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cis-Preferential requirement of a −1 frameshift product p88 for the replication of *Red clover necrotic mosaic virus* RNA1

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Abstract

The genome of *Red clover necrotic mosaic virus* (RCNMV) consists of RNA1 and RNA2. RNA1 encodes N-terminally overlapping replication proteins, p27 and p88, which are translated in a cap-independent manner. The 3′ untranslated region of RNA1 contains RNA elements essential for cap-independent translation and negative-strand RNA synthesis. In this study, we investigated how p27 and p88 were engaged in the replication of RCNMV genomic RNAs by using DNA vectors or *in vitro* transcribed RNAs in protoplasts and in a cell-free extract of evacuolated BY-2 protoplasts. Our results show a *cis*-preferential requirement of p88, but not of p27, for the replication of RNA1. This mechanism might help to facilitate a switch in the role of RNA1 from mRNA to a replication template.

Keywords: RNA-dependent RNA polymerase; Cap-independent translation; RNA replication; Divided genome; RNA virus; Positive-strand RNA virus

Introduction

The viral replicase complex performs the replication of positive-strand RNA viruses (Ahlquist, 2002; Buck, 1996). The replicase complex consists of multiple proteins, including viral proteins, RNA-dependent RNA polymerase (RdRP) and auxiliary proteins, and host factors (Ahlquist et al., 2003; van der Heijden and Bol, 2002). *cis*-Acting RNA elements also play essential roles in viral RNA replication. These RNA elements are often distributed throughout the genomic RNAs, including protein-coding regions. Generally, the 3′ proximal nucleotide sequences of viral genomic RNAs are essential to initiate negative-strand RNA synthesis. These RNA elements alone are often sufficient to function in recruiting the viral replicase supplied in *trans* for initiating RNA synthesis (Buck, 1996; Kao et al., 2001).

Also, the viral replicase in *trans* is involved in the replication of RNAs that do not encode replicase component proteins in viruses with a multipartite genome. Likewise, defective-RNAs occurring naturally or created artificially as constructed-deletion variants can be efficiently amplified by a helper virus that supplies replicase component proteins in *trans*. In contrast, *cis*-preferential function of the viral encoded proteins or a coupling between translation and replication has been reported for several viruses, including *Alfalfa mosaic virus* (AMV) (Neeleman and Bol, 1999; van Rossum et al., 1996), *Clover yellow mosaic virus* (CYMV) (White et al., 1992), *Bovine coronavirus* (Chang et al., 1994), *Cowpea mosaic virus* (van Bokhoven et al., 1993), *Poliovirus* (Hagino-Yamagishi and Nomoto, 1989; Johnson and Sarnow, 1991; Novak and Kirkegaard, 1994), *Turnip crinkle virus* (TCV) (White et al., 1995), *Tobacco etch virus* (Mahajan et al., 1996; Schaad et al., 1996), *Tobacco mosaic virus* (TMV) (Lewandowski and Dawson, 2000), *Tomato bushy stunt virus* (TBSV) (Oster et al., 1998), *Turnip yellow mosaic virus* (TYMV) (Weiland and

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Dreher, 1993), and *Rubella virus* (Liang and Gillam, 2001). The coupling between translation and RNA replication appears to play important roles in virus infection. However, the roles are poorly understood.

Red clover necrotic mosaic virus (RCNMV) is a positive-strand RNA virus in the *Dianthovirus* genus, *Tombusviridae* family (van Regenmortel et al., 2000). Its genome is divided into RNA1 and RNA2. RNA1 encodes two N-terminal overlapping non-structural proteins of 27 kDa (p27) and 88 kDa (p88). p88 has an RdRP motif (Koonin, 1991) and is produced by programmed −1 ribosomal frameshifting (Kim and Lommel, 1994 and 1998; Xiong et al., 1993b). Both p27 and p88 are required for viral RNA replication. RNA2 encodes a 35 kDa movement protein (MP) required for viral cell-to-cell movement in plants (Lommel et al., 1988; Xiong et al., 1993a). RNA2 is not required for the replication of RNA1 in protoplasts (Mizumoto et al., 2003; Osman and Buck, 1987; Paje-Manalo and Lommel, 1989), but a stem–loop structure on the protein-coding region of RNA2 is necessary for the synthesis of subgenomic RNA from RNA1 in an RNA-mediated *trans*-activation mechanism, which leads to the expression of coat protein (CP) (Sit et al., 1998; Tatsuta et al., 2005). In addition to 5′- and 3′-untranslated regions (UTRs) of RNA2, the same stem–loop structure is essential for RNA2 replication (Tatsuta et al., 2005; Turner and Buck, 1999).

RNA1 and RNA2 have no cap structure at the 5′ end (Mizumoto et al., 2003) and no poly-A tail at the 3′ end (Mizumoto et al., 2002; Xiong and Lommel, 1989). The 3′-UTR of RCNMV RNA1 contains *cis*-acting RNA elements (TE-3′ DR1) that can function as a cap-independent translational enhancer (Mizumoto et al., 2003). However, RNA2 has no such *cis*-acting RNA element. The strong link between the cap-independent translation and replication of RNA2 suggests that RNA2 functions as mRNA for translation only when generated *de novo* through the viral RNA replication machinery (Mizumoto et al., 2006). The RNA silencing suppression activity of RCNMV is also linked to viral RNA replication (Takeda et al., 2005). Thus, p27 and p88 play important roles not only in viral RNA synthesis but also in the regulation of viral gene expression, and the suppression of host resistance during viral infection.

We found previously that *in vitro* transcribed RNA1 mutants encoding either p27 or p88 do not efficiently complement each other to accumulate detectable viral RNAs in BY2 protoplasts (Takeda et al., 2005). This suggests several possibilities including *cis*-preferential requirement of p27 or p88, or both for the replication of RNA1. In this study, we investigated how p27 and p88 were engaged in the replication of RCNMV genomic RNAs in protoplasts and in a cell-free extract of evacuolated BY-2 protoplasts (BYL) by using DNA vectors or *in vitro* transcribed RNAs expressing viral proteins and RNAs. Results indicated that RNA1 mutants from which intact p88 was translated were efficiently replicated in the presence of p27 in protoplasts, whereas RNA1 mutants from which p27 alone or no functional p88 was translated were replicated to very low levels in the presence of p88 and p27, even at levels sufficient to replicate RNA2. Together with the results obtained from *in vitro* experiments in BYL, our results suggest a *cis*-preferential function of p88 in the replication of RNA1.

Results

Complementation in replication between p27-deficient and p88-deficient RNA1 mutants

DNA vector plasmids used in this study are presented in Fig. 1. Cowpea protoplasts were inoculated with pUBRC1-p27, pUBRC1-p88u, or their mixture. pUBRC1-p27 expresses RNA1 mutant that expresses p27 but not p88, and pUBRC1-p88u expresses RNA1 mutant that expresses p88 but not p27. Virus RNA accumulation was analyzed after 24 h of incubation by Northern blotting using Dig-labeled RNA probe specific to RCNMV RNA1. RNA1 accumulated in inoculation with a mixture of pUBRC1-p27 and pUBRC1-p88u (Fig. 2A, lane 3), whereas no signal specific to RNA1 was detected in inoculation with either pUBRC1-p27 or pUBRC1-p88u alone (Fig. 2A,

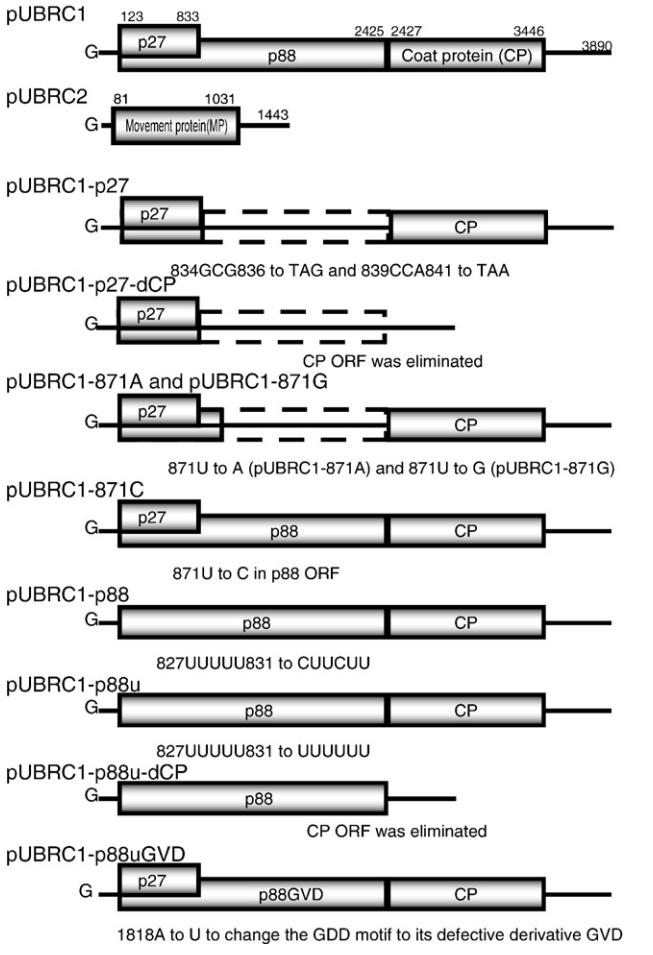

Fig. 1. Schematic representation of wild-type RCNMV RNA 1 and 2 (pUBRC1 and pUBRC2, respectively) and mutants derived from RNA 1 (referred to as the names of plasmids). These viral RNAs had extra G residue at the 5′ end. The viral RNA was initially synthesized *in vivo* from the 35S promoter of CaMV, and was cleaved by the ribozyme of satellite *Tobacco ringspot virus* to generate the appropriate 3′ end. Individual sites of the introduced mutations were explained and indicated by open triangles.

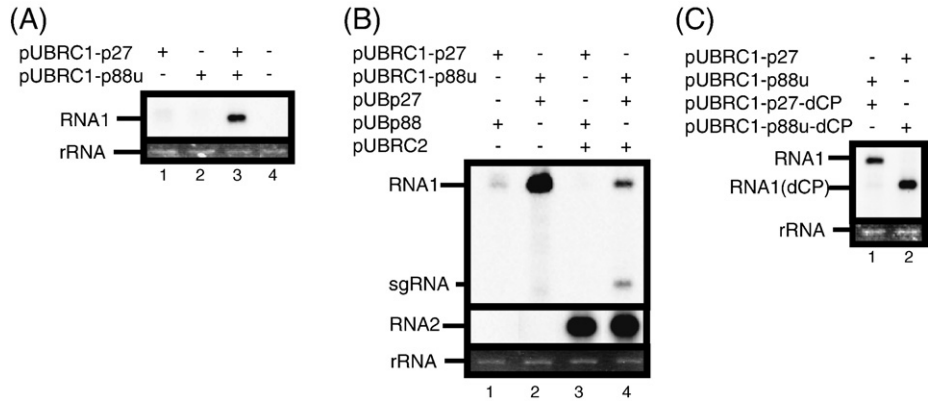

Fig. 2. Replication of RCNMV RNA1 mutants deficient in expression of either p27 or p88 by supplying their lacking component in cowpea protoplasts. Cowpea protoplasts were inoculated with plasmids, and accumulation of progeny viral RNAs were analyzed by Northern blotting after 24 h of incubation. (A) Accumulations of RNA1 mutants in single and mixed inoculation. (B) Accumulations of RNA1 mutants when p27 or p88 was supplied from protein expression vectors. pUBRC2 was also inoculated to assess and to confirm the expression of p27 and p88. (C) Accumulations of RNA1 mutants with the coat protein (CP) gene deleted. The combination of plasmids used for the inoculation is indicated above each lane.

lanes 1 and 2). Similar results were obtained by using pUBRC1-p88 whose transcripts were used in a previous study (Takeda et al., 2005). Both pUBRC1-p88u and pUBRC1-p88 were expected to express wild-type p88 alone, but differed in silent mutations to eliminate a translational termination codon for p27 (Fig. 1). These results suggested that RNA1 mutants expressing p27 or p88 alone, respectively, complemented each other for RNA replication.

Replication of p27-deficient or p88-deficient RNA1 mutants by supplying lacking replicase components in trans

We next tested whether p27-deficient and p88-deficient RNA1s could be replicated by supplying lacking replicase components from protein expression plasmids. Cowpea protoplasts were inoculated with various combinations of p27, p88, RNA1, and RNA2-expressing plasmids, and viral RNA accumulations were analyzed. RNA1 accumulated efficiently in protoplasts inoculated with a mixture of pUBRC1-p88u and pUBp27 (Fig. 2B, lane 2), although the accumulation level decreased in the presence of RNA2 (Fig. 2B, lane 4). On the other hand, the accumulations of RNA1 were extremely low or below detection limits in protoplasts inoculated with a mixture of pUBRC1-p27 and pUBp88 (Fig. 2B, lane 1, data not shown), and the accumulation level of RNA1 was consistently below detection limits in the presence of RNA2 (Fig. 2B, lane 3). Either combination of pUBRC1-p88u and pUBp27 or pUBRC1-p27 and pUBp88 supported the accumulation of co-inoculated RNA2 efficiently (Fig. 2B, lanes 3 and 4), indicating that p27 and p88 from these plasmids were sufficient to replicate RNA2. Thus, these results suggested that p88 mainly functioned in a *cis*-preferential manner in RNA1 replication, whereas p27 effectively functioned in *trans* to support the replication of RNA1.

If this idea is true, RNA1 signals detected in the coinoculation experiments of pUBRC1-p27 with pUBRC1-p88u or pUBRC1-p88 should reflect the preferential accumulation of p88-encoding RNA1. To confirm this expectation, we used pUBRC1-p27-dCP and pUBRC1-p88u-dCP (Fig. 1), because these CP-deletion mutants allow one to distinguish RNA1 mutants by size. In inoculations with a mixture of pUBRC1-p27-dCP and pUBRC1-p88u or a mixture of pUBRC1-p27 and pUCRC1-p88u-dCP, RNA1 with a size expected from pUBRC1-p88u or pUBRC1-p88u-dCP preferentially accumulated (Fig. 2C, lanes 1 and 2).

In addition, *in vitro* transcripts of RNA1 mutants expressing p88 alone corresponding to pUBRC1-p88u, pUBRC1-p88, pUBRC1-p88u-dCP, or pUBRC1-p88-dCP were efficiently replicated by coinoculation with p27-expressing plasmids (data not shown).

cis-Acting RNA elements are not involved in the failure of rescue of replication of RNA1 mutants by p88 supplied in trans

The above results do not rule out the possibility that failure in the accumulation of p88-deficient RNA1 mutants by p88 supplied in *trans* might result from the disruption of *cis*-acting replication elements by the nucleotides inserted into or substituted in those RNA1 mutants. pUBRC1-p27 has six nucleotides inserted immediately downstream of the stop codon of the p27 ORF (TAG**TA**GGC**TAA**; inserted nucleotides in bold font) (Fig. 1; Xiong et al., 1993b; Takeda et al., 2005). However, it is difficult to address the above question by gene manipulation in the region used to create pUBRC1-p27, because any nucleotide changes in the region accompany changes in amino acid sequences in p88, which may affect RNA replication. Therefore, to avoid the effects of gene manipulation on amino acid sequences and *cis*-acting replication elements, we created other p88-deficient RNA1 mutants by substituting the U at position 871, thirty nucleotides downstream of the stop codon of p27, because base-substitutions in the region of 870–875 nt have been shown not to affect RNA replication and systemic infection of RCNMV (Kim and Lommel, 1998). pUBRC1-871A and pUBRC1-871G had A and G, respectively, at position 871, which create an additional stop codon to prevent p88 expression. pUBRC1-871C had C at position 871 to create a synonymous codon for wild-type tyrosine (Fig. 1). These plasmids were inoculated into

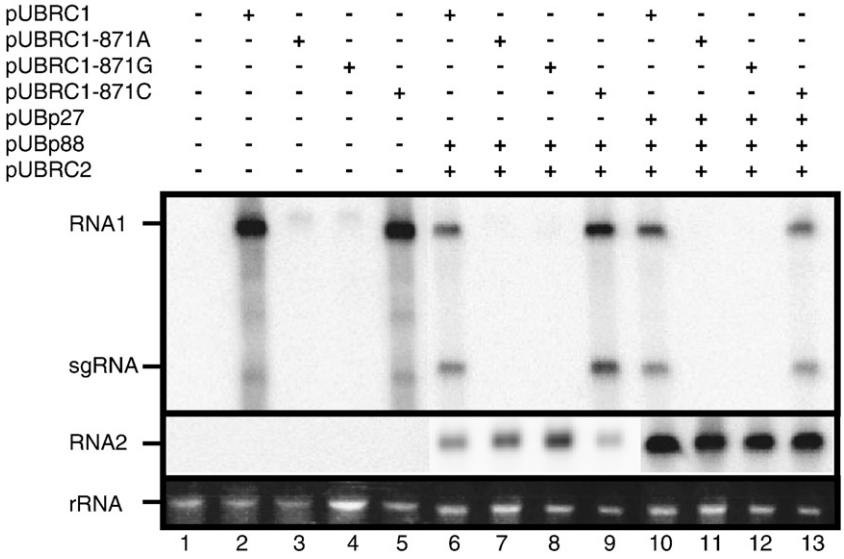

Fig. 3. Effects of single-nucleotide substitutions at the same position of RNA1 which abolish and restore the expression of p88 on its own replication in the presence or absence of p88 supplied in *trans*. Cowpea protoplasts were inoculated with various combinations of plasmids indicated above each lane, and accumulations of progeny viral RNAs were analyzed by Northern blotting after 24 h of incubation. For others, refer to the legend of Fig. 2.

cowpea protoplasts alone or together with pUBp88 or a mixture of pUBp27 and pUBp88. pUBRC2 was also used to confirm the expression of p27 and p88. In inoculation with pUBRC1-871A or pUBRC1-871G alone, RNA1 mutants did not accumulate to detectable levels, confirming the lack of expression of functional p88 from these RNA1 mutants (Fig. 3, lanes 3 and 4). In contrast, RNA1 from pUBRC1-871C accumulated to a level similar to wild-type RNA1 (Fig. 3, lanes 5). Supplementation of p88, or both p88 and p27 in *trans* did not efficiently support the replication of RNA1 mutants from pUBRC1-871A and pUBRC1-871G (Fig. 3. lanes 7, 8, 11, and 12), whereas the *trans*-supplied p88 efficiently supported RNA2 replication with co-inoculated pUBRC1-871A or pUBRC1-871G (Fig. 3. lanes 7 and 8). The successful replication of RNA1 from pUBRC1-871C suggested that single-nucleotide substitutions at this position did not affect *cis*-acting RNA elements required for replication. Together, these results suggest that the impaired ability of RNA1 mutants expressing p27 alone to be amplified by *trans*-supplied p88 is caused by a *cis*-preferential requirement of p88 for RNA1 replication.

Effects of mutations in the GDD motif on RNA1 replication in supply of wild-type p88

To investigate whether a complete translation process through p88 ORF is involved in the *cis*-preferential requirement of p88 for RNA1 replication, we used pUBRC1-p88uGVD, in which the Gly-Asp-Asp (GDD) motif was substituted to Gly-Val-Asp (GVD) (Fig. 1). The GDD motif is conserved in RdRP (Koonin and Dolja, 1993), and is required for RCNMV RNA synthesis (Bates et al., 1995). Cowpea protoplasts were inoculated with pUBRC1-p88uGVD or pUBRC1 together with or without pUBp88p and pUBRC2. In protoplasts inoculated with pUBRC1-p88uGVD, the accumulation of RNA1 was extremely low compared to that of wild-type RNA1 from pUBRC1 (Fig. 4). Similar results were obtained in coinoculation with a mixture of pUBp88 and pUBRC2 (Fig. 4). These results indicated that wild-type p88 supplied *in trans* did not complement the impaired replication of RNA1 mutant containing GVD motif. Failure of the rescue in the replication of RNA1 mutant with GVD motif by wild-type p88 suggests that the act of translation throughout the p88 ORF is insufficient by itself to recruit replication factors including p88 to RNA1, and supports the *cis*-preferential requirement of p88 for RNA1 replication. The effect of the introduced mutation in the GVD mutant on *cis*-acting RNA replication elements is discussed later.

cis-Preferential requirement of p88 for negative-strand RNA synthesis in BYL

To further investigate the functions of p27 and p88 in RNA1 replication, we analyzed negative-strand RNA synthesis in a

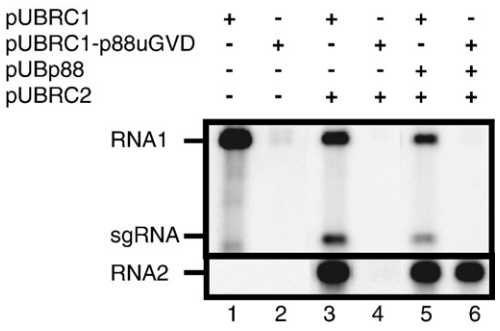

Fig. 4. Effects of a mutation in the GDD motif on RNA1 replication in the supply of wild-type p88. Cowpea protoplasts were inoculated with various combinations of plasmids indicated above each lane. For others, refer to the legend of Fig. 2.

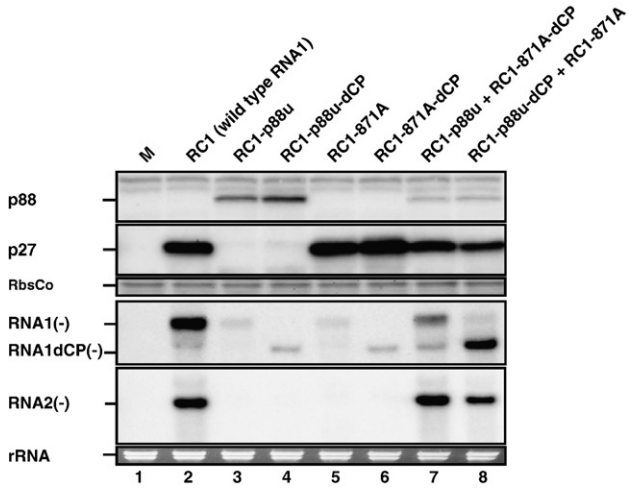

Fig. 5. Analysis of translation products and negative-strand RNA synthesis of RCNMV RNA1 mutants in a cell-free BYL system. *In vitro* transcribed uncapped RCNMV RNAs, RC1, RC1-p88u, RC1-p88u-dCP, RC1-871A, and RC1-871A-dCP corresponding to DNA vector plasmids, pUBRC1, pUBRC1-p88, pUBRC1-p88-dCP, pUBRC1-871A, and pUBRC1-871A-dCP were incubated in BYL reaction mixture at 17 °C for 4 h. Proteins and negative-strand RNAs, respectively, were analyzed by Western blotting with p27-antiserum and Northern blotting with specific probes for RNA1 and RNA2.

BYL *in vitro* system using *in vitro* transcribed RNA1 mutants. This system allows one to analyze negative-strand RNA synthesis following translation from the input RCNMV RNAs (Iwakawa et al., 2007). RNA1 transcripts having mutations corresponding to pUBRC1-871A, pUBRC1-p88u, pUBRC1-871A-dCP and pUBRC1-p88u-dCP were incubated in various combinations in the BYL *in vitro* system. Immunoblot analysis using p27-antiserum confirmed the accumulation of p27 and p88 as expected from the nucleotide sequences of RNA1 mutants (Fig. 5, lanes 3 to 8). In incubation of a mixture of RNA1 mutants corresponding to pUBRC1-p88u and pUBRC1-871A-dCP and a mixture of those corresponding to pUBRC1-p88u-dCP and pUBRC1-871A, negative-strand RNA1s with a size expected from the respective mutants expressing p88 were detected as a band with strong intensity (Fig. 5, lanes 7 and 8). RNA bands with sizes corresponding to those expected for RNA1 mutants expressing p27 were also detected in these lanes, but the intensity of the bands was similar to that of RNA1 mutants incubated alone (Fig. 5, lanes 3 to 6). Therefore, these RNAs with weak signals were unlikely to be negative-strand RNA products synthesized from the input RNA1 mutants. Together, the results obtained in the BYL *in vitro* system indicated that negative-strand RNA was synthesized only from RNA1 mutants expressing p88 and in a *cis*-preferential manner.

Discussion

Our study shows that only RCNMV RNA1, from which p88 is translated, can be an effective template for viral RNA replicase to initiate RNA synthesis in the presence of p27. This suggests that p88 is required preferentially in *cis* for the replication of RNA1, which contrasts with the efficient *trans*-acting activity of the protein for the replication of RNA2.

Possible mechanisms responsible for the coupling between translation and replication

A *cis*-preferential function of the encoded protein in viral RNA replication or coupling between translation and replication of viral RNA has been reported in genomic, defective (D), or defective-interfering (DI) RNAs of several positive-strand RNA viruses. In these phenomena, the process of translation, including the protein-coding capacity of the D or DI RNAs, may be essential for viral RNA replication; alternatively the translated protein products included in the translation process may be essential for viral RNA replication. For example, the replication of poliovirus DI RNA requires inframe deletion in the 2B non-structural protein (Johnson and Sarnow, 1991) and the replication of DI RNAs with amber mutations introduced into the 2A protein gene is not complemented in *trans* by the wild-type virus (Novak and Kirkegaard, 1994). These observations suggest a *cis*-preferential requirement of either the encoded protein or the translation process itself through the coding region, or both, for RNA replication. In the case of a prototype of CYMV D RNA, the process of translation rather than the encoded protein is considered essential for RNA replication in the presence of helper CYMV RNA. The full-length D RNAs of CYMV containing prematurely terminated fusion ORFs are not viable, whereas D RNAs containing a variety of inframe deletions and insertions in the regions of the fusion ORF of the D RNA are viable, despite the observation that these fusion ORFs are unlikely to encode functional proteins (White et al., 1992).

In the coupling between translation and replication of RCNMV RNA1, a biologically active translation product rather than translation process appears important for RNA1. This is because an RNA1 mutant with the GDD motif replaced by GVD failed to replicate in the presence of p27 and p88 (Fig. 4). Failure in the replication of this mutant suggests that the translation process through p88 ORF, including −1 frameshift, is not sufficient to recruit replication proteins and to initiate the replication of RNA1. Although the effects of the mutation introduced at position 1818 on *cis*-acting RNA replication elements in the GVD mutant cannot be completely ruled out, no or little effect on RNA1 replication of one nucleotide substitution (G to C) at position 1815 in the GDD motif (ADD in the mutant) (H. Nagano and T. Okuno, unpublished data) suggest that the impaired replication of the GVD mutant is mainly caused by amino acid change in p88, and not by nucleotide change in RNA.

No report for the occurrence of small RNA replicons derived from RNA1 in RCNMV infection supports the *cis*-preferential requirement of p88 for the replication of RNA1. This is unlike RNA2, which has *cis*-acting RNA elements that can recruit replication proteins supplied in *trans*: the 3′ UTR of RNA2 or the *trans*-activator located in the MP ORF are candidates for the *cis*-acting RNA elements of RNA2, because these elements are essential for the initiation of negative-strand RNA synthesis (Takeda et al., 2005; Tatsuta et al., 2005; Turner and Buck, 1999; H. Iwakawa and T. Okuno, unpublished results). In place of such RNA elements in RNA2, p88 may function as an element for recruiting replication factors including p27 in RNA1 replication. Binding of p88 to the proper region of RNA1 through translation

may cause a structural change in RNA1, which is required for the formation of RNA replication complexes. The regions may include RNA elements predicted in the 3′ proximal regions of RNA1 that are essential for initiating RNA synthesis (Iwakawa et al., 2007). A structural change in RNA induced by viral protein and its translation has been reported in several RNA viruses. In the RNA bacteriophage MS2, translation of the CP gene is required for the expression of the replicase protein gene. The CP gene alone is accessible to ribosomes in the full-length genomic RNA. The ribosomal binding site of the replicase gene is masked by a base pairing to an internal CP gene located at upstream of the viral genome, and the expression of the replicase protein gene is coupled to the expression of CP by the disruption of the base pairing and release of the replicase start site (Berkhout and van Duin, 1985). CP-mediated RNA structural change has also been reported in the 3′ UTR of genomic RNAs of AMV, in which CP plays an important role in initiation of viral RNA synthesis (Olsthoorn et al., 1999). In AMV, non-structural replicase component proteins encoded by genomic RNA 1 and RNA2 are required in *cis* for the replication of RNA1 and RNA2 (Neeleman and Bol, 1999; van Rossum et al., 1996).

Possible roles of the cis-preferential requirement for p88 in RNA1 replication and in RCNMV infection

What does *cis*-preferential requirement for p88 in RNA1 replication means for RCNMV? *cis*-Preferential requirement of p88 coupled with p88 translation will limit the number of RNA1 molecules that can engage in RNA replication, because a frameshifting event required to generate p88 occurs in less than 10% of translations, as assessed by the rabbit reticulocyte lysate *in vitro* translation assay or by transient expression assay of the chimeric RCNMV frameshift element-GUS construct in *Nicotiana benthamiana* protoplasts (Kim and Lommel, 1994; Kim and Lommel, 1998). We have never detected p88 in RCNMV-infected cells and in incubation of wild-type RNA1 in BYL in our experimental conditions (unpublished data; Fig. 5). In BYL, p88 was detectable only in incubation of RNA1 mutants from which p88 alone is translated. This implies that most wild-type RNA1 can engage in translation to produce a large amount of p27 by functioning as a template for ribosomes without competing with RNA replicase. In addition to frameshifting regulation, such regulation should lead to overproduction of p27 in excess to that of p88, which may be important for RCNMV RNA replication and infection.

On the other hand, *cis*-preferential requirement of p88 in RNA1 replication may function to avoid or reduce access of translation factors to RNA1 and to facilitate the replication of RNA1. Genomic RNA of positive-strand RNA viruses serves as a template for synthesis of negative-strand RNA and as a template for synthesis of viral proteins. This results in a conflict between RNA replication and translation pathways. The *cis*-preferential requirement of viral proteins for RNA replication may partly help to facilitate a switch in the role of genomic RNA from mRNA to a replication template. In polioviruses whose replication appears to be coupled with translation of encoded viral proteins (Novak and Kirkegaard, 1994), the binding of viral protein 3CD represses translation and facilitates negative-strand RNA synthesis (Gamarnik and Andino, 1998).

There remain several questions to be addressed for the roles of *cis*-preferential requirement of p88 in RNA1 replication. For example, at what frequency is p88 used for *cis*-preferential replication of RNA1? At least sufficient amounts of p88 should be available for RNA2 replication. Probably, p88 is released from RNA1 at frequencies enough to support RNA2 replication. It is also possible that RNA2 may have the ability to deprive RNA1 of translated p88.

Interestingly, the *cis*-preferential requirement of the frameshift product p88 in the replication of RCNMV RNA1 is opposite to what was found in two other members of *Tombusviridae*, TCV and TBSV. In these viruses, viral RNAs expressing pre-readthrough products, p28 and p33 alone, respectively, are preferentially replicated over those expressing readthrough products alone in their coinoculation (White et al., 1995; Oster et al., 1998; Monkewich et al., 2005). In TBSV, the preferential replication of viral RNAs expressing p33 alone does not result from a *cis*-preferential function of p33, but rather results from translation-based inhibition of a *cis*-acting RNA element (RII) that is positioned in the readthrough portion of the p92 ORF (Monkewich et al., 2005). RII is considered to function for assisting the recruitment of viral RNAs out of translation and into replication.

Thus, a *cis*-preferential function of viral replication proteins or the translation process appears to function in a different way in regulation between RNA replication and translation in different viruses.

Materials and methods

Plasmid clones

pUBRC1 (Mizumoto et al., 2006) and pUBRC2 (Takeda et al., 2005) were previously described as plasmids from which wild-type RCNMV Australian strain RNA1 and RNA2, respectively, were transcribed from the cauliflower mosaic virus 35S promoter in the introduced cells. pUBRC1-p27 was created by insertion of the *Sac*I/*Sma*I fragment containing RCNMV RNA1 mutant cDNA from pBICRC1-p27 (Takeda et al., 2005) into pUC118 at the corresponding sites. pUBRC1-p27dCP and pUBRC1-p88u-dCP were derived from pUBRC1-p27 and pUBRC1-p88u, respectively, in which the *Xho*I/*Sac*II region was replaced with that from pBICRC1-ΔCP (Takeda et al., 2005). Other mutants, pUBRC1-p88u, pUBRC1-871A, pUBRC1-871G, pUBRC1-871C, and pUBRC1-p88uGVD were created by PCR-based *in vitro* mutagenesis from pUBRC1. In short, recombinant PCR products were digested by appropriate restriction enzymes that had unique recognition sites neighboring the mutation sites in pUBRC1, and the fragments containing the mutation substituted the corresponding fragments in pUBRC1. The absence of unexpected base change was confirmed by sequencing the resulting plasmids. These RNA1 mutants transcribed from pUBRC1 plasmids with harboring mutations are summarized in Fig. 1. The plasmids for protein expression, pUBp27 and pUBp88 were previously described (Takeda et al., 2005). All infectious DNA

plasmids were prepared by using QIAGEN Plasmid Midi Kit (QIAGEN). The plasmids pUCR1-871A, pUCR1-871A-dCP, pUCR1-p88u and pUCR1-p88u-dCP were created by replacement of the *Aor*51HI-*Bsi*WI region in pUCR1 (Takeda et al., 2005) with the corresponding regions from pUBRC1-871A, pUBRC1-871-dCP, pUBRC1-p88u and pUBRC1-p88u-dCP, respectively. RNA transcripts were synthesized from these plasmids by T7 RNA polymerase after linearization by *Xma*I.

Protoplast experiments

Preparation and inoculation of cowpea (*Vigna unguiculata* cv. California Blackeye) protoplasts were previously described (Mizumoto et al., 2003). In brief, the inoculation was performed by polyethylene glycol-mediated inoculation method as described by Sheen (http://genetics.mgh.harvard.edu/sheenweb/) using PEG4000 (Fluka). Mixture of plasmids (10 μg each) or transcripts (1.5 μg each) were used as an inoculum. Inoculated protoplasts (5×10^4 cells) were incubated for 24 h at 17°C (Mizumoto et al., 2003). Total RNA and protein were extracted from infected protoplasts using TRIzol reagent (Invitrogen) and Laemmli's sample buffer (Laemmli, 1970), respectively, and subjected to Northern blot and immunoblot analysis. All inoculation experiments were repeated at least three times.

BYL in vitro experiments

Preparation of cell extracts of evacuolated tobacco BY-2 protoplasts (BYL) and cell-free *in vitro* translation and replication were done as described (Iwakawa et al., 2007). Uncapped *in vitro* transcripts (2 μg in each 1 μl) were added to 25 μl BYL. The BYL translation and replication mixture was incubated at 17 °C for 4 h. Aliquots (15 μl) of the reaction mixture were used for immunoblot analysis, and aliquots (10 μl) of the reaction mixture were used for Northern blot analysis as described previously (Iwakawa et al., 2007).

Northern blot analysis

Northern blot analysis was done as previously described (Damayanti et al., 2002). The digoxigenin (DIG)-labeled RNA probes specific to the 3′ UTRs of RCNMV RNA1 and RNA2, and negative-strand RNA1 and RNA2, were synthesized *in vitro* and used for the hybridization as previously described (Mizumoto et al., 2002). The RNA signals were detected with a luminescent-image analyzer (LAS 1000 Plus; Fuji Photo Film, Japan).

Immunoblot analysis

Immunoblot analysis of p27 and p88 was performed as previously described (Tatsuta et al., 2005) using ECL Plus Western Blotting Detection System (GE Healthcare Bio-science, USA).

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