292
Current Topics in Microbiology and Immunology

Z.F. Fu (Ed.)

The World of Rhabdoviruses

With 27 Figures and 7 Tables

Springer

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Cover figure by Zhen F. Fu

Library of Congress Catalog Number 72-152360

ISBN-10 3-540-24011-X Springer Berlin Heidelberg New York
ISBN-13 978-3-540-24011-2 Springer Berlin Heidelberg New York

Springer is a part of Springer Science+Business Media
springeronline.com

Printed in The Netherlands

Editor: Dr. Paul Roos, Heidelberg
Desk editor: Anne Clauss, Heidelberg
Production editor: Nadja Kroke, Leipzig
Cover design: design & production GmbH, Heidelberg
Typesetting: LE-TEX Jelonek, Schmidt & Vöckler GbR, Leipzig
Printed on acid-free paper SPIN 11001713 21/3150/YL – 5 4 3 2 1 0

Preface

Rhabdoviruses have a very wide host range and have been isolated from plants, insects, and almost from all vertebrates including fish and primates. The Rhabdoviridae family consists of six genera which have all been associated with diseases, either in animals or plants. While rhabdoviruses that are the etiological agents of human diseases can cause serious public health problems, other members of this family that infect domestic livestock and agricultural plants can also cause enormous economic loss. Despite the significance of rhabdoviruses for public health and agriculture, the last book exclusively devoted to these viruses was published in 1987. The first classification of rhabdoviruses was based on the distinct bullet-shaped morphology that is characteristic of members of this family. However, using modern gene technology, which allows the complete analysis of entire viral genomes, it was recently found that different rhabdoviruses exhibit not only morphological similarities but are also genetically related. In this respect, the development of reverse genetics technology and its use for studying the regulation of viral transcription and replication, deciphering the pathogenic mechanisms, and developing vaccines and gene therapy vectors probably resulted in the most significant progress in rhabdovirus research during the past 15 years.

This volume is intended to review the unique and common features of rhabdoviruses, particularly their morphological, molecular, and pathogenic characteristics, and their phylogenetic relationships.

The chapter by Fu reviews the common characteristics of rhabdoviruses, particularly from the viewpoint of phylogenetic relationships. The chapter by Dietzschold et al. summarizes the latest findings on the molecular pathogenic mechanisms of rabies. Hoffmann et al. discuss the molecular epidemiology and evolution of fish rhabdoviruses. Walker describes the epidemiology of and the diseases caused by ephemeroviruses. Warrilow summarizes how a new genus of rhabdoviruses, the Australian bat lyssaviruses, was discovered and how this genus of viruses is maintained and transmitted by insect- as well as fruit-eating bats. Redinbaugh and Hogenhout summarize recent findings on plant rhabdoviruses.

The development of reverse genetics in the 1990s has enabled the manipulation of the rhabdoviral genome and thus revolutionized the research field for rhabdoviruses. The contribution by Finke and Conzelmann reviews the development and establishment of this technology for vesicular stomatitis virus and rabies virus. Furthermore, this chapter also updates the progress of using this technology for the development of vaccines and gene therapy vectors. In addition, Brémont describes the establishment of a reverse genetics system for fish rhabdoviruses. This technology is also a powerful tool for the investigation of pathogenic mechanisms by which rhabdoviruses induce diseases in respective hosts. An example is the chapter by Dietzschold et al. in which different rabies viruses were constructed to decipher the contribution of each of the proteins in the induction of rabies. With either the minigenome or the infectious clones in the vesicular stomatitis virus system, many of the *cis*- and *trans*-elements important in the process of transcription and replication have been identified and/or confirmed, some of which are described in the chapter by Fu.

I would like to take this opportunity to thank Dr. Hilary Koprowski for constant encouragement and help during the editing of this volume and all of the contributors to this volume for their patience and enthusiasm.

March 2005 *Zhen F. Fu*

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Genetic Comparison of the Rhabdoviruses from Animals and Plants

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Abstract There are more than 160 viral species in the *Rhabdovidae* family, most of which can be grouped into one of the six genera including *Vesiculovirus*, *Lyssavirus*, *Ephemerovirus*, *Novirhabdovirus*, *Cytorhabdovirus*, and *Nucleorhabdovirus*. These viruses are not only morphologically similar but also genetically related. Analysis of viral genes shows that rhabdoviruses are more closely related to each other than to viruses in other families. With the development of reverse genetics, the functions of many *cis*- and *trans*-elements important in the process of viral transcription and replication have been clearly defined such as the leader, trailer, and the intergenic sequences. Furthermore, it has been shown that there are two entry sites for the RNA-dependent RNA polymerase: 3′ entry for leader synthesis and RNA replication, and direct entry at the N gene start sequence for transcription of the monocistronic mRNAs.

1 Introduction

Rhabdoviruses are a large family of viruses that can infect a wide range of hosts (Walker et al. 2000). Rhabdovirus has been isolated from plants, invertebrate and vertebrate animals (for reviews, see Wagner 1987; Dietzschold et al. 1996; Rose and Whitt 2000). To date there are more than 160 species of rhabdoviruses isolated (Walker et al. 2000), with new viruses being discovered constantly (Amelia et al. 2002; Kuzmin et al. 2003; Mork et al. 2004). Some of the rhabdoviruses induce severe diseases in humans and animals, including fish (Dietzschold et al. 1996; Brown 1987; Ahne et al. 2002). Others cause plant diseases, particularly in agricultural crops (Jackson et al. 1987). Previously viruses were classified as rhabdoviruses because of their distinct bullet-shaped morphology (Wagner 1987). With recent development of genetic analysis, these viruses are clearly not only morphologically similar, but also genetically related (Walker et al. 2000; Warrilow et al. 2002). This paper is intended to present the genetic relatedness of all the rhabdoviruses regardless the sources of isolation. Furthermore, recent advances in reverse genetics have defined more clearly the *cis*- and *trans*-elements that are important regulators of rhabdoviral transcription and replication.

2 Classification of Rhabdoviruses

The family of Rhabdoviridae has been classified in the order of Mononegavirales together with *Paramyxoviridae*, *Filoviridae*, and *Bornaviridae* (Murphy 1996). To date, more than 160 species of rhabdoviruses have been reported, most of which can be classified or tentatively classified in six genera and others have yet to be assigned (Walker et al. 2000). The six genera include *Vesiculovirus*, *Lyssavirus*, *Ephemerovirus*, *Novirhabdovirus*, *Cytorhabdovirus*, and *Nucleorhabdovirus*. Many of the well-known and characterized vesiculoviruses include VSV Indiana virus (VSIV), and VSV New Jersey virus (VSNJ). The less characterized vesiculoviruses include Alagoas virus (VSAV), Carajas virus (CJSV), Chandipura virus (CHPV), Cocal virus (COCV), Isfahan virus (ISFV), Maraba virus (MARAV), and Piry virus (PIRYV) (Walker et al. 2000). The Lyssavirus genus includes rabies and rabies-related viruses. Molecular comparison has divided lyssaviruses into seven genotypes (Bourhy et al. 1993; Gould et al. 1998). All the classical rabies viruses (RABV) belong to genotype 1; Lagos bat virus (LBV) genotype 2; Mokola virus (MOKV) genotype 3; Duvenhage virus (DUVV) genotype 4, European bat Lyssavirus

1 (EBLV-1) genotype 5, European bat Lyssavirus 2 (EBLV-2) genotype 6; and the newly discovered Australian bat lyssavirus (ABLV) belong to genotype 7. The Ephemerovirus genus includes bovine ephemeral fever virus (BEFV), Adelaide River virus (ARV), and Berrimah virus (BRMV) (Walker et al. 2000). The genus of Novirhabdovirus also has three well-defined species that infect aquatic hosts, i.e., infectious hematopoietic necrosis virus (IHNV), viral hemorrhagic septicemia virus (VHSV), and hirame rhabdovirus (HIRRV) (Kurath et al. 1997; Walker et al. 2000). The last two genera in the family of Rhabdoviridae are the viruses infecting plants, the Cytorhabdovirus and the Nucleorhabdovirus (Walker et al. 2000). Well-characterized cytorhabdoviruses include lettuce necrotic yellow virus (LNYV), barley yellow striate mosaic virus (BYSMV), broccoli necrotic yellow virus (BNYV), and Northern cereal mosaic virus (NCMV) (Jackson 1987; Wetzel et al. 1994). Well-characterized nucleorhabdoviruses include eggplant mottled dwarf virus (EMDV), maize mosaic virus (MMV), rice yellow stunt virus (RYSV), sonchus yellow net virus (SYNV), and potato yellow dwarf virus (PYDV) (Jackson et al. 1987; Martins et al. 1998).

3
Diseases Caused by Rhabdoviruses

The importance of rhabdoviruses is that these viruses cause severe diseases in plants and animals, including humans. For example, rabies virus causes rabies in humans as well as in all warm-blooded animals, with almost 100% mortality (Dietzschold et al. 1996). VSV causes a disease that is clinically similar to that of foot-and-mouth disease in cattle and pigs (Brown 1987). Bovine ephemeral fever virus causes a disabling viral disease of cattle and water buffalo (St George 1990). Spring viremia of carp virus (SVCV) causes a severe hemorrhagic disease of cyprinids (Ahne et al. 2002). Plant rhabdoviruses cause many plant diseases including, but not limited to, maize mosaic, rice transitory yellowing, potato yellow dwarf, and lettuce necrotic yellows (Jackson et al. 1987). Thus, rhabdoviruses not only cause diseases in humans, thus presenting public health problems, but also induce diseases in domestic and wildlife animals and fish, as well as in agricultural plants, causing economic losses.

4
Transmission of Rhabdoviruses

Many of the rhabdoviruses are transmitted by insect vectors. Plant-infecting rhabdoviruses are transmitted to their plant hosts by insect vectors, including aphids, planthoppers and leafhoppers (Jackson 1987). Several animal rhabdoviruses, for example, ephemeroviruses, and some vesiculoviruses, are also transmitted by insect vectors (Wagner 1987; Walker et al. 2000). Actually, many of the vesiculoviruses and ephemeroviruses were isolated initially from insects. For example, Cocal virus was isolated from dust mites (Jonkers et al. 1964), and Sigma virus was recognized first as a congenital infection of *Drosophila* (Printz 1973). Others can readily infect mosquitoes or mosquito cells (Gillies and Stollar 1980). Recently it has been proposed that VSV is transmitted by mosquitoes from wild to domestic animals on Ossabaw Island, although transmission by insect vectors among the wild animal population is not clear (Stallknecht 2000). The widespread ability of rhabdoviruses to infect insects has led to the suggestion that the family of Rhabdoviridae evolved from an ancestral insect virus (Nault 1997) and that the host range of rhabdoviruses is largely determined by the insect host (Hogenhout et al. 2003).

Among the rhabdoviruses, only Lyssavirus and fish rhabdoviruses (Novirhabdovirus and some vesiculoviruses) are not maintained by insect hosts. Previously, Obodhiang and Kotonkan viruses that were classified into the Lyssavirus genus were isolated from insects (Shope and Tesh 1987). These viruses are now listed as unassigned rhabdoviruses because serological and molecular data link them to viruses in the genus Ephemerovirus (Walker et al. 2000). Although it has been known for a long time that the rabies virus life cycle is maintained by carnivores (Fu 1997), now it is known that almost all the viruses in the Lyssavirus genus can be transmitted by bats. RABV (genotype 1) transmitted by vampire bats in South America has caused major economic loss in the cattle industry (Diaz et al. 1994). From the beginning of the 1990s, rabies virus strains normally circulating in the insectivorous bat population have been responsible for most human rabies cases in the United States (Krebs et al. 2000; Messenger et al. 2002; Morimoto et al. 1996). Lyssavirus genotypes 2 (LBV), 4 (DUVV), 5 (EBLV-1), and 6 (EBLV-2) have all been isolated from bats (Shope 1982). The newly discovered ALBV (genotype 7) was maintained by insectivorous as well as fruit-eating bats, the flying foxes (Gould et al. 2002). Recently serological evidence of bat rabies has been reported in the Philippines (Arguin et al. 2002) and novel lyssaviruses have been isolated in bats in Central Asia (Kuzmin et al. 2003). Obviously, further studies are needed to understand the role of bats in the evolution and transmission of lyssaviruses.

5
Genome Organization of Rhabdoviruses

Rhabdovirus genomes are among the simplistic viruses and most of them contain only five genes in the order 3′ N-P-M-G-L 5′ (Walker et al. 2000; Rose and Whitt 2000). The very 3′ end of the genome is the leader sequences and the very 5′ end of the untranslated region is the trailer sequences (Rose and Whitt 2000). Between each of these viral genes lies the intergenic sequence. The leader, trailer, and the intergenic sequences play important roles in the process of viral transcription and replication (see Sect. 3.1). In addition to these five structural proteins, two small proteins have been detected in virus-infected cells that are translated from a second reading frame within the P gene of the vesiculoviruses (Herman 1986; Spiropoulou and Nichol 1993) and lyssaviruses (Chenik et al. 1995). Some fish rhabdoviruses have an extra small gene between G and L (Kurath et al. 1985). Plant rhabdoviruses can have extra genes between P and M (Chen et al. 1998) and between G and L (Huang et al. 2003). Recently Tanno et al. (2000) reported four extra genes between P and M in Northern cereal mosaic virus. Ephemeroviruses encode extra genes but these genes were between G and L. So far six extra genes, including a second nonstructural glycoprotein (G_{NS}) and five smaller proteins ($\alpha 1$, $\alpha 2$, $\alpha 3$, β and γ), were found between the G and L genes in ephemeroviruses (Walker et al. 1992; Wang et al. 1994; McWilliam et al. 1997). The Sigma virus of *Drosophila* also has three extra genes between N and G as well as a 33-nucleotide overlap of the G gene with the preceding gene (Landes-Devauchelle et al. 1995; Teninges et al. 1993). However, the functions of these extra gene products are still unknown.

The rhabdovirus nucleoprotein (N) serves the critical function of encapsidating the genomic RNA into an RNase-resistant core that is the template for both transcription and replication (Banerjee and Chattopadhyay 1990; Blumberg et al. 1983; Wertz et al. 1987; Wunner 1991, Yang et al. 1998). By encapsidating the genomic RNA, N is thought to regulate the switch from transcription to replication. It has been calculated from the length of the genome and the number of N molecules per virion that each N molecule would cover about nine nucleotides of RNA (Wunner 1991), which has been demonstrated when the N is expressed alone (Schoehn et al. 2001; Green et al. 2000). This N–RNA complex interacts with the P–L polymerase complex during transcription and replication (Banerjee and Chattopadhyay 1990; Wertz et al. 1987). The phosphoprotein (P), on one hand, binds to N, to confer the specificity of N encapsidation of genomic RNA (Banerjee et al. 1989; Yang et al. 1998). On the other hand, P in combination with the L protein forms the RNA-dependent RNA polymerase (RdRp). The matrix protein (M) interacts with

the nucleocapsid and the cytoplasmic portion of the G, thereby facilitating virus assembly and budding (Walker et al. 2000; Wunner 1991). The M protein also plays an important role in regulating viral RNA transcription (Finke and Conzelmann 2003). The glycoprotein (G) is the only surface protein for rhabdoviruses (Rose and Whitt 2000), and it plays important roles for binding to cellular receptors, thus initiating virus infection (Dietzschold et al. 1996; Walker et al. 2000). The large protein (L) is the major component of the RdRp, which is responsible for copying the N–RNA template to produce mRNA, or complete antigenomic and genomic RNA (Baltimore et al. 1970; Emerson and Yu 1975; Rose and Whitt 2000). In addition, the L also plays roles in mRNA capping, methylation of 5′ cap structures, and polyadenylation (Baltimore et al. 1970; Banerjee 1987; Rose and Whitt 2000).

6 Genetic Comparison of Rhabdoviruses

Rhabdoviruses are not only morphologically similar, but also share serological cross-reactions among some of them. Polyclonal antisera to BEFV and ARV showed cross-reactivity with RABV N protein (Walker et al. 1994). Low-level cross-reactions have also been reported between ephemeroviruses, lyssaviruses and several unclassified rhabdoviruses isolated from cattle or insects (Calisher et al. 1989). The cross-reactivity possibly reflects the genetic relatedness. Genetic comparison has been carried out previously, particularly between one or two genes of different viruses (Gallione and Rose 1983; Tordo et al. 1986a; Poch et al. 1989, 1990). Relatedness between different rhabdoviruses was observed. Recently full-length sequences have been obtained from many of the rhabdoviruses (Iverson and Rose 1981; Schubert et al. 1985; Tordo et al. 1988; Conzelmann et al. 1990; Schutze et al. 1995, 1999; Tanno et al. 2000; Ito et al. 2001; Hoffmann et al. 2002), which makes global genetic comparison possible. The full-length of rhabdoviral genomic RNA is about 11–15 kb and comparison of each of the five structural genes among the different rhabdoviruses is presented in the following sections.

6.1 N Gene

Since N is the first gene transcribed from the genome, it has been sequenced from most of the existing rhabdoviruses. Genetic comparison of N sequences from different rhabdoviruses has been used most often for evolution and

classification studies among rhabdoviruses and for relatedness between rhabdovirus and other viruses (Tordo et al. 1986a; Barr et al. 1991; Bourhy et al. 1993; Wang et al. 1995; Amengual et al. 1997; Walker et al. 2000). The N sequences are highly homologous within each of the genera as well as among all the rhabdoviruses. Using a relatively conserved region of the N protein (119 amino acids), Walker et al. (2000) constructed a universal phylogeny of rhabdoviruses, which indicates that the N sequences from different rhabdoviruses are more closely related than the N sequences from other negative-stranded nonsegmented RNA viruses (for example, human paramyxovirus type 1). We also analyzed the N sequences from different genera of the rhabdoviruses by using the Phylip Package developed in the Department of Genome Sciences at University of Washington (http://evolution.genetics.washington.edu/phylip/doc/main.html references), which include a combination of Clustalw, SEQBOOT, PROTDIST, NEIGHBOR (Neighbor-Joining method of Saitou and Nei 1987), CONSENSE, and DRAWTREE. As shown in Fig. 1A, viruses in each of the genera are clustered together and separated from the others, similar to that described by Walker et al. (2000). Furthermore, common motifs have been found in the N sequences between rhabdoviruses and other negative-stranded nonsegmented RNA viruses, which may be involved in protein-RNA and protein–protein interactions in the virus nucleocapsid (Barr et al. 1991).

6.2 L Gene

The L genes are relatively conserved not only among the rhabdoviruses, but also among the negative-stranded nonsegmented RNA viruses. Six conserved domains (blocks I–VI) have been found (Poch et al. 1990) in which motifs related to enzymatic functions important for viral replication reside. Block I is critical for multiple polymerase function (Chandrika et al. 1995) and there is an invariant tripeptide GHP within this region. Block II is rich in basic residues and may play a role in RNA recognition or nucleotide binding (Muller et al. 1994; Smallwood et al. 1999). Block III has four conserved motifs, A–D, some of which are present in all known polymerases, implying the critical function of these motifs. For example, mutation of the GDN core sequence of motif C blocks viral transcription and replication (Schnell and Conzelmann 1995). Block IV is rich in proline residues among all the negative-stranded RNA viruses and may be involved in nucleotide binding. Block V has invariant cysteine and histidine residues that may play a catalytic role via metal binding. Block VI contains a glycine-rich motif (GXGXG) that

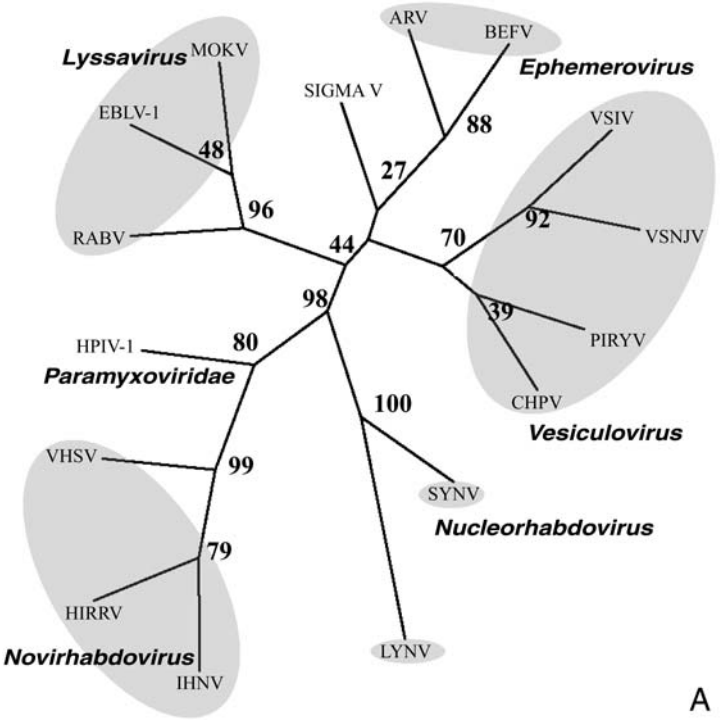


Fig. 1A–E Phylogenetic relationships between different genera of the rhabdoviruses. Rhabdoviral N (**A**), L (**B**), P (**C**), M (**D**), and G (**E**) genes were analyzed using the Phylip Package developed in the Department of Genome Sciences at the University of Washington (http://evolution.genetics.washington.edu/phylip/doc/main.html references), which include a combination of Clustalw, SEQBOOT, PROTDIST, NEIGHBOR, CONSENSE, and DRAWTREE. Wherever possible, sequences from each genus were included. Human parainfluenza virus 1 (HPIV-1) was used as the outgroup for the analyses. *ABLV*, Australia bat lyssaviruses; *ARV*, Adelaide River virus; *BEFV*, bovine ephemeral fever virus; *CHPV*, Chandipura virus; *EBLV-1*, European bat Lyssavirus 1; *HIRRV*, hirame rhabdovirus; *IHNV*, infectious hematopoietic necrosis virus; *LNYV*, lettuce necrotic yellow virus; *MOKV*, Mokola virus; *PIRYV*, Piry virus; *NCMV*, Northern cereal mosaic virus; *SIGMAV*, Sigma virus; *RABV*, rabies viruses; *SYNV*, sonchus yellow net virus; *VHSV*, viral hemorrhagic septicemia virus; *VSIV*, VSV Indiana virus; *VSNJ*, VSV New Jersey virus

may be important for polyadenylation or protein kinase activities. Using the conserved domain III sequences, Warrilow et al. (2002) analyzed the phylogenetic relationship between all the genera within the Rhabdoviridae family and found that each of the genera is clustered together. Furthermore, these authors reported that genera containing viruses that infect terrestrial

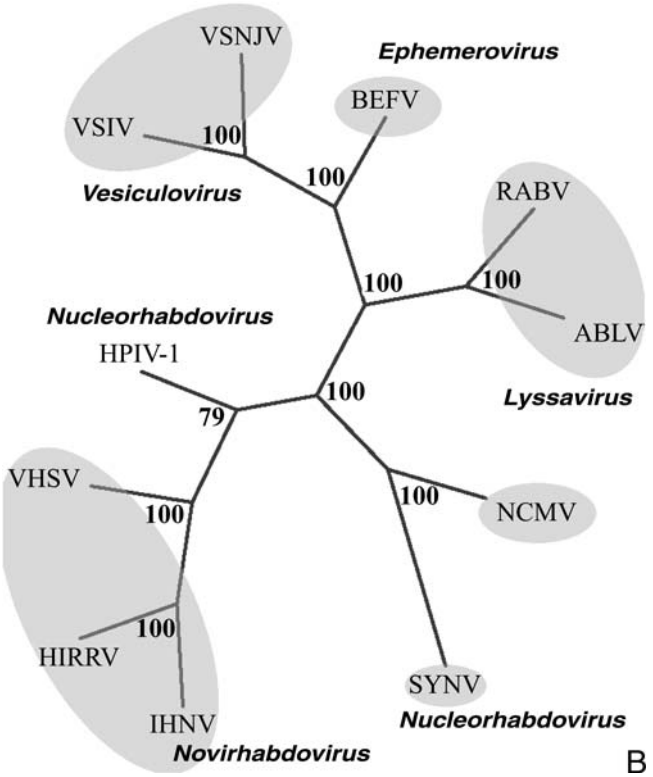


Fig. 1A–E (continued)

animals (lyssaviruses, vesiculoviruses, and ephemeroviruses) clustered more closely than those infecting fish and plants. Strong bootstrap support (>95) was found for the clustering at each of the nodes separating the genera. We analyzed the complete L sequences of representatives from each genus by using the Phylip Package and similar findings as those reported by Warrilow et al. (2002) were obtained (Fig. 1B).

6.3
P, M, and G Genes

Although sequence similarities have been reported for P, M, and G genes within some of the rhabdoviruses (Gallione and Rose 1983; Rayssiguier et al. 1986; Larson and Wunner; 1990), these sequences have not been compared among all the rhabdoviruses. Here we compared the P, M, and G sequences

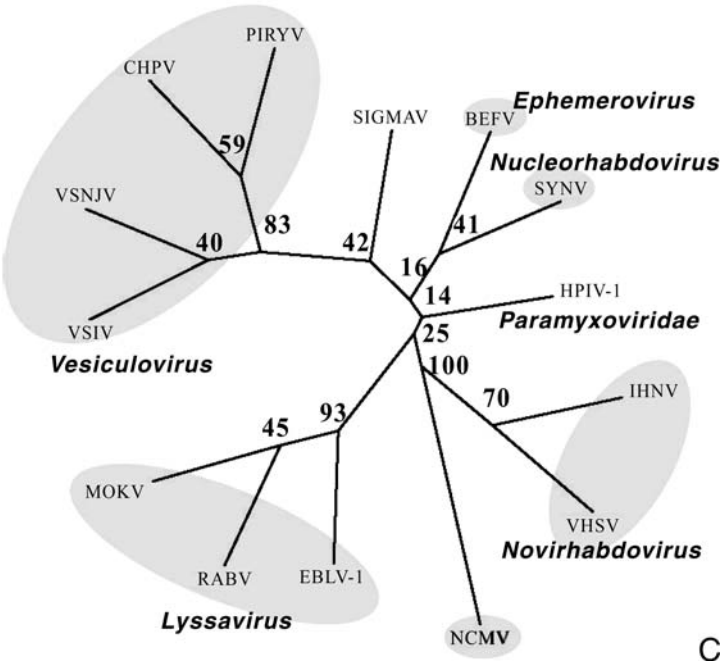
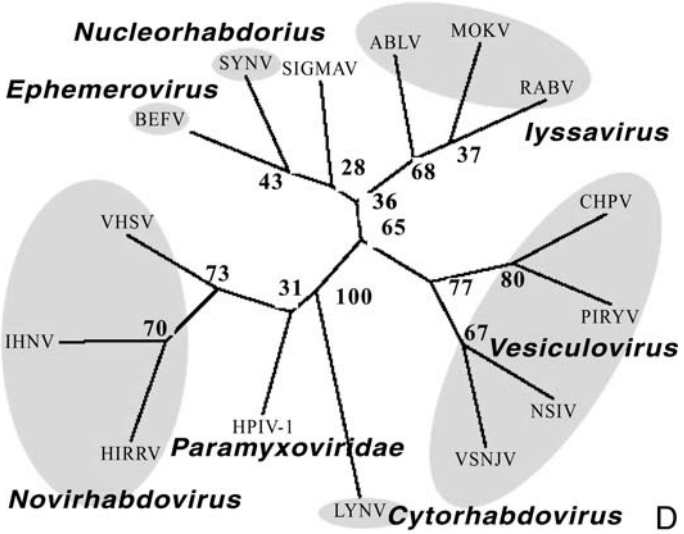


Fig. 1A–E (continued)

from all the genera within the rhabdovirus family using the Phylip Package. As shown in Fig. 1C–E, viruses in each of the genera are clustered together for each of the genes, just like the N and L genes. However, viruses that infect terrestrial animals (lyssaviruses, vesiculoviruses, and ephemeroviruses) are not always clustered together and separate from those infecting fish and plants. It is also interesting to note that the viruses transmitted by insect cells are clustered together (vesiculoviruses, ephemeroviruses, and Sigma virus) for the G genes (Fig. 1E). It is tempting to speculate that the similarities of the G may contribute to the ability of these viruses to infect insects.

7 Regulation of Rhabdoviral Transcription and Replication

The single-strand, negative-sense genomic RNA of rhabdoviruses acts as a template for both transcription and replication. During the transcription process, a positive-strand leader RNA and five or more monocistronic mRNAs are synthesized (Wertz et al. 1987, Banerjee and Chattopadhyay 1990; Wunner



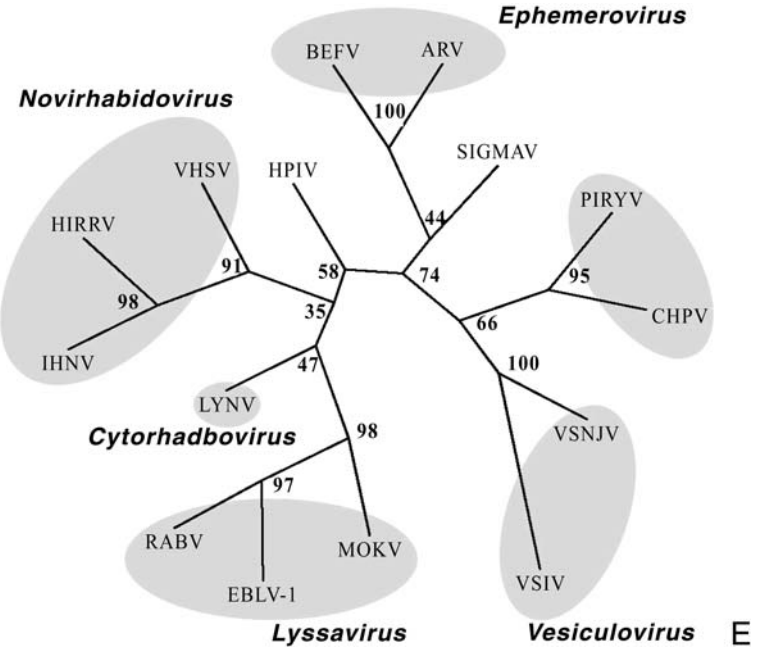


Fig. 1A–E (continued)

1991; Rose and Whitt 2000; Barr et al. 2002). Like cellular mRNA, rhabdoviral mRNAs are capped and methylated at the 5′ end and polyadenylated at the 3′ end. However, the leader RNA is neither capped nor polyadenylated. Rhabdoviral transcription initiates at the 3′ end of the genome in an obligatorily sequential manner (Abraham and Banerjee 1976; Ball and White 1976; Rose and Whitt 2000). However, the mRNAs were not produced in equimolar amounts, rather their abundance attenuated with distance from the 3′ promoter (Villarreal et al. 1976; Iverson and Rose 1981). As soon as these transcripts are translated into viral proteins, genome replication can begin. During the replication process, a full-length, positive-strand RNA (antigenome) is first synthesized, which then serves as the template for the synthesis of progeny negative-strand genomic RNA (Banerjee 1987; Banerjee and Chattopadhyay 1990; Wunner 1991). Both the genome and the antigenome are encapsidated with N and this RNA–N complex, together with P and L, forms the ribonucleoprotein (RNP) complex. The RNP is then utilized as templates for subsequent rounds of transcription, replication, or assembled into infectious particles (Wunner 1991; Rose and Whitt 2000; Barr et al. 2002).

Rhabdovirus, particularly VSV, has been used as the prototypic virus for studying the regulation of viral transcription and replication among the nonsegmented and single-stranded RNA viruses (see Barr et al. 2002 and references therein). It has been proposed that it is the availability of soluble N protein to encapsidate the nascent genomic and antigenomic RNA that switches the virus from transcription to replication (Blumberg et al. 1983; Wertz et al. 1987). In addition, many *cis*- (leader and trailer sequences, intergenic sequences) and *trans*- (viral and cellular proteins) elements have been proposed to participate in the regulation of viral transcription and replication. The most important advance in the past decade is the development of reverse genetics, which made manipulation of the rhabdoviral genome possible (Pattnaik and Wertz 1990, 1991; Schnell et al. 1994). With this technology, many of the *cis*- and *trans*-elements in the regulation of viral transcription and replication have been delineated. Recently, Barr et al. (2002) described in detail the *cis*- and *trans*-elements involved in the transcriptional control of VSV. Only the highlights are summarized in the following section.

7.1 *Cis*-elements Involved in Viral Transcription and Replication

The 3′ leader sequences of the genome act as a promoter for both transcription and replication (Banerjee and Chattopadhyay 1990; Wunner 1991; Rose and Whitt 2000). The 5′ trailer sequence of the genome acts exclusively as an origin

of replication. However, the complementary sequence of the trailer region, i.e., the 3′ end of the antigenome, acts as the promoter for synthesis of full-length negative-sense genomes. Using reverse genetics, it is now confirmed that the genomic termini of VSV were multifunctional, containing essential signals for encapsidation of RNA by N, binding of polymerase to template, transcription, replication, and assembly of infectious particles (Pattnaik et al. 1995). The 3′ and the 5′ sequences are complementary to a certain extent in all the sequenced rhabdoviruses (Iverson and Rose 1981; Schubert et al. 1985; Tordo et al. 1988; Conzelmann et al. 1990; Schutze et al. 1995, 1999; Tanno et al. 2000; Hoffmann et al. 2002). From the studies of VSV, it has been shown that the extent of complementarity between the genomic termini affected the use of the template for transcription or replication (Wertz et al. 1994) because increasing the extent of complementarity between the termini increased replication and ultimately decreased transcription. However, Li and Pattnaik (1997) reported that deletion of nucleotides 25–45 from both termini of DI RNA but maintaining the length of terminal complementarity reduced replication by about 20-fold, which may indicate that the presence of specific sequences rather than the extent of complementarity at the termini determines the efficiency of replication.

Other *cis*-elements include the highly conserved sequences at the beginning and end of each gene and the intergenic sequence (Colonno and Banerjee 1978; Rose 1980; Tordo et al. 1986b; Walker et al. 2000). Recent findings demonstrated that these *cis*-elements are important in regulation of viral transcription (Barr et al. 2002 and references therein). The arrangement of these sequences is similar in the family of rhabdoviruses and these sequences are conserved among viruses within each genus (Wunner 1991; Rose and Whitt 2000; Walker et al. 2000). For example, each of the internal gene junctions in VSV comprised the sequence 3′...AUACUUUUUUU *G/CA*UUGUCNNAG ... 5′ (Rose and Whitt 2000; Barr et al. 2002). In addition, there is a leader-N gene junction sequence that is present neither in the leader RNA nor the N mRNA (Wunner 1990; Rose and Whitt 2000; Walker et al. 2000). Deletion of these nucleotides in VSV resulted in the abrogation of mRNA synthesis from a subgenomic replicon (Whelan and Wertz 1999), although point mutations engineered throughout this region had little effect on mRNA synthesis.

Mutational studies with the start sequence of the VSV G gene (Stillman and Whitt 1997, 1999) indicated that the first three nucleotides were most critical for efficient gene expression because these nucleotides contained essential signals for processing of the nascent mRNA strand. Without this processing, most of the transcripts were prematurely terminated. The gene ending sequences consist of a tetranucleotide (AUAC in VSV) and the U_7 track (Ross and Whitt 2000). It has been shown with VSV studies that termination of the

previous mRNA is affected most if each of the first three nucleotides AUA is replaced with a C residue. As for the 5′ C residue of the tetranucleotide, any replacement resulted in a total loss of termination signaling ability (Barr et al. 1997a; Hwang et al. 1998). The function of the U7 tract has been suggested to provide a template for generation of the mRNA poly (A) tail (Schubert et al. 1980). Mutational analysis of the U tract revealed that shortening or interrupting the U7 tract abolished all termination ability of the resulting gene junction (Barr et al. 1997a). However, increasing the U-tract length had little effect on termination signaling ability. In addition, U7 tract also plays a role in signaling initiation of downstream mRNA synthesis. Either reducing or increasing the length of the U7 tract resulted in reduced downstream initiation (Hinzman et al. 2002). The U7 track has been found to be universal for each of the genes in all the rhabdoviruses (Tordo et al. 1986b; Schutze et al. 1995, 1999; Tanno et al. 2000; Hoffmann et al. 2002). However, the sequences immediately preceding the U7 track may differ from one genus to the other. For example, AC or UC are the ending sequences prior to the U7 track for lyssaviruses (Tordo et al. 1986b; Conzelman et al. 1990; Ito et al. 2001; Warrilow et al. 2002).

Likewise, the intergenic sequences are different from one genus to the other among the rhabdoviruses. However, they are more conserved within each of the genera. For example, the intergenic sequence for VSV is either GA (for N/P, P/M, G/L) or CA (M/G) (Rose 1980). However, the intergenic sequence for rabies is GA (N/P), GUCCG (P/M), GAUAA (M/G), 423 nucleotides (G/L) (Tordo et al. 1986b). The role of these intergenic sequences has been studied with regard to its roles in transcription termination and subsequent downstream transcription in VSV Indiana strain (Stillman and Whitt 1997, 1998; Barr et al. 1997b). Any dinucleotide can signal for termination. However, any mutation on the dinucleotide abolishes the downstream transcription. This finding suggested that the role of the dinucleotide in signaling termination was to position a non-U residue directly downstream of the minimum length of U tract that supported reiterative transcription, while the intergenic sequence acted to physically separate the upstream U tract from the downstream gene start sequence, which begins with the sequence 3′-UUGUC-5′ (Barr et al. 2002). It is thus clear that the gene end sequence, the nontranscribed intergenic sequence, and the gene start sequence are sufficient to direct the polymerase to terminate the upstream mRNA synthesis, to add the polyA tail, and to subsequently initiate the synthesis of downstream mRNA (Schnell at al. 1996).

7.2 *Trans*-elements Involved in Viral Transcription and Replication

Trans-elements involved in rhabdoviral transcription and replication include viral proteins and possibly some cellular proteins. Cellular proteins, for example, have been found to be packaged in rabies virions (Sagara et al. 1995, 1997) or associated with VSV RdRp (Das et al. 1998; Gupta et al. 2002). These cellular proteins may play a role in the process of viral transcription and replication. For viral proteins, it has been shown conclusively from recent reverse genetics studies that N, P, and L are absolutely required for the initiation of viral transcription and replication (Pattnaik and Wertz 1990; Conzelmann and Schnell 1994; Biacchesi et al. 2000). The minigenome or the full-length genome synthesized by transfected plasmids can only be replicated and subsequently transcribed when N, P, and L are supplied *in trans*. It is not only these viral components per se, but also the complicated interactions between these proteins and viral genomic RNA that initiates and regulates rhabdoviral transcription and replication (Banerjee 1987; Banerjee and Chattopadhyay 1990; Wertz et al. 1987; Wunner 1991). For example, rhabdoviral N encapsidates the de novo-synthesized genomic RNA, thus switching the virus from the mode of transcription to that of replication (Blumberg et al. 1983). However, N is capable of encapsidating nonspecific RNA and the N needs to interact with P for specific encapsidation of the genomic RNA (Banerjee et al. 1989; Yang et al. 1998). Likewise, L interacts with P to form the RdRp to fulfill all the required enzymatic reactions in the process of RNA transcription and replication (Banerjee and Chattopadhyay 1990). Until recently, it was generally believed that the RdRp was both the transcriptase and the replicase. Regulation of the process from transcription to replication is due to the encapsidation of the leader RNA by N, or the N–P complex.

In a recent study, Pattnaik et al. (1997) reported that transcriptionally inactive P mutants can efficiently function in replication of VSV defective interfering particle and these investigators proposed that a tripartite complex consisting of L-(N-P) protein may represent the putative replicase for synthesis of the full-length genome RNA. The tripartite complex has been purified from VSV-infected BHK cells as well as in insect cells that express L, N, and P proteins. The purified tripartite complex supported the replication of genome-sense RNA in an in vitro replication reconstitution reaction (Gupta et al. 2003). In this system, a mutant P protein (P260A) that has been shown to be inactive in transcription but active in replication (Das et al. 1997) was also capable of forming the mutant [L-(N-Pmut)] complex in both insect cells and BHK cells and supporting genome-sense RNA synthesis (Gupta et al. 2003). These studies may indicate that the transcriptase and replicase are