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Characterization of a Siberian Virus Isolated from a Patient with Progressive Chronic Tick-Borne Encephalitis

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A strain of *Tick-borne encephalitis virus* designated Zausaev (Za) was isolated in Siberia from a patient who died of a progressive (2-year) form of tick-borne encephalitis 10 years after being bitten by a tick. The complete genomic sequence of this virus was determined, and an attempt was made to correlate the sequence with the biological characteristics of the virus. Phylogenetic analysis demonstrated that this virus belongs to the Siberian subtype of *Tick-borne encephalitis virus*. Comparison of Za virus with two related viruses, a Far Eastern isolate, Sofjin, and a Siberian isolate, Vasilchenko, revealed differences among the three viruses in pathogenicity for Syrian hamsters, cytopathogenicity for PS cells, plaque morphology, and the electrophoretic profiles of virus-specific nonstructural proteins. Comparative amino acid alignments revealed 10 individual amino acid substitutions in the Za virus polyprotein sequence that were different from those of other tick-borne flaviviruses. Notably, the dimeric form of the Za virus NS1 protein migrated in polyacrylamide gels as a heterogeneous group of molecules with a significantly higher electrophoretic mobility than those of the Sofjin and Vasilchenko viruses. Two amino acid substitutions, T_{277}→V and E_{279}→G, within the NS1 dimerization domain are probably responsible for the altered oligomerization of Za virus NS1. These studies suggest that the patient from whom Za virus was isolated died due to increased pathogenicity of the latent virus following spontaneous mutagenesis.

Tick-borne encephalitis virus (TBEV) is an extremely dangerous human pathogen, causing about 10,000 to 14,000 human cases of tick-borne encephalitis (TBE) across Europe and Asia annually (http://www4.tbe-info.com/epidemiology/). Approximately 11,000 of these cases occur in Russia, and the other 3,000 occur in Western Europe. According to the most recent classification scheme, TBEV is defined as a species within the *Mammalian tick-borne virus* group, which together with the *Seabird tick-borne virus* group comprises the tick-borne viruses within the genus *Flavivirus* of the family *Flaviviridae* (34). Other species within the mammalian tick-borne virus group include *Omsk hemorrhagic fever virus*, *Powassan virus*, *Langat virus* (LGTV), *Kyasanur Forest disease virus*, and *Louping ill virus*.

The virion is 50 nm in diameter with an icosahedral capsid composed of capsid (C) protein and an RNA genome about 11 kb long. The capsid is surrounded by a lipid bilayer derived from host membranes and contains the small viral membrane (M) protein and the larger viral envelope (E) protein. The genomic positive-strand RNA is translated into a polyprotein from which three structural proteins (C, M, and E) and seven nonstructural proteins, designated NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5, are processed by cellular and viral proteases (5).

Different strains of TBEV are antigenically very closely related, and at first it was believed that only one virus circulated across Europe, Siberia, and the Far East. The existence of two different pathogenic variants, central European encephalitis virus and Russian spring summer encephalitis virus, was proposed in 1944 (6), and antigenic differences between these viruses were subsequently confirmed by serological tests (7). More recently, a third subtype of TBEV, previously called west Siberian virus, was defined based on clinical signs in humans, geographical location, and antigenic analysis (57). Nucleotide sequencing and phylogenetic analysis (8, 21, 25, 46, 56, 68, 86, 87) have now enabled these viruses to be defined as three subtypes of the same TBEV species, namely, European, Siberian, and Far Eastern, previously defined as central European encephalitis virus, west Siberian virus, and Russian spring summer encephalitis virus, respectively (34).

In humans, TBEV produces a variety of clinical manifestations. The subclinical form of infection accounts for ~85 to 95% of all TBE cases in regions of endemicity and is recognized only following serological surveys (72). Clinically recognized forms of TBE include febrile disease without neurological sequelae (80 to 98% of all TBE cases) and encephalitis of differing severity levels, the most severe form resulting in disability or death. Chronic infections occur less frequently than the other forms of TBE. In some regions of Russia, they are seen in 1 to 3% of all TBE cases, mainly in Siberia and the Far East (58, 73). The chronic form of TBE is seen in patients after a long incubation period—sometimes years after the tick bite—or in some cases as the long-term consequence of an acute neurological form of TBEV infection (relapsing form).

Although the European, Siberian, and Far Eastern virus

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subtypes all produce a variety of clinical symptoms in humans, it is believed that the severity and clinical characteristics are associated with the specific TBEV subtype (4, 85). Human infection with the Far Eastern subtype results in the most severe form of central nervous system disorder, with a tendency for the patient to develop focal meningoencephalitis or polyencephalitis accompanied by loss of consciousness and prolonged feelings of fatigue during recovery. In the most severe forms, there is major damage to neurons in different parts of the brain and spinal cord. Case fatality rates of 20 to 40% have been recorded. During these epidemics, the progressive form of TBE is rarely observed, and the disease is more severe in children than in adults. In contrast, the Siberian subtype characteristically induces a less severe acute period and a high prevalence of the nonparalytic febrile form of encephalitis. Case fatality rates rarely exceed 6 to 8%. Instead, there is a tendency for patients to develop chronic TBE. Encephalitis produced by European-subtype viruses is biphasic, with fever during the first phase and neurological disorders of different severities, which occur in 20 to 30% of patients, during the second phase. In contrast with the severe Far Eastern-subtype infections, those following infection by European-subtype strains are usually milder, mostly without sequelae; case fatality rates are often as low as 1 to 2%, and the disease is less severe in children than in adults. Chronic TBE has never been reported in Europe. Comparative experiments with sheep and monkeys have confirmed pathogenic differences between Far Eastern- and European-subtype viruses on the one hand and Far Eastern- and Siberian-subtype strains on the other (85).

Despite the abundance of studies of the mechanisms of flavivirus virulence, the reasons for the different clinical manifestations of TBEV in humans are not understood. Here, we report the isolation and molecular biological characterization of TBEV strain Zausaev (Za), which caused fatal encephalitis in a patient who died after an incubation period estimated to be 10 years. Sequence analysis demonstrated that this virus belongs to the Siberian subtype of TBEV, together with Vasilchenko (Vs) virus, which was isolated from a patient with a subclinical form of TBE (10). The results of our investigation revealed a range of biological differences between the Za and Vs viruses, including altered oligomerization of the NS1 protein, cytopathic effect (CPE), plaque morphology, and pathogenic characteristics for Syrian hamsters. The possible effects of individual mutations within the NS1 protein on the pathogenicity of Za virus and their implication in this fatal case are discussed.

MATERIALS AND METHODS

Viruses and cells. The viruses Sofjin (Sof; unknown passage history), Za (7th passage), and Vs (20th passage) were maintained as 10% suckling mouse brain suspensions. All biological experiments with viruses on PS cell cultures and in animals were carried out in the Chumakov Institute of Poliomyelitis and Viral Encephalitis of the Russian Medical Academy, Moscow, Russia.

RNA extraction and reverse transcription (RT) of viral RNA. Viral RNA was precipitated from 100 μl of Vs virus-infected mouse brain suspension by incubation with 1 ml of Catrimox (Iowa Biotechnology Corp., Oakdale, Iowa) as described previously (23, 26) and reconstituted in 50 μl of water. For first-strand cDNA synthesis (23), 11 μl of RNA and 5 μl of an appropriate primer (50 pM) were mixed and heated for 2 min at 95°C. The mixture was then chilled, and 3 μl of deoxynucleoside triphosphates (10 mM), 3 μl of dithiothreitol (0.1 mM), 1 μl of RNasin (40 U), 6 μl of 5× buffer (GIBCO-BRL, Paisley, United Kingdom), and 1 μl of the reverse transcriptase Superscript II were added. The mixture was incubated at 42°C for 2 h.

Derivation of PCR products and sequencing. Two large overlapping PCR products were produced from 10% infected mouse brain suspension under the conditions for long high-fidelity RT-PCR described previously (23, 26). The PCR products for both halves of the genome were amplified in five different tubes, pooled, and purified using the Qiagen DNA purification kit. Primers for RT-PCR and sequencing were designed as described previously for Vs virus (21, 23, 25).

Sequencing reactions were performed using a *Taq* Dye Deoxy Terminator sequencing kit (Perkin-Elmer–Applied Biosystems Inc.) as recommended by the manufacturer, and the products were analyzed using an automated Applied Biosystems 373 XL DNA sequencer.

Sequencing analysis. The nucleotide and deduced amino acid sequences of Za virus were aligned with those of other flaviviruses using ClustalX (82) and analyzed using BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html), GeneDoc (http://www.psc.edu/biomed/genedoc/gddl.htm), and MEGA (39; http://www.megasoftware.net/).

Neighbor-joining and bootstrap analyses were undertaken using ClustalX to estimate phylogenetic relationships. The resultant trees were viewed in TreeView (51). Alignments are available on request. Accession numbers for flaviviruses used for analysis, if not specified in the text, were taken from the literature (8, 14, 15, 21, 22, 28, 30, 34, 47, 49, 86, 87).

Plaque assays. PS cells in 50-ml flasks were inoculated with 10-fold dilutions of 10% infected mouse brain suspension. After 1 h of virus adsorption, the inoculum was removed and cell monolayers were overlaid with 1% Difco Agar in Earle solution containing 0.22% sodium bicarbonate, 2% fetal calf serum, and 0.002% neutral red. Plaques were observed after incubation at 37°C for 9 days.

Estimation of CPE. PS cells in 50-ml flasks were infected with Sof, Za, or Vs virus at estimated multiplicities of infection (MOI) of 1 or at 20 50% lethal doses (LD_{50})/cell. Cell destruction was observed by light microscopy at 24, 48, 72, 96, and 120 h.

Virus growth cycles in PS cells. Monolayers of PS cells in 50-ml flasks were infected with TBEV at an estimated MOI of 5 LD_{50}/cell. Each experiment was performed in quadruplicate. The inoculum (0.2 ml) was removed after 1 h, and the monolayers were washed thoroughly with medium without serum and replaced with 1 ml of medium containing 2% fetal calf serum. The supernatant media from appropriate wells were collected at 24, 48, and 72 h and frozen at −70°C. Virus titers were estimated as LD_{50} per milliliter by intracerebral (i.c.) inoculation using suckling mice.

Polyacrylamide gel electrophoresis and Western blotting. PS cell monolayers in 50-ml glass flasks were infected at an MOI of 20 PFU/cell with each virus, Sof, Za, or Vs. Radioactive labeling of protein was carried out according to the procedure described previously (91) with some modifications. The infected monolayers were washed with phosphate-buffered saline (PBS) 24 (for Sof and Za viruses) and 48 h (for Vs virus) postinfection. One milliliter of medium 199 without methionine and containing 5 μg of actinomycin D (Sigma, St. Louis, Mo.)/ml was added, and after incubation for 2 h at 37°C, the medium was replaced with 1 ml of methionine-free medium containing 5 μg of actinomycin D/ml, 190 mM NaCl, and 15 μCi of [^{35}S]methionine (Amersham, Little Chalfont, United Kingdom)/ml. After incubation for 3 h at 37°C, the cell monolayers were washed with PBS and dissolved in 200 μl of loading buffer.

The virus-specific proteins were analyzed using sodium dodecyl sulfate–7 to 15% polyacrylamide gel electrophoresis (PAGE) (40) and revealed by exposure of the dried gels to X-ray film.

For Western blotting, the proteins from the gel were transferred onto a nitrocellulose membrane, which was then soaked in blocking buffer (5% [wt/vol] skim milk–0.05% NP-40 in PBS) and incubated for 1 h at 37°C with a 1:2,000 dilution (in blocking buffer) of horse anti-TBEV hyperimmune gamma globulin (Institute of Vaccines and Sera, Tomsk, Russia). After being washed for 30 min in blocking buffer, the membrane was incubated with secondary antibody (goat anti-horse polyclonal immunoglobulin; 1:1,000 dilution in blocking buffer) conjugated with horseradish peroxidase (Sigma). Following the second wash, the nitrocellulose membrane was treated with appropriate substrate.

TBEV-specific proteins were identified by reference to previous publications (43). Since the sequences of the proteins are now known, corrections have been made to the molecular weights (MW) of proteins that were originally determined on the basis of electrophoretic mobility (EM).

Neurovirulence and neuroinvasiveness tests in mice. To estimate neurovirulence and neuroinvasiveness, 3- to 4-week-old mice (four groups with 20 animals in each) were inoculated i.c. or intraperitoneally, respectively, with 100 or 1,000 LD_{50} of Sof, Za, or Vs virus. Sick animals were euthanized at the stage of paralysis, and the results were evaluated as to the assumed mortality rate and

average survival time (AST) (the average number of days to the appearance of paralysis) of infected animals. The titer of virus recovered from the brain of each infected animal was determined as LD_{50} per milliliter following i.c. inoculation into suckling mice.

Neurovirulence and neuroinvasiveness tests in Syrian hamsters. To determine neurovirulence and neuroinvasiveness in Syrian hamsters, four groups of hamsters (3 to 4 weeks old; 60 to 80 g each; 20 animals in each group) were injected i.c. or subcutaneously (s.c.) with 100 or 10,000 LD_{50} of each virus. The animals were observed for 30 days. Sick animals were sacrificed, and the assumed mortality rate and AST were evaluated.

Virus growth cycles in brains of asymptomatic hamsters. Four groups of Syrian hamsters were inoculated i.c. or s.c. with 100 LD_{50} of Za or Vs virus. Asymptomatic animals were euthanized on days 2, 5, 8, 14, and 30 after i.c. inoculation and on days 1, 3, 5, 7, 8, 9, 14, 21, and 30 after s.c. inoculation. The titer of virus recovered from three brains (per time period) was determined as LD_{50} per milliliter following i.c. inoculation of suckling mice.

RESULTS

Isolation and serological identification of Za virus. Za virus was isolated in the Institute of Poliomyelitis and Viral Encephalitis of the Russian Medical Academy. The 34-year-old patient was admitted to the Clinic of Neurologic Diseases (Tomsk, Russia) for the first time in 1983. The patient had a very weak and thin left hand with limited movement of the fingers and prolonged uncontrollable contractions. He had no record of acute TBE but informed the clinicians that in 1973 he had removed an embedded tick from his skin. He had no record of vaccination against TBEV. The first symptoms of encephalitis appeared in 1983, i.e., 10 years after the tick bite. There had been no related record of ill health. The symptoms progressed steadily after 1983. By 1984, the limb weakness had spread to the left leg and the right hand, and the patient also started to suffer severe choking episodes. As a last resort, the serologically negative patient was immunized with inactivated TBE vaccine, but no immune response was detected. His T-cell responses were negative, and it was therefore assumed that further treatments could not make the situation worse. Therefore, he was also treated with specific gamma globulin and RNase and with interferon therapy, none of which produced an accelerated deterioration in his condition. Nevertheless, the disease progressed, and the patient died in intensive care in 1985. It should be emphasized that there were no TBEV-specific antibodies in the serum of the patient nor any detectable T-cell immune responses at any time during the illness, even though he had been immunized during the illness (unpublished data). The disease was diagnosed as seronegative progressive chronic TBE. Similar cases are often diagnosed in Siberia and the Far East (58, 73).

Several attempts had been made to isolate virus from the blood of the patient during his lifetime, but none had been successful. The virus was isolated postmortem from the medulla oblongata by i.c. inoculation of tissue homogenate into suckling white mice. Thirty percent of the animals developed symptoms of mild disease (weakness and paresis) at the first passage. Virus isolated following the fourth passage produced encephalitis in 100% of the mice, and virus from the seventh passage was used for subsequent laboratory research.

In order to reduce the risk of cross-contamination between viruses, the work on Za virus isolation was carried out in a dedicated isolation room where no work with other related viruses had been done for a considerable time.

Antigenic identification based on complement fixation, hemagglutination, neutralization, and immunodiffusion in agar demonstrated that Za virus was antigenically very similar to other Russian strains of TBEV (unpublished data).

Replication of Za virus in PS cells. The CPE produced on PS cells by Za virus was evaluated in comparison with those of the Sof and Vs strains of TBEV at estimated MOI of 1 and 20 LD_{50}/cell over the period from 24 to 120 h postinfection. For each virus, there was a direct correlation between the initial MOI and the level of cell destruction. The relative extent of CPE induced by Za virus resembled that of Sof rather than Vs virus at both virus input concentrations. These viruses completely destroyed monolayers within 48 and 96 h postinfection at the high and low MOI, respectively. Vs virus induced significantly less extensive CPE than the Za and Sof viruses, with at least 25 and 50% of the monolayer remaining intact 120 h after high and low input multiplicities, respectively.

Comparison of plaque sizes (Fig. 1A) demonstrated similarity between the Sof and Za viruses (average, 4- to 5-mm diameter on day 6), although the plaque morphologies were different: Sof virus produced clear plaques with smooth edges, whereas Za virus produced more turbid plaques with rough edges, making the plaques look like stars. Vs virus formed significantly larger plaques with smooth edges (7- to 9-mm diameter on day 6).

The growth kinetics of the three viruses in PS cells were compared (Fig. 1B). They were very similar, reaching peak infectivities 48 h postinfection.

Neuroinvasiveness and neurovirulence of Za virus. The Sof, Za, and Vs viruses induced similar mortality rates and ASTs in 3- to 4-week-old mice following i.c. or intraperitoneal inoculation with two different challenge doses of virus, 100 or 1,000 LD_{50}. The mortality rate was 100% at both virus input concentrations, and the AST was 5 to 7 days (data not shown).

Differences in pathogenicity among the Za, Sof, and Vs viruses were observed in 3- to 4-week-old Syrian hamsters. The mortality for Sof virus in these experiments was 100% with an AST of 9 days following s.c. challenge doses of either 100 or 10 LD_{50}. These results are consistent with those published previously (41).

For both Za and Vs viruses, 95% of the hamsters remained healthy following i.c. inoculation. The AST for the 5% that died with clinical symptoms of TBE was 15 to 16 days, regardless of whether the virus dose was 100 or 10,000 LD_{50}/animal. Following s.c. inoculation, Za virus produced no visible disease in hamsters during 3 months of observation, and Vs virus induced only 3% mortality during this period.

The titers of virus recoverable from the brains of asymptomatic hamsters 30 days after i.c. and s.c. inoculations were estimated for Za and Vs viruses (Fig. 2). The two viruses had similar rates of replication after i.c. inoculation; nevertheless, the average titers of Za virus were approximately 30 times higher than those of Vs virus 5 to 14 days postinfection (Fig. 2A). By day 30, both viruses had been cleared from the brain (Fig. 2A).

After s.c. inoculation, the rates of Vs and Za virus reproduction were different, but the maximum virus titers were similar and did not exceed 10^6 LD_{50}/ml (Fig. 2B). Za virus was detected earlier and moved out of the brains more rapidly than Vs virus (Fig. 2B). By day 30 postinfection, Za virus was still present in low titers while Vs virus was not detectable (Fig.

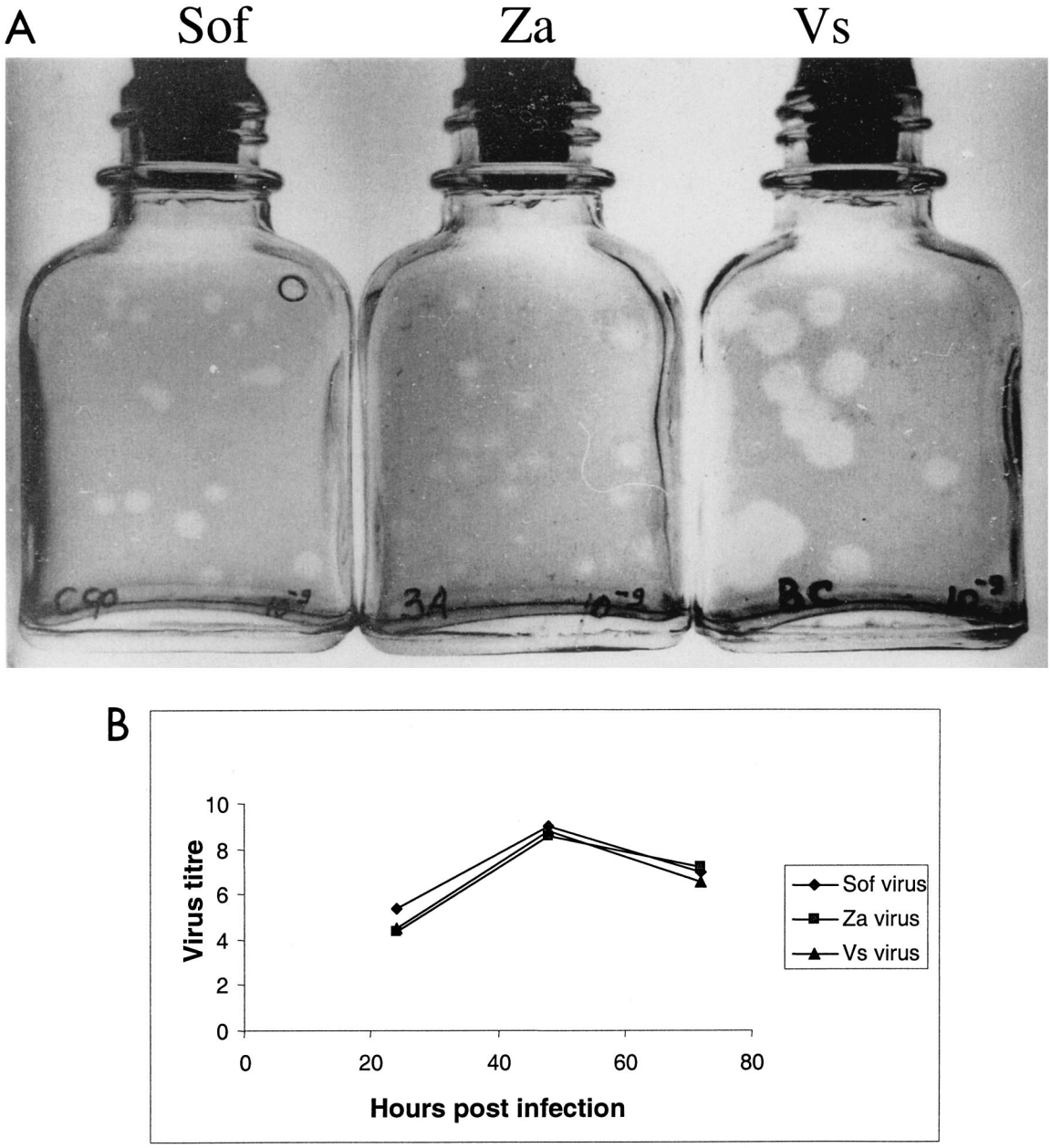

FIG. 1. Replication of Za virus in PS cells in comparison with Sof and Vs viruses. (A) Characteristics of plaques produced by Sof, Za, and Vs viruses. (B) Dynamics of virus reproduction. PS cell monolayers were infected with Sof, Za, and Vs viruses at an MOI of 5 LD_{50}/cell, and supernatant medium was collected at 24, 48, and 72 h postinfection (*x* axis). Virus titers were estimated as LD_{50} per milliliter in suckling mice (*y* axis).

2B). Sof virus killed all of the animals, reaching peak titers of 1×10^8 to 10×10^8 LD_{50}/ml.

Comparative protein profiles of Za, Vs, and Sof viruses. PAGE analysis revealed similar but not identical protein profiles for the Sof, Za, and Vs viruses (Fig. 3). Previously identified intracellular TBEV-specific proteins (42, 90, 92) in this work were redesignated (with the appropriate MW) on the basis of comparison with the equivalent proteins of mosquito-borne flaviviruses (78–80).

The NS5 (p103), NS2b-NS3 (p83), NS3 (p69), E (p53), prM (p18), NS4A (p16)m, and C (p12) proteins (Fig. 3) of the three viruses had identical EMs. The proteins NS4B (p27), NS2A (p25), and NS2B (p14) of the Sof virus were distinguishable from the equivalent proteins of the Za and Vs viruses, and this corresponds to previously revealed differences between closely related flaviviruses (93). Figure 3 shows the absence of a correlation between the MW of small proteins of these three viruses and their EMs in discontinuous gel systems, which was described previously for mosquito-borne viruses (78–80). Since no differences between cleavage sites in different virus genes were shown by alignment of the polyprotein sequences (see below), the differences in EM could probably be accounted for

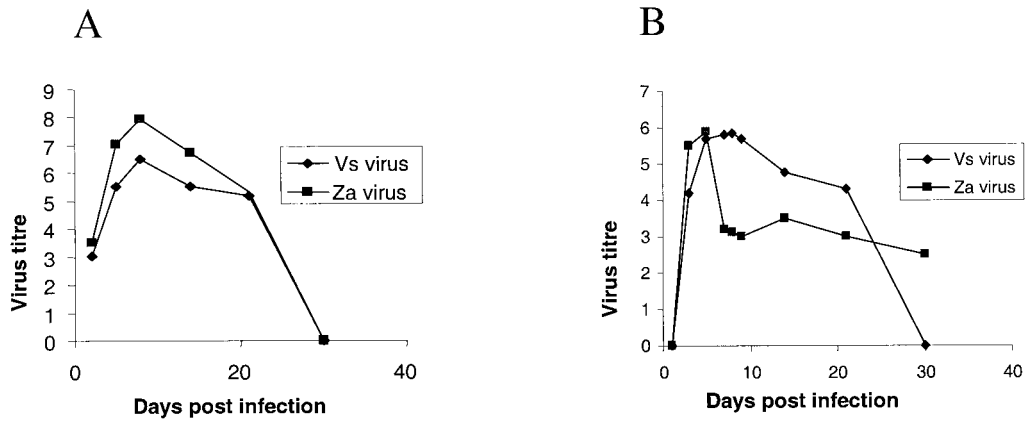

FIG. 2. Dynamics of Za and Vs virus reproduction within the brains of healthy Syrian hamsters after i.c. (A) and s.c. (B) inoculation with 100 LD_{50} of each virus. Virus titers (in LD_{50} per milliliter in suckling mice; *y* axis) had been determined on days 2, 5, 8, 14, 21, and and 30 (*x* axis) after i.c. inoculation and on days 1, 3, 5, 7, 9, 14, and 30 after s.c. inoculation.

by the differences in amino acid composition. It is likely that these differences affect the binding of sodium dodecyl sulfate and migration speed in acrylamide gels (53).

Most notably, the NS1 protein (p49) of Za virus was distinct from those of the Sof and Vs viruses. Under denaturing conditions, the NS1 proteins of all flaviviruses form dimers that degrade to monomers after being boiled for 5 min (89). The NS1 protein of Za virus in unheated samples formed a broad band or smear covering the MW range of 70,000 (70K) to 150K (Fig. 3A), and this was also revealed by Western blotting with polyclonal anti-TBEV antibodies (Fig. 3B). The EMs of dimeric molecules of Sof and Vs virus NS1 proteins were also different, but each of them formed a homogeneous band. Monomeric NS1 proteins were not always separated properly from radioactively labeled cellular proteins (Fig. 3A) but could be visualized by Western blotting (Fig. 3B). The EMs of monomeric NS1 proteins were different for all three viruses (Fig. 3B).

Nucleotide and deduced amino acid sequences of Za virus. The nucleotide and deduced amino acid sequences for the complete genome of Za virus were determined and compared with those of other tick-transmitted flaviviruses using either the whole polyprotein, the envelope E gene, or the 3′ untranslated region (3′ UTR).

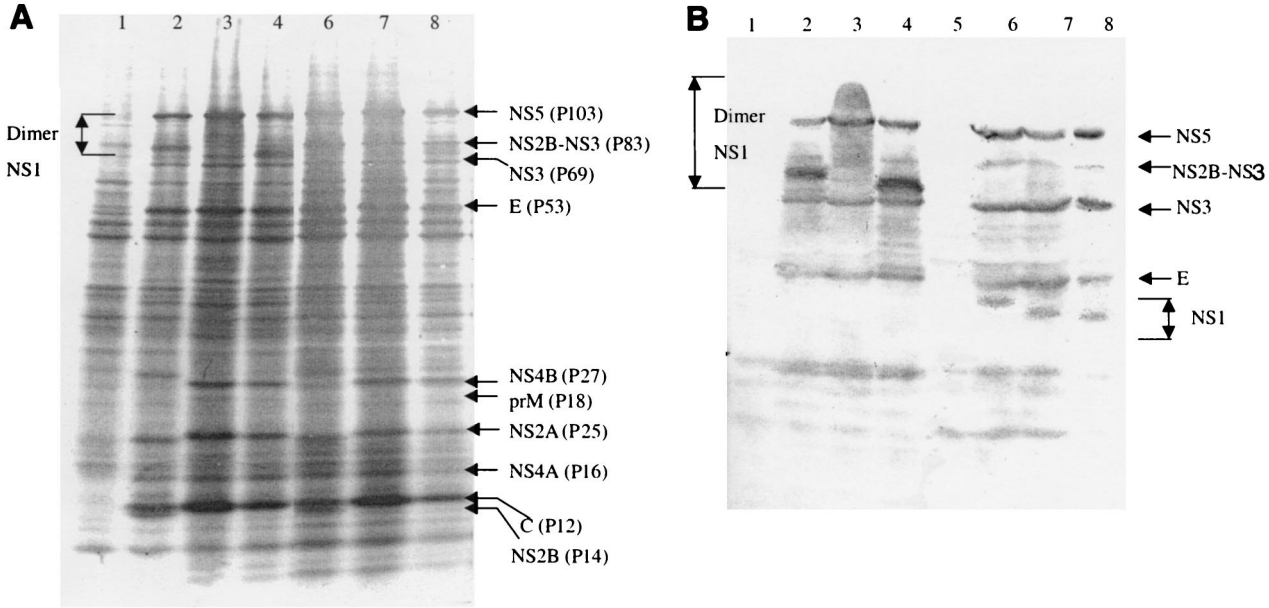

FIG. 3. Analysis of TBEV-specific proteins by PAGE (A) and Western blotting (B). Infected PS cell monolayers were lysed 48 h postinfection for Sof and Za viruses and 72 h postinfection for Vs virus. Proteins were separated by electrophoresis on 7 to 15% polyacrylamide gels. For Western blotting, the proteins were transferred onto a nitrocellulose membrane and revealed by interaction with anti-TBEV antibodies. Lanes 1 and 5 contained mock-infected PS cells; the other lanes contained PS cells infected with Sof (lanes 2 and 6), Za (lanes 3 and 7), and Vs (lanes 4 and 9) viruses. Samples 1 to 4 were unheated and samples 5 to 8 were heated for 1 min at 95°C. The virus-encoded proteins and their masses are specified.

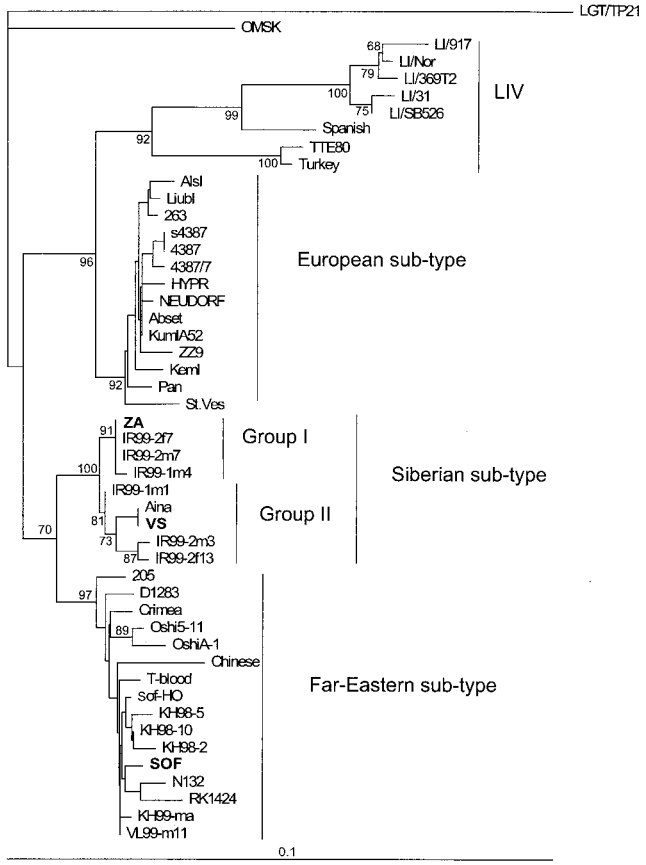

FIG. 4. E protein phylogeny showing the position of ZA virus relative to 48 other TBE virus E protein sequences (see Materials and Methods). The phylogeny was estimated using ClustalX (default settings) but invoking the "exclude gaps" and "correct for multiple substitutions" options. Bootstrap values (percentage of 1,000 replicates) of >65% are shown above or below the branches. The three viruses in this study are shown in boldface. LIV, louping ill virus.

E protein. Comparative sequence alignments and phylogenetic analysis, based on the E proteins of Za virus and 48 other tick-borne flaviviruses, showed that Za virus is a member of the Siberian subtype of TBEV (Fig. 4). This subtype also includes the Vs and Aina viruses (8, 21, 30, 34), both of which have been characterized in detail (57). However, it was previously demonstrated that the Siberian subtype of TBEV can be subdivided into two subclusters (groups I and II [Fig. 4]) on the basis of detailed phylogenetic analyses (30). Overall, the branching pattern in Fig. 4 is congruent with previously published trees (22), and the two Siberian subclusters are clearly identifiable and supported by high bootstrap values. The analyses placed the Za virus in subcluster I and the Vs and Aina viruses in subcluster II.

It was previously demonstrated that several amino acids within the E protein (so-called signatures) can differentiate tick-borne flaviviruses in accordance with their serological classifications (8, 21, 22, 24, 76, 77). Table 1 shows that two amino acids, Q_{234}-T_{431} and H_{234}-A_{431} (E-protein numeration) can subdivide the Siberian subtype into two groups (subclusters), in concordance with their phylogenetic differentiation (30). Za virus falls into the subcluster H_{234}-A_{431}, different from the subcluster Q_{234}-T_{431} for Vs and Aina viruses (Table 1). The Vs and Aina viruses have identical sequences in the E gene but differ from Za virus in three other positions corresponding to V_{119}-A_{279}-F_{349}. In these positions, Za virus has the signature A_{119}-T_{279}-S_{349}, which is identical to those of other Siberian viruses in both subclusters (Table 1). No unique amino acid substitutions in the E gene have been identified for the Za and Vs viruses.

3′ UTR. A phylogenetic analysis and a nucleotide alignment of the 3′-UTR sequence of Za virus with those of other TBEVs confirmed the grouping of this virus with the Siberian strains that form subcluster I, corresponding to H_{234}-A_{431} of the E protein. The nucleotide differences between the two Siberian subclusters are summarized in Table 2.

The alignment of Za virus with other TBEVs, which is represented schematically in Fig. 5A, revealed that, in common with many other TBEVs, Za virus has a large deletion between nucleotides 79 and 346 within the hypervariable domain of the 3′ UTR. This domain, which is not important for virus infectivity (48), was identified previously between the stop codon and the terminal conserved region (core, or C3′ UTR) (Fig. 5A) (25, 30, 86). One individual nucleotide substitution for Za virus, A_{280}→G (position 554 of the Ir-2m4 virus [Fig. 5A]), occurs within the stem of stem-loop structure 5 of the C3′ UTR (25). Folding of the 3′ UTR of Za virus using the

TABLE 1. Identification of Za virus among Siberian isolates based on alignment of E proteins

Virus name	Source of virus	Yr of isolation	Accession no.	Amino acid[a]						
				A_{83}	A_{119}	T_{128}	H_{234}	T_{279}	S_{379}	A_{431}
Stolby-1	*I. persulcatus*	Not known	AAF43697							
IR99-2m7	*I. persulcatus*	1999	AB049351							
IR99-1m4	*I. persulcatus*	1999	AB049398							
IR99-2f7	*I. persulcatus*	1999	AB049352							
Za	Human brain	1985	AF527415							
IR99-1m1	*I. persulcatus*	1999	AB049348				Q			T
Vs	Human blood	1961	M97659		V		Q	A	F	T
Aina	HCSF[b]	1963	AF091006		V		Q	A	F	T
IR99-2f13	*I. persulcatus*	1999	AB049353	T	V	I	Q			T
IR99-2m3	*I. persulcatus*	1999	AB049350	T	V	I	Q			T

[a] Column headers are the amino acids (E protein numeration) that distinguish the viruses (virus signatures). Only amino acids that differ from the signatures are shown. The virus description have been taken from references 30, 57, and 58.
[b] HCSF, human cerebrospinal fluid.

TABLE 2. Identification of Za virus among Siberian TBEV, on the basis of 3′ UTR sequence

Virus	Accession no.	Nucluotide[a]														
		A_2	C_5	C_8	C_{37}	A_{75}	G_{377}	G_{404}	T_{421}	T_{423}	T_{484}	A_{537}	A_{556}	T_{594}	C_{620}	T_{635}	C_{695}
IR99-2m7	AB049399			G													
Za	AF527415			G	T										T		
IR99-1m4	AB049398			T													G
IR99-1m1	AB049397	G	T	T	T	T	A	A	C	C	C	G	T	C	T		G
IR99-2f13	AB049400	G	T		T	T	A	A	C	C	C	G	T	C	T	C	
Vs	L40361	G	T		T	T	A	A	C	C		G	T	C	T	C	
Aina	TEU27492	G	T		T	T	A	A	C	C		G	T	C	T		

[a] The column headers represent nucleotides within the 3′ UTR, and the letters in the columns represent substitutions. The numeration corresponds to the sequence of strain IR99-1m4 of TBEV (Hayasaka, et al. no. 2930 [30]) starting from the first nucleotide after the stop codon.

MFOLD program demonstrated that this mutation does not disrupt the secondary structure of the stems and loops conserved among tick-borne flaviviruses (results not shown).

Polyproteins. Alignment of Za virus with 11 available polyprotein sequences of other tick-borne flaviviruses revealed 10 unique amino acid substitutions (Table 3). Four of these were conserved, and the other six (Table 3) possibly cause minor hydrophobicity shifts in the C, NS1, or NS5 protein. For comparison, Vs virus had nine unique substitutions, three of which mapped either in the NS3 or the NS5 gene and were nonconservative in nature. None of the amino acid substitutions altered the essential motifs important for the function of the virus-specific protease (NS2B-NS3), helicase (NS3), RNA polymerase, or methyltransferase (44).

Significantly, within the dimerization domain of the NS1 protein, Za virus differed from both the Sof and Vs viruses at two positions, viz., $T_{277} \rightarrow V$ and $E_{279} \rightarrow G$ (for the Za polyprotein, the numeration is V_{1053} and G_{1055}, respectively). Among the tick-borne flaviviruses, V_{277} was also present in this position only for Powassan virus and Kyasanur Forest disease virus (Fig. 5A). The other chemically nonconserved substitution, $E_{279} \rightarrow G$, was specific in the NS1 protein for only a few flaviviruses distantly related to the tick-borne virus group, namely, dengue virus serotype 4 (mosquito borne), cell fusing agent virus (isolated from a mosquito cell line), Apoi virus, and Rio Bravo virus (both nonvectored flaviviruses) (34). In view of the fact that both the amino acids V_{277} and G_{279} mapped in the dimerization domain of the Za virus NS1 protein and because both of them increase the hydrophobicity of this region, they are likely to be responsible for the distinct oligomerization of the NS1 protein of Za virus shown in the PAGE analysis (Fig. 3).

DISCUSSION

Although TBEV is usually thought of as being associated with severe neurological disease culminating in disability and death, approximately 85 to 95% of all infections actually result in a febrile disease with flu-like symptoms which do not progress to encephalitis. Moreover, the chronic form of TBE

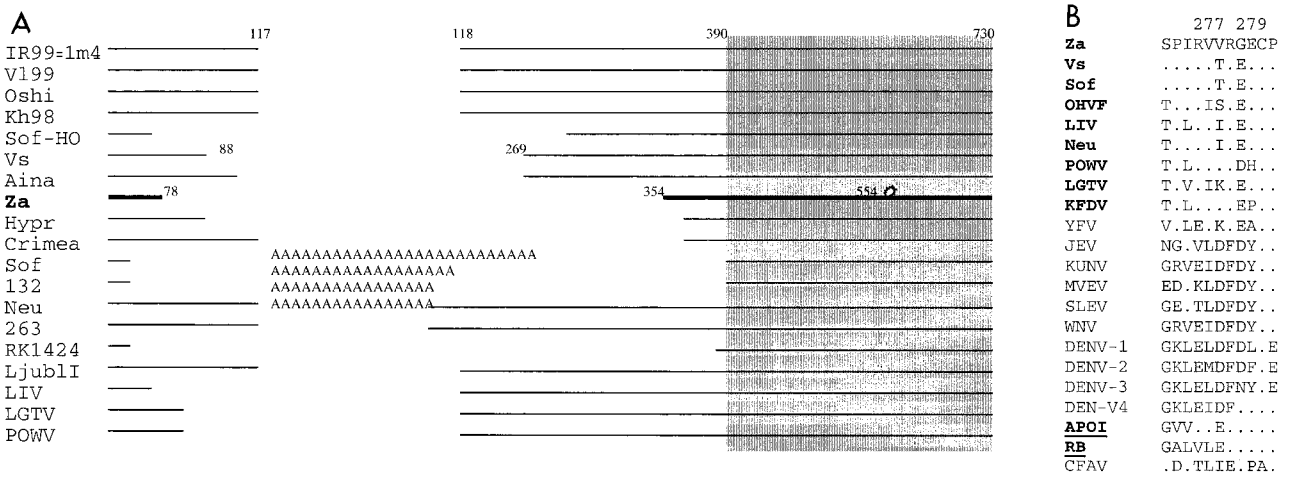

FIG. 5. Sequence analysis of Za virus RNA. (A) Schematic presentation of aligned 3′-UTR sequences of different TBEV isolates compared with that of Za virus (boldface). The numeration starts after the stop codon and refers to the longest 3′ UTR of the Siberian strain IR99-1m4 (29). The lines and gaps represent the sequences and deletions, respectively, within the hypervariable domain. The internal poly(A) sequences are shown as insertions. The conserved (C3′-UTR, or core) domain is shaded. The unique nucleotide substitution for Za virus is depicted as a star. (B) Aligned sequences of the dimerization domain of NS1 protein for tick-borne (boldface) and mosquito-borne flaviviruses and the nonvectored *Apoi virus* (APOI) and *Rio Bravo virus* (RB) (boldface and underlined). Positions 277 and 279 of the NS1 protein, in which the amino acid sequence for Za virus is different from those of the Vs and Sof viruses, are shaded. The designations of viruses with accession numbers are listed in reference 33.

TABLE 3. Individual amino acid substitutions for Za and Vs viruses in comparison with 11 tick-borne flaviviruses as revealed by polyprotein alignment

Virus protein	Protein numeration	Polyprotein numeration	Amino acids for other tick-borne flaviviruses	Individual substitutions[a]	
				Za	Vs
C	73	73	A, L	**T**	
	80	80	K, R	**M**	
PrM	2	115	L, A, M	F	
NS1	123	899	M, F, I	V	
		279	E, D	**G**	
		294	K	R	
NS3	246	1741	A	V	
	16	1505	R, Q		K
	64	1553	D, E		**N**
	186	1675	T, G		M
NS4B	195	2464	A, L, T		V
NS5	195	2706	A	**T**	
	706	3217	E	**A**	
	407	2918	S	**N**	
	51	2562	M, V		**T**
	202	2713	Q, K		R
	261	2772	V, T		I
	272	2783	K		R
	297	2808	R, K, E, H		**G**

[a] Nonconserved amino acid substitutions are in boldface.

appears to be quite rare and has been identified only in patients in Siberia and the Russian Far East.

The patient described in this paper was not the first human case of progressive chronic TBE; there have been other seropositive and seronegative patients with similar symptoms (58, 73). The patient was properly monitored from the first mild symptoms of encephalitis (weakness of the left hand) in 1983 to the comatose stage and death in 1985. During his medical examination, he remembered that he had been bitten by a tick in 1973. While it cannot be proved that the patient was not bitten during the subsequent 10-year period, he was monitored very closely for the final 2 years before he died and is therefore extremely unlikely to have been bitten during that period. All attempts to recover infectious virus from his blood during these final 2 years failed. However, postmortem, Za virus was isolated from his brain and positively identified as a strain of TBEV in serological tests. The pathomorphological features of the brain were consistent with the lesions typical of lethal TBEV infections. Our objective was to see whether we could identify distinct biological and molecular biological features of Za virus that could explain the reasons for chronic manifestations of TBE in the patient.

Comparison of the nucleotide and amino acid sequences of the polyprotein, the E gene, and the 3′ UTR of Za virus with published TBEV sequences (8, 24, 30, 75, 86) showed that Za virus belongs to the Siberian subtype of TBEV, together with the prototype viruses Vs and Aina. These results correlated with previously produced results of antigenic and serological identification of Za virus (unpublished). However, more detailed differentiation (Fig. 4 and Tables 1 and 2) placed Za virus in subcluster I within the Siberian subtype, differentiating it from subcluster II, which contains the Vs and Aina viruses.

Distinct biological properties were demonstrated for Za virus in comparison with the Sof and Vs viruses. Sof virus is a member of the Far Eastern subtype of TBEV and represents the most virulent TBEV. It was isolated in 1937 from the brain of a patient who died from acute encephalitis (94). This virus was used as the neurovirulent model in many laboratory experiments in Russia on hamsters, sheep, monkeys, and mice, killing 100% of the animals after i.c. or s.c. inoculation (41, 84). In contrast, Vs virus was isolated from a patient with subacute encephalitis from which he subsequently recovered completely (58). He was then monitored regularly for 11 years, during which time he was immunized with the live attenuated Elantcev strain of LGTV (antigenically related to but distinct from TBEV). At no time during these 11 years was any antibody either to TBEV or to LGTV detected in his serum (58). Nevertheless, infection of hamsters and monkeys with Vs virus resulted in the development of chronic encephalitis in a proportion of the animals (10, 45). Sequence analysis of the E protein demonstrated that Vs virus is identical to Aina virus, which was isolated from the cerebrospinal fluid of an 11-year-old girl who developed Kozshevnikov's epilepsy, with paresis in one hand, following the acute febrile form of the disease. Virus was isolated from her serum 108 days after the onset of disease. Virus-specific antibodies were not detected until 131 days after the beginning of symptoms. For the next 5 years, this patient suffered periodic relapses requiring hospitalization (58). Both Vs and Aina virus were serologically identified as Siberian subtypes of TBEV (57), and this was later confirmed by sequence analysis (8, 21, 25, 86).

Comparison of Za virus with the Sof and Vs viruses in PS cells demonstrated several differences. First, all three viruses had different plaque morphologies (Fig. 1A). Second, they produced different levels of CPE in PS cells: Sof virus produced 100% CPE at all input multiplicities, while Vs virus produced significant CPE only at high input multiplicities (Table 1). Za virus also demonstrated cytopathic activity, but it was milder than that produced by Sof virus. Despite these differences, growth curve experiments demonstrated that all three viruses were nearly identical in virus yield and the dynamics of virus reproduction (Fig. 1B). Therefore, the major difference among these viruses in PS cells could be in the ability to initiate apoptosis.

The other unique biological characteristic of Za virus was the distinct electrophoretic properties of the nonstructural NS1 protein (Fig. 5). Under denaturing and reducing conditions, the NS1 proteins of flaviviruses form dimers that dissociate to monomers at 95°C (89). However, the unheated Za virus NS1 protein formed heterogeneous aggregates that, under denaturing conditions, migrated with a range of masses between 70 and 150 kDa (Fig. 3). In the monomeric form, the NS1 proteins of all three viruses had different EMs. Nevertheless, there were no differences in the cleavage sites flanking the NS1 proteins. Therefore, the differences in mobility of the Sof, Za, and Vs virus NS1 proteins reflect their distinct amino acid sequences (53). Previously, it was demonstrated that flavivirus NS1 proteins form hexamers (9, 19, 20). The results presented here for Za virus suggest that the heterogeneous band with a mass ranging from 70 to 150 kDa consists of NS1 multimers. Alternatively, the increased hydrophobicity of the Za virus NS1 dimers might result in nonspecific interactions with cellular proteins, leading to a heterogeneous range of protein complexes.

Previously, it was demonstrated that the Syrian hamster had proved to be a suitable laboratory model to distinguish the different forms of human TBE caused by these viruses, i.e., acute and subacute TBE, chronic TBE with a preliminary acute period, and chronic TBE without a preliminary acute period, so-called progressive TBE (10, 13). A latent form of TBEV infection without a preliminary acute period or significant symptoms has also been shown to be reactivated by inoculation of immunosuppressants into latently infected hamsters 2 years after TBEV inoculation. Following immunosuppression, the hamsters developed typical encephalitis, and TBEV could be readily recovered from the brains of these animals (11, 12).

In neurovirulence and neuroinvasiveness tests in Syrian hamsters, Za virus was more similar to Vs than to Sof virus (Table 2), reflecting the previously established pathogenetic and phylogenetic differences between Siberian and Far Eastern TBEV.

Two mutations that might affect the pathogenetic characteristics of Za virus were identified in the dimerization domain of the NS1 protein (52) for Za virus in comparison with Sof and Vs viruses, namely, T_{277}→V and E_{279}→G (Fig. 5B). The function of this protein in virus replication is not completely understood, although interaction with viral polymerase complexes during double-stranded RNA synthesis has been implicated (44). Previous research demonstrated that destabilization of NS1 protein dimerization following point mutations affects the secretion of dimers and reduces virus replication (29, 62–64). The combination of two mutations in the NS1 protein, V_{277} and G_{279}, which we identified, is unique to Za virus, although the individual mutations are not unique (Fig. 5B). These two mutations, which are not present in the Sof and Vs viruses, could increase the hydrophobicity of the dimerization domain of NS1 protein, resulting in protein aggregation (Fig. 3).

Many inherited slow neurodegenerative diseases are associated with mutations in particular cellular proteins that fail to fold correctly and, as a result, aggregate, thus triggering apoptosis in neuronal cells (50). Extending this analogy to Za virus, increased hydrophobicity of NS1 as the consequence of two mutations could result in relatively increased aggregation of NS1 protein and eventually apoptotic death of PS cells without increasing the virus replication rate. However, the pathogenicity of Za virus in hamsters is not significantly different from that of Vs virus, and thus, NS1 protein aggregation cannot be implicated in the development of neuron apoptosis in hamsters (Fig. 2 and Table 2). Since apoptosis is triggered in a cell-specific manner (74, 83), the consequences of the replication of Za virus in PS cells, hamsters, and humans may not necessarily correlate with each other.

The influence of the 10 other individual mutations (Table 3) on the neurovirulent characteristics of Za virus cannot be excluded. Previously, it was demonstrated that virus attenuation results from the cumulative effects of several mutations, some of which have only small biological effects that might not be observed in routine biological tests (27). The titers of Za virus in the brain are higher than those of Vs virus during reproduction in hamsters (Fig. 2), and these differences might occur because of the cumulative effects of several individual mutations that, along with the mutations in the NS1 protein, relate to the higher virulence of Za virus.

Apoptosis associated with TBEV virulence has recently been reported, and a possible role for the E protein has been proposed (36, 37, 60). The envelope (E) glycoprotein of flavivirus virions has been implicated in many virus functions, namely, interaction with cell receptors during adsorption (2, 31, 35), pH-dependent fusion with cellular endosomal membranes (1, 33, 69, 81), and assembly and secretion of virion particles (33, 38, 88). This protein also induces protective immunity, interacts with virus-neutralizing antibodies (3, 32), and is involved in antibody-dependent enhancement of virus infection (17, 54, 55, 59, 70). Many point mutations that change the pathogenetic properties of flaviviruses have been identified. The crystal structure of the E protein has been determined, and the positions of amino acids that influence the pathogenetic characteristics of viruses have been mapped (66). Comparison of the E proteins of different flaviviruses has revealed hypervariable domains that can influence the virulence and antigenic properties of flaviviruses (24).

We have identified five different substitutions in the E protein between the Za and Vs viruses (Table 1), none of which is unique for Za or Vs virus and three of which are not chemically conserved (Q_{234}-H_{234}, T_{279}-A_{279}, and S_{379}-F_{379}). These differences probably have minor effects on virus reproduction, but because there are no available comparisons of the pathogenicities of the two subclusters of Siberian TBEV, it is difficult to predict the biological effects of these mutations. It is important to note that the Vs and Aina viruses have identical E protein sequences but very different clinical manifestations in humans and animals (58), and therefore for these viruses the E protein does not appear to be responsible for the different clinical outcomes.

Although the deletion in the 3′ UTR of Za virus is larger than that of Vs virus (Fig. 5A), it is not sufficiently large to destroy conserved elements of secondary structure (25, 61, 65) or to affect the virulence characteristics of TBEV (48). Different TBEV strains with long histories of laboratory passage have large deletions similar to those described above, in contrast with viruses that have short passage histories (30, 86). This deletion for Za virus might be the consequence of long-term persistence in the human body following the tick bite. We studied this virus from the seventh laboratory passage, and therefore it is possible that the deletion was the result of these passages. One individual nucleotide substitution, A_{280}→G, occurred in a conserved position within the 3′ UTRs of all tick-borne flaviviruses, but it did not change the folding pattern (25, 61, 65) and probably does not have an effect on virus reproduction.

Viruses circulating in the Irkutsk region infrequently produce fatal clinical manifestations in humans. Ninety-five percent of TBEV infections are febrile without neurological sequelae (95). Indeed, modeling of TBEV infections in Syrian hamsters supports this, since 95% of the hamsters survive i.c. inoculation of Siberian TBEV strain Vs. Moreover, virus can be found in different tissues (including brains) of healthy animals 2 years postinfection (11, 12). Therefore, it is possible that the patient with Za virus infection was infected by nonpathogenic TBEV through the tick bite and that this did not result in the development of neurological symptoms. Subsequently, dur-

ing the long period of persistence, the virus might have reached the brain and also initially failed to produce any symptoms until it had acquired two mutations in the NS1 protein that could trigger neuron apoptosis. Increased apoptotic activity of the viruses in neurons due to spontaneous mutations has been described previously (18).

Another factor that probably contributed to a fatal outcome was the absence of antibodies and of an essential T-cell immune response against TBEV in the patient at any stage of the disease (unpublished). In this sense, this case is not unique. Many viruses isolated from seronegative TBE patients have been studied, including the case of the patient from whom Vs virus was isolated. This patient was also TBEV seronegative but developed only subacute symptoms without further relapses (58). It is worth mentioning that during flavivirus infections, in addition to E protein, the NS1 protein also stimulates the development of complement-fixing antibodies that might have a protective effect through complement-mediated cytotoxicity of infected cells (16, 67, 71). In this case, the long-term persistence of Za virus in a human might be due to the failure of specific immune mechanisms contributing to the elimination of the virus.

In summary, this paper describes the molecular biological characteristics of a virus that had been isolated from a seronegative patient who died from progressive chronic TBE. Although the genomic sequence and many biological properties of this virus have been analyzed and the reasons for a fatal outcome have been proposed, many questions relating to the pathogenicity of Za virus are still not answered and need to be tested experimentally. Further research involving the introduction of mutations into the infectious clone of Vs virus (26) and studies of the biological characteristics of virus mutants could answer many of the questions related to the pathogenic properties of Za virus.

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