Transmissible gastroenteritis virus : genome and messenger RNA sequence
by Quentin Boyd Reuer

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Microbiology
Montana State University

Abstract:
The genetic structure of the pathogenic Miller strain of transmissible gastoenteritis virus (TGEV) was studied at the molecular level. Subgenomic RNAs 6 and 7 and the 3' 7.3 kb of the viral genome were reverse transcribed into cDNA. Complementary DNA clones were mapped; maps suggested that RNA 7 was a subset of RNA 6, and the maps of both subgenomic RNAs were identical to the map of the 3' region of the virion cDNA. Restriction fragments of the cDNA clones were sequenced. Common 5' leader sequences were found in RNA 6- and RNA 7-specific cDNAs but not in the corresponding region of virion cDNA. The gene encoding the matrix (E1) protein of TGEV (Miller strain) was found in the virion RNA and RNA 6 nucleotide sequence. The 29.4 kd primary product of this gene possessed a 17-residue hydrophobic leader peptide. Hydrophilicity analysis of the protein revealed internal membrane-spanning regions, an amphiphilic C-terminal half, and a hydrophilic C-terminus.

A gene encoding the nucleocapsid (N) protein of TGEV (Miller strain) was found in the virion RNA, RNA 6, and RNA 7 nucleotide sequences. The predicted molecular weight of the serine-rich polypeptide was 43.4 kd. Clusters of charged residues were found over the entire amino acid sequence of N. The sequence of virion cDNA contained the 3' 3183 bases of the TGEV (Miller strain) peplomer (E2) gene. Downstream of the E2 gene were open reading frames that may represent the coding regions of TGEV (Miller strain) RNAs 4a, 4b, and 5. Data obtained during this research suggested that TGEV RNAs form a nested set and that the primary products of the RNAs are encoded by the 5' region not found in the next smaller RNA. The presence of 5' leader sequences in RNA 6- and RNA 7-specific cDNA not found in virion cDNA indicates that TGEV subgenomic RNAs may be transcribed by a leader—primed discontinuous process. Analysis of the primary structure of TGEV (Miller strain) structural proteins demonstrates that the virulent strain differs markedly from the attenuated Purdue strain of TGEV. These data will be useful in the development of safe, effective vaccines against porcine transmissible gastroenteritis.

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Quentin Boyd Reuer

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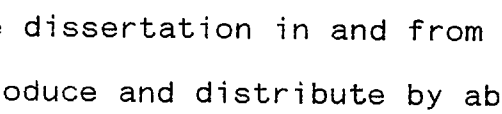
Signature 

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ABSTRACT

The genetic structure of the pathogenic Miller strain of transmissible gastoenteritis virus (TGEV) was studied at the molecular level. Subgenomic RNAs 6 and 7 and the 3' 7.3 kb of the viral genome were reverse transcribed into cDNA. Complementary DNA clones were mapped; maps suggested that RNA 7 was a subset of RNA 6, and the maps of both subgenomic RNAs were identical to the map of the 3' region of the virion cDNA. Restriction fragments of the cDNA clones were sequenced. Common 5' leader sequences were found in RNA 6- and RNA 7-specific cDNAs but not in the corresponding region of virion cDNA. The gene encoding the matrix (E1) protein of TGEV (Miller strain) was found in the virion RNA and RNA 6 nucleotide sequence. The 29.4 kd primary product of this gene possessed a 17-residue hydrophobic leader peptide. Hydrophilicity analysis of the protein revealed internal membrane-spanning regions, an amphiphilic C-terminal half, and a hydrophilic C-terminus. A gene encoding the nucleocapsid (N) protein of TGEV (Miller strain) was found in the virion RNA, RNA 6, and RNA 7 nucleotide sequences. The predicted molecular weight of the serine-rich polypeptide was 43.4 kd. Clusters of charged residues were found over the entire amino acid sequence of N. The sequence of virion cDNA contained the 3' 3183 bases of the TGEV (Miller strain) peplomer (E2) gene. Downstream of the E2 gene were open reading frames that may represent the coding regions of TGEV (Miller strain) RNAs 4a, 4b, and 5. Data obtained during this research suggested that TGEV RNAs form a nested set and that the primary products of the RNAs are encoded by the 5' region not found in the next smaller RNA. The presence of 5' leader sequences in RNA 6- and RNA 7-specific cDNA not found in virion cDNA indicates that TGEV subgenomic RNAs may be transcribed by a leader-primed discontinuous process. Analysis of the primary structure of TGEV (Miller strain) structural proteins demonstrates that the virulent strain differs markedly from the attenuated Purdue strain of TGEV. These data will be useful in the development of safe, effective vaccines against porcine transmissible gastroenteritis.

INTRODUCTION

The development of safe and effective vaccines has been a goal of molecular biologists since the advent of recombinant DNA technology. Many types of virus preparations and means of inoculation have been used in attempts to provide immunity to viral diseases. Exposure to virulent viruses and inoculation with killed virus or attenuated virus vaccines have been used, although disease development, inadequate protection, and the reversion of attenuated viruses to pathogenic forms have been dangers inherent in the use of these preparations. Risks associated with immunization might be reduced or eliminated upon employment of subunit vaccines. In order to develop reliable viral subunit vaccines, the biological and biochemical characteristics of virulent and attenuated forms of the virus must be studied. Once the genetic organization of the virus and the molecular basis of its pathogenicity are understood, genetically altered viruses that induce a protective immune response but fail to cause disease might then be constructed. The purpose of my research was to study at the molecular level the genetic

structure of the Miller strain of transmissible gastroenteritis virus (TGEV), a pathogen of swine.

Biology and Biochemistry of Coronaviruses

Members of the family Coronaviridae are spherical, pleomorphic particles 60-220 nanometers in diameter which bear characteristic club-shaped surface projections. The corona-like appearance of these projections led to the creation of the family Coronaviridae by the International Committee on the Taxonomy of Viruses in 1975 (91). Coronaviruses have been placed in antigenic groups on the basis of cross-reactivity in serological tests (Table 1) (59,76,88,95). The mammalian coronaviruses fall into two groups, while the avian coronaviruses, infectious bronchitis virus (IBV) and turkey coronavirus (TCV), compose the remaining two groups. Several strains of two coronaviruses, IBV of chickens and murine hepatitis virus (MHV), have been intensively studied and serve as models of this family.

Coronaviruses are widespread pathogens of many species of mammals and birds and cause acute and chronic diseases. Targets of infection include the gastrointestinal tract, respiratory system, liver and nervous system. Marked tissue tropism is characteristic of the coronaviruses. Investigations of the epidemiology and pathogenesis of

Table 1. Antigenic cross-reactivity among coronaviruses

Antigenic group	Virus*	Host
I	HCV-229E	Human
	TGEV	Pig
	CCV	Dog
	FECV	Cat
	FIPV	Cat
II	HCV-OC43	Human
	MHV	Mouse
	HEV	Pig
	BCV	Cow
	RbCV	Rabbit
III	IBV	Chicken
IV	TCV	Turkey
Unclassified	HECV	Human

* Abbreviations: HCV-229E, human respiratory coronavirus; TGEV, transmissible gastroenteritis virus; CCV, canine coronavirus; FECV, feline enteric coronavirus; FIPV, feline infectious peritonitis virus; HCV-OC43, human respiratory coronavirus; MHV, mouse hepatitis virus; HEV, hemagglutinating encephalomyelitis virus; BCV, bovine coronavirus; RbCV, rabbit coronavirus; IBV, infectious bronchitis virus; TCV, turkey coronavirus; HECV, human enteric coronavirus.

The table was developed with references 59,76,88,95.

coronavirus infections have been impeded by the difficulty of isolating coronaviruses from diseased hosts.

Studies of coronaviruses have demonstrated several unique features in RNA transcription, protein composition, and virus assembly (73,75,76,88). Coronaviruses multiply

exclusively in the cytoplasm of infected cells (98). Unlike many other types of viruses, coronaviruses do not induce rapid inhibition of host cell macromolecular synthesis. Productive infection of a susceptible cell by a coronavirus usually results in cell death due to fusion or lysis, although persistent coronavirus infections can readily be established *in vitro* and *in vivo*. Some persistently infected cells synthesize viral antigens and release infectious virus particles.

Coronavirions assemble by budding at internal membranes of host cells (16). Coronavirions contain an envelope derived from the endoplasmic reticulum and Golgi apparatus of host cells. Virions are released from cells by fusion of post-Golgi vesicles with the plasma membrane (53,88,91,95).

The genome of coronaviruses is a single-stranded, polyadenylated, colinear RNA of 6-8 megadaltons (62,76,88). Virion RNA is infectious and is believed to encode an RNA-dependent RNA polymerase. Negative-sense copies of the genome are synthesized by this enzyme in infected cells, and from these templates genomic and subgenomic RNAs are transcribed.

MHV and IBV produce 5-6 polyadenylated subgenomic mRNAs in infected cells (43,72,88). These RNAs are made in unequal amounts (48,80,94). Regulatory mechanisms controlling coronavirus mRNA synthesis have not been

identified. MHV and IBV mRNAs form a nested set; the RNAs possess common 3' termini and extend for different lengths in the 5' direction. Only the 5'-terminal region not found in smaller species of mRNA is translated (88,91). Coding assignments of the mRNAs of several coronaviruses have been established (35,72,82). A common 5' leader sequence at least 70 nucleotides in length has been found in the genomic and subgenomic RNAs of MHV (43,78,79). UV transcriptional mapping studies have shown that the synthesis of each MHV subgenomic RNA is initiated independently, suggesting that the RNAs are not spliced from larger precursors (34). Rather, the leader sequence is joined to the body sequence of mRNAs by discontinuous transcription (78). In this process, the leader sequence may serve as a primer for transcription of the mRNA body sequence by a virus-specific RNA-dependent RNA polymerase (43,78,79).

Coronavirus particles contain from 3 to 7 structural proteins, which can be grouped into 3 functional classes (76,88). The nucleocapsid (N) protein is a phosphorylated, basic polypeptide of 45-60 kd that encapsidates the virion RNA to form a long, flexible structure with helical symmetry (12,48,75,88). In intact virions, N protein is resistant to treatment with bromelain (12) and pronase (93), indicating that it is located internally. N protein is the only virion protein to display significant

phosphorylation (83). *In vitro* studies by Siddell et al. have detected a protein kinase activity associated with the coronavirus JHM virion that specifically phosphorylates the virion nucleocapsid protein (74).

A second class of structural polypeptides is a heterogenous transmembrane glycoprotein species, E1, or matrix (M) protein, of 25-35 kd (1,7,12,49,75,92). This protein has been intensively studied in several coronaviruses and appears to possess three domains: a glycosylated hydrophilic region that extends outside the viral envelope, a hydrophobic region which extends through the viral membrane, and a third domain that possibly interacts with viral RNA within virus particles. E1 is not transported to the plasma membrane of infected cells as are other viral glycoproteins, but accumulates in the Golgi apparatus. The protein may bind the nucleocapsid to the viral envelope as the coronavirion buds, a function in common with that of the nonglycosylated matrix proteins of orthomyxo-, paramyxo-, and rhabdoviruses. Although E1 is a membrane protein, in MHV and IBV it lacks a signal peptide that is cleaved following translocation. Instead, the matrix proteins of these coronaviruses may be inserted into membranes by recognition of the internal hydrophobic region (65). E1 is glycosylated to varying degrees; this results in the protein appearing as multiple bands in sodium dodecyl sulfate-polyacrylamide gels of infected cell lysate

or dissociated virions. The N-terminal region of E1 proteins of MHV and bovine coronavirus (BCV) possess oligo- and polysaccharides O-linked to serine and threonine residues (56,58). O-linked glycosylation, unusual among viral glycoproteins, takes place at the Golgi apparatus of infected cells. Antibodies to E1 are capable of neutralizing viral infectivity only in the presence of complement (15).

The third class of polypeptides is a large (125-220 kd) complex glycoprotein, E2, which forms the characteristic surface projections of coronaviruses (7,12,13,21,28,40, 49,70,92,93). These projections, or peplomers, are responsible for attachment of coronaviruses to cells, induction of neutralizing antibodies, and the fusion of infected cells into syncytia. The biological activity of peplomers determines the virulence and tissue tropism of coronaviruses. Almost all of E2 is located outside the viral membrane; only a small anchor region of the peplomer is embedded in the viral envelope. The molecule is transported to the plasma membrane of infected cells. Cells displaying E2 on their surface are susceptible to cell-mediated cytotoxicity. The peplomer of IBV is composed of two or three copies each of glycoproteins S1 (90 kd) and S2 (84 kd) (13). A C-terminal hydrophobic domain of S2 secures the peplomer to the viral envelope. S1, the target of neutralizing and

hemagglutinating antibodies, is non-covalently linked to S2. S1 is necessary for infectivity of the virus but need not be present for attachment of the virion to cells. Comparison of the amino acid sequences of different IBV strains suggests that the neutralization epitopes are located near the S1 N terminus.

Bovine coronavirus (BCV) virions possess two different peplomeric glycoproteins (40). A 190 kd peplomer glycoprotein appears to be composed of 100 and 120 kd subunits. A second, smaller peplomer, a dimer of 65 kd glycopeptides joined by disulfide bonds, is responsible for the hemagglutinating activity of the virus. Both peplomers can elicit the production of BCV-neutralizing antibodies.

Cleavage of the peplomeric glycoprotein may be required for coronavirus infectivity and cytopathic effects (31,88,89). Trypsinization of peplomers in vitro increases the infectivity of virus particles and dramatically increases the yield of infectious virus from cells infected with trypsinized stock virus (84,87). Cells that make infectious coronaviruses without added trypsin possibly cleave peplomer proteins with cellular proteases in the Golgi apparatus or at the plasma membrane.

Transmissible Gastroenteritis: Epizootic and Enzootic

Transmissible gastroenteritis (TGE) is a disease of swine first reported in Indiana in 1946, and it is now

found in most swine-producing countries of the world (29). The loss of animals by swine producers makes TGE economically important. The cause of the disease, transmissible gastroenteritis virus (TGEV), is a porcine coronavirus that infects and destroys the absorptive epithelial cells of the small intestine. TGEV can be transmitted to a swine unit in a number of ways, including contaminated fomites, birds, and recently recovered swine that appear healthy but are still shedding the virus. Ingestion is the normal route of exposure to the pathogen. TGEV virions display resistance to low pH, trypsin, and bile, making it possible for the virus to maintain infectivity during passage through the alimentary tract. Two forms of TGE, epizootic and enzootic have been described (29).

Epizootic TGE affects swine of all ages; morbidity is virtually 100% in exposed herds. The severity of the disease is greatest in newborn pigs which, during epidemics, can suffer mortality rates of up to 100% (5,29). Newborn animals experience vomiting, severe diarrhea, and subsequent dehydration resulting in rapid weight loss. Extreme thirst is characteristic of TGE in nursing swine. Upon autopsy, nursing piglets display villus atrophy in the jejunum and ileum and undigested milk curds throughout the gastrointestinal tract. Fluorescent antibody tests often reveal the presence of viral antigens in villus epithelial

cells. Laboratory diagnosis may also include electron microscopic examination of small intestinal tissues for the presence of coronavirus particles. There is no practical method of treating young pigs; replacement fluid therapy has been successful in treating laboratory pigs, but the method is labor-intensive and not applicable in large swine units. Clinical signs in older animals include anorexia and profuse, watery diarrhea. Lactating sows may experience agalactia. The incubation period of TGEV prior to the onset of clinical signs in infected animals is 18-24 hours. Pigs under 7 days of age usually die 2-7 days after clinical signs appear. The rate of mortality decreases as the age of swine at the time of infection increases. Mortality in 2 to 3 week old pigs approaches 20-30%, while only 3-4% of weaned pigs and fewer than 1% of adult pigs die as a result of the disease.

Enzootic TGE occurs in swine units that practice continuous or nearly continuous farrowing. Previous infection of the herds with TGEV results in establishment of adequate immunity to the virus. Later this immunity declines, and a non-explosive form of TGE develops. Pigs are usually 6 days of age or older when stricken with diarrhea, and not all pigs in the litter may be affected. Vomiting is not always present, and the rate of mortality is often low. Sows may provide some lactogenic immunity; if the level of immunity is high enough, nursing pigs are

protected until weaning. Agalactia experienced by some sows may prevent transfer of adequate colostral and milk antibody to the young. Enzootic TGE is not always recognized by pork producers and veterinarians familiar with the explosive epizootic form of the disease.

Pigs that recover from TGEV infection can shed the virus from the lungs for more than 4 months after the initial infection. Porcine alveolar macrophages are capable of supporting TGEV replication, as demonstrated by positive immunofluorescence, infectious virus release, and interferon synthesis (45). Also, TGEV may be maintained in a latent state in cells of the intestinal villi. Persistently infected animals can then shed virulent TGEV in fecal material.

There is currently no effective vaccine against TGEV. Natural infection of sows with virulent TGEV leads to production of secretory antibodies capable of neutralizing the virus, but intramuscular inoculation of the pathogenic form does not. Induction of lactogenic immunity may depend upon the route of immunization. Ingestion of TGEV results in the infection of the intestinal epithelium (5); it is possible that macrophages in nearby Peyer's patches break down the virus and present viral antigens to migrating T lymphocytes, which then pass these antigens to lymph nodes near secretory glands. By this process large amounts of protective secretory IgA can be produced and provided to

suckling pigs in milk and colostrum. Passive protection provided by immune sows usually prevents the majority of piglets in a litter from developing TGE (6,10,26,95,99). In contrast, intramuscular administration of the virus stimulates production of circulating antibodies, largely of the IgG class. Although the IgG may neutralize TGEV particles, very little of this antibody is found in the milk of sows immunized intramuscularly. Oral or intramuscular administration of attenuated TGEV particles results in secretion of virus-specific antibodies, but this response is not adequately protective against infection with virulent TGEV. Also, an inherent danger in the use of attenuated viruses as vaccines is the possibility of reversion of the viruses to pathogenic forms. TGEV subunit vaccines containing purified viral protein from virulent or attenuated strains have also failed to protect vaccinated animals (23). A greater understanding of the molecular basis of TGEV pathogenicity is necessary if a protective vaccine is to be developed.

The attenuated Purdue strain of TGEV has been studied by several laboratories. This strain was developed by repeated passage of virulent TGEV through cell culture. Although it was produced for use as a vaccine, the Purdue strain is often used in laboratory studies of TGEV because it replicates to a higher titer <u>in vitro</u> than does the low-passage Miller strain. However, because the Purdue strain

is attenuated, its properties may not accurately reflect those of the virulent virus.

Transmissible Gastroenteritis Virus Proteins

TGEV contains three major structural polypeptides (21). The nucleocapsid (N) protein is a phosphorylated molecule of approximately 50 kilodaltons that is associated with TGEV genomic RNA (21). The nucleotide sequence of the N protein gene of the Purdue strain has been determined by Kapke and Brian (38). TGEV is not of the antigenic subgroup of the coronaviruses MHV and IBV, but the predicted amino acid sequence of TGEV (Purdue strain) N protein shows an overall homology of 26 and 27% with IBV and the neurotropic JHM strain of MHV, respectively. A conserved 68 amino acid region was found to be shared by the three viruses. This region is more basic than the overall nucleocapsid protein, and may interact with genomic RNA. Other regions of the N proteins were found to share structural characteristics even though amino acid sequences differed, suggesting the existence of additional conserved funtional domains.

The second virion protein is the matrix glycoprotein E1. Using cDNA sequence data, Laude et al. predicted that the primary translation product of the TGEV (Purdue strain) E1 gene is 262 amino acids long with a molecular weight of 29.6 kd (47). A 17 amino acid leader peptide is removed

from E1 during maturation of the polypeptide. This leader sequence may direct passage of E1 into internal membranes of TGEV-infected cells, a means of localization different than that of MHV and IBV matrix proteins. Comparison of the nucleotide sequence of the TGEV (Purdue strain) E1 gene with the E1 genes of MHV (strain A59) and IBV revealed no significant homology. However, the amino acid sequences showed homologies of 38% (TGEV-MHV) and 27% (TGEV-IBV). Three potential membrane-spanning regions were found in the amino acid sequences of the three E1 polypeptides, but only one of these regions displays an equal degree of homology among the coronaviruses studied. The variance may be due to functional differentiation between the three hydrophobic segments. Binding of the hydrophilic region of E1 by antibodies in the presence of complement may result in virus neutralization (15). Two potential sites of N-glycosylation are present in this area, only one of which may be accessible to glycosylation while E1 is associated with the endoplasmic reticulum (47).

The largest TGEV structural protein is the 195-220 kd peplomer glycoprotein, E2. TGEV (Purdue strain) sequence data suggests the molecular weight of the primary translation product of the E2 gene to be 158 kd (60). The carbohydrate moiety is thus approximately 25% of the total molecular size of the polypeptide, a level in agreement with that reported for the IBV peplomer (3). E2 of the

Purdue strain is largely hydrophobic (60). Hydrophobic residues are concentrated in the core of the peplomer. An extremely hydrophobic sequence of 45 residues near the C terminus is the region of the peplomer presumed to anchor the molecule in the viral envelope. This segment has a much higher ratio (24.5%) of cysteine residues than the molecule as a whole (3.4%); this appears to be a distinctive feature of the coronaviruses (3,60). The peplomer domain immediately exterior to the viral envelope contains an eight residue segment that is perfectly conserved in TGEV (Purdue strain) and IBV (3,60). In both viruses, this region of E2 is preceded by N-glycosylation sites. Hydrophilic segments appear to be concentrated in the carboxyl half of E2. Overall homology between the amino acid sequences of IBV and TGEV (Purdue strain) peplomers is 32.3%. Regions of homology are concentrated in the carboxyl half of the molecule, while the amino half shows considerable divergence.

Possibly all complement-independent TGEV-neutralizing antibodies are directed against the peplomer (37,46). Laude et al. (46) used monoclonal antibodies to construct a map of the antigenic determinants of the TGEV peplomer protein. In this model, TGEV peplomers possess four major antigenic sites, fewer than expected of such a large protein. The neutralization-mediating domain is composed of two of these sites; both sites possess a common epitope.

Also, the sites are conserved among the different strains of TGEV tested. Laude's data suggested that the immunodominant site of the peplomer might reside within the neutralization-mediating region.

Transmissible Gastroenteritis Virus RNAs

Six viral mRNA species were detected by Jacobs et al. in porcine cells infected with the Purdue strain of TGEV (35). Their size, as determined by SDS-PAGE, was 23.6 kb (RNA 1), 8.4 kb (RNA 3), 3.8 kb (RNA 4), 3.0 kb (RNA 5), 2.6 kb (RNA 6), and 1.9 kb (RNA 7). The RNAs were translated *in vitro*. RNA 7 was shown to encode the nucleocapsid protein, while RNA 6 encoded an unglycosylated precursor of the matrix protein. A 24 kd nonstructural protein was the primary translation product of RNA 4. Translation of RNA 3 resulted in 130 and 250 kd proteins and smaller molecules that could be precipitated with a monoclonal antibody directed against the peplomer. No virus-specific translation product was identified for RNA 5.

The intracellular RNAs of swine testicle (ST) cells infected with the virulent Miller strain of TGEV were studied by Andreas Luder (personal communication). Seven virus-specific RNAs were detected. Sizes of the RNAs were predicted by their rate of migration in agarose following denaturation with glyoxal. The predicted lengths of the

RNAs were: 23.0 kb (RNA 1), 8.5 kb (RNA 3), 3.9 kb (RNA 4a), 3.6 kb (RNA 4b), 2.9 kb (RNA 5), 2.6 kb (RNA 6), and 1.8 kb (RNA 7). The full length minus-strand copy of the genome is considered to be RNA 2. Jacobs et al. did not detect the 3.6 kb RNA during their study of TGEV (Purdue strain) RNAs (35). However, the method of RNA numbering used by Jacobs et al. is also used in this thesis.

The kinetics of TGEV (Miller strain) RNA synthesis are not well understood. A proposed mechanism of coronavirus replication suggests two main phases of RNA synthesis (82). Following virion RNA-directed synthesis of an RNA-dependent RNA polymerase, a full-length minus-strand RNA is transcribed from the viral genome. Transcription of subgenomic RNAs from this template could comprise the first phase of virus-specific RNA synthesis. The second phase would occur later in the cycle of infection, when full-length virion RNA is transcribed from the minus-strand template. This may occur just before packaging of the genome and subsequent release of virions from infected cells. ST cells infected with TGEV (Miller strain) reach their maximum yield of virus at 18 h post-infection (67).

Past research has not conclusively demonstrated that TGEV produces a nested set of RNAs in infected cells. It is not known if TGEV mRNAs are transcribed by a leader-primed mechanism similar to that of MHV. Immediately preceeding the E1-encoding and N-encoding regions of TGEV

(Purdue stain) virion RNA, AACTAAAC sequences have been found (47). Laude et al. assumed these consensus sequences to be the start of mRNA transcripts, but did not assign the sequences a function in gene transcription.

In vitro translation studies have provided proof of the messenger function of TGEV intracellular RNAs (35), and identification of translation products based on electrophoretic migration and recognition by TGEV-specific antibodies suggests that the virus possesses a mechanism of expression similar to that of coronaviruses MHV and IBV. This has not been confirmed by determination of the primary structure of TGEV subgenomic mRNAs.

Goals and Experimental Design

The goal of my research was to determine the primary structure of the genome and RNAs 6 and 7 of the pathogenic Miller strain of transmissible gastroenteritis virus (TGEV). I did this by cloning and sequencing DNA copies of these RNAs. I used the sequence data to compare the structure of the virion RNA to that of the two subgenomic RNAs. Sequence data was used to identify open reading frames and predict the amino acid sequences, glycosylation sites, and functions of potential gene products. From a comparison of these results to information obtained from studies of the attenuated Purdue strain of TGEV, I

endeavored to increase our understanding of the molecular basis of TGEV pathogenicity.

MATERIALS AND METHODS

Chemicals, Media, and Buffers

Reagent grade liquid organic chemicals were obtained from J. T. Baker Chemical Co. Other chemicals and reagents were obtained from Sigma Chemical Co. unless otherwise stated in the text. Radioisotopes were purchased from New England Nuclear Corp. Calf intestinal phosphatase, T4 DNA ligase, Escherichia coli DNA polymerase I, terminal transferase, and Klenow fragment were obtained from Bethesda Research Laboratories and Promega. Cell culture media were obtained from Irvine Scientific, and sera were purchased from Hyclone Laboratories.

Cell cultures were maintained in Dulbecco's Modified Eagle's (DME) medium supplemented with 10% (vol/vol) calf serum (DME-10). Infection of cells with TGEV was done in DME supplemented with 2% (vol/vol) fetal bovine serum, 10 mM 3-(N-morpholino)propanesulfonic acid (MOPS), 10 mM N-tris-(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES), and 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) (DME-2).

The composition and pH of buffers and reaction mixtures are shown in Table 2.

Table 2. Composition and pH of buffers and mixtures.

Buffer	Composition	pH
Annealing buffer	10 mM Tris-HCl, 2 mM EDTA, 150 mM NaCl	7.6
Chase solution	2 mM dCTP, 2 mM dATP, 2 mM dGTP, 2 mM dTTP	
Chloroform	96% chloroform (vol/vol), 4% isopentanol (vol/vol)	
CIP mix	100 mM glycine, 1 mM $MgCl_2$, 1 mM $ZnCl_2$, 1 unit/ul calf intestinal phosphatase (CIP)	10.5
Citrate-urea gel buffer	25 mM citric acid, 9 M urea	3.0
Citrate-urea sample buffer	10 mM citric acid, 6 M urea, 15% (wt/vol) sucrose, 0.005% (wt/vol) bromophenol blue	3.0
Hybridization buffer	50% (vol/vol) formamide, 5X SSPE, 0.4% (vol/vol) SDS, 200 ug/ml calf thymus DNA	
Klenow 10X buffer	100 mM Tris-HCl, 500 mM NaCl	7.5
Klenow d/ddATP mix	300 µM ddATP, 33 µM dCTP, 33 µM dTTP, 33 µM dGTP, 50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol (DTT)	7.5
Klenow d/ddCTP mix	66 µM ddCTP, 1.66 µM dCTP, 33 µM dTTP, 33 µM dGTP, 50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM DTT	7.5
Klenow d/ddGTP mix	66 µM ddGTP, 33 µM dCTP, 33 µM dTTP, 1.66 µM dGTP, 50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM DTT	7.5

Table 2, continued.

Buffer	Composition	pH
Klenow d/ddTTP mix	117 µM ddTTP, 33 µM dCTP, 1.66 µM dTTP, 33 µM dGTP, 50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM DTT	7.5
Ligation buffer	66 mM Tris-HCl, 5 mM $MgCl_2$, 5 mM DTT, 1 mM ATP, 4 units/ml T4 DNA ligase	7.5
NET	10 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA	7.6
Nick translation mix	50 mM Tris-HCl, 10 mM $MgSO_4$, 0.1 mM DTT, 500 ug/ml BSA, 1 nM dTTP, 1 nM dGTP, 1 nM dCTP, 2 nM [alpha-^{32}P]-deoxyadenosine 5'-triphosphate (NEG-012A), 0.1 ug/ml DNase I, 0.1 units/ul E. coli DNA pol I	7.2
NTE	50 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA	7.2
Oligo(dT) elution buffer	10 mM Tris-HCl, 1 mM EDTA, 0.05% (vol/vol) SDS	7.5
Oligo(dT) high-salt buffer	20 mM Tris-HCl, 500 mM NaCl, 1 mM EDTA	7.6
Oligo(dT) low-salt buffer	20 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA	7.6
Oligo(dT) washing buffer	100 mM NaOH, 5 mM EDTA	
Phenol	63% phenol (vol/vol), 37% (vol/vol) 50 mM TE (pH 8.0), 7 mM 8-hydroxy quinoline	
PNE	30 mM piperazine-N,N'-bis [2-ethane-sulfonic acid] (PIPES), 100 mM NaCl, 1 mM EDTA	6.0

Table 2, continued.

Buffer	Composition	pH
Reverse transcription mix	50 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM DTT, 4 mM Na pyrophosphate, 1.25 mM dGTP, 1.25 mM dCTP, 1.25 mM dATP, 1.25 mM dTTP, 0.5 units/ul RNasin, 3 units/ul reverse transcriptase (Life Sciences)	8.3
RIP buffer	50 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, 0.2% (vol/vol) NP40, 0.05% (vol/vol) SDS, 1% (vol/vol) Aprotinin, 0.02% (wt/vol) Na azide	7.4
RT 10X buffer	340 mM Tris-HCl, 500 mM NaCl, 60 mM $MgCl_2$, 50 mM DTT	8.3
RT d/ddATP mix	3.6 µM ddATP, 250 µM dCTP, 250 µM dTTP, 250 µM dGTP, 50 mM NaCl, 34 mM Tris-HCl, 6 mM $MgCl_2$, 5 mM DTT	8.3
RT d/ddCTP mix	100 µM ddCTP, 250 µM dCTP, 250 µM dTTP, 250 µM dGTP, 50 mM NaCl, 34 mM Tris-HCl, 6 mM $MgCl_2$, 5 mM DTT	8.3
RT d/ddGTP mix	50 µM ddGTP, 250 µM dCTP, 250 µM dTTP, 250 µM dGTP, 50 mM NaCl, 34 mM Tris-HCl, 6 mM $MgCl_2$, 5 mM DTT	8.3
RT d/ddTTP mix	200 uM ddTTP, 250 µM dCTP, 250 µM dTTP, 250 µM dGTP, 50 mM NaCl, 34 mM Tris-HCl, 6 mM $MgCl_2$, 5 mM DTT	8.3
SDS-PAGE sample buffer	120 mM Tris-PO_4, 1% (vol/vol) SDS, 40% (vol/vol) glycerol, 0.02% (wt/vol) phenol red	6.7

Table 2, continued.

Buffer	Composition	pH
Second-strand synthesis mix	20 mM Tris-HCl, 5 mM $MgCl_2$, 10 mM $(NH_4)_2SO_4$, 100 mM KCl, 50 ug/ml BSA, 40 uM dGTP, 40 uM dCTP, 40 uM dATP, 40 uM dTTP, 8.5 units/ml RNase H, 230 units/ml Klenow fragment of E. coli pol I	7.5
SSC	150 mM NaCl, 15 mM Na citrate	7.0
SSPE	50 mM $NaPO_4$, 900 mM NaCl, 5 mM EDTA	7.7
Stop solution	90% (vol/vol) formamide, 20 mM EDTA, 0.3% (wt/vol) bromophenol blue, 0.3% (wt/vol) xylene cyanol	
TAE	40 mM Tris-acetate, 2 mM EDTA	8.0
TBE	89 mM Tris-borate, 89 mM boric acid	8.3
10 mM TE	10 mM Tris base, 1 mM EDTA	8.0
50 mM TE	50 mM Tris base, 50 mM EDTA	8.0
Terminal transferase mix	200 mM K cacodylate, 2 mM $MnCl_2$, 1 uM DTT, 1 mM dCTP, 1 unit/ul terminal transferase	6.9

Virus Strains and Cell Lines

The virulent Miller strain of transmissible gastroenteritis virus (TGEV) was obtained from American Type Culture Collection (ATCC VR743-1W) in the form of porcine intestinal washings and cloned twice by plaque purification. Amplification of TGEV was carried out in a

continuous swine testicle (ST) cell line established by McClurkin and Norman (52) and obtained from Dr. David Brian. TGEV used in the experiments had been passed 8 to 10 times in ST cells after plaque purification.

Cloning and Sequencing Vectors

PstI-digested, oligo(dG)-tailed pBR322 used as the cDNA cloning vector in the following experiments was purchased from Bethesda Research Laboratories. Riboprobe Gemini sequencing vector plasmids were obtained from Promega.

Virus Stocks

ST cells were grown to 90% confluency in 10 cm plastic dishes (Nunc) and were infected with TGEV at a multiplicity of infection of 3 to 5 in DME-2. Following adsorption of the virus for one h, the inoculum was aspirated and replaced with 5 ml DME 2. The infected cells were incubated at 37^{o}C until approximately 75% of the cells had lysed. The plates were scraped and the medium was collected and freeze-thawed once at -70^{o}C prior to sonication for 120 s in a Heat Systems Sonicator (model W-225R) using a cup probe at 75% power. The lysates were clarified by centrifugation at 1200 x g for 5 min and stored at -70^{o}C.

Plaque Assay

TGEV stocks were titered by plaque assay on ST cell monolayers in plastic six-well dishes (Nunc). Monolayers were infected with 0.4 ml of serial 10-fold dilutions of virus in DME-2. After an adsorption period of one h at 37°C, the inoculum was removed and replaced with 3 ml of DME-2 containing 0.75% (wt/vol) agarose (type II, Sigma). Plaque assays were incubated at 37°C until plaques became visible. Cells were fixed by adding one ml 2% (vol/vol) glutaraldehyde to each well and incubating at room temperature for at least three hours. The agarose was removed, the plates were allowed to dry, and plaques were counted. Titers were recorded as plaque-forming units per ml (PFU/ml).

Organic Extraction and Recovery of Nucleic Acids

Nucleic acids were purified by extracting twice with one volume phenol and one volume chloroform and once with one volume chloroform. Phenol as referred to in this text is equivalent to 63% (vol/vol) phenol, 37% (vol/vol) 50 mM TE (pH 8.0), 7 mM 8-hydroxy quinoline. Chloroform as referred to in this text is equivalent to 96% (vol/vol) chloroform, 4% (vol/vol) isopentanol. Aqueous and organic phases were separated by centrifugation at 4,000 x g for 5 min at room temperature.

DNA was precipitated from the aqueous phase by addition of one-fifth volume saturated ammonium acetate and two volumes 95% (vol/vol) ethanol. RNAs were precipitated by addition of one-fifth volume 5 M NaCl and 2.5 volumes 95% ethanol. RNA precipitations were incubated for at least one h at -20°C. Nucleic acids were pelleted by centrifugation at 16,300 x g for 30 min at 4°C. Pelleted material was washed with 70% (vol/vol) ethanol and dried under vacuum.

Virion RNA Production

ST cell monolayers in 10 cm plastic dishes were infected at an MOI of 3 to 5 with TGEV in DME-2. After an adsorption period of 1 h at 37°C, an additional 3 ml DME-2 was added to each dish. The plates were incubated at 37°C. To 3 of 10 dishes, 100 mCi [5,6-^{3}H]-uridine (NET-367) was added at 5 h post-infection. Alternatively, [^{32}P]-labeled virion RNA was produced by adding 375 µCi [^{32}P]-phosphoric acid (NEX-053) to each dish. When 75% of the cells had lysed, the plates were scraped with a rubber policeman and the cell lysate was collected. Following one freeze-thaw cycle at -70°C, the medium was sonicated for 120 s and clarified as described above. Virions were precipitated by the addition of 5.9 g NaCl and 50 ml 50% (wt/vol) polyethylene glycol (PEG) (mol. wt. 3350, Sigma cat. no. P3640) per 200 ml clarified lysate to give a final

concentration of 10% PEG and 2.4% NaCl (90). Following a 2 h incubation of the mixture at 4°C, the precipitate was collected by centrifugation at 10,400 x g for 45 min at 4°C. Pellets were dissolved in PNE buffer and transferred to 8.8 cm x 1.5 cm polyallomer centrifuge tubes (Seton Scientific). The suspensions were underlaid with 3.5 ml 10% (wt/vol) potassium tartrate (KT) in PNE buffer and 2 ml 33% (wt/vol) KT in PNE buffer. Centrifugation of the discontinuous gradients was carried out in a Beckman SW41Ti rotor at 160,000 x g for 150 min at 4°C. Virus particles were collected from the interface between the 10% KT and 33% KT in PNE buffer pads. The virus suspension was diluted ten-fold with PNE buffer and virions were pelleted by ultracentrifugation at 160,000 x g for 75 min at 4°C. Pellets were drained and dissolved in 2 ml NET buffer supplemented with 100 µl 200 mM vanadyl ribonucleoside complexes (Bethesda Research Laboratories), 10 µl 20 mg/ml proteinase K, 200 µl 10% (wt/vol) sodium dodecyl sulfate (SDS), 160 µl 5 M NaCl, and 100 µl 200 mM EDTA. Following a 30 min incubation at 37°C the RNA was phenol-chloroform extracted. RNA pellets were resuspended in 200 µl NET buffer. The material was loaded onto 10-30% continuous sucrose gradients in NET buffer supplemented with 0.2% (vol/vol) SDS. Following ultracentrifugation of the gradients at 160,000 x g for 3 h at 20°C, 400 µl fractions were collected with a peristaltic pump from the bottom of

the gradients. Twenty µl aliquots from each fraction were transferred to Whatman GFC glass fiber filters. The filters were dried at room temperature and placed in scintillation vials. Three ml scintillation fluid [5 g 2,5-diphenyloxazole (PPO) per liter xylene] was added to each vial and the samples were counted in a Packard LSC 460CD liquid scintillation counter. Gradient profiles were plotted using the count data and from these plots the fractions containing TGEV genome-length RNA were identified. RNA was recovered from these fractions by ethanol precipitation. RNA pellets were resuspended in NET buffer and the purity and concentration of the RNA was determined by measuring the absorbance of the suspensions at wavelengths of 260 and 280 nm in a Gilford 2600 spectrophotometer. RNA was reprecipitated and stored at -70°C.

Isolation of Polyadenylated Intracellular RNAs

Monolayers of ST cells in 10 cm plastic dishes were infected at an MOI of 5 with TGEV in DME-2. After adsorption for one h at 37°C the inoculum was aspirated and replaced with 5 ml DME-2 per dish. At six h post-infection the DME-2 was aspirated and replaced with 3 ml fresh DME-2 containing 2.5 ug/ml actinomycin D. Three of 10 dishes received 100 µCi [5,6-^{3}H]-uridine (NET-367). The incubation was continued at 37°C to 11 h post-infection.

The medium was then aspirated and one ml ice cold NTE buffer containing 0.5% (vol/vol) Nonidet P40 (NP40) (Particle Data Laboratories Ltd.) was added to each dish. Dishes were held on ice for 5 min and the cell lysates were collected. Dishes were rinsed with an additional one ml volume of NTE buffer and the rinses were pooled with the lysates. Following clarification, the lysates were treated with proteinase K, precipitated, and recovered as described above.

Polyadenylated RNA was isolated by oligodeoxythymidylic acid (oligo (dT)) cellulose chromatography. Columns consisting of approximately 2 ml oligo(dT) cellulose (type 2, Collaborative Research, Inc.) in a pasteur pipet were washed with 3 volumes of distilled water, 3 volumes oligo(dT) washing buffer, and additional distilled water as needed to bring the pH of the effluent below 8.0. RNA was resuspended in 400 µl distilled water and denatured by heating at 65^{o}C for 5 min. An equal volume of 2X high-salt oligo(dT) loading buffer was added and the suspension was passed through the column. The eluate was collected, heated at 65^{o}C for 5 min, and reapplied to the column. The column was washed with 8 ml high-salt loading buffer and 4 ml low-salt loading buffer. Polyadenylated RNA was eluted from the column by addition of 3 ml elution buffer. Eluted RNA was recovered as described above.

Urea-Agarose Gel Electrophoresis

TGEV subgenomic RNAs were purified by urea-agarose gel electrophoresis as described by Rosen et al. (63) with minor modifications. Three-fold concentrated agarose (2.1 g agarose in 70 ml distilled water) was autoclaved and added to 140 ml citrate-urea buffer and horizontal gels (8.8 cm x 25.4 cm) were poured in a Studier apparatus (85) (10 cm x 39.5 cm) in a refrigerated room. RNA was resuspended in citrate-urea sample buffer, held at room temperature for 5 min, and loaded onto the urea-agarose gels. Ribosomal RNAs from ST cells labeled with [5,6-^{3}H]-uridine (NET-367) were used as molecular weight markers. Electrophoresis was carried out at 50 volts for 16 h at room temperature. Lanes were then sliced at 0.4 cm intervals, and slices were placed in scintillation vials. Three ml aqueous scintillation fluid [33% (vol/vol) Triton X-100 (Research Products International Corp.), 66.5% (vol/vol) xylene, 0.5% (wt/vol) PPO] was added to each vial and the samples were counted using the preset tritium channel. Count data were used to plot gel profiles of lanes containing TGEV-ST RNA or ST ribosomal RNA. Migration distances of the rRNAs were used to estimate the location of TGEV subgenomic mRNAs. Gel slices from parallel lanes predicted to contain TGEV mRNAs of interest were suspended in NET buffer and melted by heating at 65^{o}C

for 5 min. Agarose slurries were extracted three times with chloroform and RNA was recovered from the aqueous fractions as described above.

In vitro Translation of Gel-Purified mRNAs

The identities of RNAs extracted from urea-agarose were confirmed by *in vitro* translation. RNA (1 to 2 µg) in 8 µl distilled water was added to a microcentrifuge tube containing 35 µl rabbit reticulocyte lysate (Promega), 1 µl methionine-free amino acid mixture (Promega), 5 µl (50 uCi) L-[^{35}S]-methionine (NEG-009A), and 1 µl (30 U) RNasin (Promega). Following incubation at 30°C for 2 h the reactions were terminated by freezing at -70°C.

Immunoprecipitation of Virus-Specific Proteins

Products of *in vitro* translation were immunoprecipitated with TGEV-specific polyclonal ascitic fluid using a modification of previously described procedures (8,67). Fifty µl of translation mixture was diluted eleven-fold in RIP buffer. Fifty µl of hyperimmune ascitic fluid was added and the mixtures were incubated for 1 h at 0°C. Immune complexes were precipitated with 50 µl 10% (vol/vol) heat- and formalin-fixed *Staphylococcus aureus* (Cowan) cells (39) by incubation at 0°C for 1 h and pelleted by centrifugation at 6,500 x g for 20 s. Pellets were washed 5 times with ice cold RIP buffer. Bound

proteins were eluted with 20 µl 1% (wt/vol) SDS, 0.02 M dithiothreitol (DTT) for 15 min at room temperature and 5 min at 60°C. S. aureus cells were removed by centrifugation at 6,500 x g for 20 s. The supernatant fluids were mixed with 20 µl SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer and stored at -20°C.

SDS-Polyacrylamide Gel Electrophoresis

Immunoprecipitated proteins were analyzed by the Laemmli method of PAGE (42). Proteins were denatured prior to electrophoresis by heating for 5 min at 65°C. Electrophoresis was in 10% (wt/vol) polyacrylamide slab gels. Gels were fixed in 10% (vol/vol) trichloroacetic acid and proteins were stained with Coomassie brilliant blue G (18). To aid in detection of labeled proteins, gels were enhanced for 30 min in Fluoro-Hance (Research Products International Corp.) according to the manufacturer's directions. Gels were dried onto Whatman 3MM paper and exposed to preflashed Kodak XAR-2 x-ray film.

The molecular weights of translation products were determined from their distance of migration in the gels relative to those of standard proteins of known molecular weight. GEL, a computer program obtained from Dr. Brian Fristensky and based on the work of Schaffer and Sederoff (68), was used in this analysis. The standard proteins were bovine serum albumin (BSA) (66 kd), ovalbumin (45 kd),

ß-glyceraldehyde-3-phosphate dehydrogenase (36 kd), carbonic anhydrase (29 kd), and trypsin inhibitor (20.1 kd).

Complementary DNA Synthesis

Synthesis of first-strand cDNA was carried out according to the protocol of Gubler and Hoffman (25). Three µg virion or subgenomic RNA and 4 µg oligo(dT) or one to 3 µg priming cDNA restriction fragment were added to a reaction mixture containing 40 ul reverse transcription mix and incubated for 60 min at 42°C. Reactions were terminated by addition of 100 µl 50 mM TE buffer. Reaction products were phenol-chloroform extracted and ethanol precipitated.

First-strand cDNA reverse transcribed from virion RNA was rendered double-stranded in a reaction mixture containing 100 µl of second strand synthesis mix and incubated at 12°C for 60 min and 22°C for 60 min. To stop the reaction, EDTA was added to a final concentration of 20 mM. Products were phenol-chloroform extracted and recovered by ethanol precipitation. First-strand cDNA copies of TGEV subgenomic RNAs were not subjected to second strand synthesis (47).

Oligodeoxycytidylate tails were added to double-stranded cDNA or cDNA:RNA hybrids in 50 µl of terminal transferase mix as described by Michelson and Orkin (54).

Reaction mixtures were incubated at 30°C for 10 min. Homopolymeric tail addition was terminated by addition of 2.5 µl 200 mM EDTA and 100 µl 10 mM TE. Tailed molecules were recovered by ethanol precipitation.

Oligo(dC)-tailed double-stranded cDNA or cDNA:RNA hybrids were annealed to PstI-digested, oligo(dG)-tailed pBR322. A vector:cDNA molar ratio of approximately 50:1 was used. Annealing was done in 50 µl annealing buffer incubated at 65°C for 15 min and 58°C for 90 min. E. coli (strain JM109 or DH5) cells rendered competent by the method of Hanahan (27) were transformed with the recombinant molecules and plated on Luria agar containing 10 µg/ml tetracycline. Colonies that developed during a 24 h incubation at 37°C were patched on Luria agar supplemented with tetracycline (10 µg/ml) or ampicillin (100 µg/ml).

Isolates resistant to tetracycline but susceptible to ampicillin were subjected to small-scale plasmid analysis (4), as follows. Isolates were inoculated into 12 ml Luria broth containing 10 µg/ml tetracycline. Cultures were incubated at 37°C with agitation for approximately 16 h and chilled in an ice bath. Cells were pelleted at 3,000 x g for 5 min at 4°C and resuspended in 650 µl 10 mM TE buffer. The cell suspensions were transferred to 1.5 ml microcentrifuge tubes (Treff Lab). Cells were pelleted at 15,600 x g for 15 s and resuspended in 120 µl ice cold 20%

sucrose (wt/vol) in 50 mM TE buffer. The mixtures were freeze-thawed once and 20 µl of 10 mg/ml lysozyme in 50 mM TE buffer was added. Following incubation at 0°C for 30 to 60 min, 400 µl 1% (wt/vol) SDS, 0.2 N NaOH was added and the tubes were incubated at 50°C for 60 min and 0°C for 10 min. The mixtures were neutralized by addition of 200 µl 3 M potassium acetate (pH 4.8); incubation at 0°C was continued for an additional 40 min. Debris was cleared from the preparations by centrifugation at 15,600 x g for 15 s. An equal volume of isopropanol was added to the supernatant fluids, the tubes were held at room temperature for 30 min, and plasmid DNA was pelleted by centrifugation at 15,600 x g for 10 min. Pellets were washed with 70% ethanol, dried under vacuum, and resuspended in 200 µl distilled water. Ten µl of this volume were run in horizontal gels consisting of 0.8% agarose (wt/vol) in TAE buffer. The lengths of cDNA inserts were predicted upon comparison of migration of the recombinant plasmids to that of unrestricted vector.

Restriction maps of these inserts were constructed as follows. Recombinant plasmids as large as vector dimers were phenol-chloroform extracted and recovered by ethanol precipitation. The plasmids were digested with the restriction endonucleases HindIII, KpnI, PstI, PvuII, XbaI, and XhoI (Boehringer Mannheim) and subjected to electrophoresis in 0.8% agarose in TAE. Bacteriophage

lambda DNA digested with restriction endonuclease HindIII was used as standards in these gels. The lengths of the lambda DNA restriction fragments were 23,130 bp, 9416 bp, 6682 bp, 4373 bp, 2322 bp, 2027 bp, and 564 bp. The lengths of cDNA restriction fragments were determined using the computer program GEL (68). Restriction maps were constructed by inspection.

Hybridization Analysis of cDNA Clones

Cloned cDNAs were screened for TGEV-specific sequences by colony (24) or slot blot hybridization. In colony hybridization studies, discs of Zeta-Probe nylon membrane (Bio-Rad) were placed on Luria agar supplemented with 10 µg/ml tetracycline. Bacteria from tetracycline-resistant, ampicillin-susceptible colonies were patched onto the discs with sterile toothpicks. The plates were incubated at 37°C for 8 h and bacterial cells on the filters were lysed by placing the membranes on Whatman 3MM paper saturated with 0.5 N NaOH for 5 min. Filters were neutralized on 3MM paper saturated with 1 M Tris-HCl (pH 8.0) for 5 min and were then incubated on 3MM paper saturated with 1 M Tris-HCl (pH 8.0), 1.5 M NaCl for 5 min, washed in 2X SSC and baked under vacuum at 80°C for 90 min.

A Hybri-Slot filtration manifold (Bethesda Research Laboratories) was used to carry out slot blot hybridizations. Plasmid DNA obtained from small-scale

plasmid isolations was denatured in 0.4 N NaOH at 70°C for one h and passed through the manifold onto nylon membranes that had been pre-wet with distilled water and 0.4 N NaOH. Membranes carrying plasmid DNA were dried at room temperature.

DNA copies of TGEV (Miller strain) RNA 6 and RNA 7 were characterized by the Southern blot procedure (77). Restricted plasmids were fractionated in 0.8% (wt/vol) agarose in TAE gels. Gels were run at 30 volts for 4 h. Following staining with ethidium bromide the gels were photographed using Polaroid Type 55 Land film. DNA was transferred to Zeta-Probe nylon membranes (Bio-Rad) by the method of Reed and Mann (61). Following DNA transfer, the membranes were baked at 80°C for one h under vacuum.

Colony and slot blots were probed with [^{32}P]-labeled TGEV virion RNA prepared as described above. Nylon membranes were prehybridized in 5 ml per filter of hybridization buffer at 42°C for one h. The radiolabeled RNA was denatured in 0.1 N NaOH at 70°C for 5 min and added to the prehybridization solution. Hybridization was carried out at 42°C for 15 h. Filters were washed in 2X SSPE, 0.1% (vol/vol) SDS at 68°C for one h and 1X SSPE, 0.1% SDS at 68°C for one h prior to exposure to x-ray film.

Southern blots were probed with labeled restriction fragments of virion RNA-specific cDNA. The fragments were separated and recovered as previously described and were

radiolabeled by nick translation (51) in a reaction mixture containing 50 µl nick translation mix and incubated at 16°C for one h. The reaction was terminated by addition of 2 µl 0.5 M EDTA and the nick-translated DNA was separated from unincorporated dNTPs by chromatography through a column containing 2 ml Sephadex G-50 in 10 mM TE. Radiolabeled DNA was recovered by ethanol precipitation. The nylon membranes were prehybridized and radiolabeled DNA fragments denatured in 0.1 N NaOH were added to the buffer. Hybridization conditions and washes were as described above.

Subcloning of cDNA Restriction Fragments

Selected cDNA restriction fragments were subcloned into pGEM sequencing vectors. The sequencing strategy was planned such that restriction fragments were overlapping. The sequence of regions not overlapped by other fragments was determined using at least two clones. Recombinant plasmids were restricted and loaded onto 0.8% low-gelling temperature agarose in TAE buffer gels. Gels were run at 40 volts for 2 h, stained with ethidium bromide, and viewed using an ultraviolet transilluminator. Gel slices containing cDNA fragments were excised, suspended in 400 µl NET, and melted at 65°C for 5 min. Agarose was removed by extracting the suspensions three times with phenol/chloroform and once with chloroform. Restriction fragments were

recovered by ethanol precipitation.

Sequencing vector plasmids were restricted, gel purified as described above, and dephosphorylated with calf intestinal phosphatase (CIP), as follows. Plasmid DNA was resuspended in 50 µl CIP mix. The reactions were incubated at 37°C for 15 min and 56°C for 15 min. An additional 50 units CIP were added and the incubations were repeated. The CIP was then inactivated by addition of 1 µl 10% (vol/vol) diethylpyrocarbonate and incubation at 68°C for 10 min. The dephosphorylated vectors were phenol-chloroform extracted and precipitated with ethanol.

Complementary DNA restriction fragments were ligated to dephosphorylated sequencing vectors in 25 µl ligation mix. Ligation mixtures were incubated overnight at 18°C. Competent E. coli (strain JM109) cells were transformed with the products and plated on Luria agar containing 100 µg/ml ampicillin. Plasmids of ampicillin-resistant transformants were characterized by small-scale plasmid analysis.

Sequencing of TGEV-Specific cDNA

The GemSeq K/RT Sequencing System (Promega), which employs the dideoxy method of chain termination (66), was used to determine the nucleotide sequence of subcloned restriction fragments. One to 2 µg plasmid DNA was resuspended in 20 µl of distilled water in a 1.5 ml

microcentrifuge tube. Two µl 2 M NaOH, 2 mM EDTA was added and the tubes were incubated for 5 min at room temperature. Reactions were neutralized by addition of 3 µl 3 M sodium acetate (pH 5.0) and 7 µl distilled water. Denatured DNA was precipitated by addition of 75 µl absolute ethanol and incubated at -20°C for 15 min. DNA was pelleted by centrifugation at 15,600 x g for 10 min. The pellets were washed with 70% ethanol and dried under vacuum. Dried pellets were resuspended in 6 µl distilled water, 1 µl 10X reverse transcriptase (RT) or Klenow buffer, and 3 µl 10 ng/ml SP6 or T7 promoter/primer. Annealing mixtures were incubated at 37°C for 90 min. Five units of Klenow fragment or avian myeloblastosis virus RT and 5 µl [^{35}S]-deoxyadenosine 5'-[alpha-thio]triphosphate (500 Ci/mmol) (NEG-034S) were added and 3 µl of this radiolabel/primer/template mixture was added to 1.5 ml microcentrifuge tubes containing 3 µl deoxy/dideoxy (d/dd) CTP, d/ddATP, d/ddGTP, or d/ddTTP. The reactions were incubated at 37°C (Klenow) or 42°C (AMV RT) for 20 min. One µl chase solution was added and the incubation was continued for an additional 20 min. Alternatively, primers were extended for 10 to 30 min in the absence of dideoxy nucleotides, followed by addition of 3 µl d/ddNTPs and incubation at 37°C (Klenow) or 42°C (AMV RT) for 20 min. The concentration of dNTPs in the extension mixture was 50 µM. All reactions were terminated by addition of 5 µl stop solution and stored at -70°C.

Reaction mixtures were electrophoresed on 32 cm X 38 cm 8 M urea, 6 or 8% polyacrylamide sequencing gels (15 or 20 ml acrylamide:N,N'-methylene-bis-acrylamide::39:1, respectively, 10 ml 10X TBE buffer, 30 ml distilled water, 50 g urea, 1 ml 10% (w/v) ammonium persulfate, and 20 µl N,N,N',N'-tetramethylethylenediamine) in TBE buffer at 1100 volts. To maximize the amount of sequence information obtained from a single sequencing reaction, some samples were loaded into adjacent wells when the bromphenol blue marker dye of the first (double-loading) or second (triple-loading) load had run off the gel. Gels were fixed in 10% (vol/vol) methanol, 10% (vol/vol) acetic acid for 15 min and dried onto Whatman 3MM paper. Kodak XAR-2 film was exposed to the gels for 120 to 168 h prior to development according to the manufacturer's instructions. Sequencing data were analyzed on an IBM personal computer using the programs of Mount et al. (55) and Fristensky et al. (20). The hydrophilicity plots were constructed with a computer program written by Dr. James Etchison that incorporated the algorithm of Hopp and Woods (33).

RESULTS

Production of TGEV Virion RNA

Full-length TGEV virion RNA was purified for use as a template in cDNA synthesis primed by a cDNA restriction fragment representative of TGEV (Miller strain) genomic RNA. ST cells were infected with TGEV, and the infections were allowed to proceed until 75% of the cells had lysed. Virions recovered from the cell lysate by PEG precipitation were purified by centrifugation to equilibrium in a discontinuous KT gradient. Coronavirions have a density in KT of 1.18 g/ml. A narrow band of virus particles formed at the interface between the 10% KT (density = 1.07) and 33% KT (density = 1.22) pads. Virus particles were recovered from the interface and the genomic RNA was partially purified by phenol-chloroform extraction. Virion RNA was purified from the extracted material by sedimentation velocity centrifugation in an isokinetic sucrose gradient. The results obtained are shown in Figure 1. Virion RNA sedimented in the lower portion of the gradient. The material sedimenting near the top of the gradient consisted of nonspecific RNAs. The yield of TGEV virion RNA, as determined by spectrophotometry, was