Chapter 5
Brome Mosaic Virus RNA Replication and Transcription

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Introduction

Brome mosaic virus (BMV) was first isolated in 1942 from bromegrass (*Bromus inermis*) and has since been documented to infect several monocot and dicot species studied in the laboratory. The impact of BMV, however, is not so much as a plant pathogen, but as a model for in-depth studies of the infection process of positive-stranded RNA viruses. As such, BMV is responsible for several firsts. (1) BMV was among the first to be translated using a cell-free system (Shih and Kaesberg, 1973), allowing studies of cap-dependent translation. (2) The BMV genome was one of the first RNA viruses for which the entire sequence was determined (Ahlquist et al., 1981, 1984a). (3) BMV was the first plant virus to be regenerated from transcripts derived from infectious cDNAs (Ahlquist and Janda, 1984; Ahlquist et al., 1984b). (4) The BMV replicase could be produced from membranes of infected plants and accept exogenously supplied transcripts for RNA synthesis, enabling the dissection of the mechanism of viral RNA synthesis (Hardy et al., 1979). (5) Recombinant BMV proteins were first demonstrated to direct replication and transcription of BMV RNA replicons in *Saccharomyces cerevisiae* (Janda and Ahlquist, 1993), allowing in-depth probing of the requirements of the host.

With regard to RNA replication, the topic of this chapter, extensive effort has been focused on the characterization of the *cis*-acting sequences, the identification of the host proteins, the assembly of the replicase, and the mechanism of RNA-dependent RNA synthesis. After an introduction in the basics of BMV molecular biology, this chapter will emphasize progress in these areas.

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BMV RNAs and Replication Proteins

BMV belongs to Bromoviridae family, member of the alphavirus-like superfamily of animal- and plant-infecting viruses. The BMV genome is composed of three mRNA-sense RNAs that are encapsidated separately, named RNA1, RNA2, and RNA3 (Fig. 5.1). RNA1 and RNA2 are monocistronic and encode replication-associated proteins 1a and 2a, respectively. RNA3 is dicistronic and encodes the movement protein 3a and the capsid protein. The capsid protein is translated from a subgenome-length RNA named RNA4 (Fig. 5.1B).

The 5′ untranslated regions (UTRs) of BMV RNA1 and RNA2 share the conserved motif of the TψC loop of tRNA, named box B, which is also found in the RNAs of other bromoviruses (Dzianott and Bujarski, 1991; Romero et al., 1991; Ahlquist, 1992). In BMV RNA3, the box B is located in the intercistronic regulatory region between the movement protein and the capsid coding sequence. In addition, the intercistronic region contains an 18–21 nucleotide oligo(A) tract, a replication enhancer and subgenomic promoter (Ahlquist et al., 1981; French and Ahlquist, 1987; Adkins et al., 1997; Fig. 5.1B). The 3′UTRs of all three BMV RNAs lack a poly(A) tail, but contain a highly conserved and tRNA-like structure, as determined by enzymatic probing and computer modeling (Perret et al., 1989; Rietveld et al., 1983; Felden et al., 1994, 1996).

The replication scheme used by BMV is fairly typical for a plus-strand RNA virus (Fig. 5.1C). After entry into the cell and translation from the BMV RNAs, the

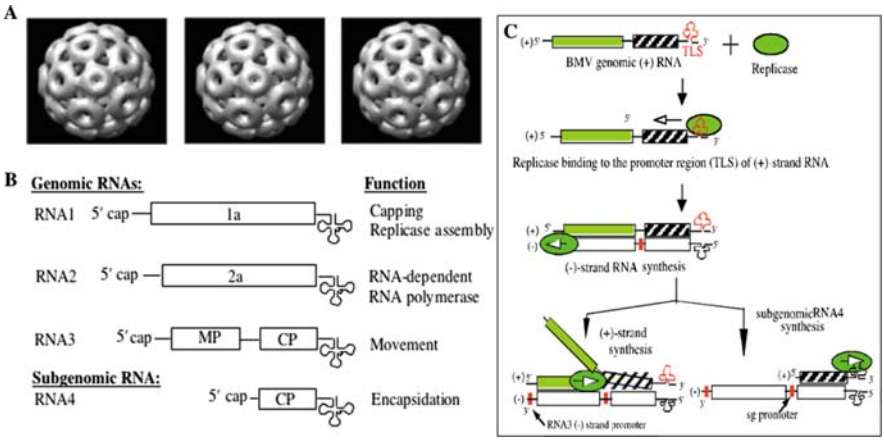

Fig. 5.1 Basic information on BMV. (**A**) Structures of the BMV virion; each virion packages one genomic RNA. The images are reconstructed from negative-stained BMV particles using the EMAN software (Sun et al., 2007). (**B**) Schematics of the BMV genomic RNAs, the subgenomic RNA4, and the functions encoded by each RNA. The cloverleaf denotes a tRNA-like structure present at the 3′ terminus of all BMV positive-strand RNAs. (**C**) Schematic of the BMV replication and transcription mechanism. The locations of the core promoters are in *red*. The BMV replicase is shown as a *green ball* and its direction of movement is denoted with an *arrow*.

1a and 2a protiens and unidentified cellular factors form the BMV replicase. The complex assembles in plant cellular membranes and will bind specifically to the BMV genomic plus-strand RNAs to first synthesize a complementary minus-strand RNA, which then serves as the template for genomic plus-strand RNA synthesis (Fig. 5.1C). The minus-strand RNA3 can also direct subgenomic RNA4 synthesis. RNA4 thus provides a simple means to allow RNA3 to be a dicistronic RNA without breaking rules for cap-dependent translation of both the movement and capsid proteins. Many of the requirements for BMV RNA replication and transcription have been elucidated.

1a and 2a are central players in BMV RNA synthesis. Cells transfected with only RNA1 and RNA2 can replicate in the absence of RNA3 or RNA4 transcription (Gopinath et al., 2005; Annamalai and Rao, 2005; French and Ahlquist, 1987). Thus, the movement and the capsid proteins encoded by BMV RNA3 are not essential parts of the BMV replicase. However, the normal accumulation of molar excesses of BMV plus-strand RNAs relative to the minus-strand RNAs is not observed in the absence of RNA3 (Marsh et al., 1991; Gopinath et al., 2005).

BMV 1a is a multifunctional protein. The N-terminal portion of 1a has a predicted secondary structure that is highly similar to known DNA and RNA methyltransferases, while the C-terminal portion contains helicase-like motifs (Ahlquist et al., 1985; Ahola and Ahlquist, 1999; O'Reilly et al., 1998). The N-terminal 516 residues have been demonstrated to have RNA capping-associated activities, including the ability to form a covalent complex and methylate a guanine nucleotide at the N7 position (Ahola and Ahlquist, 1999; Kong et al., 1999). RNA helicase activity of 1a has not been demonstrated biochemically. However, the C-terminal 424–961 amino acids of 1a, including the NTPase/hel domain, has ATPase activity (Wang et al., 2005). Mutations in both parts of the 1a protein have led to defects in BMV RNA replication.

The BMV 2a protein is an RNA-dependent RNA polymerase. 2a has a large central domain that contains all the hallmark motifs of an RNA-dependent RNA polymerase, flanked by less conserved N- and C-termini (O'Reilly and Kao, 1998; Traynor et al., 1991). The N-terminal domain of 2a interacts with the 1a protein while the C-terminal domain is dispensable for replication in protoplasts (Traynor et al., 1991). Recombinant 2a protein has recently been demonstrated to direct RNA synthesis in vitro (Wierzchoslawski and Bujarski, 2006). As with most recombinant viral RdRps, the most robust activity of 2a is in extending from a primed template. This does not mean that primer extension is the preferred mechanism of initiation by 2a, however. In fact, positive-strand BMV RNAs are capped, and the substrate for the capping reaction is a de novo initiated RNA (Kao et al., 2001).

There is abundant evidence that the BMV 1a and 2a proteins function by forming a complex. A complex is observed when 1a and 2a are expressed in rabbit reticulocytes (Kao et al., 1992; Kao and Ahlquist, 1992) and in yeast in the form of the two-hybrid assay (O'Reilly et al., 1997). 1a and 2a also co-purified with enzymatically active BMV replicase (Quadt et al., 1988) and are co-localized in the endoplasmic reticulum of plant cells, the site of BMV RNA synthesis (Restrepo-Hartwig and Ahlquist, 1996, 1999).

The interaction between 1a and 2a is species specific, as determined by examining the replication of homologous and heterologous combinations of BMV 1a and 2a and the orthologs from the closely related bromovirus, cowpea chlorotic mottle virus (Dinant et al., 1993; O'Reilly et al., 1995, 1997). Attempts to overexpress the 1a protein from the 35S Cauliflower Mosaic virus promoter can lead to the inhibition of both 1a and 2a expression in *Nicotiana benthamiana*. The inhibition acts through box B in the 5′ untranslated region of BMV RNA1 and RNA2. RNA3 is not regulated by 1a since it lacks a box B in its 5′UTR (Gopinath et al., 2005; Yi et al., 2007). These results suggest that there are mechanisms in place for BMV to regulate the translation of the replication proteins and that unregulated production may be detrimental to efficient BMV infection, perhaps through innate host responses.

Properties of the BMV Replicase

Viral nucleic acids are targets for the host innate defense systems (Meylan and Tshopp, 2006). Hence, it is likely that the process of viral RNA replication will include a number of mechanisms that are in place to prevent recognition by the host. For example, double-stranded RNA viruses, minus-sense RNA viruses, and retroviruses replicate within some form of the viral particles, the site where replication-associated enzymes have been cached (Jayaram et al., 2004). However, positive-stranded RNA viruses, due to the need for translation to precede replication, must expose their RNA. To accommodate this exposure as well to protect the viral genome, RNAs are redirected to cellular membranes once the replication proteins are available.

Indeed, for all the positive-strand RNA viruses, RNA replication is associated with intracellular membranes (Schaad et al., 1997). BMV RNA replication occurs on the perinuclear region of the endoplasmic reticulum (ER), both in its natural plant host and in the surrogate host *S. cerevisiae* (Restrepo-Hartwig and Ahlquist, 1996, 1999). The replication protein 1a is the primary viral protein determinant for the subcellular localization of the BMV replication complex (Fig. 5.2). 1a can localize to the cytoplasmic face of ER membranes in the absence of other viral factors and induces spherules serving as replication compartments sequestering viral positive-strand RNA templates in a nuclease-resistant, detergent-susceptible state (Restrepo-Hartwig and Ahlquist, 1999; Schwartz et al., 2002). Membrane flotation gradient analysis with wild type 1a and deletion mutants showed that the sequences in the N-terminal RNA capping domain of 1a mediate membrane association (den boon et al., 2001). In the absence of 1a, 2a is present in the cytoplasm in diffused distribution or in punctate spots that are not apparently associated with cytoplasmic organelles. Thus 1a appears to bring 2a into the membrane-associated replicase complex. Indeed, the interaction between the N-terminus of 2a and the C-terminal helicase-like domain of 1a leads to the formation of double-membrane layers (Chen and Ahlquist, 2000). The expression level of 2a polymerase can also modulate 1a-induced membrane rearrangements (Schwartz et al., 2004). Using monoclonal antibodies raised against the 1a and 2a proteins, it was shown that the region between

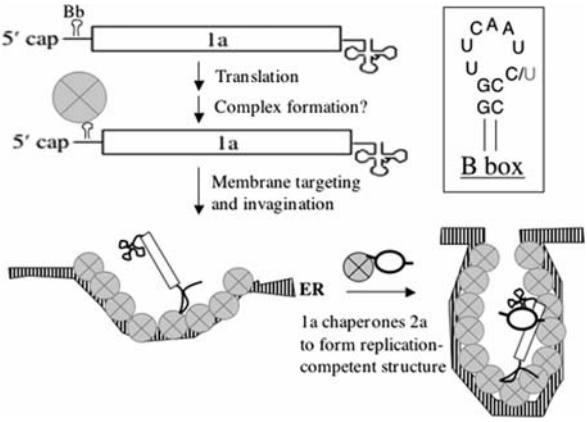

Fig. 5.2 A schematic for the assembly of the BMV replicase. The 1a protein is represented as a sectored *circle*. This complex could involve 1a–1a interaction and/or binding to cellular proteins. The 2a protein is shown as an open oval with thick lines denoting the N- and C-terminal domains. The inset shows the sequence of the Box B, which is identical in RNA1 and RNA2. For RNA3, there is a conserved change of the seventh residue in the loop from a C to a U.

the N-terminal methyltransferase domain and the C-terminal helicase-like domain of 1a and the N-terminus region of 2a protein are exposed on the surface of the solubilized replicase complex. (Dohi et al., 2002).

The capping domain and NTPase/helicase-like domain of 1a contribute to RNA templates recruitment in the formation of the BMV replicase. Mutations in the capping enzyme active site cause defects in template recruitment, negative-strand RNA synthesis (Ahola et al., 2000). Mutations in the helicase motifs in the 1a protein severely inhibited RNA replication and reduced the stability of RNA3, although they did not affect 1a accumulation, localization to perinuclear ER membranes, or recruitment of 2a polymerase (Wang et al., 2005).

The replicase must specifically recognize the viral RNAs. This interaction has been partially elucidated using the yeast system. The 1a replication protein has been demonstrated to recognize the BMV genomic RNA2 and RNA3 through the box B, then recruit the RNAs from translation to replication. RNA1 is likely recognized in a manner similar to RNA2, as they share an identical box B RNA in their 5′UTRs. In the absence of 2a, 1a can induce the association of RNA2 or RNA3 with cellular membrane by the intercistronic sequence (Janda and Ahlquist, 1998; Sullivan and Ahlquist, 1999; Schwartz et al., 2002). The RNA2 5′UTR was sufficient to confer 1a-induced membrane association although sequences in the N-terminal region of the 2a open reading frame could enhance 1a responsiveness (Chen et al., 2001). In addition to recruitment of the RNA presumably through binding the box B, 1a can also recruit RNA2 through its interaction with the N-terminal portion of 2a, presumably when the 2a is being translated from RNA2 (Chen et al., 2003).

The viral RNA is an active participant in the assembly of replicase complex. In yeast, coexpression of BMV RNA3 was required for functional BMV RNA-dependent RNA polymerase activity (Quadt et al., 1995). Deletion analysis

showed that the tRNA-like 3′UTR and the intercistronic region are minimally required for in vivo formation of functional RNA-dependent RNA polymerase. This RNA may contribute by recruiting essential host factors to participate in replicase assembly (Quadt et al., 1995). The mechanism of replicase assembly in BMV RNA1 and RNA2 may differ from that of RNA3, since they lack the intercistronic region. Once assembled, the functional BMV replicase does not need to contain RNA, as the RNA is not present in biochemically active preparations of the BMV replicase (Sun et al., 1996), suggesting that once the replicase assembles, a functional complex can be maintained through protein–protein or protein–membrane interaction.

In yeast, the 1a protein can significantly increase the stability of BMV RNA3 by binding to the intercistronic region of RNA3 (Janda and Ahlquist, 1998; Sullivan and Ahlquist, 1999). This requirement is not observed in *N. benthamiana* or barley protoplasts (Gopinath et al., 2005), indicating either that the replicases formed in yeast and plants have distinct properties or that the host degradation pathways have different access to the RNA in plants and in yeast. While this may seem counter-intuitive at first glance, an emerging theme in virus replication is that the virus is far more adaptable than one may expect. For example, flock house virus, a positive-stranded virus of insect cells, was found to replicate perfectly well in two distinct membrane locations when the membrane targeting signal sequence was altered (Miller et al., 2003).

BMV RNA Motifs and the Modes of RNA Replication and Transcription

Efficient viral RNA synthesis requires specific and coordinated interactions between the template RNA and the viral replicase, a membrane-associated complex of viral replication proteins and host-encoded factors (Kao et al., 2001; Lai, 1998). The recognition is likely to be quite complex because an RNA virus not only will express different classes of RNAs [i.e., genomic plus-strand RNA, genomic minus-strand RNA, and possibly subgenomic RNA(s)], but will do so at regulated levels and times (Buck, 1996).

Using a combination of approaches, including genetic analysis in plant protoplasts and a template-specific BMV replicase that can be extracted from BMV-infected plants, the sequences and motifs that can efficiently direct BMV RNA synthesis have been identified (Choi et al., 2004). The replicase-binding sequences are called ‘core promoters’ since they can bind the BMV replicase and direct the initiation of RNA synthesis. As is the case with core promoters for transcription from DNA templates, the viral core promoters direct a basal level of RNA synthesis that can be modulated by positive- and negative-acting sequences (Lai, 1998).

Genomic Minus-Strand Promoter

The promoter for minus-strand RNA synthesis is within the tRNA-like 3′ sequence (Dreher and Hall, 1988a,b). These secondary structures interact with each other to

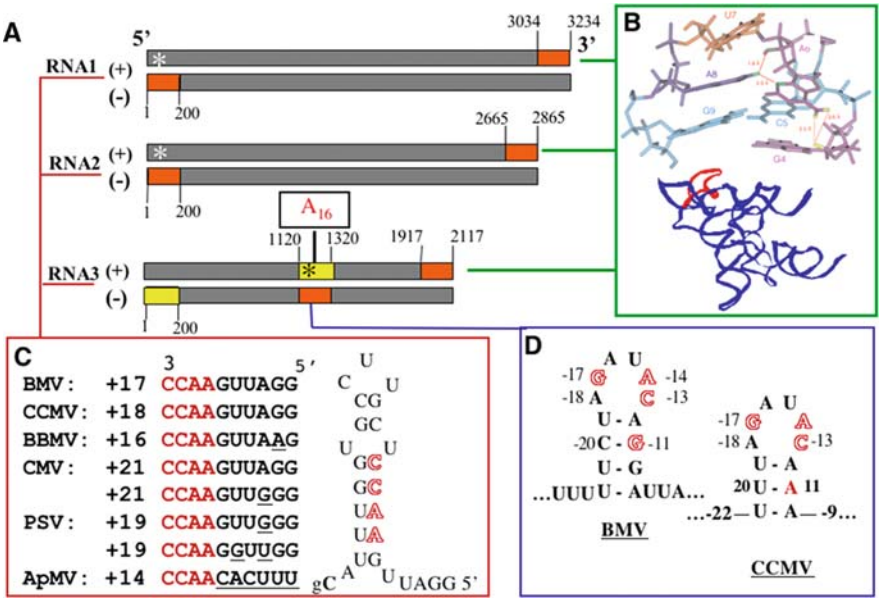

Fig. 5.3 A summary of the locations and relevant features of the three classes of BMV core promoters. (**A**) The locations of the replicase-binding RNA sequences within positive- and negative-strand BMV RNAs. (**B**) Structure of the clamped adenine motif of BMV RNA that directs the initiation of BMV minus-strand RNA synthesis. The lower structure denotes the approximate structure of the tRNA-like structure with SLC in *red*. The upper structure was determined by NMR and shows the essential features that contribute to the formation of a clamped adenine motif. (**C**) The core promoter for genomic plus-strand RNA synthesis. A conserved sequence complementary to the Box B that contains a CCAA motif is *highlighted*. The conservation of the CCAA sequence in related members of the Bromoviridae is listed along with the NMR-derived secondary structure of the RNA that can direct genomic plus-strand RNA synthesis. The nontemplated nucleotide required for genomic plus-strand RNA synthesis is in a lower case "g". (**D**) A summary of the essential residues for replicase binding in the BMV subgenomic promoter. The critical residues are in *outlined letters*. The sequence can fold into a quasi-stable hairpin in both BMV and the related virus, CCMV (cowpea chlorotic mottle virus).

contribute to mimicry of the tRNA-like tertiary structure needed for aminoacylation of the 3′ termini of BMV and CMV RNAs (Fig. 5.3B; Felden et al., 1993; Giege' 1996). A notable exception to the tRNA-like tertiary structure is a complex stem-loop named SLC. Mutations in SLC can severely reduce BMV and CMV replication in protoplasts (Dreher and Hall, 1988a; Rao and Hall, 1993) and SLC was sufficient to interact with the BMV replicase in vitro in the absence of the remainder of the tRNA-like sequence. SLC fused to the 3′ terminal 8 nt of the 3′-terminus of the tRNA-like sequence resulted in an RNA that could direct RNA synthesis in vitro (Fig. 5.3; Chapman and Kao, 1999). Mutations to SLC+8 that decreased BMV RNA replication in vivo have parallel effects on RNA synthesis in vitro (Chapman and Kao, 1999).

The solution structure of the BMV SLC was determined by NMR spectroscopy and found to be composed of two stems, separated by a flexible internal bulge

(Fig. 5.3B). The bulged portion of the RNA was dynamic (Kim et al., 2000). Related viruses also possess a bulged sequence, suggesting that a dynamic motif here in the bulge may be preferred for viral RNA replication. The terminal stem contains a tri-nucleotide loop (5′AUA3′) that is specifically required for interaction with the replicase (Kim et al., 2000). The tri-loop was found to fold into a highly ordered structure called a clamped adenine motif (CAM), with the 5′-most adenine of the tri-loop projected into the solution, primarily due to the base stacking interactions between the 3′-most adenine and the stem-closing C–G base pair (red nucleotides, Fig. 5.3B; Kim et al., 2000). A network of electrostatic interactions also stabilizes the solution-exposed 5′-adenine. Variations of the terminal loop nucleotides that were unable to form a CAM failed to direct efficient RNA synthesis by the BMV replicase (Kim and Tinoco, 2001).

A change of the 3′-most adenine of the tri-loop (5′AUA3′) to a guanine (5′ AUG 3′) resulted in wild-type levels of RNA synthesis. This change should disrupt the normal CAM. However, when the solution structure of an RNA containing the 5′AUG3′ tri-loop (the mutated nucleotide is underlined) was solved using NMR, it was found to form a dramatically altered structure that still retained a solution-exposed and clamped adenine (Kim and Kao, 2001). These studies reveal the features in the RNA core promoter required for recognition by the BMV replicase for minus-strand RNA synthesis in vitro and in vivo.

Genomic Plus-Strand Promoter

BMV genomic plus-strand RNA synthesis in vitro required an adjacent stem-loop with a short single-stranded sequence with nontemplated 3′ nucleotide (Fig. 5.3C; Sivakumaran and Kao, 1999; Sivakumaran et al., 1999). The replication of the related CMV satellite RNA also requires a nontemplated nucleotide (Wu and Kaper, 1994). Nontemplated nucleotide addition is a common property of cellular and viral polymerases (Kumar et al., 2001; Siegel et al., 1997). Therefore, the addition to BMV minus-strand RNA might be by the BMV replicase or a cellular enzyme. Furthermore, since the initiation of minus-strand RNA synthesis occurs from the penultimate nucleotide (Miller et al., 1986; Sun et al., 1996), the requirement may reflect a structural requirement for the BMV polymerase. In the ternary structure of the RdRp from bacteriophage ϕ6, a nontemplated nucleotide was required to allow proper contact between the active site and the initiation nucleotide (Bamford et al., 2005).

In addition to the 3′ initiation cytidylate, a highly conserved ca. 9 nt sequence called the cB box exists at the 5′-end of the BMV genomic core promoter from RNA1 and RNA2 (Fig. 5.3C). The cB box is found in RNA1 and RNA2 of all of the Bromoviridae except for most ilarviruses and the alfalfa mosaic virus (Sivakumaran and Kao, 2000). The cB box is complementary to the Box B that is required for replicase assembly (French and Ahlquist, 1987; Marsh and Hall, 1987; Pogue and Hall, 1992; Chen et al., 2003). In positive-strand BMV RNAs, box B is usually positioned upstream of the protein-coding sequence and interacts with the 1a protein in a way that increases the stability of the RNA (Sullivan and Ahlquist, 1999). The cB

box appears to be required in a position-specific manner; moving it one nucleotide closer to the 3′ initiation site severely reduced RNA synthesis (Sivakumaran et al., 2000). However, moving the cB box one nucleotide 5′ of its original position was less detrimental to RNA synthesis (Sivakumaran et al., 2000), indicating that some flexibility in the RNA can be used to correctly position box B relative to the replicase subunits.

The synthesis of RNA3 from BMV genomic RNA3 may be different than for other genomic RNAs. For BMV RNA3, the cB box exists in the intercistronic region, over a kilobase from the initiation site for genomic plus-strand RNA synthesis. Since RNA3 is replicated in trans by the viral replicase, it is possible that RNAs that do not encode a subunit of the replicase have different replicase recognition requirements than RNAs that can be translated to provide a subunit of the replicase.

Subgenomic Promoter

A 20 nt 3′ of the initiation cytidylate for BMV RNA4 is sufficient for an accurate initiation of RNA synthesis in vitro (Adkins et al., 1997). Additional sequences 3′ of the core promoter does affect RNA synthesis (Adkins et al., 1997; French and Ahlquist, 1988; Marsh et al., 1988), but does not influence the selection of the initiation site. Single-nucleotide changes identified that positions −11, −13, −14, and −17 relative to the +1 initiation cytidylate were required for efficient RNA synthesis (Fig. 5.3D). While some other positions within this 20 nt sequence also contributed to the level of RNA synthesis, changes at these four positions decreased RNA synthesis by up to 10-fold (Siegel et al., 1997) (Fig. 5.3D). These results suggest that the BMV subgenomic core promoter may be recognized in an RNA sequence-dependent manner, a mechanism similar to the recognition of DNA promoters by DNA-dependent RNA polymerases (Adkins et al., 1998). Consistent with this, RNAs containing nucleotide analogues at these four positions, some of which should retain normal base pairing potential of these nucleotides, significantly decreased RNA synthesis (Siegel et al., 1998). Moieties in these nucleotides of the BMV core promoter are specifically required for RNA synthesis in vitro.

Haasnoot and colleagues proposed that a stable secondary structure exist in the core promoter (Haasnoot et al., 2000). This structure is not required for RNA synthesis by the BMV replicase in vitro, but mutations that prevented stem-loop formation did reduce BMV subgenomic RNA synthesis in protoplasts (Sivakumaran et al., 2004). The stem and specific nucleotide within presumably will bind the replicase to direct the recognition of the initiation cytidylate. Whether the subgenomic core promoter is recognized after the synthesis of the full-length minus-strand RNA or can be recognized during minus-strand RNA3 synthesis is unknown.

The characterization of the three classes of core promoters from both BMV and CMV revealed some similarities and differences in replicase-promoter interaction. In general, each core promoter contains three features that are, to different extents, required for RNA synthesis. First, there is a specificity determinant (SLC

for genomic minus-strand initiation, the cB box for genomic plus-strand initiation of RNA1 and RNA2, the nucleotides upstream of the initiation site for the subgenomic promoter). It is not known whether viral or cellular subunits within the replicase interact with the specificity determinants. A second required element is the initiation site, which includes the initiation cytidylate and a few neighboring nucleotides. The viral RdRp subunit within the replicase must recognize this site, since the RdRp polymerizes nucleotides. In addition to the RdRp–initiation site interaction, the base pairing between the initiation cytidylate and the substrate GTP could contribute to specificity for initiation. Specificity recognition of the initiation complex was observed in the ternary crystal structure of the recombinant RdRp from bacteriophage ϕ6 (Butcher et al., 2001) and with a functional analysis of the template used by the recombinant RdRps from members of the Flaviviridae (Kim et al., 2000). A third requirement is the template sequence immediately following the initiation cytidylate, which can apparently alter the level of synthesis, perhaps by regulating the efficiency of the replicase transition from initiation to elongation. Plus-strand RNA viruses in the Bromoviridae generally have at least three nucleotides after the initiation cytidylate that will weakly base pair with the nascent RNA. Templates for minus-strand synthesis do not follow this trend (Table 5.1), perhaps reflecting a role in regulating the level of RNA produced.

Despite the three general requirements, there is some fluidity in each of the requirements, because several changes in each core promoter can be tolerated. Even a change of the initiation cytidylate to a uridylate can result in RNA synthesis at about 5% of wild type. This fluidity suggests that extensive molecular communication occurs between the RNA and the replicase to allow some adjustments by conformational changes (induced fit) in the interactions that lead to productive synthesis (Williamson, 2000). An induced fit mechanism provides the best explanation for the recognition of some variants of the BMV subgenomic promoters by the BMV replicase (Stawicki and Kao, 1999) and the cross-recognition of some core promoters between the replicases of BMV, CMV, and Cowpea chlorotic mottle virus (Adkins and Kao, 1998; Chen et al., 2000; Sivakumaran et al., 2000).

Replication Mechanism

The recognition of the core promoters is only the first step in successful BMV RNA replication. This process was studied in detail using the BMV replicase. Overall, the process of BMV RNA synthesis can be divided into several biochemically defined steps, consisting of initiation, abortive initiation, template commitment, elongative RNA synthesis, and termination (Fig. 5.4).

Initiation by the BMV replicase is perhaps the most distinct aspect of viral RNA replication since the nature of the linear viral templates requires that initiation take place at or near the 3′ terminus of the template. For the BMV RNA synthesis, a cytidylate penultimate to the 3′ nucleotide is preferred. The cytidylate is recognized in a sequence-specific manner and is paired with the initiation GTP or GTPi. The recognition of the GTPi has additional requirements in comparison to

Table 5.1 Host factor implicated in BMV replication

Host factors	Function	Effects on BMV replication	References
eIF3	Translation elongation factor	Stimulate (–)-Strand RNA synthesis	Quadt et al. (1993)
DED1	Translation factor	Translation of 2a RNA polymerase	Noueiry et al. (2000)
Lsm1	mRNA turnover	Recruiting RNA from translation to replication	Diez et al. (2000), Noueiry et al. (2003)
YDJ1	Chaperone	(–)-Strand RNA synthesis and assembly replicase complex	Tomita et al. (2003)
OLE1	Fatty acid desaturase	RNA replication	Lee et al. (2001)
Pus4	Pseudouridine synthase	Multiple effects on translation, replication, and systemic infection	Zhu et al. (2007)
App1	Intracellular trafficking	RNA replication and systemic infection	Zhu et al. (2007)
Lsm1p-7p Pat1p/Dhh1p	Decapping cellular RNA and RNA turnover	Recruit RNA for replication	Nouseiry et al. (2003), Mas et al. (2006)
mab1-1, *mab2-1* and *mab3-1*		RNA3 and subgenomic RNA4 replication	Ishikawa et al. (1997)
SIF2 and ~100 host factors in total		Regulate BMV RNA replication and translation	Kushner et al. (2003)

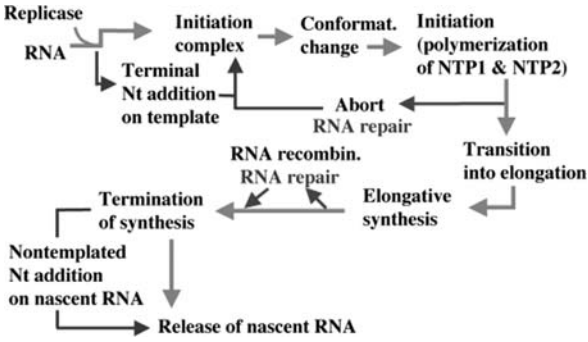

Fig. 5.4 A summary of the steps in the RNA synthesis by BMV replicase. The sequence of events proceeds from the *upper right* according to the direction of the *arrows*.

the recognition of GTP late in RNA synthesis. For example, the GTPi is required at ∼15-fold higher concentration for initiation than for elongation (Sun et al., 1996). The GTPi can also be replaced with oligonucleotide primers as long as the primers can maintain base pairing to the initiation cytidylate in the template (Kao and Sun, 1996). Similar requirements are found for several RdRps from the Flaviviridae family (Ranjith-Kumar et al., 2003), demonstrating that this is a basic property of the initiation process by RNA virus polymerases. The presence of the template and the GTPi will increase the stability of the binding by the replicase, preventing inhibition by template mimics such as heparin (Sun and Kao, 1997a,b). In fact, the stability is increased stepwise with the number of nucleotides in the initiating RNA, suggesting that the polymerase will undergo a series of transitions that lead to a productively synthesizing complex.

The transition from initiation and commitment to the template by the BMV replicase is marked by the formation of abortive initiation products (Fig. 5.4). Abortive initiation products are formed by the replicase and released before the replicase transitions to productive elongative synthesis (Carpousis and Gralla, 1980). They are typically present at molar excesses of the full-length products and, for the BMV replicase, range from 2 to 12 nt in length. Interestingly, the abundance of the abortive products decreases after 8 nt, suggesting that the ternary complex is committing to elongation at or shortly after the synthesis of a nascent RNA of 8 nt. Abortive products could have additional roles in the repair of the ends of the RNA (perhaps giving an advantage to viruses with multi-partite genomes that share common 3′ sequences). Rapid repair of short deletions in the 3′-end of the BMV genomic RNAs was observed (Hema et al., 2005).

Much less is known about the regulation of elongation and termination of BMV RNA synthesis. A basic residue in the template will trap the BMV ternary complex depending on its location. When present within the first ten nucleotides from the 3′ termini, the replicase can reinitiate. However, when present later in the template, the replicase is unable to reinitiate (Picard et al., 2005). With regard to termination, the sequence from −4 to −2 of the position from the very 3′ terminus of the template could regulate the proper termination of nascent RNA synthesis; nucleotides that allow stronger base pairing tend to promote the synthesis of a full-length RNA while nucleotides that have weaker base pairing tend to decrease the proportion of

full-length RNA synthesis and increase premature termination (Tayon et al., 2001). These steps in BMV RNA synthesis are of interest relative to the mechanism of transcription by DNA-dependent RNA polymerases. A comparison of the requirements for the steps is in Adkins et al. (1998).

Host Factors That Affect BMV Replication

Successful viral replication requires proper interaction between viral and cellular factors. Early in vitro experiments have shown that translation elongation factor EF-1A could bind to the 3′tRNA-like structure of BMV RNA1, although its function in RNA replication remains unclear (Bastin and Hall, 1976). The translational factor eIF3 was co-purified with BMV RNA replicase (Table 5.1; Quadt et al., 1993).

The identification of host factors that regulate BMV replication was accelerated by the ability of BMV to replicate in *S. cerevisiae* (Janda and Ahlquist, 1993; Noueiry and Ahlquist, 2003; Sullivan and Ahlquist, 1997). An initial screen using the mutant yeast strains has identified a number of host factors involved in cellular RNA degradation and fatty acid metabolism, such as Lsm1p-7p and OLE1 that are involved directly or indirectly in regulation of BMV replication complex assembly, template recruitment for replication, or the process of RNA synthesis. A summary of the host factors is presented in Table 5.1. Several are involved in the specific steps of BMV replication. For example, mutation of host gene Lsm1p, which encodes a protein involved in mRNA turnover and other processes, resulted in defects in an early template selection step of BMV RNA replication (Diez et al., 2000). In addition to Lsm1p, all tested components of the Lsm1p-7p/Pat1p/Dhh1p decapping activator complex, which functions in deadenylation-dependent decapping of cellular mRNAs, were required for BMV RNA recruitment (Mas et al., 2006). Additional factors have been identified to be involved in BMV RNA replication, but their exact contributions to BMV-specific processes remains to be determined.

Yeast proteome chips have also been used to identify the host proteins that could bind to the specific BMV RNA (Zhu et al., 2007). Among the ones identified to bind the BMV core promoter for minus-strand RNA synthesis are Pus4, a pseudouridylate synthase, and App1, which is associated with the actin patch. Overexpression of Pus4 and App1 resulted in the inhibition of BMV virion assembly. In all of these cases, it is important to recognize that, while it is informative to see what possibly could interact with BMV, the plant host factors homologous to the yeast proteins should be characterized in order to study an evolved interaction.

Relationship Between Replication, Encapsidation, and Translation

For BMV genomic and subgenomic RNA, viral RNAs are serving as templates for translation and replication as well as encapsidation. Thus these processes may be related and coordinated during virus infection. Coupling packaging and replication

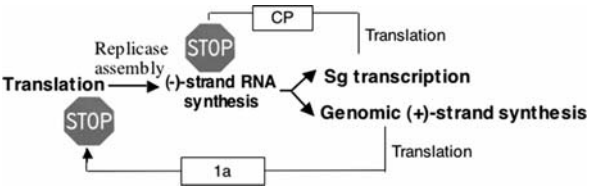

Fig. 5.5 Crosstalk between BMV RNA replication and processes required for BMV infection. The process of BMV replication is shown in *bold* and the regulatory roles of 1a or the capsid protein are denoted by *arrows*.

has been reported for some positive-strand RNA viruses, such as Poliovirus (Nugent et al., 1999), Kunjin virus (Khromykh et al., 2001), Flock House virus (Venter et al., 2005), and Venezuelan equine encephalitis virus (Vovkova et al., 2006). Recently, Annamalai and Rao (2006) demonstrated that efficient packaging of subgenomic RNA4 was functionally coupled to translation of coat protein from replication-derived mRNA, both in vitro assembly assay and *Agrobacterium*-mediated transient in vivo expression system. Packaging of RNA by the BMV CP was nonspecific in the absence of replication, while induction of viral replication increased the specificity of RNA packaging (Annamalai and Rao, 2006). Since 1a recruiting of RNA2 to replication complex required high-efficiency translation of the N-terminal half of the RNA template (Chen et al. 2003), replication and translation might be coupled since recruitment of RNA template to the replication complex is a major step in RNA replication. Recently, our lab found that efficient BMV genomic RNA1 replication required the translation of encoded protein 1a *in cis*, indicating that replication and translation of genomic RNA1 is functionally coupled (in preparation). Coupling replication and translation has been observed for poliovirus (Novak and Kirkegaard, 1994), mouse hepatitis virus (de Groot et al., 1992), and turnip yellow mosaic virus (Weiland and Dreher, 1993). The linkage among these processes may favor the viral replicase to efficiently differentiate viral RNA template from cellular RNA. A schematic for the crosstalks between different BMV processes is summarized in Fig. 5.5.

It is likely that the intersection between viral RNA replication and other processes required for infection (translation, RNA recombination, encapsidation, host innate responses) will provide fertile grounds for future research.

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