Chapter 4

TBE virology

Daniel Růžek, Kentaro Yoshii, Marshall E. Bloom and †Ernest A. Gould

Key points

- TBEV is the most medically important member of the tick-borne serocomplex group within the genus *Orthoflavivirus*, family *Flaviviridae*.
- Three antigenic subtypes of TBEV correspond to the 3 recognized genotypes: European (TBEV-EU), also known as Western, Far Eastern (TBEV-FE), and Siberian (TBEV-SIB).
  An additional 2 genotypes have been identified in the Irkutsk region of Russia, currently named TBE virus Baikalian subtype (TBEV-BKL) and TBE virus Himalayan subtype (Himalayan and "178-79" group; TBEV-HIM).
- TBEV virions are small enveloped spherical particles about 50 nm in diameter.
- The TBEV genome consists of a single-stranded positive sense RNA molecule.
- The genome encodes one open reading frame (ORF), which is flanked by untranslated (non-coding) regions (UTRs).
- The 5'-UTR end has a methylated nucleotide cap for canonical cellular translation. The 3'-UTR is not polyadenylated and is characterized by extensive length and sequence heterogeneity.
- The ORF encodes one large polyprotein, which is co- and post-translationally cleaved into 3 structural proteins (C, prM, and E) and 7 non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5).
- TBEV replicates in the cytoplasm of the host cell in close association with virus-induced intracellular membrane structures. Virus assembly occurs in the endoplasmic reticulum.
  The immature virions are transported to the Golgi complex, and mature virions pass through the host secretory pathway and are finally released from the host cell by fusion of the transport vesicle membrane with the plasma membrane.

Virus classification

Tick-borne encephalitis virus (TBEV) is the most medically important member of the tick-borne serocomplex group within the genus *Orthoflavivirus*, family *Flaviviridae* (from the Latin *flavus* – 'yellow', referring to the prototype virus, yellow fever virus).[1]

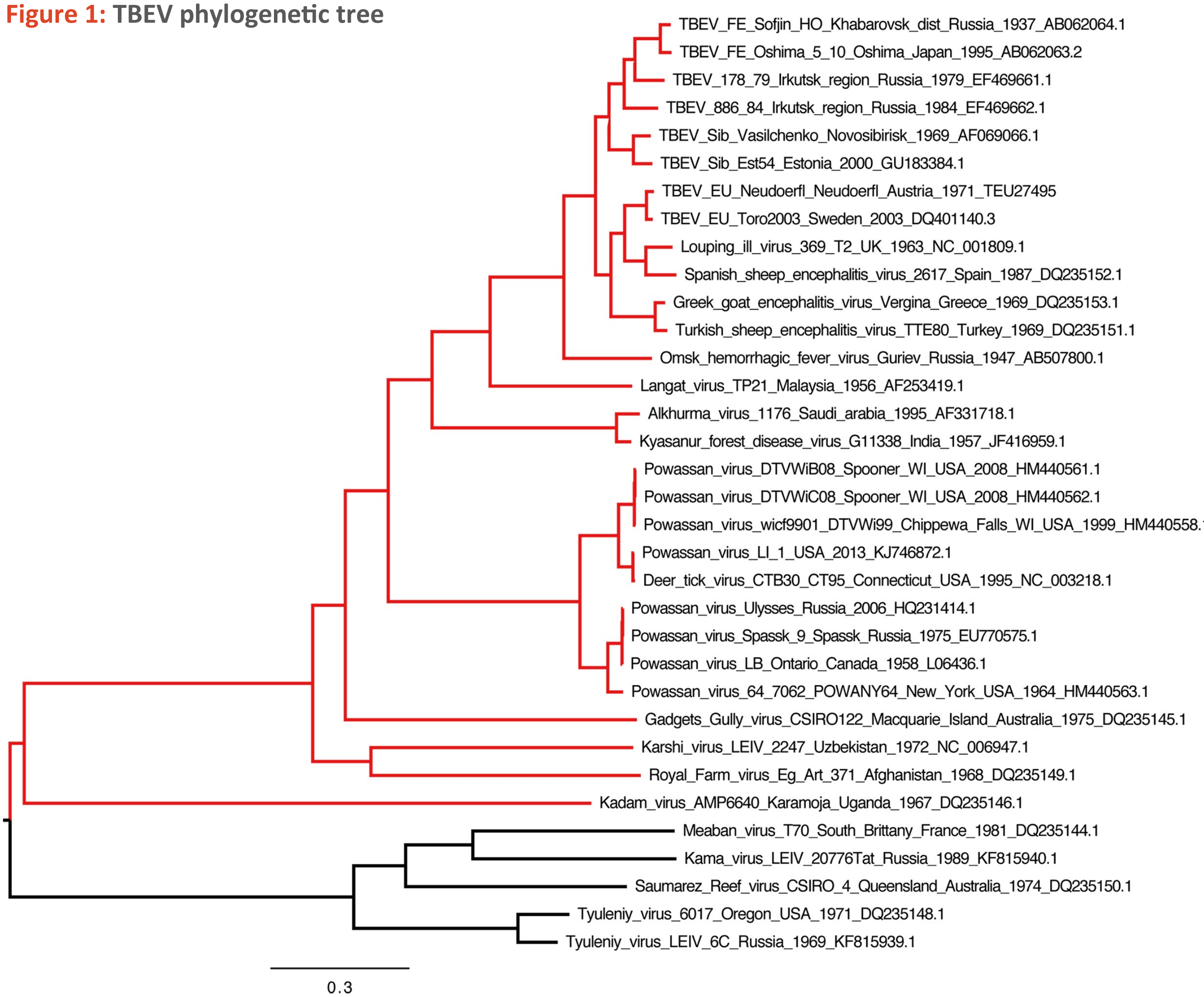
The genus *Orthoflavivirus* comprises over 70 virus species, many of which are important human pathogens.[2] Besides TBEV, these include mosquito-borne viruses such as dengue viruses, Japanese encephalitis virus, yellow fever virus, Zika virus, and many others. Virtually the entire human population lives where at least one flavivirus species is endemic.[2] Moreover, many orthoflaviviruses have recently expanded their endemic areas, being introduced to novel loci either on new continents (West Nile virus, Zika virus, etc.) or to areas with higher altitude or latitude (TBEV as an example).[3,4] For these reasons, flaviviruses pose an important threat to public and animal health. Moreover, they have high zoonotic potential because they can infect a broad range of hosts and vectors including domestic animals.

Most of the known flaviviruses are transmitted horizontally between hematophagous arthropods (ticks or mosquitoes) and their vertebrate hosts. They are therefore considered to be dual-host viruses. Depending on the recognized arthropod vector, they are divided into mosquito-borne or tick-borne viruses.

The term 'arbovirus' (an acronym from '**ar**thropod-**bo**rne **virus**') is non-taxonomic but is frequently used for viruses that cycle between vertebrates and arthropod vectors. However, not all orthoflaviviruses are arboviruses – some are vertebrate-specific (also called 'No known vector' and further divided into rodent-specific and bat-specific flaviviruses, with best-characterized representatives Rio Bravo and Modoc viruses)[5] while some are insect-specific.[6] These classifications reflect the adaptation of the viruses to particular invertebrate or vertebrate hosts, and modes of virus transmission in nature.

Tick-borne orthoflaviviruses (TBFVs) are further divided into mammalian and seabird TBFVs. While the seabird TBFV are non-pathogenic for humans, mammalian TBFV include several important human pathogens; in particular, TBEV, Kyasanur Forest disease virus (KFDV), Omsk hemorrhagic

Figure 1: **TBEV phylogenetic tree**



Phylogenetic tree illustrating the relationships between representative members of the TBEV complex (highlighted in red). Complete genome open reading frame sequences were retrieved from genbank and aligned using the gins option in mafft v7.266. The tree was constructed with RAxML v.8.2.9 using the GTR+G model of nucleotide evolution and 1,000 bootstrap replicates. The resulting tree was visualized and edited in Figtree v.1.4.1. All branches have maximum bootstrap support (not shown). The tree was midpoint rooted for visual purposes only. The lowest clade (black) contains members of the divergent seabird tick-associated virus complex (Meaban virus through Tyuleniy virus). We gratefully acknowledge the assistance of Dr John Pettersson (Zoonosis Science Center, Uppsala University, Sweden) who prepared and supplied the tree.

fever virus (OHFV), Powassan/Deer tick virus (POWV), and louping ill virus (LIV), which together with Langat virus (LGTV), for which there are no known cases of natural human disease, comprise a group known as the 'TBEV serocomplex' (Figure 1). All TBFVs are closely related antigenically and antibodies against one TBFV often cross-react with the other TBFVs, which should be taken into consideration when interpreting serological tests in areas where more than one TBFV co-circulates. The broadest cross-reactivity is seen in hemagglutination inhibition assays, whereas the highest specificity is seen in neutralization assays.[7]

Although all TBFVs are closely related genetically and antigenically, they cause diverse clinical manifestations in humans: OHFV and KFDV (including a subtype of this virus, Alkhurma hemorrhagic fever virus) induce hemorrhagic fever syndromes, while the others cause neurological disease. Importantly, the hemorrhagic fever associated TBFVs and encephalitogenic TBFVs do not form separate phylogenetic lineages and no specific determinants in the genomes of these viruses have been associated with particular disease manifestations.[8,9]

Three main antigenic subtypes of TBEV correspond to the 3 recognized genotypes: Western, also known as European (TBEV-EU; previously Central European encephalitis; prototype strain Neudoerfl), Far Eastern (TBEV-FE;

previously Russian spring-summer encephalitis; prototype strain Sofjin), and Siberian (TBEV-Sib; previously Western Siberian encephalitis; prototype strains Zausaev and Vasilchenko).[10] Two additional lineages; i.e., “178-79” and “886-84 group”, named as Baikalian TBEV (TBEV-Bkl) respectively, have been identified in Eastern Siberia and proposed as TBEV subtypes.[11] The geographical distribution and clinical significance of these newly identified genotypes remains to be determined. However, some studies indicate that 0.6-6% of TBEV strains circulating in Eastern Siberia might belong to these new genotypes.[11] Another new potential TBEV subtype (Himalayan – TBEV-Him) was identified recently in wild rodents in Qinghai-Tibet Plateau in China.[12]

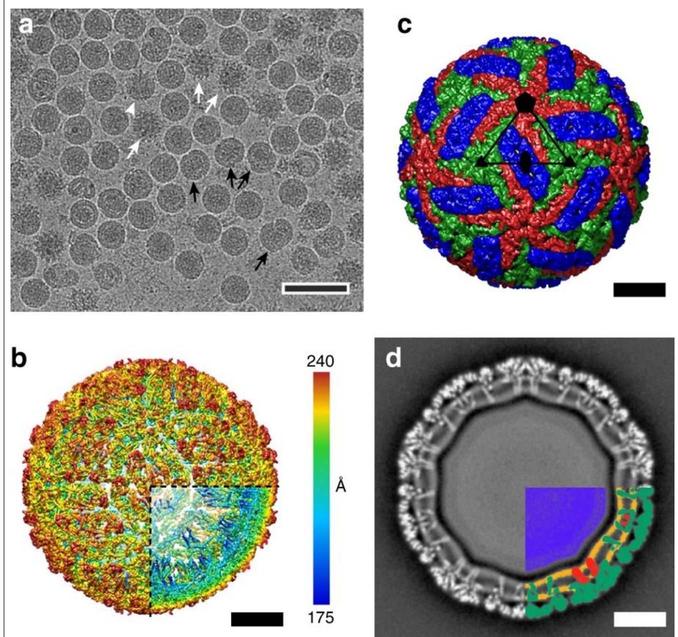
Comparison of the complete coding sequences of all recognized TBFV species led to a new taxonomic proposal, viz. the assignment of TBEV and LIV to a single species (TBEV) encompassing 4 viral types; i.e., Western TBEV (TBEV-EU); Eastern TBEV (TBEV-Sib and TBEV-FE); Turkish sheep TBEV, including Greek goat encephalitis virus subtype; and Louping ill TBEV, the latter having Spanish, British, and Irish subtypes.[13] This classification was supported by the fact that, based on antigenic properties, the European TBEV strains are more closely related to LIV than to TBEV-FE and TBEV-Sib strains.[14,15]

All TBFVs are thought to have shared a common ancestor, which diverged from mosquito-borne flaviviruses in Africa less than 5,000 years ago.[16-18] However, some studies suggest that this split might have occurred as long as 50,000 years ago.[19] The descendant TBFV species evolved and spread through Asia and then more recently westwards through Europe as they adapted to different host and tick species. [16-18] In comparison with mosquito-borne flaviviruses, TBFVs evolved nearly twice as slowly, primarily due to the long life-cycle of the Ixodes tick vector.[16,20,21] Overall, it was concluded that there is a direct correlation between genetic and geographic distance of individual TBFV species[16,22] and, furthermore, that the evolution and dispersal of these viruses is relatively slower than that of the mosquito-transmitted viruses. In addition, the evolution is not significantly influenced by migratory birds or international trade.[18]

Virion structure and morphology

Infectious TBEV virions are small spherical particles about 50 nm in diameter with no obvious distinct projections. The mature virions contain an electron-dense core approximately 30 nm in diameter which is surrounded by a lipid bilayer (Figure 2).[23,24] The nucleocapsid core consists of single-stranded positive-polarity genomic ribonucleic acid (RNA) molecule (11 kb) and the capsid protein C (12 kDa). The surface of the lipid membrane incorporates an envelope glycoprotein (E, 53K) and a membrane glycoprotein (M, 8K) (Figure 2).

Figure 2: TBEV particles



A. *Cryo-EM micrograph of TBEV particles. The sample contained mature, immature (white arrows), half-mature (white arrowheads), and damaged (black arrows) particles. Scalebar, 100 nm*
B. *B-factor sharpened electron-density map of TBEV virion, rainbow-colored according to distance from particle center. Scalebar, 10 nm.*
C. *Molecular surface of TBEV virion low-pass filtered to 7 Å. The three E-protein subunits within each icosahedral asymmetric unit are shown in red, green, and blue. Scalebar, 10 nm.*
D. *Central slice of TBEV electron density map perpendicular to the virus 5-fold axis. The virus membrane is deformed by the transmembrane helices of E-proteins and M-proteins. The lower right quadrant of the slice is color-coded as follows: nucleocapsid—blue; inner and outer membrane leaflets—orange; M-proteins—red; E-proteins—green. Scalebar, 10 nm.*

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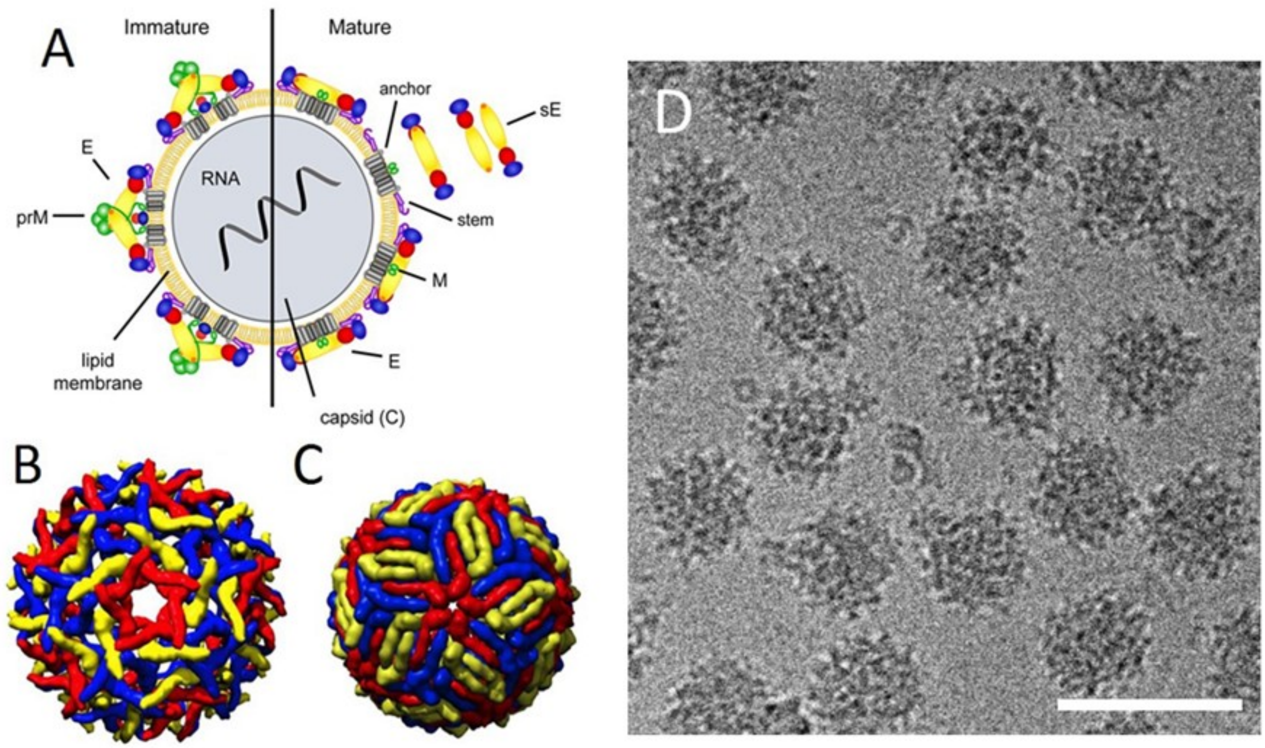
The glycosylated E protein is also a major antigenic determinant of the virus and induces immune responses in infected mammalian hosts. It also contains the sites for virus binding to receptors on the surface of susceptible host cells and subsequent pH-mediated fusion of the viral E protein with endosomal membranes during entry of viral RNA into the cell.

In the mature infectious virions, the M protein has been proteolytically cleaved from the precursor (pr)M protein. This post-translational process occurs during the maturation of nascent viral particles within the secretory pathway and immediately before release of the infectious virions from the infected cell. In immature non-infectious particles, prM and E proteins form hetero-dimers and

Figure 3



A. *Schematic model of a flavivirus particle. Left panel: immature virion, right panel: mature virion. The surface of immature particles consists of 60 spikes composed of trimers of prM-E heterodimers. Mature particles are formed after prM cleavage and contain 90 E homodimers. (From[25] (CC BY)).*

B. *Pseudoatomic cryo-EM reconstruction model of the immature flavivirus particle (PDB: 2OF6).*

C. *Pseudoatomic cryo-EM reconstruction model of the mature flavivirus particle (PDB: 3J0B).*

D. *Cryo-EM micrograph of immature TBEV particles (kindly provided by Tibor Füzik and Pavel Plevka, with permission). Scalebar, 100 nm.*

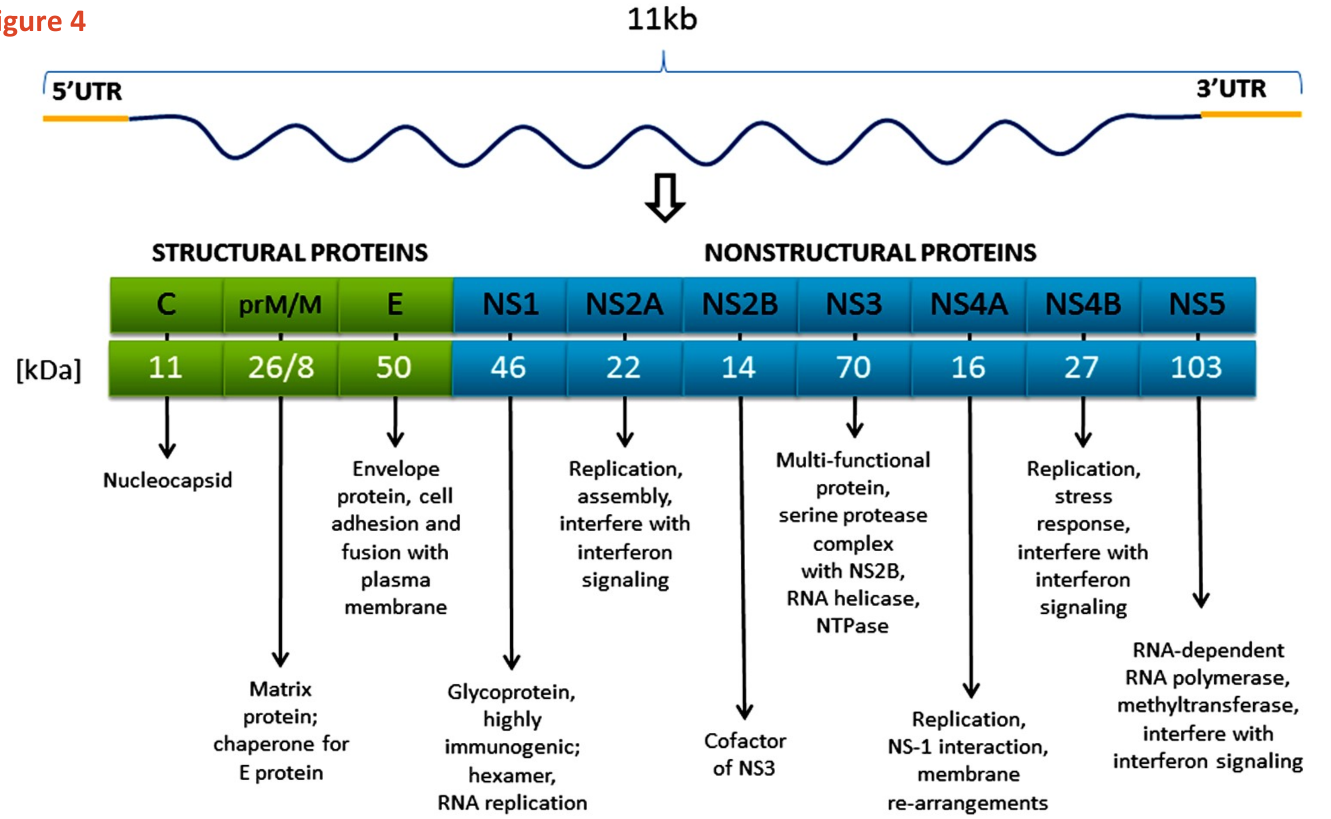
exist as trimers covering the virion surface. At this stage, the pr part of prM occludes the fusion domain of the E glycoprotein, preventing premature fusion with cell membranes within the secretory pathway (Figure 3).

In the trans-Golgi compartment, the pr is cleaved from prM by a cell furin-like protease; this is followed by the conformational change, rotation, and rearrangement of E proteins from 60 antiparallel trimers into 90 anti-parallel dimers, forming an unusual 'herring-bone' pattern with icosahedral symmetry and resulting in the viral particles being mature and fully infectious. However, the efficiency of prM cleavage varies for different flaviviruses; cleavage is therefore not always absolute. Thus, immature particles may also be released as a proportion of the infectious/non-infectious virus pool.[23]

The structure of purified mature TBEV particles has been determined at near atomic resolution of 3.3 (strain Kuutsalo-14) or 3.9 Å (strain Hypr) by reconstruction of cryo-electronmicroscopic images (Figure 2).[23,24] These studies revealed a relatively smooth outer surface of the particle, and E and M proteins organized in a similar manner to that in other flaviviruses. The surface of the TBEV virion is covered with small protrusions formed by glycans attached to the E-protein molecules.[23,24] Both E-proteins and M-proteins are anchored in the virion membrane, each by two trans-membrane helices. Viral envelope membrane is not spherical; instead the shape of the membrane closely follows the inner surface of the protein envelope and is deformed by insertions of the trans-membrane helices of E-proteins and M-proteins.[23]

Cryo-electronmicroscopic analysis was employed to explore the structure of three immature TBEV strains: Hypr, Neudoerfl, and Kuutsalo-14. The immature TBEV particle exhibited a diameter of 56 nm, with surface glycoproteins organized into characteristic spikes reminiscent of immature flaviviruses. The topology and domain assignment of prM in immature TBEV closely resembled that of the mosquito-borne Binjari virus, however was significantly different from other immature flavivirus models.[26] Recombinant sub-viral particles (RSPs) are of T-1 icosahedral symmetry formed by 30 E protein dimers. They have the same antigenic properties as wild-type virus. They can be used for vaccination purposes and represent an

Figure 4



Genome organization of TBEV and processing pathways of the polyprotein. A schematic representation of the TBEV genome with the 5' and 3' non-translated regions (NTRs) is shown in the top; the translation products are given below (adapted from[38], with permission).

established model system for flavivirus membrane fusion because they have fusion characteristics similar to those of infectious virions.[27]

Viral genome

The nucleocapsid is formed from a single viral RNA genome and multiple copies of the C protein. The RNA binding domains of the C protein molecules are located at their N- and C-termini and are separated by hydrophobic regions. The nucleocapsid is less ordered and as for other flaviviruses, no discernible symmetry was detected in cryoelectron microscopic reconstructions.[23] Instead, the C protein is arranged in a cage-like structure surrounding the viral genome. The icosahedral symmetry is, therefore, directed by surface proteins rather than by the nucleocapsid protein.

In addition to mature virions, smaller (approximately 14 nm in diameter) non-infectious particles are released from the infected cells. These particles lack nucleocapsid and consist of E and M proteins only; they are called sedimenting (70S) hemagglutinin (SHA).

Similar RSPs of a slightly larger size (approximately 30 nm in diameter) can be produced by cells expressing only prM and E proteins.[28]

The TBEV genome consists of a single-stranded positive sense RNA molecule, approximately 11 kilobases in length. The genome encodes 1 open reading frame (ORF) of over 10,000 bases, which is flanked by untranslated (non-coding) regions (UTRs). The ORF encodes 1 large polyprotein of approximately 3,400 amino acids, which is co- and post-translationally cleaved by viral and cellular proteases into 3 structural proteins (C, prM, and E) and 7 non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5)[29] (Figure 4). A second short upstream ORF is present in the 5'-UTR of some TBEV strains. However, no protein encoded by this ORF has been found in TBEV-infected cells, indicating that it is neither expressed nor present at undetectable concentrations, suggesting that this additional ORF has either a minor or no biological role in the TBEV replication cycle.[30] A common feature of all flavivirus genomes is their high purine content and low GC and UA doublet frequencies, which may influence translation of the genome and/or reflect the requirement for flaviviruses to grow in different hosts and cell types; however, a specific role for this unique genomic characteristic remains unclear.[31] A replication enhancer element (REE) has been found within the capsid gene of TBEV. The REE folds as a long stable stem-loop (designated SL6), conserved among all TBFVs. Although SL6 REE is not essential for growth in tissue culture, it acts to up-regulate virus replication.[32]

In addition to coding for the polyprotein, the genome has

RNA structural motifs that play a crucial role in the viral life-cycle.[33] In particular, the untranslated regions form secondary stem-loop structures that probably serve as cis-acting elements for genome replication, translation, and/or packaging.[33-36] The 5'-UTR contains a type 1 cap (m7GpppAmG), followed by a conserved stem-loop structure. The 3'-UTR is not polyadenylated and is characterized by extensive length and sequence heterogeneity.[37] This region of the viral genome can be divided into 2 parts: a proximal (localized behind the 'stop' codon of the ORF) and a distal ('core', the 3' terminus itself). The distal part of this region (approximately 340 nt) is highly conserved, whilst the proximal part is a noticeably variable segment with common deletions and insertions.[34-36]

RNA structural models demonstrate that flavivirus genomes, including TBFVs, form dsRNA cyclization stems or 'panhandles' at their 5'- and 3'-termini. The 'panhandle' of the TBFV group (5'CYCL) is formed by a perfectly conserved continuous 21-nucleotide sequence located in the 5'-UTR. The 5'-UTR and 3'-UTR sequences directly involved in cyclization are located downstream from the 5' Y-shaped structure and the 3' long stable hairpin, respectively. The terminal 5'-UTR and 3'-UTR regions not involved in cyclization also show homology, suggesting they are evolutionary remnants of a long cyclization domain that probably emerged through duplication of 1 of the UTR termini.[39]

5'-untranslated region

The 5'-UTR is 132 nucleotides long in most TBEV strains and its secondary structure is highly conserved among different TBEV strains.[36] Common secondary structures in this region can also be found among different flaviviruses, although the sequence is diverse.[31] The function of these conserved secondary structures is probably related to translation of the genome and in the complementary RNA strand serves as a site for initiation of synthesis of positive-stranded RNA molecules.[31]

The folding of 333 nt as a reverse complement of the 5'-end (3'-end of the negative-stranded RNA) of TBEV revealed a stem-loop pattern different from the 3'-UTR of positive-stranded RNA. However, 2 nucleotide regions in these 3'-ends are identical and conserved among all TBFVs. One of these, an 11-nt region, forms a loop within the folding pattern at the 3'-end of the negative strand and a stem at the 3'-UTR of the positive strand.[34] These structural motifs at the 5' and 3'-UTR termini could be recognition sites for viral RNA polymerase.[34]

The alignment of the 5'-UTRs of different TBFVs demonstrated an internal hypervariable domain in which Powassan virus has a deletion of 27 bases.[34] The predicted folding of the 5'-UTR sequence produces a stem-loop structure similar for all TBFV, and the 27 nt deletion in the Powassan virus has no effect on the typical 5'-UTR folding.[34] This indicates that the length of stem-loop structure 3 is not critical for virus infectivity.[34]

3'-untranslated region

The alignment of 3'-UTRs of all TBFVs revealed 2 nucleotide regions, 1 about 340 bases in length, of conserved sequence at the extreme 3'-end (designated C3'- UTR) and another hypervariable region placed between the stop codon and the C3'-UTR where even strains from a single species showed deletions of different lengths,[34] whereas some TBEV strains have a 30-250 nt long poly(A) sequence in this region.[39] Deletions or a poly(A) sequence insertion in the variable region were found in strains passaged in mammalian cell culture,[40] and deletions of different lengths were also observed in TBEV strains isolated from human patients.[41-43] It was suggested that the hypervariable region could act as a spacer separating the folded 3'-UTR structure from the rest of the genome that might be necessary for efficient binding of viral RNA polymerase and cellular factors involved in transcription 34 and may play a role in the natural transmission cycle of TBEV.[44-46] A short poly(A) tract is genetically more stable compared with the virus having a long poly(A) tract.[45]

Previous studies reported that the variable region plays no role in viral replication and virulence for laboratory mice.43 However, recent studies revealed that partial deletions and poly(A) insertion in the variable region increases TBEV virulence in the mouse model.[45,46] These data suggested that the variable region of the 3'-UTR might impact neurovirulence and function as a critical virulence factor.[45,46]

All TBFVs share a common folding pattern of secondary structures at the C3'-UTR position. RNA in this region is predicted to fold into a 3' stem-loop and it contains conserved sequence elements. However, these structures are different from those observed in mosquito-borne flaviviruses.[34] Indeed, some RNA sequences within the 3'-UTR clearly distinguish mosquito-borne from TBFVs.[37,39] Modifications within the 3'-UTR of TBEV that affect the conserved structural motifs are known to attenuate the virus without altering their antigenic specificity. Modification of this region might form the basis for live-attenuated vaccines and/or for antiviral therapeutics.[47,48]

Short direct repeat sequences (20-70 nucleotides long) in the 3'-UTR were found to be conserved for each flavivirus group or subgroup.[48] Four R1 repeats, two R2 repeats, and two R3 repeats, approximately 23, 26, and 70 nucleotides long, respectively, apparently arranged randomly, have been described in the 3'-UTR of the TBFVs.[34,47,48] These short repeats apparently originated from at least 6 long repeat sequences (LRS) approximately 200 nucleotides in length, arranged in tandem. Four of these LRS are present in the 3'-UTR and 2 in the 3' region of the ORF. Thus, it seems that evolution of the 3'-UTR and probably the ORF occurred through multiple duplications of LRS that form the basis for the development of the functionally important secondary RNA structures in the 3'-UTR. Subsequent formation of extended RNA domains evolved as promoters and enhancers of virus replication determined by the selective requirements of the vertebrate and invertebrate hosts.[39,47]

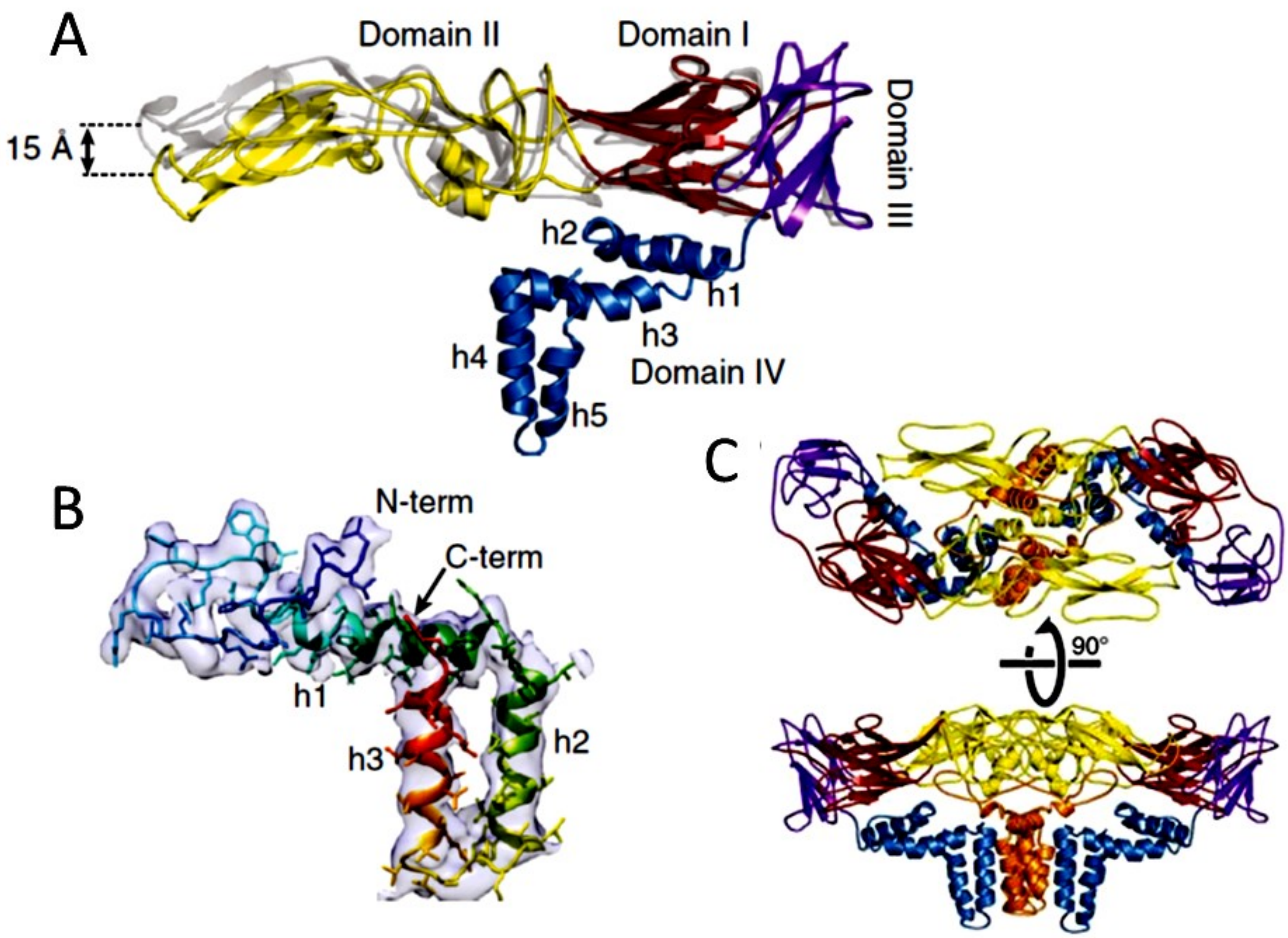
Flaviviruses, including TBFVs, are known to produce unique non-coding subgenomic flaviviral RNA (sfRNA), which is derived from the 3'-UTR. SfRNA results from incomplete degradation of viral RNA by the cellular 5'-3' exoribonuclease XRN1.49 The exoribonuclease activity stops at the highly ordered RNA secondary structures at the beginning of the 3'-UTR. SfRNA is involved in modulating multiple cellular pathways; e.g., inhibiting antiviral activity of type I interferons (IFN) and RNAi pathways, facilitating viral pathogenicity.[50]

Proteins encoded by the virus

Structural proteins

C (Capsid) protein is a relatively small (11 kDa), basic, and highly positively charged protein with low sequence homology between different flaviviruses.[51] Within the ORF

Figure 5



A. *Superposition of cryo-EM (colored) and X-ray (gray) E-protein structures. Domain I is colored in red, domain II in yellow, domain III in violet, and domain IV in blue.*

B. *M-protein rainbow-colored from N-terminus in blue to C-terminus in red with electron density map shown as semi-transparent surface. The M-protein consists of an extended N-terminal loop followed by perimembrane (h1) and two transmembrane helices (h2 and h3).*

C. *Heterotetramer of two E-proteins and two M-proteins. E-proteins are colored according to domains, and M-proteins are shown in orange.*

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that encodes the single polyprotein precursor of all structural and non-structural proteins, protein C is located at the amino-terminal end and is thus synthesized first during translation. The protein interacts with viral RNA genomes and represents a structural component of the nucleocapsid. Despite the low sequence homology among diverse flaviviruses, regions of hydrophobic and hydrophilic amino acids are conserved. The C-terminal hydrophobic domain (this domain is cleaved from mature C protein) is preceded by a hydrophilic region, and a central hydrophobic region. The N-terminus contains a hydrophilic region.[31] The central hydrophobic region mediates membrane association of the protein and the charged residues that cluster at the hydrophilic N- and C-termini presumably mediate the interaction of the protein with viral RNA.[50,51] In flavivirus infected cells, it was found that the mature C protein accumulates on the surface of endoplasmic reticulum (ER)-derived organelles named lipid droplets. The lipid droplets may play multiple roles during the viral life-cycle; i.e., they could sequester the flaviviral capsid protein early during infection and provide a scaffold for genome encapsidation.[52]

The introduction of various deletions into the TBEV genome that removed parts of the central hydrophobic domain of protein C revealed a remarkable structural and functional flexibility of this protein.[53] TBEV mutants carrying deletions in C that extended from residue 28 up to residue 43 were viable in cell culture. The mutants produced substantial amounts of subviral particles lacking capsid, and the deletions impaired the assembly or stability of the virions.[53] However, virus viability was affected when the deletions extended up to residue 48 or when the full hydrophobic domain was removed.[53] Interestingly, these deletions led to spontaneous mutations in other regions of the C protein that generally increased the C protein hydrophobicity and restored infectivity of the virus.[54]

prM protein is a glycosylated precursor of the membrane **protein M**. The carboxyl terminus of C protein serves as an internal signal sequence element leading the structural protein prM into the membrane of the endoplasmic reticulum. The viral protease NS2B-NS3 cleaves this signal sequence, releasing the N-terminus of prM protein.[53] The prM protein shows a chaperone-like activity during the envelope protein E folding.[55] The N-terminus of the pr is mainly hydrophilic and, in TBEV, contains a single N-linked glycosylation site that appears to have an important role during virion assembly and release.[31,51,56] Six cysteine residues, all disulphide-bridged, are highly conserved. The C-terminal region contains an ectodomain and 2 potential membrane-spanning domains.[57] The cleavage of prM into pr and M occurs in the Golgi complex and is mediated by furin or a furin-like enzyme[58,59] leading to a conversion from immature to mature fusogenic and fully infectious viral particles (Figure 3).[58] The pr fragment is then secreted.[51] A conserved region in the prM protein is a critical molecular determinant for the assembly and secretion of the virus.[60] The M-protein consists of an N-terminal loop and three helices (Figure 5B). The first helix is situated as a perimembrane and the last two as trans-membranes; however, the M-protein is not exposed at the surface of the viral particle due to its small size and close association with the viral envelope membrane.[23] Two M-proteins together with two E-proteins form a compact heterotetramer, which is the main building block of the virion, formed by head-to-tail dimerization of two E-M heterodimers (Figure 5C).[23]

The E protein contains the major viral antigens and is the main target for neutralizing antibodies (although antibodies directed against prM/M and NS1 also induce some protective immunity). Moreover, the E protein is responsible for specific binding to a cellular receptor and penetration of the virus into the host cell. It is also believed to be a main determinant of TBEV virulence.[61] The three-dimensional structure of the E protein was studied at the resolution of 2.0 Å by X-ray crystallography[62] (Figure 5). Comparison of the crystal structure of E protein and the structure of E protein in the virion observed by cryoelectron microscopy revealed root-mean-square deviations (RMSD) of 1.7 Å for the corresponding Cα atoms.[23] The most important difference is in the positioning of domains I–III relative to each other. Whereas in the crystal structure the domains I, II, and III are arranged in a line, in the virion the tip of domain II is bent 15 Å towards the virus membrane (Figure 5A).[23] Such a bending of the ectodomain in the virion prevents induction of premature membrane fusion mediated by the E protein.[23] The structure of TBEV E protein was found to be highly similar to E1 glycoprotein from a distantly related virus, Semliki Forest virus (family *Togaviridae*). These proteins were defined as class II virus fusion proteins, distinct from previously characterized class I fusion proteins such as hemagglutinin of influenza virus.[51]

The protein forms 2 monomers anchored in the membrane by their distal parts at physiological pH. After virus uptake by receptor-mediated endocytosis into host cells, acidic pH in endosomes triggers irreversible changes in the E protein structure including its re-arrangement to trimeric forms. This leads to the initiation of the fusion process between the viral and endosomal membrane.[63] Conserved histidines in the E protein function as molecular switches and, by their protonation at acidic pH, control the fusion process.[64]

Each E protein monomer is composed of 3 domains (I- III). Domain I is located in the central part of the protein. It is formed by 8 antiparallel beta sheets, contains the *N*-terminus of the protein, 2 disulphide bridges, and an *N*-glycosylation site. Mass spectrometric analysis was employed to examine the variations in *N*-glycosylation profiles of TBEV cultured in human neural and tick cells. The predominant asparagine-linked oligosaccharides identified on the surface of TBEV derived from human neuronal cells included high-mannose glycan with five mannose residues

($Man_5GlcNAc_2$), a complex biantennary galactosylated structure with core fucose ($Gal_2GlcNAc_2Man_3GlcNAc_2Fuc$), and a group of hybrid glycans with the composition $Gal_{0-1}GlcNAc_1Man_{3-5}GlcNAc_2Fuc_{0-1}$. In contrast, the N-glycosylation profile of TBEV grown in tick cells revealed paucimannose ($Man_{3-4}GlcNAc_2Fuc_{0-1}$) and high-mannose structures containing five and six mannose residues ($Man_{5-6}GlcNAc_2$) as the major glycans present on the viral envelope protein.[65] The function of E protein glycosylation was investigated using recombinant TBEV with or without the E protein *N*-linked glycan. The results suggested that glycosylation of the TBEV E protein is critical for the intracellular secretory process in mammalian cells but cleavage of the *N*-linked glycan after secretion did not affect virion infectivity in these cells. On the other hand, E protein glycosylation seems to play no significant role in virus reproduction in ticks.[66]

Domain II is formed of 2 long loops that extend out of domain I and form a finger-like structure. Domain II contains a number of beta sheets and 3 disulphide bridges.[62,67] Part of the domain responsible for the fusion of viral envelope with the membrane of the endosome is called the fusion peptide; this peptide mediates insertion of the E protein into the endosomal membrane resulting in fusion of viral envelope with the membrane of the endosome.[68] The initiation of fusion is crucially dependent on the protonation of 1 of the conserved histidines (His323), which works as a pH sensor at the interface between domains I and III of E, leading to the dissolution of domain interactions and to the exposure of the fusion peptide.[64]

Domain III has the typical fold of an immunoglobulin constant (IgC) molecule.[67] It contains a beta barrel composed of 7 antiparallel beta sheets. The lateral part of domain III is believed to be responsible for binding to a specific cellular receptor.[62]

Amongst the most conserved parts of the E protein, there are 12 cysteine residues forming 6 disulphide bridges with conserved localization in common with all known flaviviruses.[69]

The E protein is also considered to be a major determinant of TBEV virulence. Amino acid substitutions in E protein often cause a decrease in neuroinvasiveness, although neurovirulence is usually not reduced.[70] The highest number of attenuating mutations in the E protein was revealed in the domain that probably binds to specific cell receptors and participates in membrane fusion.[63] A number of identified substitutions causing escape of the virus from the neutralizing effect of monoclonal antibodies,[71] deficiency in the ability to agglutinate erythrocytes,[72] and a change in virus growth properties in cell cultures, mice, or ticks,[61,73-76] have been described.

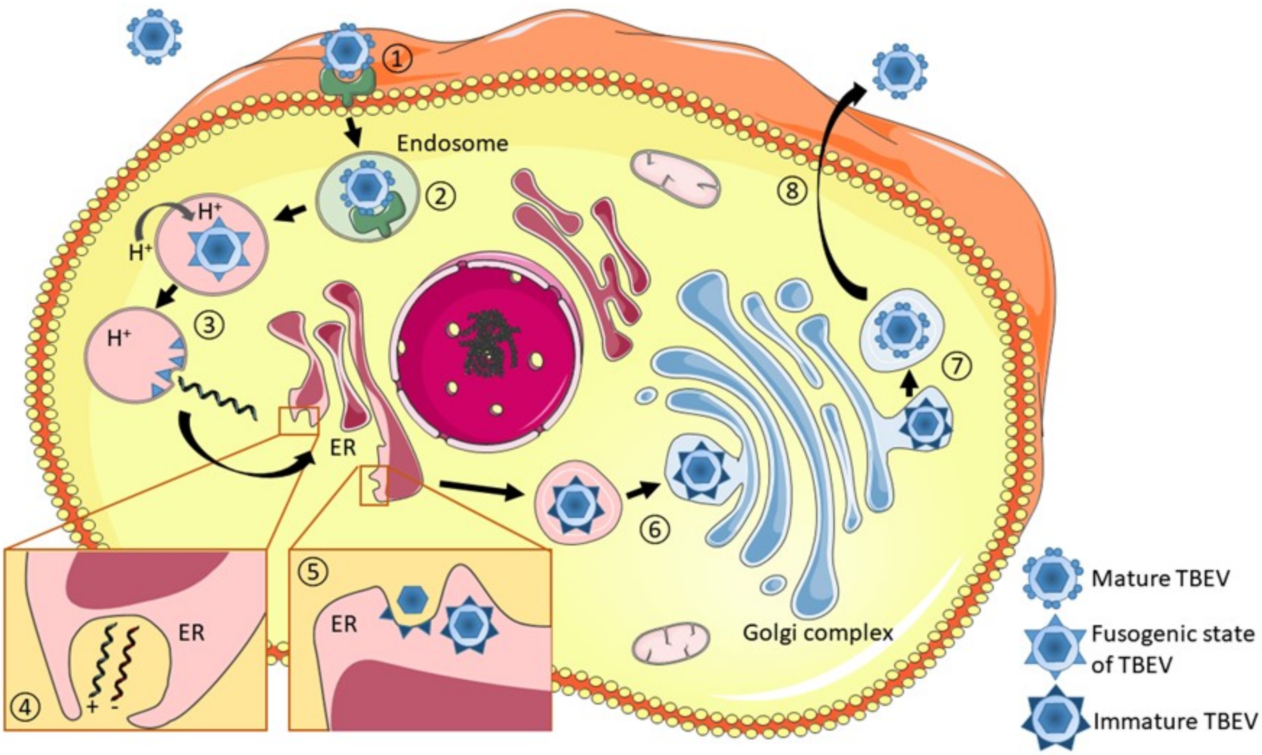
The E protein serves as the primary target and inducer of neutralizing antibodies.[27] Neutralizing antibodies can be elicited by any of the three domains of the E protein, with numerous sites across the particle's surface having the potential to induce potent neutralizing antibodies. These epitopes may include quaternary epitopes, which consist of residues from adjacent domains or adjacent E proteins on the surface.[23,77] The neutralization process by antibodies can occur through inhibition of the interaction between the E protein and the receptor on the host cell surface. Alternatively, it can involve the inhibition of post-entry processes, such as blocking the fusion of the viral envelope with the endosomal membrane. This fusion process necessitates significant reorganization of the E protein domains, which antibodies can impede, thereby preventing viral entry and infection.[23,78]

Recently, highly potent human monoclonal antibodies that target the E protein domain III have been discovered. These antibodies show great promise for use as post-exposure prophylaxis or early therapeutics for TBE.[79] Through the selection of TBEV escape variants by culturing the virus with increasing concentrations of the antibody, it was determined that a combination of two amino acid substitutions in the E protein is necessary. One substitution occurs in domain III, while the other occurs in domain II. The domain III substitution impairs formation of a salt bridge critical for antibody-epitope interaction. The substitution in domain II is not located within the antibody epitope, but it is believed to induce quaternary rearrangements of the virus surface. This rearrangement occurs due to the repulsion of positively charged residues on the adjacent domain I. Consequently, both resistance mechanisms—a substitution in domain III and one in domain II—are required for TBEV to evade neutralization by this antibody.[80]

Antibodies that target the fusion loop of the E protein, a region highly conserved among flaviviruses, often exhibit cross-reactivity across multiple flavivirus species. However, they typically do not neutralize TBEV. This is attributed to their recognition of cryptic epitopes that are not typically exposed on the surface of mature virions. Consequently, these antibodies are unable to access the endosomes where viral fusion occurs, thus limiting their neutralization capability against TBEV.[81]

A unique mechanism of TBEV infection enhancement by antibodies against E protein, which operates independently of interactions with Fcγ receptors, has been described. This mechanism involves the binding of a specific antibody to the E protein on the viral surface, particularly recognizing an epitope located at the interface of the dimeric envelope protein E. This binding event triggers the dissociation of E protein dimers and exposes the fusion loop, facilitating the exposure of a structural element that interacts with the lipids of the cellular plasma membrane. Consequently, this process enhances viral infection by promoting viral entry into host cells.[82]

Figure 6



Schematic illustration of the TBEV life cycle. (1) Infection begins with the binding of viral particles to specific cell-surface receptors, which have not yet been unequivocally identified. (2) Viral particles enter cells via endocytic pathway. (3) Low pH in the late endosome triggers conformational changes in the E proteins, leading to rearrangement of dimers to trimeric forms (fusogenic state) and the subsequent fusion of the viral envelope with endosomal membranes, which leads to virion uncoating. (4) Replication of the virus occurs through the synthesis of anti-sense (negative) RNA, which serves as the template for genome RNA production. Replication complexes are localized in membranous structures within the endoplasmic reticulum (ER). (5) Assembled nucleocapsids acquire lipid envelopes by budding into the ER lumen. (6) Immature particles pass through the Golgi complex. (7) Maturation takes place in the trans-Golgi network, involving the cleavage of prM and the reorganization of E proteins into fusion-competent homodimers, leading to a change from spiky immature to smooth mature particles. (8) Mature particles are transported in cytoplasmic vesicles and released into the extracellular space by exocytosis.

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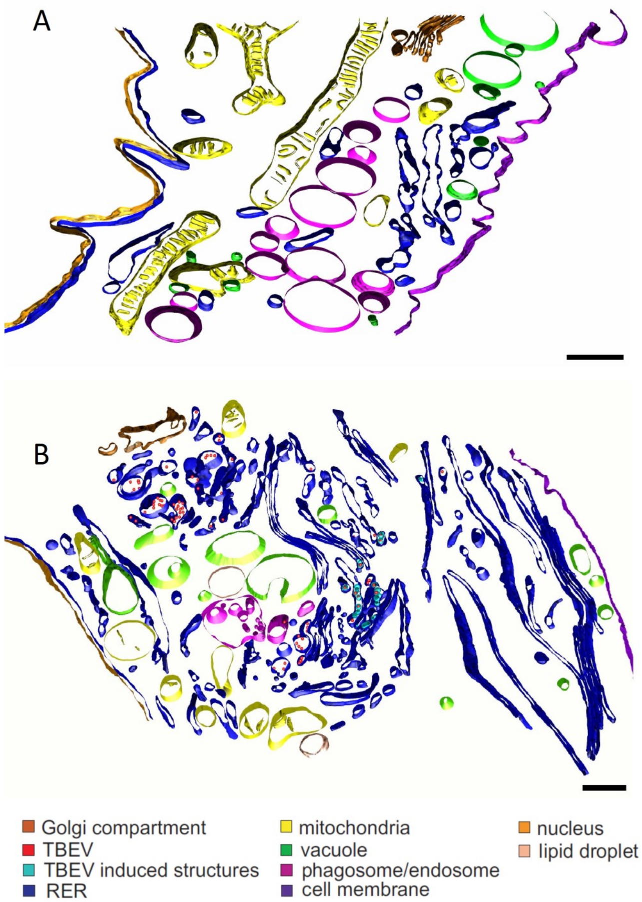
Non-structural proteins

NS1 is a glycoprotein containing 2 or 3 potential glycosylation sites and 12 conserved cysteines forming disulphide bridges.[83] It exists in dimeric forms localized freely in the cytoplasm or associated with membranes. Since the protein is highly hydrophilic and contains no transmembrane domains, its association with membranes remains poorly understood. Probably, dimerization creates a hydrophobic surface of the protein for its peripheral association with membranes.[51,84] Alternatively, some species of the protein could be anchored into the membrane by glycosyl-phosphatidylinositol.[51,85] The intra-cellular NS1 is central to viral RNA replication. The NS1 protein along with other non-structural proteins (see below) and viral RNA are targeted towards the luminal side of the endoplasmic reticulum, forming a replication complex (RC). Intracellular NS1 also interacts with various host proteins to assist viral replication, translation, and virion production; e.g., interaction of NS1 with 60S ribosomal subunits was described.[86] Secretion of NS1 protein into the extracellular space appears particularly in the form of pentamers or hexamers and occasionally as decamers or dodecamers.[87] This so-called 'soluble antigen', together with membrane-bound NS1 induces a protective immune response in the host.[88,89] NS1 protein is also known to activate the Toll-like receptors (TLRs),[90] and inhibit the complement system.[91,92]

NS2A is a small, hydrophobic protein, currently with no defined function. It is believed to play a role in forming the RC.[51] A small membrane-associated protein, NS2B, serves as a crucial co-factor for protease activity of the NS3 protein. The central hydrophilic domain of the NS2B protein possibly

Figure 7



Morphological changes in TBEV-infected mammalian cells. 3D models of mock-infected (A) and TBEV-infected human astrocytes (B). TBEV infection causes extensive morphological changes, including membrane reorganization of the endoplasmic reticulum; differences are evident in the Golgi complex, mitochondria, and phagosomes. (From[113], with permission).

interacts with the NS3 protein and it is flanked by hydrophobic regions probably anchored in the membrane.[93] The central hydrophilic region of NS2B (40 amino acids that mediate the NS2B co-factor activity) is flanked by hydrophobic regions that mediate membrane association.[51]

NS3, the second largest viral protein, is an enzyme central to virus replication and polyprotein processing. Conserved regions impart functions as a serine protease, helicase, and RNA nucleoside triphosphatase.[51] The protease activity is localized at the N-terminal domain of NS3, and this enzyme cleaves peptide bonds between NS2A-NS2B, NS2B-NS3, NS3-NS4A, and NS4B-NS5. As mentioned above, the protease activity occurs, in association with a 40-amino acid region of NS2B, resulting in the formation of a heterodimeric complex.[51,94] It was found that mutations which were mapped in close proximity to the NS2B-NS3 protease active site may determine the neuro- or non-neuropathogenicity of TBEV.[95] The C-terminal region of the NS3 protein has a helicase activity, utilizing the energy released from ATP to unwind RNA duplexes. Possible functions include elimination of complex secondary structures of viral RNA and/or resolving RNA duplexes formed during replication.[51] The C-terminal region also has RNA triphosphatase and 5'RNA phosphatase activities.[96] Due to the crucial role of NS3 protein in the virus replication process, this protein represents an excellent target for the development of specific antiviral inhibitors.[94,97]

NS4A and **NS4B** are small, hydrophobic proteins. NS4A is probably part of the replication complex.[98] NS4B, a trans-membrane protein localized to the sites of replication and nucleus, partially blocks activation of STAT1 and IFN-stimulated response element (ISRE) promoters in cells stimulated with IFN.[99] NS4A and, to a lesser extent, NS2A also block IFN signaling, and the cumulative effect of these 2 proteins together with NS4B results in robust IFN signaling inhibition.[100]

NS5 is the largest (100 kDa) and most highly conserved viral protein serving as a viral RNA-dependent RNA polymerase.[101] Its C-terminus shares sequence homology with RNA-dependent RNA polymerases of other positive-stranded RNA viruses.[51,102,103] The N-terminal domain has a function as AdoMet-dependent methyltransferase involved in the mRNA capping process, transferring a methyl group from the cofactor S-adenosyl-l-methionine onto the N7 atom of the cap guanine and onto the 2'OH group of the ribose moiety of the first RNA nucleotide.[94] The NS5 proteins form complexes with NS3 proteins, which results in stimulation of the NS3 RNA nucleoside triphosphatase activity.[51,104]

The NS5 protein is a promising target for specific antiviral inhibitors. Indeed, several nucleoside analogues targeting NS5 and causing premature termination of viral RNA synthesis were found to exhibit high inhibitory activity against TBEV.[105,106]

Apart from the main function as RNA-dependent RNA polymerase, the TBEV NS5 protein interferes with type I IFN JAK-STAT signaling.[107,108]

Replication strategy

Infection of the host cell with TBEV begins with the binding of the virus to a cell receptor (Figure 6), which has not yet been unequivocally identified. Interaction of the viral particle with cellular receptors is mediated by viral E glycoprotein. Kopecký et al.[109] identified 2 polypeptides of 35 and 18 kDa as putative vertebrate receptors for TBEV using a viroblot technique with anti-idiotypic monoclonal antibodies directed against antibodies that neutralize the infectivity of TBEV. However, the anti-idiotypic monoclonal antibodies did not bind effectively to tick cells, implying that different receptors are used by vertebrate and invertebrate cells for the binding of TBEV.[109] T-cell immunoglobulin and mucin domain 1 (TIM-1) was found to act as another cellular entry factor for TBEV.[110] It remains unclear whether TBEV uses single or multiple receptors on susceptible cells. Involvement of highly conserved glycosaminoglycans, such as heparan sulphate, during attachment and entry of flaviviruses has been suggested, but it seems likely that other host-cell receptor(s) can also mediate entry of TBEV into the host cells.[76,111] Apparently, just the ability to use multiple receptors could be responsible for the very wide host range of flaviviruses, which replicate in arthropods and in a broad range of vertebrates.[112]

In addition, in the presence of sub-neutralizing levels of specific immunoglobulins, the attachment and uptake by cells expressing Fc receptors might be enhanced, and this is called antibody-dependent enhancement.

After binding to the receptor, virus is internalized into clathrin-coated vesicles by the process of endocytosis. Acidification within the endosomal vesicle triggers conformational changes of the E proteins leading to rearrangement of the dimers to trimeric forms and subsequent fusion of the viral envelope with the membrane of the vesicle (Figure 6).[114,115] At a pH threshold of 6.5, the acidic environment triggers oligomeric rearrangement of metastable E dimers into stable trimers on the virion surface. This process exposes the fusion loop, located at the tip of domain II of the E protein.[116,117] The fusion loop interacts with the endosomal membrane, thereby mediating the initiation of the membrane fusion process.[117] The viral nucleocapsid is then released into the cytoplasm and viral RNA is uncoated. The exact mechanism of nucleocapsid uncoating remains unknown. The positive-sense viral RNA is the translational template, also functioning as a template for negative-sense RNA synthesis and formation of the double-stranded replicative intermediate.

The ratio of the newly synthesized positive-stranded RNA to negative-stranded RNA is at least 10 or 100 to 1, indicating that some regulatory mechanism must exist to produce higher numbers of positive-stranded RNA molecules.[51] The biological explanation for this is the double function of the genomic positive-strand RNA: it is used as a template both for transcription of the negative strand and translation of the viral polyprotein, while the negative strand is only transcribed into the new positive strands.[36]

The single viral polyprotein is cleaved by viral and cellular proteases into individual viral proteins. The surface structural proteins prM and E (and also NS1) are translocated into the lumen of the ER and their amino termini are liberated through proteolytic cleavage by host signalase. The newly synthesized RNA is condensed by protein C into nucleocapsids on the cytoplasmic site of ER. Viral envelope is acquired by budding of the nucleocapsid into ER.[118]

TBEV replicates in the cytoplasm in close association with virus-induced intracellular membrane structures, also called replication compartments (Figure 6). These compartments provide an optimal microenvironment for viral RNA replication by limiting diffusion of viral/host proteins and viral RNA, thereby increasing the concentration of components required for RNA synthesis, and by providing a scaffold for anchoring the replication complex.[119] These packets of vesicles have a diameter of about 80 nm and are formed as invaginations of the endoplasmic reticulum within a highly-organized network of inter-connected membranes (Figure 6).[119]

Virus assembly takes place in the endoplasmic reticulum, leading to the formation of immature particles. The immature non-infectious virions contain proteins prM and E in heterodimeric association forming spikes at the surface of the particles. These immature "spiky" virions are transported to the Golgi complex, where the pr part of the prM molecule is cleaved by the cellular protease furin, and the E protein is reorganized from trimers to form fusion-competent homodimers. The slightly acidic pH in the trans-Golgi complex leads to the conformational changes that are required for furin cleavage.[59] Interestingly, the low-pH-induced structural changes appear to be irreversible in TBEV in contrast with mosquito-borne flaviviruses, where this change seems to be reversible.[59,120] The function of prM and the pr fragment is to protect the E protein in the acidic Golgi complex and prevent premature membrane fusion at this stage of the viral life cycle.[121] The mature virions pass through the host secretory pathway and are finally released from the host cell by fusion of the transport vesicle membrane with the plasma membrane (Figure 6).[118]

TBEV infection is associated with dramatic morphological changes occurring in the infected cells (Figure 7). These include formation of smooth membrane structures, proliferation of endoplasmic reticulum, reorganization of the Golgi complex, and accumulation and convolution of membranes. Several cellular organelles are often damaged.[113,122-124] The infection is commonly cytocidal; the infected cells often die by apoptosis or necrosis,[122] but some vertebrate cell types survive the lytic crisis and become chronically infected.[125]

It was found that NS3 protein from Langat virus is able to activate cellular caspase-8 and induce apoptosis of the host cell.[109] On the other hand, tick cells do not undergo major inhibition of host macromolecular synthesis caused by the infection. No dramatic cytopathic and ultrastructural changes are seen in the infected tick cells and persistent productive infection is established in these cells.[124,126-129] However, both vertebrate and tick cells activate innate defense mechanisms against the infection.[129]

The TBEV maturation process in tick cells seems, however, to be different from that observed in vertebrate cells. In a cell line derived from the tick *Rhipicephalus appendiculatus* infected with TBEV, nucleocapsids are found in the cytoplasm and the envelope is acquired by budding on cytoplasmic membranes or into cellular vacuoles.[130]

Concluding remarks

The chapter summarized the major biological features of TBEV, focusing particularly on virus taxonomy, structure, genetics, and replication strategy in host cells. The past 2 decades have witnessed tremendous progress in our understanding of the structural, biochemical, and molecular aspects of a variety of the processes involved in morphogenesis, genome replication, maturation, and genetic basis for virulence of flaviviruses, including TBEV.

This has been made possible by the recent advances in structural and biochemical techniques, and methods of molecular biology, mainly site-directed mutagenesis. However, several key questions related to TBEV molecular biology and individual steps in the TBEV life-cycle remain unresolved. Major gaps in our understanding of the TBEV replication strategy both in mammalian and tick cells still exist. For instance, the nature of the cellular receptor for virus entry into the host cell, mechanisms of viral genome release from nucleocapsid, packaging of viral RNA by the C protein, and virus maturation remain to be identified. Except for the E glycoprotein, no structural data for the other TBEV proteins are available, and indeed the complete functional role of some proteins remains obscure. The role of specific RNA secondary structures present in TBEV untranslated genomic regions in viral RNA replication, capping, and controlling the functions of non-structural proteins, such as NS3 or NS5, need to be established. These and other unresolved problems highlight the necessity for further research into the molecular, genetic, and structural properties of TBEV. Advances in our basic knowledge of TBEV biology should promote the development of more effective methods of controlling this important human pathogen.

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Contact: ruzekd@paru.cas.cz

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