**Cis-preferential replication of the turnip yellow mosaic virus RNA genome**

(positive-strand RNA virus/coreplication/complementation/cis action)

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**ABSTRACT**

The largest open reading frame of the turnip yellow mosaic virus RNA genome encodes a 206-kDa protein that is cleaved to yield N-terminal 150-kDa (p150) and C-terminal 70-kDa (p70) proteins. Using a genomic cDNA clone capable of generating infectious transcripts in vitro, we have introduced substitution, frameshift, and in-frame deletion mutations into the regions encoding both proteins. None of the mutant RNAs was able to replicate independently in turnip protoplasts, indicating that p150 and p70 are both essential. The replication in protoplasts of most of these defective RNAs was poorly supported in trans by a coinoculated helper genome with a deletion in the coat protein gene; replication could also be supported in trans by certain defective RNAs, but this complementation was likewise inefficient in most cases. The replication in trans was more efficient for defective RNAs encoding wild-type p150 and defective p70 than for those encoding defective p150 and wild-type p70. One defective RNA with a large deletion in the p70 coding region was able to replicate efficiently, both when inoculated with the helper genome and when inoculated with a second complementing defective RNA that supplied a wild-type p70. Thus, the cis preference of replication can be overcome in some cases. A model in which p150 and p70 form a complex with the 3′ end of the RNA is proposed to explain the cis-preferential replication of turnip yellow mosaic virus RNA.

Turnip yellow mosaic virus (TYMV) is a monopartite, positive-strand RNA virus infecting crucifers. One of the three open reading frames (ORF-206) present in the 6318-nucleotide genome (Fig. 1A) encodes proteins essential for RNA replication in protoplasts (1, 2). Expression of ORF-206 involves the proteolytic maturation in cis of a precursor protein, resulting in synthesis of an N-terminal 150-kDa protein (p150) and a C-terminal 70-kDa protein (p70; Fig. 1B; ref. 3), each of which contains amino acid sequence motifs that are common to the essential nonvirion proteins of other RNA viruses. A motif of sequence GCGKT is encoded by nucleotides 3029–3043 and is present near the C terminus of p150 (Fig. 1B). This motif is thought to represent a nucleotide binding element and is associated with additional sequences resembling those found in NTP-dependent helicases (4, 5). The second motif, GDD, encoded by nucleotides 5081–5089 in the p70 coding region of TYMV RNA, is considered to be a signature sequence for RNA-dependent RNA polymerases (6, 7).

In multipartite viruses, the proteins bearing the helicase-like and polymerase-like domains clearly function in trans, because infections require a mixture of RNA components that separately encode these proteins. Likewise, in many monopartite viruses the analogous proteins appear to function in trans, because naturally occurring defective interfering RNAs, as well as experimentally constructed deletion variants that can be efficiently coreplicated with a helper genome, have been described (e.g., see refs. 8–11). In the course of our studies on the replication of TYMV RNA, we observed that several genomic RNAs with different internal deletions and frameshift mutations failed to replicate detectably in the presence of wild-type helper genome (C.-H. Tsai and J.J.W., unpublished observations). We report here investigations demonstrating that the replication of TYMV RNA is strongly cis preferential, but we also show that certain combinations of defective RNAs are able to overcome this cis limitation. Our observations suggest a model explaining the molecular basis of the cis-preferential replication of TYMV RNA, in which the newly synthesized essential replication proteins are channeled in cis to the minus-strand promoter of the template RNA.

The existence of cis-limited replication in positive-strand RNA viruses previously has been described only with poliovirus. The coreplication of deletion variants of poliovirus RNA depends on the presence of a translatable nonstructural cistron (10, 12), and mutations in the 2B nonstructural protein are noncomplementable in trans (13–15). These observations suggest that cis-preferential replication of poliovirus RNA results from the cis action of protein 2B. For tobacco mosaic virus, the possibility of cis-preferential replication has been suggested, although the present evidence is only circumstantial (16, 17). Our studies with TYMV support the idea that some degree of cis preference may be a general feature of the replication of positive-strand RNA viruses.

**MATERIALS AND METHODS**

**Materials.** Turnip (Brassica rapa cv. Just Right) plants were grown in a controlled chamber at 21°C with a 16-hr day length. Plasmid pTYMC, a cDNA clone from which infectious TYMV RNA (strain TYMC) can be transcribed in vitro, has been described (1).

Plasmid DNAs and in Vitro Mutagenesis. Deletion and frameshift mutants (shown in Fig. 1C) were constructed by using either the Klengow fragment of DNA polymerase I or mung bean nuclease to blunt restriction site overhangs (18); sequences were confirmed in each case by sequencing across the deletions. The following mutants with in-frame deletions were created from pTYMC: pTYMC-A5707-6062 by deletion between the Pvu II (position 5708) and Smal I (position 6062) restriction sites; pTYMC-A213-1759 by deletion between the filled Nco I (position 210) site and the nuclease-blunted BamHI (position 1755) site; pTYMC-A1536-1759 by ligation

Abbreviations: TYMV, turnip yellow mosaic virus; ORF, open reading frame.

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Fig. 1. Diagram of TYMC and various modified genomes studied in this paper. (A) Map of the cDNA insert of pTYMC showing the major viral ORFs and restriction sites used in this study. The T7 RNA polymerase promoter (arrow at left) permits infectious genomic RNA to be made from pTYMC linearized at the unique HindIII site. The site of proteolytic cleavage of the ORF-206 product is indicated. (B) Diagram of the features of essential replication proteins encoded by ORF-206. GCGKT and GDD elements are the core amino acids of the helicase-like and polymerase-like domains, respectively. (C) TYMC variants described in this work and a summary of their replication properties (taken from Figs. 2 and 3). Only ORF-206 and the coat protein ORF are shown. Mammalian nuclease reflects the genome nucleotides that are joined in the case of deletion variants and the ORF-206 codon changes for substitution mutants (TY-K982S and TY-G1331R). Solid bars represent deleted sequences and stippled bars are out-of-frame sequences prior to reading frame termination for TYMC-Δ3278-3283 and TYMC-Δ4085-4090. ORF-206 and coat protein ORF sequences are fused in mutant TYMC-Δ4085-6062. Genomes used as common coinoculation partners are in boldface in the diagram, with the corresponding number at the head of a column of the table on the right. The table shows total accumulations of defective genomes (relative to wild-type TYMC infections) in turnip protoplasts when the indicated defective RNA was coinoculated with helper genome (1) or with the complementing defective RNAs (2) and (3). Accumulation of helper genome (relative to TYMC infections) when inoculated alone is given in the top cell of column 1.

To create the frameshift mutants pTYMC-Δ3278-3283 and pTYMC-Δ4085-4090, plasmid pTYMC was digested at the XhoI (position 3279) and SalI (position 4086) sites, respectively, and blunt ended with mung bean nuclease prior to recircularization.

Construction of the amino acid substitution mutants pTY-K982S (nucleotides 3038 and 3039, AA → UC; Lys-982 → Ser) and pTY-G1663R (nucleotides 5081-5083, GGC → AGA; Fig. 1) was by PCR-mediated megaprimer site-directed mutagenesis (19) utilizing a subclone of pTYMC. The entire mutagenized fragment was sequenced prior to subcloning into pTYMC.

Inoculation of Transcripts onto Protoplasts and Plants. DNA templates were linearized at the unique 3' HindIII site (Fig. 1A) and were transcribed to generate capped transcript RNAs (1). Protoplasts were prepared from turnip plants and inoculated as reported for Chinese cabbage protoplasts (1). Inoculated protoplasts subsequently were incubated for 30 hr under constant light at 25°C. A fraction of the protoplasts harvested after incubation were lysed and used to mechanically inoculate turnip plants. Protoplasts (4 × 10^5) were resuspended to 10 μl in inoculation buffer (10 mM Tris-HCl, pH 7.2/0.9 mM potassium phosphate/3 mM sodium phosphate/14 mM NaCl/3 mg of bentonite per ml) and rubbed onto the carborundum-dusted leaves of 2-week-old turnip plants.

Tissue Extraction and Blot Hybridization. Protoplasts and plant tissue were harvested and extracted by grinding in cold extraction buffer (10 mM Tris-HCl, pH 8.0/0.1 M NaCl/1 mM EDTA/1% SDS/3 mg of bentonite per ml) and immediately extracting twice with buffer-saturated phenol/chloroform. Total nucleic acids were recovered from the aqueous phase by ethanol precipitation, glyoxalated, electrophoresed in 1% agarose gels, and blotted to nylon membranes and probed as described (1), with modifications from ref. 20. After exposure to film, the viral RNAs were quantitated by laser scanning densitometry.

**RESULTS**

Inefficient Coreplication of Defective TYMC Derivatives in the Presence of Helper Genome. After initial experiments in which TYMC RNA (Cornell strain of TYMV) was used as helper genome, an RNA lacking two-thirds of the coat protein ORF (TYMC-Δ5707-6062 RNA; Fig. 1C) was used as the helper genome in further experiments. TYMC-Δ5707-6062 RNA replicated in turnip protoplasts, accumulating (+)-sense genomic RNA to levels 10% those of wild-type TYMC infections 30 hr postinoculation (Fig. 2) and accumulating (−)-sense genomic RNA to levels similar to those in TYMC infections (data not shown). Because normal levels of (−)-sense genomic RNA accumulated in protoplasts infected with TYMC-Δ5707-6062, the decreased accumulation of (+)-sense genomic RNA may be due to increased degradation of the nonecapsidated replication product. A hybridization probe complementary to coat protein ORF sequences absent from the helper genome (Pvu II (position 5708)/Smal I (position 6062)) was used to specifically detect defective coreplication substrates bearing alterations in the essential ORF-206 (Fig. 2).

Variant RNAs to be used as coreplication substrates (described in Fig. 1C) were designed to contain alterations in the p150 or p70 coding regions of ORF-206 that would not perturb the normal proteolytic processing at the p150/p70 junction (thought to be located between amino acids encoded by nucleotides 3851-3877; see Fig. 1B; ref. 3). As expected from our knowledge of the location of the TYMV protease domain (Fig. 1B; ref. 3), all mutant RNAs (except TYMC-Δ3278-3283 RNA) directed the synthesis of ORF-206 products that were
Normally processed after in vitro translation in rabbit reticulocyte lysates (data not shown).

None of the mutant RNAs with alterations in ORF-206 shown in Fig. 1C replicated to levels detected by blot hybridization when independently inoculated into turnip protoplasts (shown for TY-K982S and TY-G1663R in Fig. 3A and for TYMC-A4085-5406 RNA in Fig. 3B). However, when mutant RNAs TYMC-A1536-1759, TY-K982S, TY-G1663R, and TYMC-A4085-5406 were separately coinoculated with helper genome TYMC-D5707-6062 RNA, coreplication was detected (Fig. 2). The presence of subgenomic RNAs, absent in the inoculum, verified that replication of all defective genomes except TYMC-D3278-3283 was occurring. Although the level of coreplication varied widely between the different defective genomes tested, most of the defective RNAs were inefficiently coreplicated (genomic RNA accumulations < 20% relative to that of the helper genome) (Fig. 2). By stripping and reprobing the blots with a probe complementary to the 3' 259 nucleotides that detects both helper and defective RNAs, it could be shown that the varying levels of defective RNA accumulation were not the result of varying helper RNA levels (data not shown). There was thus no indication that any of the mutant genomes tested were defective interfering RNAs.

![Fig. 2. Coreplication of defective TYMC RNAs in the presence of TYMC-D5707-6062 helper genome.](image)

**Fig. 2.** Coreplication of defective TYMC RNAs in the presence of TYMC-D5707-6062 helper genome. (A) Blot probed with sequences absent from helper genome. Turnip protoplasts (4 × 10⁶) were inoculated with the RNAs indicated (3 μg of TYMC or helper RNA and equimolar amounts of the defective RNAs), incubated for 30 hr at 25°C, and harvested. Nucleic acid samples corresponding to 4 × 10⁶ protoplasts were analyzed in a blot probed with (-)sense sequences between the Pst I (position 5708) and Sma I (position 6062) sites of TYMC (see Fig. 1). Accumulations of defective genomic RNAs relative to the levels of wild-type genomic RNA present in TYMC-infected cells are indicated below lanes and were quantitated by densitometrically scanning similar blots from at least three replicate experiments, using appropriate dilutions of RNA extracts. One-tenth the amount of extract (representing 4 × 10⁶ protoplasts) was analyzed in the left lane, as indicated. The migration positions of genomic (g) and subgenomic (sg) RNAs are marked. (B) Blot reprobed with sequences complementary to the 3' 259 nucleotides of TYMC RNA common to all the TYMC RNA variants.

**Fig. 3.** Complementation in protoplasts between two defective RNAs. (A) Complementation between TY-K982S RNA and various defective RNAs encoding mutant p70. Blot probed with sequences complementary to the 3' 259 nucleotides of TYMC RNA to detect (+) strand genomic (g) and subgenomic (sg) RNAs. Lanes were loaded with RNAs from 4 × 10⁶ protoplasts inoculated with the RNAs specified and harvested 30 hr postinoculation (except for K982S + A4085-6062, which was harvested at 36 hr). Accumulations of total defective genomic RNAs relative to TYMC infections are indicated below the lanes (means of at least four experiments). (B) Complementation between TYMC-A4085-5406 RNA and defective RNAs encoding mutant p150. Blot probed as in A, with lanes analyzing RNA from 8 × 10⁶ cells harvested 30 hr postinoculation (h.p.i.).

Fig. 3A shows the results from a typical experiment in which TY-K982S RNA was coinoculated with a series of RNAs encoding defective p70 and wild-type p150. Among the defective RNAs with mutations restricted to the p70 coding region (TY-G1663R, TYMC-A4085-4090, TYMC-A4085-4587, and TYMC-A4085-5406 RNAs), the accumulations of viral RNAs increased progressively with deletion size. Replication was observed for all the inoculation mixtures studied, but, as observed in the helper coinoculation experiments of Fig. 2, the efficiency varied widely. The total accumulation of genomic RNAs 30 hr postinoculation relative to those of TYMC-infected protoplasts ranged from 1% for the TY-K982S + TY-G1663R RNA inoculation to 49% for the TY-K982S + TYMC-A4085-5406 RNA inoculation (Fig. 3A). Coinoculation of TY-K982S + TYMC-A4085-6062 RNAs resulted in inefficient complementation, with genomic RNA accumulations at 30 hr postinoculation in protoplasts to only 0.2% relative to TYMC RNA. TYMC-A4085-6062 RNA has an extensive deletion removing most of both the p70 and coat protein coding regions, as well as the highly conserved "tmbox" RNA sequence present upstream of the initiation site for subgenomic RNA transcription (21). The decreased amount of subgenomic RNA, and hence of coat protein, in coinfections of this mutant with TY-K982S RNA may account for the low accumulations of viral RNA.

To compare the complementation efficiencies for defective RNAs encoding mutant p150 (TYMC-A213-1759, TYMC-A1536-1759, and TY-K982S RNAs), these RNAs were coinoculated with TYMC-A4085-5406 RNA (Fig. 3B). Defective RNAs bearing deletions in the p150 coding region were replicated in trans less efficiently than the substitution mutant TY-K982S.

**Biased Accumulation in Favor of Those Defective RNAs Encoding Wild-Type p150.** Blots such as those in Fig. 3 involving coinoculations with defective RNAs that could be separated electrophoretically showed that RNAs encoding wild-type p150 accumulated to levels higher than their com-
plication partners encoding wild-type p70. TYMC-Δ4085-5406 RNA accumulated to levels 10.5-fold higher than TY-K982S RNA after coinoculation of these RNAs, with a similar skewing observed for (−)-strand RNAs (data not shown). The progeny RNAs present in protoplasts coinoculated with TY-K982S and TY-G1663R RNAs were studied in order to determine the ratio of these two complementing RNAs of equal size. Unfractionated RNA extracted from protoplasts was reverse transcribed and the regions surrounding the K982S and G1663R mutations were used as PCR amplified and sequenced. For three independent inoculations analyzed in this way, the sequence indicated a clear excess of the TY-G1663R RNA over TY-K982S RNA (data not shown). The results emphasize the generality of the preferential accumulation of the defective RNA encoding wild-type p150 and relate to the higher accumulations of p150 mutants than p70 mutants in the helper inoculation experiments of Fig. 2.

Replication of Two Coinoculated Defective RNAs Results from Complementation Rather Than Reversion or Recombination. Throughout multiple replicates, coinoculation experiments involving two defective RNAs yielded highly consistent results. This suggested the absence of reversion or recombination, events that rely on replication errors and are relatively infrequent and often random. An absence of gross recombination was indicated by the fact that only the expected electrophoretic pattern of progeny RNAs (corresponding to the inoculum mixture) was observed (Fig. 3). However, since several progeny RNAs comigrate with wild-type TYMC RNA during agarose gel electrophoresis, we wished to verify that wild-type RNA was absent from the progeny of complementation experiments.

A fraction of the protoplasts harvested from all experiments was lysed and inoculated onto turnip plants, which were then inspected for disease symptoms after 2 weeks. This is a potent assay for the presence of wild-type RNA. Local lesions or systemic symptoms failed to appear for all but one inoculum combination: plants inoculated with protoplasts infected with TY-K982S + TYMC-Δ4085-5406 RNAs developed delayed and distinctive symptoms that differed from those caused by TYMC. Both input RNAs were present in infected tissue (data not shown), and further studies have shown that this combination of defective RNAs in fact constitutes a novel bipartite form of TYMC (to be reported elsewhere). Since the tests described above have indicated the absence of wild-type RNAs in the coinoculation experiments, it can be concluded that the replication observed after coinoculation with two defective RNAs was indeed due to complementation rather than some genetic alteration that regenerated wild-type or pseudo-wild-type RNA.

**DISCUSSION**

Using defective TYMC RNAs with mutations in ORF-206, we have shown that both of the proteolytic cleavage products (p150 and p70) are essential for RNA replication in turnip protoplasts. The defective substitution mutants we have studied support the assignement of p150 as a helicase and p70 as a polymerase; the substitution mutation of TY-K982S RNA (GCGKT to GCGST) is analogous to the GXGKT to GXGRT substitution that inactivated the RNA helicase activity of RAD3S protein from yeast (22), and the substitution mutation of TY-G1663R RNA (GDD to RDD) is analogous to the substitution of the glycine residue in the GDD element of poliovirus RNA-dependent RNA polymerase with various amino acids, which resulted in large losses of polymerase activity (23).

**TYMC RNA Replication Is Characterized by a Cis- Preferential Mechanism.** The most striking observation of our studies has been that the replication of most defective TYMC RNAs could be rescued in trans only inefficiently. This was true whether defective RNAs were coinoculated with a helper genome or with another potentially complementing defective RNA. The generality of the observations makes it highly unlikely that the inefficient replication in trans is due to the loss of cis-acting RNA elements (e.g., promoter sequences), to an instability or high turnover of the mutant RNA, or to a gross alteration of ribosomal trafficking that is somehow detrimental to replication (24), although one or more of these factors may pertain with certain defective RNAs. Rather, it seems clear that the TYMV replicational machinery displays a cis preference, such that the essential replication proteins made from a given genomic mRNA molecule assemble most efficiently into a replication complex on that same RNA molecule, facilitating the switch in the role of the RNA from mRNA to replicational template.

Figs. 2 and 3 show that the efficiency of replication in trans varied considerably, depending on the type and location of the mutation. Indeed, even though most defective RNAs replicated inefficiently in trans, TYMC-Δ4085-5406 RNA, which lacks most of the p70 coding region (Fig. 1C), could be efficiently replicated in trans in most contexts. This demonstrates that the observation of efficient replication in trans of a particular virus-derived RNA does not necessarily indicate the absence of a cis-preferential replication mechanism for that virus. Thus, it is possible that some degree of cis-preferential replication will be found to exist for many RNA viruses, including multipartite viruses and those that support the replication of defective interfering RNAs.

**A Model for Cis- Preferential Replication of TYMV RNA.** The exceptional ability of TYMC-Δ4085-5406 RNA to overcome the usual cis limitation of replication that is observed in the TYMV system provides insight into the mechanism by which TYMV RNAs are cis-preferentially replicated. The most noteworthy feature of TYMC-Δ4085-5406 RNA is the absence of almost all of the p70 coding region. We suggest that the lack of an encoded p70 is the key property that permits TYMC-Δ4085-5406 RNA to be replicated in trans. We propose a model (Fig. 4) in which the cis-preferential replication is due to the interaction of newly synthesized p150 and p70 preferentially with the RNA genome from which they have been made, resulting in the channeled formation of a replication initiation complex in cis. A p150/p70 complex, formed directly after the proteolytic release of these two products from the p206 precursor, is proposed to interact with the viral RNA molecule made from a given genomic mRNA in cis. As indicated by the skewed ratio of complementing defective RNAs in favor of the RNA encoding a wild-type p150, even in infections involving TYMC-Δ4085-5406 (Fig. 3), p150 appears to possess some cis interaction with the RNA and is presumed to make primary contact with the RNA template in the postulated p150/p70 complex (Fig. 4). We suggest that the dissociation rate of the p150/p70/template initiation complex is relatively slow, preventing ready mixing of subunits and replacement of a defective protein by an active molecule in trans. Although the identity of the subunits present in TYMV replication complexes is not clear (25), there is evidence in related viruses for the presence of helicase-like and polymerase-like proteins analogous to the TYMV p150 and p70 proteins in replication complexes (26, 27).

The above scenario explains the poor coreplication of defective RNAs with substitution mutations (TY-K982S and TY-G1663R RNAs) as resulting from cis inhibition due to formation of enzymatically inactive p150/p70 complexes. The mutations of these RNAs are expected to inactivate essential enzymatic activities without affecting other domains involved in p150/p70 complex formation and protein-template interaction. Other defective RNAs with small in-frame deletions (TYMC-A1536-1759 and TYMC-Δ4085-4587...
mechanisms may pertain to individual defective RNAs; in particular, the low accumulation of mutant TYMC-A4085-4090 RNA after mixed inoculations may be due to rapid RNA turnover, since frameshift mutations commonly increase mRNA turnover rates (28).

Future experiments could test the model of Fig. 4. A mechanism such as that proposed would clearly be beneficial in the early stages of an infection, by facilitating the assembly of replication complexes when few viral RNAs and proteins are present, by helping to prevent the use of host RNAs as templates, and by culling out defective RNAs from the replication pool.

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