GENOMICS. TRANSCRIPTOMICS

Changes in the Genome of the Tick-Borne Encephalitis Virus during Cultivation

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Abstract—The tick-borne encephalitis virus (TBEV) strain C11-13 (GenBank acc. no. OQ565596) of the Siberian genotype was previously isolated from the brain of a deceased person. TBEV C11-13 variants obtained at passages 3 and 8 in SPEV cells were inoculated into the brains of white mice for subsequent passages. Full genome sequences of all virus variants were analyzed by high-throughput sequencing. A total of 41 single nucleotide substitutions were found to occur mainly in the genes for the nonstructural proteins NS3 and NS5 (GenBank MF043953, OP902894, and OP902895), and 12 amino acid substitutions were identified in the deduced protein sequences. Reverse nucleotide and amino acid substitutions were detected after three passages through mouse brains. The substitutions restored the primary structures that were characteristic of the isolate C11-13 from a human patient and changed during the eight subsequent passages in SPEV cells. In addition, the 3'-untranslated region (3'-UTR) of the viral genome increased by 306 nt. The Y3 and Y2 3'-UTR elements were found to contain imperfect L and R repeats, which were probably associated with inhibition of cellular XRN1 RNase and thus involved in the formation of subgenomic flaviviral RNAs (sfRNAs). All TBEV variants showed high-level reproduction in both cell cultures and mouse brains. The genomic changes that occurred during successive passages of TBEV are most likely due to its significant genetic variability, which ensures its efficient reproduction in various hosts and its broad distribution in various climatic zones.

Keywords: tick-borne encephalitis virus, viral genome, 3'-untranslated region, nucleotide substitutions, adaptive substitutions, reverse mutation, cell culture, mouse

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INTRODUCTION

The tick-borne encephalitis virus (TBEV) causes encephalitis, which often causes severe damage to the central nervous system, including lethal outcomes [1, 2]. TBEV is endemic in many regions of Central Eurasia and has recently been detected in new geographical areas, probably because changes arise in the species ranges of ixodid ticks, which are TBEV vectors [3–5].

TBEV (*Orthoflavivirus encephalitidis*) belong to the genus *Orthoflavivirus* of the family Flaviviridae (https://ictv.global/taxonomy) [2]. The genus includes spherical enveloped RNA viruses that are relatively simple in structure and 40–60 nm in size. The TBEV genome is a single-stranded (+)RNA of 9200–11 500 nt. Genomic RNA codes for a single polyprotein, which is processed by viral and cell proteases to yield three structural and seven nonstructural viral proteins. Flaviviral genomic RNA is flanked by 5'- and 3'-untranslated regions (UTRs), which are of principal importance for the initiation of virus replica-

tion and the formation of a replication complex including viral RNA-dependent RNA polymerase [6, 7]. The 5'- and 3'-UTRs are thought to determine the virus replication rate, RNA polarity, genome encapsidation, and RNase resistance [8–11].

Three main TBEV genotypes are recognized: European, Far Eastern, and Siberian [12]. Himalayan and Baikal TBEV genotypes have recently been proposed to isolate [4, 13–15]. The level of homology between different TBEV genotypes is 82–85% for whole-genome nucleotide sequences and 92–96% for amino acid sequences [16, 17]. Genetic differences between TBEV isolates are substantial even within the same TBEV genotype. For example, the strain Glubinnoe/2004, which is highly pathogenic to humans and belongs to the Far Eastern genotype, differs from the prototype strains 205 and Sofjin by 53–57 amino acid substitutions [16].

A high genetic variability of TBEV makes it possible to hypothesize that the genetic potential allows

Table 1. TBEV C11-13 variants examined in this study

TBEV strain	History	Source	GenBank acc. no.
C11-13_1p	1 passage in SPEV cells	This work	OQ565596
C11-13_3p	3 passages in SPEV cells	Described in [21]	KP644245
C11-13_8p	8 passages in SPEV cells	This work	MF043953
C11-13_3/3m	C11-13_3 → 3 passages in mice	This work	OP902894
C11-13_8/3m	C11-13_8 → 3 passages in mice	This work	OP902895

TBEV to change substantially and to adapt to new cell types and new host species. TBEV may consequently be easy to introduce into new biotopes. For example, more than 40 wild bird species have been found to TBEV in Tomsk and its suburbs [18−20].

We studied the genetic variation of the highly pathogenic TBEV strain C11-13 of the Siberian genotype during its adaptation to culture in laboratory conditions.

EXPERIMENTAL

Virus preparation. The TBEV strain C11-13 of the Siberian genotype was isolated in 2013 from a brain suspension of a patient who died from tick-borne encephalitis in the Moshkovskii region of the Novosibirsk oblast; the strain has been described earlier [21]. All experiments with virus materials were carried out in compliance with the biosafety regulations as per Sanitary Regulations and Norms 3.3686-21.

Cell line. A SPEV cell culture was obtained from the cell culture bank of the State Research Center of Virology and Biotechnology "Vector" and maintained in DMEM supplemented with 10% fetal bovine serum (FBS) (Gibco, United States) and 80 μg/mL gentamycin sulfate.

Laboratory animals. We used female and male two- and three-day-old outbred suckling mouse (body weight 2−3 g), which were obtained from the breeding facility of the State Research Center of Virology and Biotechnology "Vector." All procedures with animals were carried out in compliance with the effective documents "Rules for Working with Experimental Animals" (https://docs.cntd.ru/document/456016716) and "Guidelines on Rearing and Use of Laboratory Animals" [22].

Serial passaging of TBEV. Virus suspensions obtained after three or eight passages of the TBEV strain C11-13 in cultured SPEV cells [21] were injected intracerebrally in outbred mice. Further passaging was performed using 10% brain suspensions obtained from the mice on days 3−4 after infection. The suspensions were prepared using 50 mM borate (pH 9.0) containing 150 mM sodium chloride. The TBEV variants examined in this work are summarized in Table 1.

TBEV isolation and titration. A virus-containing specimen was applied to a SPEV cell monolayer. The culture was incubated at room temperature for 1 h, supplemented with DMEM containing 2% FBS and 80 μg/mL gentamycin sulfate, and incubated at 37°C for 7 days. Titration of TBEV-containing specimens in SPEV cell cultures were carried out in 96-well culture microplates. Virus concentrations were estimated by a tissue cytopathic effect assay ($TCID_{50}$/mL) in SPEV cells and ELISA. Virus preparations were stored in aliquots at −40°C. The infectious titer of the virus was calculated by the Kerber method [23].

ELISA. TBEV testing in the culture medium was performed by ELISA, using 96-well plates (Nunk, Denmark) sensitized with anti-TBEV mouse monoclonal antibody 10H10 (SRC VB "Vector", Russia) according to a published protocol [24]. The virus bound to the plate was detected using biotinylated anti-TBEV mouse monoclonal antibody EB1 (SRC VB "Vector") and a streptavidin−peroxidase conjugate (ICN, United States).

RNA isolation and reverse transcription−PCR. Total nucleic acid was isolated from virus-containing material with an ExtractRNA kit (Evrogen, Russia); the first DNA strand was synthesized using a MMLV RT kit (Evrogen) as recommended by the manufacturer. PCR was carried out using a BioMaster LR HS-PCR kit (BioLabMix, Russia) and oligonucleotide primers specific to TBEV RNA [6, 16, 25]. PCR was run on a T100 thermal cycler (Bio-Rad Laboratories, United States) and included 94°C for 5 min; 40 cycles of 94°C for 10 s, 58°C for 20 s, and 72°C for 30 s; and 72°C for 7 min.

Sanger sequencing and high-throughput sequencing (NGS). Sanger sequencing of DNA fragments was carried out on an ABI 3130×1 automated sequencer (Hitachi, Japan). Full-genome nucleotide sequences were established by NGS. The first cDNA strand was synthesized using a NEBNext Ultra Directional module; the second strand, using a NEBNext Ultra Directional RNA Second Strand Synthesis module (NEB, United States). DNA libraries were analyzed using a MiSeq instrument (Illumina, United Kingdom). Adaptors were trimmed and duplicate reads eliminated using SAMtools (v. 0.1.18, https://www.htslib.org/download). Extended sequences were de novo assembled using the MIRA assembler (v. 4.9.6, https://linux-packages.com/ubuntu-impish-indri/package/mira-assembler). Results were processed using the specialized software pack-

ages MEGA 7/10 (PSU, United States) and Lasergen 7 (Invitrogen, United States).

Imperfect repeats were sought in virus genomes with the use of the diagonal of a local nucleotide sequence similarity matrix (Unipro UGENE, v. 45.1; https://ugene.net/ru/download-all.html).

RESULTS

Genetic Analysis of C11-13 TBEV Variants after In Vitro−In Vivo Passaging

A set of TBEV variants has previously been obtained after several successive passages of the TBEV strain C11-13 in SPEV, HEK293, and Neuro-2a cells [21]. First amino acid substitutions in viral proteins were detected after three passages. The number of amino acid substitutions changed no longer after eight passages. TBEV variants have accumulated 9 amino acid substitutions after passages in SPEV cells, 14 after passages in HEK293 cells, and only 4 after passages in Neuro-2a cells.

In this work, C11-13 TBEV variants obtained after three and eight passages in SPEV cells were thrice passaged through the brains of outbred white mice. The resulting isolates C11-13_3/3m and C11-13_8/3m, respectively, were examined by full genome sequencing (Table 2). In total, 41 nucleotide substitutions were identified in the virus genome and associated with TBEV adaptation to SPEV cells and subsequent passaging through mouse brains. The majority of the nucleotide substitutions occurred in the viral genes for nonstructural proteins. Only nine substitutions were mapped to the capsid (C) and envelope (E) protein genes. The greatest number of substitutions (14) was observed for the NS5 gene with a nonsynonymous-to-synonymous nucleotide substitution ratio (dN/dS) of 0.12. A total of 32 nucleotide substitutions were detected in the nonstructural genes, and nine of them caused amino acid substitutions. Maximal numbers of amino acid substitutions were observed in the NS3 and NS5 proteins (four and two substitutions, respectively). The dN/dS ratio was high (2.0) in NS1 and minimal (0.12) in NS5. The dN/dS ratio tended to be lower in the case of passaging through mouse brains compared with cell cultures.

It is of interest to note that seven reverse amino acid substitutions were found in the variants C11-13_3/3m and C11-13_8/3m after three passages through mouse brains. The resulting amino acid residues were characteristic of the C11-13 virus strain isolated from a human patient, but were changed during TBEV passaging (eight passages) in SPEV cells. The following viral proteins acquired substitutions during adaptation from SPEV cells to mice as a new host: E (Q367R), NS1 (N1067D), NS2b (R1488Q), NS3 (D1511N and F1906S), and NS5 (M2561K and S2925F).

Note that the NS5 gene underwent reverse nucleotide substitutions (two blocks of three synonymous substitutions each and two nonsynonymous substitutions) and five new ones. In addition, mutations were detected in the E (D347N and H699Q) and NS3 (T1890S) proteins.

Replication activity substantially increased during serial passaging in cultured cells and through mouse brains in all TBEV variants (C11-13_3p, C11-13_8p, C11-13_3/3m, and C11-13_8/3m) compared with the initial isolate (C11-13_1p). For example, the virus titer has increased by two orders of magnitude, from 10^6 to 10^8 $TCID_{50}$/mL, as the first amino acid substitutions arose in NS3 (H1745Q) and NS5 (S2925F) after the third passage of the strain C11-13 in SPEV cells (C11-13_3p) [21]. A virus titer of 10^8 $TCID_{50}$/mL was observed for the variant C11-13_8p obtained after eight passages in SPEV cells and all virus variants obtained after the third passage through mouse brains (C11-13_3/3m and C11-13_8/3m) as measured in 10% mouse brain homogenates on day 7 after infection.

Genome Size and 3'-UTR Length in the TBEV Variants

An increase in 3'-UTR length was detected after adaptation of TBEV C11-13 to in laboratory conditions (Table 3). We have previously shown that a variable region of the 3'-UTR is 107 nt in the genome of the C11-13 strain isolated from the human brain (C11-13_1p) [7]. The region was found to increase by 37 nt after eight passages in SPEV cells (C11-13_8p) and 306 nt after passaging in mice as compared with the clinical isolate. It is important to note that a conserved 3'-UTR region was 320 nt in all of the virus variants obtained.

A variation in 3'-UTR length has been described for a number of laboratory TBEV strains and found to reach a maximum of 767 nt in the European genotype (strain Neudoerfl) [7]. A 3'-UTR sequence alignment relative to the strain Neudoerfl is shown in Fig. 1. The 3'-UTR length increased because identical fragments were inserted in the LRS4 and LRS3 sequences in the enhancer region of the variable part. The enhancer is between the stop codon and the promoter in the flavivirus genome and involves both conserved and variable parts of the 3'-UTR. The insertion probably restored the hairpins SL10−SL14, thus making the region more structured. A similar organization of the respective 3'-UTR region has been observed in the Siberian-genotype TBEV strain PT-122, which has been isolated from the liver of a Blyth's reed warbler (*Acrocephalus dumetorum*) and sequenced previously (KM019545).

The role of the enhancer function may not be crucial for virus viability in experimental conditions, but be of importance in natural environments and contribute to virus adaptation to new hosts.

Table 2. Nucleotide and amino acid substitutions that were found in the genome of the strain C11-13 adapted to SPEV cells after additional passaging through the brains of outbred white mice

TBEV gene	Nucleotide/amino acid substitution (Nt/AA)[a]	C11-13_1p	C11-13_8p	C11-13_3/3m	C11-13_8/3m	dN/dS[b]
c	Nt240-AA80/K	A	A	G	G	0/1
e	Nt1039-AA347/D → N	G	G	G	A	3/5 (0.6)
	Nt1100-AA367/Q → R	A	G	A	A	
	Nt1704-AA568/V	A	A	G	G	
	Nt1740-AA580/K	A	G	A	A	
	Nt1743-AA581/G	C	T	C	C	
	Nt1932-AA644/I	T	T	C	T	
	Nt2097-AA699/H → Q	C	C	C	G	
	Nt2379-AA793/E	A	A	G	G	
ns1	Nt3199-AA1067/N → D	A	G	A	A	2/1 (2)
	Nt3498-AA1166/G	T	A	A	A	
	Nt3502-AA1168/L → V	C	G	G	G	
ns2a	Nt3810-AA1270/F	C	T	T	T	0/3
	Nt4044-AA1348/F	C	T	C	C	
	Nt4090-AA1364/L	C	T	T	T	
ns2b	Nt4350-AA1450/E	G	G	G	A	1/1 (1)
	Nt4463-AA1488/R → Q	G	A	G	G	
ns3	Nt4863-AA1621/D	G	A	G	G	4/6 (0.67)
	Nt4531-AA1511/D → N	G	A	G	G	
	Nt4629-AA1543/H	T	T	C	T	
	Nt4635-AA1545/T	A	A	G	G	
	Nt5235-AA1745/H → Q	T	A	A	A	
	Nt5634-AA1878/I	T	T	C	C	
	Nt5658-AA1886/E	G	G	G	A	
	Nt5668-AA1890/T → S	A	A	A	T	
	Nt5697-AA1899/F	C	T	C	C	
	Nt5717-AA1906/F → S	T	T	C	C	
ns5	Nt7685-AA2562/M → K	T	A	T	T	2/12 (0.12)
	Nt8562-AA2854/A	C	T	C	C	
	Nt8586-AA2862/G	A	G	A	A	
	Nt8628-AA2876/Q	G	A	G	G	
	Nt8774-AA2925/S → F	C	T	C	C	
	Nt9003-AA3001/L	C	C	T	T	
	Nt9060-AA3020/E	A	A	G	G	
	Nt9126-AA3042/F	C	C	C	T	
	Nt9456-AA3152/L	G	A	A	A	
	Nt9514-AA3172/V	G	G	A	A	
	Nt9540-AA3180/R	A	A	A	G	
	Nt9744-AA3248/G	G	A	G	G	
	Nt9750-AA3250/A	C	T	C	C	
	Nt10170-AA3390/K	A	G	A	A	
	Total reversions to the initial isolate			18	20	

[a]Amino acid substitutions are shaded dark gray; nucleotide substitutions, light gray.
[b]Proportion (ratio in parentheses) of nonsynonymous and synonymous nucleotide substitutions.

Table 3. RNA genome size and 3'-UTR length in the C11-13 TBEV variants

TBEV strain	Genome size, nt	3'-UTR length, nt		
		variable region	conserved region	total
C11-13_1p	10801	107	320	427
C11-13_3p[a]	10838	144	320	464
C11-13_8p	10838	144	320	464
C11-13_3/3m	11 107	413	320	733
C11-13_8/3m	11 107	413	320	733

[a]Described previuosly [21].

Detection and Mapping of Imperfect Repeats in the 3'-UTR

Two 60-nt sequences with 57% homology to each other were found near the enhancer in the 3'-UTR of the TBEV genomic RNA (Fig. 2). These imperfect repeats were detected using the diagonal of the local nucleotide sequence similarity matrix (Unipro UGENE, v. 45.1). Similar repeats with 52−57% homology were observed in other viruses (Fig. 3). A nucleotide sequence searched for imperfect repeats is arranged along the X and Y axes. In particular, a prototype sequence of the strain Glubinnoe/2004 was used in Fig. 2.

It is important to note that analogous imperfect repeats in the 3'-UTR of genomic DNA were observed in the viruses Powassan, West Nile, Kunjin, dengue 1-2, yellow fever virus and Omsk hemorrhagic fever virus, which belong to Flaviviridae, and the Semliki Forest virus, which is an alphavirus. A sequence alignment of the espective genome regions of the viruses is shown in Fig. 3.

It is probably not accidental that imperfect repeats occur in the enhancer 3'-UTR region. This region was found to increase to affect the genomic RNA size and the 3'-UTR length in the C11-13 TBEV variants obtained after successive passages in cells and through the brains of white mice.

DISCUSSION

Certain RNA viruses efficiently overcome the species barrier because a far higher variability is characteristic of their RNA genomes compared with the genomes of DNA viruses [26, 27]. A high mutation rate of RNA viruses is thought to be a starting point in facilitating virus reproduction in a new cell type or a new host species; i.e., the rate provides a structural basis for a high transmissivity and a broad host range of RNA viruses [28−30]. Many flaviviruses, including TBEV, are spread through vast areas and reproduce in various invertebrates (mosquitoes and ticks) and vertebrates [31]. Natural and climatic conditions may also substantially vary between natural foci of infections. Flaviviruses efficiently circulate in various climatic zones from circumpolar to equatorial ones. This means that their replication occurs in a broad temperature range [18, 19]. Genetic heterogeneity between TBEV genotypes is at least 18%, which corresponds to 2000−2200 nt of the total TBEV genome. A high genetic variation is thought to allow TBEV to circulate in various natural biotopes of Northern Eurasia and to adapt to new invertebrate and hot-blooded hosts while retaining its potential as a human pathogen [1].

In this study, the genetic variation of the Siberian TBEV genotype was evaluated with the highly pathogenic isolate C11-13, which was isolated from the brain of a deceased person and examined during passaging in cells and through the brains of outbred white mice. Whole-genome NGS was used to establish the nucleotide sequence of the viral genome and to monitor the formation of adaptive mutations over time.

Nucleotide substitutions of the genes for both structural and nonstructural TBEV proteins were detected as early as three to eight passages in cultured SPEV cells and three passages through mouse brains. The finding indicates that the field (clinical) TBEV isolate rapidly and efficiently adapted to a new host. The majority of the substitutions arose in the non-structural protein genes and respective proteins. Only 9 out of the 41 adaptive mutations identified in the TBEV genome affected the structural protein genes. Maximal numbers of nucleotide substitutions were found in the *ns3* and *ns5* genes. A similar pattern was observed for amino acid sequence mutations; i.e., three substitutions were detected in the E structural protein and nine, in nonstructural proteins.

Flaviviral structural proteins have been shown to act as important virulence factors [31−33]. The E protein was earlier thought to play a key role in TBEV virulence [34]. For example, structural changes in the E protein have been reported to affect the neurovirulence and neuroinvasiveness of tick-borne flaviviruses in several studies [31, 35−37]. Partial genome sequencing has been used in some of the studies. Rumyantsev et al. [38] have shown more recently by reverse genetic experiments and whole-genome sequence analysis that the virulence of the TBEV strains Sofjin-HO and Oshima 5-10 is not associated
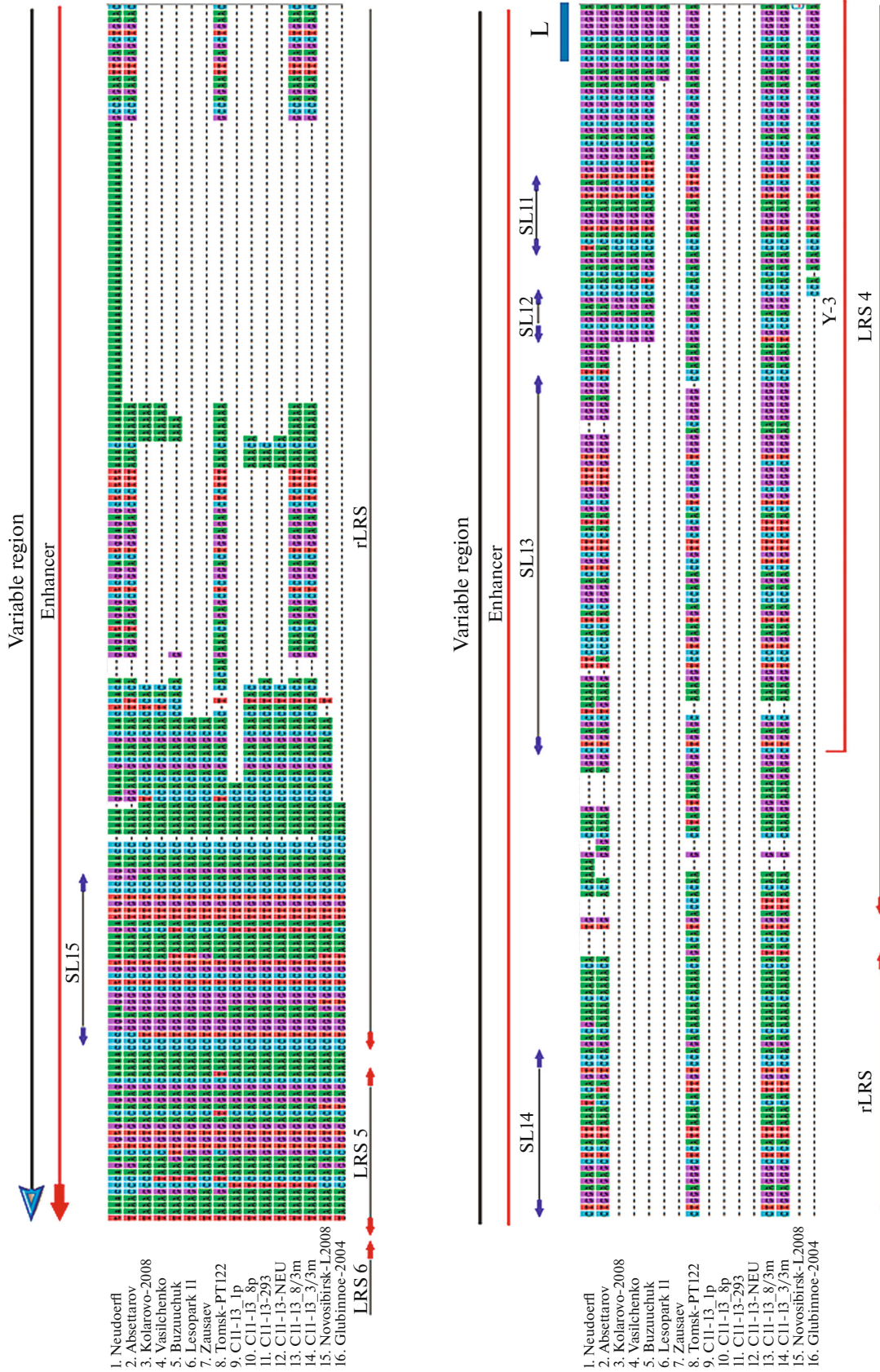
Fig. 1. Schematic organization of the TBEV 3'-UTR. Prototypes used in the comparison included the (1, 2) European, (3–6) Siberian, and (7, 8, 15, 16) Far Eastern genotypes of TBEV. Variants l1 and l2 are the C11-13 TBEV variants that have been obtained in normal human embryonic kidney cells (HEK293) and mouse neurons (Neuro-2a), respectively (GenBank MF043954 and MF043955, respectively). Deletions in the 3'-UTR are shown with dashes. L and R, imperfect repeats found in the TBEV genomic RNA (repeat length 60 nt, 57% identity with the respective region of the strain Glubinnoe/2004 (DQ862460)); SL1–SL15, stem-loop UTR structures; LRS1–LRS6, long repeat sequences; rLRS, region of long repeat sequences; Y1–Y3, Y-shaped UTR structures; 3' LSH, 3'-terminal long stable hairpin. The nucleotides adenine (A), thymine (T), cytosine (C), and guanine (G) are color coded green, red, blue, and purple, respectively, as commonly accepted in MEGA 7/10 (PSU, United States).
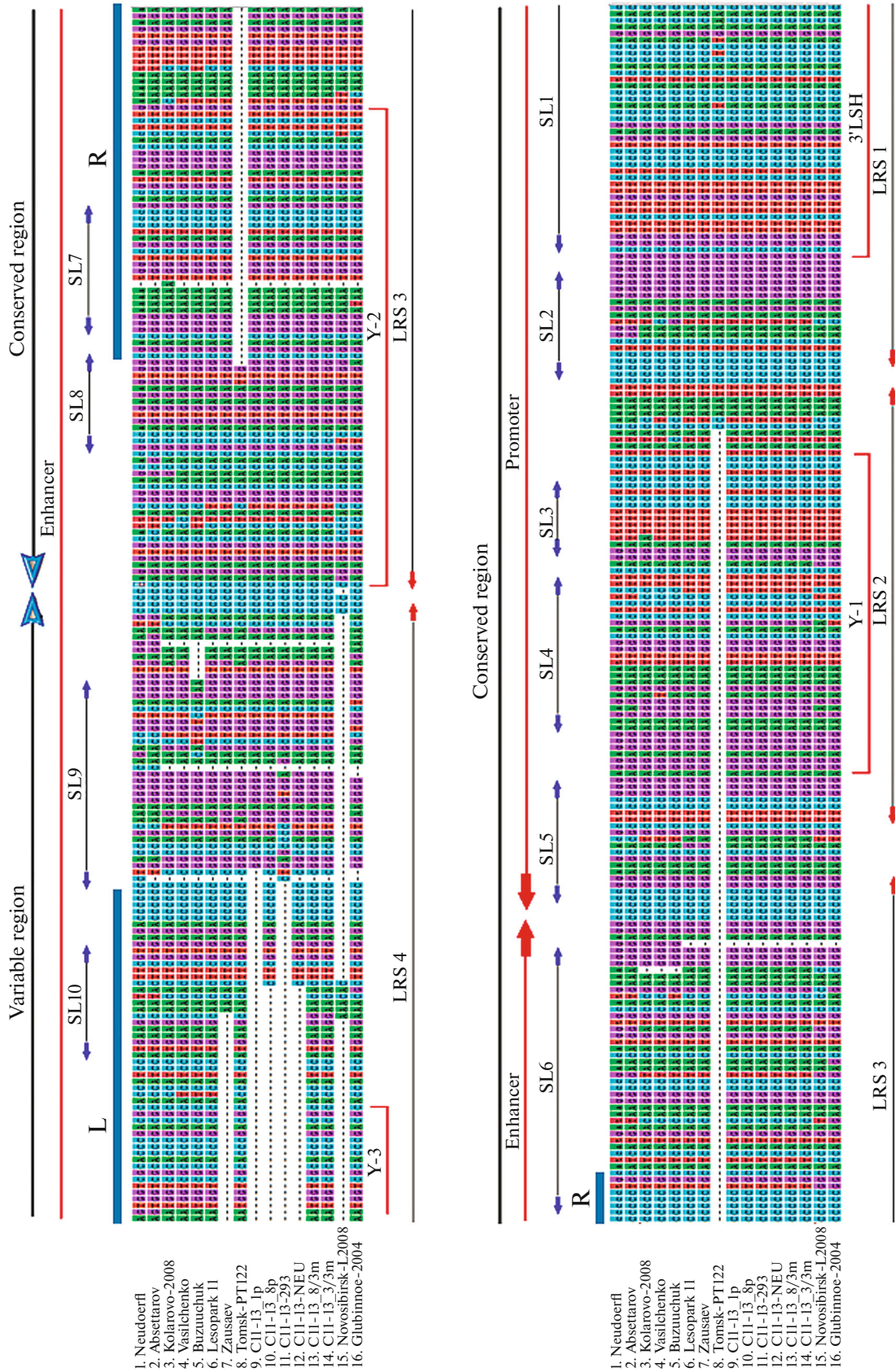
Fig. 1. (Contd.)

Fig. 2. Mapping of imperfect repeats in the variable region of the 3'-UTR of the TBEV genomic RNA (prototype strain Glubinnoe/2004, GenBank DQ862460). The mapping was performed using Unipro UGENE v. 45.1 at a 60-nt length of nucleotide sequences to be compared.

TBEV, strain Glubinnoe/2004 (DQ862460); $x = 10\,473$, $y = 10610$
CGACGGGGAAATGGTCGCACCCGACGCACCATCCATGAAGAAACGCTTCGTGAGACCCCCC
CGACGGG**AATGGTCG**CCCGACG*A*****C******AAAC**T**G*GA**CCC**C
CGACGGGATAATGGTCGATCCCGACGTAGGGCACTCTGTCAAACTTTGTGAGACCCCCTGC

Powassan virus, strain Partizansk/2006 (EU543679); $x = 10\,062$, $y = 10141$
GACAACAGAAGACATGCTGCGCATCTGGAACAAAGTCTGGATTTTGGACAACCCTCACATG
GA**AC*G*AGACA*****CG*ATCTG**A*AAA**C*GGAT*T*G*A***CCTCAC*TG
GATGAGTGGAGAGACATCCCCTATCTGCCAAAAACACAGGACCTGGTATGTTCCTCACTTG

West Nile virus, strain Vol-1 (AF317203); $x = 10\,496$, $y = 10650$
GTAGACGGTGCTGCCTGCGACTCAACCCCAGGAGGACTGGGTGAACAAAGCCGCGAAGTGATCCATGTAAGCCCT
G**GA**GTGC*G*CTGCGA*****CCCCAGGAGGACTGGGT*AACAAAG*C*********CC******GCCCT
GCGGAGAGTGCAGTCTGCGATAGTGCCCCAGGAGGACTGGGTTAACAAAGGCAAACCAACGCCCCACGCGGCCCT

Dengue virus, serotype 1, isolate NC 001475.2 (ON103302); $x = 10\,441$, $y = 10526$
CACGGTGTAGCAGACTAGCGGTTAGAGGAGACCCCTCCCATGACACAACGCAGCAGCGGGGCCCGAGCA
C*C**TG*A**AGACTAG*GGTTAGAGGAGACCCC*CCCA**A*A*AAC*****A**G**GC**G**CA C
TCCTTGCAAAGGACTAGAGGTTAGAGGAGACCCCCCGCAAATAAAAACAGCATATTGACGCTGGGAGA

Dengue virus, serotype 2, isolate THSTI-TRC-DENV2-12 (OP809583) $x = 10\,461$, $y = 10548$
GCATGGCGTAGTGGACTAGCGGTTAGAGGAGACCCCTCCCTTACAAATCGCAGCAACAATGGGGGCCCAAGG
G**T**C*****GGACTAG*GGTTAGAGGAGACCCC*C***ACAAA***CAGCA*******G*****AA*G
GTCTCACTGGAAGGACTAGAGGTTAGAGGAGACCCCCCAAAAACAAAAAACAGCATATTGACGCTGGGAAAG

Semliki Forest virus, strain Smithburt (MT121983); $x = 11\,252$, $y = 11426$
ACTACTTGAACAGAAAACTGGAAAACAGAAAAAGTTAGGGTAGGCAATGTTAGTATATTATACCTCTATTATAA
A*TA*T**A*CA**AAA***GAAAA*AGAAAAAGTTAGGGTAGGCAATG**A*T****TA*******A*T**AA
ATTAATATAGCAAGAAAACCGAAAATAGAAAAAGTTAGGGTAGGCAATGGCATTGATATAGCAAGAAAATTGAA

Japanese encephalitis virus, strain Beijing-1 (JEVBEICG); $x = 10\,484$, $y = 10635$
GATACTGGGTAGACGGTGCTGCCTGCGCCTCAGTCCCAGGAGGACTGGGTTAACAAATCTGACAACGGAAAGTG
G***C*********GG***TGC***C**C*CAG*CCCAGGAGGACTGGGTTA*CAAA*C*G***A********G
GTGCCACTCCGCTGGGGAGTGCGGCCTGCGCAGCCCCAGGAGGACTGGGTTACCAAAGCCGTTGAGCCCCCACG

Yellow fever virus, strain SP14/BRA/2018 (MZ604876); $x = 9276$, $y = 9512$
GGAAATCTTGAACTACATGAGCCCACACCATAGGAAGTTGGCGCTGGCTGTAATGGAAAT
G*A*A*C*TCA*C*AC*TGA*****CA*C**A**A*G*TGGCGG*GGC*G**ATGG**AT
GAACACCATCACCAACCTGAAGGTTCAACTGATTAGGATGGCGGAGGCAGAGATGGTGAT

Omsk hemorrhagic fever virus, strain Br-354/11661 (OL689688); $x = 10\,380$, $y = 10493$
ACAGACAGCCATAGAGCAAAACCCGGAGGGCTCTTTAAACATTGTCCGGAAATGAAAAACCAAAACAA
A*A**C****AT*GAGC**AACC*GGA*GGCTC**AAA*AT*TCC*GAAA*G***AA***A**CAA
AAAAGCCAGAATTGAGCTTAACCTGGAAGGCTCACAAAATATATTCCAGAAAAGCTGAAAACAGTCAA

Kunjin virus, isolate CH16514C (KX394389); $x = 10\,496$, $y = 10650$
TGAGTAGACGGTGCTGCCTGCGACTCAACCCCAGGAGGACTGGGTGAACAAAGCTGCGAAGTGATCCATGTAAGCCCT
T**G**GA**GTGC*G*CTGCGAC****CCCCAGGAGGACTGGGTGAACAAAG**G*******TCC*****GCCCT
TCTGCGGAGAGTGCAGTCTGCGACAGTGCCCCAGGAGGACTGGGTGAACAAAGGCGAATCAACGTCCCACGCGGCCCT

Fig. 3. Imperfect repeats found in the variable rregion of the 3'-UTR of genomic RNA in flaviviruses and alphaviruses. X and Y are the coordinates of a nucleotide sequence corresponding to the matrix diagonal in Unipro UGENE v. 45.1. Imperfect repeat homology was 52−57% according to Unipro UGENE v. 45.1.
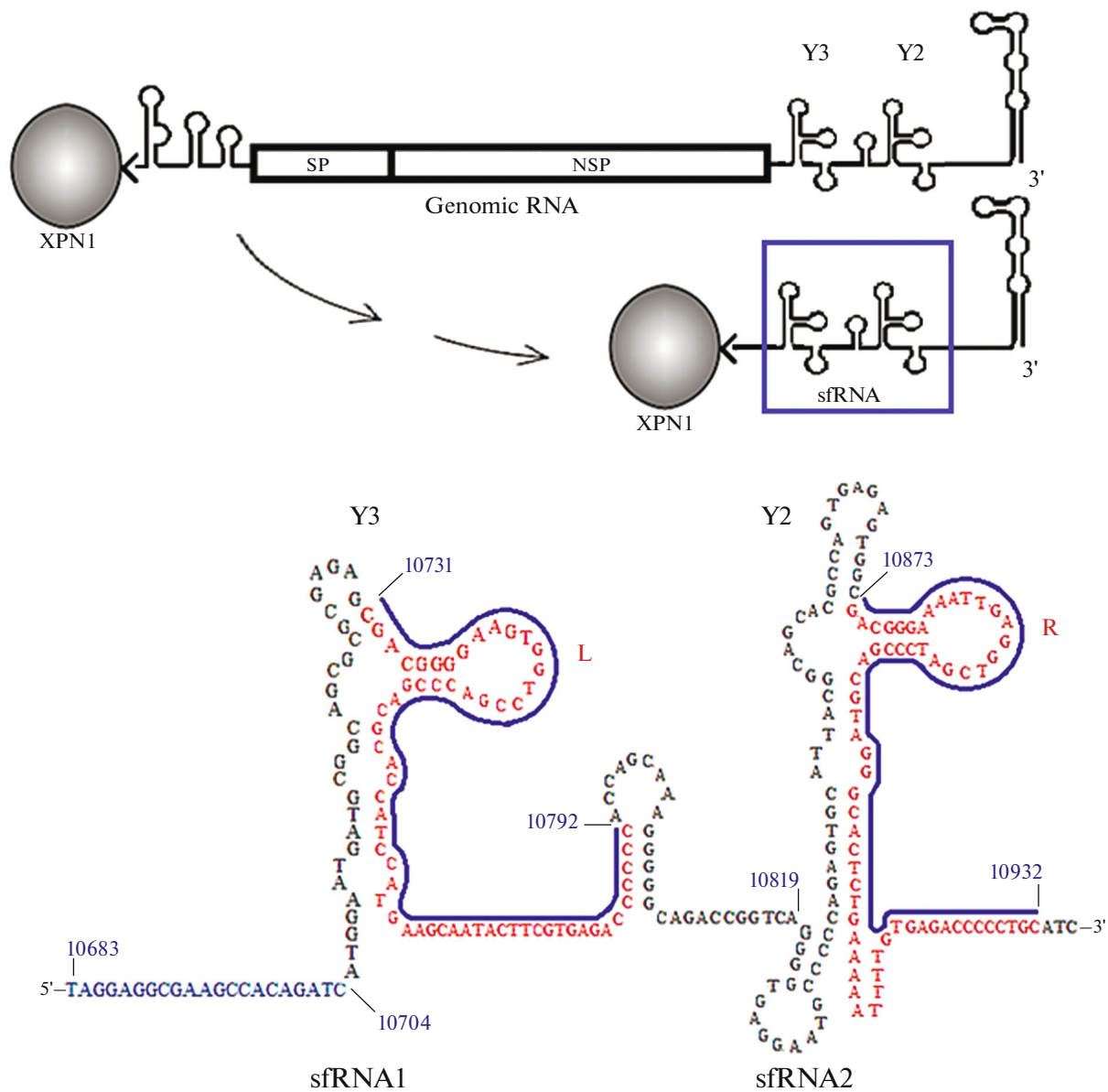
Fig. 4. Mechanism of viral RNA cleavage by exoribonuclease XRN1 to produce TBEV sfRNA. The imperfect repeats L and R (underlined) have 52−57% homology and form the right part of the loops Y3 and Y2, respectively. SP and NSP are, respectively, the genes for structural and nonstructural flaviviral proteins.

with structural proteins, but it tightly related to the genes for nonstructural proteins and their 3'-UTRs. Our data obtained by whole-genome NGS agree with the above findings and the hypothesis that nonstructural proteins play an important role in TBEV adaptation to new cell types and new hosts.

It is of interest that seven reverse amino acid substitutions were detected in the variants C11-13_3/3m and C11-13_8/3m after three passages through mouse brains; i.e., the variants adapted to SPEV cells (C11-13_3 and C11-13_8) returned to the initial isolate obtained from a human brain. The *ns5* gene was found to accumulate six synonymous and two nonsynony-

mous reverse nucleotide substitutions and five new mutations in the TBEV variants obtained after passaging through mouse brains.

We observed that the variable region of the 3'-UTR increased substantially during passaging through mouse brains as compared with the TBEV variant preliminarily adapted to SPEV cells. The 3'-UTR length increased by 306 nt in the variants C11-13_3/3m and C11-13_8/3m. Shorter inserts of 22−37 nt in the variable part of the 3'-UTR have earlier been observed to arise during serial TBEV passaging in SPEV, Neuro-2A, and HEK293 cells [7, 21]. The flaviviral 3'-UTR is known to be highly structured and to con-

tain various structures, such as stem-loops, hairpins, and dumbbell and Y-shaped elements [25, 30]. The 5'- and 3'-UTRs are known to ensure the interaction of viral genomic RNA with RNA-dependent RNA polymerase to initiate the synthesis of a new viral RNA. The conserved part of the 3'-UTR is possibly responsible for the host specificity in flaviviruses. Recent studies have shown that the 3'-UTR is a source of at least two noncoding RNAs that affect flavivirus replication and/or pathogenesis. These are subgenomic flaviviral RNA (sfRNA) and microRNA (KUN-miR-1), and their formation involves exoribonuclease 1 (XRN1) [39—46]. XRN1 recognizes a unique triple-stranded ring-like structure formed by 5'-UTR Y-shaped elements and a 3'-UTR pseudoknot and produces sfRNA. Then sfRNA hinders the innate immunity response (by inhibiting interferon induction), decreases XRN1 and Dicer activities, and directly interacts with the viral replication complex to regulate its activity [47—50]. Structural elements of the 3'-UTR are also capable of blocking XRN1 activity. It is thought that sfRNA terminates degradation of viral genomic RNA approximately 525 nt away from the 3'-UTR end. This prevents complete degradation of viral genomic RNA and leads to sfRNA accumulation in infected cells [43, 51]. The Y3 and Y2 3'-UTR elements are, respectively, at the end of the variable region and at the start of the conserved region. The elements most likely act to ensure the formation of sfRNA2 and sfRNA3, respectively.

The imperfect nucleotide repeats L and R found in the enhancer region of TBEV genomic RNA are probably involved in regulating cell XRN1 activity through the Y2 and Y3 elements (Fig. 4). An increase in 3'-UTR length may be critical for virus genome replication during TBEV adaptation to laboratory culture conditions and, eventually, may ensure efficient TBEV reproduction in various cells and in passages through mouse brains.

To summarize, point nucleotide substitutions were observed to arise in the TBEV genome during passaging in cell cultures and through mouse brains. The substitutions occurred mostly in the nonstructural protein genes, and some of them caused mutations in the amino acid sequence of the polyprotein products. The majority of substitutions reverted during passaging through mouse brains to the initial TBEV strain, which was isolated from a human brain. In addition, a substantial increase in the length of the variable part was observed in the 3'-UTR. We assume that the increase is associated with inhibition of cell XRN1, which is involved in producing sfRNA1 and sfRNA2. The adaptive mutations detected in our work most likely act to ensure a high reproduction level of TBEV in cells and the white mouse brain.

Multiple TBEV genomic changes of the kind may play an important role in TBEV survival in natural conditions, which change continuously, and in virus adaptation to new host species.

ABBREVIATIONS

3'-UTR, 3'-untranslated region; TBEV, tickborne encephalitis virus; ORF, open reading frame.

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ETHICS APPROVAL AND CONSENT TO PARTICIPATE

All procedures with animals were carried out in compliance with the effective documents "Rules for Working with Experimental Animals" (https://docs.cntd.ru/document/456016716) and "Guidelines on Rearing and Use of Laboratory Animals" (Washington, 1996). The study was approved by Ethics Committee no. 1 at State Research Center of Virology and Biotechnology "Vector" (protocol no. 1-04.2021 dated April 30, 2021).

CONFLICT OF INTEREST

The authors of this work declare that they have no conflicts of interest.

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