

Genetic Basis for the Pathogenesis of Transmissible Gastroenteritis Virus

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Intracellular RNAs of an avirulent small-plaque (SP) transmissible gastroenteritis virus variant and the parent virulent Miller strain of transmissible gastroenteritis virus were compared. Northern RNA blotting showed that the Miller strain contained eight intracellular RNA species. RNAs 1, 2(S), 5, 6(M), 7(N), and 8 were similar in size for both viruses; however, the SP variant lacked subgenomic RNAs 3 and 4. Instead, the SP virus contained an altered RNA species ($\Delta 4$) that was slightly smaller than RNA 4. S1 nuclease protection experiments showed a deletion of approximately 450 nucleotides in the SP genome downstream of the peplomer S gene. Sequencing of cDNA clones confirmed that SP virus contained a 462-nucleotide deletion, eliminating the transcriptional recognition sequences for both RNAs 3 and 4. These RNAs encode open reading frames A and B, respectively. An alternative consensus recognition sequence was not readily apparent for the $\Delta 4$ RNA species of SP virus. Since open reading frame A is missing in SP virus, it is not essential for a productive infection. The status of the potential protein encoded by open reading frame B is not clear, because it may be missing or just truncated. Nevertheless, these genes appear to be the contributing entities for transmissible gastroenteritis virus virulence, SP morphology, tissue tropism, and/or persistence in swine leukocytes.

Transmissible gastroenteritis virus (TGEV) belongs to the family *Coronaviridae*. The *Coronaviridae* are pleomorphic enveloped viruses that contain a large, positive-sense, single-stranded RNA genome (18). During coronavirus replication, a full-length negative-sense template RNA is first synthesized from the viral genome. This negative-strand RNA serves as a template for transcription of viral genomic and subgenomic RNAs. The subgenomic mRNAs are a nested set sharing common 3' poly(A)⁺ termini and extending for different lengths in the 5' direction (19). The 5' end of each subgenomic mRNA contains a short 60- to 72-nucleotide (nt) RNA leader sequence that primes transcription and is complementary to the 3' end of the negative-strand template RNA (12). This leader RNA-primed mechanism of transcription (11) has been extensively studied in two other coronaviruses, mouse hepatitis virus and infectious bronchitis virus. The leader RNA and the body sequences of each subgenomic mRNA are joined during transcription. The fully dissociated leader RNA binds to an intergenic recognition sequence and serves as a primer for the transcription of subgenomic mRNAs, and only the 5'-most open reading frame (ORF), which is not present in the smaller subgenomic mRNAs, is translated. However, a recent report suggested that negative-sense subgenomic RNAs also could serve as templates for the synthesis of TGEV subgenomic mRNAs (17). Whether this occurs with mouse hepatitis virus is not clear, since apparently contradictory results have been obtained (13, 16).

At least 8 intracellular mRNAs are synthesized during a TGEV productive infection (17, 20). Although each mRNA except mRNA 8 is polycistronic in coding capacity, apparently only the unique region of each mRNA is translationally active; thus, each mRNA gives rise to only a single polypeptide. The translation products from mRNAs are as follows: from the full-length mRNA, a polyprotein yielding one or

two polymerases; from mRNA 2(S), the peplomer or spike protein (S); from mRNA 3, a 7.9-kilodalton protein; from mRNA 4, a 27.7-kilodalton protein; from mRNA 5, a 9.3-kilodalton protein; from mRNAs 6(M), 7(N), and 8, integral membrane, nucleocapsid, and 14-kilodalton polypeptides, respectively (Fig. 1). Currently, it is not clear whether the full-length mRNA that encodes the polymerase gene(s) is identical to the genomic RNA that is encapsidated in the virion. In this paper, we have adopted the nomenclature for subgenomic RNAs suggested by the coronavirus study group in 1989. Thus, the previously identified mRNAs 4a and 4b of the Miller PP3 strain have been renamed mRNAs 3 and 4, respectively (20).

TGEV causes a fatal diarrheal disease in piglets infected during the first few weeks of life. In a susceptible herd, the mortality rate of nursing piglets can approach 100%. One of the virulent TGEV isolates, the Miller strain, was shown to be consistently lethal when 10^3 PFU were administered to suckling pigs (21). An attenuated, small-plaque (SP) mutant derived from the Miller strain caused a persistent infection in a porcine leukocyte cell line but was cytolytic for a swine testicular (ST) cell line, generating titers of about 10^6 PFU/ml (23). Three-day-old susceptible baby pigs vaccinated orally and intranasally with 2×10^8 PFU of the SP mutant showed no clinical signs typical of transmissible gastroenteritis. The SP mutant replicates in cells of the lamina propria rather than the absorptive epithelial cells of the small intestine (24). Vaccination of pregnant swine by the oral-intranasal and/or the intramammary routes resulted in solid lactogenic immunity. Mortality of suckling piglets, which approaches 100% when piglets are challenged with virulent TGEV, was reduced to 14 to 34% when these piglets were nursing vaccinated dams (23, 25). In this report, we compared the genomic make-up and mRNA expression of the avirulent SP virus and the virulent plaque-purified Miller virus (PP3) in an attempt to understand the unique properties exhibited by the SP variant.

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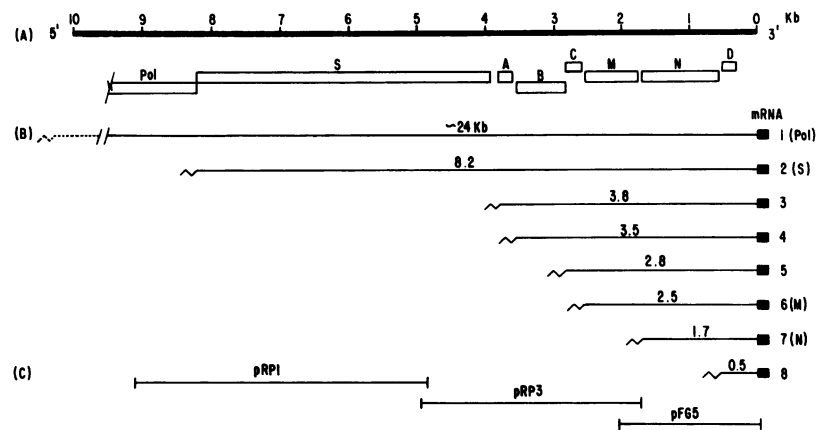


FIG. 1. Schematic diagram of the 3'-coterminal nested-set arrangement of TGEV intracellular RNAs. (A) Organization of known genes and ORFs at the 3' end of the TGEV genome. The major viral structural proteins are the spike or peplomer glycoprotein (S), the integral membrane glycoprotein (M), and the nucleocapsid protein (N). A potential minor structural protein is encoded by ORF C (19, 20). The gene products of ORFs A, B, and D are putative nonstructural proteins. (B) Transcriptional map of intracellular mRNAs 1 to 8 that share a common 3' end. The filled boxes indicate polyadenylation of each RNA at the 3' end, and the squiggle at the 5' end represents the leader sequence that primes transcription. The first ORF at the 5' end of each TGEV RNA, which is not present in the next smaller RNA species, encodes the unique translation product of that mRNA. (C) Relative positions of plasmids pRP1, pRP3, and pFG5.

MATERIALS AND METHODS

Virus and cell culture. The Miller virus stock, the sixth passage in piglets, was maintained under liquid nitrogen as an intestinal homogenate. The PP3 virus was derived from the Miller virus stock after three rounds of plaque purification on ST cells.

The SP TGEV variant originated from a persistently infected swine leukocyte cell culture infected with the virulent Miller TGEV after the third passage in piglets. This virus gives a SP size when plated on ST cells (23).

The persistently infected leukocyte cell line (22) and the ST cell line were grown in a modified Eagle minimal essential medium supplemented with bovine fetal serum (10%), lactalbumin hydrolysate (0.25%), sodium bicarbonate (0.22%), sodium pyruvate (0.01%), and gentamicin sulfate (50 μ g/ml).

Preparation of RNAs. Whole-cell total RNAs were prepared from infected ST cells or from persistently infected swine leukocytes by the guanidinium isothiocyanate method (5) and the CsCl cushion centrifugation method (6). Poly(A)⁺ RNAs were selected by oligo(dT)-cellulose chromatography (1).

Plasmids. Plasmids pRP1 (4,256 base pairs) and pRP3

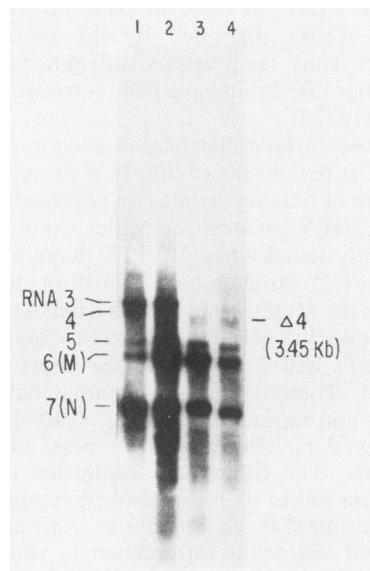


FIG. 2. Northern blot of intracellular oligo(dT)-selected RNA from TGEV-infected cells. Lanes: 1, Miller TGEV poly(A)⁺ RNA; 2, PP3 poly(A)⁺ RNA; 3, SP poly(A)⁺ RNA from infected ST cells; 4, SP poly(A)⁺ RNA from a persistently infected swine leukocyte cell line. The blot was probed with nick-translated FG5 from the 3' end of the TGEV genome. The prototype Miller virus RNAs are indicated on the left, and the altered RNA species ($\Delta 4$) is indicated on the right.

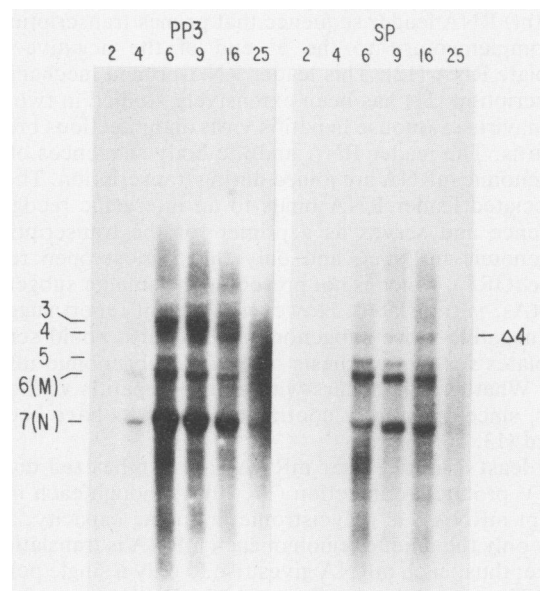


FIG. 3. Northern blot analysis of total intracellular RNAs of PP3- and SP-infected ST cells at various times p.i. The RNAs used at different times p.i. (in hours) are shown above the lanes. The RNA species are indicated in the margin. Nick-translated pFG5 was used as the probe.

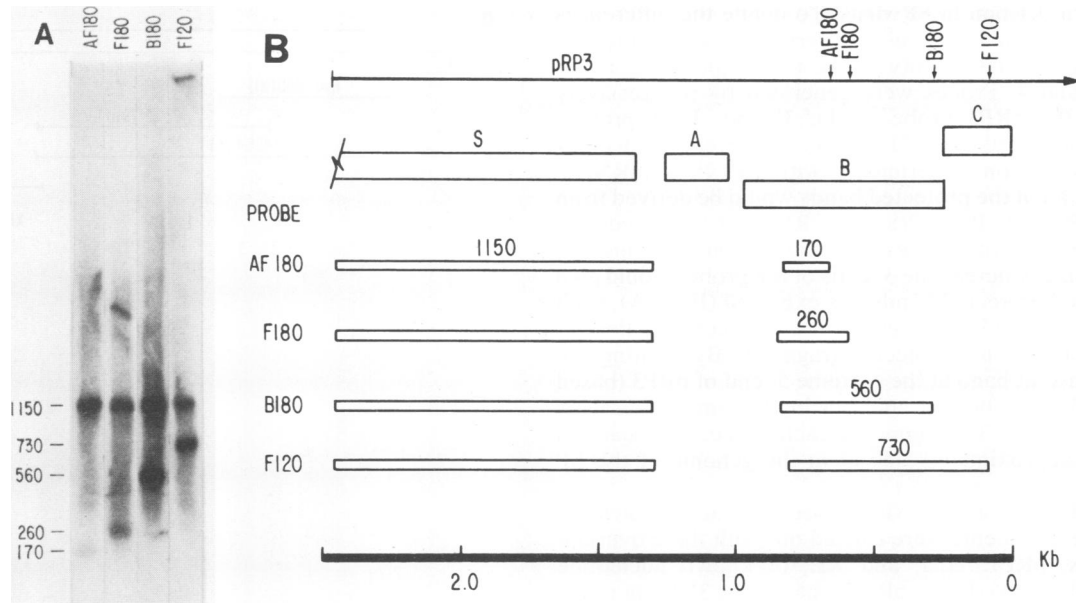


FIG. 4. (A) Localization of the deletion in the SP virus genome. Four subclones, AF180, F180, B180, and F120, of different sizes were constructed by unidirectionally shortening the plasmid RP3 at the 3' end. 32 P-labeled runoff transcripts were prepared for each subclone, hybridized to oligo(dT)-selected SP RNA, S1 nuclease digested, and analyzed by agarose gel electrophoresis. One constant and one variably sized protected fragment were derived for each subclone. The size of each protected fragment is indicated in the margin. (B) Schematic diagram of the results of the S1 nuclease protection experiment. The ORFs, the 5' end of pRP3, and the sizes of the unidirectional subclones AF180, F180, B180, and F120 are shown. By positioning the 1,150-nt constant band at the extreme 5' end of pRP3 and placing the smaller variable bands at the 3' terminus of each subclone, a deletion of approximately 450 nt is revealed.

(3,232 base pairs) were cDNA clones of the Miller PP3 isolate of TGEV cloned into the *Eco*RI site of the pBlue-script phagemid (Stratagene) and overlap each other by 72 bases (Fig. 1C). Plasmid pFG5 was derived from the Purdue strain of TGEV. Plasmids F120, B180, F180, and AF180 are subclones of pRP3 (see Fig. 4B); and plasmids Hpa and XE are subclones of pRPI (see Fig. 5A).

Time course experiment. Parallel cultures of confluent ST cells in 75-cm² flasks (Corning Glass Works) were infected with the PP3 virus or with SP virus at a multiplicity of infection of approximately 1. At selected times postinfection (p.i.), the medium was removed, the cells were rinsed with cold PBS (Ca²⁺ and Mg²⁺ free), and a guanidinium isothiocyanate solution was added for total cellular RNA preparation.

RNA analysis. Northern blot and S1 nuclease protection analyses were carried out as previously described (4). RNA concentrations were determined spectrophotometrically. In all cases, equivalent amounts of RNA of each sample were used in the same experiment.

cDNA cloning and sequencing. cDNA was prepared from SP genomic RNA by using the oligonucleotide 5'-CATAG CACAATAGCG-3', which is complementary to the 5' end of the integral membrane M gene, as a specific primer and cloned into the *Eco*RI site of the pBluescript phagemid vector. Nucleotide sequencing was carried out using the dideoxy-chain termination method (15) on double-stranded plasmids (7). Unidirectional deletions were constructed at either end of the cDNA insert by the exonuclease III-S1 nuclease method (8).

RESULTS

Subgenomic RNA pattern. Four preparations of oligo(dT)-selected TGEV-infected cell RNAs were examined by

Northern RNA blot analysis. These RNA samples were generated from (i) parent Miller virus-infected ST cells at 9 h p.i., (ii) PP3 virus-infected ST cells at 9 h p.i., (iii) a 5-day-old persistent leukocyte culture of SP virus, and (iv) SP virus-infected ST cells at 9 h p.i. Results of a Northern blot after hybridization with a nick-translated probe, FG5, derived from the 3' end of the genome is shown in Fig. 2. The Miller virus poly(A)⁺ RNA patterns in ST cells before and after plaque purification are identical. Likewise, the poly(A)⁺ RNA patterns of the SP virus in leukocytes or ST cells are indistinguishable. For Miller virus, the presence of subgenomic RNAs 3, 4, 5, 6(M), and 7(N) were evident; subgenomic mRNAs 1, 2(S), and 8 could be detected upon longer exposure (see Fig. 5C). For SP virus, RNAs 5, 6(M), and 7(N) were readily located; whereas RNAs 3 and 4 were no longer detectable. Instead, a 3.45-kilobase RNA species ($\Delta 4$) slightly smaller than RNA 4 became noticeable.

Viral RNA pattern during a productive infection. To further illustrate the genetic basis underlying the difference in RNA patterns between the PP3 and SP viruses, parallel ST-cell cultures were infected with each virus. At the indicated times p.i., total RNAs were isolated and examined by Northern blot analysis with nick-translated FG5 probe. Viral RNAs could be detected by 4 h p.i. in both the PP3 virus- and SP virus-infected cell cultures (Fig. 3). From 6 to 16 h p.i., the RNA pattern generated by both viruses remained at approximately the same level; at 25 h p.i., both RNA patterns had degenerated. The ratio of RNA 6(M) versus RNA 7(N) derived from the PP3 virus was less than that derived from the SP virus, and the amount of mRNA 8 was reduced in SP virus-infected cells (see Fig. 5C). The results confirmed that RNAs 3 and 4 were not detected at any time during the entire SP replicative cycle and indicated the appearance of a smaller $\Delta 4$ RNA species.

Mapping a deletion in SP virus. To define the differences between the PP3 and SP viruses observed above, we focused on the region in the vicinity of the transcription signal for mRNAs 3 and 4. Probes were generated by progressively shortening the pRP3 probe at the 3' end. Four probes, AF180, F180, B180, and F120 (Fig. 4B), were used in S1 nuclease protection experiments with SP virus mRNAs. Essentially, all of the protected bands would be derived from full-length RNA 1, RNA 2(S), and RNA $\Delta 4$. We predicted that the 5' ends of the probes would give protected bands of a constant size, whereas the 3' ends of the probes would give variably sized protected bands. As expected (Fig. 4A), each probe yielded a 1,150-nt fragment in addition to a different-sized (170- to 730-nt) protected fragment. By plotting the 1,150-nt constant band at the extreme 5' end of pRP3 (based on the result from below), and placing the smaller variable bands abutting the 3' terminus of each probe, we located a deletion of approximately 450 nt in the genome of the SP virus.

S genes of Miller and SP viruses are colinear. S1 nuclease protection experiments were carried out with three overlapping probes, pRP1, Hpa, and XE, of known nucleotide sequences (Fig. 5A). Probe pRP1 contains the 3' 930 nt of the polymerase gene and the 5' three-quarters of the S gene and overlaps probe RP3 by 72 nucleotides. The entire lengths of both the PP3 and SP RNAs were protected by all three probes (Fig. 5B). In addition, the sizes of the PP3 and SP genomic RNA and RNA 2 (S) were indistinguishable by Northern blot analysis (Fig. 5C). These results indicate that there is no significant difference in the mRNA 2(S) peplomer gene of PP3 and SP viruses and that no large differences occur in the unique region of RNA 1 encoding the polymerase gene(s).

Intergenic recognition sequences for the transcription of mRNAs 3 and 4 are deleted in SP virus. The approximately 450-nt deletion deduced by S1 mapping indicated that the complete ORF A (unique region of mRNA 3) and the 5' one-fifth of ORF B have been deleted from the SP virus genome (Fig. 4). A question arises as to whether a consensus transcription initiation sequence is present (possibly from 5' of ORF A) and functions as the transcriptional initiation signal for the SP mRNA $\Delta 4$. To investigate this possibility, cDNA clones that contained the deletion of SP virus genome were isolated and sequenced. The nucleotide sequences of the PP3 and SP viruses were aligned and compared (Fig. 6). The SP virus had a 462-nt deletion, which is in close agreement with the S1 experiment estimates. Also, SP RNA contained a 16-nt insertion in the noncoding region immediately 5' of the deletion. As indicated, both transcription recognition signals ($\overset{\text{A}}{\text{T}}\text{TCTAAAC}$) for ORF A and ORF B were absent in the SP virus genome.

DISCUSSION

In this study, we used Northern blot and S1 nuclease protection analyses to survey and compare the genomic organization and subgenomic mRNAs of the virulent PP3 virus and the avirulent SP virus. Both viruses are derived from the virulent Miller strain of TGEV. Northern blot analysis showed that the sizes of TGEV mRNAs 1, 2(S), 5, 6(M), 7(N), and 8 from both viruses are similar, whereas mRNAs 3 and 4 are missing from the SP virus. S1 nuclease protection experiments and DNA sequencing analyses confirmed that there is a 462-nt deletion in the genome of the SP virus. This deletion eliminated the transcription recognition signal ($\overset{\text{A}}{\text{T}}\text{TCTAAAC}$) for both the ORF A and ORF B;

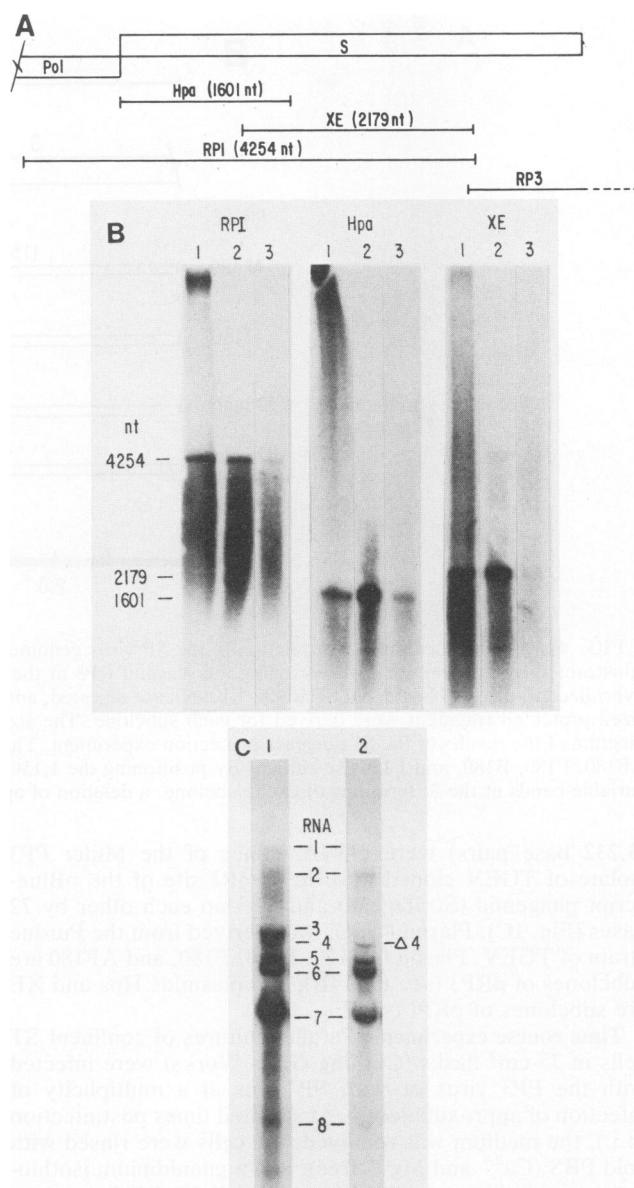


FIG. 5. (A) Schematic diagram of the 5' end of the S gene, the 3' end of the Pol gene, and the relative positions and sizes of plasmid RP1 and subclones Hpa and XE. (B) S1 nuclease fragments protected with runoff transcripts generated with pRP1, pHpa, and pXE were analyzed on a 1% agarose gel after glyoxal-dimethyl sulfoxide treatment and heat denaturation. Lanes: 1, size of the input probe; 2, fragment protected with PP3 RNA; 3, fragment protected with SP RNA. (C) Northern blots of SP and PP3 total intracellular RNAs. SP and PP3 RNAs 1, 2(S), 5, 6(M), 7(N), and 8 comigrated. PP3 RNAs 3 and 4 are not present in the SP RNA pattern; instead, there is a smaller $\Delta 4$ RNA species. Lanes: 1, PP3 virus RNA; 2, SP virus RNA.

consequently, mRNAs 3 and 4 were not synthesized during an SP virus infection. Thus, the gene products of ORF A and of an intact ORF B are dispensable and not required for virus replication. When the cDNA sequences of PP3 and SP were aligned, we noted that there was an insertion of 16 nt present downstream of the S gene in the noncoding region of the SP genome. Interestingly, the same 16-nt sequence was also present in the genome of the Purdue strain of TGEV (14). We

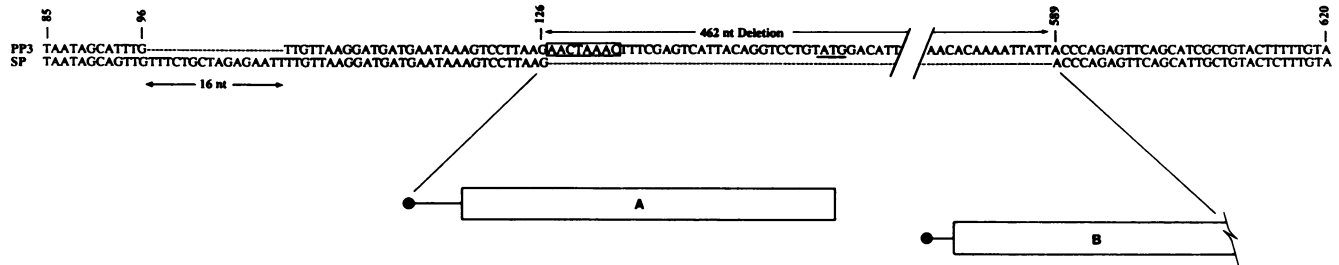


FIG. 6. Comparison of cDNA sequences of SP and PP3 strains of TGEV. The sequences begin at position 85 downstream of the S gene to position 620 into ORF B (see Fig. 2 of reference 20). The SP sequence contains a 16-nt insertion in the noncoding region and a 462-nt deletion from positions 126 to 589. The PP3 transcription initiation sequence for ORF A is boxed, and the methionine initiation code for ORF A is underlined. The 462-nt deletion in the SP genome eliminates all of ORF A, the 5' one-fifth of ORF B, and the accompanying intergenic consensus sequences (●) for both ORFs.

suspect that this 16-nt deletion occurred during the propagation of the parent Miller virus in culture to generate the PP3 virus.

In all coronaviruses studied to date, the unique 5'-end sequences of genomic-length RNA 1 encode one or two viral RNA-dependent RNA polymerases for the synthesis of viral RNAs. For the prototype coronavirus, infectious bronchitis virus which has been completely sequenced, the 5' portion of the genome is occupied by two large ORFs encoding two putative polymerases (2). We have sequenced the 3' 930 nt of the ORF immediately upstream of the peplomer S gene of TGEV and have shown that it has extensive homology with the polymerase F2 gene of IBV (R. D. Wesley, *Adv. Exp. Med. Biol.*, in press). Thus the polymerase(s) of TGEV, like those for infectious bronchitis virus, might be the only gene products encoded by full-length mRNA 1. As yet, we do not have probes to examine the 5' end of the TGEV genome in great detail; however, Northern blot analysis has shown that the full-length RNAs of PP3 and SP viruses are similar in size, suggesting that no major deletions or insertions have occurred in the 5' region of either genome. The large S gene encoded by RNA 2(S) has been examined in more detail by Northern blot and S1 nuclease protection analyses. The results indicate that the S gene sequences are extremely homologous for both viruses (Fig. 4A and 5B). Additional Northern blot and S1 nuclease analyses have shown that no large deletions or insertions are present in SP viral sequences downstream of the 462-nt deletion. It appears that the 462-nt deletion is the only prominent feature in the SP genome.

We have examined the available ORF A and ORF B DNA sequences for other TGEV strains (3, 9, 20). The Miller PP3, British FS772/70, and Purdue-115 strains are virulent for baby pigs (3, 10, 20). The sizes of the ORFs B in all three strains is 244 amino acids, with only a few single-amino-acid substitutions among the strains. For the SP virus, the N-terminal part of ORF B is missing, allowing for a potentially truncated gene B product. The size of ORF A is considerably more variable. The ORFs A in Purdue-115 and Miller PP3 are 71 and 72 amino acids, respectively. There is a 29-nt deletion in Miller PP3 that caused a frame shift and a new termination codon such that the last six amino acids of ORF A are different. The British strain FS772/70 contained a base change resulting in a termination codon that shortened ORF A to 61 amino acids. ORF A is not present in the attenuated SP virus genome due to the 462-nt deletion. Currently we are studying another TGEV variant, PRCV Ind/89, which infects cells of the upper respiratory tract and few, if any, cells of the gastrointestinal tract (21A). S1 nuclease protection experiments have indicated that at a

minimum ORF A is deleted in PRCV-Ind/89. These observations are consistent with our hypothesis that ORFs A and B are functionally important in the pathogenesis of TGEV.

Previous studies showed that the SP virus differs from the virulent parental Miller strain of TGEV in four important aspects: (i) site of replication in the intestine of baby pigs, (ii) persistence in swine leukocytes, (iii) nonlethality for baby pigs at high dosage, and (iv) small plaque size on ST cells. The fact that the most noticeable feature of the SP virus is a deletion that eliminated the two transcriptional signals, the entire ORF A and 5' portion of ORF B, indicates that the gene products of ORF A and ORF B are not required for virus replication; we suspect that they may play a role in plaque morphology, tissue tropism, virulence, and persistence. Experiments to introduce ORFs A and B individually and/or jointly into the SP virus genome will enable us to assess the contribution of these genes in the pathogenesis of TGEV.

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