Vesicular Stomatitis Virus

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Historical Perspective

Vesicular stomatitis viruses (VSVs) are transmitted naturally by arthropods to a broad range of animal species. A clinically significant acute disease is manifest in domesticated animals, notably cattle, horses, and pigs, and it is characterized by fever and the appearance of vesicular lesions in the mouth, tongue, udder teats, and hoof coronary bands. Symptoms are therefore similar to those following infection with the apthovirus, foot-and-mouth disease virus (FMDV) and, consequently, rapid diagnosis is important in livestock. VSV infection was first described in the USA in 1916, following an epidemic in cattle and horses. However, reports from 1862 describe a clinically similar disease in army horses during the American Civil War. Today the virus is distributed throughout the Americas and is enzootic in Central America. In Panama, estimates suggest that up to 29% of the human population has been exposed to the virus as judged by the presence of neutralizing antibodies. Infection of humans can result in a mild febrile illness, but is generally asymptomatic. VSVs have been described rarely outside the Western Hemisphere.

Taxonomy and Classification

VSVs have a nonsegmented negative-sense RNA genome and are assigned to the order *Mononegavirales*. Within this order, they are assigned to the family *Rhabdoviridae*, based upon their characteristic 'bullet' shape. Within the rhabdovirus family, they are further assigned to the genus *Vesiculovirus*. A list of the nine currently recognized species assigned to the genus is provided in **Table 1**, along with their geographic distribution and sources of the virus in nature. The *Eighth Report of the International Committee on the Taxonomy of Viruses* lists an additional 19 members tentatively assigned to this genus. The type species is *Vesicular stomatitis Indiana virus*. Vesicular stomatitis Indiana virus (VSIV) has been widely studied in laboratories as a prototype of all the *Mononegavirales* and will be the primary focus of this article. Much is known about the replication and molecular biology of VSIV. VSIV was isolated following an outbreak of a vesicular disease of cattle in Richmond, Indiana, in 1925. The infectious agent was maintained by serial passage in animals and eventually became the Indiana serotype of VSV. *Vesicular stomatitis New Jersey virus* is also classified as a species in the genus *Vesiculovirus*. It was isolated following an outbreak in cattle in 1926 and is serologically and genetically distinct from VSIV. Vesicular stomatitis Alagoas virus (VSAV) was isolated from domesticated animals in Alagoas Brazil during an outbreak of VSV.

Structure of VSV

Particles

A schematic of VSIV is shown in **Figure 1** along with an electron micrograph showing virus particles. The particles appear bullet-shaped and are approximately 180 nm long and 70 nm in diameter. The particles comprise 74% protein, 20% lipid, 3% RNA, and 3% carbohydrate. The virus possesses a lipid envelope that is decorated with trimeric spikes of the 67 kDa attachment glycoprotein (G). The interior of the particle contains a ribonucleoprotein (RNP) core of the genomic RNA complexed with the viral nucleocapsid (N) protein. This N-RNA core is associated with the RNA-dependent RNA polymerase (RdRp), the viral components of which are a 241 kDa large (L) protein and a tetramer of a 29 kDa accessory

Table 1 Geographic distribution and sources of natural isolation of viruses representing the nine recognized species in the genus *Vesiculovirus*

Virus	*Geographic distribution*	*Source*
Carajas virus (CJSV)	Brazil	Phlebotomine sandflies
Chandipura virus (CHPV)	India, Nigeria	Mammals, sandflies
Cocal virus (COCV)	Argentina, Brazil, Trinidad	Mammals, mosquitoes, mites
Isfahan virus (ISFV)	Iran, Turkmenistan	Sandflies, ticks
Maraba virus (MARAV)	Brazil	Phlebotomine sandflies
Piry virus (PIRYV)	Brazil	Mammals
Vesicular stomatitis Alagoas virus (VSAV)	Brazil, Columbia	Mammals, sandflies
Vesicular stomatitis Indiana virus (VSIV)	Americas	Mammals, mosquitoes, sandflies
Vesicular stomatitis New Jersey virus (VSNJV)	Americas	Mammals, mosquitoes, midges, blackflies, houseflies

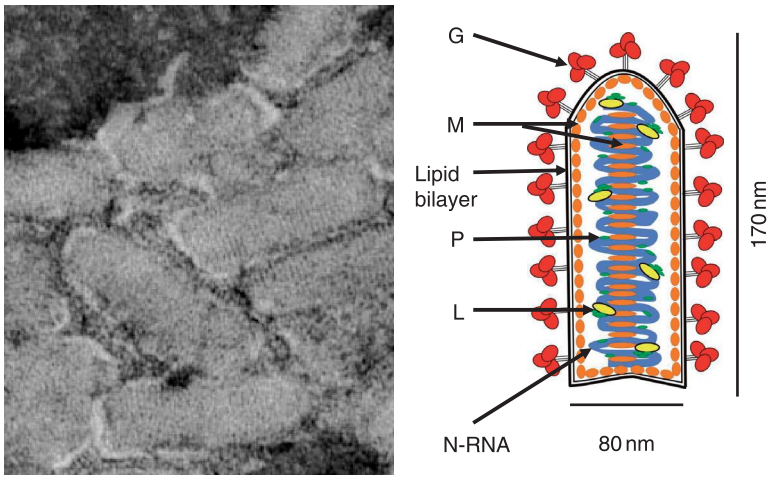

Figure 1 Electron micrograph of VSIV particles and a schematic illustration of the virion. At left, a negative-stained image of a group of virus particles is shown. At right, a schematic illustration of the virion is shown along with the dimensions of a viral particle. N-RNA, nucleocapsid protein coated RNA; P, phosphoprotein; M, matrix protein; G, attachment glycoprotein; L, large polymerase subunit. Kindly provided by David Cureton, Harvard Medical School.

phosphoprotein (P). Together, these form the internal helical nucleocapsid. Another major structural component of the virus particles is the 26 kDa matrix (M) protein, which is located below the membrane and associated with the nucleocapsid. The approximate composition of the particle is one molecule of RNA, 1200 copies of N, 500 copies of P, 1800 copies of M, 1200 copies of G, and 50 copies of L. The role of these proteins in viral replication is described in more detail below.

Genome

VSIV has a nonsegmented, negative-sense RNA genome of 11 161 nt. The genome comprises a 50 nt 3′ leader region (le), five genes that encode in order the N, P, M, G, and L proteins, and a 59 nt 5′ trailer region (Figure 2). The 5′ and 3′ ends of the genome are not modified and contain a 5′ triphosphate and 3′ hydroxyl. A key feature of all mononegaviruses is that the RNA genome is not found naked within infected cells. Instead, it is present as a ribonucleoprotein complex, in which it is completely covered by the viral nucleocapsid protein. This N-RNA template, associated with the viral L and P proteins, comprises the transcription-competent core of the virus, and delivery of this complex is required to initiate the infectious cycle. Consequently, in contrast to positive-sense RNA viruses, the naked RNA is not infectious.

Viral Replication Cycle

The replication cycle (Figure 3) should be considered a continuum of events. However, it is convenient to divide the cycle into the following three stages.

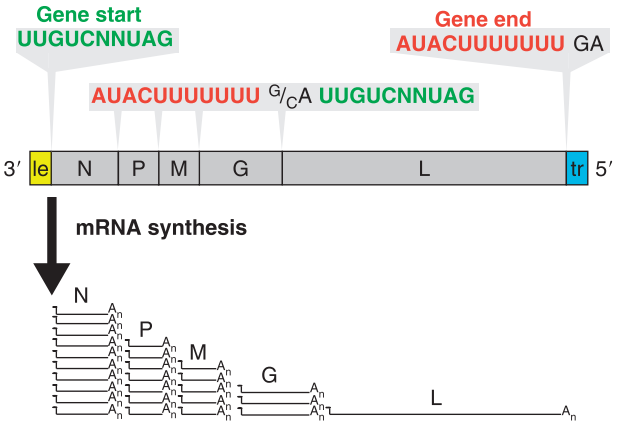

Figure 2 A schematic illustration of the VSIV genome highlighting the polymerase regulatory elements. The genome is represented 3′–5′ as a series of boxes, comprising le = leader region, N = nucleocapsid, P = phospho, M = matrix, G = glyco, L = large polymerase, and tr = trailer region. The conserved *cis*-acting elements that regulate polymerase activity during mRNA synthesis are shown for emphasis. Colors: green, the conserved residues of the gene-start sequence; red, the conserved residues of the gene-end sequence; and black, the nontranscribed residues of the gene junction. The leader and trailer regions also contain key elements that regulate polymerase activity and serve as promoters as described in the text.

Attachment, Entry, and Uncoating

Attachment of VSV to host cells is mediated by the glycoprotein which binds to the surface of cells. Given that VSV G can mediate infection of almost all cells in culture, either the receptor for VSV must be widely distributed, or the virus may be able to utilize multiple surface molecules for attachment. Phosphatidylserine (PS) was long thought to be the receptor for VSV but

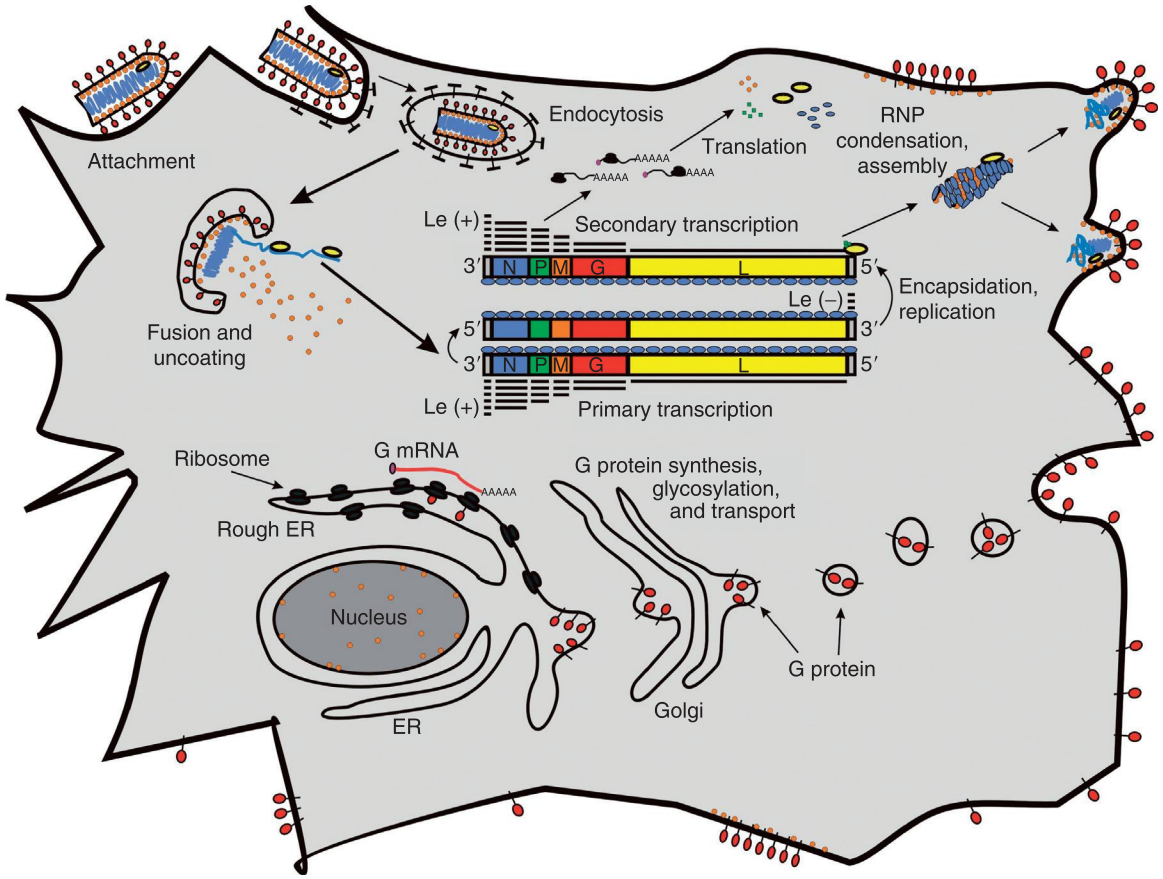

Figure 3 A schematic illustration of the replication cycle of VSV. The replication cycle (described in the text) is depicted showing attachment of virus to the cell, internalization, release of the viral core into the cytoplasm, primary viral mRNA synthesis, mRNA translation, genomic replication, secondary viral mRNA synthesis, assembly, and budding of infectious particles. Kindly provided by David Cureton, Harvard Medical School.

recent studies have questioned this finding. Importantly, the PS binding site in VSV G is internal to the trimer in its prefusion form. Following attachment to the cell, the virus is internalized via clathrin-dependent endocytosis and delivered to an early endosome. The pH threshold necessary to trigger the conformational alterations in VSIV G that promote fusion of the viral and cellular membranes is approximately 6.2. This pH is reached in the early endosome, and viral and cellular membranes fuse to release the transcription-competent RNP core into the cytoplasm of the cell. Recent work has called into question this conventional view of the VSV entry pathway and has posited a new model for viral entry. In this model, fusion and RNP release are spatially and temporally separated. Specifically, the low pH encountered during endocytic transport triggers fusion and the delivery of the RNP core into an intralumenal vesicle within an endosomal carrier vesicle. A subsequent (cell-mediated) fusion event is then required to fuse the membrane of the intralumenal vesicle with the limiting membrane of the cell and deliver the RNP into the cytoplasm to initiate the infectious process. Irrespective of the precise route by which virus enters the cell, the end result is the delivery of the transcription-competent core into the cytoplasm. This is accompanied by the release of M protein, which can migrate to the nucleus where it plays a role in inhibiting host gene expression.

Gene Expression

Following the delivery of the transcription-competent core into the cytoplasm, RNA synthesis can begin. The replication cycle of VSV is entirely cytoplasmic, occurring efficiently in enucleated cells. The demonstration that purified VSV particles contain a functional RdRp that is active *in vitro* has led to major advances in our understanding of viral gene expression. During RNA synthesis, the polymerase uses the encapsidated genomic RNA as template in two distinct reactions: (1) transcription of five mRNAs that encode the N, P, M, G, and L proteins; and (2) replication to yield full-length antigenomic, and then genomic RNA strands. Our current

understanding of gene expression is summarized as follows. In response to a specific promoter element that is provided by sequences within the 3′ leader region and the conserved residues of the first (N) gene-start (3′-UUGUCNNUAG-5′) sequence, the RdRp initiates mRNA synthesis. Synthesis commences at the N gene-start sequence and generates an mRNA that is capped, methylated, and polyadenylated, each of these reactions occurring co-transcriptionally. Termination at the end of the N gene is achieved by the polymerase recognizing a highly conserved sequence element referred to as the gene-end 3′-AUACUUUUUUUG/C-5′. This sequence signals the polymerase to stutter on the U_7 tract to generate the polyA tail, and leads to termination of mRNA synthesis. Termination at the end of the N gene is essential for the polymerase to be able to transcribe the P gene. A poorly understood event that is localized to a short region at the N–P gene junction results in the synthesis of approximately 30% less P mRNA than N mRNA. This sequential and polar synthesis of the viral mRNAs continues through the entire genome and provides a gradient of viral mRNA synthesis such that N > P > M > G > L. These products of mRNA synthesis are illustrated in Figure 2.

Among the notable steps of mRNA synthesis are the unusual mechanisms by which the 5′ and 3′ ends of the RNA are formed. Each stage of mRNA cap formation is distinct from those employed in other systems. Specifically, the 5′ end of the pppApApCpApG mRNA is capped by an unusual ribonucleotidyltransferase activity that transfers the monophosphate RNA onto GDP derived from GTP to form the *Gpp*pApApCpApG mRNA cap structure. In contrast, all other capping reactions are catalyzed by an RNA guanylytransferase that transfers GMP onto the 5′ end of a diphosphate RNA through a reaction that involves a covalent enzyme GMP intermediate. The VSV L protein is responsible for this novel reaction, which appears to involve a covalent intermediate between L and the viral mRNA. The resulting *Gpp*pApApCpApG cap structure is then methylated at guanine N-7 and ribose 2′-O positions to yield 7^{m}G*pp*pA^{m}pApCpApG. These activities are also provided by the L protein, and again differ from conventional mRNA cap methylation reactions. For VSIV, the two enzymatic activities have been shown to share a single binding site for the methyl donor, *S*-adenosyl-L-methionine (SAM). In contrast, other cap methylation reactions are normally executed by two distinct proteins with separate binding sites for the methyl donor SAM. Formation of the 3′ end of the RNA is also unusual. Specifically, polyadenylation occurs in a pseudo-templated fashion in which the polymerase complex reiteratively transcribes the conserved U tract present at the end of each VSV gene.

The viral mRNAs are efficiently translated by the host translation machinery, but how they compete with cellular mRNAs for translation is not well understood. Viral protein synthesis is essential for replication of the genomic RNA. Ongoing translation provides a continuous supply of soluble N protein that drives the encapsidation of the nascent RNA chain. This process is intimately linked to genomic RNA replication which first results in the production of a full-length encapsidated complementary antigenome RNA. This antigenome can then serve as template to produce more progeny genomic RNAs for use as templates for further mRNA synthesis in a process referred to as secondary transcription.

Precisely how the different polymerase activities are regulated in infected cells remains poorly understood. Two functionally distinct pools of polymerase have been purified from cells. One initiates internally at the N gene-start sequence and functions as the viral transcriptase. A second complex initiates at the 3′ end of the genome and functions as the viral replicase. These complexes are reported to differ in their composition such that the transcriptase comprises the viral P and L proteins, together with several cellular proteins including translation elongation factor-1α, heat shock protein 60, and the host cell RNA guanylyltransferase. In contrast, the replicase is reported to comprise the viral N, P, and L proteins.

In addition to the species of RNA described above, two short leader RNAs are generated during RNA synthesis: a 47 nt Le+ from the 3′ end of the genomic RNA and a 45 nt Le– from the 3′ end of the antigenomic RNA. The function of these RNAs is poorly understood, although a role for the Le+ in the shutoff of host gene expression has been described. A long-standing model for the regulation of RNA synthesis in VSV postulates that polymerase initiates all RNA synthesis at position 1 of the genome, and during synthesis of Le+ a crucial regulatory decision is made to either terminate leader and initiate mRNA synthesis at the N gene start, or alternatively to read through the leader–N gene junction and synthesize the full-length antigenome. The obligatory requirement for protein synthesis to provide a source of N protein to encapsidate the nascent RNA during genome replication led to the suggestion that N protein availability switches polymerase activity from transcriptase to replicase.

In recent years, there has been an accumulation of evidence that conflicts with this model. Specifically, a VSV mutant containing a single amino acid change in the template-associated N protein produces an excess of N mRNA over Le+ *in vitro*, suggesting that polymerase can synthesize N independently of Le+. In another series of experiments, recombinant VSVs, containing a 60 nt gene inserted between the leader region and the N gene, were employed. The recombinant viruses were examined to determine the effect of altering the potential number of

ultraviolet (UV)-induced dimers between adjacent uracil residues. Such dimers block progression of the polymerase. These experiments showed that changing the UV sensitivity of the Le+ had no effect on the sensitivity of the 60 nt mRNA in infected cells, suggesting that polymerase can initiate synthesis internally at the first gene start. In addition, two separate pools of polymerase can be isolated from infected cells, one that initiates internally at the N gene start and the second that initiates at the 3′ end of the genome. These findings support the hypothesis that mRNA synthesis can initiate independently of leader synthesis, and show that polymerase function is not simply switched by N protein levels. However, viral gene expression is controlled, it results in the exponential amplification of the input genomic RNA, yielding progeny genomes that can be assembled into infectious particles, the next phase of the viral replication cycle.

Assembly and Budding

Assembly of infectious virus particles involves many critical interactions. Our current understanding of this intricate process is that the matrix (M) protein complexes with the RNP core and represses transcription of viral mRNAs. This condensed RNP complex acquires a lipid envelope that has been modified by the insertion of an externally oriented glycoprotein. Details of how the RNP is transported to the site of budding and how the matrix interacts with and condenses the RNP are poorly understood. The M protein is only found associated with RNPs at sites of viral budding, and how genomic RNPs (rather than antigenomic RNPs) are specifically selected for budding is unclear. The M protein contains two 'late domains' (PTAP and PPPY motifs) that appear to be critical in the late phase of the assembly-release pathway. Amino acid substitutions in the PPPY motif result in the accumulation of bullet-shaped virions that are stalled at a late stage of virus budding. This motif, and similar motifs in proteins from other enveloped viruses, target virus for budding through interaction with components of the endosomal sorting complex required for transport or ESCRT pathway. The release of infectious particles completes the replication cycle and provides progeny virions for infection of the next cell.

The kinetics of the viral replication cycle are rapid. In mammalian cells in culture, one infectious particle can produce 10 000 infectious progeny within 8 h. While much remains to be explored about the biology of VSV, it is one of the best understood animal viruses. Studies on VSV should continue to prove informative in understanding how enveloped viruses enter and bud from their host cells, and how nonsegmented negative-strand RNA viruses (mononegaviruses) express their genetic information.

Functions of the Viral Proteins

Nucleocapsid protein

The N protein coats the viral genomic RNA and the positive-sense antigenomic replicative intermediate to form ribonucleoprotein (RNP) complexes. The interaction with the N protein renders the RNA resistant to cleavage by ribonucleases. It is in this form that the RNA is presented to the polymerase to serve as template. The N protein comprises 422 amino acids and has a molecular weight of approximately 48 kDa. The crystal structure of a complex of 10 molecules of N protein bound to 90 nt of RNA has been solved, revealing that N has a bilobed structure with RNA bound between the lobes. The structure indicates that N protein must be either transiently displaced or substantially remodeled during copying of the RNA genome by the viral polymerase.

Phosphoprotein

P is a multiply phosphorylated acidic protein of 265 amino acids that functions as an essential polymerase cofactor and plays an additional role in maintaining N protein in a soluble form necessary for RNA encapsidation. Sequence analysis has identified three domains of P. An acidic N-terminal domain (domain I) of 150 amino acids contains phosphorylation sites at Ser 60, Thr 62, and Ser 64. Phosphorylation of these residues by the host casein kinase II leads to the oligomerization of P protein and together with the large polymerase protein L, assembly of the polymerase complex. Domain I is separated from domain II by a highly variable hinge region comprising residues 150–210. Domain II (residues 210–244) contains additional phosphorylation sites at Ser 226, Ser 227, and Ser 233 that appear to be important for RNA replication. Domain III is basic and comprises the C-terminal 21 amino acids. A crystal structure of a fragment comprising amino acids 107–177 of the P protein has been solved, providing evidence that P protein functions as a tetramer. In addition to P, two proteins (C and C′) are produced from the P gene. These proteins are small (55 and 65 amino acids, respectively), are highly basic, and have not been detected in virus particles. Recombinant viruses that are unable to produce C and C′ replicate normally in cultured mammalian cells. The role of these proteins is unclear but may be important for infections in insects and/or mammalian hosts *in vivo*.

Matrix protein

The matrix (M) protein is the major structural component of virus particles. M is a small 229-amino-acid multifunctional protein. It condenses viral RNPs and drives budding of virus particles from the host plasma membrane. In addition, M downregulates host gene expression by directly interacting with Rae 1, thus inhibiting nuclear transport. The crystal structure of amino acids 48–229 of

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