1 **The Brome Mosaic Virus 3' Untranslated Sequence Regulates RNA**
2 **Replication, Recombination, and Virion Assembly**

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Abstract

The 3' untranslated region in each of the three genomic RNAs of *Brome mosaic virus* (BMV) is highly homologous and contains a sequence that folds into a tRNA-like structure (TLS). Experiments performed over the past four decades revealed that the BMV 3' TLS regulates many important steps in BMV infection. This review summarizes in vitro and in vivo studies of the roles of the BMV 3' TLS functioning as a minus-strand promoter, in RNA recombination, and to nucleate virion assembly.

Introduction

Brome mosaic virus (BMV) belongs to the alphavirus-like superfamily of positive-strand RNA viruses (Kao and Sivakumaran, 2000). In nature BMV is a minor pathogen of monocots, with only sporadic observation in cereal crops (Lane, 1981). However, it has been a valuable model system to study RNA virus infection. BMV is also among a number of viruses whose genome contains a 3' untranslated region (UTR) that folds into tRNA-like structures (TLS) (Hall et al., 1972). Extensive analyses have revealed that the BMV 3' UTRs regulate many important steps in BMV infection. This review will summarize studies of the roles of the BMV 3' UTR on BMV infection.

The BMV genome is divided into three capped RNAs, designated RNA1, RNA2 and RNA3 (Kao and Sivakumaran, 2000). RNA1 encodes a capping methyltransferase and a helicase-like domain needed to process the BMV RNA and the membrane-encased RNA replication factories (Ahlgquist et al., 2005). RNA2 encodes the BMV RNA-dependent RNA polymerase responsible for RNA synthesis. RNA3 encodes two proteins required for systemic movement of the virus in plants and encapsidation of viral RNAs. The 5' proximal first cistron within RNA3 codes for the movement protein required for cell-to-cell spread. The 3' proximal second cistron codes for the coat protein (CP), 180 copies of which encapsidate each BMV genomic RNAs into three physically and morphologically indistinguishable stable virions (Rao, 2006a). The CP is translated from a subgenomic RNA (RNA4) that is transcribed from the minus-strand RNA3 by internal initiation (Miller et al., 1985). Subgenomic RNA4 is encapsidated in the same viral particle with RNA3. All three subsets of BMV virions are arranged in a T=3 icosahedral particles (Lucas et al., 2002).

The BMV 3' UTR

All BMV RNAs contain a 3' UTR with three RNA pseudoknots followed by a ~200-nt sequence that can fold into a TLS (Fig. 1A) (Dreher, 2009). The deletions of two or more of the three pseudoknots in RNA3 reduced the replication of RNA3 and affected the stability and translation of the RNA (Gallie and Kobayashi, 1994; Lahser et al., 1993). The sequences 5' of the conserved TLS also affected RNA replicase binding and modulated initiation of RNA synthesis (Chapman et al., 1998).

The TLS at the 3' portion of the BMV RNAs has been of interest since it raises questions about the origin of BMV RNAs, the roles of cellular proteins in translation, and possible interactions between the 5' and 3' UTR that can regulate translation and RNA synthesis. Indeed, the RNAs of BMV and the Turnip Yellow Mosaic Virus can bind the translation initiation factor 1a (EF-1 α) (Bastin and Hall, 1976; Dreher, 1999). The TLS has also been shown to regulate the translation of BMV RNAs, perhaps serving to recruit ribosomes to facilitate efficient translation and to regulate the timing of translation and replication (Barends et al., 2004).

The resemblance to the aminoacyl acceptor stem of the TLS was missed in the initial modeling of the RNA sequence until a pseudoknot, was added to form the aminoacylation acceptor stem that contained the 3'-CCA (Fig. 1) (Dasgupta and Kaesberg, 1977; Rietveld et al., 1983). The RNA folding has been verified and extended by chemical and enzymatic probing followed by NMR spectroscopy analysis (Felden et al., 1994a). These secondary structures interact with each other to contribute to mimicry of the tRNA-like tertiary structure (Felden et al., 1993; Giege, 1996).

The TLS has been extensively studied for aminoacylation *in vitro* (Dreher and Hall, 1988b; Fechter et al., 2001). However, the role of aminoacylation of the BMV RNAs on replication remains unclear. Mutant BMV RNAs that are unable to be aminoacylated are competent to direct RNA synthesis using partially purified BMV replicase (Dreher et al., 1984; Dreher and Hall, 1988a; Dreher and Hall, 1988b). BMV RNA3 with mutations that disrupt aminoacylation replicated to near wild-type levels in plant protoplasts and in plants (Dreher et al., 1989). It is possible that aminoacylation may not be required for all three BMV RNAs. Mutations in RNA2 that prevented aminoacylation were not replicated in protoplasts (Rao and Hall, 1991).

BMV 3' UTR and RNA replication

A key activity of the TLS for BMV infection is to direct the synthesis of the minus-strand RNA (Dreher and Hall, 1988a; Dreher and Hall, 1988b). As the first step in the replication of a positive-strand RNA virus genome, minus-strand RNA synthesis is subject to multiple levels of regulation. These include the recruitment of the replication machinery to the RNA, the presentation of an accessible 3' initiation sequence to the replicase complex to ensure the complete synthesis of the viral genome, and regulation of the appropriate levels of RNA synthesis during the infection cycle.

Stem-loop C (SLC) within the BMV TLS determines specific recognition by the BMV replicase. SLC appears to be a motif absent in the canonical tRNA-like tertiary structure, leading to speculation that it was added to the tRNA structure (Felden et al., 1994b). Also, it does not participate in recognition of the aminoacyl transferase (Fig. 1B). Mutations in SLC severely reduced BMV replication in protoplasts (Dreher and

Hall, 1988b; Rao and Hall, 1993). Mutation of the comparable sequence within the Cucumber mosaic virus TLS was also deleterious for minus-strand RNA synthesis (Sivakumaran et al., 1999). In vitro, an RNA containing only SLC could bind the BMV replicase. However, SLC did not direct RNA synthesis by the BMV replicase unless the last 6 to 8 nt of the 3'-terminus of the TLS were added to the 3' terminus of SLC (Chapman and Kao, 1999). These results suggest the specificity motif must be properly positioned relative to the sequence that directs initiation to allow proper viral RNA synthesis. In fact, molecular modeling positioned the SLC to the same spatial plane as aminoacylation stem and the 3' terminal CCA sequence that contain the initiation sequence (Felden et al., 1994b). The sequence then regulates the early steps in RNA synthesis (Sun et al., 1996; Sun and Kao, 1997a; Sun and Kao, 1997b). Furthermore, addition of SLC to a bona fide tyrosine tRNA resulted in an RNA that directs RNA synthesis, but only when SLC was positioned in the same plane as the aminoacyl acceptor stem (Zhang et al., 2003).

SLC contains two elements that are critical for directing RNA synthesis by the BMV replicase, a central bulge and a terminal hairpin that contained a 3-nt loop (triloop). Deletion of the bulge residues or mutations that affected the RNA triloop sequence (5' AUA) decreased BMV RNA replication *in vivo* and on RNA synthesis *in vitro* (Chapman and Kao, 1999; Kim et al., 2000). NMR analysis of a hairpin containing this triloop revealed that it folded into a highly ordered structure where the 5'-most adenine of the triloop was projected into the solution, due to the base stacking interactions between the 3'-most adenine, the uridyate of the triloop, and the stem-closing C–G base pair (Fig. 1C) (Kim et al., 2000). A network of electrostatic interactions stabilizes the

solution-exposed 5'-adenine, which has been named a clamped adenine (CAM). Variations of the terminal loop nucleotides that were unable to form the CAM failed to direct efficient RNA synthesis by the BMV replicase (Kim and Tinoco, 2001). Only a change of the 3'-most adenine of the triloop to a guanine (the triloop sequence of 5' AUG 3') resulted in robust level of RNA synthesis. This mutant triloop was found to retain a CAM, confirming its importance for BMV minus-strand RNA synthesis (Kim and Kao, 2001). These studies of the BMV TLS resulted in the first structure of a RNA virus core promoter and revealed the specificity requirements to initiate BMV minus-strand RNA synthesis *in vitro*. In addition, the structure of the BMV subgenomic core promoter also contains a motif structurally similar to the CAM (Skov et al., 2012).

While the reductionist approach has defined key elements in the BMV TLS that are responsible for replicase recognition, it is important to point out that other motifs within RNA3 contribute to proper levels of BMV RNA synthesis. The 3' UTR binds the replicase better than SLC alone (Chapman et al., 1998). Furthermore, replacement of the 3' UTR of BMV RNA3 with the comparable sequence from CMV did not result in efficient BMV RNA replication despite the CMV 3'UTR containing SLC. The intergenic region in BMV RNA3 also contributes to BMV RNA replication (French and Ahlquist, 1987). The intergenic sequence could direct the assembly of the BMV RNA replicase (Quadt et al., 1995).

An important question in viral RNA synthesis is which factor recognizes the promoter. Protoplasts transfected with only RNA1 and RNA2 that encode the replication proteins can replicate the two RNAs, indicating that the replicase can recognize the BMV RNAs. However, the level of RNA synthesis was low and the ratio of the minus-

and plus-strand RNAs was different than those during a wild-type infection. These results suggest that RNA3 or the proteins encoded by RNA3 are not required for activities of the BMV replicase. Instead, either RNA3 or its encoded proteins play a modulatory role in RNA synthesis. In a screen of a protein array containing both host and the BMV CP, the protein that binds best to the RNA hairpin containing the CAM was the BMV CP (Zhu et al., 2007).

Confirmation that BMV CP contributes to minus-strand RNA synthesis came from the use of *Agrobacterium* system to reconstitute systemic BMV infection in *Nicotiana benthamiana*. BMV replication and systemic infection in plants can be reconstituted by infiltration with agrobacteria that harbor the cDNAs of the three BMV RNAs (Annamalai and Rao, 2005b; Gopinath et al., 2005). In addition, expression of the 1a and 2a proteins can direct the replication of BMV RNA3 and the transcription of subgenomic RNA4 (Gopinath et al., 2005). Intriguingly, co-expression of the BMV CP along with RNA1 and RNA2 stimulated minus-strand RNA1 and RNA2 accumulation (Yi et al., 2009). Co-expression of the CP did not affect the accumulation of minus-strand RNA3 or subgenomic RNA4. These results demonstrate that the BMV CP participates in the regulation of minus-strand RNA synthesis, possibly by contributing to the recognition of the promoter for minus-strand RNA synthesis. A regulatory role for the CP is an emerging theme in RNA virus replication (Ni et al., 2014).

The observation that the requirements for the synthesis of minus-strand RNA3 differed from the replication of minus-strand RNA1 and RNA2 suggests that the TLSs for the three BMV RNAs are not functionally equivalent, despite their high conservation in sequence. First, the three TLS differ at several nucleotides. Second, a swap of the 3'

UTRs between the BMV RNAs resulted in RNAs that are replication defective (Duggal et al., 1992). It was also found that removal of the TLS from BMV RNA3 had a more modest effect on the translation of the MP and CP while the same removal had more severe effects on the translation of the replication proteins from BMV RNA1 and RNA2 (Barends et al., 2004). Selected changes in BMV RNA1 sequence was observed when it is passaged in different plant hosts (Ni and Cheng Kao, 2013).

TLS functions in 3' end repair

Similar to canonical tRNAs where the 3' CCA sequence can be repaired by nucleotidyltransferases, the BMV 3'TLS can be repaired by host tRNA nucleotidyltransferase. Minus-strand RNA synthesis *in vitro* uses the penultimate cytidylate as the template nucleotide (Miller et al., 1986; Sun et al., 1996). Therefore, deletion of two or more 3'-terminal nucleotides was necessary to significantly decrease RNA synthesis. In barley protoplasts transfected with the deleted RNA3, however, BMV was replicated normally and without noticeable delay in minus-strand RNA synthesis (Rao et al., 1989). Sequence analysis revealed that progeny had acquired the correct 3' -CCA termini, suggesting rapid turnover of the 3'-terminal nucleotides and repair, likely by a cellular tRNA nucleotidyltransferase.

A second repair mechanism could be at play. Hema et al. observed that the 3' CCA sequence of RNA1 plays an important role in the repair of the other two BMV RNAs. Indeed, repair was significantly decreased when RNA1 had suffered a loss of the two terminal nucleotides (Hema et al., 2005). It is possible that synthesis from RNA1 could generate the sequences that are responsible for the repair of the other two RNAs.

Recently, it was demonstrated that BMV RNA1 is preferentially replicated early after introduction into plants (Vaughan et al., 2014). The mechanism for RNA1's role in the repair of the other RNAs is not clear, but BMV RNAs are known to produce abortively initiated RNA products during RNA synthesis. It is possible that the aborted RNAs from RNA1 could serve to prime RNA synthesis from the other RNAs and thus restore the 3' CCA sequence.

BMV 3' TLS and RNA recombination

During replication, the accuracy with which the viral genomes are copied by viral replicase plays an important role in establishing a successful infection in susceptible hosts (Domingo et al., 1996). However, the lack of proofreading activity during RNA synthesis by viral RdRp results in highly heterogeneous populations of RNAs (quasispecies) that held together in a dynamic equilibrium (Domingo et al., 1996; Drake and Holland, 1999; Rao and Hall, 1993). In addition to polymerase error, RNA recombination is a major driving force to generate genetic variation in RNA viruses (Bujarski et al., 1994; Domingo et al., 1996; Kirkegaard and Baltimore, 1986; Nagy and Simon, 1997; Rao and Hall, 1993). Whether it is polymerase error or RNA recombination, natural selection is likely to maintain favorable consensus sequences for optimal replication of viral genomes (Domingo et al., 1996).

The contribution of the BMV 3' UTR to RNA recombination was first documented by (Bujarski and Kaesberg, 1986). In this study, inoculation of wild-type BMV RNA1 and RNA2 and along with RNA3 mutant M4 that bears a 20-nt deletion within the 3' TLS exhibited normal levels of aminoacylation, enhanced adenylation of the TLS, and

replicase template activity. However, M4 was unstable in prolonged infections in barley plants and regained the wild-type structure by recombination with the 3'UTR sequence of either RNA1 or RNA2 (Bujarski and Kaesberg, 1986). During replication of RNA viruses, recombination is envisioned to restore defective sequences that play a crucial role in replication. Therefore, the selective disadvantage for the M4 mutant is not obvious since RNA3 bearing M4 is hyperactive in minus-strand synthesis both *in vitro* and *in vivo* (Bujarski et al., 1986). However, more direct evidence for the restoration of 3' sequences by recombination that restored minus-strand RNA synthesis emerged when the 3' TLS was deleted from RNA2 and co-inoculated to barley protoplasts along with the wild-type counterparts. The 2a protein encoded by RNA2 needs to assemble into a functional replicase along with the 1a protein encoded by RNA1 (Kao and Ahlquist, 1992). The requirement of 2a to perform optimal replication selected for an RNA2 sequence generated by recombination that occurred as early as 24 h (Rao and Hall, 1990). Additional *in planta* experiments on the replicative competence of RNA3 bearing several 3' TLS mutants with varied minus-strand synthesis activity or genomic RNA1 defective in the continued expression of 1a demonstrated that recombination between viral molecules provides an important mechanism for the restoration of required biological activity (Chapman and Kao, 1999; Kwon and Rao, 2012; Rao, 2006b; Rao and Hall, 1993; Rao et al., 1990).

As mentioned above, the 3' TLS for each of the three BMV genomic RNA is highly homologous but contain distinctive nucleotides (Fig. 1). We note that the nucleotide differences are maintained during replication *in planta*, likely due to the requirement for different amounts of the BMV genomic RNA being optimal for viral RNA functions

(Duggal et al., 1992). Nevertheless, the type of host plant could affect recombination between the 3' ends between BMV genomic RNAs. Consequently, BMV RNAs passaged from *Nicotiana benthamiana* to either barley or wheat revealed recombination of the 3' untranslated region of RNA1 with that of RNA2 or RNA3 (Ni et al., 2014).

Using agroinfiltration that can be manipulated to express different BMV RNAs, the relative contributions of (+)- and (-)-strand RNA inocula to recombination of the BMV 3' TLS mutants was determined (Kwon and Rao, 2012). The polarity of the inoculated RNA was found to influence the type of recombinants generated. For example, when BMV RdRp reinitiates synthesis on a given B3 TLS mutant, nonhomologous recombinants longer than the inoculum template are generated. In contrast, re-initiation of synthesis from (-)-strand inoculum templates had generated homologous recombinants of native WT B3 size (Fig. 2).

Role of BMV 3' TLS in virus assembly

In BMV, genomic RNA1 and RNA2 are packaged independently into two virions while genomic RNA3 and its subgenomic RNA4 are co-packaged into a third virion (Rao, 2006a). Given the physical homogeneity of these three virions, the mechanism by which the capsid protein (CP) regulates this balanced distribution of four BMV progeny RNAs into three individual virions is elusive. However, the ease with which BMV virions can be reassembled *in vitro* from dissociated CP and RNA allowed the identification of RNA sequences required for virion assembly and genome packaging. BMV virions are stabilized by RNA-CP interactions (Fox et al., 1994). Although empty capsids can assemble *in vitro* at low pH (~5), they do not form *in vitro* under physiological conditions

of low salt and neutral pH and are not observed *in vivo* (Fox et al., 1994; Lane, 1981). At neutral pH, various polyanions can act to nucleate CP polymerization, and non-BMV RNAs can be assembled into capsids *in vitro*. However, when BMV RNA is present in RNA mixtures, it is selectively encapsidated (Cuillel et al., 1979). Further, differential encapsidation has been discerned among the BMV RNAs *in vitro*, with RNA4, followed by RNA3, showing the greatest affinity for assembly (Herzog and Hirth, 1978).

The 3' TLS of BMV served to nucleate assembly of virus particles (Fig. 3). When incubated with dissociated CP subunits, BMV genomic and subgenomic RNAs lacking the 3' TLS failed to assemble into virions and assembly was restored by the addition of 201-nt sequence encompassing the TLS to the assembly mixture (Choi et al., 2002) (Fig. 3). tRNAs from wheat germ and yeast supplied *in trans* were similarly active in promoting the assembly of truncated BMV RNAs into virions (Choi et al., 2002). However, virions assembled from truncated BMV RNAs in the presence of tRNAs or TLS-containing sequences did not incorporate the latter molecules (Choi et al., 2002). Analysis of BMV virion formation using agroinfiltration to dissect replication from packaging demonstrated that the 3'TLS has distinct ability to promote RNA packaging of the three BMV genomic RNAs (Annamalai and Rao, 2005b). Despite interplay between minus-strand promoter activity (Dreher and Hall, 1988a; Hema and Kao, 2004) and packaging of all four BMV RNAs by the 3' TLS *in vitro* (Choi et al., 2002), additional *in vivo* experiments designed to clarify the discriminative role of viral TLS revealed that the packaging requirement of the 3' TLS for RNA3 is distinct from that of RNA1 and RNA2. That is, the *cis*-acting BMV 3' TLS is obligatory for directing the packaging of RNA3, but not RNA 1 and RNA2, into virions (Annamalai and Rao, 2007).

Despite the importance of 3' TLS in RNA encapsidation, this role does not appear to be conserved among bromoviruses. *In vitro* reassembly assays performed with dissociated *Cowpea chlorotic mottle virus* (CCMV) CP and homologous and heterologous assembly of (CCMV) CP into virions did not depend on its 3' TLS (Annamalai and Rao, 2005a). Efficient virion assembly was observed when the three genomic RNAs of either CCMV or BMV lack the 3' TLS. Furthermore, CCMV CP efficiently packaged and assembled into icosahedral virions of expected size when either an RNA transcript of *Xenopus* elongation factor 1 of 1800 nt or a 2199-nt transcript of the genomic RNA3 of *Cucumber mosaic virus* was provided as a substrate (Annamalai and Rao, 2005a). These observations exemplify that a packaging requirement of 3' TLS is not conserved among bromoviruses. The genomes of several other plant RNA viruses also terminate in 3' TLS (Dreher, 2009). It remains to be determined whether virion assembly in non-bromoviruses is dependent on 3'TLS.

Concluding Comments

The 3' UTR of BMV is a central regulator for multiple processes in BMV infection, including translation, minus-strand RNA synthesis, RNA recombination, and RNA encapsidation for virion formation. There is also significant evidence that 3' UTRs of the three BMV RNAs have a number of significant functional differences, despite their sequences being nearly identical. While studies of the BMV 3'UTR have spanned decades, much remains to be learned as to how the 3' UTR contributes to BMV infection. A structure of the BMV 3' UTR to atomic resolution, as recently determined for the TLS of TYMV (Colussi et al., 2014), will significantly contribute to our understanding

of how structure influences the various BMV processes. It will also be important to identify the host factors that interact with each of the 3' UTRs of the BMV RNAs. Finally, given that the 3' UTR participates in just about every major step in BMV infection, it is important to learn the conformational changes within the 3' UTR needed to accomplish the proper sequences of events in BMV infection.

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References Cited

- Ahlquist, P., Schwartz, M., Chen, J., Kushner, D., Hao, L., Dye, B.T., 2005. Viral and host determinants of RNA virus vector replication and expression. *Vaccine* 23(15), 1784-1787.
- Annamalai, P., Rao, A.L., 2005a. Dispensability of 3' tRNA-like sequence for packaging cowpea chlorotic mottle virus genomic RNAs, *Virology*. Vol. 332, pp. 650-658.

347 Annamalai, P., Rao, A.L., 2005b. Replication-independent expression of genome
 348 components and capsid protein of brome mosaic virus in planta: a functional role
 349 for viral replicase in RNA packaging. *Virology* 338(1), 96-111.

350 Annamalai, P., Rao, A.L., 2007. In vivo packaging of brome mosaic virus RNA3, but not
 351 RNAs 1 and 2, is dependent on a cis-acting 3' tRNA-like structure. *J Virol* 81(1),
 352 173-181.

353 Barends, S., Rudinger-Thirion, J., Florentz, C., Giege, R., Pleij, C.W., Kraal, B., 2004.
 354 tRNA-like structure regulates translation of Brome mosaic virus RNA. *J Virol*
 355 78(8), 4003-4010.

356 Bastin, M., Hall, T.C., 1976. Interaction of elongation factor 1 with aminoacylated brome
 357 mosaic virus and tRNA's. *J Virol* 20(1), 117-122.

358 Bujarski, J.J., Ahlquist, P., Hall, T.C., Dreher, T.W., Kaesberg, P., 1986. Modulation of
 359 replication, aminoacylation and adenylation in vitro and infectivity in vivo of BMV
 360 RNAs containing deletions within the multifunctional 3' end. *EMBO J* 5(8), 1769-
 361 1774.

362 Bujarski, J.J., Kaesberg, P., 1986. Genetic recombination between RNA components of
 363 a multipartite plant virus. *Nature* 321(6069), 528-531.

364 Bujarski, J.J., Nagy, P.D., Flasiński, S., 1994. Molecular studies of genetic RNA-RNA
 365 recombination in brome mosaic virus. *Adv Virus Res* 43, 275-302.

366 Chapman, M.R., Kao, C.C., 1999. A minimal RNA promoter for minus-strand RNA
 367 synthesis by the brome mosaic virus polymerase complex. *J Mol Biol* 286(3),
 368 709-720.

369 Chapman, M.R., Rao, A.L., Kao, C.C., 1998. Sequences 5' of the conserved tRNA-like
 370 promoter modulate the initiation of minus-strand synthesis by the brome mosaic
 371 virus RNA-dependent RNA polymerase. *Virology* 252(2), 458-467.

372 Choi, Y.G., Dreher, T.W., Rao, A.L., 2002. tRNA elements mediate the assembly of an
 373 icosahedral RNA virus. *Proc Natl Acad Sci U S A* 99(2), 655-660.

374 Colussi, T.M., Costantino, D.A., Hammond, J.A., Ruehle, G.M., Nix, J.C., Kieft, J.S.,
 375 2014. The structural basis of transfer RNA mimicry and conformational plasticity
 376 by a viral RNA. *Nature* 511(7509), 366-369.

377 Cuillel, M., Herzog, M., Hirth, L., 1979. Specificity of in vitro reconstitution of brome grass
 378 mosaic virus. *Virology* 95(1), 146-153.

379 Dasgupta, R., Kaesberg, P., 1977. Sequence of an oligonucleotide derived from the 3'
 380 end of each of the four brome mosaic viral RNAs. *Proc Natl Acad Sci USA*
 381 74(11), 4900-4904.

382 Domingo, E., Escarmis, C., Sevilla, N., Moya, A., Elena, S.F., Quer, J., Novella, I.S.,
 383 Holland, J.J., 1996. Basic concepts in RNA virus evolution. *FASEB J* 10(8), 859-
 384 864.

385 Drake, J.W., Holland, J.J., 1999. Mutation rates among RNA viruses. *Proc Natl Acad*
 386 *Sci U S A* 96(24), 13910-13913.

387 Dreher, T.W., 1999. Functions of the 3'-Untranslated Regions of Positive Strand Rna
 388 Viral Genomes. *Annu Rev Phytopathol* 37, 151-174.

389 Dreher, T.W., 2009. Role of tRNA-like structures in controlling plant virus replication.
 390 *Virus Res* 139, 217-229.

391 Dreher, T.W., Bujarski, J.J., Hall, T.C., 1984. Mutant viral RNAs synthesized in vitro
392 show altered aminoacylation and replicase template activities. *Nature* 311(5982),
393 171-175.

394 Dreher, T.W., Hall, T.C., 1988a. Mutational analysis of the sequence and structural
395 requirements in brome mosaic virus RNA for minus strand promoter activity. *J*
396 *Mol Biol* 201(1), 31-40.

397 Dreher, T.W., Hall, T.C., 1988b. Mutational analysis of the tRNA mimicry of brome
398 mosaic virus RNA. Sequence and structural requirements for aminoacylation and
399 3'-adenylation. *J Mol Biol* 201(1), 41-55.

400 Dreher, T.W., Rao, A.L., Hall, T.C., 1989. Replication in vivo of mutant brome mosaic
401 virus RNAs defective in aminoacylation. *J Mol Biol* 206(3), 425-438.

402 Duggal, R., Rao, A.L., Hall, T.C., 1992. Unique nucleotide differences in the conserved
403 3' termini of brome mosaic virus RNAs are maintained through their optimization
404 of genome replication. *Virology* 187(1), 261-270.

405 Fechter, P., Giege, R., Rudinger-Thirion, J., 2001. Specific tyrosylation of the bulky
406 tRNA-like structure of brome mosaic virus RNA relies solely on identity
407 nucleotides present in its amino acid-accepting domain. *J Mol Biol* 309(2), 387-
408 399.

409 Felden, B., Florentz, C., Giege, R., Westhof, E., 1994a. Solution structure of the 3'-end
410 of brome mosaic virus genomic RNAs. Conformational mimicry with canonical
411 tRNAs. *J Mol Biol* 235(2), 508-531.

412 Felden, B., Florentz, C., McPherson, A., Giege, R., 1994b. A histidine accepting tRNA-
 413 like fold at the 3'-end of satellite tobacco mosaic virus RNA. *Nucl Acid Res*
 414 22(15), 2882-2886.

415 Felden, B., Florentz, C., Westhof, E., Giege, R., 1993. Non-canonical substrates of
 416 aminoacyl-tRNA synthetases: the tRNA-like structure of brome mosaic virus
 417 genomic RNA. *Biochimie* 75(12), 1143-1157.

418 Fox, J.M., Johnson, J.E., Young, M.J., 1994. RNA/protein interactions in icosahedral
 419 virus assembly. *Semi Virology* 5, 51-60.

420 French, R., Ahlquist, P., 1987. Intercistronic as well as terminal sequences are required
 421 for efficient amplification of brome mosaic virus RNA3. *J Virol* 61(5), 1457-1465.

422 Gallie, D.R., Kobayashi, M., 1994. The role of the 3'-untranslated region of non-
 423 polyadenylated plant viral mRNAs in regulating translational efficiency. *Gene*
 424 142(2), 159-165.

425 Giege, R., 1996. Interplay of tRNA-like structures from plant viral RNAs with partners of
 426 the translation and replication machineries. *Proc Natl Acad Sci U S A* 93(22),
 427 12078-12081.

428 Gopinath, K., Dragnea, B., Kao, C., 2005. Interaction between Brome mosaic virus
 429 proteins and RNAs: effects on RNA replication, protein expression, and RNA
 430 stability. *J Virol* 79(22), 14222-14234.

431 Hall, T.C., Shih, D.S., Kaesberg, P., 1972. Enzyme-mediated binding of tyrosine to
 432 brome-mosaic-virus ribonucleic acid. *Biochem J* 129(4), 969-976.

433 Hema, M., Gopinath, K., Kao, C., 2005. Repair of the tRNA-like CCA sequence in a
 434 multipartite positive-strand RNA virus. *J Virol* 79(3), 1417-1427.

435 Hema, M., Kao, C.C., 2004. Template sequence near the initiation nucleotide can
 436 modulate brome mosaic virus RNA accumulation in plant protoplasts. *J Virol*
 437 78(3), 1169-1180.

438 Herzog, M., Hirth, L., 1978. In vitro encapsidation of the four RNA species of brome
 439 mosaic virus. *Virology* 86(1), 48-56.

440 Kao, C.C., Ahlquist, P., 1992. Identification of the domains required for direct interaction
 441 of the helicase-like and polymerase-like RNA replication proteins of brome
 442 mosaic virus. *J Virol* 66(12), 7293-7302.

443 Kao, C.C., Sivakumaran, K., 2000. Brome mosaic virus, good for an RNA virologist's
 444 basic needs. *Mol Plant Pathol* 1(2), 91-97.

445 Kim, C.H., Kao, C.C., 2001. A mutant viral RNA promoter with an altered conformation
 446 retains efficient recognition by a viral RNA replicase through a solution-exposed
 447 adenine. *RNA* 7(10), 1476-1485.

448 Kim, C.H., Kao, C.C., Tinoco, I., Jr., 2000. RNA motifs that determine specificity
 449 between a viral replicase and its promoter. *Nat Struct Biol* 7(5), 415-423.

450 Kim, C.H., Tinoco, I., Jr., 2001. Structural and thermodynamic studies on mutant RNA
 451 motifs that impair the specificity between a viral replicase and its promoter. *J Mol*
 452 *Biol* 307(3), 827-839.

453 Kirkegaard, K., Baltimore, D., 1986. The mechanism of RNA recombination in
 454 poliovirus. *Cell* 47(3), 433-443.

455 Kwon, S.J., Rao, A.L., 2012. Emergence of distinct brome mosaic virus recombinants is
 456 determined by the polarity of the inoculum RNA. *J Virol* 86(9), 5204-5220.

457 Lahser, F.C., Marsh, L.E., Hall, T.C., 1993. Contributions of the brome mosaic virus
 458 RNA-3 3'-nontranslated region to replication and translation. *J Virol* 67(6), 3295-
 459 3303.

460 Lane, L.C., 1981. Bromoviruses. In: Kurstak, E. (Ed.), *Hand book of plant virus*
 461 *infections and comparative diagnosis*. Elsevier Bimedical Press, Amsterdam, pp.
 462 333-376.

463 Lucas, R.W., Larson, S.B., McPherson, A., 2002. The crystallographic structure of
 464 brome mosaic virus. *J Mol Biol* 317(1), 95-108.

465 Miller, W.A., Bujarski, J.J., Dreher, T.W., Hall, T.C., 1986. Minus-strand initiation by
 466 brome mosaic virus replicase within the 3' tRNA-like structure of native and
 467 modified RNA templates. *J Mol Biol* 187(4), 537-546.

468 Miller, W.A., Dreher, T.W., Hall, T.C., 1985. Synthesis of brome mosaic virus
 469 subgenomic RNA in vitro by internal initiation on (-)-sense genomic RNA. *Nature*
 470 313(5997), 68-70.

471 Nagy, P.D., Simon, A.E., 1997. New insights into the mechanisms of RNA
 472 recombination. *Virology* 235(1), 1-9.

473 Ni, P., Cheng Kao, C., 2013. Non-encapsidation activities of the capsid proteins of
 474 positive-strand RNA viruses. *Virology* 446(1-2), 123-132.

475 Ni, P., Vaughan, R.C., Tragesser, B., Hoover, H., Kao, C.C., 2014. The Plant Host Can
 476 Affect the Encapsidation of Brome Mosaic Virus (BMV) RNA: BMV Virions Are
 477 Surprisingly Heterogeneous. *J Mol Biol* 426(5), 1061-1076.

478 Quadt, R., Ishikawa, M., Janda, M., Ahlquist, P., 1995. Formation of brome mosaic virus
 479 RNA-dependent RNA polymerase in yeast requires coexpression of viral proteins
 480 and viral RNA. *Proc Natl Acad Sci U S A* 92(11), 4892-4896.

481 Rao, A.L., 2006a. Genome packaging by spherical plant RNA viruses. *Annu Rev*
 482 *Phytopathol* 44, 61-87.

483 Rao, A.L., 2006b. Sensitivity of brome mosaic virus RNA1 replication to mutations in the
 484 3' tRNA-like structure implies a requirement for sustained synthesis of replicase
 485 protein 1a. *Arch Virol* 151(4), 721-733.

486 Rao, A.L., Dreher, T.W., Marsh, L.E., Hall, T.C., 1989. Telomeric function of the tRNA-
 487 like structure of brome mosaic virus RNA. *Proc Natl Acad Sci U S A* 86(14),
 488 5335-5339.

489 Rao, A.L., Hall, T.C., 1990. Requirement for a viral trans-acting factor encoded by
 490 brome mosaic virus RNA-2 provides strong selection in vivo for functional
 491 recombinants. *J Virol* 64(5), 2437-2441.

492 Rao, A.L., Hall, T.C., 1991. Interference in trans with brome mosaic virus replication by
 493 RNA-2 bearing aminoacylation-deficient mutants. *Virology* 180(1), 16-22.

494 Rao, A.L., Hall, T.C., 1993. Recombination and polymerase error facilitate restoration of
 495 infectivity in brome mosaic virus. *J Virol* 67(2), 969-979.

496 Rao, A.L., Sullivan, B.P., Hall, T.C., 1990. Use of *Chenopodium hybridum* facilitates
 497 isolation of brome mosaic virus RNA recombinants. *J Gen Virol* 71 (Pt 6), 1403-
 498 1407.

499 Rietveld, K., Pleij, C.W., Bosch, L., 1983. Three-dimensional models of the tRNA-like 3'
 500 termini of some plant viral RNAs. *EMBO J* 2(7), 1079-1085.

501 Sivakumaran, K., Kim, C.H., Tayon, R., Jr., Kao, C.C., 1999. RNA sequence and
502 secondary structural determinants in a minimal viral promoter that directs
503 replicase recognition and initiation of genomic plus-strand RNA synthesis. *J Mol*
504 *Biol* 294(3), 667-682.

505 Skov, J., Gaudin, M., Podbevsek, P., Olsthoorn, R.C., Petersen, M., 2012. The
506 subgenomic promoter of brome mosaic virus folds into a stem-loop structure
507 capped by a pseudo-triloop that is structurally similar to the triloop of the genomic
508 promoter. *RNA* 18(5), 992-1000.

509 Sun, J.H., Adkins, S., Faurete, G., Kao, C.C., 1996. Initiation of (-)-strand RNA
510 synthesis catalyzed by the BMV RNA-dependent RNA polymerase: synthesis of
511 oligonucleotides. *Virology* 226(1), 1-12.

512 Sun, J.H., Kao, C.C., 1997a. Characterization of RNA products associated with or
513 aborted by a viral RNA-dependent RNA polymerase. *Virology* 236(2), 348-353.

514 Sun, J.H., Kao, C.C., 1997b. RNA synthesis by the brome mosaic virus RNA-dependent
515 RNA polymerase: transition from initiation to elongation. *Virology* 233(1), 63-73.

516 Vaughan, R., Tragesser, B., Ni, P., Ma, X., Dragnea, B., Kao, C.C., 2014. The tripartite
517 virions of the brome mosaic virus have distinct physical properties that affect the
518 timing of the infection process. *J Virol* 88(11), 6483-6491.

519 Yi, G., Letteney, E., Kim, C.H., Kao, C.C., 2009. Brome mosaic virus capsid protein
520 regulates accumulation of viral replication proteins by binding to the replicase
521 assembly RNA element. *RNA* 15(4), 615-626.

Zhang, X., Kim, C.H., Sivakumaran, K., Kao, C., 2003. Stable RNA structures can repress RNA synthesis in vitro by the brome mosaic virus replicase. *RNA* 9(5), 555-565.

Zhu, J., Gopinath, K., Murali, A., Yi, G., Hayward, S.D., Zhu, H., Kao, C., 2007. RNA-binding proteins that inhibit RNA virus infection. *Proc Natl Acad Sci U S A* 104(9), 3129-3134.

Figure legends

Fig. 1. A) The secondary structure of the BMV 3' UTR and motifs required for RNA synthesis. A) The secondary structure of the 3' UTR for BMV RNA3. The termination codon for the BMV CP at the 5'-most region of the UTR is in bold. Nucleotides that participate in base-pairing in the RNA pseudoknots are bracketed, with the bonds indicated by the dashed lines. B) A proposed tertiary structure of the BMV 3' UTR. The structure is adapted from the one proposed by Fechter et al. (2001). The portion of the 3' TLS that resembles a canonical tRNA-like structure is shown in blue. SLC, the core promoter for minus-strand RNA synthesis is colored red. C) Secondary and tertiary structure of the RNA hairpin from SLC that forms the core promoter for minus-strand RNA synthesis. The structure is from the PDB 1ESH and drawn using Chimera. The clamped adenine and its ribose are in orange. The cytidylate and guanylate residues within the stem of SLC that stabilized the clamped adenine are colored, respectively, blue and teal. The red lines denote bonds that are within 3.5 Å of the clamped adenine.

Fig. 2 Schematic representation of 3'TLS mediated RNA recombination in BMV involving (A) end-to-end switching and (B) internal template switching copy-choice mechanism. For details refer to Kwon and Rao (2012).

Fig. 3 tRNA-mediated virion assembly. Schematic representation of BMV virion assembly in the presence (A) and absence (B) of 3'TLS.

Figure(s)
Figure 1

Figure 2

A. End-to-end template switching

B. Internal template switching

Figure 3

Fig. 3