Viral Genome Replication

Craig E. Cameron · Matthias Götte ·
Kevin D. Raney
Editors

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Editors
Craig E. Cameron
Pennsylvania State University
University Park
PA, USA
cec9@psu.edu

Matthias Götte
McGill University
Montreal, Quebec
Canada
matthias.gotte@mcgill.ca

Kevin D. Raney
University of Arkansas for Medical Sciences
Little Rock, AR
USA
raneykevind@uams.edu

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Preface

Currently, there is no single source that permits comparison of the factors, elements, enzymes and/or mechanisms employed by different classes of viruses for genome replication. As a result, we (and our students) often restrict our focus to our particular system, missing out on the opportunity to define unifying themes in viral genome replication or benefit from the advances in other systems. For example, extraordinary biological and experimental paradigms that have been established over the past 5 years for the DNA replication systems of bacteriophage T4 will likely be of great value to anyone interested in studying a replisome from any virus. These studies could easily go unnoticed by animal RNA and DNA virologists. It is our hope that this monograph will cross-fertilize and invigorate the field, as well as encourage students into this area of research.

The monograph has been divided into eight parts. Chapters appearing in Parts I–VI are intended to compare and contrast the replication and/or transcription processes and corresponding "players" of the indicated family of viruses. We are interested in the sequence of events that lead to production of mRNA and progeny genomes as well as the *cis*-acting elements and *trans*-acting factors and enzymes (viral and cellular) that are required for these processes. Chapters appearing in Part VII are intended to provide a more biochemical and biophysical perspective of the replication and/or transcription process. Chapters appearing in Part VIII are intended to provide a practical perspective on viral replication and its inhibition.

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Contributors

Diego E. Alvarez, PhD Molecular Virology Laboratory, Fundación Instituto Leloir, Av Patricias Agentinas 435, Buenos Aires 1405, Argentina, dalvarez@leloir.org.ar

Graciela Andrei, PhD Laboratory of Virology, Rega Institute for Medical Research, Katholieke Universiteit Leuven, B-3000 Leuven, Belgium, graciela.andrei@rega.kuleuven.ac.be

Todd Appleby, PhD Gilead Sciences, 333 Lakeside Drive, Foster City, CA 94404, USA, Todd.Appleby@gilead.com

Matthew P. Badtke, PhD Department of Molecular, Cellular and Developmental Biology, University of Michigan, Ann Arbor, MI 48109, USA, badtkemp@umich.edu

George A. Belov, PhD Picornavirus Replication Section, Laboratory of Infectious Disease, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20982, USA, gbelov@niaid.nih.gov

Stephen J. Benkovic, PhD Department of Chemistry, Pennsylvania State University, 414 Wartik Laboratory, University Park, PA 16802, USA, sjb1@psu.edu

Kathleen Boyle, PhD Microbiology & Mol Genetics, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, WI 53226, USA, kboyle@mcw.edu

Erik De Clercq, PhD Laboratory of Virology, Rega Institute for Medical Research, Katholieke Universiteit Leuven, B-3000 Leuven, Belgium, erik.declercq@rega.kuleuven.be

Paul Digard, PhD Department of Pathology, University of Cambridge, Tennis Court Road, Cambridge, CB2 1QP, United Kingdom, pd1@mole.bio.cam.ac.uk

James Dolan, PhD Department of Microbiology and Immunology, Northwestern university Feinberg School of Medicine, 303 E. Chicago Ave., Chicago, IL 60611, USA, j-dolan@northwestern.edu

Ellie Ehrenfeld, PhD Picornavirus Replication Section, Laboratory of Infectious Disease, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20982, USA, eehrenfeld@niaid.nih.gov

Debra Elton, PhD Centre for Preventative Medicine, The Animal Health Trust, Lanwades Park, Kentford, Newmarket, Suffolk, CB8 7UU, United Kingdom, debra.elton@aht.org.uk

Cristina Ferrer-Orta, PhD Institut de Biologia molecular de Barcelona (CSIC), Parc Cientific de Barcelona, Baldiri i Reixac 15, E-08028 Barcelona, Spain, cfocri@ibmb.csic.es

Claudia V. Filomatori, PhD Molecular Virology Laboratory, Fundacion Instituto Leloir, Av Patricias Agentinas 435, Buenos Aires 1405, Argentina, cfilomatori@leloir.org.ar

Román Galetto, PhD Cellectis SA, 102 avenue Gaston Roussel, 93235 Romainville, France, roman.galetto@cellectis.com

Andrea V. Gamarnik, PhD Molecular Virology Laboratory, Fundacion Instituto Leloir, Av Patricias Agentinas 435, Buenos Aires 1405, Argentina, agamarnik@leloir.org.ar

Reuben S. Harris, PhD Department of Biochemistry, Molecular Biology and Biophysics, University of Minnesota, 321 Church Street South East, 6-155 Jackson Hall, Minneapolis, MN 55455, USA, rsh@umn.edu

Fred L. Homa, PhD Department of Molecular Genetics and Biochemistry, University of Pittsburgh, W1256 BSTWR, 200 Lothrop Street, Pittsburgh, PA 15261, USA, flhoma@pitt.edu

Nestor G. Iglesias, PhD Molecular Virology Laboratory, Fundacion Instituto Leloir, Av Patricias Agentinas 435, Buenos Aires 1405, Argentina, giglesias@leloir.org.ar

C. Cheng Kao, PhD Department of Biology, Indiana University, Bloomington, IN 47405, USA, ckao@indiana.edu

Rebecca S. LaRue, PhD Department of Biochemistry, Molecular Biology and Biophysics, 321 Church Street South East, 6-155 Jackson Hall, Minneapolis, MN 55455, USA, larue005@umn.edu

Stuart F.J. Le Grice, PhD HIV Drug Resistance Program, National Cancer Institute – Frederick, Building 535, Room 312, P.O. Box B, Frederick, MD 21702-1201, USA, slegrice@ncifcrf.gov

Jonathon Leis, PhD Department of Microbiology and Immunology, Northwestern university Feinberg School of Medicine, 303 E. Chicago Ave., Chicago, IL 60611, USA, j-leis@northwestern.edu

Brett D. Lindenbach, PhD Section of Microbial Pathogenesis, Yale University School of Medicine, 295 Congress Ave., BCMM 354C, New Haven, CT 06536-0812, USA, brett.lindenbach@yale.edu

Shenping Liu, PhD Exploratory Medicinal Sciences, Pfizer Inc., Eastern Point Rd., Groton, CT 06340, USA, shenping.liu@pfizer.com

David Loakes, PhD Medical Research Council, Laboratory of Molecular Biology, Hills Road, Cambridge, CB2 2QH, United Kingdom, David.Loakes@MRC-LMB.CAM.AC.UK

Bruno Marchand, PhD Department of Molecular Microbiology and Immunology, University of Missouri School of Medicine, 471d Life Sciences Center, 1201 E. Rollins Drive, Columbia, MO 65211-7310, USA, marchandb@missouri.edu

Sarah M. McDonald, PhD Department of Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892, USA, mcdonaldsa@niaid.nih.gov

Jennifer T. Miller, PhD HIV Drug Resistance Program, National Cancer Institute, Frederick, MD 21702, USA, jtmiller@ncifcrf.gov

Peter D. Nagy, PhD Department of Plant Pathology, University of Kentucky, 201F Plant Science Building, Lexington, KY 40546, USA, pdnagy2@uky.edu

Matteo Negroni, PhD Unité de Régulation Enzymatique des Activités Cellulaires, CNRS-URA 2185, Institut Pasteur, Paris, France; Present address: Architecture et reactivité de l'ARN, Université de Strasbourg, CNRS, IBMC, 15 rue René Descartes, 67084, Strasbourg, France, m.negroni@ibmc.u-strasbg.fr

Scott W. Nelson, PhD Department of Biochemistry, Biophysics, and Molecular Biology 1210 Molecular Biology Building Iowa State University Ames, IA 50011, USA, swn@iastate.edu

Smita S. Patel, PhD Department of Biochemistry, Robert Wood Johnson Medical School, 675 Hoes Lane, Piscataway, NJ 08854, USA, patelss@umdnj.edu

John T. Patton, PhD Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892, USA, jpatton@niaid.nih.gov

Aniko V. Paul, PhD Department of Molecular Genetics and Microbiology, SUNY at Stony Brook, Nicolls Road, Stony Brook, NY 11794-5222, USA, apaul@notes.cc.sunysb.edu

Judit Pogany, PhD Plant Pathology, University of Kentucky, 201F Plant Science Building, Lexington, KY 40546, USA, jpoga2@uky.edu

Vaishnavi Rajagopal, PhD Department of Biochemistry, Robert Wood Johnson Medical School, 675 Hoes Lane, Piscataway, NJ 08854, USA, rajagova@umdnj.edu

Stefan G. Sarafianos, PhD Department of Molecular Microbiology and Immunology, University of Missouri School of Medicine, 471d Life Sciences Center, 1201 E. Rollins Drive, Columbia, MO 65211-7310, USA, sarafianoss@missouri.edu

Stanley G. Sawicki, PhD Department of Medical Microbiology and Immunology, University of Toledo, College of Medicine, 3055 Arlington Avenue, Toledo, OH 43614, USA, stanley.sawicki@utoledo.edu

April J. Schumacher, PhD Department of Biochemistry, Molecular Biology and Biophysics, 321 Church Street South East, 6-155 Jackson Hall, Minneapolis, MI 55455, USA, schu1480@umn.edu

I-hung Shih, PhD Gilead Sciences, 333 Lakeside Drive, Foster City, CA 94404, USA, Ihung.Shih@gilead.com

Robert Snoeck, PhD Laboratory of Virology, Rega Institute for Medical Research, Katholieke Universiteit Leuven, B-3000 Leuven, Belgium, Robert.Snoeck@rega.kuleuven.be

Michelle M. Spiering, PhD Department of Chemistry, 104 Chemistry Building, The Pennsylvania State University, University Park, PA 16802, USA, mms36@psu.edu

Mark D. Stenglein, PhD Department of Biochemistry, Molecular Biology and Biophysics, 321 Church Street South East, 6-155 Jackson Hall, Minneapolis, MN 55455, USA, sten0171@umn.edu

John E. Tavis, PhD Molecular Microbiology and Immunology, St Louis University School of Medicine, 1100 South Grand Blvd., St Louis, MO 63104, USA, tavisje@slu.edu

Timothy L. Tellinghuisen, PhD Department of Infectology, The Scripps Research Institute, Scripps Florida, 5353 Parkside Drive, RF-2, Jupiter, FL 33458, USA, tellint@scripps.edu

Laurence Tiley, PhD Department of Veterinary Medicine, University of Cambridge, Madingley Road, Cambridge, CB3 0ES, United Kingdom, lst21@cam.ac.uk

Kathleen Too, PhD Medical Research Council, Laboratory of Molecular Biology, Hills Road, Cambridge, CB2 2QH, United Kingdom, kathleentoo@gmail.com

Juan C. de la Torre, PhD Department of Immunology and Microbial Science, The Scripps Research Institute IMM-6, 10550 N Torrey Pines Road, La Jolla, CA 92037, USA, juanct@scripps.edu

Paula Traktman, PhD Department of Microbiology and Molecular Genetics, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, WI 53226, USA, ptrakt@mcw.edu

Anne-Mieke Vandamme, PhD Katholieke Universiteit Leuven, Laboratory for Clinical and Epidemiological Virology, AIDS Reference Laboratory, Rega Institute

and University Hospitals, Minderbroedersstraat 10, B-3000 Leuven, Belgium, annemie.vandamme@uz.kuleuven.ac.be

Kristel Van Laethem, PhD Katholieke Universiteit Leuven, Rega Institute for Medical Research, Clinical and Epidemiological Virology, Minderbroedersstraat 10, 3000 Leuven, Belgium, kristel.vanlaethem@uz.kuleuven.ac.be

Nuria Verdaguer, PhD Institut de Biologia molecular de Barcelona (CSIC), Parc Cientific de Barcelona, Baldiri i Reixac 15, E-08028 Barcelona, Spain, nvmcri@ibmb.csic.es

Sandra K. Weller, PhD Department of Molecular, Microbial and Structural Biology, University of Connecticut Health Center, 263 Farmington Avenue, Farmington, CT 06030-3205, USA, weller@nso2.uchc.edu

Michaela Wendeler, PhD HIV Drug Resistance Program, National Cancer Institute, Frederick, MD 21702, USA, mwendeler@ncifcrf.gov

Sean P.J. Whelan, PhD Department of Microbiology and Molecular Genetics, Harvard Medical School, 200 Longwood Avenue, Boston, MA 02115, USA, swhelan@hms.harvard.edu

Eckard Wimmer, PhD Molecular Genetics & Microbiology, SUNY at Stony Brook, Nicolls Road, Stony Brook, NY 11794-5222, USA, ewimmer@ms.cc.sunysb.edu

Guanghui Yi, PhD Department of Biology, Indiana University, Bloomington, IN 47405, USA, guanghuiyi@tamu.edu

Weidong Zhong, PhD Gilead Sciences, Inc., 333 Lakeside Drive, Foster City, CA 94404, USA, weidong.zhong@gilead.com

Zhihao Zhuang, PhD Department of Chemistry & Biochemistry, 214A Drake Hall, University of Delaware, Newark, DE 19716, USA, zzhuang@udel.edu

Part I
Genome Replication Strategies

Chapter 1
Model of Picornavirus RNA Replication

Aniko V. Paul, George A. Belov, Ellie Ehrenfeld, and Eckard Wimmer

Introduction

The virus family *Picornaviridae* represents a large number of human and animal pathogens, which can cause a variety of diseases ranging from the benign (common cold) to the serious (poliomyelitis). These small non-enveloped plus-stranded RNA viruses have been grouped into nine genera of which five are well known: Enterovirus, Rhinovirus, Hepatovirus, Cardiovirus, and Aphthovirus. The life cycle of picornaviruses begins with attachment to a susceptible host cell, entry, and the delivery of the RNA genome into the cytoplasm (Semler and Wimmer 2002). The RNA is translated into a large polyprotein, which is processed into functional precursor and mature proteins. The nonstructural proteins of the virus and cellular proteins assemble with the parental RNA to form replication complexes on the surface of membranous vesicles where RNA replication takes place. The progeny RNA are encapsidated prior to being released from the host cell.

The RNA genome of picornaviruses (∼7500 nucleotides) contains a long 5′ nontranslated region (5′NTR), a single open reading frame, and a short 3′ NTR followed by a poly(A) tail (Fig. 1.1). At the 5′-end the RNA is covalently linked to a tyrosine residue in a small peptide called VPg. Picornaviruses use the same basic steps to replicate their genomes as other plus-strand RNA viruses. First the parental RNA is copied into a complementary minus strand yielding a double-stranded replicative intermediate. The minus strand then serves as the template for the production of progeny plus strands. There is also an important difference, however, between the RNA replication strategy of picornaviruses and of other plus-strand RNA viruses. While most other plus-strand RNA viruses start the synthesis of their RNA strands by de novo initiation, picornaviruses use a uridylylated form of the VPg peptide as primer for the production of both plus- and minus-strand RNAs. The enzyme primarily responsible for RNA synthesis is the RNA-dependent RNA polymerase,

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A.V. Paul (✉)
Department of Molecular Genetics and Microbiology, SUNY at Stony Brook,
Nicolls Road, Stony Brook, NY 11794-5222, USA
e-mail: apaul@notes.cc.sunysb.edu

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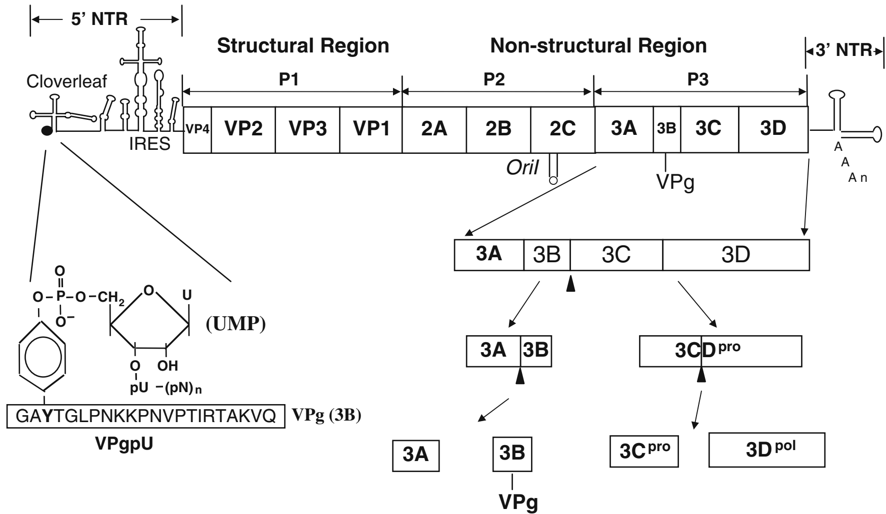


Fig. 1.1 Genomic structure of PV and processing of the P3 domain of the polyprotein. The single-stranded RNA genome of PV is shown with the terminal protein VPg at the 5′-end of the 5′NTR and the 3′NTR with the poly(A) tail. The 5′NTR contains a cloverleaf-like structure and a large IRES element. The attachment site of the 5′-terminal UMP of the RNA to the tyrosine of VPg is shown enlarged. The *oriI* element is located in the coding region of $2C^{ATPase}$. The polyprotein contains structural (P1) and nonstructural (P2 and P3) domains. The *vertical lines* within the polyprotein box represent proteinase cleavage sites. Processing of the P3 domain is shown enlarged.

which requires not only viral but also cellular proteins and *cis*-acting RNA elements to achieve complete replication of the viral RNA genomes.

In this review an attempt will be made to summarize what is known predominantly about the genome replication of poliovirus, the prototype of *Picornaviridae*. Because of the limited scope of this article we will neither be able to discuss in detail the current literature available on all picornavirus RNA replication nor to acknowledge the contribution of every investigator. Principally, progress in five areas have greatly advanced our understanding of poliovirus genome replication during the last 15 years: (i) the development of a de novo cell-free poliovirus replication system, (ii) the elucidation of the mechanism of VPg uridylylation, (iii) the discovery of *cis*-acting genomic RNA structures, (iv) the identification of cellular proteins essential for RNA synthesis, and (v) the characterization of cellular membranous structures involved in genome replication. We suggest that the reader consults previous review articles listed for some early references that could not be accommodated in this article. Finally, we should emphasize that the proposed models of RNA replication are highly speculative and are expected to change as more information accumulates.

Viral and Cellular Factors Involved in Replication

Viral Proteins

The single open reading frame of picornavirus RNAs is translated into a large polyprotein, which is processed by viral proteinases into a variety of precursor and mature proteins (Fig. 1.1). The polyprotein consists of three domains. The P1 domain contains the structural proteins that make up the capsid of the virus while the nonstructural proteins (P2 and P3) are involved in RNA replication and in promoting changes in cellular metabolism. It has been known for a long time that all of the nonstructural proteins of poliovirus have functions in RNA replication. Since picornavirus genomes have a limited coding capacity the virus has adapted to use the genetic information encoded in the RNA multiple times in the form of different precursor and mature proteins. For example, evidence has been presented suggesting that minus-strand RNA synthesis requires large precursors of P2 proteins (P2/P3 or 2BC/P3) (Jurgens and Flanegan 2003).

1. Proteins of the P2 domain. The P2 domain of the polyprotein is processed into a precursor (2BC) and mature proteins ($2A^{pro}$, 2B, and $2C^{ATPase}$) (Leong et al. 2002; Paul 2002; Skern et al. 2002). Protein $2A^{pro}$ is a proteinase in entero- and rhinoviruses whose primary function is to separate the structural and nonstructural domains of the polyprotein but it also has functions in the inhibition of cellular translation and transcription and in RNA replication. The roles of proteins 2B and of its precursor 2BC in RNA replication are not well understood but it is known that they are related to the biochemical and structural changes that occur in the infected cell (Egger et al. 2002; Paul 2002; see below). Expression of 2B in mammalian cells leads to a block of secretory transport, disassembly of the Golgi complex, permeabilization of the plasma membrane, and induction of membrane proliferation and rearrangements. Expression of 2BC results in membrane rearrangements leading to the formation of vesicles. The most conserved protein among picornaviruses is a membrane-bound polypeptide $2C^{ATPase}$ (Leong et al. 2002; Paul 2002). Biochemical and genetic studies have implicated this protein in a variety of functions during the viral life cycle such as uncoating, host cell membrane rearrangements, RNA replication, and encapsidation. The protein contains N- and C-terminal amphipathic helices and RNA-binding domains. There is an N-terminal membrane-binding domain and a cysteine-rich Zn^{++}-binding domain near the C-terminus. In vitro purified $2C^{ATPase}$ exhibits ATPase activity, which is blocked by guanidine hydrochloride, a potent inhibitor of RNA replication in vivo (Pfister et al. 2000 and refs. therein). Although the protein contains conserved motifs typical of helicases so far no helicase activity of the protein has been detected.

2. Proteins of the P3 domain. The proteins derived from the P3 domain are directly involved in RNA replication (Cameron et al. 2002; Leong et al. 2002; Paul 2002). Initial cleavage of the P3 domain yields two relatively stable and very important precursors, 3AB and $3CD^{pro}$. In vitro biochemical studies have shown

that the small 3AB protein has multiple functions in RNA replication: (a) 3AB stimulates the polymerization activity of RNA polymerase $3D^{pol}$; (b) 3AB is a non-specific RNA-binding protein, which, however, forms a specific complex with proteinase $3CD^{pro}$ at either the 5′-cloverleaf structure or at the 3′NTR of the viral RNA; (c) 3AB stimulates the autoprocessing of $3CD^{pro}$; (d) the membrane-bound form of 3AB is required for processing by $3CD^{pro}$; (e) 3AB has nucleic acid chaperone and helix destabilizing activities (DeStefano and Titilope 2006). Yeast two-hybrid and biochemical analyses have indicated that 3AB strongly interacts with $3D^{pol}$ and the sequences primarily responsible for this interaction reside in the 3B domain (Y3, K9K10, R17) of the protein (Paul 2002; Paul et al. 2003a). Three amino acids (F377, R379, V391) on the surface of $3D^{pol}$ in a hydrophobic patch were recently identified as binding partners of 3AB (Lyle et al. 2002). Protein 3AB has the propensity to dimerize and form oligomers in solution with both the N-terminal and hydrophobic domain of 3A involved in these interactions (Paul 2002; Strauss et al. 2003). Our recent studies with synthetic membranes suggest that the hydrophobic anchor sequence of 3A forms a mixture of transmembrane and non-transmembrane topographies but adopts only a non-transmembrane configuration in the context of the 3AB protein (Fujita et al. 2007).

Proteolytic processing of 3AB by $3CD^{pro}$ yields 3A and VPg (Leong et al. 2002; Paul 2002). The 3A protein is 87 amino acids long and consists of a soluble cytosolic domain (58 residues), which forms a symmetric dimer (Strauss et al. 2003), a 22-residue long hydrophobic and membrane-binding domain followed by seven additional residues at the C-terminus. The 3A protein inhibits ER to Golgi membrane and secretory protein traffic and induces specific translocation of some ADP Ribosylation Factors (ARF) proteins to membranes (Belov et al. 2005). Studies by the yeast and mammalian two-hybrid systems showed that 3A multimerizes and interacts with $2C^{ATPase}$ and 2B (Teterina et al. 2006; Yin et al. 2007). Mutants resistant to Enviroxime, an antiviral drug that blocks PV RNA replication, map to the 3A sequences supporting a critical role for 3A (or 3AB) in RNA replication.

The VPgs of all picornaviruses are small peptides 21–24 amino acids in length with an absolutely conserved Tyr at position 3. Tyr3 links VPg via a phosphodiester bond to the 5′-terminal UMP of the genome (Fig. 1.1; Wimmer et al. 1993; Paul 2002; Paul et al. 2003a). Entero- and rhinovirus VPgs contain several fully or highly conserved amino acids (Y3, G5, P7, K9, K10, P14, R17), which are required for function in vivo. Interestingly, when two VPgs are introduced in tandem into the PV genome the resulting virus, which has a quasi-infectious growth phenotype, retains only the N-terminal VPg. The replacement of PV VPg with that of HRV14 or HRV16, but not with that of HRV2, results in viable poliovirus (Cheney et al. 2003; Paul et al. 2003a). In contrast to other picornaviruses, foot-and-mouth disease virus (FMDV) encodes in tandem, and uses at random, three distinct VPg peptides (3B1–3B3), which are 23 or 24 amino acids long (Nayak et al. 2005). Each of the VPgs can be uridylylated in vitro although 3B3 is the best substrate for FMDV $3D^{pol}$. Recently two different kinds of structures were proposed for PV VPg. The first structure was predicted by computational modeling and was found to have two

antiparallel B strands with the N- and C-termini of the peptide located in close proximity (Tellez et al. 2006) The second structure, determined by NMR, consisted of a large loop (residues 1–14) from which the reactive tyrosine (Y3) projects outward, and of an α-helix (residues 18–21) at the C-terminus (Schein et al. 2006). The amino acids conserved in the VPgs of picornaviruses were located on the same face of the structure as Y3.

The second important precursor of the P3 domain is $3CD^{pro}$, which together with $3C^{pro}$ processes most of the entero- and rhinovirus polyprotein into precursor and mature proteins (Leong et al. 2002; Paul 2002). $3CD^{pro}$ possesses no polymerase activity but it has essential functions in RNA replication as a RNA-binding protein. The RNA-binding domain of the protein is located in $3C^{pro}$ but the $3D^{pol}$ domain of the protein modulates this activity. The crystal structure of PV $3CD^{pro}$ revealed a poorly ordered polypeptide linker between the structurally conserved $3C^{pro}$ and $3D^{pol}$ domains (Marcotte et al. 2007). $3CD^{pro}$ forms several important RNA/protein complexes that are required in RNA replication and these will be discussed later. Studies with the in vitro translation/RNA replication system of Molla et al. (1991) indicated a role for PV $3CD^{pro}$ also in virus maturation, which required both the RNA-binding activity of the $3C^{pro}$ domain and the integrity of interface I in the $3D^{pol}$ domain (Franco et al. 2005).

Processing of the $3CD^{pro}$ precursor yields proteinase $3C^{pro}$ and RNA polymerase $3D^{pol}$. Crystal structures of several picornavirus $3C^{pro}$ proteins (HAV, PV1, HRV14, HRV2) were published and shown to contain a protein fold similar to serine proteinases such as chymotrypsin (Skern et al. 2002). The structure of the PV $3C^{pro}$ protein indicated the formation of dimers and this was confirmed by biochemical experiments (Pathak et al. 2007).

The RNA polymerase $3D^{pol}$ of picornaviruses possesses two major types of synthetic activities in vitro (Cameron et al. 2002; Paul 2002). It elongates RNA or DNA primers on homopolymeric or heteropolymeric RNA templates or catalyzes the covalent attachment of UMP to the hydroxyl group of tyrosine in VPg (Paul et al. 1998). The second reaction requires an RNA template, which can be either poly(A) or an adenylate residue in the *cis*-replicating RNA element *oriI*. The products of the reactions are VPgpU and VPgpUpU, the primers for the synthesis of plus and minus-RNA strands. Crystal structures have been determined for a number of picornavirus RNA polymerases (PV, HRV14, HRV16, HRV1B, and FMDV) and these are discussed by N. Verdaguer and colleagues in another chapter of this book. These structures display a common architecture characteristic of all RNA polymerases, which is that of a right hand with finger, thumb, and palm domains. The purified PV RNA polymerase has been found to exhibit a high level of cooperativity with respect to RNA binding and template usage, suggesting that polymerase/polymerase interactions are important for function. The dimerization/oligomerization of PV $3D^{pol}$ was confirmed by both the yeast and mammalian two-hybrid analysis (Teterina et al. 2006 and refs. therein) and such interactions were also observed in the crystal structure of the protein.

Cellular Proteins

Since plus-strand RNA viruses possess small RNA genomes that encode only a limited number of proteins they seek to supplement their existing synthetic capabilities with cellular proteins (Paul 2002). Several lines of evidence, involving both genetic and biochemical approaches, suggest that this is the case. First, it is known that the replication of RNA viruses is cell-type specific suggesting their dependence on cell-specific factors. Second, a number of host proteins have been identified that interact with viral genomic RNAs or replication proteins and some of these are essential to viral RNA replication.

1. PCBP. Poly(rC)-binding protein 2 (PCBP2), also known as hnRNP E2, or αCP-2, has functions both in the translation and in the replication of PV RNA and possibly also in RNA stability (Paul 2002; Walter et al. 2002). PCBP2 is an RNA-binding protein with a strong preference for poly(rC) sequences. It contains three hnRNP K-homology domains, the first and third of which mediate poly(rC) binding. The protein has been shown to form homodimers and to interact with other hnRNP proteins. For picornavirus RNA containing type I IRES elements, PCBP2 binds to domain IV of the IRES that is essential for translation initiation. In addition, PCBP2 binds to stem-loop B of the 5′-cloverleaf and an adjacent C-rich region in the spacer between the cloverleaf and the IRES (Toyoda et al. 2007). Together with $3CD^{pro}$, this interaction is required for viral RNA synthesis.

2. Sam68. Previous studies using yeast two-hybrid analyses have identified cellular protein Sam68 that interacts with PV $3D^{pol}$ and is relocalized from the nucleus to the cytoplasm upon PV infection (Paul 2002). No function has as yet been assigned to Sam68 in poliovirus replication.

3. Nucleolin. This nuclear protein was found to interact with the 3′NTR of wt PV RNA but not with the RNA of replication-defective mutants (Paul 2002). As with Sam68, no function has as yet been assigned to nucleolin in poliovirus replication.

4. Poly(A)-binding protein (PABP). Herold and Andino (2001) have observed that human PABP interacts in vitro with PV $3CD^{pro}$, PCBP2, and the 3′NTR-poly(A). These observations led to the proposal that the PV genome circularizes via an interaction of PABP, $3CD^{pro}$, and the 5′ cloverleaf on one hand and of PABP and the 3′NTR-poly(A) of the genome on the other.

5. Heterogeneous nuclear ribonucleoprotein C (hnRNP C). This cellular protein that is abundant in the nucleus belongs to a family of RNP motif RNA-binding proteins (Brunner et al. 2005). Using GST-pull down assays it was demonstrated that hnRNPC1 binds to PV $3CD^{pro}$, as well as to the P2 and P3 precursors of the nonstructural proteins. In addition, hnRNPC can be co-immunoprecipitated with PV plus and minus-strand RNA in HeLa extracts suggesting a possible role for hnRNP C in plus-strand RNA synthesis.

6. Reticulon 3. Using yeast two-hybrid analyses, a cellular ER-associated protein, reticulon 3, was recently identified as an interacting partner of enterovirus 71 $2C^{ATPase}$ (Tang et al. 2006). The N-terminal domain of $2C^{ATPase}$, which has both RNA- and membrane-binding activity, was found to interact with reticulon 3. Reduced production of reticulon 3 by RNA interference reduced the synthesis of

viral proteins, replicative double-stranded RNA, and plaque formation. Reticulon 3 could also interact with the $2C^{ATPase}$ proteins of PV and CAV16, suggesting that it may be a common factor for the replication of enteroviruses. The function of reticulon 3 was proposed to be to anchor the $2C^{ATPase}$ protein to the membranes but its role needs to be further studied.

7. Other host proteins. The replication of PV in the in vitro translation/replication system and in Xenopus oocytes was found to be dependent on one or more unknown cellular factors. There are numerous other host cell proteins that have been identified through their ability to interact with *cis*-acting RNA elements in the picornavirus genomes (Paul 2002). However, it is not clear that these RNA/protein interactions are biologically important for picornavirus RNA replication.

Cis-*Acting RNA Elements*

The genomes of plus-strand RNA viruses harbor a large amount of genetic information of which much resides in highly structured RNA elements. Most studies in the past concentrated on the role of the 5′NTR and 3′NTR in RNA replication and only recently has the importance of internal *cis*-replicating elements been recognized (Paul 2002).

1. The 5′ cloverleaf (oriL). The 5′-terminal sequences of entero- and rhinovirus RNAs contain a cloverleaf structure (stem-loops A-D) in which the terminal UMP is covalently linked to the hydroxyl group of a tyrosine in the genome-linked protein VPg (Figs. 1.1 and 1.2A). The cloverleaf forms two essential RNP complexes with $3CD^{pro}$ in the presence of either PCBP2 or protein 3AB (Paul 2002). Stem-loop B binds either PCBP or 3AB while a tetra loop in stem-loop D interacts with $3CD^{pro}$ (Rieder et al. 2003). Mutations that disrupt complex formation abolish RNA replication but do not affect translation. Interestingly, not only the C residues in stem-loop B of the cloverleaf are required for PCBP binding and RNA replication but also an adjacent C-rich sequence in the spacer between the cloverleaf and the IRES (Toyoda et al. 2007). Thus, this short segment of spacer sequence is an essential part of the 5′-terminal *cis*-acting element (*oriL*) of the poliovirus genome. The solution structure of a consensus entero- and rhinovirus cloverleaf stem-loop D was determined by NMR and was shown to have an elongated helical stem capped by a UACG tetra loop with a wobble UG closing base pair (Du et al. 2004).

2. The 3′NTR-poly(A) (oriR). The heteropolymeric regions of the 3′NTR in different picornaviruses are very diverse and their functions are unknown although genetic evidence supports their role in RNA replication (Fig. 1.2C; Agol et al. 1999; Paul 2002). A "kissing interaction" between stem-loops X and Y of the PV 3′NTR was found to be important for RNA replication.

The poly(A) tail of picornaviruses is genetically encoded (Wimmer et al. 1993) unlike the poly(A) tails of cellular mRNAs, which are added post-transcriptionally. Efficient RNA replication and infectivity of the viral RNA requires the presence of a poly(A) tail with at least 20 nt (Silvestri et al. 2006). A detailed analysis of the poly(A) tail of CVB3 revealed that while the poly(A) tail is about 80 nt long

A. Stem-loop C, Stem-loop B, Stem-loop D, Stem-loop A

PV1(M) +cloverleaf with C-rich region of spacer

B.

PV1(M) *oriI* [cre(2C)}

C. Stem-loop Y, Stem-loop X

PV1(M) 3’NTR-poly(A)

Fig. 1.2 The nucleotide sequence and structure of the PV1 *cis*-replicating elements. (**A**) The 5′-terminal cloverleaf followed by a C-rich region in the spacer between the cloverleaf and the IRES. (**B**) The PV *oriI* [cre(2C)] element and (**C**) the 3′NTR with the poly(A) tail. The conserved entero- rhinoviral cre(2c) sequences are indicated in *bold*.

the complementary poly(U) tract contains only about 20 nts (van Oij et al. 2006a). The 3′NTR controls the length of the poly(A) tail and ensures efficient minus-strand RNA synthesis but apparently it has no effect on poly(U) length.

3. The internal origin of replication (oriI or cre). Analyses of picornaviruses genomes revealed an important *cis*-acting RNA element mapping either to the coding sequences or to the 5′NTR (Fig. 1.2B; Paul 2002). First discovered in the coding sequence of capsid protein VP1 of human rhinovirus 14 (HRV14) (McKnight and Lemon 1998), *oriI* elements have subsequently been identified in $2C^{ATPase}$ of poliovirus and coxsackie virus B3, in $2A^{pro}$ of HRV2, and in the capsid protein VP2 of cardioviruses (for refs. see van Oij et al. 2006b). An exception is the *oriI* of FMDV, which was found to be located in the 5′NTR (Mason et al. 2002). These *oriIs* all consist of a small RNA stem-loop structure made of quite diverse nucleotide sequences. Entero- and rhinovirus *oriIs*, however, contain a conserved motif (Fig. 1.2B; $G_1XXXA_5A_6A_7XXXXXXA_{14}$), which is critically important for function (Yang et al. 2002; Yin et al. 2003). Within this motif, the A_5 residue templates the linkage of both UMPs to VPg by a "slide back" mechanism in a reaction catalyzed by $3D^{pol}$ and stimulated by $3CD^{pro}$ (Fig. 1.3; Paul 2002; Paul et al. 2003b). The products are VPgpU and VPgpUpU, the primers for RNA synthesis. The solution structure of a 33-nt segment the HRV14 *oriI* was recently determined by NMR spectroscopy (Thiviyanathan et al. 2004). It contains a large open loop with 14 nucleotides that derives stability from base-stacking interaction. The two conserved adenylates are oriented to the inside of the loop. Interestingly, the poliovirus *oriI* structure can be moved to different positions within the genome without affecting function (Yin et al. 2003). Recent studies by Crowder and Kirkegaard (2005) have shown that mutants of the PV *oriI* can inhibit PV replication in a *trans*-dominant manner in vivo.

4. The Internal Ribosomal Entry Site (IRES). The poliovirus IRES is located in the 5′NTR between nucleotides 124 and about 630 whose primary function is to promote cap-independent translation (Wimmer et al. 1993; Paul 2002). Numerous

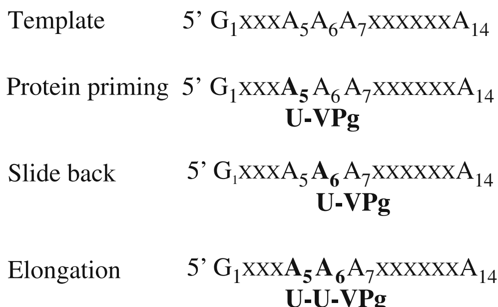


Fig. 1.3 The "slide back" mechanism of VPg uridylylation. The first UMP is linked to VPg on the A_5 template nucleotide of the PV1 *oriI*. VPgpU slides back to hybridize with A_6 and the second UMP is templated again by A_5 yielding VPgpUpU. Nucleotides A_5 and A_6 involved in the reaction are shown in *bold*.

genetic studies suggest that the IRES also contains signals for RNA replication in stem-loops II, IV, and V. However, other results are difficult to reconcile with a direct role of the IRES in RNA replication. For example, the IRES of PV1 can be replaced with totally different IRESes from EMCV or HCV but the resulting chimeras have growth properties similar to that of wt poliovirus (Gromeier et al. 1996 and refs. therein). Furthermore, using the in vitro translation/RNA replication system Murray et al. (2004) showed that poliovirus RNA replication was not absolutely dependent on the IRES although the replication of genome length viral RNAs was stimulated by the presence of the IRES in the template RNAs.

5. The cloverleaf at the 3′-end of minus strands. Using 3′-terminal fragments of PV minus-strand RNA, the binding of both cellular and viral ($2C^{ATPase}$, 2BC) proteins derived from virus-infected cell extracts has been demonstrated (Paul 2002). The biological significance of some of these RNA/protein interactions is not yet known. Sharma et al. (2005) recently demonstrated with in vitro translation/RNA replication reactions that the 5′-terminal sequence of stem A in the plus strand, and consequently the 3′-terminal sequence of the minus strand, was required for the efficient plus-strand RNA synthesis.

Membrane Structures

1. Morphological organization of replication complexes. The complexity of the numerous factors that participate in viral RNA synthesis requires that some mechanism exist to topologically coordinate and concentrate the multiple components to function in concert. All positive-strand RNA viruses, including picornaviruses, induce the reorganization of membranes from various sub-cellular organelles (endoplasmic reticulum (ER), Golgi, endosomes, etc.) to form functional scaffolds on which genome replication occurs. In most cases new virus-induced structures are formed that appear by electron microscopy as clusters of heterogeneous sized vesicles concentrated near the nucleus and eventually occupying nearly all the cytoplasm (Fig. 1.4).

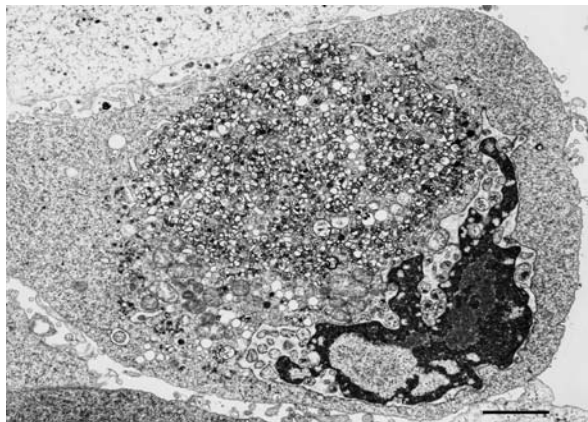


Fig. 1.4 Electron microscopic picture of PV-infected Hep-2 cells. Numerous vesicles can be seen 9 hours post-infection. The bar represents 2 μm. The picture is a gift of K. Bienz and D. Egger. It should be noted that Jackson et al. 2005 have observed some double membrane vesicles in PV-infected cells, which are not apparent on the picture shown here.

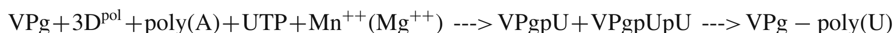
The most detailed account of the development of this membrane remodeling has been obtained for poliovirus (Egger et al. 2002; Egger and Bienz 2005). Characteristic vesicles were detected by electron microscopy at 2 h p. i., initially associated with the ER and then clustered in the perinuclear region. Replicating RNAs were located in electron-dense patches in close vicinity to budding vesicles on modified ER and later associated with vesicles. When lysates from infected cells were analyzed by density gradient centrifugation, polymerase activity co-purified with smooth membranes. These replication complexes looked like loosely associated rosettes of membranous vesicles surrounding more dense structures, where actual replication sites were located. When provided with nucleotides and optimal reaction conditions, they could support RNA replication in vitro. It is not yet known how the replication complexes are attached to the membranes but the hydrophobic domains of 3AB, 3A and 2BC, 2B and $2C^{ATPase}$, the latter possibly in conjunction with reticulon 3, are likely to mediate membrane binding.

2. Viral proteins involved in membrane remodeling. Expression of all poliovirus nonstructural proteins from non-replicating RNA constructs resulted in membrane rearrangements typical of those found in infected cells (Egger et al. 2002; Egger and Bienz 2005), indicating that viral proteins alone are sufficient to induce characteristic vesicles. Among individual virus proteins that might perform this function, attention was drawn to proteins with intrinsic membrane-targeting properties. Domains in proteins 2B, $2C^{ATPase}$, and 3A and their precursors confer the ability to bind to membranes. Expression of these individual proteins in cells caused intracellular membrane modifications, and when 2BC was co-expressed with 3A, the ultra structure and biochemical properties of the induced vesicles appeared very similar to vesicles found during normal infection. Nevertheless, when cells expressing individual proteins were infected with poliovirus, the pre-formed vesicles were not used in virus replication. This result could mean either that replication vesicles must be formed in *cis*, close to the place of RNA translation, or that vesicles induced by expression of a single viral protein are not the same as those formed when all poliovirus proteins are present. It has been suggested that expression of poliovirus proteins may modify early steps of the secretory pathway (Belov and Ehrenfeld 2007; Egger and Bienz 2005) and/or autophagy (Jackson et al. 2005) but the precise cellular pathways that are utilized in virus-induced membrane remodeling have not yet been elucidated and are currently under investigation in several laboratories.

VPg Uridylylation and RNA Synthesis In Vitro

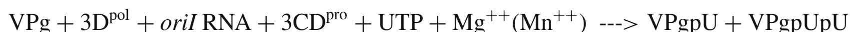
With Purified Proteins

Purified poliovirus RNA polymerase catalyzes the uridylylation of VPg on a poly(A) template yielding VPgpU and VPgpUpU. These precursors are elongated into VPg-linked poly(U), the 5′-end of minus strands (Paul et al. 1998).



$$VPg + 3D^{pol} + poly(A) + UTP + Mn^{++}(Mg^{++}) \text{ ---> } VPgpU + VPgpUpU \text{ ---> } VPg - poly(U)$$

The enzyme can also use an *oriI* containing PV RNA as template for VPg uridylylation but this reaction requires the stimulatory activity of $3CD^{pro}$ or $3C^{pro}$ (Paul 2002; Pathak et al. 2002).



$$VPg + 3D^{pol} + \textit{oriI}\ RNA + 3CD^{pro} + UTP + Mg^{++}(Mn^{++}) \text{ ---> } VPgpU + VPgpUpU$$

The elongation of the uridylylated VPg precursors in vitro into minus-strand RNA on a PV plus-strand RNA template is very inefficient suggesting that other factors are also required for this process (Paul 2002). In contrast, when PV RNA or another poly(A)-tailed RNA template is incubated with purified $3D^{pol}$ and an oligo(U) primer full-length minus strands can be synthesized.

With Crude Replication Complexes

When crude replication complexes (CRCs) isolated from poliovirus-infected cells are supplied with UTP in vitro they synthesize VPgpU and VPgpUpU in a reaction that is sensitive to the presence of detergents (NP40) (Paul 2002). The uridylylated VPg precursors can be chased into both double- and single-stranded viral RNAs.

With In Vitro Translation/RNA Replication Complexes

As discussed above, dissection and reconstitution of individual steps (partial reactions) that are part of the overall RNA replication mechanism can be performed in vitro with purified components, or analyzed after isolation of replication complexes from infected cells. An additional method for studying viral RNA replication in vitro was developed by Molla et al. (1991) and modified by Barton et al. (2002).

Uridylylation of VPg to form VPgpU and VPgpUpU occurs in the extract in excess of their utilization as primer for RNA chain elongation. Both positive- and negative RNA strands synthesized in vitro are linked to VPg; however, there is some controversy regarding the requirement for *oriI* to serve as template for VPg uridylylation to prime synthesis of negative strands in vitro (see below).

Although uridylylation of VPg can be catalyzed by $3D^{pol}$ in a defined reaction devoid of any membranes (Paul 2002; Nayak et al. 2005), VPg uridylylation formed after translation of poliovirus RNA in HeLa cell extracts was completely eliminated by treatment with non-ionic detergents, suggesting that in vivo this reaction is tightly coupled to the replication complex associated with membranes (Egger et al. 2002; Fogg et al. 2003; Paul 2002). These data, in conjunction with the demonstration that addition of detergent prevented initiation of synthesis of new molecules by replication complexes isolated from infected cells, suggest that the initiation reaction is the membrane-requiring step of viral RNA synthesis. Indeed, addition of even

mild detergent abolishes synthesis of poliovirus in the HeLa cell-free extract (Molla et al. 1993). Although membranes are essential for picornavirus RNA replication, their organization into the morphological structures found in infected cells seems to be unnecessary for replication in vitro. Rosettes or vesicle structures typical of poliovirus replication complexes isolated from infected cells were not seen in cell extracts that actively synthesized viral RNA (Fogg et al. 2003).

Proposed Model of Picornavirus RNA Replication

Since virus-infected cells contain both VPgpUpU- and VPg-linked plus- and minus-strand RNAs (Paul 2002), there is little doubt that protein-priming is involved in the initiation of both RNA strands. This hypothesis is supported by the observation that the PV RNA polymerase is strictly primer dependent.

Model of Minus-Strand RNA Synthesis

Prior to minus-strand RNA synthesis translation must be terminated because the ribosomes and the RNA polymerase would have to proceed on the same template but in opposite directions (Paul 2002). It was proposed that the switch from translation to replication occurs when the concentration of $3CD^{pro}$ reaches a critical level. At that time $3CD^{pro}$ interacts with the cloverleaf and sequesters PCBP2 from the IRES thereby shutting off translation and promoting minus-strand RNA synthesis. One problem with this model is that for the most part protein synthesis and RNA replication co-exist in the infected cell (Agol et al. 1999).

Plus-strand RNA viruses initiate negative strand RNA synthesis at the 3′-end of the genome, which is the poly(A) tail in picornavirus RNAs (Agol et al. 1999; Paul 2002). However, the poly(A) tail cannot be the sole determinant of the initiation of negative strand RNA synthesis since the RNA polymerase must discriminate between cellular mRNAs and the viral RNA. For many years it was assumed that the 3′NTR was the only site of recognition in picornavirus RNAs by $3D^{pol}$. This hypothesis was difficult to accept after it was found that the PV 3′NTR can be replaced by the 3′NTR of HRV14 or even deleted and still yield viable virus (Brown et al. 2005). An alternate model was proposed by Herold and Andino (2001) in which the specificity of selection was provided by the viral cloverleaf, which interacted with PCBP2 and $3CD^{pro}$ on the one hand and PABP bound to the poly(A) on the other, thus linking the ends of the viral RNA and effectively circularizing it. This model was based on the observation that all of these *cis*-acting elements and proteins interact in vitro and are required for efficient minus-strand RNA synthesis. In addition, the involvement of a circularized genome in RNA replication is supported by the observation that the 5′ cloverleaf is required in *cis* for minus-strand RNA synthesis (Barton et al. 2001).

Currently two models are being considered to explain the mechanism of VPg-primed negative strand RNA synthesis. According to the first model VPg is

Fig. 1.5 Model of PV minus-strand RNA synthesis. (**A**) VPg is uridylylated on the poly(A) tail and VPgpU is elongated into minus-strand RNA. (**B**) VPg is uridylylated on the *oriI* and VPgpUpU is transferred to the 3′-end of poly(A) before elongation into minus strands. See the text for details of the model.

uridylylated on the poly(A) tail of PV RNA and the resulting VPgpU is immediately elongated into minus strands (Murray and Barton 2003; Morasco et al. 2003). This model is supported by several lines of evidence. First, purified $3D^{pol}$ catalyzes the uridylylation of VPg in vitro on a poly(A) template yielding VPgpUpU, which is elongated into VPg-linked poly(U) (Paul et al. 1998). Second, the length of the poly(A) tail on PV RNA is an important determinant of minus-strand RNA synthesis both in the in vivo and in the in vitro translation/RNA replication system (van Oij et al. 2006a). Third, mutations in the *oriI* of PV RNA that destroy its structure inhibit viral growth in vivo and VPg uridylylation in vitro translation/RNA replication reactions but have no effect on minus-strand RNA synthesis in the same system (Murray and Barton 2003; Morasco et al. 2003).

In the second model VPgpUpU is made on the PV *oriI* and is subsequently translocated to the 3′-end of the poly(A) tail where it is used as primer for minus-strand RNA synthesis. This model is supported by studies of minus-strand RNA synthesis in the in vitro translation/RNA replication system by point mutants of CVB3 *oriI*. van Oij et al. (2006b) have observed that point mutations in the *oriI* RNA, which do not affect its structure, inhibit both plus and minus-strand RNA synthesis. These investigators proposed that in the in vitro system poly(A) is only used as an alternate template to *oriI* for the uridylylation of VPg when the structure of the *oriI* is disrupted. Under these conditions no RNP complex can form, which would sequester the replication proteins.

Figure 1.5 illustrates both models of minus-strand RNA synthesis in which either the poly(A) tail (A) or the *oriI* (B) is the template for uridylylation of VPg. In each case the first step is the circularization of the genome followed by processing of $3CD^{pro}$ to yield $3C^{pro}$ and $3D^{pol}$. The RNA polymerase forms a complex with VPg, derived from membrane-bound 3AB, and uridylylates it on the poly(A) tail (A). VPgpUpU is elongated into VPg-linked poly(U) and minus-strand RNA (A). In model B the VPgpUpU made on the *oriI* is translocated to the poly(A) tail where it is elongated into VPg-linked poly(U) and minus-strand RNA. The final product according to both models is a double-stranded replicative form.

Model of Plus-Strand RNA Synthesis

It has been generally accepted that the double-stranded RF structure formed after minus-strand RNA synthesis is a true intermediate in replication (Paul 2002). Therefore, before plus-strand synthesis can begin the end of the RF has to be unwound. It has been proposed that $2C^{ATPase}$ is responsible for the unwinding of the ends of the duplex molecule because the protein has a conserved helicase motif as well as ATPase activity. However, no helicase activity has been found to be associated with this protein. It is more likely that the unwinding of the end of the RF and the formation of the plus- and minus-strand cloverleaves is facilitated by the binding of a complex of viral and cellular proteins. Since the double-stranded form of picornavirus RNA is infectious it has also been suggested that a cellular helicase is responsible for unwinding the end of the RF.

The in vitro reaction in which VPgpUpU is made on the PV *oriI* with purified protein 3D^{pol}, 3CD^{pro}, and synthetic VPg has been thoroughly characterized (Paul 2002). Subsequently, studies with the in vitro translation/RNA replication system have significantly enhanced our understanding of the relationship between VPg uridylylation and RNA replication. First, these studies have provided convincing evidence that the VPgpUpU precursors used for PV plus-strand synthesis are produced on the *oriI* [cre(2C)] RNA (Murray and Barton 2003; Morasco et al. 2003). Second, they showed that the synthesis of VPgpUpU requires membranes (Fogg et al. 2003). Murray and Barton (2003) have proposed that during minus-strand RNA synthesis the circularized genome is disassembled and 3CD^{pro} translocates to and enhances the formation of the *oriI* structure where VPg is then uridylylated by 3D^{pol}. The priming of plus-strand RNA synthesis by VPgpUpU is quite inefficient (Murray and Barton 2003). It is estimated about 500 molecules of VPgpUpU and about 20 plus strands are made for each minus-strand RNA. While the elegant studies using the in vitro translation/replication system have yielded important clues of poliovirus genome replication, their validity in vivo has not been confirmed in all cases.

Figure 1.6 illustrates the proposed model of plus-strand RNA synthesis. Before the synthesis of minus-strand RNA starts or reaches the 2C^{ATPase} coding sequences a dimer of 3CD^{pro} binds to the upper stem of the *oriI* and destabilizes it (Pathak et al. 2007; Yang et al. 2004; Yin et al. 2003). 3D^{pol} is then recruited to the *oriI* by

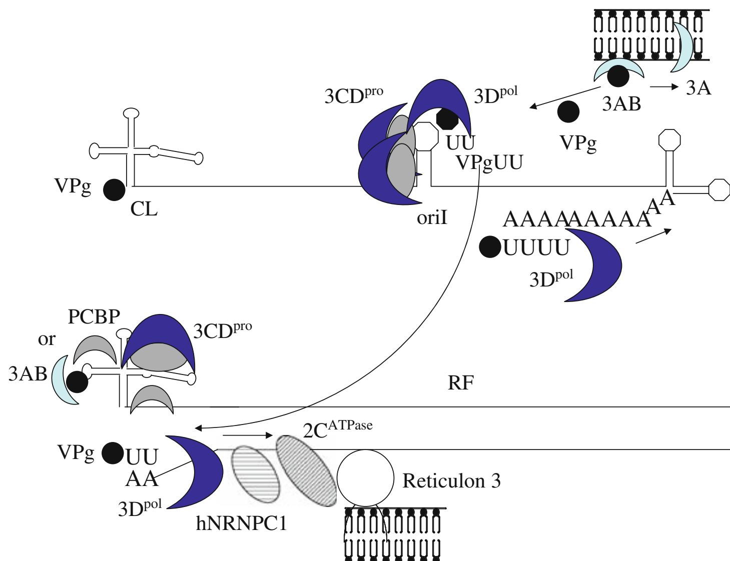


Fig. 1.6 Model of PV plus-strand RNA synthesis. The end of double-stranded RF is unwound by the binding of cellular and viral proteins. VPg is uridylylated on the *oriI* and VPgpUpU is transferred to the 3′-end of minus strands. VPgpUpU primes plus-strand RNA synthesis. See the text for details of the model.