A Simple Strategy to Assemble Infectious RNA and DNA Clones

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1. INTRODUCTION

The availability of infectious full-length cDNA clones is important for the molecular genetic analysis of the structure and function of RNA virus genomes (Ahlquist et al., 1984; Boursnell et al., 1987). Infectious cDNA clones for a number of positive-stranded RNA viruses have been developed, advancing our understanding of the molecular mechanisms of viral replication and pathogenesis, and has resulted in novel approaches for heterologous gene expression and vaccine development. Clearly, a full-length infectious construct of TGEV would enhance our understanding of all aspects of TGEV biology by providing a means for reverse genetic analysis.

2. METHODS AND MATERIALS

2.1 Mutagenesis, Cloning, and Assembly of a Full Length TGE Infectious Clone

The TGEV genome (Purdue strain, ATCC VR-763) was cloned from infected swine testicular cells (ST) and PCR mutagenesis was utilized to

The Nidoviruses (Coronaviruses and Arteriviruses).
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generate unique junction sites at the 5' and 3' end of each cloned fragment (Figure 1). Standard recombinant DNA techniques were used to remove unwanted amino acid changes associated with reverse transcription or naturally occurring quasispecies variation and a consensus sequence was determined (Yount et al., submitted).

We isolated five cDNA subclones spanning the entire TGEV genome (designated TGEV A, B, C, DE and F) (Figure 1). The A fragment contains a unique 5' T7 promoter and the F fragment contains a 3' poly-T tail. Appropriately sized cloned fragments were isolated as described by Yount et al.

The pTGEV A, C, DE and F clones were stable in plasmid DNA's in *E.coli*. However, the B fragment was unstable and only a few slow growing isolates were obtained, all of which contained deletions or insertions in the wildtype sequence at a region consistent with those noted by other investigators (position 9973) (Denison et al., 1999; Eleouet et al., 1995). A unique BstxI site was engineered into the unstable region of the B fragment by primer-mediated mutagenesis (Yount et al., submitted) and used to bisect this fragment into B1 and B2 fragments (Figure 1). The region of instability, as suggested by locations of deletions and insertions, maps in or near the TGEV poliovirus 3C-like protease (3-Clpro) motif and may be bactericidal or unstable in microbial cloning vectors.

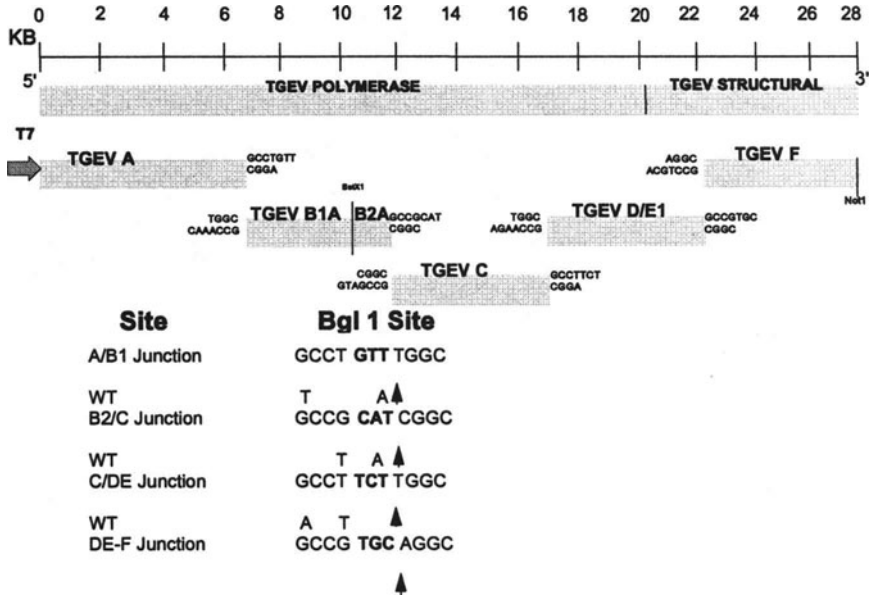

Figure 1. Assembly Strategy of TGEV Clone. Using RT-PCR and unique oligonucleotide primer mutagenesis, five clones spanning the entire TGEV genome were isolated using standard recombinant DNA techniques.

A full-length cDNA construct was assembled in two ligation steps. Each cDNA clone is flanked by unique Bgl1 sites and will only assemble with the appropriate adjacent subclone. The TGEV A and B1, B2 and C, and the DE-1 and F fragments were ligated overnight in the presence of T4 DNA ligase (Sambrook et al., 1989). Directionally assembled products were isolated from agarose gels and the TGE A/B1, B2/C3-2, DE-1/F fragments religated overnight. The final products were purified and capped-T7 transcripts were synthesized and analyzed as described by Yount et al.

2.2 Transfection, Virus Isolation, and Identification of Marker Mutations

Baby Hamster Kidney (BHK) cells were transfected with TGEV transcripts alone, TGEV + TGEV N gene transcripts, or just TGEV N gene transcripts and co-cultured with ST cells. Viral progeny were isolated as described by Yount et al.

Mutations introduced into the TGEV genome in order to engineer unique Bgl1 and BstX1 restrictions sites were identified in TGEV 1000 derived virus by standard molecular biology techniques as described by Yount et al.

3. DIRECTIONAL ASSEMBLY OF LARGE GENOMES AND CHROMOSOMES

Conventional restriction enzymes, such as Not1 and EcoR1, cleave identical DNA on average every 65,000 bp (Sambrook et al., 1989) and leave sticky ends that assemble with similarly cut DNA fragments in the presence of DNA ligase. Because of this, they rarely are appropriate choices for assembling large intact genomes or chromosomes. However, a second subclass of restriction enzymes (i.e. Bgl1, BstX1) also recognize palindrome sequences but leave random sticky ends of one to four nucleotides in length that are not complementary to most other sticky ends generated with the same enzyme at other sites in the DNA. Because a three nucleotide variable overhang is generated following cleavage with Bgl1, 64 different variable ends can be generated, which assemble only with the appropriate 3 nucleotide complementary overhang generated at an identical Bgl1 site. We hypothesized that a sequential series of smaller DNA subclones that are flanked by unique Bgl1 junctions could be directionally assembled into an intact, full-length infectious cDNA clone. To test this hypothesis, we assembled a full-length infectious clone of a coronavirus, thereby demonstrating its potential applications for assembling other large genomes or chromosomes *in vitro*.

4. TRANSFECTION AND RECOVERY OF INFECTIOUS VIRUS

The efficiency of electroporation of full length TGEV transcripts into BHK cells was low, as indicated by indirect fluorescent assay (IFA), and only evident when electroporated with TGEV N gene transcripts (Figure 2). Supernatants from transfected BHK/ST cell co-cultures were passaged onto ST cells and CPE was observed at each passage only with supernatants derived from the TGEV+N transfected cultures (Figure 3). Virus isolated from these cultures formed plaques in agar (Figure 4). In addition, no significant differences in the replication of wildtype TGEV or TGEV 1000 derived viruses were noted in ST cells, and all viruses replicated to titers that approached 1 x 10^8 PFU/ml within 28 hrs (data not shown).

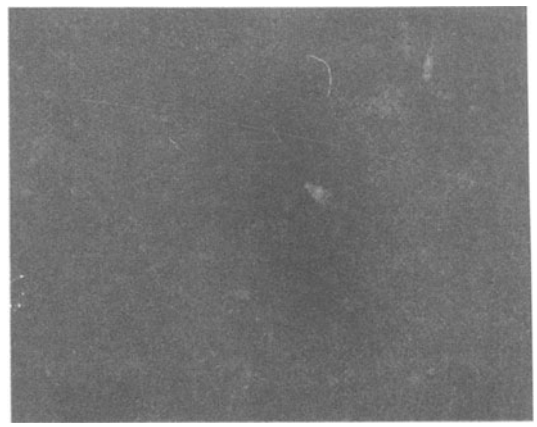

Figure 2. BHK Cell Electroporation Efficiency as Indicated by IFA. BHK cells were electroporated with TGEV transcripts alone, TGEV + TGEV N gene transcripts, or TGEV N gene transcripts alone.

4.1 Identification of Marker Mutations

Infectious virus derived from transfected cultures should each contain the four unique interconnecting junction sequences used in the construction of the infectious construct (Figure 1). Results from restriction fragment length polymorphism (data not shown) and sequencing analysis (Figure 5) of virus derived from passage one (icTGEV-1) and passage three (icTGEV-3) demonstrated that the appropriate marker mutations were absent from the wildtype TGEV genome and present in TGEV 1000 derived virus.

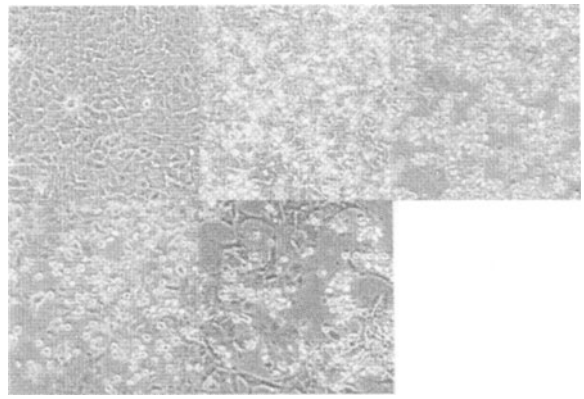

Figure 3. CPE typical of TGEV infection is evident at each passage. (A) uninfected ST cells, (B) passage 1, (C) passage 2, (D) passage 3, (E) passage 4

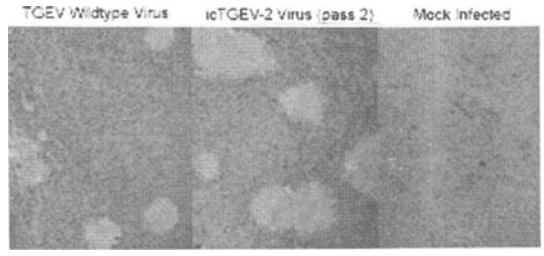

Figure 4. Plaque Assay. TGEV wildtype virus and TGEV 1000 derived virus were plaque assayed on ST cells. Mock infected cells were included as a negative control.

5. CONCLUSION

Until recently, full length infectious clones have not been assembled because of size constraints in bacterial cloning vectors, regions of coronavirus chromosomal instability/toxicity in bacterial vectors, the requirement for a vector system which allows for simple reverse genetic applications, and the inability to drive full length transcripts *in vitro*. In this manuscript, we describe a simple and rapid approach to systematically assemble a full-length infectious coronavirus cDNA from a panel of six smaller subclones. Directional assembly methods should be appropriate for constructing full-length infectious constructs of other large RNA viruses, such as filoviruses and other coronaviruses, DNA genomes of herpesviruses, adenoviruses, and poxviruses, and those viral genomes which are unstable in prokaryotic vectors (Boursnell et al., 1987; Delmas et al., 1992; Peters et al.,

1996). In addition, the systematic assembly of large chromosomes or minichromosomes approaching several million base pairs in length is theoretically possible.

Clone	Junction	Sequence
icTGE-1	B2/C	TCCAGC**C**GCATC**G**GCTACA
wildtype		T A
icTGE-1	C/DE	CAAGGCCTT**C**TT**G**GCACAT
wildtype		T A
icTGE-1	DE/F	AGTAGC**C**GT**G**CAGGCTAGA
wildtype		A T
icTGE-3	B2/C	TCCAGC**C**GCATC**G**GCTACA
wildtype		T A
icTGE-3	C/DE	CAAGGCCTT**C**TT**G**GCACAT
wildtype		T A
icTGE-3	DE/F	AGTAGC**C**GT**G**CAGGCTAGA
wildtype		A T

Figure 5. Sequence analysis of marker mutations at fragment junctions of clones at passage 1 and 3. Marker mutations are underlined and wildtype nucleotides are shown.

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