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Turnip Yellow Mosaic Virus RNAs with Anticodon Loop Substitutions That Result in Decreased Valylation Fail To Replicate Efficiently†

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Single and multiple nucleotide substitutions have been introduced into the anticodon loop of the tRNA-like structure of turnip yellow mosaic virus (TYMV) genomic RNA. We studied the effects of these mutations on in vitro valylation and on replication in Chinese cabbage protoplasts and plants. Only those mutants capable of efficient and complete valylation showed efficient replication in protoplasts and gave rise to systemic symptoms in whole plants. Mutants that accepted valine inefficiently (in some cases V_{max}/K_m values were $<10^{-3}$ relative to wild-type values) replicated to levels 200- to 500-fold below wild-type levels in protoplasts (estimated on the basis of coat protein and genomic RNA levels). These mutants could not support systemic spread in plants. In one plant inoculated with TYMC-A55 RNA, which replicates poorly in protoplasts, systemic symptoms developed after a delay. The reversion in replication was accompanied by improved valine acceptance and the appearance of a U57 second-site mutation. Our results indicate a correlation between valine acceptance activity and viral yield. Possible roles for valylation are discussed, and the results are compared with those of similar studies with brome mosaic virus which suggested that tyrosylation is not crucial for brome mosaic virus replication (T. W. Dreher, A. L. N. Rao, and T. C. Hall, J. Mol. Biol. 206:425–438, 1989).

Turnip yellow mosaic virus (TYMV) has a positive-sense single-stranded RNA genome 6.3 kb long. In common with a number of other plant viruses, the 3′ region of TYMV RNA has distinctive characteristics similar to those of free tRNAs. The virion RNA, which terminates in 3′-CC_{OH}, can be adenylated by (CTP,ATP): tRNA nucleotidyltransferase (19). The resulting -CCA_{OH} terminus can be specifically and efficiently valylated by valyl-tRNA synthetase (ValRS) from wheat germ (6) or yeast cells (14). The valylated viral RNA can in turn form a ternary complex with GTP and elongation factor EF-Tu from *Escherichia coli* (17) or EF-1α from wheat germ (18). These properties are associated with a distinct domain encompassing the 3′ 82 nucleotides of the viral RNA, which can be folded into a tRNA-like structure whose probable three-dimensional conformation is similar to the L conformation of cytoplasmic tRNAs (Fig. 1; 10, 28). Despite the functional mimicry, sequence similarities to higher eukaryotic $tRNA^{Val}$ are limited to the 3′-ACCA end, a valine anticodon with 3′-flanking bases (57-<u>CAC</u>AC-53; anticodon is underlined), and a degenerate version (38-UGCA-35) of the conserved TψCPu of tRNAs (ψ = pseudouridine, Pu = purine; 31). These features are shown in Fig. 1; note that nucleotides are numbered from the 3′ end of the adenylated RNA.

The role that the tRNA mimicry plays in the replication cycle of the virus remains incompletely understood. It is known that TYMV RNAs become valylated during replication in Chinese cabbage (20). Thus, TYMV RNA is an active substrate in vivo for host ValRS, and most likely also for host (CTP,ATP):tRNA nucleotidyltransferase, since the virion RNA inoculum lacks the 3′ adenosine residue necessary for aminoacylation. A leading question is whether the in vivo valylation is merely coincidental, or whether it is a necessary element of the replication machinery or of its control. We have previously addressed this question with brome mosaic virus (BMV), another positive-sense RNA virus. In BMV, the tyrosine-charging tRNA-like structure has been shown to function as the promoter for negative-strand synthesis (24), suggesting a close relationship between tRNA mimicry and RNA replication. However, tyrosylation may not be a key part of that interdependence, since mutants that were expected on the basis of in vitro characterization to be tyrosylated inefficiently in vivo replicated fairly well in barley protoplasts (9). Rather than tyrosylation, substrate activity for tRNA nucleotidyltransferase may be a more crucial function: the BMV tRNA-like structure acts as the 3′ telomere of the positive strand, and host tRNA nucleotidyltransferase most likely acts as a telomerase to maintain intact 3′ termini during replication (26).

To further investigate the role of aminoacylation in viral replication, the TYMV system offers advantages over BMV. In particular, the discoveries that valine identity determinants of *E. coli* $tRNA^{Val}$ are present in the anticodon and that limited nucleotide changes can alter the aminoacylation specificity of this tRNA (30) suggested that noncharging and mischarging TYMV RNAs might be constructed fairly readily. It was thought that such mutants would provide more convincing results than the partially charging BMV RNA mutants previously studied (9). We report here on mutant TYMV genomic RNAs carrying substitutions in the anticodon loop, some of which have very low valine-accepting activities in vitro. The results show that valine acceptance of viral RNAs is an important criterion for the amplification of TYMV RNA.

MATERIALS AND METHODS

Materials. Chinese cabbage (*Brassica pekinensis* cv. Wong Bok) plants were grown in a growth chamber under 16-h day length at 20°C. Sources for materials were as

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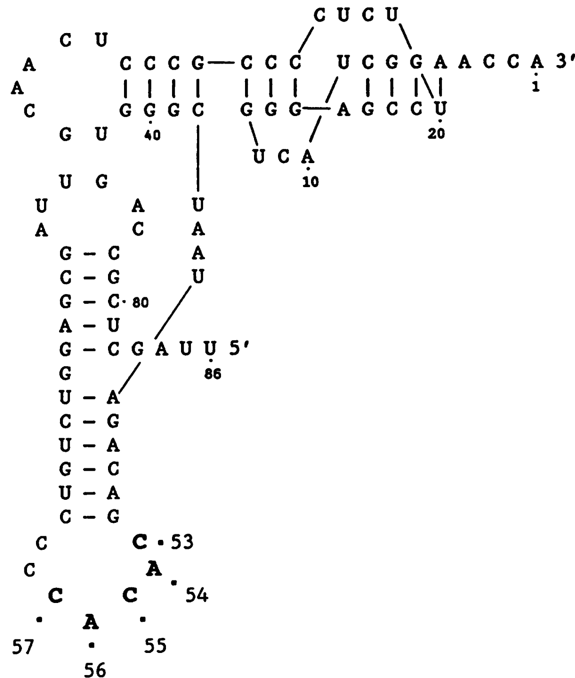

FIG. 1. Proposed L conformation of the tRNA-like structure of TYMV RNA showing the nucleotides at which substitutions have been made (shown in bold). They comprise the valine anticodon (57-CAC-55) and two further anticodon loop nucleotides conserved among valylatable viral and transfer RNAs. Note that nucleotides are numbered in ascending order from the 3'-CCA end, since the 5' end of the fragment shown is connected to a further 6.2 kb of genomic sequence.

follows: T7 RNA polymerase, Bethesda Research Laboratories; m^7GpppG cap analog, New England BioLabs; Inhibit-Ace RNase inhibitor, 5 Prime-3 Prime, Inc. (West Chester, Pa.); T7 DNA polymerase (Sequenase) and reverse transcriptase, United States Biochemical; *Thermus aquaticus* DNA polymerase, Promega; restriction enzymes, Gibco-BRL, Boehringer-Mannheim, and New England BioLabs; and Macerase and Cellulysin, Calbiochem. Synthetic deoxyoligonucleotides were made by automated phosphoramidite synthesis and purified on 20% polyacrylamide–7 M urea gels.

Cloning, in vitro site-directed mutagenesis, and transcription. In preparation for mutagenesis, a 258-bp *Sma*I-*Hin*dIII fragment from the 3' end of the TYMV genomic cDNA clone pTYMC (33) was transferred to an M13 mp18 vector. Single-stranded deoxyuridine-containing template DNA was generated in *E. coli* CJ236 and used for in vitro mutagenesis, directed by the appropriate 5'-phosphorylated synthetic deoxyoligonucleotides (7, 21). Mutant clones were screened either by direct sequencing or by plaque hybridization, and the sequence of the entire TYMV insert was verified prior to subcloning back to the genomic pTYMC clone as *Sma*I-*Hin*dIII fragments.

Plasmid DNAs were prepared from large cultures, and the mutant sequences were confirmed by double-stranded DNA sequencing (5). Transcripts labeled with [α-^{32}P]UTP (0.1 Ci/mmol) were prepared from DNA templates linearized with *Hin*dIII and analyzed as described previously (33).

Protoplast and plant inoculations. Young healthy leaves (3 to 4 g) were taken from 6-week-old Chinese cabbage plants that had been held in the dark for 3 days to deplete starch grains. Protoplasts were prepared and inoculated (5 μg of transcript RNA per 4 $\times$ 10^5 cells) as described previously (33) except that protoplasts were released after incubation of leaf slices in the hydrolytic enzymes overnight at 23°C. Inoculated protoplasts were incubated under constant light at 23°C for 22 or 48 h prior to harvest.

Three-week-old Chinese cabbage plants with two true leaves were used for inoculations of whole plants. Each leaf was mechanically inoculated with 10 μl of RNA transcript (0.25 mg/ml) in 50 mM glycine–30 mM K_2HPO_4 (pH 9.2)–1% bentonite–1% Celite. At times, plants were inoculated with a suspension of protoplasts harvested 48 h postinoculation (5 $\times$ 10^4 cells per leaf). Plants were held as described above and scored for symptom appearance and virus levels.

Analysis of viral products by Western immunoblotting and Northern (RNA) blotting. The levels of coat protein in harvested protoplasts were analyzed in Western blots as described previously (33). Results were quantitated by scanning laser densitometry with reference to a dilution series of virus. In most blots, the distribution of the horseradish peroxidase-labeled secondary antibody was detected by using the chromogenic substrate 4-chloro-1-naphthol (detection limit of 2 ng of coat protein). Where necessary, about 10-fold-higher sensitivity was achieved by using chemiluminescent development (ECL; Amersham International).

RNA was extracted from protoplasts, glyoxalated, electrophoresed, and transferred to nylon membranes as described previously (33). The hybridization probe was a ^{32}P-labeled RNA transcript complementary to 0.9 kb at the 3' end of TYMV RNA. Both full-length genomic and subgenomic RNAs could be detected (33). RNA levels were quantitated by scanning laser densitometry or with a β-emission radioisotope scanner (Ambis Systems, San Diego, Calif.).

RNase protection assays of RNAs labeled in vivo. Radiolabeled RNAs were extracted from protoplasts which had been cultured in medium containing carrier-free $^{32}P_i$ (200 μCi/ml; 23). To preclude the detection of input (inoculated) RNA, both the inoculum and the analytical probe were nonradioactive. The deproteinized RNA extracts were mixed with the 0.9-kb RNA probe (see Fig. 4) and denatured at 85°C for 5 min in the presence of 80% formamide, 40 mM piperazine-*N*,*N*'-bis(2-ethanesulfonic acid) (PIPES; pH 6.7), 0.4 M NaCl, and 1 mM EDTA (in a 30-μl reaction volume). After hybridization overnight at 55°C, 300 μl of RNase solution containing 0.3 M NaCl, 10 mM Tris-HCl (pH 7.5), 5 mM EDTA, RNase A (40 μg/ml), and RNase T_1 (2 μg/ml) was added to the samples, which were then incubated at 30°C for 1 h. RNases were removed by treatment with 50 μg of proteinase K per ml in 0.5% sodium dodecyl sulfate (SDS) at 37°C for 30 min. After phenol-chloroform extraction, the samples were ethanol precipitated with carrier RNA and then analyzed by electrophoresis on 1% agarose gels and autoradiography.

Virion RNA extraction and characterization. Virus was isolated from infected plants as described by Lane (22) and quantitated spectrophotometrically. Virion RNA was prepared from virus by two phenol-chloroform extractions. To assess the stability of mutated sequences during replication in plants, the region encompassing the tRNA-like structure was sequenced by dideoxy-chain termination. This was done either directly with reverse transcriptase and a primer hybridizing to the 3' end or after further DNA amplification of the 3' region of the genome via the polymerase chain reaction (34). The valine acceptances of the virion RNAs were studied by determining the amount of [^{3}H]valine bound

TABLE 1. Properties of TYMV mutants with altered anticodon loops[a]

Group	Mutant	Anticodon loop sequence	In vitro valylation properties[b] (264-nt-long 3′ RNAs)		Replication in Chinese cabbage relative to wt (genomic transcripts as inocula)				
					Protoplasts, 48 h postinoculation			Plants	
			mol of val/mol of RNA[c]	Relative V_{max}/K_m	CP[d]	Genomic RNA[e]	Subgenomic RNA[e]	Virion[f]	Score[g]
A	wt	59-CCCACAC-53	1.01	1.00	1.0	1.0	1.0	1.0	9/9
	G57	. . G	0.98	0.70	0.7	0.6	0.6	1.1	5/5
	A57	. . A	0.98	0.62	1.6	1.1	1.3	0.7	3/3
	U57	. . U	—	—	1.5	0.9	0.8	1.8	5/5
	G54	. . . . . G .	0.99	0.74	0.8	1.0	0.8	0.8	3/3
	C54	. . . . . C .	1.05	0.21	0.24	0.3	0.2	0.5	1/1
	A53	. . . . . . A	0.92	0.088	0.12	—	—	NSS	0/7
B	U55	. . . . U . .	0.66	0.062	0.002–0.005	D	ND	NSS	0/3
	A55	. . . . A . .	0.34	0.010	0.015–0.025	D	ND	NSS(r)	1(r)/3
	G55	. . . . G . .	0.09	0.0033	≤0.002	D	ND	—	—
	U55/C54	. . . . UC .	0.14	0.0079	0.002–0.005	—	—	—	—
	U55/A53	. . . . U . A	0.010	$<10^{-3}$	ND	—	—	—	—
	U55/C54/A53	. . . . UCA	0.007	$<10^{-3}$	ND	D	ND	—	—
	G56	. . . G . . .	0.008	$<10^{-3}$	0.002	D	ND	NSS	0/2
	U56	. . . U . . .	0.005	$<10^{-3}$	≤0.002	D	ND	NSS	0/2
	U56/A55	. . . UA . .	0.001	$<10^{-3}$	ND	D	ND	NSS	0/2
	C56/A55	. . . CA . .	<0.001	$<10^{-3}$	≤0.002	D	ND	NSS	0/2
	G57/U56/G55	. . GUG . .	0.003	$<10^{-3}$	ND	D	ND	—	—
	U53	. . . . . . U	1.06	0.54	0.015–0.025	0.055	0.029	(r)	1(r)/5

[a] All data represent averages of three or more experiments unless otherwise indicated. nt, nucleotide; wt, wild type; —, no experiments performed; ND, results either undetectable or too low for a reliable estimate; NSS, no systemic symptoms observed and no coat protein detected in upper leaves; D, double-stranded genomic RNAs detected by in vivo labeling (Fig. 4), verifying a low level of RNA synthesis; (r), reversion of phenotype observed after 2 weeks, resulting from second-site suppressor mutations (see text).

[b] Valylation catalyzed by wheat germ ValRS; standard errors for K_m/V_{max} are 20 to 25%.

[c] Extent of valylation after 60 min at 0.6 μM RNA.

[d] Coat protein (CP) levels determined in Western blots (see Fig. 3a); standard error = 20 to 40%.

[e] TYMV RNA levels determined in Northern blots (see Fig. 3b); standard error = 20 to 40%.

[f] TYMV levels determined after virion purification from systemically infected leaves; wild-type yield was 1.1 mg/g (fresh weight).

[g] Number of plants with systemic symptoms/number of plants inoculated.

in reactions catalyzed by ValRS present in an extract from wheat germ (6).

Valylation of genome-length transcripts. To make full-length genomic RNA transcripts capable of aminoacylation, the restriction overhangs were removed from *Hin*dIII-linearized pTYMC and selected mutant derivatives by treatment with mung bean nuclease (6). The treated DNAs were extracted with phenol-chloroform and ethanol precipitated and then used as templates for the synthesis of genomic transcripts (as described above except that cap analog was omitted). The resultant transcripts were valylated as described above. The 3′ termini were analyzed by 3′ labeling with [5′-^{32}P]cytidine-3′,5′-bisphosphate, complete digestion with a mixture of RNases, and two-dimensional thin-layer chromatography (9).

RESULTS

Generation of TYMC RNA mutants with substitutions in the anticodon loop. For two reasons, our strategy for obtaining mutants with altered valylation properties has focused on substitutions of the nucleotides 56-ACAC-53 in the anticodon loop of TYMV RNA. First, these nucleotides are present in all known valylatable eukaryotic RNAs, including valine-specific tRNAs (31) and tymoviral RNAs (32). Protection experiments comparing the interaction of yeast ValRS with TYMV RNA and $tRNA^{Val}$ have suggested that these conserved nucleotides in the viral RNA are functionally analogous to the tRNA anticodon loop (13). Second, studies with *E. coli* $tRNA^{Val}$ have shown that the anticodon is a major site recognized by ValRS in discriminating its cognate tRNA from others (30).

Oligonucleotide-directed mutagenesis was used to introduce single and multiple substitutions involving each position in the anticodon loop from C53 through C57 (Fig. 1; Table 1). Nucleotide C57 corresponds to the wobble base of tRNA, which is variable in different $tRNA^{Val}$ sequences (31). Nucleotides A56 and C55 complete the anticodon. The residues corresponding to the 3′-flanking nucleotides A54 and C53 are invariant in eukaryotic $tRNA^{Val}$; among all known eukaryotic tRNAs, the former base is always a purine and the latter is never a guanine (15, 31). These observations were considered in designing the mutants discussed below.

Mutations were made in an M13 clone containing the 3′ 258 bp of TYMV cDNA and were subcloned into pTYMC after sequencing of the complete insert (Materials and Methods). This eliminated the possibility of introducing unwanted mutations outside the mutagenic target region. Clone pTYMC contains the entire genomic cDNA of TYMV adjacent to a T7 promoter at the 5′ end and with a unique *Hin*dIII site at the 3′ end. Infectious TYMC RNA can be made with T7 RNA polymerase from *Hin*dIII-linearized pTYMC in the presence of m^7GpppG cap analog (33). Such transcripts made from pTYMC and its mutant derivatives (referred to as, e.g., TYMC-U55) were used in the replication studies described below. These transcripts had four extra nonviral nucleotides (AGCU, directed by the *Hin*dIII restriction

overhang) at the 3′ end and were thus not valylatable. Studies with a number of viruses have shown that such short 3′ extensions do not alter the infectivity of transcripts (1). In barley, the 3′ ends of BMV RNAs undergo rapid turnover, with repair to a 3′-CCA terminus probably being a function of host tRNA nucleotidyltransferase (26). Presumably, the 3′ ends of inoculated TYMC transcripts are processed in vivo in a similar way to produce a valylatable terminus.

In vitro valylation characteristics of mutant TYMV RNAs. Mutations introduced into pTYMC as described above were also introduced into the vector mpT7YSma, from which short (264-nucleotide-long) RNAs spanning the tRNA-like structure can be transcribed with T7 RNA polymerase (6). These transcripts end in 3′-CCA and, unlike the genomic transcripts, are appropriate substrates for ValRS. To distinguish them from the genomic transcripts mentioned above, these short RNAs are referred to as, e.g., TY-U55. In studies reported elsewhere, the valylation properties of anticodon loop mutants have been described in detail (8a). Table 1 contains a summary of those data: valylation stoichiometries and V_{max}/K_m values. The latter parameter can be taken as an indication of the valylation efficiency of the mutant.

To verify that the valylation studies using short 3′ RNAs (Table 1) were generally valid for genome-length RNAs, the valylation of the genomic transcripts of a selection of mutants was studied. Full-length RNAs with 3′-CCA termini were transcribed from *Hin*dIII-linearized DNA templates after treatment with mung bean nuclease to remove the restriction overhang (Materials and Methods). The valylation of the genomic mutants paralleled that of the respective short transcripts (compare Fig. 2a with Table 1), indicating that the accessibility or conformation of the tRNA-like structure is not significantly perturbed by the presence of 6 kb of upstream RNA. The valylation data of Table 1 are thus readily applicable to the genomic transcripts used in this study.

One of the goals of our mutational study was to obtain mischarging mutants. Preliminary studies have failed to detect mischarging of the mutant RNAs listed in Table 1. Further work is needed to obtain such mutants.

Only those mutants capable of efficient valylation are able to replicate systemically in plants. Chinese cabbage plants were inoculated with capped full-length wild-type and mutant transcripts, and the appearance of symptoms was checked over the 2-week period postinoculation. Systemic symptoms (indicated by symptoms in noninoculated upper leaves) appeared 7 days postinoculation only in plants inoculated with RNAs capable of efficient valylation: wild type, wobble base mutants (TYMC-G57, -U57, and -A57), and nucleotide 54 mutants (TYMC-G54 and -C54; Table 1, group A). The yields of the mutant viruses from symptomatic upper leaves were comparable to the yield of wild-type TYMC. Interestingly, TYMC-U57 replicated to significantly higher levels than TYMC. The appearance of symptoms was similar for all infected plants.

Sequencing of the tRNA-like structures of progeny RNAs showed that the mutant sequences were preserved (not shown). The mutant virion RNAs could also be valylated to the same extent as wild-type RNA in reactions catalyzed by wheat germ ValRS (Fig. 2b). The valylation properties of these progeny virion RNAs are consistent with those of the short transcripts listed in Table 1.

Replication of TYMC mutants in protoplasts. The whole plant infectivity studies described above divided the mutants into two groups, those capable and those incapable of

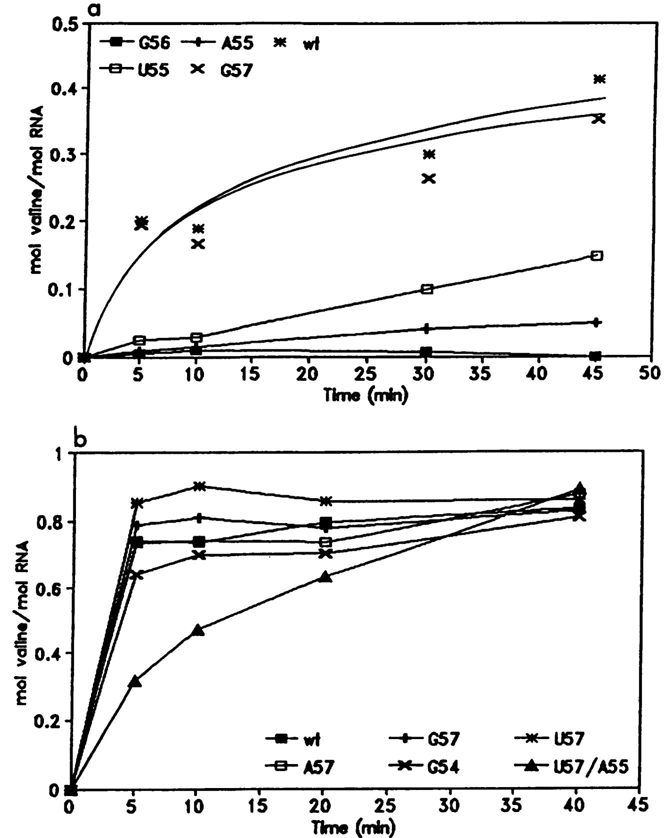

FIG. 2. Valylation in vitro of selected mutant genomic RNAs with wheat germ ValRS. (a) Valylation of transcripts (0.6 μM) made from pTYMC templates linearized with *Hin*dIII and treated with mung bean nuclease to remove restriction overhangs (Materials and Methods). These templates are designed to generate transcripts that have 3′-CCA termini capable of accepting valine. (b) Valylation of progeny virion RNAs (0.6 μM) extracted from systemically infected leaves of plants. wt, wild type. Mutants are identified in Table 1.

systemic spread. To investigate further the differences between the two groups, the mutants were inoculated onto Chinese cabbage protoplasts, and the levels of viral products were then analyzed after 48-h, or in some cases 22-h, incubations. At harvest, a fraction of the cells was used to detect coat protein in Western blots (Fig. 3a), while another fraction was used to detect genomic and subgenomic RNAs in Northern blots (Fig. 3b).

As in the whole plant studies, the replication behavior in protoplasts divided the mutants into two groups (A and B), whose characteristics are listed in Table 1. The members of the first group replicated well, producing yields of coat protein and viral RNAs that were at least 10% of wild-type levels (Table 1). This group included all those able to produce systemic symptoms in plants (TYMC-G57, -A57, -U57, -G54, and -C54) but in addition mutant TYMC-A53. The latter mutant has the poorest valylation properties of this group (compare V_{max}/K_m values in Table 1), although it can be completely valylated (Table 1). For members of this group, there was a direct relationship between the four parameters investigated: valylation efficiency, coat protein accumulation in protoplasts, viral RNA accumulation (genomic and subgenomic) in protoplasts, and yield of virus in systemically infected leaves. For mutant TYMC-A53, the

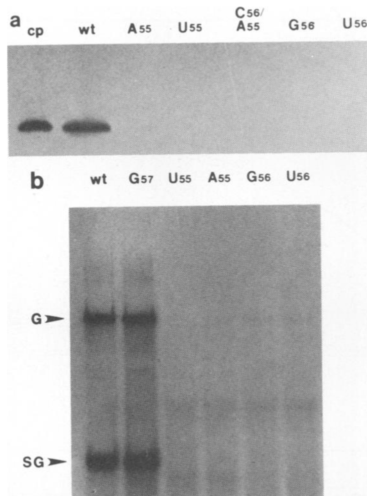

FIG. 3. Replication of TYMV mutants in Chinese cabbage protoplasts. Representative experiments that have contributed to the quantitative data in Table 1 are shown. Protoplasts (4×10^5 cells) were inoculated with 5 μg of TYMC (wt) or mutant genomic transcripts, as indicated. (a) Detection of coat protein (20 kDa) in Western blots made after separation of proteins on 14% polyacrylamide-SDS gels. Extracts were made from 2×10^5 protoplasts harvested 48 h after inoculation, and blots were developed by using horseradish peroxidase-linked second antibodies and 5-chloro-1-naphthol color reagent. Lane cp, marker coat protein (50 ng of TYMV virions). (b) Detection of viral genomic (G; 6.3 kb) and subgenomic (SG; 0.7 kb) RNAs in Northern blots. RNAs were extracted from 2×10^5 protoplasts harvested 22 h after inoculation. The hybridization probe was a ^{32}P-labeled RNA transcript complementary to 0.9 kb at the 3′ end of genomic RNA.

replication rate in single cells may be too low to overcome the plant's defenses, resulting in limitation to inoculation foci and no systemic spread. The inability to spread systemically was confirmed by the absence of coat protein in Western blots of upper leaves; low levels of coat protein were detected in the inoculated leaves.

Group B (Table 1) included all other mutants studied. They replicated poorly in protoplasts (Fig. 3) and corresponded to those mutants unable to replicate systemically in plants. Except for TYMC-U53, these mutants had substitutions within the anticodon, at nucleotides 55 and 56 and combinations including those nucleotides. Most group B mutants replicated to such low levels in protoplasts that viral products were difficult to detect; coat protein was detectable after 48 h but not 22 to 24 h postinoculation. Mutant TYMC-A55 replicated to the highest levels (about 50-fold lower than wild type), while the other anticodon mutants yielded at most 200 times less than wild type (Table 1). The detection and quantitation of viral products for these mutants was most accurate in Western blots analyzing for coat protein, chemiluminescent development providing ca. 0.2-ng sensitivity. Northern blots were not useful because small amounts of inoculum genomic RNA were detected and because elevated background interfered with subgenomic RNA detection.

None of the group B RNAs except TY-U53 could be efficiently valylated with wheat germ ValRS, having V_{max}/K_m values at best 16 times lower than those of wild-type RNA (for TY-U55) and in the other cases far lower. These RNAs could not be completely valylated in standard long (60-min) reactions (Table 1). For these mutants, inefficient valylation correlates to basal levels of replication, and the ranking in replication generally follows the ranking in valylation efficiency.

There were two atypical members of group B: TYMC-U55 and -U53. Both replicated poorly yet could be completely valylated (TY-U55 only in the presence of excess ValRS). Clearly, the ability to valylate efficiently is not the sole criterion for efficient replication.

Evidence that all mutants are able to replicate to some degree. The detection of low levels of coat protein in protoplasts inoculated with RNAs of group B mutants indicates that replication of input RNA has occurred. Coat protein is not translated from genomic RNA, being translated instead from the 0.7-kb subgenomic RNA that is colinear with the 3′ region of the genomic RNA. In BMV, subgenomic RNA arises in a transcriptional event that uses negative strand as its templates (25). Presumably, the same mechanism is used by TYMV, and thus the appearance of coat protein implies the generation of subgenomic RNA via negative strand from the mutant input RNAs. Nevertheless, the possibility that coat protein may arise by translation of unreplicated 3′ RNAs generated by fragmentation of the inoculum was considered. We therefore studied the in vivo labeling of replicating RNAs to obtain stronger evidence that group B mutants are capable of slow replication.

Unlabeled RNA transcripts were inoculated to protoplasts, which were then incubated in phosphate-free medium supplemented with carrier-free $^{32}P_i$. Protoplasts were harvested, and the RNAs were extracted, denatured, annealed to an excess of nonradioactive 0.9 kb negative-sense RNA probe, and then treated with RNase under conditions that protect double-stranded RNA. Digestion products were analyzed by gel electrophoresis and autoradiography. This assay detected radiolabeled double-stranded genomic RNA, positive-sense genomic strands (0.9-kb protected band), and subgenomic RNA (0.7-kb protected band; Fig. 4). Note that this assay does not permit quantitation of amounts of RNAs synthesized during the labeling period, since the specific activities of ^{32}P-labeled nucleotides vary with time until intracellular pools become saturated. Consistent with the results of Western detection, all group B mutants tested showed evidence of replication in this assay, in the form of double-stranded genomic RNAs and the radiolabeled 0.9-kb protected band that is indicative of positive-sense genomic RNA. Unfortunately, signals were not strong enough to permit detection of the 0.7-kb RNA indicative of radiolabeled subgenomic RNA for group B mutants. This band was evident in extracts of protoplasts inoculated with TYMC or TYMC-U57 RNAs (Fig. 4).

Three negative control inoculations produced no evidence for radiolabel in similar products after incubation in protoplasts: mock inoculation (Fig. 4); inoculation with uncapped genomic RNA transcripts, which are known to be unstable in cells (1); and inoculation with a deleted genomic RNA missing part of the nonstructural gene necessary for replication, TYMC-ΔKpn^{2366}-Kpn^{3248} (not shown). The detection of radiolabeled double-stranded RNAs and coat protein in protoplasts makes it clear that replication, albeit at a low level, has occurred after inoculation with group B mutants. This conclusion is based on the use of techniques that are not confounded by the detection of input inoculum.

Coincident phenotypic reversion of replication and valylation. In two instances, systemic symptoms appeared in plants inoculated with mutants that consistently fail to replicate well enough to support systemic spread: TYMC-A55 and -U53. In both cases, the plants had been inoculated

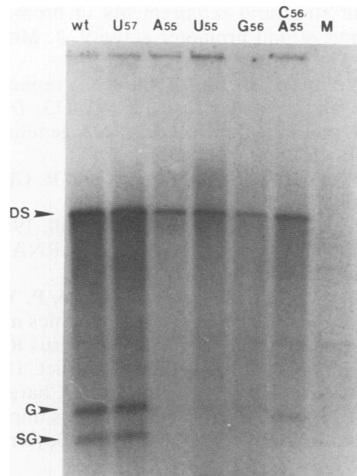

FIG. 4. In vivo-labeled RNAs. Inoculated protoplasts were incubated for 24 h in medium containing $^{32}P_i$. The in vivo-labeled RNAs were extracted and analyzed in an RNase protection assay explained in the text. Three forms of viral RNA, separated here by electrophoresis on 1% agarose gels, were protected: genomic double-stranded RNA (DS), the 0.9-kb protected fragment of positive-sense genomic RNA (G); and the protected subgenomic RNA (SG; 0.7 kb). The lane markings indicate the various inocula used. Lane wt, wild type; lane M, no RNA. The protected genomic band recovered from mutant TYMC-C56/A55 migrates slightly fast as a result of RNase cleavage of the probe opposite the double mutation.

with lysed protoplasts harvested 48 h after inoculation, and systemic symptoms appeared after a delay of 2 to 3 weeks rather than the normal 7 days. On reinoculation onto plants, the newly acquired phenotypes were stable and similar to wild type with respect to symptom appearance and virus yield (0.5 mg/g [fresh weight] in both cases, versus 1.1 mg/g for wild-type TYMC). Virion RNAs from both progeny could be valylated to completion. This represents no change in the aminoacylation properties of the progeny arising from TYMC-U53 inoculation, since TY-U53 valylates highly efficiently. For the progeny RNA arising from TYMC-A55, however, the extent of valylation was greatly improved over that of the parent (1.00 versus 0.34 mol of valine/mol of RNA; Fig. 2b).

The 3′ regions from these progeny were cloned, and three separate clones were sequenced. In no case was the phenotypic reversion due to a simple reversion at the mutated locus to the wild-type sequence, defining the progeny as second-site suppressor mutants (SSSM). The A55-SSSM was shown to contain a C57-to-U57 transition in addition to the original A55 mutation. All three clones had the same sequence. No sequence differences between U53-SSSM and U53 were found in the 3′ 259 nucleotides of the clones examined, suggesting the presence of a second mutation outside the 3′ region.

DISCUSSION

Interdependence between valylation and replication. The study of a number of mutants with a range of valine identities has shown clearly that valine acceptance activity and the amplification of TYMV RNA are correlated. Only those mutants capable of complete valylation in vitro replicated systemically in plants (Table 1, group A). Further, when a second mutation arose fortuitously from TYMC-A55 RNA inoculum, resulting in the acquisition of the ability to spread systemically in plants, there was a concomitant improvement in valylation efficiency. Some exceptions to the correlation have been observed, but these concern mutants that replicate poorly yet valylate well (TYMC-U53 and -U55) rather than the reverse. Thus, valine acceptance is an important, but not the sole, criterion for replication. Anticodon loop mutant RNAs that valylate but fail to replicate well may, for instance, be promoter mutants, may be subject to mischarging or to base modification by tRNA-specific enzymes (29), or may have decreased stability or half-lives resulting from the introduction of fragmentation sites or endoribonuclease recognition elements (2). Valylation is also not obligatory for replication per se, since all mutants supported low levels of RNA synthesis (Fig. 4). Rather, valylation is able to enhance those low replication rates to the rates observed in wild-type infections.

The mechanism by which the ability of TYMV RNA to be valylated influences the yield of virion RNA has not been established. RNA synthesis may be directly influenced by the presence or absence of bound valine. On the basis of the finding that the tRNA-like structure of BMV RNA functions as the negative-strand promoter (24), it has been hypothesized that aminoacylation may play a role in modulating negative-strand initiation rates (8). Our results are consistent with such a suggestion and invite speculation that host elongation factor might, as with Qβ replicase (3), be part of the replication complex (8, 16). As with tRNA, elongation factor binds preferentially to aminoacylated viral RNA (18). An alternative role for valylation may be in regulating the function of a particular RNA as a template for negative-strand synthesis or translation (12). Perturbation of the normal partitioning of positive-sense RNAs between these functions could be detrimental to RNA amplification. A third mechanism by which valylation might be necessary for efficient amplification of viral RNA is by stabilizing the 3′ end against the action of exonucleases. However, because viral RNAs are such efficient substrates for host (CTP,ATP): tRNA nucleotidyltransferase, which maintains intact 3′ termini of tRNAs, this enzyme is thought to be responsible for stabilizing viral 3′ ends (26). Decreased stability by the endonucleotyic mechanism mentioned above could not be generally applicable to all mutants because of its sequence specificity. Finally, valylation is clearly not involved in packaging, since the virion RNAs lack the 3′-terminal A residue that is necessary for aminoacylation (4).

It should be noted that at present, the correlation discussed above relates valylation determined in vitro to replication in vivo. It is unfortunately not easy to quantify the valylation status of viral RNAs in vivo, especially for poorly replicating mutants. Surprisingly, there are very few studies relating in vitro and in vivo aminoacylation of tRNAs. In one relevant study, yeast elongator $tRNA^{Met}$ was shown to be mischarged with phenylalanine by yeast phenylalanyl-tRNA synthetase with a k_{cat}/K_m only 200-fold lower than that of the cognate $tRNA^{Phe}$ (11), yet there is adequate discrimination in vivo. The very poor valylation that we have observed for several TYMV RNA mutants in vitro makes it unlikely that these become significantly valylated in vivo. It is also unlikely that the mutants capable of systemic spread do not become readily valylated in vivo. We are thus confident that the valylation data presented in Table 1 for these mutants reasonably predict their valylation potential in cells; nevertheless, the in vivo valylation status of mutant RNAs will need to be determined experimentally.

Comparison with BMV. Experiments conducted with

BMV have previously led to the conclusion that efficient tyrosylation is not crucial for replication (9). For example, two BMV mutant RNA3s (Δ5′ and 5′-AGA) had initial in vitro tyrosylation rates 5 and 3%, respectively, of the wild-type rate and could not be completely aminoacylated, yet these mutants replicated to levels greater than 50% of the wild-type level. Comparable TYMV mutants are TYMC-A55 and -U55/C54, with initial valylation rates (at 150 nM) 4% of the wild-type rate and final valylation levels 34 and 14%, respectively, of the wild-type rate. These mutants yielded at best 2.5% of the wild-type level. The differences between the two viral systems remain to be clarified. Although the experimental strategies of the two studies were similar, the BMV study involved the replication in *trans* of the mutant, while the present study used 3′ mutations *cis* to the nonstructural protein-encoding sequences (a *trans* replication system could not be established). The BMV studies have since been extended to include the positioning of 3′ mutations on RNA2, a genomic RNA encoding a protein essential for replication. Negative mutations are rather more detrimental in that context (27), but this fact does not change previous conclusions (9).

The differences between the conclusions derived from the BMV and TYMV studies may have other explanations. In neither case has the in vivo aminoacylation status of the mutant viral RNAs been established. This deficiency is more serious in interpreting the BMV results, since no mutants comparable to the range of very poorly valylating mutants described in this report were obtained. On the other hand, a detailed characterization of promoter elements in the TYMV RNA will assist in interpreting the results in this study. Finally, it is possible that the role and relevance of aminoacylation are not the same among the various plant RNA viruses that exhibit tRNA mimicry. More research is needed to elucidate this phenomenon genetically and biochemically.

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