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Transfer RNA Mimicry in a New Group of Positive-Strand RNA Plant Viruses, the Furoviruses: Differential Aminoacylation between the RNA Components of One Genome

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Recent sequencing of the genomes of several furoviruses—fungus-transmitted rod-shaped positive-strand plant viruses—has suggested the presence of tRNA-like structures (TLSs) at the 3′ ends of the genomic RNAs. We show here that the genomic RNAs of soil-borne wheat mosaic virus (SBWMV), beet soil-borne virus (BSBV), potato mop-top virus (PMTV), peanut clump virus (PCV), and Indian peanut clump virus (IPCV) all possess functional TLSs that are capable of high-efficiency valylation. While the SBWMV, BSBV, and PMTV TLSs are similar to those found in tymoviruses, the PCV and IPCV TLSs harbor an insertion of about 40 nucleotides between the two halves of the TLS. The valylated SBWMV and BSBV RNAs formed tight complexes with wheat germ EF-1α · GTP (K_d = 2 to 11 nM), whereas valylated PMTV, PCV, and IPCV RNAs bound EF-1α · GTP weakly ($K_d \geq 50$ nM). The TLS of PCV RNA2 differs from PCV RNA1 in lacking the major valine identity nucleotide in the anticodon and consequently is capable of only very inefficient valylation. This is the first case of differential aminoacylation between the RNA components of one genome.

Key Words: fungus-transmitted; valylation; valyl-tRNA synthetase; soil-borne wheat mosaic virus; beet soil-borne virus; potato mop-top virus; peanut clump virus; turnip yellow mosaic virus; translation elongation factor EF-1α.

INTRODUCTION

Over the last few years, the nucleotide sequences of the genomic RNAs of a number of rod-shaped positive-strand RNA plant viruses that are transmitted by plasmodiophoromycetous fungi have been reported. The entire bipartite genomes of soil-borne wheat mosaic virus (SBWMV; Shirako and Wilson, 1993), peanut clump virus (PCV; Manohar *et al.,* 1993; Herzog *et al.,* 1994), and beet soil-borne virus (BSBV; Koenig *et al.,* 1996, 1997; Koenig and Loss, 1997) have been determined, while partial genomes of two other viruses have been sequenced: RNA 1 of Indian peanut clump virus (IPCV; Miller *et al.,* 1996) and RNAs 2 and 3 of potato mop-top virus (Scott *et al.,* 1994; Kashiwazaki *et al.,* 1995). Although the genome designs of these five viruses currently grouped as furoviruses (Brunt and Richards, 1989) appear sufficiently distinct to necessitate their classification into separate genera (Torrance and Mayo, 1997), they do have in common 3′-terminal sequences that suggest the presence of tRNA-like structures. This contrasts with the 3′-poly(A) tail present on each of the genomic segments of beet necrotic yellow vein virus (BNYVV; Bouzoubaa *et al.,* 1987), the best-studied rod-shaped fungus-transmitted plant virus.

Transfer RNA-like structures (TLSs) exist at the 3′ end of tymoviral, tobamoviral, bromoviral, cucumoviral, and hordeiviral genomic RNAs; these TLSs are specific for aminoacylation by valine, histidine, or tyrosine (Mans *et al.,* 1991; Florentz and Giegé, 1995). The authors of most of the furoviral sequencing studies mentioned above recognized the presence of candidate TLSs reminiscent of that present in turnip yellow mosaic virus (TYMV) RNA, but there have been no experimental verifications of tRNA mimicry to date. The similarity to the TYMV TLS is marked for SBWMV, PMTV, and BSBV RNAs, but less so for PCV and IPCV RNAs. We show now that this is due to an insertion of some 40 nucleotides between the two halves of the TLSs of PCV and IPCV RNAs and that, despite these insertions, PCV and IPCV RNA1 can be efficiently aminoacylated with valine, as can the other furoviral RNAs. This expands for the first time in over 15 years the number of groups among the positive-strand RNA plant viruses known to possess genomic TLSs. The discovery that PCV RNA1, but not RNA2, can be efficiently valylated is the first time that differential tRNA mimicry has been observed among the genomic RNAs of a single virus and has implications for the role of tRNA mimicry in viral biology.

RESULTS

Synthesis of 3′ fragments of seven furoviral genomic RNAs

In studying the tRNA-like characteristics of viral RNAs, it has been convenient to use short 3′ RNAs generated from cDNA clones or PCR products representing the TLS

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(Dreher *et al.,* 1984, 1992). For at least some of the furoviral RNAs, this is the only possible approach, due to the difficulty in isolating virion RNA (e.g., see Koenig *et al.,* 1996). A concern with the use of 3′ RNA fragments of the genomic RNA, however, is that the creation of an arbitrary 5′ terminus can lead to nonnative folding, with the possibility that upstream sequences may interfere with the folding of the TLS and influence its activities (Mans *et al.,* 1990). This could be avoided by using precise TLS constructs, but it may not be correct to assume that the efficiency of aminoacylation is similar for the genomic RNA and the TLS alone. Consequently, two 3′ fragments were designed for each of the furoviral RNAs under investigation (PCV RNAs 1 and 2, IPCV RNA1, SBWMV RNAs 1 and 2, BSBV RNA3, PMTV RNA2): one RNA comprising the predicted TLS, the other including additional viral sequence immediately upstream. The former RNAs (referred to as SBWMV1-TLS etc. in subsequent Tables and figures) and their predicted TLS conformation are shown in Fig. 1 Note that in the case of PCV1, PCV2, and IPCV1, a stable stem-loop (5′-SL in Fig. 1) was present immediately upstream of the predicted TLS (these RNAs are referred to as PCV1-SLTLS etc.). This was added after initial results suggested that a transcript comprising the 3′ 124 nucleotides of PCV1 RNA (the TLS alone) may not be correctly folded (not shown).

The design of the second family of longer RNAs took into account the predicted secondary structure of the 3′ regions of each genome (STAR algorithm; Abrahams *et al.,* 1990). The 5′ ends were placed at a G residue coinciding with the upstream boundary of a predicted structural element or, when no such G was available, the 5′-SL stem-loop element shown in Fig. 1 was placed at the 5′ end of the transcript (PMTV2-148, PCV1-319, and PCV2-318 RNAs). This structural element is very stable due to the four G-C basepairs and the GAAA tetraloop (Chowrira *et al.,* 1994) and thus provides an excellent initiation sequence for T7 transcription without influencing the folding of downstream regions. For technical reasons, the long form of IPCV1 3′-RNA was not obtained (Materials and Methods).

The furoviral RNAs, except PCV RNA2, can be efficiently valylated

In initial experiments, the valylation activities of the two forms of each furoviral 3′ RNA were compared, using wheat germ valyl-tRNA synthetase (ValRS), in both TM (Dreher *et al.,* 1992) and IV (physiological conditions; Dreher *et al.,* 1996) buffers. Overall valylation rates were slower in the high ionic strength IV buffer, and the differences between the two buffer conditions were more marked for the viral RNAs than for a transcript corresponding to higher plant (lupine) $tRNA^{Val}$ (representative curves shown in Fig. 2). The valylation profiles were very similar for the pairs of RNAs in TM buffer (Fig. 2A), while the longer forms showed faster and higher levels of valylation in IV buffer (Fig. 2B); the exception was PMTV2, the long version of which valylated poorly (not shown), presumably due to misfolding. Both forms of PCV RNA2 showed very low levels of valylation that were not always clearly above background (not shown). Since the valylation of the TLS and longer forms were similar in TM buffer, we chose to characterize in detail the valylation of the more convenient TLS RNAs under these conditions.

The kinetic constants, V_{max} (RNA) and K_m, for the valylation of the furoviral TLS or SLTLS RNAs by wheat germ ValRS were determined in parallel with those for the $tRNA^{Val}$ transcript. Excluding PCV RNA2, the furoviral RNAs were very efficient substrates for ValRS, with V_{max}/K_m values (a measure of substrate efficiency) close to that for the $tRNA^{Val}$ transcript (Table 1). PMTV2-TLS and PCV1-SLTLS showed K_m values similar to that for $tRNA^{Val}$, while the other viral RNAs (excluding PCV2-SLTLS) had K_m values about threefold higher than that for $tRNA^{Val}$; for the SBWMV RNAs, the elevated K_m values were matched by elevated V_{max} relative to $tRNA^{Val}$. The high efficiency of valylation was unexpected for PCV1-SLTLS and IPCV1-SLTLS RNAs in view of their more complex proposed structures compared to the TYMV paradigm (Fig. 1).

The sole exception to efficient valylation was PCV2-SLTLS, whose valylation was very poor, with highly elevated K_m and low V_{max} values (Table 1), resulting in a difference of 1300-fold in the V_{max}/K_m values for the two PCV RNAs. There are only two nucleotide differences between these RNAs: the substitution of a D-loop C residue with U (resulting in a new U:A basepair) and the absence of the middle A of the GAC anticodon in PCV RNA2. This latter change represents removal of the major valine identity nucleotide, whose substitution in TYMV RNA resulted in V_{max}/K_m decreases of 5000- and 9100-fold (Dreher *et al.,* 1992).

The furoviral RNAs also possess a low level of histidine identity

Although tymoviral RNAs are considered specifically aminoacylatable with valine (Mans *et al.,* 1991; Florentz and Giegé, 1995), some histidine identity has been observed for TYMV RNA when using yeast histidyl-tRNA synthetase (HisRS; Rudinger *et al.,* 1992). We were unable to observe any histidylation of the furoviral RNAs when using the HisRS activity of our wheat germ aminoacyl-tRNA synthetase preparation at the highest practicable concentration (52 μg protein/ml; not shown). Very high levels of pure yeast HisRS were, however, able to catalyze the histidylation of most of the furoviral TLSs to levels above 0.4 mol histidine per mole RNA (Table 2). PCV-SLTLS RNAs 1 and 2 were about equally active, consistent with the location of histidine identity elements

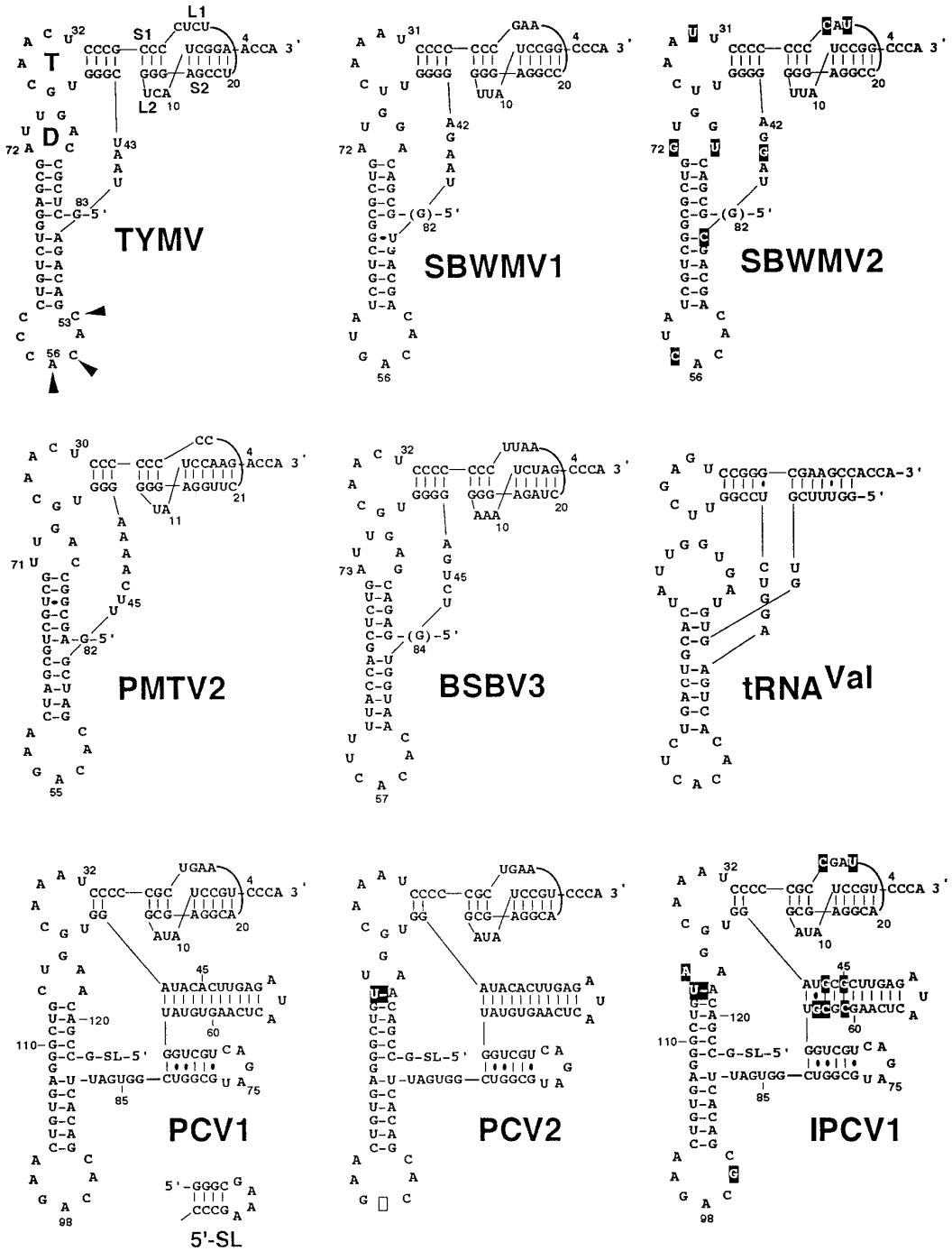

FIG. 1. Proposed structures of furoviral TLSs compared to the TLS of TYMV RNA and lupine tRNA^{Val}. The sequences shown correspond to the 3′ TLS fragments of the genomic RNAs as used in the experiments reported here. The 5′-most G residue of the SBWMV1, SBWMV2, and BSBV3 TLS RNAs, shown in parentheses, is not part of the viral sequence and was added to provide consistency among the TLSs. Although all TLSs are shown with an entire CCA 3′ end as used in our experiments, it is not certain that the 3′ terminal A is present on all virion RNAs: it is known to be present on the PCV RNA 3′ ends (Manohar *et al.,* 1993; Herzog *et al.,* 1994), IPCV (Miller *et al.,* 1996), and on the majority of SBWMV RNA 3′ ends (Shirako and Wilson, 1993), but the situation is unknown for BSBV (Koenig *et al.,* 1996, 1997) and PMTV (Scott *et al.,* 1994); note that only a small proportion of TYMV virion RNAs possess the 3′ terminal A (Briand *et al.,* 1977). The main features of the TLS are indicated on the TYMV structure: the analogues of the T and D loops of tRNAs, helical segments S1 and S2 and connecting loops L1 and L2 of the acceptor stem pseudoknot, and the main valine identity nucleotides (arrowed) in the anticodon loop that govern recognition by ValRS (Dreher *et al.,* 1992). Note that numbering is from the 3′ A. Nucleotides in the SBWMV2 TLS that differ from the SBWMV1 sequence are shown in reverse shading, as are nucleotides in the PCV2 and IPCV1 TLSs that differ from the sequence of PCV1 RNA; the single deletion (relative to the PCV1 TLS) in the anticodon of PCV RNA2 is shown as an empty box. The stem/loop structure (5′-SL) shown below the PCV1 TLS is present at the 5′ end of the PCV1, PCV2, and IPCV1 TLS RNAs and is represented by "SL" in each structure. The tRNA^{Val} transcript has a CAC anticodon in place of the IAC (I = inosine) anticodon in the mature tRNA (Barciszewska and Jones, 1987).

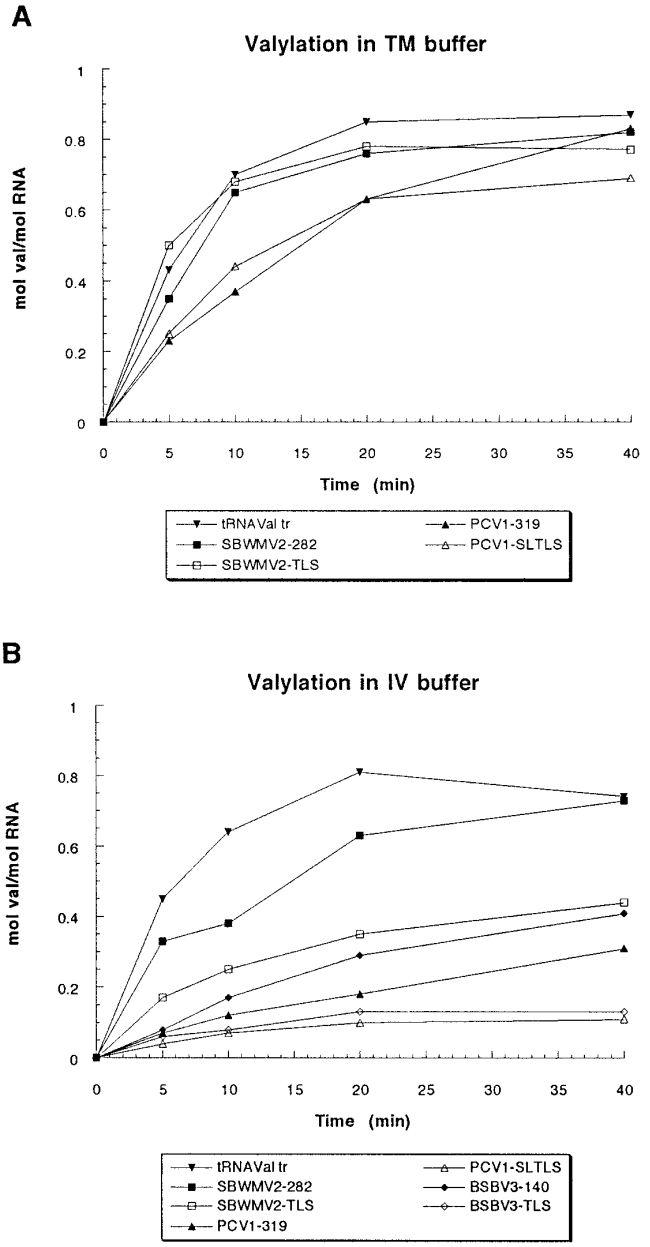

FIG. 2. Comparison of the valylation profiles of long and short 3′ fragments of selected furoviral genomic RNAs. RNAs comprising the TLS (as shown in Fig. 1) or RNAs containing an additional sequence upstream of the TLS (identified by length in nucleotides) were valylated with wheat germ ValRS in TM buffer (A) or IV buffer (B). The RNAs (identified in the legends) were present at 0.1 μM, and all reactions were conducted in the presence of the same ValRS concentration (13 μg total protein/ml).

predominantly in the aminoacyl acceptor stem (Himeno *et al.,* 1989; Rudinger *et al.,* 1997), where these RNAs are identical (Fig. 1). PMTV2-TLS consistently bound less histidine, even though the same RNA preparation was shown fully capable of valylation. Similar observations have been made with eggplant mosaic tymovirus (EMV; Dreher and Goodwin, 1998). The PMTV and EMV TLSs both differ from the TYMV/SBWMV/BSBV TLSs in that the acceptor/T arms of the former RNAs are built of three helical segments 3, 3, and 6 bp in length, rather than 4, 3, and 5 bp; there are also differences in the length of the pseudoknot loops (Fig. 1). We suggest that these differences in the acceptor stem result in conformational differences that make the RNAs of one class of viral TLS better substrates for yeast HisRS than the other.

Interaction of furoviral RNAs with wheat germ EF-1$\alpha \cdot$GTP

Viral RNAs and the $tRNA^{Val}$ transcript were preparatively valylated with [^{3}H]valine and incubated in binding reactions with activated wheat germ EF-1$\alpha \cdot$GTP. The formation of ternary complex (aminoacyl-tRNA·EF-1α/GTP) was followed in a ribonuclease protection assay, exploiting the fact that the binding of EF-1$\alpha \cdot$GTP protects the normally accessible 3′ end and attached amino acid against ribonuclease attack. Binding constants (K_d) were determined from binding curves in which the concentration of active EF-1$\alpha \cdot$GTP varied in 2-fold dilutions from 250 nM to 0.05 nM. The valylated $tRNA^{Val}$ transcript and the valylated SBWMV1-TLS and BSBV3-TLS RNAs all formed tight complexes with EF-1$\alpha \cdot$GTP, with K_d values between 1.5 and 2.9 nM (Table 3). The binding of valylated SBWMV2-TLS was 4- to 5-fold lower (K_d = 10.8 nM).

Valylated PMTV2-TLS, PCV1-SLTLS, and IPCV1-SLTLS RNAs were bound with considerably weaker affinity and to low extents at the highest EF-1$\alpha \cdot$GTP concentration used (about 15% of [^{3}H]valyl-RNAs present bound; binding curves were incomplete, whereas binding plateaus were reached for the other RNAs studied). Due to incomplete binding curves with the EF-1$\alpha \cdot$GTP concentrations available to us, K_d values could not be determined reliably. However, in parallel studies (Dreher and Goodwin, 1998), we have observed similarly weak EF-1$\alpha \cdot$GTP binding for the valylated TLS of eggplant mosaic tymovirus (EMV) and estimated the K_d for that interaction to be 57 nM. As indicated in Table 3, we thus expect the K_d values for the above RNAs to be near or above 50 nM.

To test whether the weak EF-1$\alpha \cdot$GTP binding of the above RNAs was due to the absence of required upstream sequences, the binding of longer forms of two of these RNAs, PMTV2-148 and PCV1-319, was also tested. Both valylated RNAs behaved similarly to their shorter forms in binding assays (not shown). This also confirmed that the 5′ stem/loop present in the PCV1-SLTLS and IPCV1-SLTLS RNAs (5′-SL; Fig. 1) did not interfere with EF-1α binding; in PCV1-319 RNA, this feature is no longer adjacent to the TLS, but at the 5′ terminus, some 180 nucleotides away. As a further control, PMTV2-TLS RNA was valylated with pure yeast ValRS (gift of Drs. C. Florentz and R. Giegé, Strasbourg) and used directly in binding assays without the usual phenol/chloroform extraction and ethanol precipitation. This preparation of valylated PMTV2-TLS RNA behaved the same as the

TABLE 1

Kinetic Constants for the Valylation of Furoviral TLSs by Wheat Germ ValRS

	$tRNA^{Val}$ tr[a]	SBWMV1-TLS	SBWMV2-TLS	BSBV3-TLS	PMTV2-TLS	PCV1-SLTLS	PCV2-SLTLS	IPCV1-SLTLS
K_m (nM)	86	255	265	236	63	65	3550	216
Relative V_{max}	1.00	3.39	3.21	1.51	1.01	0.68	0.028	0.73
Relative V_{max}/K_m	1.00	1.14	1.04	0.55	1.38	0.90	0.00068	0.29

Note. Valylations were performed in TM buffer using a partially purified extract from wheat germ. Data are derived from five independent experiments, except two for PCV2-SLTLS. Errors are ±20%.

[a] Lupine $tRNA^{Val}$ *in vitro* transcript.

normal preparations in binding assays. These control experiments make it clear that some of the furoviral RNAs are indeed unable to form tight complexes with EF-1α·GTP.

DISCUSSION

tRNA mimicry in a new group of viruses

The discovery that furoviral RNAs are capable of efficient valylation adds a new group of viruses to the family of plant positive-strand RNA viruses that exhibit tRNA mimicry. The valine specificity is also characteristic of the tymoviruses, and indeed the 3′-terminal regions of the furoviral RNAs can be folded into TLSs of the TYMV type (Fig. 1). The furoviral RNAs have TLSs of three structural subtypes, two of which closely resemble tymoviral counterparts: the SBWMV and BSBV TLSs correspond to the TYMV TLS, with an acceptor/T arm built of 4:3:5 bp, while the PMTV TLS corresponds to that of eggplant mosaic tymovirus in having an acceptor/T arm built of 3:3:6 bp (Fig. 1).

The PCV and IPCV TLSs are variants that are not known among the tymoviruses. The remarkably efficient valylation of these RNAs (Table 1) supports the folding of these RNAs into a basic TYMV-like TLS, into which, however, an element of some 40 nucleotides has been inserted (Fig. 1). It is proposed that nucleotides 84-GG-83 are basepaired to nucleotides 29-CC-28 to complete the 4-bp T stem and that nucleotides 88-UAGU-85 are analogous to the unpaired strand joining the two halves of the TYMV TLS (nucleotides 46-UAAU-43). Sequence covariation between the PCV and IPCV TLSs (Fig. 1) supports the existence of the predicted long stem in the TLS insertion element. The spatial disposition of the two stem/loops inserted within the TLS is uncertain, but it is interesting to note that interaction with ValRS is not perturbed, although interaction with EF-1α, which is thought to bind along the acceptor/T arm as EF-Tu does (Nissen *et al.,* 1995), is weak.

The tymoviruses and furoviruses are not closely related on the basis of the sequences of their replication proteins (Shirako and Wilson, 1993; Herzog *et al.,* 1994; Koenig and Loss, 1997). The close relationship between their TLSs, however, suggests that recombination may have occurred between a tymovirus and a progenitor of the furoviruses, perhaps as a result of a mixed infection. The genomes of the furoviruses indicate that recombination involving these viruses, a virus related to barley stripe mosaic hordeivirus (BSMV), tobacco rattle tobravirus (TRV), and/or beet necrotic yellow vein virus has likely been a strong feature of their evolution (Kashiwazaki *et al.,* 1995; Koenig *et al.,* 1996). The TYMV-like TLS of sunnhemp mosaic tobamovirus is also thought to have arisen as a result of recombination, between a tobamo- and a tymovirus (Meshi *et al.,* 1981). The distinct

TABLE 2

Histidylation of Viral RNAs by Yeast HisRS

RNA	Plateau level (mol his/mol RNA)[a]
SBWMV1-TLS	0.60
SBWMV2-TLS	0.40
BSBV3-TLS	0.65
PMTV2-TLS	0.07
PCV1-SLTLS	0.65
PCV2-SLTLS	0.79
$tRNA^{His}$ tr[b]	1.16

[a] RNAs (100 nM) were incubated with 180 nM yeast HisRS in TM buffer containing 10 μM [^{3}H]histidine for 80 min at 30°C; mean of four determinations; error, ±5%. The same RNAs (except PCV2-SLTLS) were valylated to completion when incubated with wheat germ ValRS and valine under similar conditions.

[b] Lupine $tRNA^{His}$ *in vitro* transcript (Skuzeski *et al.,* 1996).

TABLE 3

Binding Constants for the Interaction of Valylated RNAs with Wheat Germ EF-1α·GTP

RNA	K_d (nM)
val-$tRNA^{Val}$ (tr, lupine)	2.3 ± 0.4
val-SBWMV1-TLS	2.9 ± 0.5
val-SBWMV2-TLS	10.8 ± 2.0
val-BSBV3-TLS	1.5 ± 0.3
val-PMTV2-TLS	Weak; ≥50
val-PCV1-SLTLS	Weak; ≥50
val-IPCV1-SLTLS	Weak; ≥50

genome design as well as the presence of the 3′ TLS emphasizes the phylogenetic distinction of the viruses studied here from BNYVV and related rod-shaped fungus-transmitted viruses with polyadenylated genomes.

As indicated above, the furoviruses possess genes with strong relationships to BSMV and TRV. BSMV RNA has a TLS similar to that of bromo- and cucumoviruses in structure and in tyrosine aminoacylation (Agranovsky *et al.,* 1981; Loesch-Fries and Hall, 1982). TRV does not possess a TLS capable of aminoacylation, but does have a 3′-pseudoknotted acceptor/T arm mimic that makes the RNA a substrate for 3′ adenylation by CCA-NTase (van Belkum *et al.,* 1987). With the inclusion of the furoviruses, the presence of tRNA mimicry is clearly an important characteristic among the so-called "tobamo lineage" of Sindbis-like viruses, a grouping that includes the above-mentioned viruses as well as the tobamo-, bromo-, and cucumoviruses (Koonin and Dolja, 1993). The only known instance of TLSs outside this group is the tymoviruses, placed by Koonin and Dolja (1993) within the "tymo lineage" of the Sindbis-like viruses. Thus, all the known viruses with TLSs are Sindbis-like viruses encoding polymerase, helicase, and methyltransferase proteins. The presence of a TLS is not, however, tightly linked to phylogeny based on the polymerase or helicase proteins, since the closest relative of the tymoviruses is oat blue dwarf marafivirus, which has a poly(A) tail (Edwards *et al.,* 1997), while the closest relative of the bromo- and cucumoviruses is alfalfa mosaic virus, which has a 3′ structure that binds coat protein (Houser-Scott *et al.,* 1994; Reusken and Bol, 1996). This suggests that the role of the TLS in the biology of these viruses can rather easily be replaced by functions associated with a different type of 3′ terminus.

Differential aminoacylation between the two genomic RNAs of PCV

PCV has proven interesting not only in the complexity of its TLS, but also in the differential aminoacylation between RNAs 1 and 2. This appears to be a unique case of 3′ differentiation, since the sequences and properties of 3′ termini are normally very similar among the RNA components of a single virus. The deletion of the single anticodon nucleotide in RNA2 does not appear to be a sequencing or cloning error (Manohar *et al.,* 1993), and genomic transcripts corresponding to the published sequences of RNAs 1 and 2 (bearing the different anticodon loop sequences) are infectious (C. Fritsch, pers. comm.). It remains to be determined, however, whether this feature is a property of all isolates of the virus. Note that the reported absence of a TLS at the 3′ end of PMTV RNA3 is considered by the authors to require further corroboration (Kashiwazaki *et al.,* 1995).

In all previously studied multipartite viruses with aminoacylatable RNAs—the bromoviruses, cucumoviruses, and BSMV—each of the RNAs is capable of high levels of tyrosylation (Loesch-Fries and Hall, 1982; Mans *et al.,* 1991). For alfalfa mosaic virus, each genomic RNA has a similar structure at the 3′ end that includes high-affinity coat protein binding sites that are involved in activating the genome to an infectious state (Matthews, 1991). There are identical sequences at the 3′ ends of TRV RNAs (Matthews, 1991), and there is no report of a virus with RNA components both lacking and possessing a poly(A) tail.

Although PCV RNA2 could barely be valylated, this RNA did have histidine accepting activity similar to that of PCV RNA1 and each of the other furoviral RNAs except PMTV RNA2 (Table 2). Since this histidylation is dependent on very high levels of yeast HisRS, the histidine identity of the furoviral RNAs should be considered a background property of questionable relevance *in vivo.* In contrast, the valylation of all the furoviral RNAs except PCV RNA2 is so efficient (relative to $tRNA^{Val}$) that it is to be expected that these RNAs become valylated *in vivo.* Likewise, the ability of some of the valylated furoviral RNAs (SBWMV and BSBV RNAs; Table 3) to form tight complexes with EF-1α·GTP indicates that this interaction is likely to occur *in vivo.* This issue is less certain with the PMTV, PCV, and IPCV RNAs, which bind EF-1α·GTP less tightly.

The fact of differential valylation between the PCV RNAs makes this virus a particularly useful one for future studies on the role of aminoacylation. While it remains to be seen whether valylation is important for the replication of RNA1, this is evidently not the case for RNA2. It may be relevant that RNA1 encodes the replication proteins and is likely to be the only RNA necessary for replication in single cells. Thus, it may be that valylation plays a role in a genomic RNA encoding essential replication proteins, but not in an RNA that is replicated *in trans.* This is the case for brome mosaic virus, in which mutations that decrease the efficiency of tyrosylation have no apparent effect when placed on the *trans*-relicated RNA3 (Dreher *et al.,* 1989) but are inviable when placed on RNA2, which encodes the RNA-dependent RNA polymerase (Rao and Hall, 1991).

MATERIALS AND METHODS

Preparation of RNAs

Viral RNAs corresponding to the 3′ ends of the genomic RNAs were made by transcription with T7 RNA polymerase from DNA templates generated by PCR (according to Tsai and Dreher, 1993). The primer pairs used in the PCR reactions for the various RNAs are listed in Table 4; all downstream primers were purified by denaturing PAGE (20%). RNAs representing the TLS of each viral RNA were made in the presence of 10 mM 5′ GMP and 1 mM GTP in order to generate 5′-monophosphate termini (Sampson and Uhlenbeck, 1988), while the longer

TABLE 4

Deoxyoligonucleotides Used as PCR Primers to Generate Transcriptional Templates

RNA transcript	Upstream (+) sense primer	Downstream (−) sense primer
SBWMV1-TLS	T7-GGCGACAGGTAG[a]	TGGGCCGGATAACCCTC
SBWMV1-278	T7-GACCGCCTGATTA	TGGGCCGGATAACCCTC
SBWMV2-TLS	T7-GGCGACTGGTGG	TGGGCCGGATAACCCTC
SBWMV2-282	T7-GGGCAAGTCTGTCACTTT	TGGGCCGGATAACCCTC
BSBV3-TLS	T7-GGAGACGAGTTA	TGGGCTAGATTTCCCTCTA
BSBV3-140	T7-G	TGGGCTAGATTTCCCTCTA
PMTV2-TLS	T7-GAGCGGCCAGT	TGGTCTTGGATACCCTC
PMTV2-148[b]	T7-G	TGGTCTTGGATACCCTC
PCV1-SLTLS	T7-5′-SL-GCCGACAAG[c]	TGGGACGGATATCGCTCCGTTTCA
PCV2-SLTLS	T7-5′-SL-GCCGACAAG[c]	GTTTCA
IPCV1-SLTLS	T7-5′-SL-GCCGACAAG[c]	TGGGACGGATATCGCTC
PCV1-319[b]	T7-5′-SL-TAAACTGTAAGGTTTTTGT[c]	TGGGACGGATATCGCTC
PCV2-318[b]	T7-5′-SL-ATTACTTGTTTATGTGTGT[c]	TGGGACGGATATCGCTC

Note. The DNAs used as PCR templates are indicated in the text.

[a] T7 refers to the untranscribed part of the T7 promoter: TAATACGACTCACTATA.

[b] Number reflecting RNA length includes the 12-nucleotide 5′-SL sequence at the 5′ end.

[c] 5′-SL refers to a stable stem and GAAA tetraloop (shown in Fig. 1) placed at the 5′ end of the transcript: GGGCGAAAGCCC.

RNAs (designated by the RNA length in nucleotides) were made in the presence of 2 mM GTP to generate 5′-triphosphate termini. Previously described clones containing cDNAs representing SBWMV RNAs 1 and 2 (p3H5, p309; Shirako and Wilson, 1993; gift of Dr. Yukio Shirako, Tokyo) and PCV RNAs 1 and 2 (p1-47, p2-12Δ; Manohar *et al.,* 1993; Herzog *et al.,* 1994; gift of Drs. Ken Richards and Christiane Fritsch, Strasbourg) were used as templates in PCR reactions.

To generate IPCV, BSBV, and PMTV RNAs, long deoxyoligonucleotides representing the viral 3′-RNA sequence and fused T7 promoter were synthesized by standard automated phosphoramidite chemistry and used as PCR templates with the relevant terminal primers listed in Table 4. The PCR template DNAs were a 157-mer comprising T7 promoter with 140 nucleotides (3′) of BSBV3 RNA; a 165-mer comprising the T7 promoter, a 12-nucleotide-long 5′-stem/tetraloop structure (5′-SL in Fig. 1) and 136 nucleotides (3′) of PMTV RNA2; a 213-mer comprising the T7 promoter, the 5′-stem/tetraloop structure, and 184 nucleotides (3′) of IPCV RNA1 (synthesis failed after about 170 cycles from the 3′ end).

The PCR products of BSBV, PMTV, PCV, and IPCV sequences were ligated into the *Sma*I site of pUC19 for cloning. The sequences of cloned inserts were determined and shown to be free of mutations in the TLS regions shown in Fig. 1. Aliquots of the large preparations of transcript used in kinetic studies were also subjected to direct dideoxy sequencing with AMV reverse transcriptase (Life Sciences, St. Petersburg, FL) and the (−) sense primers listed in Table 4; no mutations were found.

Transcripts (5′ GMP) corresponding to the unmodified sequence of lupine $tRNA^{Val}$ (Barciszewska and Jones, 1987) were made with T7 RNA polymerase from a synthetic clone pTVAL linearized at a Bst_{NI} site placed precisely at the 3′ end.

All transcripts were purified by denaturing PAGE (8%), recovered by electroelution, and dialyzed against water. RNA concentrations were determined spectrophotometrically, and adjusted for the presence of nontemplated 3′-nucleotides on about 40% of transcripts (Dreher *et al.,* 1992).

Aminoacylation assays

Valylation and histidylation assays were performed in TM buffer [25 mM Tris–HCl (pH 8.0), 2 mM $MgCl_2$, 1 mM ATP, 0.1 mM spermine] (Dreher *et al.,* 1992) or IV buffer [30 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-ethanesulfonic acid)/KOH (pH 7.5), 100 mM potassium acetate, 2.5 mM magnesium acetate, 1.5 mM ATP] (Dreher *et al.,* 1996) in the presence of 10 μM [^{3}H]valine (10–25 Ci/mmol) or 10 μM [^{3}H]histidine (12 Ci/mmol) at 30°C. The partially purified aminoacyl-tRNA synthetase preparation from wheat germ, the determination of ^{3}H-labeled amino acid incorporation, and the methods for determining kinetic parameters were as previously described (Dreher *et al.,* 1992). ValRS (11 μg total protein/ml) was present in the concentration range in which initial rates were proportional to enzyme concentration. Pure yeast HisRS (gift of Drs. C. Florentz and R. Giegé, Strasbourg, France) was used in some experiments.

EF-1α binding assays

RNAs (30–50 pmol) were valylated in TM buffer as above with [^{3}H]valine (46–47 Ci/mmol; New England Nuclear) and wheat germ ValRS for 30 min. The reaction mixtures were acidified by adjusting to 75 mM sodium acetete (pH 5.2), deproteinized with phenol/chloroform

equilibrated to pH 5.2 with sodium acetete, and ethanol precipitated (Louie *et al.,* 1984). After recovery by centrifugation, the valylated RNAs were redissolved in 5 mM sodium acetate (pH 5.2) and stored at −80°C until use.

Purified wheat germ EF-1α (Lax *et al.,* 1986; gift of Dr. Karen Browning, University of Texas, Austin) was activated by incubation with 20 μM GTP in EF buffer [40 mM HEPES (pH 7.5), 100 mM NH_4Cl, 10 mM $MgCl_2$, 1 mM DTT] plus 25% (v/v) glycerol at 30°C for 20 min. Various concentrations of EF-1$\alpha\cdot$GTP were incubated together with valylated RNAs (2–5 nM) in EF buffer containing 12.5% glycerol and 0.5 mg/ml fragmented salmon sperm DNA on ice for 15 min (Nagata *et al.,* 1976). The amounts of ternary complex were estimated with a ribonuclease protection TCA filter precipitation assay (Dreher *et al.,* 1998) adapted from Louie *et al.* (1984). Binding constants (K_d) were determined computationally from binding curves comprising data from 14 concentrations of EF-1$\alpha\cdot$GTP (0-250 nM), and duplicate or triplicate binding assays were repeated at least twice. The concentration of active EF-1$\alpha\cdot$GTP was determined from binding experiments in the presence of excess [^{3}H]val-tRNA^{Val} transcript.

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