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Infectious cDNA Clones of Langat Tick-Borne Flavivirus That Differ from Their Parent in Peripheral Neurovirulence

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Tick-borne flavivirus strain Langat TP21 (LGT TP21) recovered from ticks, is naturally attenuated for humans but retains demonstrable neurovirulence and peripheral virulence ("neuroinvasiveness") for mice. Previously a mutant, strain E5, less virulent for mice was derived from LGT TP21. Multiple attempts to prepare a full-length infectious TP21 cDNA from cDNA fragments cloned in *E. coli* were uniformly unsuccessful. A more informative sequence than that obtained from these cloned cDNA fragments and similar E5 cDNA fragments was derived from RT-PCR fragments that had not been cloned in *E. coli.* Comparison of the RT-PCR consensus sequence of TP21 and E5 identified only seven amino acid differences that might be responsible for the observed difference in virulence of these strains for mice. Eleven independent infectious cDNA clones of TP21 were recovered using two overlapping long RT-PCR fragments. Importantly, low-titered virus used to prepare cDNA as template for PCR was harvested early in the growth cycle to minimize the frequency of deletion mutants that accumulated late in infection. The four analyzed rescued clones exhibited clone-specific minimal divergence from the consensus sequence but this limited variation was associated with diminished peripheral virulence for immunocompetent mice. Manipulation of these clones should facilitate elucidation of LGT virulence.

INTRODUCTION

There are more than 60 antigenically related, positive-strand RNA viruses in the arthropod-borne flavivirus genus of the family *Flaviviridae*, many of which are important human pathogens. The tick-borne encephalitis virus complex of flavivirus family includes tick-borne encephalitis virus (TBEV, formerly called Russian spring-summer encephalitis virus), Kyassanur forest disease, Langat, Louping ill, Negishi, Omsk hemorrhagic fever, and Powassan viruses (Calisher *et al.,* 1989; Monath and Heinz, 1996). These viruses are endemic throughout most of the Northern Hemisphere, and except for Langat, cause human disease of varying severity that can have mortality rates as high as 20 to 30%. Tick-borne encephalitis remains a pressing public health problem in Eastern Europe and Russia, where 9,000–12,000 patients are diagnosed annually. A significant increase in mortality was recorded in 1956 and 1964, when morbidity reached 4,500–4,600 per 100,000 persons (Gaidamovich, 1995).

The tick-borne encephalitis flaviviruses share envelope glycoprotein epitopes that often induce cross-resistance among viruses of the group. Approximately three decades ago, these properties of antigenic cross-reactivity and the subsequent recognition of virulence polymorphism suggested that successful immunization might be achieved using a live, naturally attenuated tick-borne flavivirus (Il'enko *et al.,* 1968; Price *et al.,* 1970; Mayer *et al.,* 1976). The impetus for this approach was the recovery of a virus from ticks in Malaysia, namely Langat virus (LGT), strain TP21, that did not appear to be associated with human disease under natural conditions (Gordon Smith, 1956). Immunization of animals and human volunteers with LGT strain E5, which is a more attenuated derivative of LGT TP21, induced a high level of virus-neutralizing antibodies against various members of TBEV complex such as Powassan, Kyassanur forest disease, and TBEV (Price *et al.,* 1970; Price and Thind, 1973). Nonetheless, TP21 exhibited neurovirulence and neuroinvasiveness ("peripheral virulence") when tested in mice (Gordon Smith, 1956; Thind and Price, 1966a; Pletnev and Men, 1998). But this was significantly less compared with that of the very virulent Far Eastern strains of TBEV that produce human disease that has a 20 to 30% rate of mortality. Several LGT strains, which were partially attenuated for mice and monkeys, were isolated and studied in the United States, Russia, and Czechoslovakia (Nathanson *et al.,* 1968; Price *et al.,* 1970; Mayer *et al.,* 1976; Smorodincev and Dubov, 1986). One such strain, designated Yelantsev, was studied extensively in over 600,000 vaccinees in Russia as an experimental live vaccine against TBEV during the early 1970s (Smorodincev and Dubov, 1986). For this reason, we chose LGT as a foundation for construction of a satisfactorily attenuated live virus vaccine by introducing site-specific mutations into the full-length infectious clone of LGT, i.e., a

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cDNA clone from which infectious RNA can be transcribed *in vitro*.

Full-length infectious clones have become available for a number of other flaviviruses (reviewed in Mandl *et al.,* 1997), and these clones have provided the foundation for elucidating the molecular biology of the tick-borne flaviviruses. In this report we describe the construction and generation of stable LGT TP21 full-length infectious cDNA clones, which can be used for genetic analysis of tick-borne flavivirus neurovirulence and neuroinvasiveness and for the development of an attenuated live virus vaccine against these viruses. Viruses derived from the infectious clones were found to vary with respect to genomic sequence and virulence for mice. A single amino acid change in structural protein preM and another in E were associated with a significant reduction in neuroinvasiveness of one of the rescued TP21 clones in SCID mice, which are highly permissive for this important manifestation of tick-borne flavivirus virulence.

RESULTS

Consensus sequences of TP21 and E5 genome

The complete nucleotide sequence of the wild-type LGT virus (TP21 strain) genome and its more attenuated derivative, strain E5, recovered following multiple passages in chick embryo tissue, was determined previously from cDNA fragments cloned in *E. coli* (Pletnev and Men, 1998). Initial attempts to prepare infectious full-length cDNA clones of LGT TP21 from these cDNA fragments derived from RNA of a high-titered virus suspension of TP21 were uniformly unsuccessful (Fig. 1A). Twelve stable individual full-length cDNAs were assembled in plasmids, but RNAs transcribed from these cDNA clones were not infectious for simian *Vero* or $LLCMK_2$ cell culture for reasons not understood at that time. It was possible that this was a result of spontaneous mutations in the LGT genome that had occurred during virus amplification in *Vero* cells or during amplification of full-length cDNA clones in the bacterial vector.

For this reason, we decided to reexamine the sequence of the LGT genome by directly sequencing RT-PCR cDNA fragments without prior cloning in bacteria. Four overlapping cDNA fragments representing the full-length genome of TP21 or E5 virus were produced using high-fidelity PCR, and the sequence of these overlapping fragments was determined. The sequence of each virus was determined twice, once with fragments that were derived from a virus suspension with a titer of 3.8×10^3 PFU/ml (TP21) or 1.2×10^4 PFU/ml (E5) and once with fragments derived from a virus suspension that was harvested 1 day later and titered 2.2×10^6 PFU/ml (TP21) or 4.0×10^6 PFU/ml (E5). Consensus sequences of both LGT strain genomes were found to differ from the previously published sequences determined from cDNA fragments cloned in *E. coli* (Table 1). The TP21 and E5 genomes were both 10,943 nucleotides (nt) in length and contained a 130 nt 5′ noncoding region and a 568 nt 3′ noncoding region. The sequence of the 5′ termini of both LGT strains was identical. This was also the case for the 3′ termini. The consensus sequences of TP21 and E5 derived by RT-PCR were thought to be more informative for identifying strain-specific mutations that might be responsible for differences in biological characteristics than were sequences derived from single DNA fragments cloned in *E. coli*.

There were 12 nucleotide differences in the consensus sequence of the two LGT strains (TP21 and E5), of which seven produced an amino acid substitution in the envelope structural protein E or nonstructural protein NS3 or NS5 (Table 1). Among the seven amino acid changes in RT-PCR consensus sequence of TP21 and E5, four amino acid differences ($Asn_{389} \rightarrow$ Asp in E, $Asn_{22} \rightarrow$ Ser, $Phe_{248} \rightarrow$ Tyr, and $Phe_{317} \rightarrow$ Leu in NS3) were also observed previously when TP21 and E5 cDNA fragments were cloned in *E. coli* (Pletnev and Men, 1998). Three additional amino acid substitutions ($Phe_{119} \rightarrow$ Val in E protein and $Ser_{422} \rightarrow$ Thr and $Arg_{542} \rightarrow$ Lys in NS5 protein) were identified in the consensus sequence determined from RT-PCR fragments of TP21 and E5. Another six amino acid differences, previously detected in the sequence of cloned fragments of TP21 and E5 (Pletnev and Men, 1998), were not found in RT-PCR consensus sequences. The somewhat greater variability observed with the cloned cDNA probably reflects bacterial selection during the cloning procedure and/or sequence heterogeneity in the viral RNAs that were employed for cDNA cloning in *E. coli*.

Infectious full-length TP21 cDNA

Having failed to assemble an infectious cDNA of the TP21 genome in bacteria (Fig. 1A), we attempted to circumvent the difficulties associated with cloning cDNA in a bacterial vector by preparing infectious full-length cDNA using long PCR. Also, we investigated the possibility that spontaneous mutations of the LGT genome might be greater for virus that attained a high titer during extended growth in cell culture. Full-length cDNA of TP21 virus was produced (Fig. 1B and Fig. 2) when high-fidelity PCR was performed using a positive-sense primer that contained SP6 polymerase promoter immediately upstream of the first 22 nts of LGT sequence, and a negative-sense primer that was complementary to the LGT nts 10,921–10,943 of the 3′ terminus. The latter primer contained an *Eco*RV cleavage site immediately following the 3′ end sequence. As shown in Fig. 2, the dominant PCR product 1A (lane 1, band A) derived from low-titered virus (3.8×10^3 PFU/ml) was approximately 11 kb in length. In contrast, the PCR product 2A (lane 2, band A) derived from high-titered virus (2.4×10^9 PFU/ml) contained very little full-length cDNA, while the major prod-

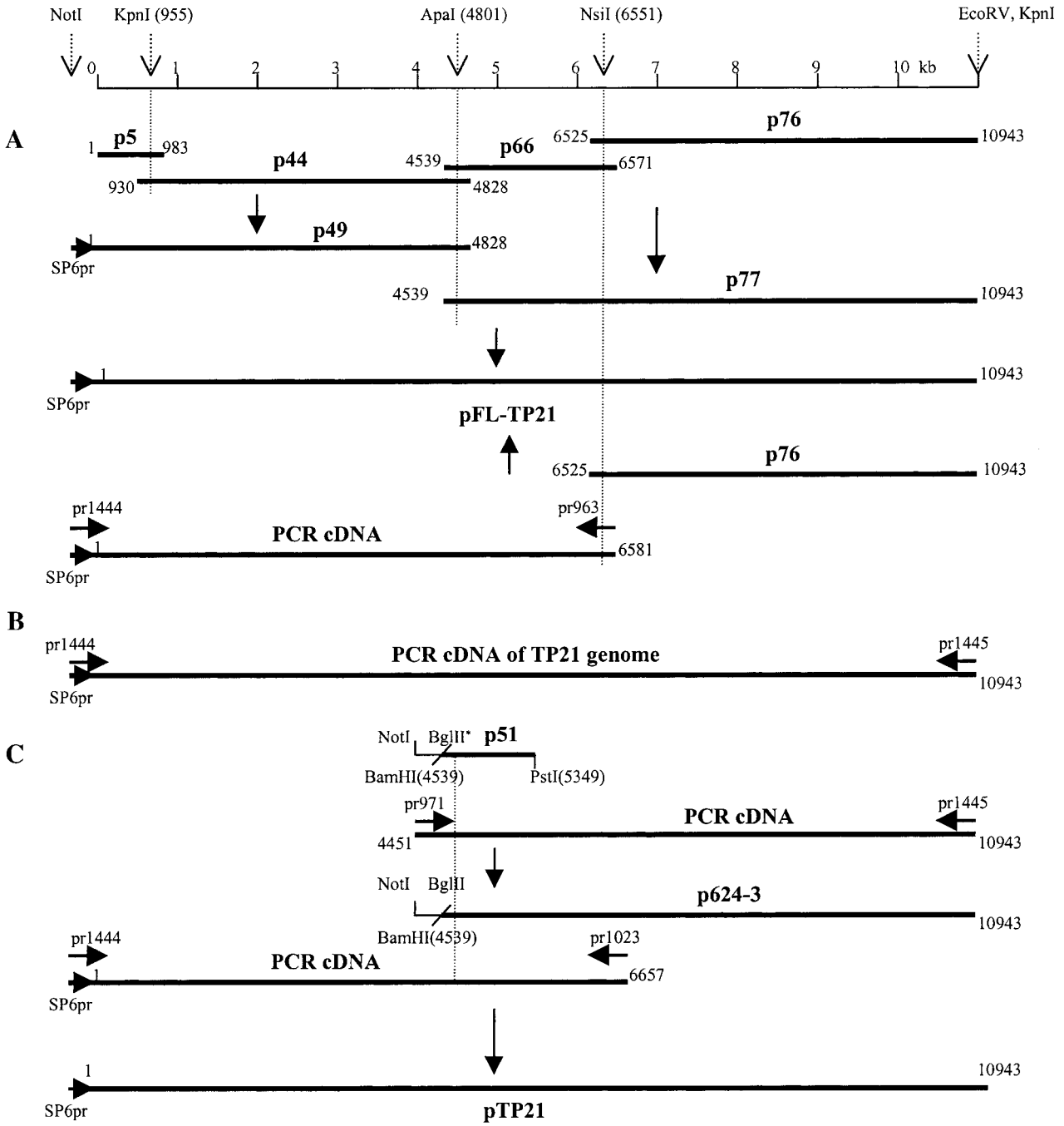

FIG. 1. Construction of full-length cDNA of LGT TP21 genome. (A) The assembly of full-length cDNA of TP21 in a plasmid was performed using the cDNA segments that were cloned and sequenced as described earlier (Pletnev and Men, 1998) or were derived by long PCR. (B) Construction of full-length cDNA by a single long PCR. (C) The assembly of full-length cDNA from two cDNA segments of the genome that were derived from the low-titered virus preparation (3.8×10^3 PFU/ml). Position of the cleavage site of *Not*I, *Kpn*I, *Apa*I, *Nsi*I, and *Eco*RV in the cDNA shown in (A) or (C) by dashed lines. Solid lines indicate PCR cDNA fragments or cloned fragments of TP21 genome. Short horizontal arrows indicate position of SP6 promoter or position of primer; vertical solid arrows indicate subsequent steps in cloning strategy. The numbers at the ends of LGT cDNA fragments represent the first and the last nucleotide positions of the genome, respectively. NT numbering derived from the results of RT-PCR sequence of TP21 genome (see Table 1). *Note:* *The junction of *Bgl*II and *Bam*HI fragments in plasmid p51 or p624-3 eliminated both *Bgl*II and *Bam*HI cleavage sites.

uct was considerably shorter, approximately 4 kb in length (lane 2, band B). Sequence analysis indicated that this fragment (lane 1 or 2, band B) represented a truncated LGT genome extending from nt 1 to 3779 that was joined to the last 23 nts of the 3′end of genome present in the negative-sense primer (oligo 1445), which was used for RT and PCR. It should be noted here that the last seven nucleotides at the 3′ end of oligo 1445 were also complementary to the LGT genome sequence at nucleotide positions 3780 to 3786. It is possible that the shorter products (lane 1 or 2, band B) were produced by binding of the primer to this alternative site on the viral

TABLE 1

Differences between the Genomic Sequence of LGT Strains TP21 and E5 As Determined by Sequence Analysis of Fragments of Virus Genome Initially Cloned in *E. coli* or Tested Directly after Derivation by RT-PCR

Region	NT position	AA change	Cloned fragments[a]			RT-PCR fragments[b]			Infectious clones[c]
			TP21		E5	TP21		E5	
5′NCR	35		G		C	C		C	C
	61		—		G	G		G	G
C	371	Thr_{80} > Pro	A	—	C	A		A	A
	461	Leu_{110} > Ile	C	—	A	C		C	C
preM	514		G		U	G		G	G
	957	Val_{160} > Ala	U		U	C		C	C
E	**1,325**	**Phe_{119} > Val**	U		U	**U**	—	**G**	***U***[d]
	1,327		C		A	C		A	C
	1,342		G		A	G		A	G
	1,437	Thr_{156}/Ile	C/U		C	C		C	C
	1,567		A		U	U		U	U
	1,823	Gly_{285} > Ser	G	—	A	A		A	A
	1,968	Phe_{333} > Ser	U	—	C	C		C	C
	2,135	**Asn_{389} > Asp**	A	—	G	**A**	—	**G**	***A***
NS1	3,008	Val_{184} > Met	G	—	A	A		A	A
	3,403		C		U	C		U	C
NS2A	3,635	Pro_{41} > Ala	C	—	G	G		G	G
	3,637		G		C	C		C	C
	3,964		G		A	G		G	G
NS3	**4,662**	**Asn_{22} > Ser**	A	—	G	**A**	—	**G**	***A***
	5,340	**Phe_{248} > Tyr**	U	—	A	**U**	—	**A**	***U***
	5,374		C		U	C		U	C
	5,546	**Phe_{317} > Leu**	U	—	C	**U**	—	**C**	***U***
NS5	8,878		U		U	U		G	U
	8,928	**Ser_{422} > Thr**	G		G	**C**	—	**G**	***G***
	9,288	**Arg_{542} > Lys**	G		G	**G**	—	**A**	***G***
3′NCR	10,516–10,517		AC		—	AC		AC	AC
	10,601		—		C	C		C	C
	10,635		—		U	U		U	U
	10,688–10,689		CG		CG	GC		GC	GC
	10,761–10,762		AG		AG	CA		CA	CA
	10,838–10,839		GU		GU	UC		UC	UC

Note. — Denotes a coding difference between TP21 and E5 at indicated position was determined for cloned genome segments and/or RT-PCR genome fragments. ↔ Indicates the same coding difference was detected using these two methods of sequence analysis.

[a] Sequence analysis of the cDNA fragments of LGT genome cloned in *E. coli* was described previously (Pletnev and Men, 1998).

[b] Consensus sequence of viral genome was determined by sequencing RT-PCR fragments derived from two separate preparations of virus which differed 10^2- to 10^3-fold (see Materials and Methods). Dominant base is shown at each indicated nucleotide position of genomic consensus sequence. A lesser amount of another base was occasionally detected at certain positions as indicated by double assignment, as for example at nt 1437. Nucleotide differences in consensus sequences between TP21 and E5 strains of LGT that resulted in a coding change difference are shown in bold letters. Numbering of nucleotides and amino acids is the same for strains TP21 and E5, because these viruses have the same number of nucleotides and amino acids.

[c] Sequence of rescued 636, 649, 656, and 689 virus genomes was determined by analysis of cDNA fragments which were obtained by RT-PCR.

[d] Sequence of four rescued cDNA LGT clones at a position where TP21 and E5 differed in amino acid consensus sequence is underlined.

genome during amplification by RT-PCR, rather than to an altered 3′ terminal sequence selected for by high-multiplicity passage.

The approximately full-length RT-PCR cDNA fragments (band A, lane 1 or 2; Fig. 2) were digested with *Eco*RV, and RNA transcripts from these templates were tested for infectivity in *Vero* cells. Evidence of infection in *Vero* cells was detected by IFA on Day 12, using LGT-specific antibodies. At that time, 80–90% of cells transfected with RNA from PCR product 1A were positive, while only a few IFA-positive cells were observed when RNA transcripts from PCR product 2A were used. This indicated that infectious cDNA was recovered most effectively when low-titered virus suspension was used as a source of full-length cDNA of the genome. This probably reflects alteration in the viral RNA genome that occurs with higher frequency during the more prolonged period of virus replication required to achieve a high titer. For this reason, viral RNA from a low-titered TP21 virus suspension harvested 2 days postinfection was used for construction of full-length TP21 cDNA.

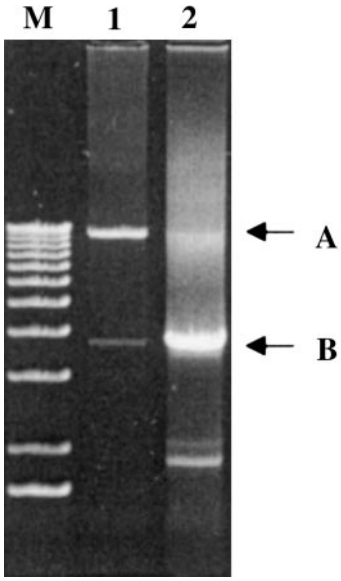

FIG. 2. Analysis of PCR-amplified cDNA from TP21 genome by 0.7% agarose gel electrophoresis. RT-PCR products were synthesized using RNA of TP21 virus that was isolated from low-titered virus (lane 1; titer of 3.8×10^3 PFU/ml on *Vero* cells) or high-titered virus (lane 2; titer of 2.4×10^9 PFU/ml on the same cell line). PCR was performed using oligos 1444 and 1445 as primers under conditions described under Materials and Methods, and 10 μl of reaction mixture was loaded on gel. Fragments of approximately 11 kb in length (band A) represent complete or nearly compete full-length genome cDNA. It was isolated from the gel and used for transcription of RNAs that were then used for transfection of *Vero* cells in culture. Shorter fragments approximately 4 kb in length (band B) were sequenced after extraction from gel. Molecular weight markers are displayed in lane M. The next-to-the-top marker corresponds to 11 kb.

Recovery of LGT viruses from cloned cDNA and their characterization

Two overlapping cDNA fragments (Fig. 1C) were prepared by long PCR using RT product derived from RNA of low-titered TP21 virus stock (3.8×10^3 PFU/ml). The PCR product (~6.1 kb) representing the 3′ region of the genome was cloned in bacterial vector p5′-2(*Not*I, *Xho*I, Δ*Hin*dIII) in two steps as described under Materials and Methods and illustrated in Fig. 1C. The resulting clone p624-3 that contained LGT nts from 4539 to the end of genome was selected, based on restriction enzyme analysis. Subsequently, the remaining 5′ sequence of TP21 genome (~4.8 kb) together with the SP6 promoter, present at the 5′ end immediately upstream of the LGT sequence, was generated by long PCR and ligated into the *Not*I and *Apa*I digested p624-3 plasmid. Cloning of this construct in *E. coli* yielded 28 stable full-length LGT TP21 cDNA clones. However, some polymorphism was observed among the stable full-length LGT cDNAs with respect to restriction enzyme digestion pattern. The sequence of four of the plasmids (pTP21-636, pTP21-649, pTP21-656, and pTP21-689) was analyzed and was found to conform very closely to the consensus sequence of TP21.

Before producing runoff transcripts, the plasmid DNA template was linearized at the *Eco*RV cleavage site that is present three nucleotides downstream of the 3′ end of LGT TP21 sequence. As a consequence, the RNA transcripts contained three additional nucleotides GAU at the 3′ terminus as well as an additional G residue at the 5′ terminus. Full-length RNA generated by SP6 polymerase from 28 different plasmids was tested for infectivity by transfection of hamster BHK, or simian *Vero,* or $LLCMK_2$ cells. Eleven individual LGT TP21 cDNA clones were infectious. Evidence of virus infection was detected by IFA with LGT-specific hyperimmune mouse ascitic fluid (HMAF) in 80–100% of transfected cells on Day 5. Additional evidence that the recovered viruses were LGT was provided by comparison of LGT-specific proteins, which were produced by parental TP21 or its progeny cDNA viruses in infected BHK, *Vero,* or $LLCMK_2$ cells as demonstrated by immunoprecipitation assay with LGT-specific HMAF or monoclonal antibodies (data not shown). The specific infectivities of RNA transcripts that were derived from three different cDNA clones ranged from 8×10^3 to 2×10^5 PFU/μg. This was significantly less than the infectivity of TP21 virion RNA, which was 4×10^6 PFU/μg measured on *Vero* cells under the same experimental conditions. Stock preparations of rescued TP21 clones were produced by passaging the virus in *Vero* cells and harvesting the supernatant medium of infected cultures. Virus titer of four rescued TP21 clones: TP21-636, TP21-649, TP21-656, and TP21-689 (designated 636, 649, 656, and 689) measured by plaque assay on $LLCMK_2$ cells varied from 1.2×10^7 to 2.4×10^8 PFU/ml 7 days postinfection.

The rescued cDNA-derived LGT clones produced characteristic LGT TP21 plaques 3.5 mm in diameter on $LLCMK_2$ cells 7 days postinfection, except for the 649 virus that produced small plaques with an average diameter of 0.8 mm. Virus replication was further analyzed by monitoring the virus titer on Days 0, 1, 2, 3, 4, and 5 after infection of *Vero* cells. Significant differences were not observed between the growth of recovered LGT clones and their parental virus.

Nucleotide sequence analysis of recovered viruses

The four viruses recovered from the transfection of *Vero* cells (i.e., clones 636, 649, 656, and 689) were amplified by further passage in *Vero* cells, and virion RNA was used for RT-PCR. Subsequent sequencing of the complete genome of rescued virus was performed using four overlapping PCR fragments without prior cloning in *E. coli*. Mutations in RNA viruses such as LGT can accumulate during RT-PCR, bacterial cloning of a cDNA genome, and/or during adaptation and propagation of virus in cell culture. To gain a better understanding of the source of genetic variability of the newly recovered LGT viruses: (1) sequence analysis of the 5′-half of the genome (nts 1–5300) of each rescued virus was performed twice in independent experiments in which virus was

TABLE 2

Sequence Variation That Occurred during the Rescue of Infectious LGT TP21 from Plasmid cDNA

Nucleotide position (genome)	Protein and amino acid position	Rescued virus genomes[a]			
		636	649	656	689
A_{590}	preM; Met_{38}			G, **Val**	
C_{1309}	E			U	
A_{1893}	E; Asp_{308}			C, **Ala**	
C_{2083}	E	U			
U_{2230}	E		C		C
C_{2282}	E; His_{438}	U, **Tyr**			
A_{3001}	NS1		G		G
C_{3599}	NS2A; Pro_{29}		U, **Ser**		U, **Ser**
G_{3777}	NS2A; Arg_{88}		A, **Lys**		
C_{4192}	NS2A		U		
G_{4254}	NS2B; Ser_{17}		A, **Asn**		
C_{4299}	NS2B; Ala_{32}	U, **Val**			C/U, **Val**
A_{4374}	NS2B; Asn_{57}		C, **Thr**		
C_{4429}	NS2B			U	

[a] Differed from consensus sequence (determined by RT-PCR) at the indicated positions of the viral genome. (The same coding and noncoding differences were also present in plasmid cDNA used for virus rescue unless underlined.)

grown, viral RNA isolated, and subjected to RT-PCR; the 3′-half of the viral genome was sequenced in a similar manner but only once; and (2) the nucleotide sequence of the viral insert in each of the four plasmids, from which infectious RNA transcripts were derived, was also determined.

Analysis of the four selected infectious clones of TP21 revealed six differences in amino acid sequence from the consensus sequence of E5 previously determined by RT-PCR of its genome fragments (Table 1). These differences were observed at positions: 119 and 389 of E; 22, 248, and 317 of NS3; and 542 of NS5. Thus, the four rescued clones contained the TP21 consensus sequence in 6 of the 7 positions at which TP21 differed from its E5 derivative. Each infectious clone had Thr at position 422 of NS5, similar to E5, instead of the Ser residue of TP21 NS5 (Table 1).

There were three conserved nucleotide changes identified in the 3′-half of the genome of each of these four recovered viruses not shown in Table 1. First, a change of $A_{10,436} \rightarrow G$ occurred in the 3′ noncoding region, and the other two changes were found in nonstructural protein genes NS3 ($A_{5357} \rightarrow G$) and NS5 ($G_{9734} \rightarrow A$), which caused the substitution $Thr_{254} \rightarrow Ala$ in the NS3 protein and substitution $Asp_{691} \rightarrow Asn$ in the NS5 protein. Nucleotide residues G_{5357}, A_{9734}, and $G_{10,436}$ present in the genome of the recovered viruses were also found in the plasmid DNA from which each of the viruses was derived and in the intermediate construct, plasmid p624-3. This suggests that these changes occurred during cloning in *E. coli* and were advantageous for amplification of plasmids containing LGT sequences or these differences might reflect the "quasispecies" of a positive-strand RNA virus. In support of the latter explanation it should be noted that nucleotide variability G/A at position 9734 or position 10,436 had been observed in the consensus sequence of TP21 RT-PCR genome derived from high-titered virus stock.

Sequence analysis also identified the presence of a few additional unique differences from the TP21 consensus sequence in the 5′-half of the genome of each of the four rescued clones (Table 2). Fourteen of the 18 nucleotide differences from the consensus sequence of TP21 were also present in the plasmid DNA from which the four clones were derived. This provided evidence that the clones were indeed derived from cDNA. Of the total of 18 nucleotide differences observed between the consensus sequence of TP21 and the sequence of the four rescued virus genomes, 10 produced an amino acid substitution in structural protein preM or E or in the nonstructural protein NS2A or NS2B. At least three nucleotide changes ($C_{4299} \rightarrow U$ in clones 636 and 689; $A_{590} \rightarrow G$ and $C_{4429} \rightarrow U$ in clone 656 underlined in Table 2) occurred during RNA transcription and transfection of *Vero* cells, or propagation of virus in cell culture, because these mutations were not present in the plasmid DNA templates from which these viruses were derived.

Clone 636 exhibited three nucleotide differences from the TP21 consensus sequence, only two of which resulted in an amino acid change. A substitution $His_{438} \rightarrow Tyr$ located near the transmembrane region of envelope protein E occurred at a position that is highly conserved among all mosquito-borne and tick-borne flaviviruses (Pletnev *et al.,* 1990; Gritsun *et al.,* 1995). Another amino acid change, $Ala_{32} \rightarrow Val$ in the nonstructural protein NS2B, was also present in a significant proportion of virions of clone 689.

Clone 649 was more distinct from parental TP21 virus than the other viruses, because its virus genome contained seven nucleotide differences (Table 2). Three of

these mutations were silent, while the other four caused an amino acid substitution in NS2A or NS2B protein. Possibly these unique mutations were responsible for fourfold reduction in plaque size of 649 clone on $LLCMK_2$ cells compared with the parental TP21 and the other rescued viruses. It is interesting that clones 649 and 689 shared three common nucleotide changes at positions 2230, 3001, and 3599. One of these mutations caused the replacement of Pro_{29} in the N-terminal region of NS2A protein by a Ser residue that is conserved among the TBEV-complex viruses (Pletnev *et al.,* 1990).

An amino acid substitution in structural proteins preM ($Met_{38} \rightarrow Val$) and E ($Asp_{308} \rightarrow Ala$) as well as two silent mutations were identified in clone 656. Since the three-dimensional structure and function of the N-terminal part of preM protein is not known, the role of $Met_{38} \rightarrow Val$ change in preM protein is difficult to assess. Substitution of Asp → Ala at position 308 occurred in domain III of the E protein, which has been proposed to play a role in neurovirulence of tick-borne and mosquito-borne flaviviruses in mice (reviewed in Rey *et al.,* 1995; McMinn, 1997). Also, it was observed earlier that louping ill virus, a TBEV-complex flavivirus, exhibited decreased neuroinvasiveness in mice following a single amino acid substitution Asp → Asn in E protein at position 308 (Jiang *et al.,* 1993). Thus, clone 656 of LGT may offer another opportunity to investigate the effect of mutation (Asp → Ala) at position 308 of E on neuroinvasiveness in mice.

Evaluation of cDNA-derived viruses in mice

Mice were employed as an experimental model to compare recovered LGT clones and their parental virus with respect to level of neuroinvasiveness, i.e., the capacity of virus to spread from peripheral site to central nervous system and cause encephalitis. Initially, adult outbred Swiss mice were injected intraperitoneally (ip) with 10^4 or 10^6 PFU of each virus and mortality was recorded for 28 days (Table 3). Previously, wild-type LGT TP21 strain was shown to be virulent for 3-week-old Swiss mice with an intraperitoneal LD50 of $10^{3.7}$ PFU (Pletnev and Men, 1998). Two clones, 649 and 689 virus, exhibited lower peripheral virulence than their LGT TP21 parent because only 12.5 or 40%, respectively, of adult mice inoculated ip with 200 LD50 (10^6 PFU) developed symptoms of encephalitis and died. Death did not occur when mice were inoculated with 10^4 PFU of 649 virus. These two viruses shared only one common amino acid change $Pro_{29} \rightarrow Ser$ in NS2A protein, which might be associated with reduced peripheral neurovirulence of these rescued viruses in normal mice. The remaining two rescued clones, 636 and 656 virus, together with the attenuated LGT E5 strain were even more attenuated than parental LGT TP21 with respect to neuroinvasiveness. This indicates that these clones and LGT E5 were at least 200-fold less neuroinvasive in normal immunocompetent mice than their LGT TP21 parent.

TABLE 3

Neuroinvasiveness of Parental LGT Strains and cDNA-Derived LGT TP21 Virus Clones in Adult Swiss Mice

Virus	Mortality[a] of mice after ip inoculation with 10^4 or 10^6 PFU of virus	
	10^4	10^6
TP21	5/5 (100%)	4[b]/5 (80%)
E5	0/5	0/5
cDNA-derived TP21 clone		
636	0/5	0/5
649	0/5	1/8 (12.5%)
656	0/5	0/5
689	nt[c]	2/5 (40%)

[a] Number of mice that died/number of mice tested (% mortality).
[b] A mouse survived ip inoculation with TP21 but was paralyzed for 5 days and then recovered.
[c] nt, not tested.

In an earlier study (Pletnev and Men, 1998), the attenuated E5 strain derived from TP21 exhibited peripheral neurovirulence in adult mice only when the amount of virus inoculated was increased to 10^7 PFU or when a more sensitive test system, such as SCID mice, was used to measure neuroinvasiveness. To evaluate neurovasiveness of the more attenuated rescued viruses (clones 636 and 656), SCID mice, that are at least 10^6-fold more sensitive than normal mice for detection of peripheral neurovirulence, were inoculated ip with 10^2 PFU (Fig. 3) (Pletnev and Men, 1998). Clone 636 did not appear to differ in neuroinvasiveness from its TP21 parent in SCID mice. Clone 656 also killed all inoculated mice during the observation period, but the survival time was increased at least twofold. The last 656-inoculated mouse died on Day 34 postinfection, which was 21 days later than the last death of TP21-inoculated mice. This delay in death suggests that clone 656 replicated and spread more slowly in immunodeficient mice. For this reason this clone was studied in greater detail. Its LD50 was evaluated by inoculating groups of five SCID mice ip with 1, 10, or 100 PFU. The LD50 estimate obtained in this manner was 40 PFU. Thus, clone 656 was 10^4-fold less neuroinvasive than TP21 (estimated LD50 for SCID mice of 0.004 PFU) and 6.6×10^2-fold less pathogenic than E5, the attenuated TP21 derivative (estimated SCID mice LD50 of 0.06 PFU) (Pletnev and Men, 1998). However, the level of attenuation of clone 656 was considerably less than that achieved by LGT/Dengue chimeric viruses (Pletnev and Men, 1998) as well as TBEV/DEN4 chimeras (Pletnev *et al.,* 1992, 1993; Dr. J. Huggins, USAMRIID, personal communication). The lack of detectable neuroinvasiveness of the TP21/DEN4 and E5/DEN4 chimeras for SCID mice was confirmed when mice inoculated ip with a dose of 10^5 PFU survived the observation period (Fig. 3).

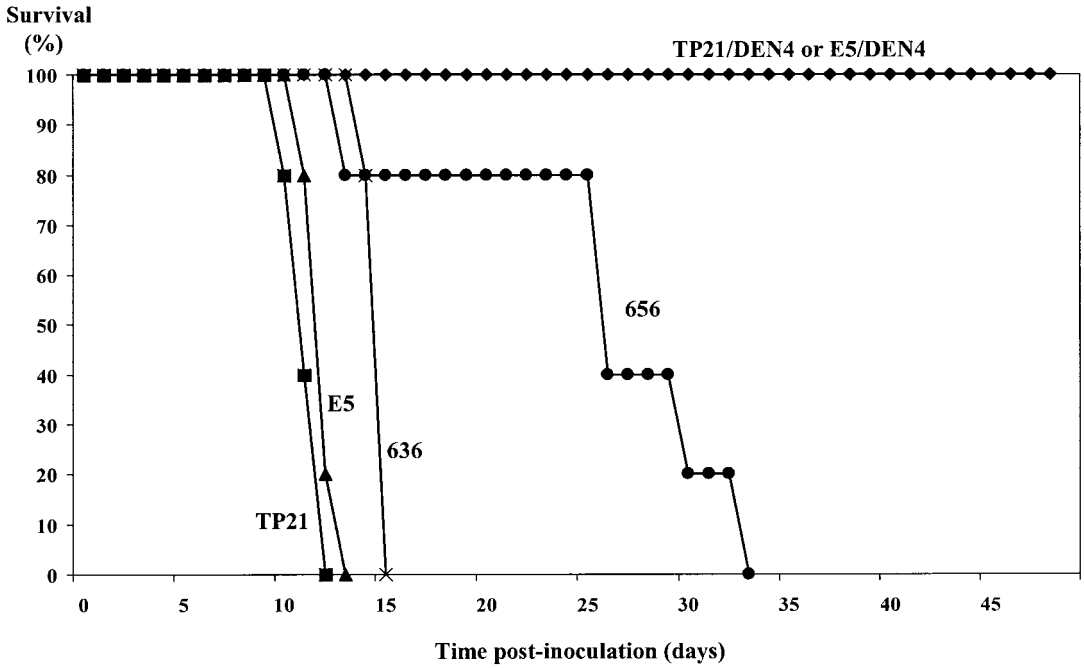

FIG. 3. Test for neuroinvasiveness of two infectious cDNA-derived clones of LGT TP21 in SCID mice. Comparison of mortality following ip inoculation of 10^2 PFU of clone 636 or 656 with that of the uncloned TP21 parental virus and its more attenuated E5 derivative. The previously described TP21/DEN4 and E5/DEN4 chimeras, which were infectious for normal mice, served as virus controls that were fully attenuated in SCID mice. For this reason the chimeras were inoculated ip with a higher dose (i.e., 10^5 PFU).

DISCUSSION

Sequence analysis of RT-PCR-produced cDNA fragments of the genome of wild-type LGT TP21 strain and its attenuated derivative strain E5 allowed us to identify mutations that might be responsible for differences in peripheral neurovirulence of these strains in mice and monkeys as well as differences in growth rate in HeLa cells (Thind and Price, 1966a,b; Nathanson *et al.,* 1968; Price and Thind, 1973). Only seven amino acid differences in the consensus sequences of the polyproteins of these strains were identified (Table 1); four of these changes were observed previously when cDNA fragments of both strains of LGT were cloned in *E. coli* (Pletnev and Men, 1998). Only two of the seven amino acid differences ($Phe_{119} \rightarrow Val$ and $Asn_{389} \rightarrow Asp$) were located in structural protein E. Mutation $Asn_{389} \rightarrow Asp$ was located on the lateral surface of domain III of the E protein, and corresponds to a site at which mutation is thought to attenuate TBEV or Murray Valley encephalitis virus for mice (for reviews, see Rey *et al.,* 1995; Monath and Heinz, 1996; McMinn, 1997).

Identification of one or more mutations responsible for the increased attenuation of LGT strain E5 compared with its parent, LGT strain TP21, can now be approached using reverse genetics. Our success in rescuing LGT TP21 from viral cDNA will allow us to investigate the molecular basis for the observed difference in neuroinvasiveness of LGT TP21 and its tissue culture passage derivative LGT E5 in mice and monkeys (Thind and Price, 1966a,b; Nathanson *et al.,* 1968). Initially, attempts were made to construct full-length infectious cDNA clones of TP21 from cDNA fragments that had been cloned previously in *E. coli* for sequence analysis. RNA transcripts of full-length cDNA clones constructed from these DNA segments uniformly lacked infectivity. The failure of these full-length cDNAs to serve as a template for infectious RNA was probably a manifestation of deviation from the consensus sequence that was favored by the high titer of the virus used to clone cDNA in *E. coli* and the actual cloning procedure in this bacterium. This explanation is consistent with the 18 nucleotide differences (Table 1) that were identified between the sequence of the cloned cDNA fragments of TP21 genome derived from high-titered virus stock and the consensus sequence of TP21 RT-PCR fragments that were not previously cloned in *E. coli* and that were derived from a low-titered virus suspension that had been harvested early in its multicycle growth curve. To minimize the frequency of spontaneous mutation during virus preparation, a low-titered TP21 virus suspension was used for construction of a full-length cDNA genome from long RT-PCR fragments. Success in generating infectious RNA from full-length cDNA of TP21 genome was achieved when this strategy was employed. Virus rescue was considerably more efficient when low-titered virus (3.8×10^3 PFU/ml) was used to prepare full-length cDNAs by long PCR than when high-titered virus (2.4×10^9 PFU/ml) was employed for this purpose. Subsequently, when full-length cDNA, derived from a low-titered TP21 suspension, was constructed from two long overlapping RT-PCR fragments and cloned

in *E. coli*, the RNA transcripts from 11 of the 28 stable cDNA clones were infectious in simian cell culture.

Three nucleotide changes ($C_{4299} \rightarrow U$ in clones 636 and 689; $A_{590} \rightarrow G$ and $C_{4429} \rightarrow U$ in clone 656; Table 2) were identified when the sequence of four of the rescued viruses was compared with the sequence of plasmid DNAs from which the four viruses were recovered. Presumably these four changes resulted from mutations that occurred during rescue of infectious virus from plasmid DNA. Two passages of the recovered viruses were performed before their genomes were sequenced. It is possible that during these passages mutations may have accumulated in the virus population, resulting in differences between sequences of recovered genome and the sequence of cDNA template from which the viruses were derived. However, in a recent unpublished study we determined the genomic sequence of a chimeric Langat E5/TP21 virus recovered from two independent RNA transfections of *Vero* cells. Two separate RNA transcripts were prepared from a cDNA clone and used to transfect *Vero* cells. When the recovered viruses were passaged twice in this cell line, the consensus sequence of the viral genome was determined. We found that sequence of the recovered virus genome in both cases was identical and was also identical to that of the cDNA template. This observation indicates that selection does not take place rapidly during propagation of virus in cell culture. Also, the long-term propagation of cDNA-derived Langat virus strain E5 in *Vero* cells was investigated (data not shown). We found that the first variation in consensus sequence of recovered virus genome appeared only after 11 passages (or about 77 days of virus replication in cell culture posttransfection). Although genomic changes occurred during long-term cultivation of the cDNA-derived E5 virus, we are inclined to consider that the changes we observed in the genome of the recovered TP21 virus clones 636, 656, and 689 take place at an early stage, namely synthesis of RNA transcripts, in which the rate of mutation is determined by the error rate of the SP6 DNA-dependent RNA polymerase ($\sim 10^{-4}$ errors per nucleotide).

The remaining 14 nucleotide changes from the TP21 consensus sequence identified in the rescued viruses were also present in the plasmid DNA from which the four clones were derived (Table 2). This means that these mutations occurred earlier, i.e., before or during assembly of the RT-PCR fragments into full-length plasmid cDNA, possibly even as early as the spontaneous development of sequence polymorphism in the virus suspension ("quasispecies") used for RT-PCR amplification.

It should be noted that six of the seven amino acids of the consensus sequence that differentiated LGT TP21 from its E5 derivative were retained in each of the rescued TP21 clones (Table 1), whereas the seventh TP21/E5 variant amino acid (Lys_{542} in NS5) conserved in each of the clones was that of E5.

The four rescued viruses contained the TP21/E5 consensus sequence (i.e., sequence common to both viruses, Table 1) with the exception that each of the four clones had three nucleotide changes ($A_{5357} \rightarrow G$, $G_{9734} \rightarrow A$, and $A_{10,436} \rightarrow G$), which were also present in plasmid p624-3 and its derivatives, i.e., the full-length cDNA clones from which viruses were recovered. It is possible that these changes in each of the recovered viruses were responsible for the decreased peripheral neurovirulence observed in Swiss mice compared with parental TP21 virus. Clones 649 and 689 caused encephalitis and death in normal mice that were inoculated ip only when a large dose, 10^6 PFU, was used (Table 3). It is interesting that both 649 and 689 virus did not differ from TP21 virus in the amino acid sequence of their structural proteins (Tables 1 and 2), but these rescued viruses shared one common change, $Pro_{29} \rightarrow Ser$, in nonstructural NS2A protein. The presence of mutations in structural proteins of clones 636 and 656 was associated with a somewhat greater reduction of neuroinvasiveness in normal mice. Both of these rescued viruses lacked detectable neurovirulence when immunocompetent mice were inoculated ip with 10^6 PFU. In contrast, when SCID mice were inoculated ip, clone 636, which contained mutation $His_{438} \rightarrow Tyr$ in E protein and $Ala_{32} \rightarrow Val$ in NS2B protein, did not appear to differ from its TP21 parent in neuroinvasiveness; both viruses were highly neuroinvasive. Thus, these two mutations in clone 636 effected a decrease in neuroinvasiveness for normal mice, but did not ablate this property completely when tested in highly permissive SCID mice. During a previous study we observed that SCID mice are 10^6- to 10^7-fold more permissive than normal mice for detection of neuroinvasiveness (Pletnev and Men, 1998).

In SCID mice, clone 656 (LD50 of 40 PFU) was 10^4-fold less neuroinvasive compared with parental TP21 virus (LD50 of 0.004 PFU). However, when SCID mice were inoculated ip with 10^2 PFU (2.5LD50) of clone 656, mean survival time for mice increased twofold. Attenuation of clone 656 is probably the result of unique mutations in structural proteins ($Met_{38} \rightarrow Val$ in preM and $Asp_{308} \rightarrow Ala$ in E protein). The role of each of these substitutions in attenuation of LGT neuroinvasiveness is currently under study. It is of interest, however, that attenuation of another tick-borne flavivirus, louping ill virus, was associated with single amino acid mutation in E protein at position 308 (Asp → Asn) or 310 (Ser → Pro) (Jiang *et al.,* 1993). In addition, mutations, which were located at or near position 308 in E protein, and which had an effect on virulence of mosquito-borne flaviviruses were also observed in yellow fever virus and Japanese encephalitis virus (Schlesinger *et al.,* 1996; McMinn, 1997; Ni and Barrett, 1998). These findings taken together with our observation of demonstrable attenuation of clone 656 suggest that this site plays an important role in virulence.

We also attempted to achieve greater attenuation of LGT by engineering two mutants of clone 656 (data not shown). The transfer of mutation $His_{438} \rightarrow Tyr$ from clone

636 into the sequence of E protein of clone 656 to replace its corresponding sequence, compromised the infectivity of the resulting chimera. Infection by the chimeric 656/636 virus was initiated in 100% of RNA-transfected simian cells but infection did not proceed to maturation and release of infectious virus. The RNA transcripts from another construct, which contained the mutation $Pro_{29} \rightarrow$ Ser in NS2A gene of clone 649 with remaining sequences derived from clone 656, generated viable virus in $LLCMK_2$ cells. A study in Swiss mice showed that this chimeric mutant, similar to its parent 656 virus, did not cause death or encephalitis when normal mice were inoculated ip with a dose of 10^6 PFU. Also, the two viruses did not differ when tested in SCID mice; both produced fatal encephalitis after a prolonged inoculation interval. This indicated that neuroinvasiveness for SCID mice was not reduced when the mutations from clone 656 and the mutation in NS2A protein in clone 649 were combined in a single virus.

Finally, the four recovered viruses exhibited a spectrum of peripheral neurovirulence in mice probably as a result of the different pattern of mutations identified by sequence analysis of the rescued virus genomes. Two rescued viruses (649 and 689) exhibited moderately less neuroinvasiveness for immunocompetent adult Swiss mice compared with their parent TP21, while the remaining two clones (636 and 656) appeared to be neuroinvasive only in SCID mice. The 656 virus retained neuroinvasiveness for SCID mice but it appeared to be attenuated in these immunodeficient mice with respect to its TP21 parent or the E5 strain (Pletnev and Men, 1998). The attenuated E5 strain was initially selected from the TP21 strain by multiple passages in chick embryo cell culture as a potential live virus vaccine candidate to protect against illness caused by the members of TBEV complex (Price *et al.,* 1970). E5 exhibited reduced neurovirulence for monkeys, which was less than that of the 17D vaccine strain of yellow fever virus (Nathanson *et al.,* 1968). The availability of clone 656 infectious cDNA provides a foundation for further studies designed to remove the remaining vestige of neuroinvasiveness for immunodeficient mice, as had been achieved previously by construction of Langat/Dengue chimeric viruses (Pletnev and Men, 1998).

MATERIALS AND METHODS

Cells and virus preparations

Simian *Vero*, $LLCMK_2$, and BHK cells were purchased from the American Type Culture Collection. Cells were grown in MEM with 1% glutamine, 10% fetal bovine serum, 50 μg/ml gentamicin, 0.25 μg/ml fungizone at 37°C, and 5% CO_2. Virus stocks of the Langat (LGT) wild-type strain TP21, its further attenuated E5 mutant, and their TP21/DEN4 and E5/DEN4 chimeras were prepared in *Vero* cells as described previously (Pletnev and Men, 1998).

Vero cells in 150-cm^2 tissue culture flasks were infected with TP21 or E5 virus at a multiplicity of infection of 0.01. After absorption at 37°C for 1 h, virus inoculum was removed and fresh medium was added. The content of a single flask was harvested on Day 1, 2, or 5 following virus infection, and virus titer was determined by plaque assay on *Vero* cells, which were stained with neutral red to visualize plaques 7 days postinfection. Titer of these three virus stocks was 3.8×10^3, 2.2×10^6, and 2.4×10^9 PFU/ml for TP21 and 1.2×10^4, 4×10^6, and 1.2×10^9 PFU/ml for E5.

Reverse-transcription

Virus in supernatant of cell culture medium was precipitated by 8% polyethylene glycol 8000 (US Biochemical Corp., Cleveland, OH) and 0.4 M NaCl overnight at 4°C and collected by centrifugation. Total RNA was extracted from virions using TRI Reagent (Molecular Research Center, Inc., Cincinnati, OH). Reverse-transcription (RT) was performed with SuperScript II Preamplification system (Life Technologies, Gaithersburg, MD) and (1) an oligonucleotide (oligo) (1445) 5′-GCCTGCGGAG*GGTACCGATATC*AGCGGGTGTTTTTCCGAGACACG that is complementary to the LGT sequence at its 3′ terminus, i.e., nucleotides (nts) 10,921–10,943, and contains the *Eco*RV and *Kpn*I site immediately following the LGT 3′ end sequence or (2) an oligo (1087) 5′-CTATGGCCAGGTGGAAAGCCGC that is complementary to the LGT sequence at nts 7256–7277. The latter oligo was used to facilitate transcription of the 5′ end of the genome. Before reverse-transcription, 5–10 μg of RNA and 100 ng of primer were incubated at 70°C for 5 min and then chilled on ice. RT reaction mixtures contained this heat-denatured RNA plus ingredients of Superscript II kit and 200 U reverse-transcriptase in a final volume of 100 μl. The reaction mixtures were incubated at 42°C for 2 h and then frozen and used as a template to generate double-stranded DNA by polymerase chain reaction (PCR) for construction and cloning of cDNA TP21 in *E. coli* or for sequence analysis of RT-PCR-derived viral genome.

PCR

The standard PCR mixture used to produce double-stranded cDNA contained 0.5 μM each of primer pairs, 200 ng plasmid DNA, or 5–10 μl of RT product as a template, 400 μM of dNTPs, 1× Buffer, and 5 U Takara LA *Taq* DNA polymerase (Takara LA PCR kit, PanVera Co., Madison, WI) in a total reaction volume of 100 μl. The reaction mixture was preheated to 94°C for 2 min and then subjected to 30 cycles, each cycle at 98°C for 20 s and 68°C for 15 min.

Sequence analysis of viral genome

The complete sequence of (1) the TP21 genome, (2) the genome of its more attenuated derivative strain E5, and (3) TP21 virus recovered from cDNA was determined

by sequence analysis of four overlapping RT-PCR cDNA fragments, directly derived from virus RNA. The oligo 1445 or 1087 was used as a primer to obtain the first-strand cDNA by reverse-transcription as described earlier. PCR was performed to amplify the four overlapping genome fragments: A (nt 1 to 4192), B (nt 3491 to 7277), C (nt 6131 to 9669), and D (nt 8857 to 10,943) using appropriate primers and Takara LA PCR kit. Primers for PCR and sequence analysis were designed using previously published LGT TP21 strain sequence (GenBank accession no. M86650 and M73835)(Mandl *et al.,* 1991; Iacono-Connors and Schmaljohn, 1992; Pletnev and Men, 1998). PCR products were purified in an agarose gel and isolated using a Qiagen gel extraction kit. Sequence of RT-PCR fragments was determined using Big-Dye Terminator Cycle Sequencing Ready Reaction (PE Applied Biosystems/ABI Prism, Foster City, CA) and a model 310 Genetic Analyzer.

To determine 3′- and 5′-end sequences of viral genome, RNA from rescued virus or from parental TP21 or E5 virus was treated with tobacco acid pyrophosphatase (Epicentre Technol. Co., Madison, WI) to cleave off the cap structure; the 5′ and 3′ termini of viral RNA were then joined using T4 RNA ligase (New England Biolabs, Beverly, MA). Oligo 979 (complementary to LGT sequence at 1149–1167 nt) was used to generate first-strand cDNA by RT. A double-stranded cDNA fragment containing the 5′- and 3′-end junction of genome was amplified by PCR using the primer pair oligo 916, positive-sense primer containing nts 10,349–10,382 of the LGT sequence and oligo 907, negative-sense primer complementary to 955–983 nts. The final PCR product, 1578 nt in length, was then sequenced.

Methods used to construct full-length cDNA clones of LGT TP21

Three different methods were employed (Figs. 1A, 1B, and 1C):

Method A. Subcloned fragments of TP21 genome from high-titered virus preparation. This method employed four overlapping cDNA fragments (Fig. 1A) previously cloned in *E. coli* for use in determining the complete nucleotide sequence of LGT TP21 genome (Pletnev and Men, 1998). These plasmid clones, namely p5 (LGT nts 1 to 983), p44 (nts 930 to 4828), p66 (nts 4539 to 6571), and p76 (nts 6525 to 10,943) were derived from a LGT cDNA library prepared from a high-titered virus suspension, 1.8×10^9 PFU/ml. The DNA fragment p5 was amplified by PCR using the sense primer (oligo 1444) 5′-GAAGGTGGTCTTTGCGGCCGCATCATACACATACGATTTAGGTGACACTATAGAGATTTTCTTGCGCGTGCATGC, which included the *Not*I site and the SP6 promoter immediately upstream of the 1–22 nts of the 5′ end of genome, while the negative-sense primer (oligo 907) 5′-GGGTGCATCTCGACGCGTAGGCCGGTACC was complementary to LGT nts 955–983 and contained *Kpn*I cleavage site. The PCR product was digested by a *Not*I and a *Kpn*I and ligated to similarly digested p44. The resulting large subgenomic clone (p49), representing the TP21 genome from its 5′ terminus to nt 4828 and containing a unique *Apa*I cleavage site at position 4801, was sequenced in its entirety. The *Eco*RV and *Kpn*I cleavage sites were incorporated into the TP21 cDNA immediately downstream of the 3′ end of genome during of construction of p76 plasmid. LGT cDNA fragments from p66 and p76 were joined to form a larger cDNA clone by ligation using a unique *Nsi*I restriction site (position 6551 of LGT genome) and a *Pvu*I site of the vector. The resulting clone p77 containing the 3′-half of the genome (from nt 4539 to the 3′ end of genome plus *Eco*RV and *Kpn*I site) was sequenced and then used for assembly of the final plasmid construct. Construction of full-length TP21 cDNA was completed by ligation of plasmid p77, which had been digested with *Not*I and *Apa*I, and the *Not*I-*Apa*I fragment of p49. Ten stable individual full-length cDNA clones were identified after transfection of *E. coli* strain BD1528 with ligation mixture. Screening of plasmids indicated that these stable clones exhibited the expected restriction pattern when tested with *Eco*RI, *Pst*I, and *Sal*I.

In addition, a separate set of full-length cDNA clones was prepared using the plasmid cDNA designated p76 (nts 6525 to 10,943) ligated to a long PCR cDNA fragment that included the 5′ terminal nucleotide (nts 1 to 6571) and overlapped p76 (Fig. 1A). The longest cDNA clones produced in this manner by ligation were cloned in *E. coli*. Two stable full-length cDNA clones were recovered.

Before RNA transcription, each of the 12 pFL-TP21 plasmids selected for apparent genome length were digested with *Eco*RV and precipitated with ethanol after phenol-chloroform extraction of proteins.

Method B. Long RT-PCR cDNA of viral genome. Method B employed long RT-PCR in a separate attempt to derive viral genome-length cDNAs. Two different virus suspensions were used as the source for viral cDNAs; one suspension harvested on Day 2 had a low titer (3.8×10^3 PFU/ml), while the other suspension harvested on Day 5 had a high titer (2.4×10^9 PFU/ml). The PCR mixture contained primers (oligo 1444 and 1445), 10 μl of RT product as a template, and Takara LA PCR kit ingredients, including 5 U DNA polymerase. The PCR products, approximately 11 kb in length, were separated from lower molecular weight DNA by electrophoresis (Fig. 2) in an agarose gel and isolated from gel using a Qiagen gel extraction kit. Before use as a template for RNA transcription, the PCR product was digested by *Eco*RV and purified by phenol-chloroform extraction. RNA transcripts ($\sim$1 μg) of these cDNAs were then transfected into *Vero* cell culture, which was then monitored for evidence of infection by immunofluorescence.

Method C. Construction of full-length cDNA clones from two overlapping PCR fragments derived from low-titered virus. This method employed two overlapping cDNA fragments that included the entire sequence of

TP21 (Fig. 1C). These fragments were derived from a virus suspension of low titer (3.8×10^3 PFU/ml). For the 5′-half of LGT genome, a PCR product was generated by using the sense primer (oligo 1444), which included the *Not*I site, the SP6 promoter, and 1–22 nts of LGT sequence and the negative-sense primer (oligo 1023) 5′-CCCAGGGTTGCAAGCCCCAGG that was complementary to LGT nts 6637–6657. PCR was employed to derive the 3′-half of LGT genome using the positive-sense primer (oligo 971) 5′-TTGCACCTGACTGAACTGGAG that was complementary to LGT nts 4451–4471 and oligo 1445 as a negative-sense primer.

Initially, the full-length cDNA genome was constructed by joining these two PCR fragments using *Not*I and *Kpn*I cleavage sites of p2A(*Xho*I) vector (Bray and Lai, 1991) and a unique *Apa*I site, present in both RT-PCR fragments (Fig. 1). However, this strategy to prepare full-length cDNA failed because the clones were unstable in *E. coli*. Subsequently, the two long fragments were assembled into a stable full-length cDNA in a two-step cloning procedure.

During the first step, the PCR product representing the 3′-half of genome (nts 4451 to 10,943) was cloned in *E. coli* using plasmid p51 as a vector. Plasmid p51 was created by inserting a small *Bam*HI-*Pst*I fragment (nts 4539 to 5349) of TP21 cDNA, which was obtained by PCR using appropriate primers, into a unique *Bgl*II and *Pst*I site of the p5′-2(*Not*I, *Xho*I, Δ*Hin*dIII) vector (Cahour *et al.,* 1995). In addition, plasmid p51 contained a *Not*I cleavage site, SP6 promoter, and the first 88 nts of Dengue type 4 virus genome. It was used as a vector because it possessed a unique *Apa*I cleavage site. The PCR product (LGT nts 4451–10943) was digested with *Apa*I and *Kpn*I and then cloned into the *Apa*I-*Bam*HI-digested region of the p51 vector together with a *Kpn*I-*Bam*HI fragment derived from p5′-2A(*Xho*I). The latter fragment derived from p5′-2A(*Xho*I) (Bray and Lai, 1991) was used only to facilitate cloning of the PCR *Apa*I-*Kpn*I fragment into p51 vector, which does not contain a unique *Kpn*I site. After transformation of bacteria with ligation mixture, a clone (p624-3) that contained LGT nts from 4539 to the 3′ end of genome was selected based on its restriction pattern.

During the second step, the PCR product representing the 5′-half of genome (nts 1 to 6657) was digested with *Not*I and *Apa*I and then cloned into p624-3, generating full-length cDNA clones of pTP21. Twenty-eight individual full-length LGT TP21 cDNA clones were stable in the plasmid vector when propagated in *E. coli* strain BD1528. However, some polymorphism was observed among the stable full-length LGT cDNAs with respect to restriction enzyme digestion pattern. The viral sequences of four plasmids that served as template for infectious RNA transcripts were verified. These four plasmids were designated as pTP21-636, pTP21-649, pTP21-656, and pTP21-689 and the corresponding number was used to designate rescued virus.

RNA transcription, transfection, and recovery of virus

Each of the 28 stable pTP21 plasmids containing full-length TP21 cDNA produced by Method C (Fig. 1) was linearized with *Eco*RV, extracted with phenol-chloroform, and ethanol-precipitated. For *in vitro* RNA synthesis, the transcription reaction mixture contained 5 μg of linearized DNA; 1 mM cap analog $m^7G(5')ppp(5')G$ (New England BioLabs, Beverly, MA); 0.5 mM each ATP, CTP, and UTP; 10 mM DTT; 1× polymerase buffer; 100 U of RNase inhibitor; 50 U of SP6 RNA polymerase (Promega, Madison, WI) in a volume of 100 μl. The reaction mixture was incubated at 37°C for 1 h, and the DNA template was then digested with 3 U of RQ1 DNase (Promega, Madison, WI) for 10 min at 37°C. The typical yield of RNA was approximately 10 μg as determined by agarose gel electrophoresis analysis.

RNA transcripts of the full-length LGT constructs were used to transfect subconfluent monolayers of simian *Vero* or $LLCMK_2$ cells or hamster BHK cells in the presence of transfection reagent LipofectAmine (Gibco BRL, Gaithersburg, MD) or DOTAP (Boehringer Manheim, Indianapolis, IN) as described previously (Pletnev *et al.,* 1993). On Day 5 and again on Days 10, 15, and 20, cells were split and passaged. Cells cultured in slide chambers were examined on each of these occasions by immunofluorescence assay (IFA) for the presence of LGT proteins using a LGT-specific mouse antiserum or LGT-specific hyperimmune mouse ascitic fluid (HMAF). When 80–100% of cells were positive as indicated by IFA, the contents of infected T-75 flasks were collected, frozen, and later used for characterization of cDNA-derived LGT virus. These recombinant LGT viruses were amplified twice in simian *Vero* cells, after which viral RNA was isolated and reverse-transcribed for cDNA amplification and sequence analysis. The procedures used for plaque assay, analysis of replication in cell culture, and radioimmunoprecipitation of virus-specific proteins were described earlier (Pletnev *et al.,* 1992, 1993).

Evaluation of cDNA-derived viruses in mice

Peripheral neurovirulence ("neuroinvasiveness") of *Vero* cell culture-derived parental and cloned TP21 viruses was evaluated in 3-week-old outbred Swiss mice that were inoculated by the intraperitoneal (ip) route in groups of five with 10^4 or 10^6 PFU of virus and observed for 28 days for fatal or nonfatal encephalitis. A considerably more sensitive assay for neuroinvasiveness of LGT virus that used immunodeficient (SCID) mice was also employed for analysis of this important virulence phenotype (Pletnev and Men, 1998). In this assay, female 3-week-old C.B.-17 Icr/*scid*/*scid* mice (Taconic Farms, Germantown, NY) in groups of five were inoculated ip with (1) 10^2 PFU parental TP21, or E5, or TP21 derived

from cDNA; or (2) with 10^5 PFU of chimeric TP21/DEN4 or E5/DEN4 virus; or (3) decimal dilutions of rescued recombinant virus ranging from 1 to 10^2 PFU. These mice were observed for mortality for 7 weeks.

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