Chapter 14
Host Factors Promoting Viral RNA Replication

Peter D. Nagy and Judit Pogany

Abstract Plus-stranded RNA viruses, the largest group among eukaryotic viruses, are capable of reprogramming host cells by subverting host proteins and membranes, by co-opting and modulating protein and ribonucleoprotein complexes, and by altering cellular pathways during infection. To achieve robust replication, plus-stranded RNA viruses interact with numerous cellular molecules via protein–protein, RNA–protein, and protein–lipid interactions using molecular mimicry and other means. These interactions lead to the transformation of the host cells into viral "factories" that can produce 10,000–1,000,000 progeny RNAs per infected cell. This chapter presents the progress that was made largely in the last 15 years in understanding virus–host interactions during RNA virus replication. The most commonly employed approaches to identify host factors that affect plus-stranded RNA virus replication are described. In addition, we discuss many of the identified host factors and their proposed roles in RNA virus replication. Altogether, host factors are key determinants of the host range of a given virus and affect virus pathology, host–virus interactions, as well as virus evolution. Studies on host factors also contribute insights into their normal cellular functions, thus promoting understanding of the basic biology of the host cell. The knowledge obtained in this fast-progressing area will likely stimulate the development of new antiviral methods as well as novel strategies that could make plus-stranded RNA viruses useful in bio- and nanotechnology.

Introduction

Plus-stranded (+)RNA viruses replicate their genomes by manipulating host cells and transforming them into viral "factories." Unraveling the interactions between viruses and their host cells as a function of time can contribute greatly to our

P.D. Nagy (✉)
Department of Plant Pathology, University of Kentucky, 201F Plant Science Bulding, Lexington, KY 40546, USA
e-mail: pdnagy2@uky.edu

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understanding of the dynamics of viral infections. (+)RNA viruses replicate their genomes in a two-step process: through the production of minus-strand replication intermediates, followed by the production of (+)RNA progeny via the use of the (–)RNA template. Interestingly, replication is an asymmetric process leading to a 20- to 100-fold excess of the new (+)RNA progeny. All known (+)RNA viruses assemble their own replicase complexes (RCs), likely containing both viral- and host-coded proteins (Ahlquist, 2002; Ahlquist et al., 2003; Buck, 1996; Nagy and Pogany, 2006; Noueiry and Ahlquist, 2003; Shi and Lai, 2005; Strauss and Strauss, 1999). In addition, replication takes place in membraneous compartments derived from intracellular organelles, such as the endoplasmatic reticulum (ER), mitochondrion, vacuole, Golgi, chloroplast, and peroxisome (Salonen et al., 2005). Some viruses actively induce the formation of novel cytoplasmic vesicular compartments, using COPII-coated or possibly autophagosomal membranes (Cherry et al., 2006; Egger and Bienz, 2005; Kirkegaard and Jackson, 2005; Rust et al., 2001). Thus, replication of (+)RNA viruses is a complex process that involves numerous interactions among viral RNA, viral-coded, and host-coded proteins and host membranes (lipids). Dissecting the functions of the various replication-associated or replication-modulating molecules in (+)RNA replication is one of the major frontiers in current virus research. The picture emerging is that the mechanism of genome replication, and the functions of viral and host factors, might be somewhat analogous among various (+)RNA viruses in spite of their diverse genome organizations and gene expression strategies. Also, most of the previously identified host factors are conserved genes, suggesting that (+)RNA viruses might selectively target conserved host functions as opposed to species-specific factors. Such a strategy would help viruses broaden their host range by expanding infections to new host species. Altogether, host factors play crucial roles in all steps of (+)RNA replication. Host factors are also key determinants of the host range of a given virus and affect virus pathology, host–virus interactions, as well as the evolution of the virus. Host factors could also be potent antiviral targets. Studies on host factors also contribute insights into their normal cellular functions, thus promoting understanding of the basic biology of the host cell.

The host factors characterized to date play diverse roles during (+)RNA replication, including mediating intracellular transport of viral proteins and viral RNA, as chaperones facilitating correct folding of viral proteins, as helicases or RNA chaperones assisting the folding of the viral RNA, facilitating the switch from translation to replication by promoting template recognition/selection, and as lipid metabolism enzymes driving membrane proliferation.

This review provides an overview of our current understanding of the role of host factors that facilitate (+)RNA virus replication. Major challenges remain to resolve further what roles the identified host factors play during (+)RNA virus replication. Studies aimed at identifying and dissecting all the replication-associated factors are expected to increase the number and efficiency of our methods to interfere with successful viral replication/infection.

Approaches

Since (+)RNA viruses can potentially co-opt most of the ~20,000–30,000 host proteins (whether animal or plant) for their replication, it is a daunting task to identify those proteins, which are actually subverted by a given (+)RNA virus. Numerous approaches have been developed during recent years to identify host factors, and we will briefly describe only a selected number of ways that yielded the most fruitful hits.

Systems Biology

Possibly, the most powerful means to identify host factors involved in (+)RNA replication are based on genome-wide approaches. Intensive (high-throughput) screens include systematic analysis of most genes available in the genome of a particular host. Yeast (*Saccharomyces cerevisiae*) is particularly useful for this approach since it has a small genome-size (~5,800 genes) and a reduced level of redundancy among host genes. In addition, yeast is the best-known model for eukaryotic cell with the highest percentage of characterized genes in the genome useful to study the aspects of virus–cell interactions. Also, the genome-wide screens can be performed with single-gene deletion (YKO) and the essential gene (yTHC) libraries of yeast. For this strategy to succeed, it is necessary to launch (+)RNA virus replication in yeast, usually based on plasmid-driven expression of viral RNA/proteins. Yeast as a host for Brome mosaic virus (BMV) was pioneered by the Ahlquist laboratory, and subsequently was adapted for studying replication of Flock house virus (FHV) and tombusviruses of plants, such as Tomato bushy stunt virus (TBSV) (Ishikawa et al., 1997b; Janda and Ahlquist, 1993; Panavas and Nagy, 2003; Panaviene et al., 2004; Pantaleo et al., 2003; Price et al., 2000). The genome-wide screens of 80% of yeast genes with BMV and 95% of yeast genes with TBSV led to the identification of over 100 yeast genes for both viruses that affected their replication (Jiang et al., 2006; Kushner et al., 2003; Panavas et al., 2005b). Interestingly, only a small set of yeast genes for BMV and TBSV overlapped, suggesting that these distantly related (+)RNA viruses use and/or are being affected by mostly different set of host genes for their replication (Jiang et al., 2006; Panavas et al., 2005b). Genome-wide screens with the yeast libraries have also been performed to identify host factors affecting RNA recombination in the case of TBSV, further illustrating the usefulness of this approach (Cheng et al., 2006; Serviene et al., 2006, 2005).

A different genome-wide approach was performed with Drosophila C virus (DCV) based on RNA interference (RNAi) and led to down-regulation of the expression of 21,000 (91%) of the *Drosophila* genes (Cherry et al., 2005). This resulted in the identification of 112 host genes affecting DCV replication (Cherry et al., 2005, 2006). More than half of the identified genes were ribosomal genes, suggesting that DCV replication depends greatly on the host translation machinery. Another large-scale RNAi-based approach was performed with hepatitis C

virus (HCV) (Ng et al., 2007). Among the 4,000 human genes targeted, Ng et al. found nine cellular genes whose depletion led to 60% or more inhibition of HCV replication.

Genomic Random Mutagenesis

Extensive mutagenesis of the host genome via treatment with a mutagenic chemical, fast-neutron irradiation, UV-treatment, or transposon insertions have also been used to obtain libraries of mutated hosts. Testing for viral (+)RNA replication, followed by positional cloning, genetic complementation, or other approaches can lead to identification of host genes affecting virus replication. Indeed, this approach was used to identify several yeast genes affecting BMV replication and *Arabidopsis* TOM1/2/3 genes affecting Tomato mosaic virus (ToMV) accumulation (Diez et al., 2000; Ishikawa et al., 1997a; Lee et al., 2001; Tsujimoto et al., 2003; Yamanaka et al., 2000).

Proteomics

Recent technological advances with mass-spectrometry (MS)-based identification of proteins present in ribonucleoprotein complexes have made it possible to dissect the composition of purified viral replicase complexes. Two-step affinity purification of a tombusvirus replicase complex from yeast, followed by 2D gel-electrophoresis and MALDI-TOF analysis of proteins cut from 2D gels led to the identification of four host proteins, namely the Ssa1/2p molecular chaperone (a yeast homologue of Hsp70 proteins), Tdh2/3p (glyceraldehyde-3-phosphate dehydrogenase, a metabolic protein with RNA-binding activity), Pdc1p (pyruvate decarboxylase), and Cdc34p E2 ubiquitin-conjugating enzyme (the later is unpublished, Li and Nagy), which were missing in the control samples (Serva and Nagy, 2006).

Similarly, a purified Tobacco mosaic virus (TMV) replicase preparation contained at least four host proteins, based on silver-staining of SDS-PAGE gels (Osman and Buck, 1997). Western blotting led to the identification of an RNA-binding protein, GCD10, which is one of the subunits of the ten-component eIF-3 complex (Osman and Buck, 1997). Another host protein in the TMV replicase might be translation elongation factor 1A (eEF1A), which was found to bind to the TMV 126K replication protein based on co-immunoprecipitation (Yamaji et al., 2006).

A highly purified replicase preparation for BMV contained two viral-coded and ~10 host proteins, based on silver-stained SDS-PAGE analysis (Quadt et al., 1993). One of the host proteins identified was the p41 subunit of the eIF-3 complex. The function of p41 in the BMV replicase is currently unknown.

To identify host proteins interacting with the non-structural protein 3 (nsP3) replication protein of Sindbis virus (SIN), an alphavirus, a green fluorescent protein (GFP)-tagged nsP3 was expressed from the viral genome, followed by immunoaffinity purification with anti-GFP antibody on magnetic beads and mass-spectrometry of the isolated proteins (Cristea et al., 2006). This, and a similar approach by

Frolova et al. (2006), led to the identification of 59 cellular proteins, of which 35 were specific to nsP3. Additional research confirmed a role for the following proteins in SIN replication: G3BP1 and G3BP2a (Ras-GTPase activating protein SH3-domain-binding proteins), hnRNP-1A, -A3, -A2/B1, and -G (heteronuclear ribonucleoproteins), and the 14-3-3 family of proteins (tyrosine-3-monooxygenase/tryptophan 5-monooxygenase activation proteins), which are phosphoserine-binding adaptor proteins (Cristea et al., 2006). The actual functions of the identified nsP3–host protein interactions are not yet known in SIN replication.

Yeast Two-Hybrid (YTH) Screens

Due to the large number of studies using YTH screens, we cannot cover the entire area. Instead, we demonstrate the usefulness of this approach by discussing findings with HCV and poliovirus (PV). For example, NS5A of HCV was screened against an interferon-induced human hepatocyte cDNA library, leading to the discovery of the 33kDa human vesicle-associated membrane protein-associated protein (hVAP-A, also called hVAP-33) (Tu et al., 1999). hVAP-A also interacts with NS5B of HCV (Tu et al., 1999). Another YTH screen with the HCV NS5A, using human brain and liver cDNA libraries, led to the identification of the immunophilin, termed FKBP8, which is a human FK506-binding protein (Okamoto et al., 2006). When the NS5B RdRp protein was used as a bait, p68 helicase was identified as an interactor from a human spleen cDNA library (Goh et al., 2004). A screen with the HCV NS5B, using a human liver cDNA library, led to the identification of eIF4AII (a DEAD box RNA helicase) that partially co-localized with NS5B in infected cells (Kyono et al., 2002). Another YTH screen with the HCV NS5B protein, using a human hepatocyte cDNA library, led to the identification of ubiquitin-like protein hPLIC1, which functionally connects the ubiquitination machinery to the proteosome and could be involved in regulation of NS5B stability and HCV replication (Gao et al., 2003). Using a human thymus cDNA library against HCV NS5B RNA-dependent RNA polymerase (RdRp) as a bait, Kim et al. identified eukaryotic translation initiation factor 4A (isoform 2, named eIF4AII) and septin 6, a GTP-binding protein involved in membrane dynamics, trafficking and cytoskeletal remodeling (Kim et al., 2007). Depletion of septin 6 by RNAi decreased HCV replication, suggesting that septin 6 is a necessary host factor for HCV (Kim et al., 2007). In the case of PV, Kirkegaard et al. found that Sam68 (Src-associated in mitosis, 68kDa) interacted with the 3D RdRp protein, which resulted in re-localization of Sam68 to PV-induced cytoplasmic vesicles/membranes, the site of PV replication (McBride et al., 1996).

Co-purification of RNA–Protein Complexes

One of the most popular approaches for identifying RNA-binding host proteins is based on purification of the viral RNA (with or without UV-crosslinking), followed by MS-based identification of the host proteins. Using the untranslated regions (UTRs) of a coronavirus RNA for UV-crosslinking, Lai, Leibowitz, Hogue, and

their colleagues identified numerous RNA-binding host proteins, including heterogeneous nuclear ribonucleoprotein (hnRNP) A1, polypyrimidine-tract-binding (PTB) protein, poly(A)-binding protein (PABP), and mitochondrial aconitase (m-aconitase) (Li et al., 1997; Nanda and Leibowitz, 2001; Shi and Lai, 2005; Spagnolo and Hogue, 2000). Also, the hnRNP-C that interacts with the (–)RNA of PV has been identified (Brunner et al., 2005; Roehl and Semler, 1995). Using West Nile virus (WNV) (+) and (–)RNAs, Brinton et al. identified several host proteins that were specifically UV-crosslinked to the viral RNA (Li et al., 2002). These cellular proteins included T-cell intracellular antigen-1 (TIA-1), TIA-1-related protein (TIAR), and eEF1A. In the case of bovine viral diarrhea virus (BVDV), a pestivirus related to HCV, various portions of the BVDV genomic (g)RNA were used in UV-crosslinking experiments with bovine and human cellular extracts (Isken et al., 2003). This work led to the identification of three RNA-binding proteins, named NF90 (nuclear factor 90, also called NFAR-1), NF45, and RNA helicase A (RHA), which are RNA-binding proteins containing double-stranded RNA-binding motifs.

Chemical Virology

A cell culture-based screen with a library of chemicals can lead to potent antiviral inhibitors, which, in turn, can be used to identify the potential cellular or viral targets of these inhibitors (Watashi and Shimotohno, 2007). For example, studies with the immunosuppressant cyclosporin A (CsA) that suppresses HCV RNA replication, led to the discovery of cellular peptidyl–prolyl *cis–trans* isomerase (PPIase), termed CyPB, which is a cellular cofactor for HCV replication (see below) (Watashi et al., 2005).

Additional approaches, such as MS-based profiling for up- and down-regulated proteins in FHV-infected cells (Go et al., 2006), protoarrays with thousands of purified recombinant host proteins (Zhu et al., 2007), and the numerous DNA microarrays used to identify up- and down-regulated cellular genes by viral infections will not be discussed here.

Molecular Interaction Between the Host and (+)RNA Virus During Replication

Most of the known host factors inhibit replication when absent, or present in reduced amount, suggesting that these genes facilitate (+)RNA virus replication by providing useful functions, directly or indirectly (see below). Identified host factors are known to be involved in various cellular processes, such as metabolism/modifications of RNAs, lipids, and proteins; in protein intracellular transport/targeting; or, in general metabolism (Kushner et al., 2003; Panavas et al., 2005b). It is intriguing to note that a large set of host genes affecting (+)RNA virus replication is unique for any given virus, suggesting that (+)RNA viruses have developed different ways to utilize the

immense resources of cells. In spite of the differences in the host genes involved, we predict that many of the different genes might provide mechanistically similar functions during replication of various (+)RNA viruses. For example, molecular chaperones, albeit different members of the chaperone family, have been found to affect BMV, TBSV, FHV, HCV, and coronavirus replication (Tomita et al., 2003; Castorena et al., 2007; Kampmueller and Miller, 2005; Nanda et al., 2004; Okamoto et al., 2006; Serva and Nagy, 2006).

The genome-wide screens with BMV, TBSV, and DCV confirmed that (+)RNA viruses depend greatly on the intracellular components of infected hosts for robust viral replication. It appears that the interactions between host cells and (+)RNA viruses are complex, likely including numerous replication-associated host factors with direct or indirect roles. Overall, the identified host factors likely belong to one of the following three groups. (i) Those that may directly interact with the viral RNA(s) or replication proteins and perform essential functions for the virus. This group of host factors also include host membranes/lipids, various components of the intracellular transport and trafficking system, the translation apparatus, and possibly intracellular compartments, such as the ER, peroxisome, and vesicles, which (+)RNA viruses require and/or utilize to complete their replication. For example, the Hsp70 molecular chaperone, which is present in the tombusviral RC, might be directly involved in replicase assembly (Serva and Nagy, 2006). (ii) Those host factors that indirectly affect (+)RNA virus replication via influencing the amount and/or activity of those host factors, which are directly involved [group (i) above]. These indirect host factors may affect the competition between the virus and the host for limited cellular resources, host proteins, and intracellular compartments. For example, transcription factors could affect the amount of host factors available in the infected cells, thus indirectly affecting (+)RNA virus replication. (iii) The third group of host factors includes direct inhibitory factors, such as components of the host innate and general antiviral defense mechanisms, which affect virus replication by destroying/modifying viral RNAs or viral replication proteins in targeted or in general manners. For example, Ngl2p endoribonuclease, the yeast Xrn1p, and the *Arabidopsis* Xrn4p 5'–3' exoribonucleases were found to affect degradation, and thus stability, of TBSV RNA (Cheng et al., 2007, 2006; Serviene et al., 2005). The second and third groups of host factors will not be discussed further in this chapter.

The Replication Cycle of (+)RNA Viruses Consists of Six Distinct Steps

The viral (+)RNA has to participate in three-to-five competing processes required for successful infection. Activities include translation to produce viral proteins, replication, transcription [to produce subgenomic (sg)RNA for some viruses], encapsidation, and cell-to-cell movement (in the case of plant RNA viruses). These processes are highly regulated and compartmentalized to avoid collision between

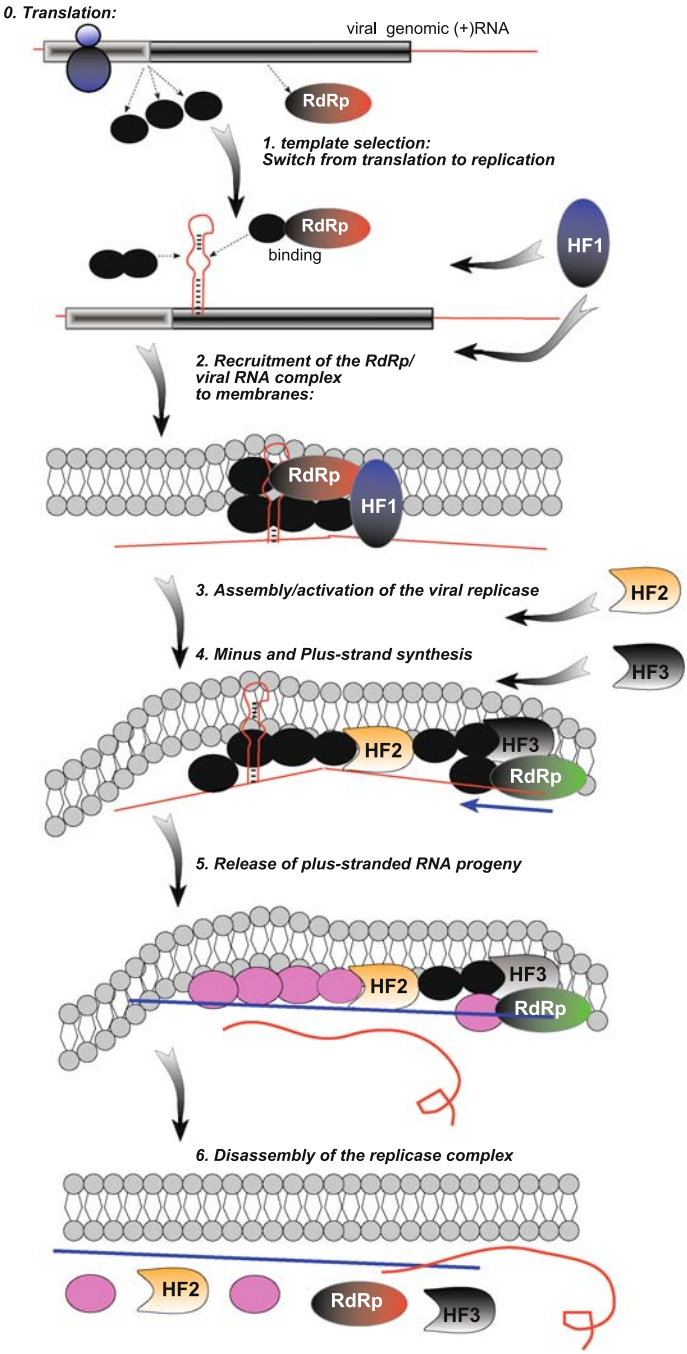

Fig. 14.1 (continued)

the ribosome and the viral RC. Recent advances in knowledge about (+)RNA virus replication have resulted in the division of the replication cycle into six separate steps (Fig. 14.1). These include (1) recruitment/selection of the viral (+)RNA template for replication (the switch of viral RNA from translation to replication); (2) targeting of viral replication proteins to the site of replication; (3) assembly of the functional viral replicase complex on intracellular membraneous surfaces; (4) synthesis of viral RNA progeny; (5) release of viral (+)RNA progeny from the site of replication; and (6) disassembly of the viral RC. In the following subchapter, we will discuss our current knowledge on the role of host factors in each of those steps.

Roles of Host Factors in Various Steps of Viral RNA Replication

Step 1. Roles of Host Proteins in the Selection of Viral (+)RNA Template for Replication and the Switch from Translation to Replication

During translation, the viral (+)RNA is used as a mRNA to produce replication proteins and other viral-coded proteins. It has been estimated that a single viral RNA is translated 1,000–10,000 times (Quinkert et al., 2005). However, unlike host mRNAs, the viral (+)RNA has to be recruited for replication and saved from RNA degradation. Both host- and viral-coded proteins have been documented to participate in the regulation of the switch from translation to replication. Translation of the replication proteins and the selection of the viral gRNA for replication likely takes place in the cytoplasm, whereas replication of (+)RNA viruses occurs on the cytoplasmic faces of various organelle-derived membrane surfaces (Ahlquist et al., 2003; Buck, 1996; Salonen et al., 2005). Therefore, the viral gRNA, together with viral and host factors, must be transported/recruited to the site of replication. Current models involve specific template selection (specific binding of viral and/or host proteins to the template RNA) as a key step in regulation of (+)RNA replication.

The recently emerging picture is that viral replication proteins can bind selectively to viral (+)RNA, which likely leads to selection/recruitment of the viral RNA

Fig. 14.1 A general model showing six separate steps during (+)RNA virus replication. The RC is shown schematically, but the RC likely contains more protein components. The *red* coloring for the RdRp suggests that the RdRp is inactive (replication incompetent) at the beginning, while it gets activated (shown in *green* color) during the replicase assembly process, possibly with the help of host factors and the viral (+)RNA. Viral-coded auxiliary replication proteins are shown as *black* circles, whereas they are shown as *pink* circles after their putative inactivation, possibly via phosphorylation. The entire RC may become inactivated by phosphorylation and/or ubiquitinaton prior to disassembly. *Red line* indicates plus-stranded, while *blue line* shows the minus-stranded viral RNAs. *Small circles* show the subcellular membraneous compartment used for replication. RdRp is the viral-coded RNA-dependent RNA polymerase, whereas HF stands for a host factor.

from translation into replication. This has been shown for the 1a protein of BMV, the p33 replication protein in tombusviruses, the 126K protein of TMV, and the 3CD protein of PV (Gamarnik and Andino, 2000; Osman and Buck, 2003; Pogany et al., 2005; Wang et al., 2005b). In the case of TBSV, highly specific binding of the p33 replication protein to a conserved sequence forming a long hairpin structure with an internal C•C mismatch in the TBSV gRNA (termed the p33 recognition element, p33RE) is essential for TBSV RNA replication in yeast or virus replication in plants (Monkewich et al., 2005; Pogany et al., 2005). Additional in vitro and in vivo data firmly support the role of the p33:p33RE interaction as the major factor in selection of TBSV RNA for replication from the diverse RNA pool present in the host cell. Interestingly, the selection of viral RNA templates for replication is mechanistically similar to viral RNA encapsidation, which is also based on the requirement of a specialized viral protein, the coat protein, leading to packaging of the viral RNA into virions that are mostly inaccessible for other processes.

The involvement of the viral replication proteins in selection of the viral RNA for replication does not exclude host proteins also from contributing to this early step in replication. For example, recruitment of BMV RNAs for replication is affected by Lsm1p, which belongs to the seven member Sm-like family of proteins (Diez et al., 2000; Noueiry et al., 2003). Lsm1p, together with Lsm2-7p, form the Lsm1p-7p heptameric ring, which is involved in mRNA turnover. Diez et al. found that Lsm1p-7p, which together with Pat1p and Dhh1p, is called the decapping activator complex, that moves cellular mRNAs from translation to degradation in the cytoplasmic processing bodies (termed P-bodies), and that might function as a key regulator to switch BMV RNAs from translation to replication (Mas et al., 2006). According to this model, the Lsm1p–7p/Pat1p/Dhh1p complex could refold the BMV RNAs allowing separation from ribosomes and translation factors and the binding of the BMV 1a replication protein to viral RNA. Then, the viral (+)RNA, likely in association with the replication proteins and host factors, ends up in the P-body that may serve as a place to pre-assemble the BMV RC prior to transport to the ER, where maturation likely occurs on the membranes (the site of BMV replication) (Beckham et al., 2007).

In the case of PV, a central role for the host poly(rC)-binding protein-2 (PCBP-2) in RNA template recruitment has been postulated. Based on this model, the full-length PCBP-2 can bind to stem-loop 4, which is part of the internal ribosome entry site (IRES), in PV RNA as well as to the host protein SRp20 (involved in RNA trafficking) to promote the cap-independent translation of the PV RNA. Due to protease activity of the PV-coded 3CD or 3C, PCBP-2 is cleaved proteolytically, removing the KH3 RNA-binding site. The cleaved form of PCBP-2, however, still contains KH1-2 RNA-binding sites and it retains its ability to bind to stem-loop 1 in the PV RNA, which is critical for replication by bringing together the 3′ and 5′ ends of the PV RNA via interaction with the poly(A)-binding protein (PABP) (Perera et al., 2007; Walter et al., 2002). PCBP also binds to the spacer sequence located between the cloverleaf and the IRES, which is required for the replication (Toyoda et al., 2007). In addition to the cellular proteins, the PV-coded 3CD RdRp precursor regulates RNA template selection and switching of the viral RNA from translation

to replication by binding to the coverleaf-like structure at the 5′ UTR and affecting genome circularization (Gamarnik and Andino, 2000; Herold and Andino, 2001). The resulting genome circularization is predicted to be critical for PV minus-strand synthesis (Gamarnik and Andino, 1998, 2000; Herold and Andino, 2001; Perera et al., 2007).

Polypyrimidine-tract-binding protein (PTB) may play a central role in translation and replication of HCV. Since PTB was found to bind to the HCV RNA and since it is re-localized to detergent-resistant membrane fractions, the sites of HCV replication, Lai et al. suggested that PTB or its truncated (proteolytically cleaved) versions might facilitate the switch from translation to replication (Aizaki et al., 2006). In addition, PTB is also critical for HCV RNA synthesis, because antibody-based depletion of PTB inhibited HCV RNA synthesis in a cell-free system and small interfering (si)RNA-based depletion of PTB inhibited HCV accumulation in infected cells (Aizaki et al., 2006; Chang and Luo, 2006).

The switch of coronavirus RNA from translation to replication may depend on two host factors, the PTB protein and hnRNP A1, which, via their interaction with each other, were proposed to mediate 5′–3′ crosstalk of the UTRs in the coronavirus (+)RNA (Choi et al., 2002; Li et al., 1997; Shi and Lai, 2005). The resulting genome circularization might facilitate RNA replication, as suggested above for PV. An additional hnRNP protein, termed synaptotagmin-binding cytoplasmic RNA-interacting protein (SYNCRIP), may also be involved in the switch to replication, via inducing conformational changes in the highly structured coronavirus RNA that could facilitate RNA replication (Choi et al., 2004). Interestingly, PTB and hnRNP-A1 have also been found to play roles in HCV replication (Aizaki et al., 2006; Kim et al., 2007). Thus, it is possible that these host factors could play similar roles in gRNA circularization or the structural rearrangement of the viral RNA during HCV replication, as proposed above for coronaviruses.

Recruitment of BVDV RNA from translation into replication was proposed to depend on three interacting cellular factors: NF90, NF40, and RHA (Isken et al., 2003). Based on RNA competition studies that included various regions of the (+)RNA genome of BVDV, Behrens et al. proposed that NF90/NF40/RHA bind to both the 3′ and the 5′ UTRs, leading to circularization of the BVDV (+)RNA (Isken et al., 2003). This could be the signal to switch from translation to replication. Indeed, RHA has helicase activity that may facilitate the refolding of the viral RNA to make it suitable for replication. Also, NF90/NF40/RHA proteins are part of the antiviral response, thus their recruitment for BVDV replication in the cytoplasm can weaken the antiviral response that takes place in the nucleus. The NF90/NF40/RHA proteins together with NF110, might also promote the circularization of HCV (+)RNA via facilitating the interaction between the 5′ UTR and the 3′ UTR. Moreover, these proteins are re-localized to the site of HCV replication, suggesting that their roles could be similar in HCV as in BVDV RNA replication (Isken et al., 2007).

Overall, the selection of the viral (+)RNA for replication in concert with switching from translation to replication (i) guarantees that the viral (+)RNA avoids degradation; (ii) prevents collision between the ribosome and the viral RC, which

must use the same RNA, though progressing in opposite directions; and (iii) facilitates the specific selection of the authentic viral (+)RNA for replication.

Step 2. Targeting of Essential Viral Replication Proteins and the Viral RNA to the Site of Replication

Translation and then selection of the viral (+)RNA for replication likely take place in the cytoplasm, whereas replication of (+)RNA viruses occurs on the cytoplasmic surfaces of various intracellular membranes (Ahlquist, 2002; Ahlquist et al., 2003; Burgyan et al., 1996; Egger and Bienz, 2005; Navarro et al., 2004; Panavas et al., 2005a; Rubino and Russo, 1998; Salonen et al., 2005). Therefore, the viral (+)RNA and the viral-coded replication proteins must be targeted to those subcellular compartments (Fig. 14.1, step 2). The viral gRNA is likely recruited in *cis* by the newly produced replication proteins, which could bind to the gRNA present in the same location (*cis*-binding) (Neeleman and Bol, 1999; Novak and Kirkegaard, 1994; Oster et al., 1998; Panaviene et al., 2003; Vlot et al., 2003; Weiland and Dreher, 1993) during natural infections. Altogether, recruitment in *cis* may be a more efficient process than recruitment in *trans* that could secure the effective transportation of the limited amount of viral gRNA to the site of replication. The picture emerging from recent studies with several (+)RNA viruses is that both viral- and host-coded proteins participate in the intracellular targeting/transport step.

In the case of some plant (+)RNA viruses, existing evidence supports a master role for the replication proteins, such as the BMV 1a protein and the TBSV p33 replication protein, in intracellular targeting of the viral RdRp as well as the viral (+)RNA, likely in the form of multimolecular complexes, to the site of replication (Fig. 14.1). Formation of the multimolecular complexes including the replication proteins, viral RNA, and some host factors, in the cytoplasm would likely facilitate efficient transport and co-localization of all these essential components to the same replication sites maximizing the assembly of fully functional RCs. Such organization can greatly increase the probability of successful replicase assembly. The pre-organization of replication factors into multicomponent complexes could be especially important at the beginning of infections when limited amounts of viral factors are available.

Recruitment of the viral replication proteins to the intracellular membranes, the sites of (+)RNA virus replication, is likely guided by host proteins involved in intracellular transport. For example, replication of ToMV depends on two *Arabidopsis* proteins, termed TOM1 and TOM3, seven-pass membrane proteins, which interact with the 130K helicase-like replication protein of TMV (Yamanaka et al., 2002, 2000). TOM1/3 are also part of the ToMV RC and they likely act as anchors of the RC to the membrane via their transmembrane domains. Mutations or RNAi-based depletion of TOM1/3 severely inhibited ToMV replication in *Arabidopsis* plants (Yamanaka et al., 2002, 2000). Interestingly, the RNAi-driven depletion of the homologous proteins in tobacco greatly inhibited the accumulation of several

tobamoviruses related to ToMV, but not the more distantly related Cucumber mosaic virus, suggesting that TOM1/3 genes have conserved functions in diverse hosts (Asano et al., 2005).

Transport of BMV replication proteins and the (+)RNAs to the sites of replication is affected by Lsm1p-7p together with Pat1p and Dhh1p, which might facilitate localization of protein and the viral RNAs to the P-bodies, where pre-assembly of the BMV RC could take place prior to final transport to the ER, which is the site of BMV replication (Beckham et al., 2007).

hVAP-A, a SNARE-like protein, was shown to interact with the HCV NS5A and NS5B replication proteins (Tu et al., 1999). The interactions may be important for the association of these viral replication proteins with intracellular membranes, the site of HCV replication, due to hVAP-A serving as a membrane receptor. Also, a geranylgeranylated cellular protein, named FBL2, might be involved in the recruitment of the HCV NS5A replication protein to the intracellular membranes. The interaction between the geranylgeranylated FBL2 and the HCV NS5A is essential for HCV replication, based on depletion of FBL2 by RNAi and the expression of the dominant negative mutant of FBL2 that both led to inhibition of HCV accumulation (Wang et al., 2005a).

Recruitment of coronavirus RNA might be affected by interaction with molecular chaperones. Leibowitz et al. have shown that mitochondrial aconitase (m-aconitase), mitochondrial HSP70 (mtHSP70), HSP60, and HSP40 form a complex, probably in the cytosol, which may be involved in stabilizing the viral RNA and/or its recruitment to replication via binding to the coronavirus 3′ UTR (Nanda et al., 2004; Nanda and Leibowitz, 2001). Though m-aconitase and mtHSP70 are mostly localized to the mitochondria, these proteins are translated in the cytoplasm and they could be hijacked by coronavirus RNA prior to their translocation to mitochondria.

At this time, our knowledge on the transport/trafficking of the viral (+)RNA and viral replication proteins is incomplete. Future works employing cellular and biochemical approaches are expected to advance this area of research for many (+)RNA viruses.

Step 3. Role of Host Factors in the Assembly of the Viral Replication Complex

(+)RNA virus replication takes place on intracellular membraneous compartments (Salonen et al., 2005) or via the active induction of the formation of novel cytoplasmic vesicular compartments (Cherry et al., 2006; Egger and Bienz, 2005; Kirkegaard and Jackson, 2005). These compartments (virosomes) contain the membrane-bound viral RC, which is the key enzyme in (+)RNA replication. The RC has to perform many functions during replication, including recognition of minus- and plus-strand initiation promoters located at the 3' terminus of (+) or (–)RNA, de novo (primer-independent) or primer-dependent initiation, as well as the synthesis of complementary RNA strands, strand separation, and the repair of viral

RNAs with damaged termini (Dreher, 1999; Kao et al., 2001; Nagy et al., 1997; Rao et al., 1989). In addition, the viral RC has to recognize additional regulatory RNA elements, such as any replication silencer or replication enhancer, which either down- or up-regulate RNA synthesis (Nagy and Pogany, 2006). Also, the viral RC is involved in the production of sgRNAs in some viruses (Buck, 1996, 1999; White, 2002). Moreover, the activity of the RC leads to genetic mutations and RNA recombination via template-switching during RNA synthesis, which affects RNA virus evolution (Cheng and Nagy, 2003; Kim and Kao, 2001; Nagy et al., 1995; Nagy and Simon, 1997; Nagyet al., 1998; Roossinck, 2003; Wierzchoslawski and Bujarski, 2006). Although it is currently unknown how the viral RC can perform so many activities, it is possible that the multi-functionality is due to the elaborate composition of the viral RC.

Molecular Composition of the Viral Replicase Complex

The viral RC shows surprising complexity as it contains both viral- and host-derived components (Serva and Nagy, 2006). Quantitative analysis of the HCV RC revealed that hundreds/thousands of viral nonstructural (NS) proteins are present in a protease/nuclease-resistant spherule containing ~1 minus-strand and ~5 plus-stranded RNAs (Quinkert et al., 2005). Electron microscopy (EM) and immuno-EM images of the sites of BMV replication, containing the active viral RC, revealed that the sites of BMV replication consist of 50–60nm spherule-like structures with cellular membranes surrounding the replication proteins and the viral RNA (Schwartz et al., 2002). Interestingly, individual spherules contain small openings (membraneous necks), which likely serve as gates for transportation of molecules between the spherules and the cytoplasm (Schwartz et al., 2002). In a broad sense, we can regard one separate spherule as one active/matured RC. Studies on the molecular composition of a single spherule based on immuno-labeled images revealed that one spherule could contain 25-fold more 1a replication protein than $2a^{pol}$, whereas the actual number and nature of host molecules within single spherules are currently unknown. Current models predict that highly organized protein:protein and protein:RNA complexes, with the help of cellular membranes, facilitate the formation of an active (+)RNA virus RC.

Regulation of the Activities of the Viral Replicase Complex

Some RdRp proteins, such as the tombusvirus $p92^{pol}$, the BMV $2a^{pol}$, Alfalfa mosaic virus (AlMV) P2, and the HCV NS5B, are nonfunctional before the assembly of the RC (Panaviene et al., 2005; Panaviene et al., 2004; Quadt et al., 1995; Vlot et al., 2001), suggesting that these RdRps have to become "activated" in cells, likely during the assembly process (Fig. 14.1, step 3). Therefore, assembly of the viral replicase could be an important regulatory step in (+)RNA virus replication. Moreover, co-expression of (+)RNA has been shown to enhance replicase assembly/activity by ~40- to 100-fold for TBSV and was required for the BMV replicase in yeast (Panaviene et al., 2005, 2004; Quadt et al., 1995) and the AlMV replicase in plants

(Vlot et al., 2001). Thus, the viral (+)RNA may serve as a platform to facilitate replicase assembly. It is also possible that initiation of minus-strand synthesis might lead to the stabilization of the viral RC.

Roles of Host Factors Within the Viral Replicase Complex

In addition to the *cis*-acting RNA factors, *trans*-acting factors are needed for the assembly of the functional viral RC. These include both viral and host factors that likely contribute to replicase assembly. For example, cellular chaperones may play essential roles in the assembly of viral replicases. The documented cases include Ydj1p, J-domain protein of the Hsp40 chaperone family that was found to affect minus-strand synthesis of BMV in yeast, but not the 1a-mediated recruitment of viral RNA or $2a^{pol}$ to the site of replication (Tomita et al., 2003). Also, Ydj1p affected the amount of $2a^{pol}$ present as a soluble protein and a reduced amount of Ydj1p in yeast led to aggregation of a fraction of $2a^{pol}$. Based on these data and the known property of Ydj1p, Ahlquist and co-authors proposed that Ydj1p is involved in the assembly and/or activation of the BMV RC prior to minus-strand synthesis (Tomita et al., 2003). Serva and Nagy proposed a similar function for the Hsp70 proteins (termed Ssa1p and Ssa2p in yeast) in folding/activation of tombusvirus RC (Serva and Nagy, 2006). Indeed, deletion of *SSA1/2* led to fourfold decrease in TBSV replication in yeast and reduced the amount of the replication proteins, suggesting that Hsp70 might stabilize the replication proteins. Also, Ssa1/2p were found to bind to the p33 replication protein and be present within the highly purified tombusvirus RC (Serva and Nagy, 2006).

Another example for the role of a cellular chaperone has been found with FHV, which requires Hsp90 for production of protein A RdRp (Castorena et al., 2007; Kampmueller and Miller, 2005). When Hsp90 was inhibited with geldanamycin, protein A was present at ~20% of its normal level, leading to inefficient assembly of the FHV RC and a reduced rate of RNA synthesis (Castorena et al., 2007). The actual mechanism of Hsp90-assisted translation/stabilization of protein A is currently unknown.

Moreover, the human FKBP8 immunophilin has been shown to interact with the HCV NS5A, which is a multifunctional phosphoprotein, and with the Hsp90 chaperone (Okamoto et al., 2006). These interactions were postulated to play a role in stabilization of the HCV RC. RNAi-based knock-down experiments with FKBP8 and the use of an inhibitor of Hsp90 demonstrated significant reduction in HCV replication in human hepatoma cell lines (Okamoto et al., 2006).

Picornaviruses, such as PV or DCV, induce a cytoplasmic vesicular compartment where RNA replication takes place (Cherry et al., 2006; Egger and Bienz, 2005). The formation of the vesicular compartment by DCV depends on the COPI host protein (Cherry et al., 2006). Depletion of COPI via RNAi reduced cytoplasmic vesicles by 2.5-fold, suggesting that the COPI coatomer is critical for the formation of the DCV-induced vesicular compartment. The induction of COPI-based vesicle formation could be regulated by small GTPase ADP-ribosylation factor 1 (Arf1). Indeed, the PV RC recruits Arfs, which, in GTP-bound form (the membrane-associated

active form), could recruit other cellular proteins to change membrane curvature, induce transport vesicles from intracellular membrane organelles, and stimulate phospholipase D activity that modifies the lipid composition of membranes (Belov and Ehrenfeld, 2007; Belov et al., 2007). PV 3A has been shown to recruit GBF1, a GEF (guanine exchange factor) to facilitate Arf1-activation/localization to the membrane, whereas the PV-coded 3CD protein binds to BIG1/2 GEFs (Belov et al., 2007). Thus, it is likely that PV 3A and 3CD proteins act synergistically to modify the cellular membrane traffic pathway to facilitate the formation of the PV RC (Belov and Ehrenfeld, 2007). Additional data from Wessels et al. revealed that modulation of the Arf-activating pathway by PV might also lead to inhibition of cellular secretion to ensure survival in the infected animal (Wessels et al., 2006a,b). Moreover, reticulon 3 cellular protein, which interacts with the 2A protein of several picornaviruses, might also be involved in modulating the assembly of the viral RC (Tang et al., 2007).

The assembly of the functional tobamovirus RC likely depends on several *Arabidopsis* proteins, including the membrane proteins TOM1 and TOM3, the four-pass membrane protein TOM2A, and the basic protein TOM2B (Tsujimoto et al., 2003; Yamanaka et al., 2002, 2000). Whereas TOM1/3 have been proposed to participate in the recruitment of the TMV 130K replication protein via direct interaction; TOM2A/2B do not interact with 130K, but they interact with TOM1/3. All these host proteins are likely part of the TMV RC on the vacuolar membrane (i.e., tonoplast in plants) based on co-localization studies (Hagiwara et al., 2003). While TOM1/3 might act as anchors for the 130K and 180K RdRp to the membrane via their transmembrane domains, the roles of TOM2A/2B remain uncharacterized (Yamanaka et al., 2002, 2000). TOM2A might be involved in organization of the TMV RC via its interaction with TOM1/3 and the membrane or, alternatively, it could facilitate recruitment of additional, as yet unknown, host factors (Hagiwara et al., 2003; Tsujimoto et al., 2003).

Assembly of the HCV RC might take place on the surfaces of detergent-insoluble lipid rafts, consisting of cholesterol- and sphingolipid-rich microdomains within the subcellular membranes. The cellular hVAP-A, a vesicle membrane transport protein associated with HCV NS4B, NS5A, and NS5B replication proteins, was shown to play a critical role in the assembly of the HCV RC on lipid rafts (Gao et al., 2004). hVAP-A is partially associated with lipid rafts and dominant negative versions of hVAP-A or RNAi-driven depletion of hVAP-A resulted in relocation of NS5B from detergent-resistant to detergent-sensitive membranes. In addition, the NS5A and hVAP-A interaction is also critical for HCV RC assembly, based on the observation that hypophosphorylated NS5A can interact with hVAP-A, leading to efficient replication, whereas the hyperphosphorylated NS5A cannot interact with hVAP-A resulting in reduced replication (Evans et al., 2004). More recently, hVAP-B, a ubiquitous VAP-A-like protein, has also been implicated in HCV replication (Hamamoto et al., 2005). hVAP-B interacts with both NS5A and NS5B of HCV and it is likely part of the HCV RC because specific antibody against VAP-B inhibited HCV replication in vitro. Furthermore, depletion of hVAP-B by RNAi inhibited HCV replication. Unlike overexpression of hVAP-A,

the overexpression of hVAP-B enhanced HCV replication, suggesting that hVAP-B is the limiting factor in the formation of the heterodimer between hVAP-A and hVAP-B, which could be the most effective form in facilitating HCV replication (Hamamoto et al., 2005). Overall, hVAP-A and likely hVAP-B play roles in targeting NS5B to the membrane and the assembly of the RC on lipid rafts (Gao et al., 2004).

The assembly of the HCV RC on membranes might also be affected by Rab5, an early endosome protein, which was found to associate with an "ER-derived membraneous web" (re-arranged membrane structure), the site of HCV replication. Rab5, which is a Ras-type small GTPase involved in membrane fusion, was found to associate with the HCV NS4B, the web-inducing protein. Reduction of Rab5 protein expression reduced HCV replication and web formation (Stone et al., 2007).

Another major group of host factors that affect the assembly of the viral RC is those proteins that affect the lipid composition of intracellular membranes used by particular (+)RNA viruses. For example, Ole1p of yeast, a Δ9 fatty acid desaturase, is known to affect the amount of unsaturated fatty acids, which are important for membrane flexibility/fluidity (Lee and Ahlquist, 2003; Lee et al., 2001). Consistent with this observation, in the *ole1*-mutant yeast, the membranes surrounding the BMV 1a-induced spherules, the site of BMV replication, were stained poorly by osmium tetroxide, which specifically binds to unsaturated fatty acids. Additional work defined that the BMV 1a was still able to induce membrane proliferation by 25% in the *ole1*-mutant yeast, but the activity of the assembled RC was reduced, likely due to the altered binding of the BMV 1a replication protein to the membrane with reduced ratio of unsaturated fatty acids (Lee and Ahlquist, 2003).

The generation of new membrane surfaces via the fatty acid biosynthesis pathway also has a major effect on DCV replication (Cherry et al., 2006). For example, depletion of the regulator of fatty acid metabolism, HLH106, and the fatty acid synthase CG3523 (performing a rate limiting step in fatty acid biosynthesis) blocked the formation of the DCV-induced vesicular compartment and altered Golgi structure. Also, carulenin, a fatty acid synthase inhibitor, blocked DCV and PV replication in tissue culture (Cherry et al., 2006). The cellular Arfs proteins also regulate the enzymatic activities of proteins involved in lipid metabolism and cytoskeleton function. Down-regulation of COPI and several genes involved in fatty acid metabolism also affected TBSV replication in yeast (Jiang et al., 2006; Panavas et al., 2005b). Altogether, these observations support the role of de novo fatty acid/membrane synthesis in picornavirus replication, where a twofold increase in total membrane surface area within infected cells has been noted.

Role of Viral Replicase Complex Assembly in Template Specificity

The elaborate assembly of the viral replicase could be an important specificity factor (a secondary, safe-guarding step behind the template selection step, see above) to prevent efficient replication of some defective viral RNAs, cellular, and/or heterologous viral RNAs. Regulation of RC assembly might ensure that efficient replication could occur only for those RNAs that contain all the required *cis*-acting elements

for the assembly of the viral replicase. Thus, RC assembly might be a "safeguard" mechanism against wasting limited viral/host components on amplification of defective viral RNA templates, which, when present in large amounts, could also trigger antiviral responses (Szittya et al., 2002).

Altogether, the assembly of functional replicase seems to be a highly regulated event during (+)RNA virus infections (Fig. 14.1, step 3). Both *cis*-acting RNA elements and *trans*-acting viral and host factors contribute to the fidelity of the assembly process, which is highly controlled in order to guarantee the presence of appropriate factors and that the assembly takes place only at the correct intracellular space and at the right time.

Step 4. The Synthesis of Viral RNA Progeny

After the assembly of the viral RC, the replicase must efficiently recognize *cis*-acting elements in the viral RNA to be able to synthesize the progeny RNA in a regulated fashion. First, the full-length minus-stranded complementary RNA is produced, then, the plus-stranded RNA progeny are synthesized in a 20- to 100-fold excess amount. Production of both (–) and (+)RNAs requires initiation of RNA synthesis at specific sites, called promoter (initiation) elements, by the viral RC either de novo (i.e., independent of primers) or via using protein primers (Dreher, 1999; Kao et al., 2001; Nagy and Pogany, 2000; Paul et al., 2000, 1998; White and Nagy, 2004). Although all (+)RNA viruses code for their own RdRp and usually one-to-six auxiliary proteins, such as helicase and methyltransferase, host-coded factors are predicted to participate in each step of RNA synthesis as described for selected (+)RNA viruses below.

Host Factors Affecting RNA Binding by the Viral RdRp

Host factors may potentially affect the conformation of the viral RdRp, which in turn could alter the template activity of the RdRp. Indeed, cyclophilin B (CyPB), a cellular peptidyl–prolyl *cis–trans* isomerase (PPIase), was found to regulate the RNA-binding ability of the HCV NS5B RdRp protein (Watashi et al., 2005). RNAi-based knock-down of the CyPB level inhibited HCV RNA accumulation by fivefold. Moreover, both a cyclosporin A and a point mutation in NS5B inhibited the interaction of NS5B with CyPB, causing a significant decrease in HCV replication. These findings argue that CyPB interaction with NS5B is critical during HCV replication.

Host Factors Affecting Minus-Strand RNA Synthesis

eEF1A, a eukaryotic translation elongation factor, was found to bind to the 3′ UTR of (+)RNA of WNV, a flavivirus, based on RNase footprinting and nitrocellulose filter-binding assays (Blackwell and Brinton, 1997; Brinton, 2001). Deleterious RNA mutations introduced at the eEF1A binding sites decreased minus-strand synthesis, strongly suggesting that eEF1A plays a critical role in WNV replication.

Moreover, eEF1A is co-localized with the WNV RC in the infected cells, indicating that eEF1A facilitates the interaction between the viral replicase and the 3′ UTR of the viral gRNA (Davis et al., 2007). Similarly, eEF1A binds to the 3′ end of Turnip yellow mosaic virus (+)RNA, which enhances translation, but represses minus-strand synthesis (Matsuda et al., 2004). The role of eEF1A in virus replication might be rather broad since eEF1A was reported to bind to TMV (+)RNA, the TMV replicase (Yamaji et al., 2006), to the NS5A replication protein of BVDV (Johnson et al., 2001), NS4A of HCV (Kou et al., 2006), Gag polyprotein of HIV-1 (Cimarelli and Luban, 1999), the TBSV RNA, and p33 replication protein (Li and Nagy, unpublished). It is also part of the RC for vesicular stomatitis virus, a negative-stranded RNA virus (Qanungo et al., 2004). Therefore, the highly conserved eEF1A could play a major role in the replication of several RNA viruses via its interactions with viral RNAs and viral replication proteins. The high abundance of eEF1A in cells might facilitate its recruitment into virus replication.

A human RNA helicase, p68, is redistributed from the nucleus to the cytosol during HCV infections due to its interaction with NS5B RdRp of HCV (Goh et al., 2004). RNAi-based depletion of p68 was found to lead to a reduction in minus-strand HCV RNA accumulation, suggesting that p68 might act as a transcription factor for HCV replication (Goh et al., 2004).

Host Factors Regulating Plus-Strand RNA Synthesis

hnRNP-C binds specifically to the 3′ end of the PV (–)RNA and to poly(U) and oligo(U) stretches in the PV RNA, via its RRM domain, and it also interacts with the replication protein 3CD (Brunner et al., 2005). Immunoprecipitation of hnRNP-C led to co-purification of both (+) and (–) PV RNAs. Although hnRNP-C is a nuclear protein, it is redistributed to the cytoplasm, probably due to PV-infection driven alterations in nucleocytoplasmic trafficking (Gustin and Sarnow, 2001). Semler et al. suggested that hnRNP-C and PV protein 2C NTPase/helicase and/or 2BC precursor bind to the PV stem-loop structure at the 3′ terminus of (–)RNA. They also proposed that hnRNP-C might recruit the 3CD to the (–)RNA template. The RNA chaperone activity of hnRNP-C could facilitate the folding of (–)RNA or the replicative dsRNA intermediate into a replication-compatible conformation that leaves the two terminal adenines exposed via binding to a 3′ proximal uridine stretch (Brunner et al., 2005).

The stress granule proteins named T-cell intracellular antigen-1 (TIA-1) as well as the TIA-1-related protein (TIAR), containing three RRM RNA-binding motifs, have been found to bind specifically to the 3′ terminal stem-loop in WNV and to the NS3 replication protein (Emara and Brinton, 2007; Li et al., 2002). Brinton et al. proposed that specific binding of TIAR and, to lesser extent, TIA-1 could promote the binding of the WNV to the minus-strand template, which could lead to efficient plus-strand synthesis. In addition to the proposed transcription factor roles of TIAR and TIA-1, the recruitment/sequestration of these proteins for WNV replication might also inhibit the formation of stress granules and P-bodies, thus potentially inhibiting the shut-off of host protein translation and mRNA degradation (Emara and Brinton, 2007).

Regulation of Asymmetrical RNA Synthesis by Host Factors

One of the hallmark features of (+)RNA viruses is the asymmetric nature of viral RNA synthesis, which leads to 10- to 100-fold more (+)RNA progeny than the (–)RNA replication intermediate (Buck, 1996). Wang and Nagy (unpublished) found that the key co-opted host protein for TBSV replication that regulates asymmetrical RNA synthesis is glyceraldehyde-3-phosphate dehydrogenase (GAPDH, or Tdh2/3p in yeast). GAPDH, which is present in the tombusvirus RC (Serva and Nagy, 2006), is a highly conserved, very abundant protein that is ubiquitous in nature (Sirover, 1999). GAPDH is a key component of cytosolic energy production, but it also displays many additional cellular activities, including roles in apoptosis, endocytosis, nuclear tRNA transport, vesicular secretory transport, nuclear membrane fusion, modulation of the cytoskeleton, DNA replication and repair, maintenance of telomere structure, and transcriptional control of histone gene expression (Sirover, 1999, 2005). GAPDH also binds to various RNAs, such as AU-rich sequences at the 3′ terminus of mRNAs, which can lead to stabilization of the RNA in the cell (Bonafe et al., 2005). Interestingly, down-regulation of GAPDH inhibited TBSV replication in yeast and in plants and resulted in the production of (+) and (–)RNAs in a 1:1 ratio, instead of the hallmark asymmetric RNA synthesis. Thus, the replication of TBSV became double-stranded RNA virus like when only a limiting amount of GAPDH was present in the viral RC. Moreover, GAPDH was relocalized from the cytosol to the peroxisomal membrane surface, the site of TBSV RNA synthesis. Based on in vitro and in vivo data, it has been proposed that GAPDH promotes asymmetric RNA synthesis by selectively retaining the (–)RNA template in the viral RC, thus allowing efficient access of the (–)RNA replication intermediate to the viral RC. On the contrary, (+)RNA progeny, which are not bound by GAPDH, get released from the RC into the cytosol (Wang and Nagy, unpublished). Thus, a cellular metabolic enzyme can regulate asymmetric viral RNA synthesis, explaining this hallmark feature of (+)RNA viruses.

Regulation of Subgenomic RNA Synthesis by Host Factors

A number of (+)RNA viruses express a set of their genes via producing sgRNAs from the gRNA (via a minus-stranded intermediate) (Lin and White, 2004; Pasternak et al., 2006, 2001; Sawicki et al., 2007; Snijder, 2001; White, 2002). In addition to the viral replication proteins, host proteins are also predicted to participate in regulation of sgRNA synthesis. Accordingly, Hardy and his colleagues found that hnRNP-K, a predominantly nuclear poly(C)-binding protein with 3 KH RNA-binding domains, co-precipitated with the SIN sgRNA, but not with the gRNA (Burnham et al., 2007). hnRNP-K also interacted with SIN nonstructural proteins and it was redistributed to the membrane fraction and co-localized with the SIN RC in infected cells. Interestingly, the amount of mitogen-activated protein kinase (MAPK) is up-regulated in SIN-infected cells and MAPK-based phosphorylation of hnRNP-K can lead to cytosolic accumulation of hnRNP-K, suggesting that the MAPK pathway could be involved in the re-localization of hnRNP-K for SIN

replication. Overall, the interaction of hnRNP-K with the sgRNA and the SIN nonstructural proteins might lead to up-regulation of the sgRNA synthesis that produces ~fourfold more sgRNA than gRNA (Burnham et al., 2007).

Step 5. Release of Viral RNA from Replication

After completion of the new (+)RNAs, they must be released from replication in order to participate in additional functions, such as new rounds of translation, replication, or packaging (Fig. 14.1, step 5). Time point studies with TBSV RNA revealed that a significant portion of the (+)RNA was associated with the sites of replication at an early time point (12 hours), while most (+)RNA was cytosolic, thus released from replication, at a later time (48 hours) (Panavas et al., 2005a). On the other hand, the (–)RNA was associated with the p33 replication protein on the peroxisomal membrane surface at both early and late time points (Panavas et al., 2005a). These observations suggest that the release of RNA progeny from replication is a highly regulated event.

Unfortunately, the escape mechanism of viral (+)RNA from replication is currently not known. It has been proposed that post-translational modification, such as phosphorylation, of host or viral proteins within the RC might play a role. For example, phosphorylation of serine/threonine residues in the vicinity of the RNA-binding domain of the TBSV p33 replication protein was shown to reduce RNA-binding capacity of p33 (Shapka et al., 2005; Stork et al., 2005). If phosphorylation takes place reversibly, then the same RC could release the viral (+)RNA progeny (Fig. 14.1, step 5), followed by new rounds of RNA synthesis and release. The cellular kinase involved in phosphorylation of TBSV p33 might be protein kinase-C (PKC) like, based on in vitro experiments. Overall, the above model does not exclude that other processes, such as replicase disassembly might also play a role in viral (+)RNA release.

Step 6. Disassembly of the Viral Replicase

The viral RC likely becomes inactivated and goes through disassembly at the end of replication (Fig. 14.1, step 6). Also, disassembly of the RC might promote the release of the (+)RNA progeny from replication. Phosphorylation and/or additional post-translational modifications might be involved in RC disassembly. A genome-wide screen in yeast identified host genes that could affect the ubiquitination pathway, such as *BRE1* and *RAD6* (Panavas et al., 2005b). Thus, ubiquitination of replication proteins may alter the stability of the virus RC. Also, it has been postulated that an unidentified cellular kinase that phosphorylates the HCV NS5A protein could inhibit its interaction with hVAP-33, thus leading to disassembly of the HCV RC (Evans et al., 2004). Subsequent work identified the human p70S6K and related

kinases as the likely candidates for hyperphosphorylation of NS5A (Coito et al., 2004). Overall, our current knowledge is poor about RC disassembly.

Future Directions

In spite of recent major efforts in studies of host–virus interactions and viral replication, our knowledge in many areas is still incomplete. However, the application of systems biology approaches and the availability of complete sequences for many host genomes, in combination with development of in vitro approaches and yeast as a model host, will likely lead to rapid advances in identification and characterization of host factors involved in (+)RNA virus replication. The combined use of genetics, biochemistry, and cell biology will help dissect the detailed functions of subverted host proteins. Also, proteomics-based analysis of the viral RC is expected to lead to identification of host proteins recruited into (+)RNA virus replication. Determination of 3D structures of viral RdRp and the auxiliary replication proteins with bound RNAs, as well as high resolution imaging of viral RCs, will likely unravel the mechanism and regulation of (+)RNA replication. Proteomics approaches should also accelerate identification of various post-translational modifications of viral and host proteins that could affect and/or regulate their functions during the replication process. These advances will lead to a better understanding of virus replication and host:(+)RNA virus interactions, which are key aspects of viral pathogenesis.

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