavirus replication are equivocal and we therefore needed to establish the effects of this antibiotic on the TGEV system beginning with the effect of actinomycin D on cellular RNA synthesis. Initially, the minimum concentration of actinomycin D required to inhibit cellular RNA synthesis was determined by labeling cells with radioactive uridine in the presence of varying concentrations of actinomycin D. Our results, illustrated in Fig. 4, establish that cellular RNA synthesis was inhibited over 95% at a concentration of 0.1 ug/ml. Concentrations of 0.1 to 2.0 ug/ml actinomycin D were used in further studies. Using a concentration of 0.5 ug/ml, approximately 90% of cellular RNA synthesis was inhibited by 1 h after adding actinomycin D to a newly confluent monolayer of cells (Fig.5). This rate of host RNA shutdown is consistent for data for other mammalian cell systems in which actinomycin D was used in concentrations of 0.1 to 5 ug/ml (6, 25, 35).

Actinomycin D Inhibition of TGEV Production

The effect of actinomycin D on the replication of TGEV as measured by the yield of infectious virus particles was measured as described in Table 2. At most, there was a 90% inhibition of virus production. This is consistent with published reports for the inhibition of human coronavirus (27), and reports for other coronaviruses (40). It is not possible to say from these data whether actinomycin D affects TGEV replication directly or whether the affect is indirect by way of an interference with other supportive cellular metabolic processes.

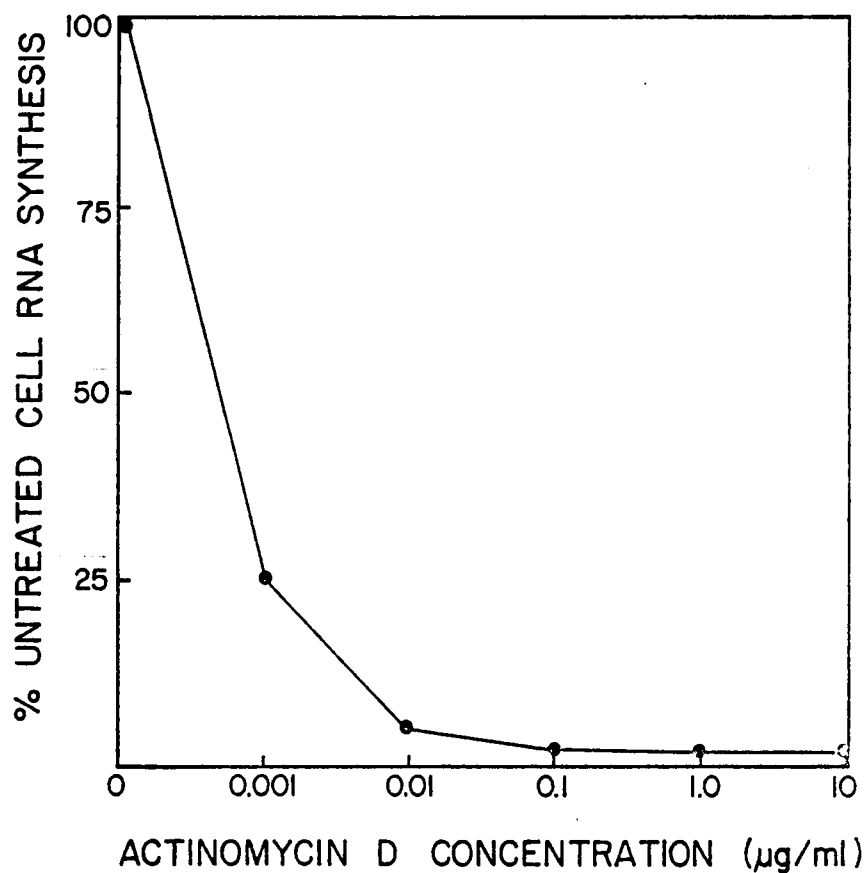


Fig. 4. Actinomycin D inhibition of cellular RNA synthesis. Cells in 30 mm plates were treated with concentrations of actinomycin D ranging from 0 to 10 μg/ml in growth medium. Three plates were used for each concentration. After 5 hours of treatment, 4 μCi of [³H] uridine (33 Ci/mmol) was added per plate and the cells were incubated for 30 min at 37°C. Cells were then scraped into ice cold 5% (wt/wt) trichloroacetic acid - 0.01 M sodium pyrophosphate and the precipitate was collected on Whatman GF/F glass filters. Radioactivity was quantitated by scintillation spectrophotometry.

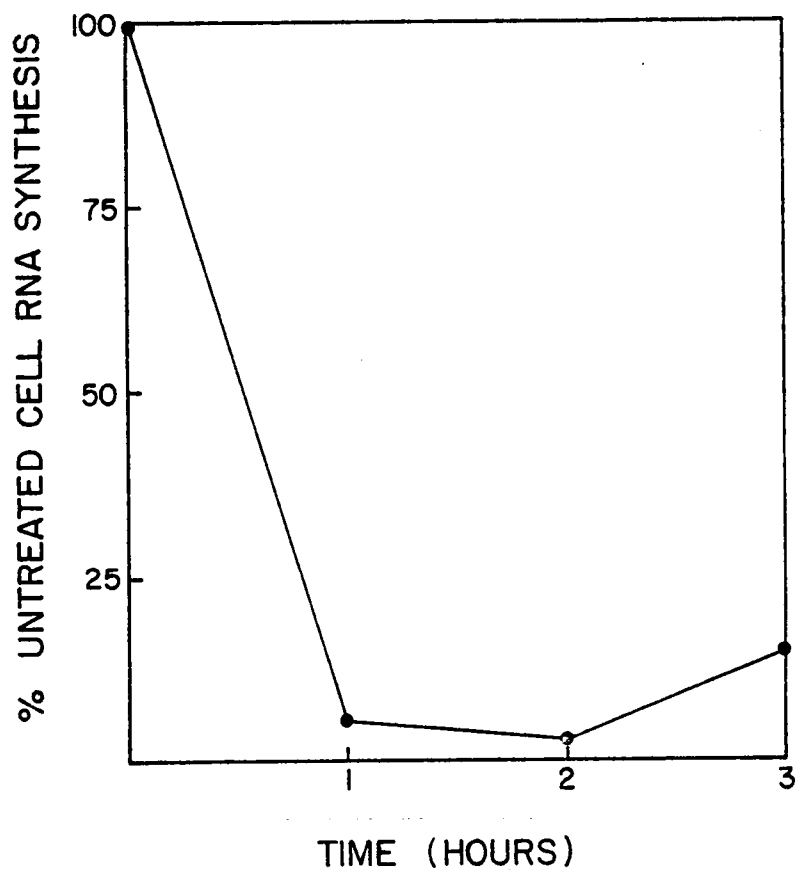


Fig. 5. Kinetics of actinomycin D inhibition of cellular RNA synthesis. Cells in 30 mm plates were treated with $0.5 \mu\text{g}$ actinomycin D per ml in growth medium for 0.5 h, 1.5 h, and 2.5 h. Three plates were used for each time point. Ten Ci of [^3H] uridine (33 Ci/mmol) was added per plate and the cultures were incubated for 30 min at 37°C . Acid precipitable radioactivity in each plate was assayed for as described in Fig. 4.

Table 2. Actinomycin D inhibition of TGEV production.

Treatment ^a	Titer (pfu/ml)
None	1.4×10^8
Actinomycin D	2.0×10^7

^aCells in 150 cm² plastic flasks were infected at a multiplicity of 10 pfu/cell and virus was allowed to adsorb for 1 h at 37°C. At the end of the adsorption period, cells were rinsed and refed with medium containing 2% fetal calf serum. This medium contained 0.1 ug actinomycin D per ml for those cultures being treated with actinomycin D. All cultures exhibited 100% cytopathic effect at 23 hours p.i. Virus was harvested by one freeze-thaw cycle and supernatant fluids were clarified at 10,000 x g for 20 min. Virus was titered from the clarified supernatant as described in the text.

Kinetics of In Vivo Viral RNA Synthesis

The time during the growth cycle of the virus at which viral RNA synthesis occurs at a maximal rate was determined by pulse-labeling infected cells with an RNA-specific radiolabel following a 1-h pretreatment with actinomycin D (Fig. 6). The 1-h actinomycin D pretreatment period was chosen based on the rate of host cell RNA shutdown (Fig. 5) and was considered to be the minimum interval of time required to yield unambiguous results. The rate of RNA synthesis was found to be maximal between 4 and 6 hours postinfection with a peak rate of synthesis at around 5 hours postinfection. The rate of RNA synthesis at times outside this period were markedly less. Mock-infected cells showed no such increase throughout the 20-h labeling period, indicating that the observed RNA synthesis is for certain induced by the viral infection and probably represents virus-specific RNA synthesis.

Actinomycin D Inhibition of TGEV RNA Synthesis

Since actinomycin D partially inhibits the replication of some coronaviruses (27, 40), it was important to determine whether this inhibition could be seen in the synthesis of viral RNA. If actinomycin D inhibits the synthesis of coronaviral RNA, then treatment of cells with actinomycin D prior to isolating RNA-synthesizing complexes would probably be of little value in differentiating viral from cellular polymerase activities. Such actinomycin D treatment of cells was shown to be beneficial in the study of picornaviral RNA polymerases (6, 25, 35). To determine the effect of actinomycin D on TGEV RNA synthesis, infected cells were incubated with actinomycin D at

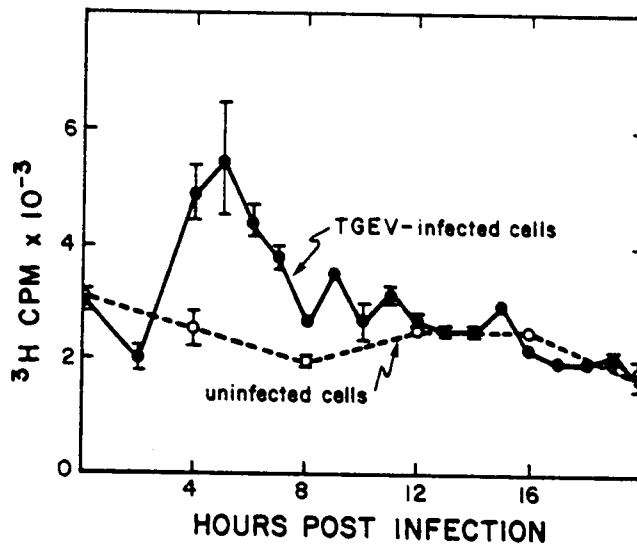


Fig. 6. Kinetics of viral RNA synthesis. Cells in 30 mm plates were infected simultaneously with TGEV at a multiplicity of 10 pfu/cell, or mock infected with balanced salt solution, and refed with medium containing 2% fetal calf serum. At 1 hour prior to labeling, actinomycin D was applied to the cells at a concentration of 0.1 μ g/ml and kept there for the duration of the RNA labeling period. Four Ci [³H] uridine (33 Ci/mmol) was added per plate at various times postinfection for 1-h pulses. Periods were timed from the beginning of virus adsorption. Duplicate cultures were used for each time point. Acid-precipitable radioactivity in each culture was assayed for as described in Fig. 4.

increasingly longer intervals prior to the time of [^3H] uridine pulse-labeling. Because 5 h postinfection is the observed time for the maximal rate of TGEV RNA synthesis, this time was chosen for pulse-labeling. At 5 h postinfection when separate infected cultures had been actinomycin D pretreated for periods of 1, 2, 3, 4, and 5 h, all cultures were pulse-labeled with [^3H] uridine for 15 minutes and the number of acid-precipitable counts in each culture was determined at the end of the pulse period. Relative to the amount of TGEV RNA synthesis occurring at the end of a 1-h actinomycin D pretreatment period (100%), and to the amount of TGEV RNA synthesis occurring in uninfected cells (0%), cells pretreated for progressively longer periods demonstrated progressively less TGEV RNA synthesis. The amount of reduction was approximately 40% for a 2-h pretreatment period, 70% for a 3-h pretreatment period, and essentially 100% reduction for periods of 4- and 5-h pretreatment (Fig. 7). Actinomycin D, therefore, significantly inhibits the synthesis of TGEV RNA. Because of these results, pretreatment periods of less than 2 hours were used in further studies of in vivo viral RNA synthesis. For the isolation of in vitro RNA-synthesizing complexes, best results were realized with no actinomycin D. While it is still not possible to say from this data whether the effect of actinomycin D on TGEV replication is a direct one on viral RNA synthesis, it is clear that the mechanism of inhibition functions at an early event in the growth cycle of the virus.

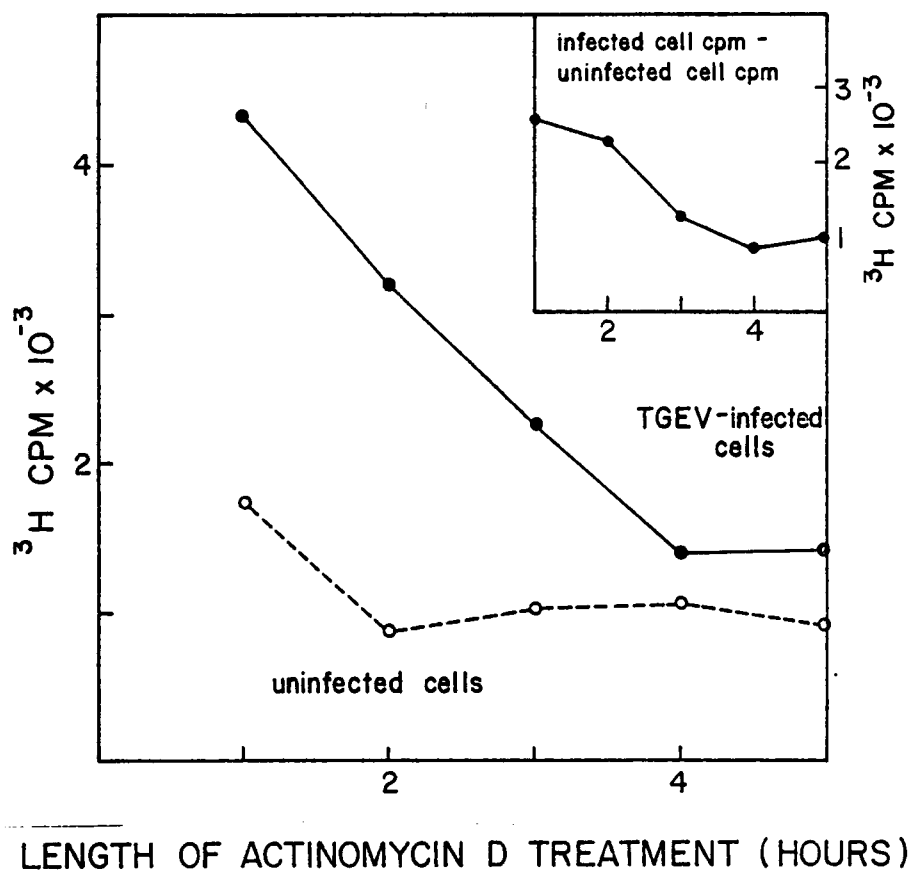


Fig. 7. Rate of viral RNA synthesis as a function of the actinomycin D treatment period. Cells in 30 mm plates were infected with TGEV at a multiplicity of 10 pfu/ml or mock infected with a balanced salt solution, and refed with medium containing 2% fetal calf serum. Starting at the end of the 1 h adsorption period, duplicate cultures of each infected and uninfected cells were made $1.0 \mu\text{g/ml}$ with actinomycin D. At one hour intervals thereafter, duplicate cultures of infected and uninfected cells were likewise treated. At 5 h postinfection, all cultures were simultaneously labeled with 4 Ci $[^3\text{H}]$ uridine (33 Ci/mmol) per plate, for 15 min at 37°C . Acid precipitable radioactivity in each culture was assayed for as described in Fig. 4. The inset illustrates the number of counts incorporated by infected cells minus the number of counts incorporated by uninfected cells.

Intracellular Location of Viral RNA Synthesis

For the purpose of isolating a crude cell fraction that would contain the TGEV RNA-synthesizing machinery and possibly enhance the feasibility of measuring a viral RNA-dependent RNA polymerase in vitro, we sought to isolate the crude cell fraction which contained TGEV-specified RNA at the end of a short pulse period. Essentially, the experimental protocol employed for isolation of picornavirus and togavirus replication complexes was followed (6, 25, 35, 37). To locate the intracellular site of viral RNA synthesis, infected cells were pulse-labeled at 5 h postinfection with [^3H] uridine, immediately fractionated into the nuclear, postnuclear, and soluble fractions, and the fractions were analyzed for acid-precipitable radioactivity (Table 3). The largest quantity of [^3H] uridine incorporation was found in the postnuclear fraction of infected cells which contained 80% of all the precipitable counts in the infected cells. This fraction also contained 50 times more counts than were contained by membranous materials of the type contained in the postnuclear fraction. Successive washes of the nuclear fraction with 0.3 M sucrose followed by pelleting, decreased the contamination as monitored by light microscopy and there was a coincidental decrease in the amount of acid-precipitable radioactive material. We concluded 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

Using an RNA polymerase assay that was optimized for the detection of the cell-associated picornaviral RNA-dependent RNA polymerase (1)

Table 3. Intracellular location of viral RNA synthesis.^a

Fraction	³ H uridine incorporation (cpm) [% of total] ^b	
	Uninfected ^c	Infected
Nuclear	11,200 [43%]	165,700 [19%]
Postnuclear	13,680 [53%]	693,960 [80%]
Soluble	880 [4%]	3,200 [1%]

^aA roller culture of 2×10^8 cells was infected at a multiplicity of 10 pfu/cell, and an identical culture was mock infected with a balanced salt solution. Both cultures were refed with medium containing 2% fetal calf serum at the end of 1 h. 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 ug/ml. At 5 h p.i. this medium was replaced with 15 ml per bottle of medium containing 1.0 ug actinomycin D per ml and 100 uCi [³H] uridine (8 Ci/mmol) per ml. After 0.5 h of incubation at 37°C, cells were scraped from the bottles and fractionated as described in the text. Aliquots of each cellular fraction were assayed for acid-precipitable radioactive materials.

^bPercentages for each fraction were calculated as percentage of the counts incorporated into all three fractions.

^cThe amount of acid-precipitable, [³H] uridine-labeled, material in uninfected cells was less than 3% of that found in infected cells.

we sought an RNA polymerase activity in the postnuclear fraction. Ten to 100-fold greater activity was found over any similar activity detectable in the postnuclear fraction from uninfected cells (Table 4). While we have rigorously examined many requirements for optimal enzymatic activity which are discussed below, two requirements became apparent only after examination of the data from several separate experiments. Firstly, enzymatic activity was detectable in cells at 5 hours postinfection only if a MOI of greater than 2 PFU/cell was used. Secondly, contrary to the reported advantage of using aged tissue culture cells (6 to 9 days after reaching confluency) for growing high-titered virus (33), the highest enzymatic activity was obtained from cells that were relatively young (1 to 2 days after reaching confluency). For obtaining high enzymatic activity it was critical to use cells of excellent physiological health as judged by (a) a rapid growth rate (a doubling time of less than 24 h), 9b) lack of refractile cytoplasmic granules, (c) a high seeding efficiency (i.e., few floating dead cells at 12 h following cell passage), and, (d) a small cell diameter (20 μ m) at confluency.

Characteristics of the Enzyme Activity

The [^3H]UMP-incorporating activity was first characterized to determine its requirements for a divalent cation, the presence of a reducing agent, and the presence of an energy-activating system. The activity was then characterized to determine whether it was in fact an RNA-dependent RNA polymerase (Table 5). It can be seen that the polymerizing activity was reduced by 25% in the absence of Mg^{++} , and

Table 4. RNA polymerase activity in the sedimentable postnuclear fraction of TGEV-infected cells.^a

Origin of postnuclear fraction	³ H]UMP incorporation	
	cpm/mg protein/hr	fmole/mg protein/hr
Uninfected cells	112	2.5
Infected cells	6385	145

^aSeparate roller cultures, each containing 2×10^8 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.

Table 5. 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	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 ug/ml dextran sulfate ^{be}	129	94
Heated product plus DNase ^{bcf}	137	100
Heated product plus RNase ^{bcg}	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

Table 5. cont'd

^aFor these reactions the assay was performed on a postnuclear fraction prepared in the following manner. Cells were harvested and lysed and the postnuclear fraction was prepared as described in the text. The supernatant 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 MgCl₂ - 40 ug/ml dextran sulfate 500 - 0.28% sodium deoxycholate to a final concentration of 5-10 mg/ml as assayed by the Biorad protein assay (Biorad). Samples were stored at -80°C.

^b[³H]UTP was used as the labeled nucleoside triphosphate.

^c[³H]GTP was used as the labeled nucleoside triphosphate.

^dThe postnuclear fraction for this reaction was resuspended in 0.25 M sucrose - 0.28% sodium deoxycholate.

^eThe postnuclear fraction for this reaction was resuspended in 0.25 M sucrose - 0.28% sodium deoxycholate. Dextran sulfate to a final concentration of 200 ug/ml was added at the start of the reaction.

^fAt the end of the 60 min reaction, the entire mixture was heated at 100°C for 2 min, cooled to 37°C, and treated with 50 ug/ml DNase I (Worthington) in a final volume of 1 ml for 15 min at 37°C. The reaction was terminated and assayed for acid-precipitable radioactivity as described in the text.

^gAt the end of the 60 min reaction, the entire reaction mixture was heated at 100°C for 2 min, cooled to 37°C and treated with 20 ug/ml RNase A (Sigma) and 20 ug/ml RNase T₁ (Sigma) in a final volume of 1 ml for 15 min at 37°C. The reaction was terminated and assayed for acid-precipitable radioactivity as described in the text.

was reduced by 85% when Mn^{++} was substituted for Mg^{++} . The activity 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 not required for full activity. No decrease in activity was observed when phosphoenolpyruvate and phosphoenolpyruvate kinase were omitted from the reaction mixture, so 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 a barrier of 20% sucrose (36). The presence of 20 ug actinomycin D per ml in the reaction failed to inhibit $[^3H]$ UMP incorporation and therefore precludes the possibility that the product was made from a DNA template. Because there was a complete loss of activity in the absence of all three supplementary nucleoside triphosphates when $[^3H]$ UMP was used as the labeled compound, there is apparently a template requirement for the activity. It is therefore unlikely that we are merely detecting a polyuridylic acid synthetase activity. When dependence on individual nucleoside triphosphates 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 $[^3H]$ GTP was used as the labeled nucleoside triphosphate a substantial dependence on each of the supplementary nucleoside triphosphates was observed (Table 5). These observations can most readily be explained by the dependence of the activity on a template. Since the enzyme is not inhibited by actinomycin D, we conclude that the template is RNA. The heat-denatured

product was totally resistant to DNase but was 73% degraded by a mixture of pancreatic and T₁ RNases. We conclude that the product of the polymerizing activity is RNA.

Because of the reported beneficial effects of dextran sulfate as an RNase inhibitor in studies of RNA-polymerizing systems (37), it was included in our reaction mixture. Because we considered the inhibition seen with 10 ug/ml to be minor, dextran sulfate was routinely included in the reaction mixture for its possible protective effects on the integrity of the reaction products. To determine whether dextran sulfate has an inhibitory effect on the in vitro polymerase reaction, we measured the amount of product made in the absence of dextran sulfate. Removing dextran sulfate, which is normally present in the reaction mixture at a concentration of 10 ug/ml, resulted in a 27% enhancement of activity (Table 5). Increasing the dextran sulfate concentration 20-fold resulted in only a 4% inhibition of the reaction.

It can be seen from Fig. 8 that the optimum Mg⁺⁺ concentration for the polymerase reaction is 5 mM. 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 kinetics of the [³H]UMP-incorporating activity at 37°C are shown in Fig. 9. The incorporation increase rapidly for the first 10 min and then decreases by more than 50% to remain constant for at least 50 min. For studies in which the RNA product was analyzed (Part III of this thesis), reaction times were normally held to less than 15 minutes to minimize the likelihood of RNase degradation.

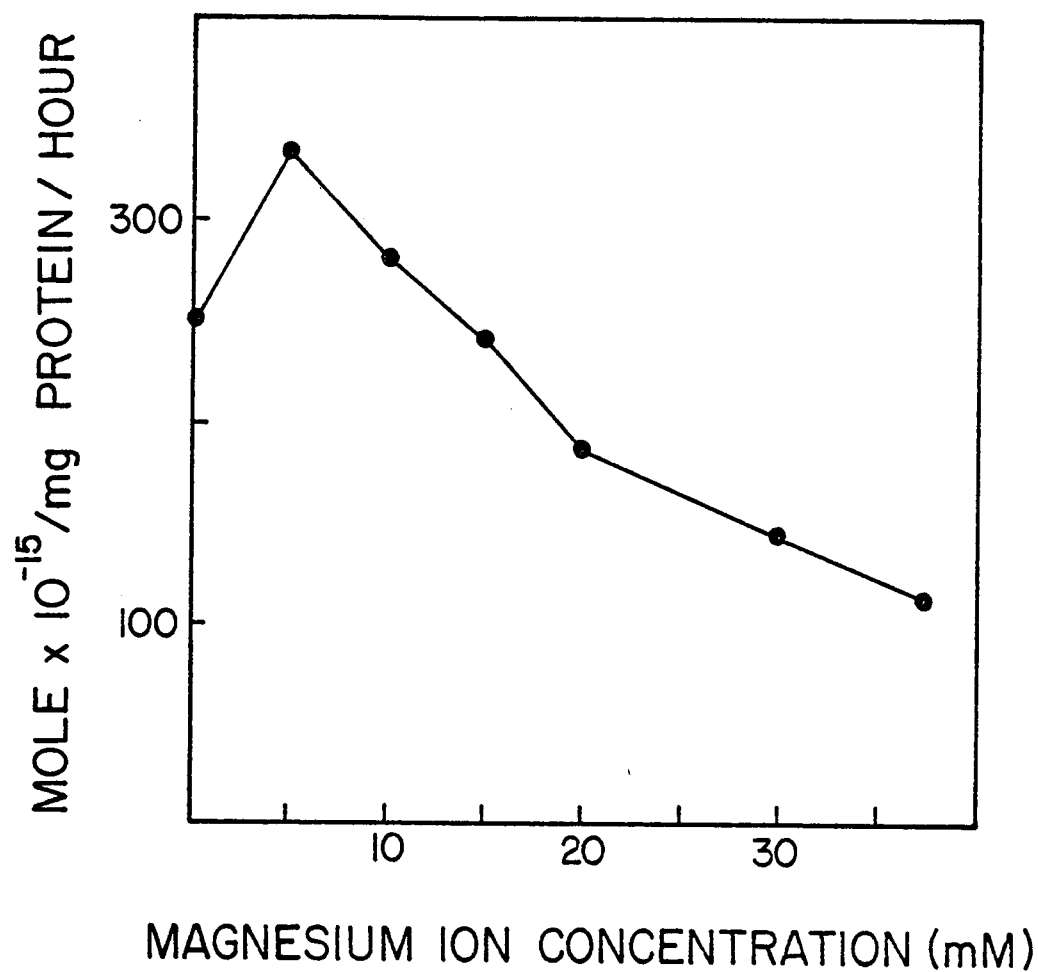


Fig. 8. RNA polymerase activity as a function of magnesium ion concentration. $[^3\text{H}]$ UMP incorporation was measured as a function of Mg^{++} concentration. The preparation of the postnuclear fraction and the reaction conditions were as described in Table 5.

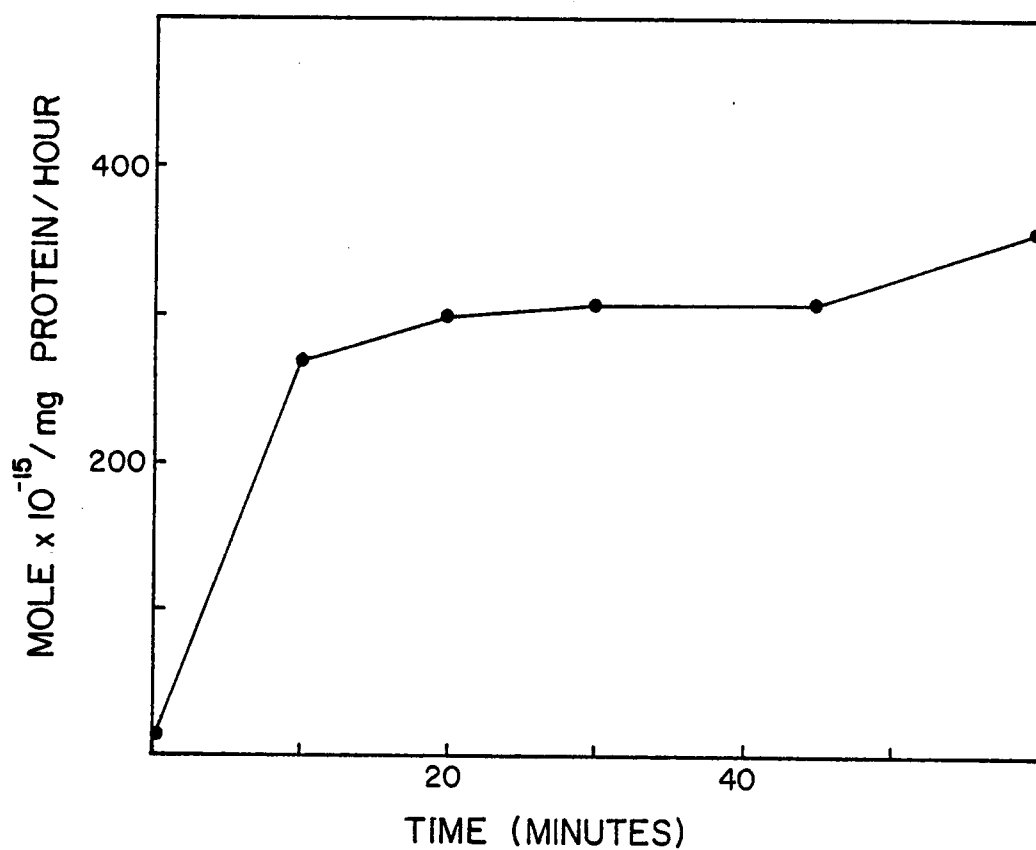


Fig. 9. Kinetics of the in vitro polymerase reaction. $[^3\text{H}]$ UMP incorporation was measured as a function of time. The preparation of the postnuclear fraction and the reaction conditions were as described in Table 5.

Isolation of the TGEV Replication Complex by Isopycnic Sedimentation

The RNA-synthesizing complexes, often referred to as replication complexes, of both positive-strand picornaviruses (10, 11, 13, 14, 15, 16, 34) and togaviruses (19, 20, 21, 22, 23, 41), and the negative-strand myxoviruses (12, 17, 39) and rhabdoviruses (24), are all found to have an integral association with cytoplasmic membranous structures. By definition, the replication complex is comprised of the viral RNA polymerase, viral RNA template, and nascent viral RNA molecules. Information about the intimate membrane association of the replication complex for other viruses has been derived from sedimentational and electron-microscopic studies (16, 21, 23). In an effort to determine whether the TGEV replication complex, as characterized by nascent in vivo RNA synthesis and in vitro RNA polymerase activity, is membrane associated, we analyzed its behavior in both isopycnic and rate zonal sedimentation studies. The method used to fractionate cell components by isopycnic sedimentation was one devised by Bosman et al. (8). In this method, lysed cells are centrifuged to remove nuclei and the resultant supernatant is sedimented in a sucrose gradient until the various particulate and membranous structures are separated on the basis of their buoyant densities. Lighter smooth membranes, such as the smooth endoplasmic reticulum and the plasma membranes, band toward the top of the gradient with buoyant densities of 1.08 to 1.15 g/cm³, whereas membranes with particulate structures attached, such as the rough endoplasmic reticulum, band toward the bottom with buoyant densities of 1.15 to 1.24 g/cm³. Free ribosomes are generally the lowermost band with a buoyant density of 1.25 g/cm³.

Using this technique, membranes having viral structures attached, e.g., partially assembled virions, may often be separated as discrete bands away from bands of other cellular membrane structures (14, 21).

The postnuclear fractions from infected and uninfected cells were fractionated on discontinuous sucrose gradients as described in Fig. 10. The appearance of both gradients was essentially identical (Fig 10a). Distinctly visible bands were seen at buoyant densities of 1.09, 1.15, 1.18, 1.21, and 1.25 g/cm³. The band at 1.09 g/cm³ was very thin and translucent. The bands at 1.15 g/cm³ (the widest band) and 1.21 g/cm³ were very wide and translucent. The band at 1.18 g/cm³ was relatively narrow and consisted of what visually appeared to be large aggregates of particulate matter. The band at 1.25 g/cm³ was opaque and flocculant.

To determine whether viral RNA could be found in any of these resolved cellular structures, infected cells were pulse-labeled with [³H] uridine at 5 hours postinfection in the presence of actinomycin D. The postnuclear fraction was isolated and sedimented on a discontinuous isopycnic gradient as described in Fig. 10. The gradients were fractionated and the fractions analyzed for absorbance at 254 nm, density, and incorporation of label into TCA-precipitable material. The postnuclear fraction from uninfected cells contained less than 10% of the acid-precipitable counts as found in infected cells. When the radioactive material from each fraction of the gradient was quantitated, greater than 90% of the radioactivity found in the infected cells was found in the bands with buoyant densities of 1.15 and 1.18 g/cm³ (Fig. 10b). The corresponding bands in the gradient from uninfected cells contained no such acid-precipitable radioactivity, indicating that these bands

Fig. 10. Isopycnic sedimentation of the postnuclear fraction from cells pulse-labeled in vivo with [^3H] uridine. 2×10^8 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. Both cultures were refed with a medium containing 2% fetal calf serum at the end of a 1 h adsorption period. At 3 h postinfection, timed from the beginning of the adsorption period, actinomycin D was added to make a final concentration of $1.0 \mu\text{g/ml}$. At 5 h postinfection, $1000 \mu\text{Ci}$ [^3H] uridine (33 Ci/mmol) was added per culture and cells were incubated for 5 min at 37°C . Cells were then harvested and fractionated as described in the text except that reticulocyte standard buffer, RSB (0.01 M Tris-hydrochloride ($\text{pH } 7.4$) - 0.01 M NaCl - 0.0015 M MgCl_2) was used for Dounce homogenization of the cells. The postnuclear fraction was then made 30% sucrose (wt/wt) and suspended in a total volume of 10 ml. A discontinuous sucrose gradient, prepared by layering 3 ml of 65% sucrose (wt/wt), 6 ml of 48% sucrose (wt/wt), 6 ml of 45% sucrose (wt/wt), 5 ml of 38% sucrose (wt/wt), 10 ml of the postnuclear fraction in 30% sucrose (wt/wt), 5 ml of 25% sucrose (wt/wt), and 3 ml of RSB, was centrifuged at $50,000 \times g$ for 17 h at 4°C in a Sorvall AH-627 rotor, and fractionated from the top using an Isco gradient harvester equipped with a UV monitor. The fractions were monitored for absorbance at a wavelength of 254 nm and 10 μl aliquots of each fraction were assayed for acid-precipitable radioactivity. a) Schematic representation of the visible appearance of the gradients. b) Graphs depict the absorbance, buoyant density, and radioactivity in 10 μl aliquots of each fraction.

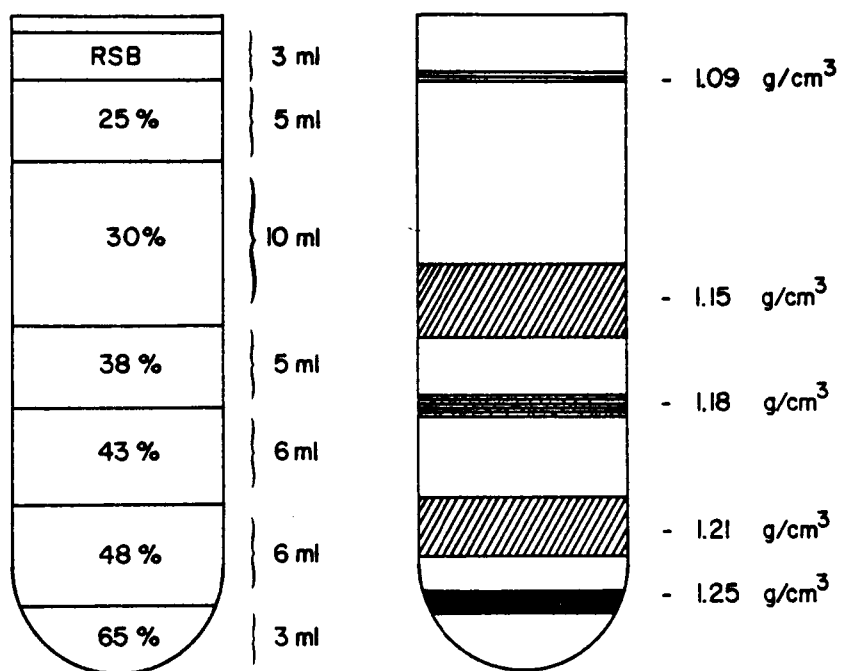


Fig. 10a

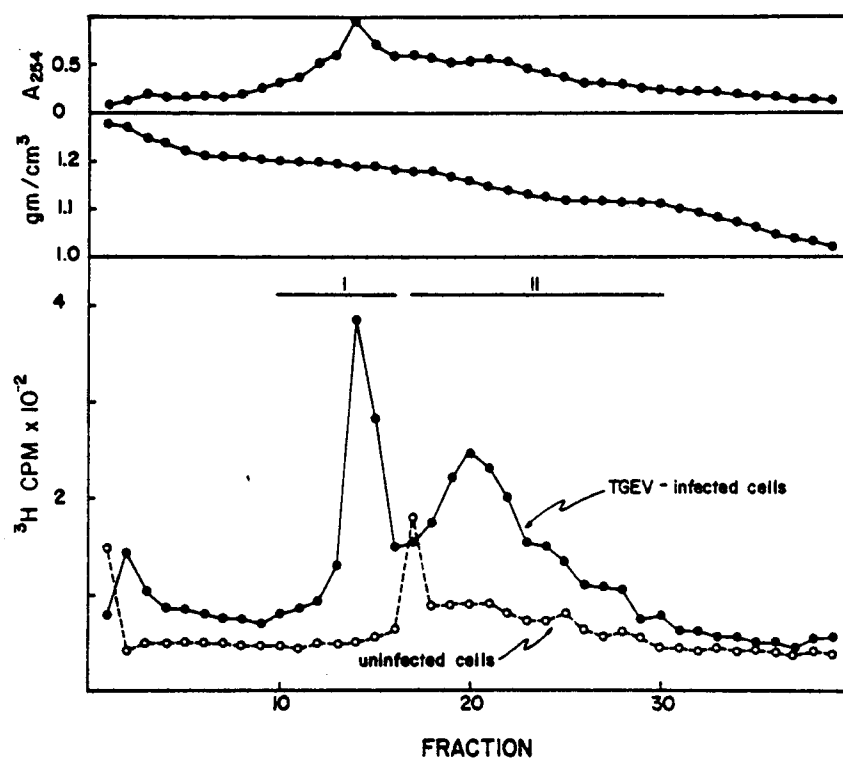


Fig. 10b

in the gradient from infected cells contained viral-specified RNA. We know that the postnuclear fraction from infected cells contains virus-specified RNA species (Part III of this thesis). In the isopycnic gradient from uninfected cells, only one fraction near the middle of the gradient contained radioactive material. The significance of this peak is questionable since it was not seen in other uninfected cell preparations from other experiments. The radioactivity in isopycnic sucrose gradients prepared from uninfected cells is usually distributed evenly throughout the gradient.

Isolation of the TGEV Replication Complex by Rate Zonal Sedimentation

In an effort to confirm the membrane association of the TGEV replication complex, the postnuclear fraction of infected cells was sedimented on a 15-40% (wt/wt) sucrose gradient following the procedure of Plagemann (36). When the postnuclear fraction from cells pulse-labeled with [^3H] uridine at 5 hours postinfection was sedimented, a large peak of radioactivity was found near the middle of the gradient (Fig. 11). No such material was seen in the gradient prepared from uninfected cells. Therefore, virus-specific RNA is probably contained within this peak. To examine this possibility, material within this band was pelleted and the radioactive RNA was extracted and analyzed by electrophoresis. Virus-specific RNA species were found (Part III of this thesis). The postnuclear fraction from infected, but unlabeled, cells was also sedimented in parallel and fractions corresponding to the radioactive peaks in the radiolabeled preparation were pooled. The material was pelleted and assayed for RNA-dependent RNA polymerase

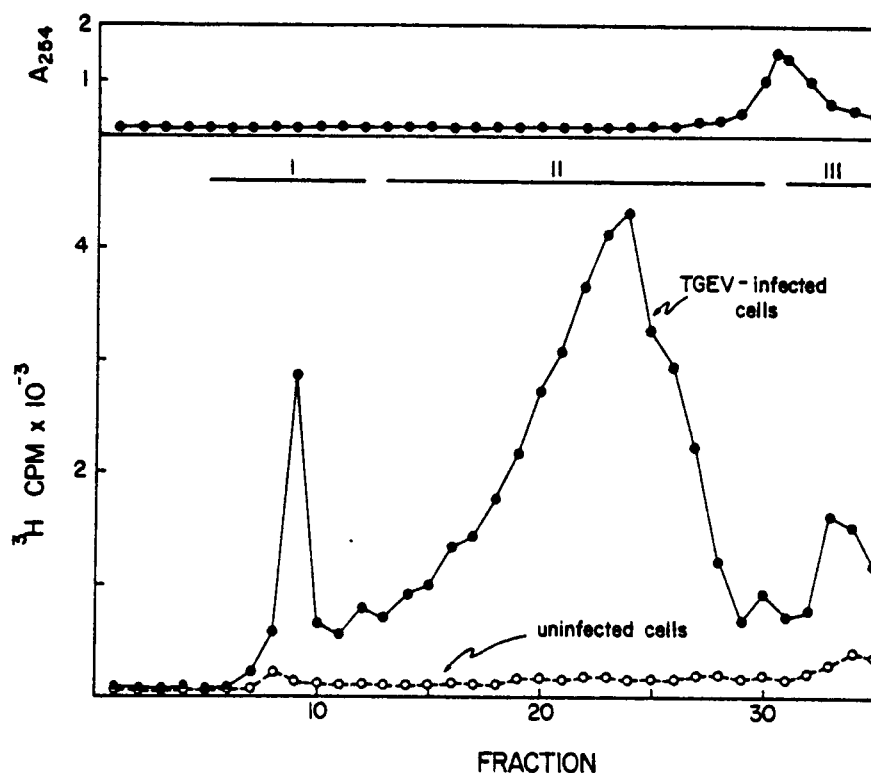


Fig. 11. Rate zonal sedimentation of the postnuclear fraction from cells pulse-labeled in vivo with [^3H] uridine. 2×10^8 each of infected and uninfected cells were prepared as described in Fig. 10 except that they were swollen and lysed by Dounce homogenization in 3 ml of 0.3 M sucrose. The postnuclear fraction was prepared as described in the text and was immediately layered onto a gradient consisting of 2.5 ml of 50% sucrose (wt/wt), 2.5 ml of 45% sucrose (wt/wt), 2.5 ml of 43.5% sucrose (wt/wt) and 28 mls of a 40% to 15% (wt/wt) continuous sucrose gradient. All sucrose solutions were made in 0.01 M Tris-hydrochloride (pH 7.4). Sedimentation was at $74,000 \times g$ for 1.5 h at 4°C in a Sorvall AH-627 rotor, and the gradients were fractionated and each fraction was analyzed spectrophotometrically for absorbance at 254 nm, and for acid-precipitable radioactivity.

activity (Table 6). This material, as represented by pool II (see Fig. 11 and Table 6), contained more than 60% of the total activity found in the gradient, and contained 20-fold more activity than the corresponding pool prepared from uninfected cells. Taken together, this data indicates that the TGEV replication complex is a membrane-associated structure.

Effect of Deoxycholate On Sedimentation Rate of the TGEV Replication Complex.

The replication complexes are shown in the picornavirus and togavirus systems to be dissociable from membranes by treatment with deoxycholate (1, 3, 19). This treatment drastically changes the sedimentation properties of the replication complex. Both in vivo labeled viral RNA species and the viral RNA polymerase, as assayed in vitro, sediment as separate smaller complexes with sedimentation coefficients of approximately 60 S and 350 S after deoxycholate treatment as compared to approximately 500 S prior to deoxycholate treatment (2, 10). We, in like manner, sought to dissociate the TGEV replication complex from rapidly-sedimenting membrane structures by this method. Initially, the postnuclear fraction of infected cells that had been pulse-labeled with [^3H] uridine at 5 hours postinfection was treated to contain a final concentration of 1% deoxycholate and then sedimented on a rate zonal gradient in the presence of 1% deoxycholate. Fig. 12a illustrates that this gradient contained a peak of acid-precipitable labeled material at the top of the gradient. A parallel gradient prepared from pulse-labeled uninfected cells exhibited no such peak (Fig. 12a). These results when compared with those illustrated in

Table 6. RNA polymerase activity in the rate zonally-sedimented postnuclear fraction.^a

Sample	<u>[³H]UMP incorporation</u>			
	<u>fmole/mg protein/hr</u>		<u>total cpm incorporated</u>	
	uninfected	infected	uninfected	infected
Pool I	64	121	1,359	268
Pool II	52	1,068	232	4,702
Pool III	40	280	259	280

^a 2×10^8 of each infected and uninfected cells were prepared and fractionated as described in Fig. 11 except that they were not isotopically labeled. All procedures were done in parallel with the isotopically-labeled preparations and pools were made of the fractionated gradients as described in Fig 11. Material in the pools was pelleted by sedimentation at 55,000 x g for 1.5 h at 4°C in a Sorvall AH-627 rotor, resuspended in 0.25 M sucrose - 0.0015 M MgCl₂, and assayed for protein content and polymerase activity using protocols described in the text.

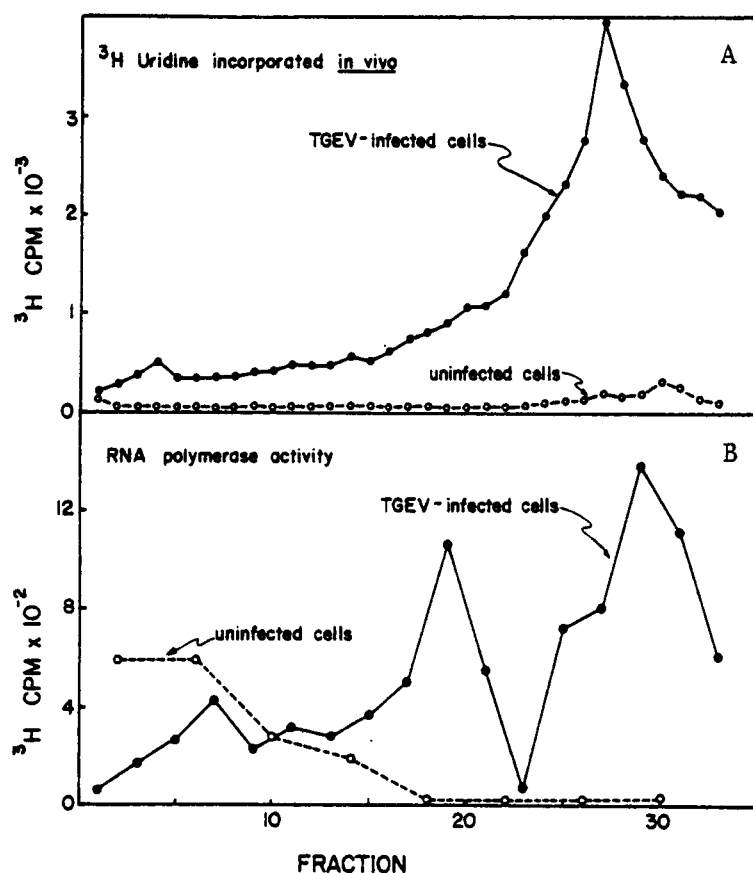


Fig. 12. Rate zonal sedimentation of deoxycholate-solubilized postnuclear fraction. a) 2×10^8 each of infected and uninfected cells were prepared as described in Fig. 10 except that the postnuclear fraction was resuspended in 0.25 M sucrose - 0.0015 M MgCl_2 and immediately made 1% (wt/wt) final concentration 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 $95,000 \times g$ for 1.5 h at 4°C in a Beckman SW-41 rotor. The gradients were fractionated and analyzed spectrophotometrically for absorbance at 254 nm, and for acid-precipitable radioactivity. b) 2×10^8 each of infected and uninfected cells were treated and fractionated as described in part a) except that they were not isotopically labeled. Each preparation was sedimented on a rate zonal gradient containing 1% (wt/wt) sodium deoxycholate in parallel with the gradients described in part a). Selected fractions of the gradient were assayed for $[^3\text{H}]\text{UMP}$ -incorporating activity.

Fig. 11, demonstrate that the TGEV replication complex, as characterized by radiolabeled nascent viral RNA, acquires a much smaller sedimentation coefficient (approximately 230 S) after deoxycholate treatment when compared to no treatment (approximately 600 S). Secondly, the post-nuclear fraction prepared from infected, unlabeled cells was treated with deoxycholate and sedimented in an identical rate zonal sucrose gradient that contained deoxycholate. Fractions were then collected and assayed for RNA-dependent RNA polymerase activity (Fig. 12b).

^3H UMP-incorporating activity was found in two distinct peaks of the gradient. Whether these represent two different polymerases or two forms of the same polymerase has not been determined, but the phenomenon of both "heavy" and "light" complexes has been described in the picornavirus system (2). The uninfected cell preparation demonstrated no polymerase activity in the upper half of the gradient, but did exhibit some activity in the bottom two fractions of the gradient. This may represent a cellular RNA polymerase, perhaps of mitochondrial origin, that is insensitive to actinomycin D (36). It is perhaps this activity that we are detecting in crude postnuclear fractions (e.g., Table 6). If it does represent a distinct cellular polymerase activity, the deoxycholate-sucrose gradient procedure may be a way to successfully separate it for further study. In a comparison of the sedimentation rates of the polymerase activities before and after deoxycholate treatment (Figures 11 and 12, and Table 6) it can be seen that treatment with deoxycholate retards the sedimentation rate. We conclude that the TGEV replication complex is intimately associated with cell membrane structures in a fashion similar to that of the class IV viruses.

CHAPTER IV

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. To our knowledge, this is the first report of a coronavirus-specified RNA polymerase. The absence of this enzyme(s) on the virion along with an infectious single-stranded RNA genome are properties shared by the picornaviruses and togaviruses (4). Many questions comparing the nature of the three virus systems are therefore raised in our analyses of the TGEV enzyme activity. The induction of an RNA-dependent RNA polymerase is demonstrated by the fact that uninfected cells contain little or no actinomycin D-insensitive [^3H]UMP- or [^3H]GTP-incorporating activity, whereas TGEV-infected cells do. The activity is located primarily in the sedimentable particulate fraction of the cell cytoplasm and this suggests that the enzyme is part of a membrane-associated replication complex. The template is not DNA based on the insensitivity of the activity to actinomycin D. The template therefore must be RNA. The product of the polymerase is RNA based on the fact that it is digested by T_1 and pancreatic RNases, but not by DNase. The requirements for maximal polymerase activity are similar to those required by the picornaviral and togaviral enzymes. There is a requirement for all three nucleoside triphosphates to obtain full enzymatic activity. While we have not rigorously ruled out the possibility

of a poly (U) polymerase or a poly (G) polymerase, the dependence on all three nucleoside triphosphates suggests that at least the classical poly (U) polymerase is not being observed since the classical poly (U) polymerase shows enhanced polymerization in the absence of the other ribonucleoside triphosphates (25, 35). The TGEV-induced polymerase requires magnesium for optimal activity and this divalent cation requirement cannot be fulfilled by manganese. This behavior is also shared by the picornaviral and togaviral enzymes.

For purposes of further characterizing the membrane association of the TGEV polymerase and to characterize the in vivo and in vitro products (Part III of this thesis), several conditions were examined to maximize the synthesis of an in vitro product. We learned that an ATP-generating system was not required. This is apparently true because the particulate RNA-synthesizing complex is separated from soluble cytoplasmic ATPases by sedimentation through aqueous sucrose (35). Dextran sulfate, while it demonstrated some inhibition of polymerase activity, was included because of reports that it serves to protect the integrity of single-stranded RNA templates (1, 37). Since cellular DNA-dependent RNA polymerase activity can interfere with the measurement of RNA-dependent RNA polymerase measurement (1, 37), actinomycin D is used by some investigators prior to the time of cytoplasmic extraction to minimize the interference. We found that this was not beneficial when studying TGEV-infected cells. Critical measurement of TGEV-specific RNA synthesis in vivo demonstrated a significant inhibition by actinomycin D. Because of this, actinomycin D was included only in the in vitro polymerase reaction. It is not clear

from our studies whether the effect of actinomycin D is a direct one on the viral polymerase, or an indirect one on cellular biochemical activities.

Viral RNA synthesis, and thus polymerase activity, takes place in association with smooth membranes of density 1.15 and 1.18 g/cm³ as judged by [³H] uridine-incorporation into these bands in the presence of actinomycin D, and by the presence of viral-specific RNA species extracted from these bands. No such [³H] uridine incorporation or RNA species were found in uninfected-cell membrane bands obtained in the same manner. Membrane association of the replication complex was further indicated by the results of rate zonal centrifugation of the postnuclear fraction. The rate zonal gradient of [³H] uridine pulse-labeled postnuclear fraction exhibited a broad peak of TCA-precipitable cpm sedimenting at an approximate sedimentation coefficient of 600 S. Analysis of this peak from unlabeled gradients revealed that it contained 20-fold more polymerase activity than an equivalent membrane pool from uninfected cells, and it contained 60% of the total activity in the gradient. In addition, the RNA species extracted from this membrane peak are virus specific (Part III of this thesis). This data combined with the data from the isopycnic gradients, argues that TGEV RNA replication takes place in association with cytoplasmic smooth membranes. To substantiate this argument, postnuclear fractions from uninfected and infected cells were solubilized with sodium deoxycholate and centrifuged in a rate zonal gradient. Solubilization of the postnuclear fraction with deoxycholate alters the sedimentation

characteristics of the polymerase activity and the in vivo-labeled [^3H] uridine-containing material (Figures 11 and 12).

Rather than one broad peak of polymerase activity sedimenting at an approximate sedimentation coefficient of 600 S, there are two activities, one sedimenting at approximately 500 S, and the other at approximately 160S. This property of the TGEV replication complex again shows striking similarity to the picornaviral and togaviral counterparts. Arlinghaus and Polatnick for foot-and-mouth disease virus (2), and Caliguirri for poliovirus (16), report the existence of membrane-associated "heavy" (100-300 S) and "light" (20-70 S) replication complexes. The "light" complexes contained double-stranded viral RNA, whereas the "heavy" complexes contained mostly single-stranded viral RNA. We did not examine the content of the TGEV heavy and light replication complexes. The alteration in the sedimentation coefficient for the polymerizing complex in the in vivo-labeled [^3H] uridine-containing material probably represents the dissolution of membranes bound to viral replication complexes. This result, taken together with the isopycnic and rate zonal gradient data, argues that TGEV RNA replication takes place in close association with cytoplasmic membranes.

Studies are currently in progress to characterize TGEV structural and nonstructural proteins. Whether the cell-associated RNA-dependent RNA polymerase is abundant enough to be detected by electrophoretic methods remains to be determined. Purification of the replication complex as we have described should serve to enrich for the coronavirus-specified polymerase.

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CORONAVIRUS REPLICATIONAL STRATEGY

III. IN VIVO AND IN VITRO PRODUCTS OF THE CORONAVIRAL RNA-DEPENDENT RNA POLYMERASE

CHAPTER I

INTRODUCTION

At least three species of the coronavirus family, including the prototype avian coronavirus, are known to be positive-strand viruses on the basis of an infectious, polyadenylated, single-stranded RNA genome. These are the avian infectious bronchitis virus (11, 18), the mouse hepatitis virus (8), and the porcine transmissible gastroenteritis virus (TGEV) (3). We have shown that in the case of TGEV, a virion-associated RNA-dependent RNA polymerase apparently does not exist. However, a viral-specified RNA-dependent RNA polymerase is detectable in cytoplasmic membranous structures as part of a membrane-bound replication complex (Part II of this thesis). These properties are consistent with those found in other positive-strand viruses which possess an infectious RNA genome, namely the picornaviruses and togaviruses. The TGEV RNA polymerase activity that we detect (possibly a mixture of two or more activities) appears in all fundamental respects to be similar to the picornaviral and togaviral counterparts.

The replicational strategies of picornaviral and togaviral RNA are apparently the same in that full-length progeny plus-strand RNA is made from full-length minus strand template. The transcriptional strategies are, however, fundamentally different. Picornavirus mRNA, which is apparently identical to genomic RNA, is translated as one full-length polyprotein with individual proteins arising from the posttranslational modification of the molecule. Togaviruses, on the other hand, employ two sizes of mRNA, one that is full-length and

apparently identical to genomic RNA which serves to synthesize non-structural proteins, and one that is approximately third-length which serves to synthesize structural proteins. For comparative purposes we wished to examine the replicational and transcriptional strategies for coronavirus RNA.

A recent report on the intracellular mRNA-like species found during the replication of one coronavirus, the avian infectious bronchitis virus, suggests that the coronaviral transcriptional strategy is indeed different than that for picornaviruses or togaviruses (23). In this study, six intracellular, polyadenylated, single-stranded, RNA species were found, five of which apparently function as sub-genomic mRNA's for the synthesis of viral proteins. The sixth species is the same as virion genomic RNA and may or may not function as an mRNA beyond the initial step in virus replication. The five polyadenylated RNA species are all known to contain sequences found in the virion RNA and are, therefore, subgenomic. It is known, furthermore, that the sequences for any given species are found in the next larger species. Whether this pattern of transcription holds true for all coronaviruses is not known although at least two functional sizes of mRNA are known to exist for the JHM strain of the mouse hepatitis virus (19).

In this paper we describe studies that show that TGEV replicates by synthesizing five major species and six minor species of single-stranded, polyadenylated, RNA. These presumably each function as mRNA. In addition, a possible double-stranded intermediate is described. We also describe experiments which show that isolated membrane-associated

replication complexes are able to synthesize RNA in vitro and that these transcripts have molecular weights the same as three of the species made in vivo. This coronavirus, apparently replicates by transcribing and translating multiple mRNA species, and the mechanism for regulating the length of at least some of the subgenomic transcripts apparently resides within the membrane-bound replication complex.

CHAPTER II

MATERIALS AND METHODS

Cells and Virus

The swine testicle cell line, (ST), was grown in monolayer cultures as previously described (Part II of this thesis). Virus stocks of the Purdue strain of TGEV were prepared as previously described (Part II of this thesis).

Preparation of the Postnuclear Fraction

Batches of $2-8 \times 10^8$ cells (representing 1 to 4 confluent roller bottles) were infected at a multiplicity of 10 PFU/cell and were refed with growth medium containing 2% fetal calf serum. At 5 h postinfection (timed from the beginning of the adsorption period), bottles 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 min, and disrupted using 15 strokes in a tight-fitting glass Dounce homogenizer. Cellular disruption and nuclear breakage were monitored by light microscopy. Nuclei and large debris were pelleted at 650 x g for 7 min and the supernatant was layered onto 20 ml of 0.01 M Tris-hydrochloride (pH 7.4)-10 ug/ml dextran sulfate (Sigma)-20% sucrose (wt/wt) in a 38-ml centrifuge tube. The pellet formed by centrifugation at 60,000 x g for 1.5 h in a Sorvall AH-627 rotor was drained and

resuspended in a solution of 0.25 M sucrose-0.0015 M MgCl_2 -40 ug/ml dextran sulfate 500-2.8 mg/ml sodium deoxycholate to a final protein concentration of 5 to 10 mg/ml as assayed by the Biorad protein assay (Biorad). The preparation was aliquoted and stored at -80°C . Samples retained approximately 75% of their activity after storage for one month.

RNA Polymerase Reaction

The assay for the cell-associated RNA-dependent RNA polymerase was essentially as we described previously (Part II of this thesis). In a standard reaction, 50 ul of sample (at a protein concentration of 5 to 10 mg/ml) was assayed in a 200 ul solution (total volume) containing: 50 mM Tris-hydrochloride (pH 8.0); 5 mM magnesium acetate; 10 mM phosphoenolpyruvic acid; 40 ug/ml phosphoenolpyruvate kinase; 17.5 mM 2-mercaptoethanol; 20 ug/ml actinomycin D; 1.25 mM $[5-^3\text{H}]$ UTP (5.2×10^4 cpm/pmole, ICN Radiochemical Division, Irvine, Ca.); 0.5 mM each of ATP, CTP, and GTP. RNA was extracted from the reaction product as described below.

Extraction of Polymerase Product RNA

For analysis of the in vitro polymerase reaction products, the reaction was terminated after 15 minutes by the addition of 0.25 volume of 5X TNE (where TNE is 0.01 M Tris-hydrochloride (pH 7.5)-0.1 M NaCl-0.001 M EDTA)-10% sodium dodecyl sulfate and 0.1 mg crystalline proteinase K. The mixture was incubated at 37°C for 15 min and then phenol extracted one time with an equal volume of TNE-saturated phenol. The RNA was precipitated at -20°C using 2 volumes

of 95% ethanol for at least 5 h. The precipitate was dissolved in 0.5 ml of 0.01 M Tris-hydrochloride (pH 7.5) and reprecipitated after the addition of 55 μ l of 2 M NaAc. This step was repeated twice. The RNA precipitate was dissolved in phosphate buffer, pH 8.0 (0.0018 M Na_2HPO_4 -0.002 M NaH_2PO_4) and stored at -80°C . All solutions were treated with 0.1% diethylpyrocarbonate for 24 h prior to use with RNA.

Preparation of Cellular and Viral Marker RNA Species

[^3H] uridine-labeled cellular and viral RNA marker species were prepared as previously described (3). [^{14}C] uridine-labeled HeLa cell RNA was a gift from J. Martin, University of Tennessee, Knoxville.

Agarose Gel Electrophoresis and Fluorography

The method of Pinder, et al., was used to analyze RNA by electrophoresis in agarose gels (13). RNA samples were heated in 2.2 M formaldehyde-50% formamide (wt/wt)-0.018 M Na_2HPO_4 -0.002 M NaH_2PO_4 , for 5 min at 60°C immediately prior to electrophoresis. Electrophoresis buffer was 2.2 M formaldehyde-0.0018 M Na_2HPO_4 -0.002 M 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 50 ma. 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 (Hoeffer Instruments) under vacuum and low heat. Gels were fluorographed by the method of Laskey and Mills (9).

CHAPTER III

RESULTS

Virus-Specific RNA In Infected Cells

We have previously established that TGEV RNA is synthesized at a maximal rate between 4 and 6 h postinfection in ST cells (Part II of this thesis). To examine the RNA species made within this interval of time, infected cells were labeled with ^3H uridine in the presence of actinomycin D between 4.5 and 5 h postinfection and the RNA was extracted from whole cells by the method of Erikson, et al. (5), and examined under denaturing conditions by electrophoresis on agarose gels (Fig. 1). Five major species having apparent molecular weights of 6.8-, 3.15-, 1.40-, 0.94-, and 0.66×10^6 were seen. In addition, 3 minor species having apparent molecular weights of 6.2-, 1.55-, and 1.05×10^6 were also seen. No RNA could be observed in extracts from uninfected cells even though the total rate of RNA synthesis under this condition was 3% of that in infected cells (Fig. 1). When extracts of infected cells were examined on agarose gels under non-denaturing conditions, only five species corresponding roughly in molecular weight to the major species seen on denaturing gels were observed (data not shown). No minor species were observed in the nondenaturing gels.

Virus-Specified RNA Associated With the Replication Complex

We have previously established that TGEV-specified RNA synthesis occurs on membrane-associated cytoplasmic structures (Part II of this

thesis). This association is apparent for RNA synthesized in vitro as well as for RNA synthesized in vivo. To determine whether the RNA species found in these structures are the same as those extracted from whole infected cells, two approaches were taken. In the first, RNA in the crudely prepared postnuclear fraction was studied. In the second, the membrane-associated RNA containing structures were purified by isopycnic sedimentation prior to RNA extraction and analysis.

To examine the RNA species in the crudely prepared postnuclear fraction, infected cells were pulse-labeled with [^3H] uridine at 5 h postinfection in the presence of actinomycin D and the postnuclear fraction was prepared in the presence of dextran sulfate to inhibit RNA degradation. RNA analyzed by electrophoresis under denaturing conditions on agarose gels displayed five major RNA species which had the same electrophoretic mobilities as the five major species extracted from whole cells (Fig. 1). In addition, there are seven minor species, three of which migrate the same as the three minor species in whole cell extracts. The minor species have apparent molecular weights of 6.2-, 1.55-, 1.05-, 0.64-, 0.39-, 0.34-, and 0.24×10^6 . Molecular weights were estimated by extrapolation and interpolation from a plot of molecular weight vs relative mobility for known single-stranded RNA markers (Fig. 2). Approximately 5% of the total radioactivity was observed as unresolved species migrating with 4 S RNA.

To examine the RNA species in isopycnicly sedimented membranous structures, infected cells were pulse-labeled with [^3H] uridine at 5 h postinfection in the presence of actinomycin D, and the postnuclear fraction was isopycnicly sedimented using the method of Bosman, et al.

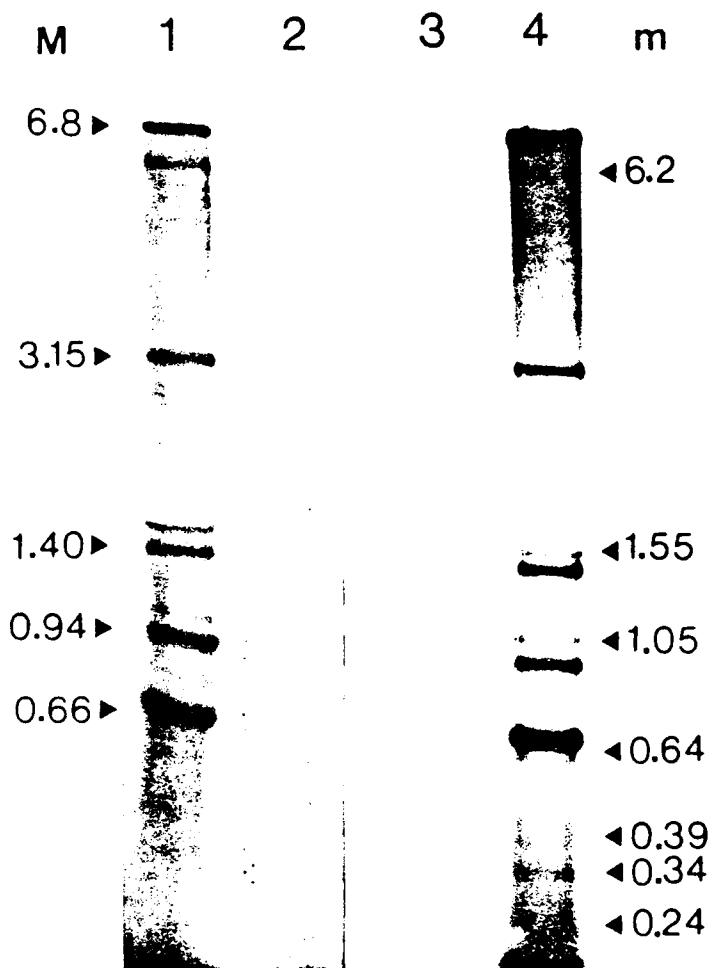


Fig. 1. Electrophoresis of viral-specified cytoplasmic RNA on a denaturing agarose gel. 2×10^8 cells in roller culture were infected at a multiplicity of 10 PFU/cell and an identical culture was mock-infected with a balanced salt solution. At 3 h p.i., timed from the beginning of adsorption, actinomycin D was added to make a final concentration of 1.0 $\mu\text{g}/\text{ml}$. At 5 h p.i., 1000 μCi [^3H] uridine was added per culture and cells were incubated for 0.5 h at 37°C . Lane 1; RNA extracted from infected whole cells. Lane 2: RNA extracted from uninfected whole cells. Lane 3: RNA extracted from the postnuclear fraction of uninfected cells. Lane 4: RNA extracted from the postnuclear fraction of infected cells. The conditions for gel electrophoresis are described in the text. The gel was fluorographed as described in the text. M and m indicate the molecular weight of the major and minor species respectively.

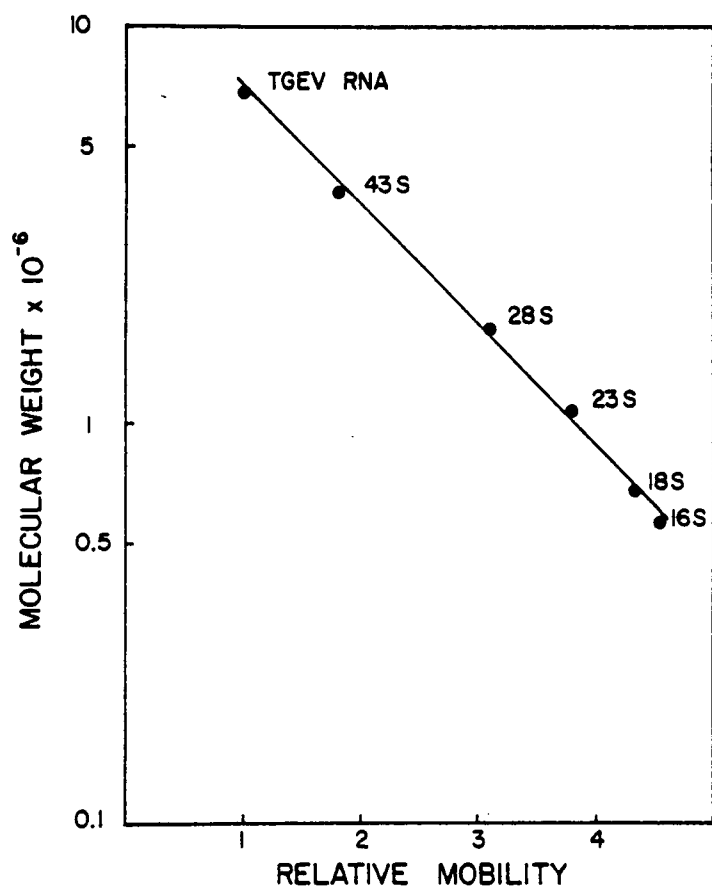


Fig. 2. Plot of the logarithm of the molecular weight versus the relative mobility for reference RNA molecules. Conditions of electrophoresis are the same as those described in Fig. 1.

(2). Two discrete membranous bands containing virus-specified RNA were observed, one at a density of 1.15 g/cm^3 , and the other at a density of 1.18 g/cm^3 (Fig. 3). RNA from each of these bands was analyzed separately by electrophoresis on nondenaturing polyacrylamide-agarose gels (Fig. 4). The 1.15 g/cm^3 band contained five RNA species of approximately 7.0-, 3.8-, 1.64-, 1.05-, and 0.55×10^6 molecular weight, which probably corresponds to the major species observed in extracts of the whole cell and of the postnuclear fraction. We cannot be certain of this correspondence, however, since no attempt was made to destroy the secondary structure of the RNA in the polyacrylamide gel. The 1.18 g/cm^3 band contained three RNA species with approximate molecular weights of 3.8-, 1.05, and 0.55×10^6 . It thus appeared that genome-length RNA and the species corresponding to 1.4×10^6 were missing or were present in amounts too small to be distinguished as a peak. From each band, greater than 5% of the radioactivity migrated as unresolved species with 4 S 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 0.3 M NaCl (Fig. 5). It is possible that double-stranded replicative intermediates were present, but in too few numbers to be detected by our method.

Polyadenylation of Intracellular Virus-Specified RNA

To determine whether virus-specified RNA is polyadenylated, infected cells were pulse-labeled with $[^3\text{H}]$ uridine at 5 h post-infection in the presence of actinomycin D, and RNA was extracted

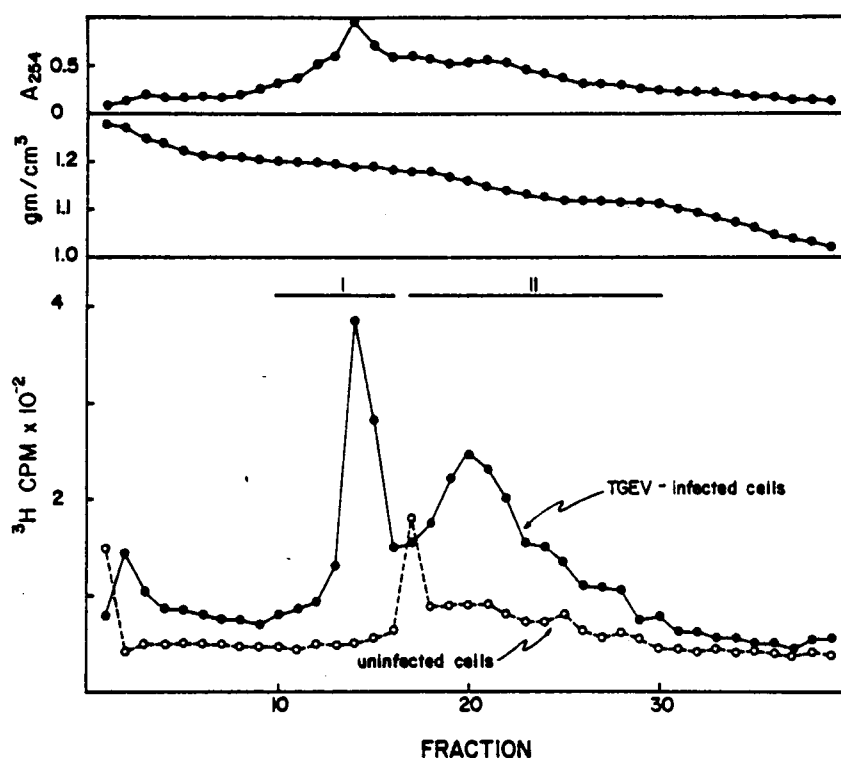


Fig. 3. Isopycnic sedimentation of the postnuclear fraction from cells pulse-labeled *in vivo* with [^3H] uridine. 2×10^8 cells in roller culture were infected at a multiplicity of 10 PFU/cell and an identical culture was mock infected with a balanced salt solution. Both cultures were refed with a medium containing 2% fetal calf serum at the end of a 1 h adsorption period. 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 $\mu\text{g}/\text{ml}$. At 5 h p.i., 1000 μCi [^3H] uridine was added per culture and cells were incubated for 0.5 h at 37°C . Cells were then harvested and fractionated as described in the text except that reticulocyte standard buffer, RSB (0.01 M Tris-hydrochloride (pH 7.4)-0.01 M NaCl-0.0015 M MgCl_2) was used for Dounce homogenization of the cells. The postnuclear fraction was then made 30% sucrose (wt/wt) and suspended in a total volume of 10 ml. A discontinuous gradient, prepared by layering 3 ml of 65% sucrose, 6 ml of 48% sucrose, 6 ml of 45% sucrose, 5 ml of 38% sucrose, 10 ml of 30% sucrose (containing the sample), 5 ml of 25% sucrose, and 3 ml of RSB, was centrifuged at $50,000 \times g$ for 17 h at 4°C in a Sorvall AH-627 rotor, and fractionated from the top using an Isco gradient harvester equipped with a UV monitor. The fractions were monitored for absorbance at 254 nm wavelength, and 10 lambda aliquots of each fraction were assayed for acid-precipitable radioactivity. Graphs depict the absorbance at 254 nm, buoyant density, and radioactivity in 10 lambda aliquots of each fraction. Sedimentation is from right to left. Pool I contains material having a peak of radioactivity at a buoyant density of $1.18 \text{ g}/\text{cm}^3$. Pool II contains material having a peak of radioactivity at a buoyant density of $1.15 \text{ g}/\text{cm}^3$.

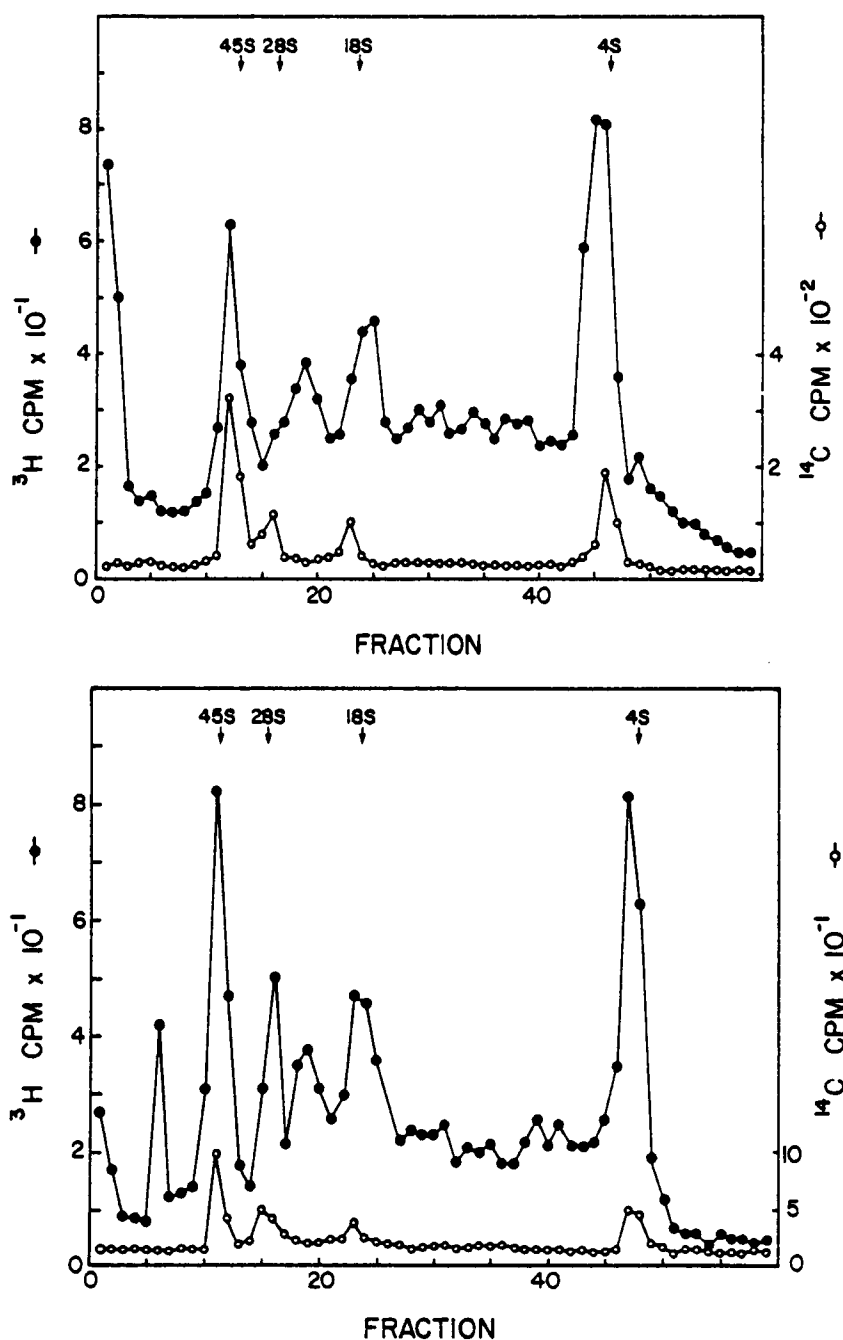


Fig. 4. Electrophoresis of viral-specified RNA isolated from isopycnicly-fractionated cytoplasmic structures. Materials in pools I and II described in Fig. 3 was separately diluted to 38 ml in RSB and pelleted by centrifugation at $90,000 \times g$ for 2 h at 4°C . RNA was then extracted as described in the text and electrophoresed on cylindrical gels of 2.0% polyacrylamide-0.5% agarose as previously described (3). A. RNA from pool I (buoyant density of 1.18 g/cm^3), closed circles. B. RNA from pool II (buoyant density 1.15 g/cm^3), closed circles. In both graphs the open circles depict the migration of $[^{14}\text{C}]$ uridine-labeled RNA reference species.

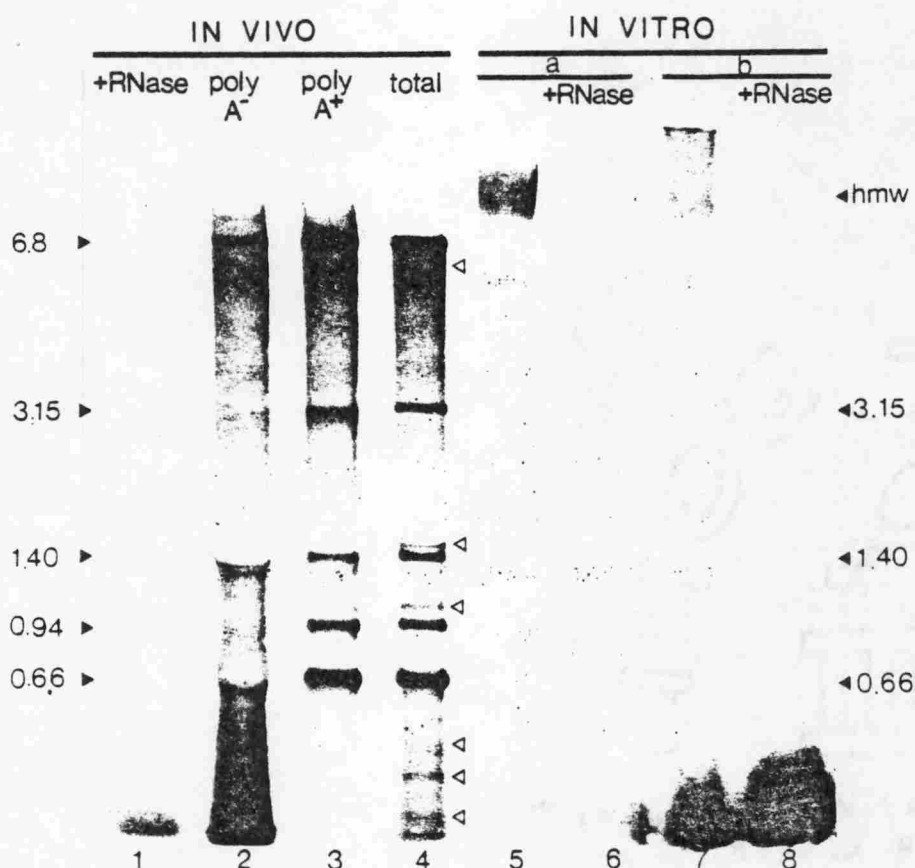


Fig. 5. RNase susceptibility of intracellular viral-specific RNA and *in vitro* polymerase products. The radiolabeled postnuclear fraction of infected cells was prepared as described in Fig. 1. The RNA was extracted and electrophoresed on denaturing agarose gels after the following treatments. Lane 1: digestion with 20 units T₁ RNase per ml-0.5 M NaCl for 1 h at 37°C. Lanes 2 and 3: oligo (dT)-cellulose chromatography using the procedure we have previously described in the text. Lane 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 treatment. Lane 5: no treatment. Lane 6: RNase digestion as described in Lane 1. Lane 7: no treatment. Lane 8: RNase digestion as described in Lane 1.

from the postnuclear fraction and chromatographed on oligo (dt)-cellulose. All five major RNA species and six of the minor RNA species are polyadenylated by this criterion (Fig. 5, 6, 7). In addition, some of the 6.8-, 1.4-, and 0.66×10^6 molecular weight species appear to be nonpolyadenylated as well (Fig. 5, 6, 7). The nonpolyadenylated species may be minus-strand RNA's, or they may reflect incomplete transcription or processing of the 3' end of the molecule. Alternatively, they may represent breakage of the 3' end of the RNA molecule.

Molar Ratios of the Viral-Specified RNA Species

The molar ratios of the intracellular RNA species were estimated by analyzing microdensitometer tracings of the fluorograms. The results are given in Table 1.

Viral RNA Species Synthesized During the In Vitro Polymerase Reaction

Initial analyses of the in vitro products were made by rate zonal sedimentation comparison with marker RNA species sedimented in parallel (Fig. 8). The sedimentation profile of the nondenatured products revealed 13 peaks having sedimentation coefficients of 4S or less, 12S, 16S, 24S, 32S, 36S, 42S, 53S, 56S, 67S, 70S, and 79S. Species ranging between 10S and 38S fell within two broadly sedimenting peaks. Approximately 5% of the radioactivity sedimented to the bottom of the gradient in the possible form of aggregates. In order to make correlations between these species and those observed by gel electrophoresis, it was first necessary to determine whether they were single- or double-stranded. In vitro polymerase products were treated with RNase in 0.5 M NaCl and sedimented in parallel. Greater than 80% of the RNase-treated RNA sedimented as apparent double-stranded RNA forms

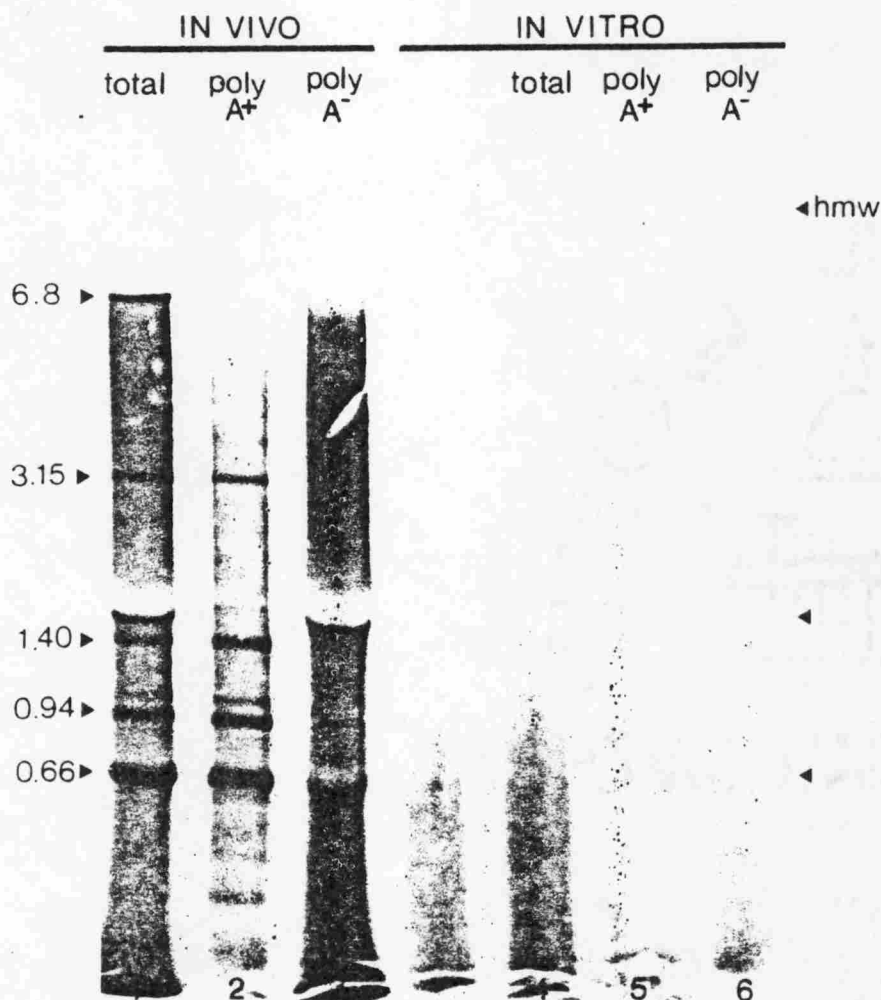


Fig. 6. Polyadenylation status of intracellular viral-specific RNA and *in vitro* polymerase products. The radiolabeled postnuclear fraction of infected cells was prepared as described in Fig. 1. The RNA was extracted and electrophoresed on denaturing agarose gels after the following treatments. Lane 1: no treatment. Lanes 2 and 3: chromatography on oligo (dT)-cellulose. Radiolabeled *in vitro* reaction products were extracted as described in the text and electrophoresed after the following treatments. Lane 4: no treatment. Lane 5 and 6: chromatography on oligo (dT)-cellulose.

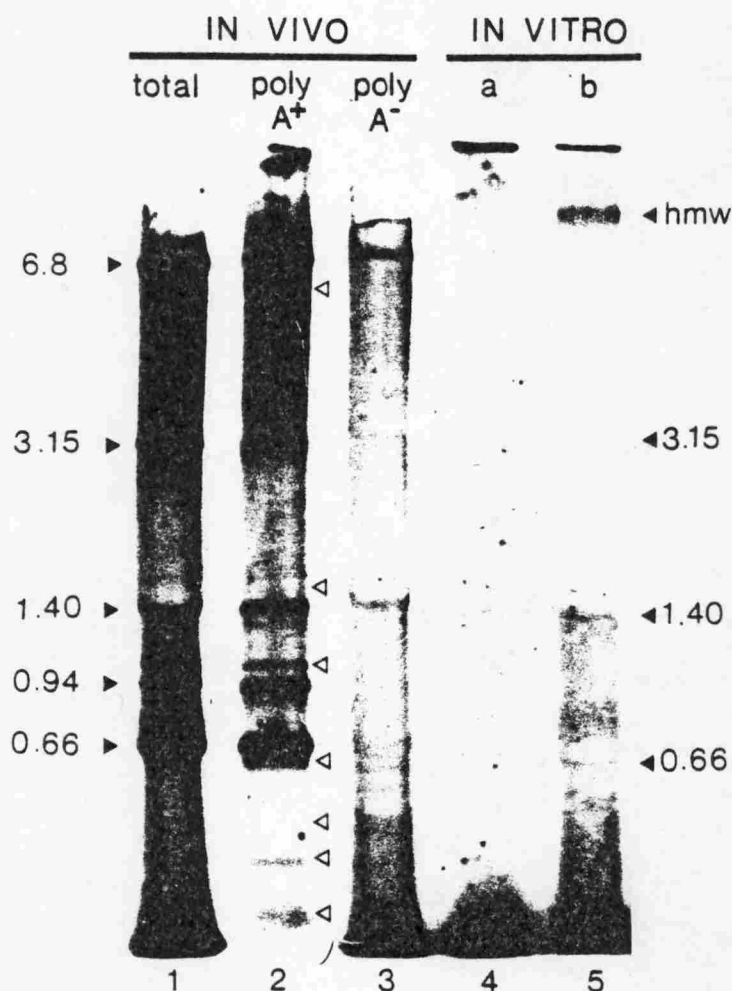


Fig. 7. Electrophoresis of viral-specified cytoplasmic RNA and *in vitro* polymerase products. The radiolabeled postnuclear fraction of infected cells was prepared as described in Fig. 1. The RNA was extracted and electrophoresed on denaturing agarose gels after the following treatments. Lane 1: no treatment. Lanes 2 and 3: oligo (dT)-cellulose chromatography. In lanes 4 and 5, radiolabeled *in vitro* reaction products from two separate experiments (a and b) were extracted as described in the text and electrophoresed after no further treatment.

Table 1. Molar ratios of the in vivo synthesized viral-specified major RNA species.

RNA species (x 10 ⁶)	molar ratio
6.8	1.00
3.15	0.43
1.40	0.46
0.94	0.87
0.66	2.83

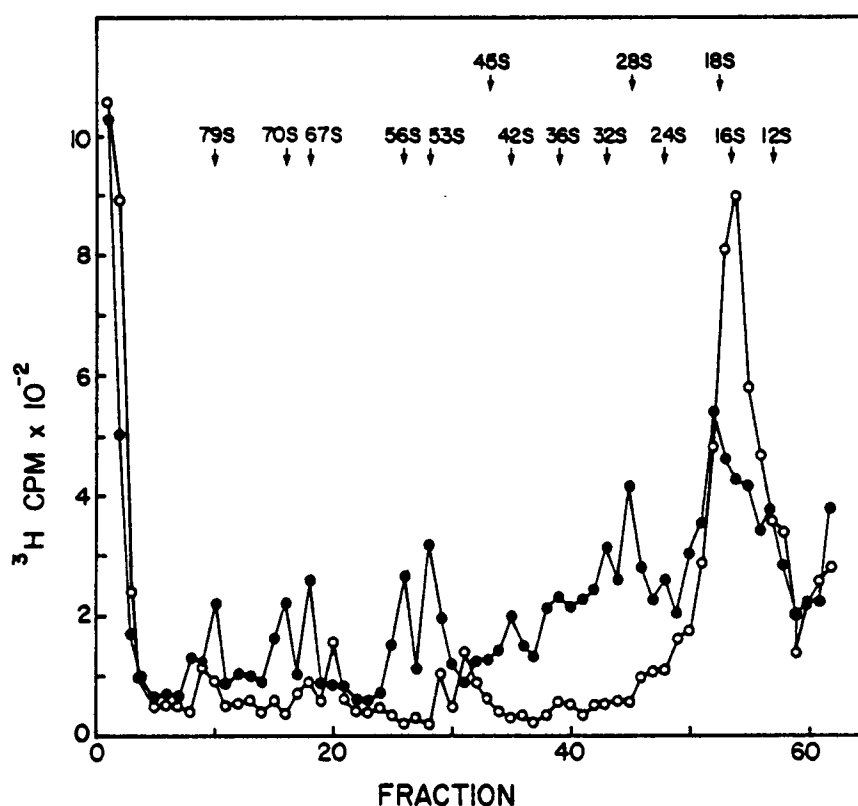


Fig. 8. Rate-zonal sedimentation of *in vitro*-transcribed polymerase products. An *in vitro* polymerase reaction was carried out as described in the text. After 20 min of incubation the reaction was divided into two equal aliquots. To the first (closed circles) was added 10% SDS in 5X TNE, to make a final concentration of 2% SDS in TNE, and 0.1 mg of proteinase K, and the sample was incubated 15 min at 37°C before centrifugation. To the second aliquot (open circles) was added 20X SSC (where SSC is 0.15 M NaCl-0.015 M sodium citrate) to make a final concentration of 0.3 M NaCl, and RNase T₁ and RNase A to make a final concentration of 25 ug/ml each, and the mixture was incubated 15 min at 37°C before centrifugation. Samples were layered onto a 38 ml 15-40% (wt/wt) sucrose gradient made up in NETS buffer (0.15 M NaCl-0.01 M EDTA-0.01 M Tris-hydrochloride (pH 7.4)-0.2 % SDS). Gradients were centrifuged at 23,000 x g for 1.5 h at 23°C. Marker positions were obtained from a separate gradient sedimented in parallel.

between 12S and 18S (Fig. 8). This indicates that species sedimenting at greater than 24S prior to RNase treatment are probably single-stranded species. Four minor peaks sedimenting at approximately 49S, 51S, 65S, and 81S following RNase treatment could represent double-stranded species. Whether any of the apparently double-stranded species represent true replicative intermediates or replicative forms was not investigated. Because the reaction products were not deproteinized before RNase treatment, we cannot rule out that apparent double-stranded forms are in fact single-stranded species protected in some manner by protein. Using the equation of Spirin (22), the single-stranded in vitro products of 4S, 12S, 16S, 24S, 32S, 36S, 42S, 53S, 56S, 67S, 70S, and 79S have corresponding molecular weights of 0.03-, 0.3-, 0.5-, 1.2-, 2.2-, 2.9-, 4.0-, 6.5-, 7.3-, 10.6-, 11.6-, and 15.0×10^6 respectively.

In order to make 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 (Fig. 5, 6, 7). By these analyses, three major in vitro transcripts with apparent molecular weights of 3.15-, 1.40-, and 0.66×10^6 molecular weight were observed. These are seen to comigrate with three of the five major RNA species made in vivo (Fig. 5, 6, 7). Digestion of the in vitro products with T_1 RNase in 0.5 M NaCl demonstrates that each of these species is single-stranded (Fig. 5). Oligo (dT)-cellulose chromatography of the in vitro products reveals that at least the 1.40- and 0.66×10^6 molecular weight species are nonpolyadenylated (Fig. 6). However, preliminary evidence (data not shown) suggests that the 3.15×10^6 molecular weight transcript is polyadenylated.

CHAPTER IV

DISCUSSION

We have previously reported that success in finding the coronaviral, cell-associated, RNA-dependent RNA polymerase in infected cells may be critically dependent on the timing of such a search (Part II of this thesis). A kinetic study demonstrated that greater than 80% of virus-specified RNA synthesis occurs between 4 and 6 h postinfection. Consequently, all studies on both in vivo and in vitro polymerase products were made using cells between 4 and 6 h postinfection. The species of viral RNA synthesized as well as the behavior of the polymerase may be quite different if studied outside of this interval of time.

In an effort to determine the viral specificity of the polymerase activity, in vitro-synthesized products were compared to RNA species made in vivo within the interval between 4 and 6 h postinfection. When cells were pulse-labeled in vivo, 5 major single-stranded, polyadenylated RNA species with molecular weights of 6.8-, 3.15-, 1.40-, 0.94-, and 0.66×10^6 were made. On first examination, these would appear to correspond to the 5 intracellular single-stranded RNA species described for the avian infectious bronchitis virus (23), and would immediately suggest that the strategy of TGEV replication (a mammalian coronavirus) and avian infectious bronchitis virus (an avian coronavirus) are very similar. This, along with the positive-strand polarity of the genomic RNA, suggests that we can certainly go beyond merely a morphological criterion for placing these two viruses into the same family. In addition, 6 minor species of single-stranded, polyadenylated,

intracellular species of RNA were also found with molecular weights of 6.2-, 1.05-, 0.64-, 0.39-, 0.34-, and 0.24×10^6 which were not reportedly found in the avian system. Whether these reflect a fundamental difference between TGEV and the avian infectious bronchitis virus, or whether similar RNA species were present but not detected by Stern and Kennedy, is not yet known. Experiments which can distinguish unique nucleotide sequence arrangements within RNA molecules are needed to determine whether each RNA molecule identified so far is indeed unique, or whether some might represent variants with only a different secondary structure. Also present in infected cells were three species of RNA with molecular weights of 6.8-, 1.40-, and 0.66×10^6 which were not polyadenylated. It is possible that these represent truly minus-strand forms of virus-specific RNA, or alternatively, they may be positive-strand species on which the poly (A) tract was too short to anneal to oligo (dT)-cellulose or they were missing altogether. Nucleotide sequence information is required to distinguish between these possibilities. The absence of RNase-resistant forms suggests that double-stranded RNA forms are, if present, escaping our detection. It can therefore be argued that minus-strand species are probably present in only very small numbers.

When the products of the in vitro polymerase reaction were examined by electrophoresis on denaturing gels 4 species were seen. The largest was greater than 6.8×10^6 and appeared to be partially RNase resistant. It is the only candidate for a double-stranded form and is a possible replicative intermediate. The three smaller species have apparent molecular weights of 3.15-, 1.40-, and 0.66×10^6 .

Assuming that the information described by Stern and Kennedy for the avian infectious bronchitis virus is also true for TGEV, namely that sequences within the smallest mRNA are contained within the next larger species and so on through to the largest mRNA species, and considering the information we have described for TGEV, we wish to speculate on the most likely coronaviral replication strategy.

With respect to RNA replication, we would predict that what appears to be the simplest scheme is employed by coronaviruses, especially in the light of the replicational strategy used by picornaviruses and togaviruses. Namely, full-length genomic RNA serves as a template for full-length positive strand genomic RNA. This mechanism remains pure speculation until a full-length minus-strand can be identified. The candidate for such a molecule is the 6.8×10^6 molecular weight nonpolyadenylated species we have described. Sequence studies requiring oligonucleotide fingerprinting or hybridization techniques are necessary to demonstrate the minus-strand polarity of this molecule. Full-length minus-strand RNA species must evidently be a minority species since double-stranded RNA products were not seen in our in vivo-labeled RNA. While it is conceivable that ligation (i.e., splicing) of short pieces of RNA could give rise to either minus-strand or positive-strand full-length molecules, since this process is known to exist in eucaryotic cells, such a mechanism would be cumbersome and we judge it to be unlikely.

With respect to RNA transcription, i.e., the synthesis of mRNA species, it is tempting to speculate that subgenomic messages are generated from a full genome-length minus-strand RNA. This is speculation again, however, since no full-length minus-strand has yet

been identified. At least four other strategies are possible based on molecular mechanisms known to exist in other viral and cellular systems. Each of these four strategies are discussed in terms of models and the most likely model for coronaviruses is discussed in light of existing data. The models are designated as 1) the multiple replicative intermediate model, 2) the posttranscriptional cleavage model, 3) the internal termination model, and 4) the internal initiation model.

The multiple replicative intermediate model employs the use of multiple mRNA-length, minus-strands as templates for mRNA synthesis. This model, at the level of transcription, is conceptually similar to the segmented minus-strand myxoviruses. This model states that sub-genomic minus-strands, in addition to a full-length minus-strand, arise from a full-length positive-strand template. We think this model is unlikely since multiple double-stranded intermediates were not found on denaturing gels following RNase digestion in high salt. While three abundant species of nonpolyadenylated RNA were observed, they were apparently positive-stranded RNA species which were missing their poly (A) tracts since truly minus strands in this abundance should have yielded some double-stranded intermediates.

The posttranscriptional cleavage model states that a full-length positive-strand transcript is made which becomes processed possibly by endonucleolytic cleavage, exonucleolytic cleavage, or scission and subsequent ligation, to yield subgenomic mRNA's. While as yet no viral precedent exists for this kind of processing, the endonucleolytic processing of ribosomal RNA serves as a precedent (1). If endonucleolytic processing were true, one would expect to find discrete

nonpolyadenylated byproducts of endonucleolysis in approximately equimolar amounts to the polyadenylated species. As our data show, gel patterns before and after oligo (dT)-cellulose chromatography are essentially identical when comparing unfractionated and polyadenylated species. In addition, the fractionated nonpolyadenylated species are of primarily 3 sizes. We therefore think that processing by endonucleolytic cleavage is unlikely. With exonucleolytic processing, we would expect to find heterogeneous RNA patterns in gels containing the polyadenylated species since our pulse time are long enough to create exonucleolytic heterogeneity (15 to 30 minutes). Based on the degree of heterogeneity we see, we are not able to rule out exonucleolytic processing, but we do not favor this model primarily because it would require heretofore undescribed intricate control mechanisms (stop signals) for controlling the exonuclease(s). We are likewise unable to rule out RNA splicing as a mechanism for the synthesis of mRNA. Sequence data is required to determine the significance of this mechanism. Nucleolytic processing in general is not supported by our in vitro data since single-stranded polymerase products corresponding in size to in vivo-synthesized single-stranded products are made in the presence of 100 ug/ml dextran sulfate, a potent RNase inhibitor. When dextran sulfate was omitted, only a single peak of RNase-resistant material sedimenting at 18-20S was observed. Unless processing RNases are unique in their sensitivity to dextran sulfate, they should be at least partially inhibited by the dextran sulfate in the in vitro reaction mixture. If this is so, we would expect to see the accumulation of full-length positive-strand precursors to the subgenomic mRNA. At no time have we been able to

detect full-length viral RNA in gels of the in vitro transcripts, even when higher amounts of dextran sulfate (200 ug/ml) were used. Therefore, we suggest that posttranscriptional nucleolytic processing is not the mode of production of coronaviral subgenomic mRNA's.

The internal termination model states that subgenomic mRNA's are produced from initiation of transcription at the 3' end of a full-length minus-strand template and that multiple termination sites exist along the template. Transcript size is then determined by different readthrough potentials. Precedence for this transcriptional mechanism exists in the papovavirus (10) and adenovirus (16) systems, although in these cases, DNA serves as the template. The primary argument against this model comes from the data of Stern and Kennedy which says that the common sequences within all the subgenomic mRNA's are at the 3' terminal end. Synthesis of mRNA's all initiating at the 3' end of a common template would result in transcripts having common sequences at their 5' terminal end and not their 3' terminal end. Internal termination is therefore an unlikely mechanism for generating subgenomic mRNA species.

The internal initiation model states that subgenomic mRNA species are derived by initiation of transcription at different internal sites along a full-length minus-strand RNA template. The negative-strand template may support one active site at a time, or alternatively, it may be able to support multiple active initiation sites producing subgenomic mRNA's. This would appear to depend on the type of transcriptional control. There is precedence for internal initiation

during the synthesis of alphavirus mRNA (20, 21), and this mechanism may also function during rhabdovirus replication (4, 17). Synthesis of the 26S alphavirus mRNA is regulated by an interconversion protein which converts the replicative intermediate to an altered configuration which results in a "jamming" of the polymerase and a resultant exposure of an internal initiation sequence. We favor the internal initiation model for two essential reasons. Firstly, it is most consistent with conserved sequences at the 3' end of subgenomic mRNA's as described by Stern and Kennedy for the avian coronavirus. Secondly, we were able to find only one, large, possible replicative intermediate after in vitro transcription. This suggests the existence of only one template for viral mRNA synthesis.

It is not clear to us why only 3 of 5 major transcripts were obtained during the in vitro transcriptase reaction. Perhaps these species were present in levels too low for detection by our methods. In any case, preferential synthesis of three species suggests that a control mechanism is operating in vitro. We are not certain that these represent plus-stranded sequences. Our data suggests that the two smaller in vitro transcripts (i.e., the 1.40-, and 0.66×10^6 molecular weight species) are not polyadenylated in vitro. If in fact the transcripts represent positive (i.e., mRNA) strands then polyadenylation must be an event that is separate from transcription. On the other hand, preliminary evidence suggests that the 3.15×10^6 molecular weight species is polyadenylated in vitro (data not shown). This would support the argument that for this species, polyadenylation is a transcriptional event (i.e., is synthesized from a template), or is an event that is closely juxtaposed to transcription in time and space.

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