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# Spontaneous Mutations Restore the Viability of Tick-Borne Encephalitis Virus Mutants with Large Deletions in Protein C

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**The capsid protein, C, of tick-borne encephalitis virus has recently been found to tolerate deletions up to a length of 16 amino acid residues that partially removed the central hydrophobic domain, a sequence element conserved among flaviviruses which may be crucial for virion assembly. In this study, mutants with deletion lengths of 19, 21, 27, or 30 residues, removing more or all of this hydrophobic domain, were found to yield viable virus progeny, but this was without exception accompanied by the emergence of additional mutations within protein C. These point mutations or sequence duplications were located downstream of the engineered deletion and generally increased the hydrophobicity, suggesting that they may compensate for the loss of the central hydrophobic domain. Two of the second-site mutations, together with the corresponding deletion, were introduced into a wild-type genetic backbone, and the analysis of these "double mutants" provided direct evidence that the viability of the deletion mutant indeed depended on the presence of the second-site mutation. Our results corroborate the notion that hydrophobic interactions of protein C are essential for the assembly of infectious flavivirus particles but rule out the possibility that individual residues of the central hydrophobic domain are absolutely required for infectivity. Furthermore, the double mutants were found to be highly attenuated and capable of inducing a protective immune response in mice at even lower inoculation doses than the previously characterized 16-amino-acid-residue deletion mutant, suggesting that the combination of large deletions and second-site mutations may be a superior way to generate safe, attenuated flavivirus vaccine strains.**

*Tick-borne encephalitis* (TBE) *virus* is a representative of the genus *Flavivirus* (family *Flaviviridae*), which also includes several other important human pathogens, such as yellow fever virus, Japanese encephalitis virus, West Nile virus, and the dengue viruses (33). Flaviviruses are small, round, enveloped particles that contain only three structural virus proteins, i.e., the membrane-anchored surface proteins M and E and the capsid protein, C (18). The last is a relatively small protein (approximately 11 kDa) with a large content of positively charged amino acid residues that shows significantly less sequence homology among members of the genus than do the other two structural proteins (21). Nevertheless, a number of characteristics of its amino acid sequence, including the clustering of basic residues in certain sections of the sequence, the presence of two distinctive hydrophobic segments located approximately in the center of the sequence and at the carboxy-terminal end, and a predicted high propensity to adopt a predominantly alpha-helical conformation, are generally maintained among different flaviviruses, suggesting an overall conserved structural and functional organization of protein C (14, 18, 25).

In the absence of protein C, the surface proteins M (which is first synthesized as a precursor protein, prM) and E can assemble into capsidless subviral particles (1, 15, 26). These are smaller than virions and typically also arise in variable amounts as by-products of normal flavivirus infections (31) but probably do not contain nucleic acids (32). Although protein C presumably plays an essential role in the packaging of the viral genome (a single positive-stranded RNA molecule approximately 11 kb long that encodes all proteins in a single long open reading frame) and the assembly of infectious virus particles, very little is known about the molecular basis of its functionality (18).

In contrast to the viral surface proteins, for which considerable tertiary and quaternary structure information is available (5, 16, 30), the structural organization and oligomeric arrangement of protein C is still unknown. The well-documented icosahedral symmetry of the outer surface of flaviviruses suggests that the flavivirus nucleocapsid is also icosahedral, but cryo-electron microscopy analyses of dengue virus indicate that the capsid protein is poorly ordered or that the orientation of the core as a whole is somewhat variable relative to the external glycoprotein scaffold (16).

The present knowledge of molecular determinants that govern the packaging of the viral genome is also limited. No distinct sequence elements of protein C have so far been found to be critical for packaging, although two regions (within the amino-terminal third of the protein and around residues 80 to 100, respectively) that are especially rich in arginine and lysine residues have been shown experimentally to be involved in RNA binding (13). A specific packaging signal on the genomic RNA also has not been identified.

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TABLE 1. Primers used for mutagenesis

| Orientation | Sequence[a] | Amino acids deleted |
|---|---|---|
| Sense | 5′-TTTACGCGT-Δ-TGGCATGCCGTAGCCGGCACC-3′ | Q28–L46 |
| Sense | 5′-TTTACGCGT-Δ-AGAAACCCCGTATTGAAGGCG-3′ | Q28–A54 |
| Sense | 5′-TTTACGCGT-Δ-GTATTGAAGGCGTTCTGG-3′ | Q28–P57 |
| Antisense | 5′-AGCGTAAACCGGTGCCAAAC-3′ | |

[a] Restriction enzyme recognition sequences (ACGCGT, *Mlu*I; ACCGGT, *Age*I) are underlined; Δ, position of the deletion relative to the wild-type sequence.

Moreover, the processes that lead to the proper envelopment of the nucleocapsid with a membrane containing the viral surface proteins are essentially unresolved. The predicted cytoplasmic domains of proteins M and E are very short, and it is therefore questionable whether these elements are capable of providing the necessary specific association with the nucleocapsid. It has been proposed that hydrophobic interactions between protein C and the membrane may be crucial for virus assembly (14, 25). In the primary translation product, the carboxy-terminal end of protein C, which functions as an internal signal sequence for the subsequent protein, prM, spans the membrane of the endomplasmic reticulum (ER). This hydrophobic tail is removed during polyprotein processing by the action of the viral protease NS2B/3 (18). Membrane association of the mature protein C may be mediated by its second hydrophobic element, the central hydrophobic domain, which exhibits features reminiscent of a signal sequence and has been proposed to be inserted in the membrane in a hairpin conformation (25). Thus, it is feasible that the principle contacts between the nucleocapsid and the envelope proteins may occur within the membrane.

In a recent study (14), the analysis of protein C of TBE virus was begun using a site-specific-mutagenesis approach. The introduction of various deletions into the full-length genome of TBE virus that increasingly removed parts of the central hydrophobic domain of protein C revealed a remarkable structural and functional flexibility of this protein. In the case of TBE virus, the central hydrophobic domain extends from residues 34 to 51 and is predicted to fold as an alpha-helix essentially over its entire length. Mutants carrying deletions extending from residue 28 up to residue 43 were found to be viable in BHK-21 cells, whereas expanding the deletion up to residue 48 resulted in the loss of viability. That study also indicated that the deletions impaired the assembly or stability of infectious virions, whereas all of the mutants produced substantial amounts of capsidless subviral particles, which are excellent immunogens (7).

In this study, we describe the isolation of viable mutants of TBE virus carrying deletions extending to residues 46, 48, 54, and 57, demonstrating that infectious particles can be formed even if the entire central hydrophobic domain of protein C is removed. All of the viable mutants, however, acquired additional mutations within protein C, and these generally increased the protein's hydrophobicity. For two examples, experimental evidence is provided that these mutations are responsible for the restoration of viability of the deletion mutants. The biological characterization of these two mutants shows them to be promising candidates for further vaccine development studies.

## MATERIALS AND METHODS

**Virus and plasmids.** Western subtype TBE virus strain Neudoerfl has been characterized in detail, and its complete genomic sequence (21, 22) is available under GenBank accession number U27495. This strain was used as the wild-type control in all experiments, and all of the mutants were derived from it. Genome-length RNA was synthesized in vitro from an infectious cDNA clone of TBE virus strain Neudoerfl (20) or its mutated derivatives. Plasmid pTNd/c contains a full-length genomic cDNA insert, and RNA transcribed from this plasmid was used as the wild-type control in RNA transfection experiments. Plasmid pTNd/5′ contains cDNA corresponding to the 5′ one-third of the genome. Mutations were engineered into plasmid pTNd/5′ and then recloned into the full-length plasmid pTNd/c.

**Cloning and sequencing procedures.** The deletion mutants were constructed following the same experimental strategy applied in a previous study (14). Briefly, the *Mlu*I-*Age*I fragment of plasmid pTNd/5′ was replaced by a PCR fragment containing the desired mutation(s). The sequences of the mutagenic sense primers and the wild-type antisense primer that were used for the construction of mutants C(Δ28-46), C(Δ28-54), and C(Δ28-57) are listed in Table 1. For the construction of plasmids containing both a deletion and a second-site mutation, appropriate fragments were prepared by reverse transcription (RT)-PCR from virions harvested from cell culture supernatants infected with the passaged mutant strain containing the desired mutations. As before, *Mlu*I and *Age*I were used for fragment swapping to introduce the mutations into plasmid pTNd/5′. The mutations were subsequently introduced into the full-length cDNA clone pTNd/c by taking advantage of the unique cleavage sites for the restriction enzymes *Sal*I, located upstream of the TBE 5′ end, and *Sna*BI, at position 1883 in the TBE genome sequence (20). Plasmids were amplified in *Escherichia coli* strain HB 101, and small- and large-scale plasmid preparations were made using Qiagen purification systems. All constructs were checked by sequence analysis, including at least the region synthesized by PCR and the vicinity of the utilized insertion sites. Sequence analysis of viral genomic RNA was performed by RT-PCR as described previously (38). Sequencing was performed using an automated DNA-sequencing system (ABI).

**RNA transcription and transfection and measurement of protein E expression.** RNA was transcribed from full-length cDNA clones using T7 polymerase (Ambion) and introduced into BHK-21 cells by electroporation under the same conditions described previously (20). Under these conditions, RNA transcribed from the wild-type clone pTNd/c yields between $10^5$ and $10^6$ infectious units/μg. Transfection of cells was performed using between 10 and 30 μg of RNA. Protein E expression was detected by indirect immunofluorescence staining after fixation of cells with acetone-methanol (1:1) using a polyclonal rabbit anti-protein E serum and fluorescein isothiocyanate-conjugated anti-rabbit antibody. The release of protein E into cell culture supernatants was monitored by a four-layer enzyme-linked immunosorbent assay (ELISA) (10). Protein E concentrations were measured by means of a previously described quantitative ELISA after the proteins were denatured with sodium dodecyl sulfate (SDS-ELISA) (9).

**Cell cultures.** BHK-21 cells, porcine kidney (PS) cells, and primary chicken embryo (CE) cells were grown under standard conditions (11, 20). Plaque assays were performed on PS cells as described previously (11). Infectivity titers of mouse brain suspensions were determined on BHK-21 cells (for which no plaque assay is available) by endpoint dilution infection experiments. Virus preparations were diluted in 0.5-log-unit steps and used to infect cells in 96-well culture plates. The culture medium was tested for virus production at 3 and 6 days postinfection by a four-layer ELISA. Viral growth curves were determined on CE cells essentially as described elsewhere (20). Briefly, cells were infected at a multiplicity of infection (MOI) of approximately 1, and virus released from the cells within 1-h periods was collected from the supernatant at several times postinfection. Infectivity released into the supernatant was quantified by duplicate endpoint dilution infection experiments on CE cells.

**Passaging experiments.** Passages on BHK-21 cells were performed by transferring aliquots (200 μl) of cell culture supernatants cleared of cell debris and insoluble material by low-speed centrifugation to fresh BHK-21 cells grown in 24-well cluster plates. In the cases indicated in the text, protein E concentrations were determined by SDS-ELISA (9) and then adjusted to contain the desired amount of viral protein. In some passaging experiments, samples were diluted prior to infection of new BHK-21 cells to achieve a smaller MOI.

Litters of suckling mice (age, 0 to 1 day) were inoculated intracerebrally with 20-μl aliquots of undiluted cell culture supernatants per mouse. The mice were sacrificed at the time of onset of severe clinical symptoms (4 to 8 days postinfection), and 20% (wt/vol) pooled suspensions of the suckling mouse brains were prepared. For the second passage, these suspensions were diluted 10-fold and used to infect suckling mice and subsequently to prepare virus suspensions as before. In some passaging experiments, mice were also inoculated with 10- or 100-fold dilutions of cell culture supernatants, and suspensions were prepared from individual mice and transferred to new litters of mice.

**Animal model.** The characterization of mutant viruses in the animal model was performed as in previous studies (19, 23, 24). Briefly, groups of 10 5-week-old (body weight, approximately 15 to 17 g) outbred Swiss albino mice were inoculated subcutaneously, and survival was recorded for 28 days. The mice were then bled, and seroconversion was detected by a TBE virus antibody ELISA (8). For the determination of the 50% lethal dose ($LD_{50}$) and the 50% infectious dose ($ID_{50}$), mice were inoculated with various doses ranging from $10^0$ to $10^6$ PFU. The calculation of $LD_{50}$s and $ID_{50}$s was performed by the method of Reed and Muench (29). Infection of mice was determined on the basis of the detection of a TBE virus-specific antibody response in the sera of surviving mice. Surviving mice without detectable seroconversion were scored as uninfected. To test whether seroconverted mice had developed protective immunity, the mice were inoculated with a challenge dose of >100 $LD_{50}$s of the highly virulent TBE virus strain Hypr (37).

**Computer-assisted sequence analysis.** Transmembrane segment prediction analysis was done using the program Toppred2 (http://bioweb.pasteur.fr/seqanal/interfaces/toppred.html) (Kyte-Doolitle scale; kingdom *Eucaryotae*; full window size, 21; core window size, 11; cutoff for putative transmembrane segments, 0.6) (4, 35).

## RESULTS

**Generation of capsid deletion mutants.** A set of five mutants of TBE virus carrying deletions in protein C was employed in this study. The amino acid sequence of protein C contains two characteristic hydrophobic regions, i.e., the central hydrophobic domain and the carboxy-terminal signal sequence for the translocation of protein prM, and four predicted alpha-helical regions (14). As illustrated in Fig. 1, the deletions start with the same residue (Q28) preceding the central hydrophobic domain and helix I and increasingly remove part or all of this domain. In a previous study (14), the 16-residue deletion mutant [C(Δ28-43)] was shown to be viable in BHK-21 cells, whereas the 21-residue deletion mutant [C(Δ28-48)] could not be passaged in these cells but produced subviral particles. Three additional deletions have now been engineered, one of them with an intermediate length of 19 residues [C(Δ28-46)] and two larger ones [C(Δ28-54) and C(Δ28-57)] that removed all of the central hydrophobic domain and helix I and some additional flanking amino acid residues. All of the deletions were introduced into the full-length cDNA clone of TBE virus strain Neudoerfl (20).

**Isolation of viable mutant virus progeny.** Full-length RNAs of the five deletion mutants were transcribed in vitro and introduced into BHK-21 cells by electroporation. RNAs derived from the wild-type infectious cDNA clone and a replication-deficient mutant carrying a large deletion in the polymerase gene (ΔNS5) (14) were used as controls. Intracellular expression of protein E was determined by immunofluorescence 2 days postelectroporation (Fig. 2, left column). All of the mutants, as well as the wild-type control, were found to be competent for protein E expression, whereas no protein E was detected in the case of the replication-deficient control RNA. Analysis of the cell culture supernatants by ELISA demonstrated that all of the mutants exported significant amounts of protein E, as did the wild-type control.

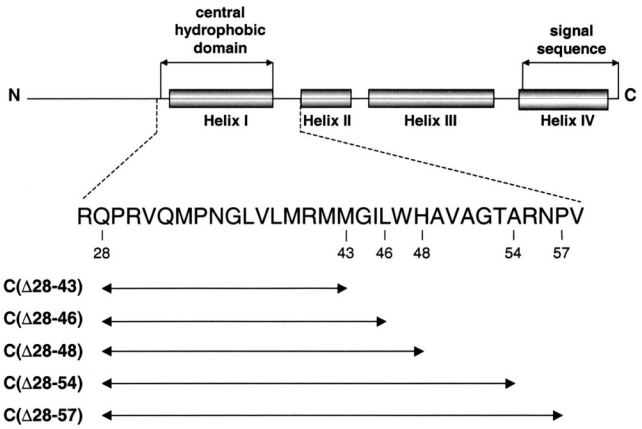


FIG. 1. Positions of introduced deletions in protein C. The schematic (top) illustrates the locations of four predicted alpha-helices and the two hydrophobic domains (the central hydrophobic domain and the signal sequence directing protein prM into the lumen of the ER) (14). An expanded view (below) shows the wild-type amino acid sequence of the region carrying the deletions, the exact positions of which are indicated by arrows and sequence numbers. The designations of the corresponding viral mutants are also shown.

The protein E concentrations of these supernatants were measured using a quantitative ELISA system, and then aliquots containing equal amounts of protein E (20 ng) were transferred to fresh BHK-21 cell cultures to assay for the presence of infectious particles. Infection of cells was determined after 2 days by immunofluorescence and ELISA (Fig. 2, middle column). The wild-type and mutant C(Δ28-43) efficiently infected these new cells, and protein E was released in considerable amounts. In contrast, only a few positive cells and no significant amount of exported protein E were detected in the case of mutant C(Δ28-46). No evidence for infectivity was obtained for any of the other mutants or the negative control. Subsequently performed additional cycles of transferring supernatants to fresh cells and monitoring infection showed that, as expected, both wild-type virus and mutant C(Δ28-43) could be repeatedly passaged in BHK-21 cells. In spite of its low efficiency in the first round of infection, mutant C(Δ28-46) could also be successfully carried through these additional passages, during the course of which its infectivity apparently increased (not shown). No infectious particles could be recovered in the cases of the other three mutants in these additional passaging experiments.

It has been shown previously that intracranial inoculation of suckling mice is the most sensitive infection system for TBE virus. Suckling mice can be infected at approximately 100-fold-higher dilutions of virus than commonly used cell cultures such as BHK-21 cells (23). To assess the viability of the capsid deletion mutants in this system, litters of suckling mice were inoculated with aliquots of supernatants harvested from BHK-21 cells transfected with the mutant RNAs. In contrast to

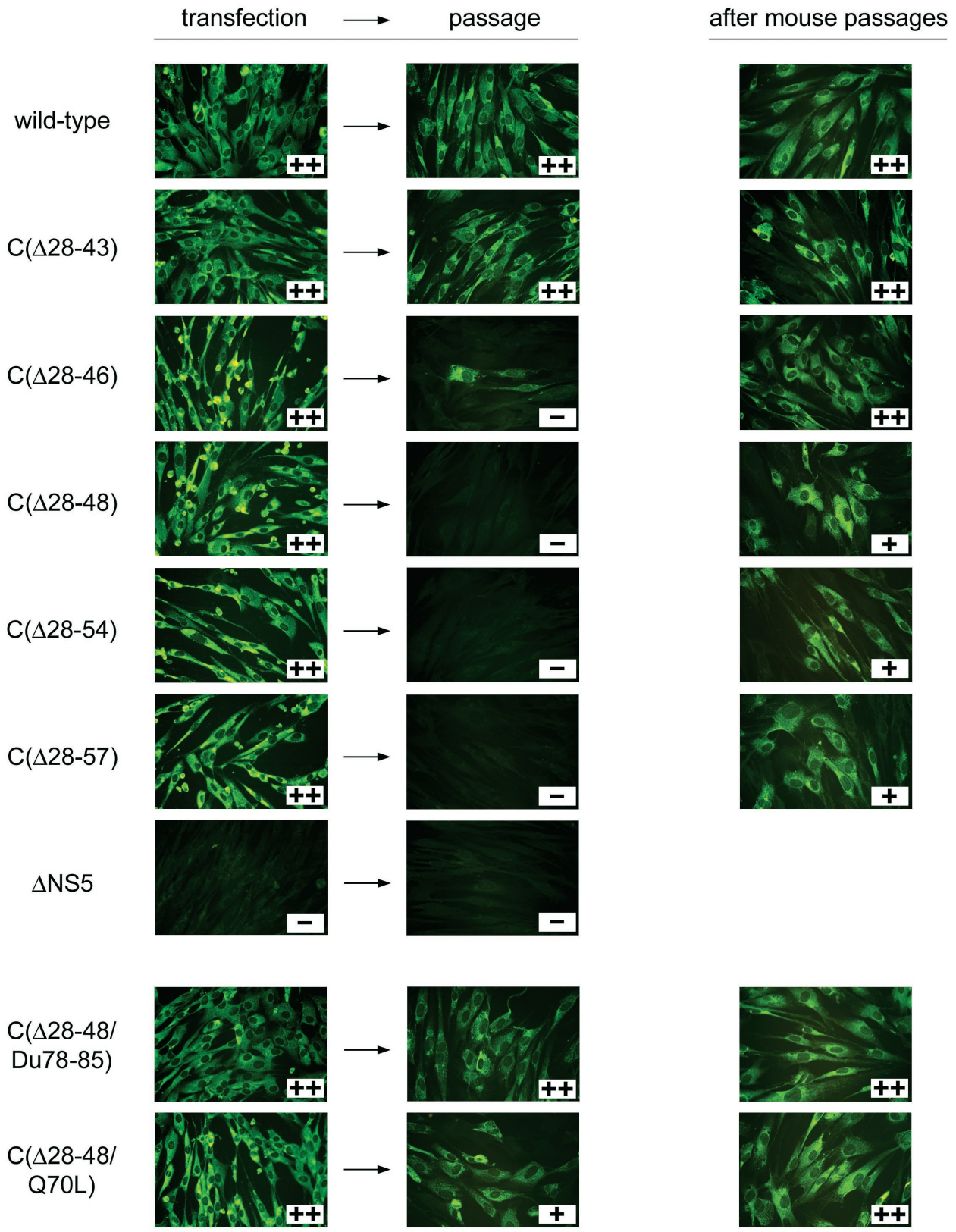
transfection
passage
after mouse passages
wild-type
C(Δ28-43)
C(Δ28-46)
C(Δ28-48)
C(Δ28-54)
C(Δ28-57)
ΔNS5
C(Δ28-48/ Du78-85)
C(Δ28-48/ Q70L)
++
+
−

FIG. 2

the corresponding experiment in BHK-21 cells, all five of the mutants successfully infected these mice, as revealed by the appearance of typical encephalitic symptoms in almost all of the inoculated animals. The period until the onset of severe symptoms varied between 4 days in the case of mutant C(Δ28-43) and approximately 8 days for the mutants with larger deletions. Virus was harvested from the brains of the diseased animals and subjected to a second round of infection of suckling mice. Virus suspensions prepared from this second suckling mouse passage were then tested for the capability to infect BHK-21 cells. As before, infection of the cells was monitored by immunofluorescence and ELISA. The results, shown in Fig. 2 (right column), demonstrated that all of the mutants were now able to infect these cells. Limiting-dilution experiments on BHK-21 cells indicated infectivity titers of these brain suspensions ranging between $4 \times 10^6$ and $4 \times 10^7$ IU/ml for the five deletion mutants compared to a titer of $3 \times 10^8$ IU/ml determined for a similarly prepared brain suspension of suckling mice infected with wild-type virus. All of the mutants could now be repeatedly passaged in BHK-21 cells (not shown).

In summary, fully viable virus progeny were obtained from all of the five deletion mutants if two passages in suckling mice were performed. In contrast, only the two smallest deletions yielded viable virus when passages were performed solely in BHK-21 cells.

**Appearance of additional mutations in protein C.** To investigate the possibility that genetic alterations may have arisen during the passages, the protein C coding region of the mutants was sequenced by RT-PCR. Sequences were determined after the viruses had been subjected to two passages in suckling mice and three subsequent passages in BHK-21 cells. In addition, the two mutants with smaller deletions, C(Δ28-43) and C(Δ28-46), were also analyzed after having been subjected to three passages in BHK-21 cells only.

These sequence analyses confirmed the presence of the engineered deletions in all of the passaged mutants but also revealed the presence of additional mutations that apparently had arisen during the passages. Several further passaging experiments were performed, and the derived virus isolates were also sequenced. In summary, these experiments yielded a multitude of different spontaneous mutations within protein C, all of which are summarized in Fig. 3. In most of the cases, single point mutations had evolved, but there were also combinations of two or three point mutations or in-frame sequence duplications. All of the observed nucleotide changes caused alterations at the amino acid level, suggesting that they were not stochastic events but had evolved under selective pressure. Only in the case of the mutant with the smallest deletion, C(Δ28-43), were no additional mutations detected in protein C, irrespective of whether it had been passaged only in BHK-21 cells or in suckling mice and cell culture. In the cases of the other four deletion mutants, additional mutations arose without exception in each passaging experiment. In the case of mutant C(Δ28-46), the same mutation was selected both when it was passaged only in BHK-21 cells and when it was passaged in mice and cell culture. Some of the mutations, such as K79I or T81M, occurred more than once and in combination with different engineered deletions. All of the mutations, as depicted in Fig. 3, were located downstream of the engineered deletion [with the exception of the sequence duplication found in mutant C(Δ28-57), which included amino acid residues on both sides of the deletion]. Notably, amino acids were generally changed into more hydrophobic residues, e.g., polar or charged residues, such as Q, T, or K, were replaced by hydrophobic residues, such as L, M, or I, or the small hydrophobic amino acid V was replaced by a larger nonpolar residue, F.

**Second-site mutations in protein C restore viability.** To obtain direct evidence that the second-site mutations in protein C were responsible for restoring the viability of the deletion mutants, we took advantage of the infectious cDNA clone to construct mutants that contained a deletion and a second-site mutation in protein C in a defined wild-type genetic background. Two "double mutants" were constructed, each carrying the 21-amino-acid-long deletion present in mutant C(Δ28-48) in combination with two types of additional mutations that occurred during the passaging of this mutant (Fig. 3), i.e., a point mutation (Q70L) and a duplication (I78 to L85). The resulting double mutants were designated C(Δ28-48/Q70L) and C(Δ28-48/Du78-85).

Transfection of BHK-21 cells with the RNAs of these mutants transcribed in vitro from the correspondingly mutated full-length cDNA clones and detection of protein E expression by immunofluorescence and ELISA confirmed their functional integrity (Fig. 2, left column, bottom two images). Aliquots of supernatants standardized as before to contain 20 ng of protein E were transferred to fresh BHK-21 cells, and infection was monitored by immunofluorescence and ELISA. In contrast to the mutant carrying only the deletion [C(Δ28-48)], the double mutants efficiently infected BHK-21 cells (Fig. 2, middle column). Two additional passages in BHK-21 cells confirmed the full viability of these double mutants (not shown). This result demonstrated that the second-site mutations within protein C restored the viability of the deletion mutant.

The double mutants were also passaged twice in suckling mice to prepare high-titer stocks (the infectivity titers amounted to $5 \times 10^7$ and $1 \times 10^8$ PFU/ml, respectively) for subsequent biological characterizations. Sequence analysis of the entire structural protein coding region performed after passages in BHK-21 cells or suckling mice revealed no further

FIG. 2. Viability of mutants in BHK-21 cells. (Left column) In vitro-transcribed RNAs of the wild-type or mutant genomes, as indicated, were introduced into BHK-21 cells by electroporation and tested by immunofluorescence for intracellular expression of protein E 48 h posttransfection, and protein E released into the supernatants was detected by ELISA. The results of ELISA are given in the insets as follows: −, optical density (OD) < 0.1; +, OD = 0.1 to 1.0; ++, OD > 1.0. (Middle column) Supernatants containing equal amounts of protein E (20 ng, except ΔNS5, in which undiluted supernatant was used) were used to inoculate fresh BHK-21 cells as indicated by the arrows. Infection of these cells was detected by immunofluorescence and ELISA 48 h postinoculation as before. (Right column) Supernatants from transfected BHK-21 cells were passaged twice in suckling mice. Then, BHK-21 cells were infected with a 1,000-fold dilution of virus suspensions prepared from the second suckling mouse passage, and infection was determined 48 h postinoculation as before.

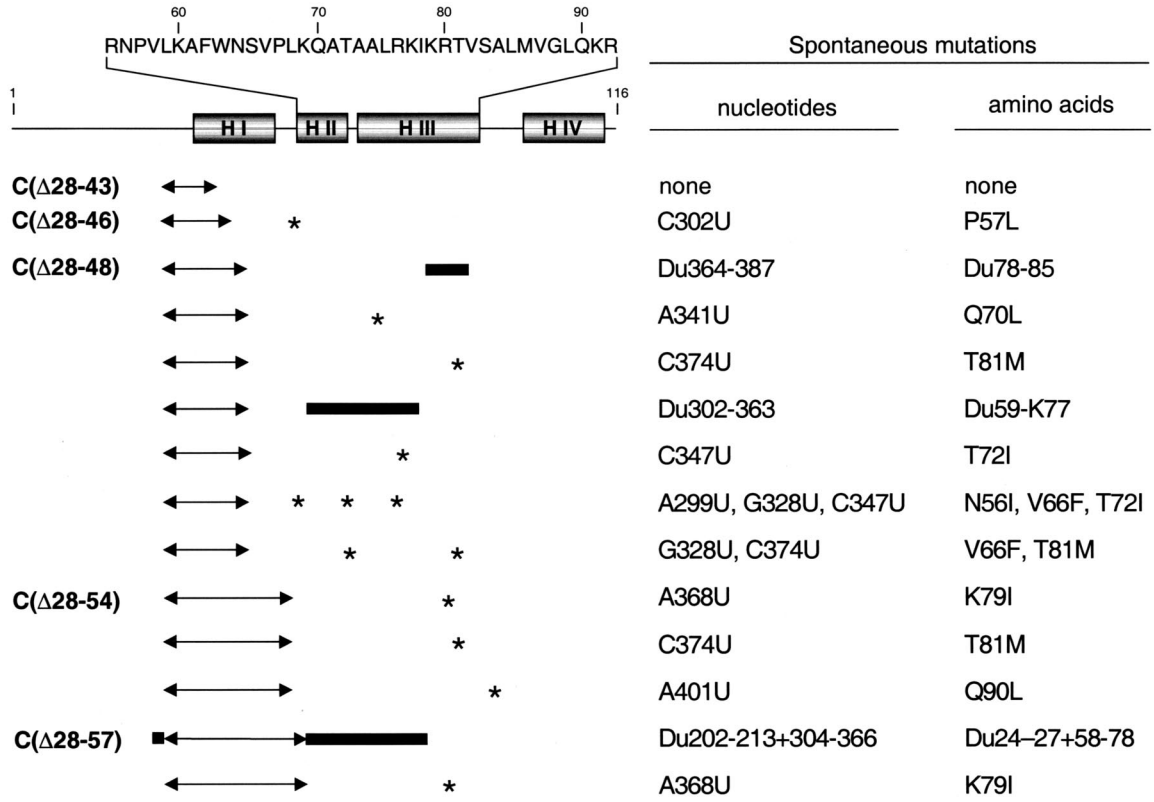


FIG. 3. Spontaneous mutations in capsid deletion mutants of TBE virus. The positions of the engineered deletions (arrows) and the additional acquired mutations (the asterisks indicate point mutations; the bars represent sequence duplications) are depicted relative to a schematic of the protein C amino acid sequence showing the four predicted alpha-helices (H I to H IV). An expanded view (above) shows the wild-type amino acid sequence of the region where the additional mutations occurred. The designations of the deletion mutants are listed on the left. The exact information about the additional mutations on both the nucleotide and the amino acid levels is given on the right. Du, duplication. The position numbers correspond to the RNA genomic sequence of TBE virus (nucleotides) or the amino acid position in protein C. Each line represents the result of an individual passaging experiment (two suckling mouse passages and three subsequent BHK-21 cell passages). With mutant C(Δ28-46), the same P57L mutation was also obtained by passaging it in BHK-21 cells alone.

sequence alterations, indicating that these double mutants were genetically stable.

**Biological characterization of mutants C(Δ28-48/Q70L) and C(Δ28-48/Du78-85).** Plaque tests performed on PS cells showed that the two mutants produced clear plaques, but significantly smaller than those of the wild-type virus (the diameters of mutant plaques were <1.5 mm compared to 2 to 3 mm with wild-type virus). To quantitatively assess the mutants' growth behavior in cultured cells, CE cells were infected at an MOI of approximately 1, and the release of newly formed infectious virus particles during 1-h periods was monitored between 9 and 30 h postinfection. The resulting growth curves obtained for wild-type virus and the two mutants C(Δ28-48/Q70L) and C(Δ28-48/Du78-85), shown in Fig. 4, demonstrated that the release of infectious particles was clearly reduced and delayed compared to that of the wild-type virus control. Nevertheless, both mutants ultimately reached plateau values that were only 10- to 100-fold lower than the wild-type level.

The pathogenicity and immunogenicity of the mutants was assessed in the adult mouse model using the same experimental approach that had been employed earlier to test various mutants of TBE virus, including the protein C deletion mutant C(Δ28-43). Upon peripheral inoculation, virulent neuroinva-

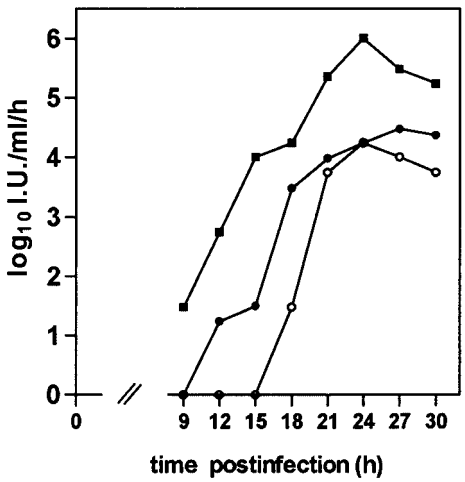


FIG. 4. Single-round growth curves on CE cells. Cells were infected with wild-type virus (■), mutant C(Δ28-48/Du78-85) (●), or mutant C(Δ28-48/Q70L) (○) at an MOI of approximately 1. The release of infectious particles into the cell culture supernatant during 1-h periods was monitored between 9 and 30 h postinfection.

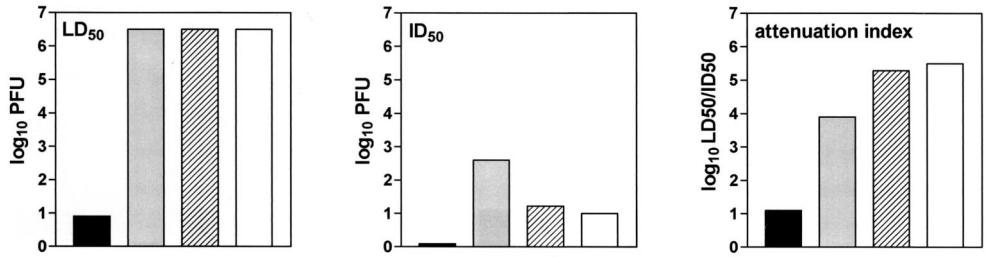


FIG. 5. Virulence (neuroinvasiveness; $LD_{50}$) and peripheral infectivity ($ID_{50}$) of wild-type virus (solid bars), mutant C(Δ28-43) (shaded bars), mutant C(Δ28-48/Du78-85) (hatched bars), and mutant C(Δ28-48/Q70L) (open bars). The $LD_{50}$ (left) and $ID_{50}$ (middle) were determined after peripheral inoculation of adult mice as described in Materials and Methods. The attenuation index (right) was calculated as the $LD_{50}/ID_{50}$ ratio. The values for mutant C(Δ28-43) were taken from a previous study (14) and are included here to allow a comparison.

sive TBE virus strains cause lethal encephalitis in almost all infected animals, whereas attenuated strains cause symptomless infections that induce a specific antibody response. Inoculation of groups of mice with various doses of virus allows the determination of the $LD_{50}$ as a parameter of neuroinvasiveness, and from the determination of seroconversion in surviving mice, the $ID_{50}$ can be calculated. With regard to vaccine development, strains with a high $LD_{50}$ and a low $ID_{50}$, conveniently expressed as the $LD_{50}/ID_{50}$ ratio (called the attenuation index), are most desirable. The results obtained for mutants C(Δ28-48/Q70L) and C(Δ28-48/Du78-85) are shown in Fig. 5 in comparison to wild-type virus and the previously analyzed mutant C(Δ28-43). Both of the double mutants were highly attenuated. Mutant C(Δ28-48/Du78-85) did not cause disease in any of the mice up to the highest inoculation dose of $10^6$ PFU. In the case of mutant C(Δ28-48/Q70L), 1 mouse out of 10 was killed at the maximum inoculation dose and none at any lower dose. The $LD_{50}$s shown in Fig. 5 were calculated under the assumption that the next-higher inoculation dose ($10^7$ PFU) would have killed all of the mice, but since this is very unlikely to be the case, the true $LD_{50}$s are probably even higher than those shown in the figure. With respect to peripheral infectivity (and thus the induction of seroconversion), mutants C(Δ28-48/Q70L) and C(Δ28-48/Du78-85) were found to be superior to mutant C(Δ28-43), yielding attenuation index values of $>10^5$. A challenge experiment using a lethal dose of the virulent TBE virus strain Hypr showed that all of the mice that had seroconverted upon inoculation with the mutants were fully protected against disease, indicating that the observed antibody response correlated with the development of protective immunity. Consequently, the 50% protective doses of these mutants are equal to the $ID_{50}$s shown in Fig. 5.

## DISCUSSION

TBE virus is capable of forming infectious particles with a capsid protein lacking almost one-third of its amino acid sequence. In a previous study, it was demonstrated that deletions starting in front of the central hydrophobic domain and removing approximately half of this domain did not abolish infectivity (14). The isolation of mutants with even larger deletions that remove most or all of the central hydrophobic domain, as reported in this study, underscores the remarkable flexibility of nucleocapsid formation of this virus. However, in contrast to the smaller deletion mutants, the viability of larger deletion mutants seems to depend on the emergence of additional mutations downstream of the deletion in protein C. A direct causal linkage between such second-site mutations and viability was demonstrated in this study for two selected examples, but the fact that similar spontaneous mutations without exception accompanied the isolation of viable mutants with large deletions strongly suggests that the mutations were essential for viability in all of these cases. Although growing the mutants in suckling mouse brains was an essential step to obtain viable virus progeny, we do not believe that the spontaneously emerging mutations represented a mechanism of adaptation to this specific host, because in the case of mutant C(Δ28-46), which could also be propagated in BHK-21 cells without prior mouse passages, the same second-site mutation arose in cell culture. Rather, the significantly higher susceptibility of suckling mice to TBE virus infection was instrumental for the successful isolation of viable virus progeny.

It has been proposed that the central hydrophobic domain may mediate membrane association of the nucleocapsid, and this may be an important determinant for the assembly of infectious particles (14, 25). The membrane topology of a protein is determined by a number of factors, particularly the arrangement of hydrophobic amino acid residues, which can insert into the membrane, and positively charged residues, which cause the protein chain to remain on the cytoplasmic side of the ER membrane (positive-inside rule) (34, 36) and can provide additional charge interactions with negatively charged head groups of phospholipids in the membrane (2, 27). The application of an algorithm that takes these factors into account to predict the membrane topology of proteins (Toppred2) (4, 35) indeed predicts that the central hydrophobic domain (and in addition the carboxy-terminal signal sequence of protein prM) will be inserted into the membrane (Fig. 6). A striking common feature of all of the observed spontaneously arising point mutations was that they replaced polar with nonpolar residues or a hydrophobic residue that has a small side chain, such as Val, with Phe, which is known to particularly favor membrane integration (3). The sequence duplications also introduced additional hydrophobic and positively charged residues. Applying Toppred2 to the sequences of the deletion mutants with and without the corresponding second-site mutations revealed that in all cases the additional mutations resulted in an increased probability of membrane association compared to the sequences carrying only the dele-

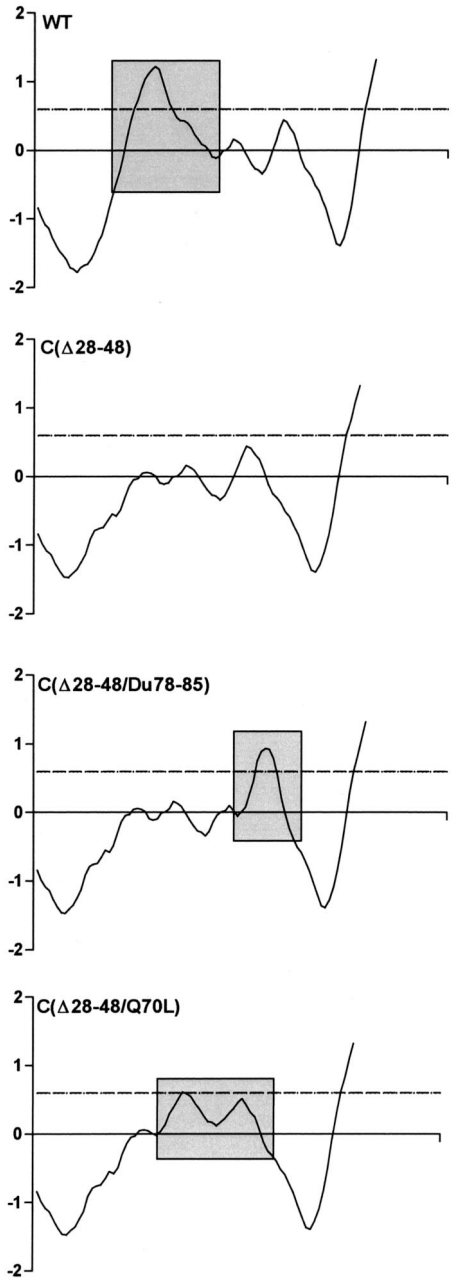


FIG. 6. Prediction of membrane topology using the computer program Toppred2. The graphs depict the results obtained for the protein C sequences of the wild-type (WT) TBE virus and the indicated mutants. Values of 0.6 or higher (the cutoff value is indicated by a dashed line) are indicative of membrane association. The shaded boxes represent potential internal membrane-associated segments.

tion. This is exemplified by the analysis of mutants C(Δ28-48), C(Δ28-48/Du78-85), and C(Δ28-48/Q70L) shown in Fig. 6. Although it is clear that any structure prediction from sequence data faces serious limitations, it is likely that the increase in hydrophobicity and the ability to interact with membranes play an important role in the compensating effects of these mutations. The results further rule out the possibility that individual residues of the central hydrophobic domain are absolutely required for viability, e.g., by providing specific protein interactions with the membrane-spanning domains of the viral surface proteins.

The viability of TBE virus mutants with highly truncated capsid proteins is reminiscent of another member of the family *Flaviviridae*, GB-C virus (or hepatitis G virus) (12). The genomic sequence of this close relative of hepatitis C virus (genus *Hepacivirus*) originally appeared to lack a core protein gene, but more recent evidence has indicated the existence of a nucleocapsid that is formed by a highly truncated core protein (39). Interestingly, this virus frequently infects humans but hardly ever causes disease (12). Viable mutants carrying deletions in the capsid protein have also been generated in the case of alphaviruses (6, 28), which apparently have many structural similarities to flaviviruses (17).

With regard to the potential use of protein C deletion mutants for vaccine development, it is important to note that the second-site mutations were capable of increasing infectivity but did not restore the neuroinvasiveness of TBE virus. The attenuation index values determined for the two double mutants, C(Δ28-48/Q70L) and C(Δ28-48/Du78-85), in the adult mouse model are the highest obtained so far for any mutant of TBE virus (19, 23). It is very likely that the principle of introducing deletions into the capsid protein to achieve stable attenuation is also applicable to other flaviviruses. Combining such deletions with mutations of the kind observed in this study may help to further improve the infectivity, and thus the immunogenicity, of such vaccine candidates.

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