Specific Valylation of Turnip Yellow Mosaic Virus RNA by Wheat Germ Valyl-tRNA Synthetase Determined by Three Anticodon Loop Nucleotides†

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ABSTRACT: The valylation by wheat germ valyl-tRNA synthetase of anticodon loop mutants of turnip yellow mosaic virus RNA has been studied. RNA substrates 264 nucleotides long were made by T7 RNA polymerase from cDNA encompassing the 3′ tRNA-like region of genomic RNA. Substitution singly, or in combination, of three nucleotides in the anticodon loop resulted in very poor valylation (V_{max}/K_M less than 10^{-3} relative to wild type). These nucleotides thus represent the major valine identity determinants recognized by wheat germ valyl-tRNA synthetase; their relative contribution to valine identity, in descending order, was as follows: the middle nucleotide of the anticodon (A56 in TYMV RNA), the 3′ anticodon nucleotide (C55), and the 3′-most anticodon loop nucleotide (C53). Substitutions in the wobble position (C57) had no significant effect on valylation kinetics, while substitutions of the discriminator base (A4) resulted in small decreases in V_{max}/K_m. Mutations in the major identity nucleotides resulted in large increases in K_M, suggesting that wheat germ valyl-tRNA synthetase has a lowered affinity for variant substrates with low valine identity. Comparison with other studies using valyl-tRNA synthetases from *Escherichia coli* and yeast indicates that the anticodon has been phylogenetically conserved as the dominant valine identity region, while the identity contribution of the discriminator base has been less conserved. The mechanism by which anticodon mutations are discriminated also appears to vary, being affinity-based for the wheat germ enzyme, and kinetically-based for the yeast enzyme [Florentz et al. (1991) *Eur. J. Biochem. 195,* 229–234].

Turnip yellow mosaic virus (TYMV)[1] has a 6.3-kb (+)-sense RNA genome whose 3′ domain shares several characteristics with tRNA (Hall, 1979; Haenni et al., 1982). The viral RNA can be efficiently and specifically valylated by valyl-tRNA synthetases (ValRS) from sources as diverse as *Escherichia coli* (Pinck et al., 1970), yeast (Giegé et al., 1978), and wheat germ (Dreher et al., 1988). Further, TYMV RNA is valylated in plant cells during the replication cycle (Joshi et al., 1982), indicating successful competition for ValRS in the presence of high levels of tRNA. We are interested in TYMV RNA as an alternative substrate for ValRS, from whose study new characteristics of aminoacylation specificity may be learnt. By example, the efficient valylation of TYMV RNA, whose only known modified bases are in the 5′ cap, was the first demonstration that the presence of hypermodified bases is not necessary for aminoacylation (Briand et al., 1977; Silberklang et al., 1977). Our studies on the tRNA mimicry of TYMV RNA are also motivated by our desire to understand the relationship between valylation and replication in this virus (Tsai & Dreher, 1991). In this report, we present an analysis of the contribution that key nucleotides make to the valine identity of TYMV RNA as perceived by a homologous higher plant enzyme, wheat germ ValRS. This work parallels and extends our studies with TYMV RNA and yeast ValRS (Florentz et al., 1991). To date, several studies have revealed the identity elements of specific tRNAs [reviewed in Normanly and Abelson (1989) and Schulman (1991)], including *E. coli* $tRNA^{Val}$ (Schulman & Pelka, 1988; Tamura et al., 1991), but only two have so far addressed identity in higher eukaryotic systems [$tRNA^{Ala}$, Hou and Schimmel (1989); $tRNA^{Phe}$, Nazarenko et al. (1992)].

There is evidence from structure-probing experiments using nucleases and chemical reagents that the 3′ 82 nucleotides of TYMV RNA can fold into a structure that closely resembles the L-conformation of tRNAs [Figure 1; Rietveld et al. (1983) and Dumas et al. (1987)]. In this proposed structure, the similarities to canonical tRNAs comprise the 3′-CCA_{OH} terminus, a 12 base pair amino acid acceptor arm, and seven-membered loops analogous to the pseudouridine and anticodon loops. A valine anticodon CAC is present in the latter loop, whose sequence 59-CCCACAC-53 is similar to the CUIACAC (anticodons underlined; I = inosine) of lupine $tRNA^{Val}$ (Barciszewska & Jones, 1987) and CTAACAC of an *Arabidopsis thaliana* $tRNA^{Val}$ gene (Gokhman & Zamir, 1990), to date the only higher plant $tRNA^{Val}$ sequences available (Sprinzl et al., 1991). The sequence within the pseudouridine loop in the viral RNA (38-UGCA-35) deviates somewhat from the conserved sequence found in tRNAs (TFCPu; F = pseudouridine, Pu = purine). The remainder of the tRNA-like structure differs widely in sequence from eukaryotic $tRNA^{Val}$'s. An important feature specific to the amino acid acceptor arm of the viral RNA is the presence of a pseudoknot near the 3′ terminus (Pleij et al., 1985) (Figure 1).

In characterizing the valine identity of TYMV RNA, we have focused for a number of reasons on the nucleotides of the anticodon loop. This region has been shown to interact unambiguously with yeast ValRS (Florentz & Giegé, 1986)

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[1] Abbreviations: ValRS, valyl-tRNA synthetase; TYMV, turnip yellow mosaic virus; F, pseudouridine.

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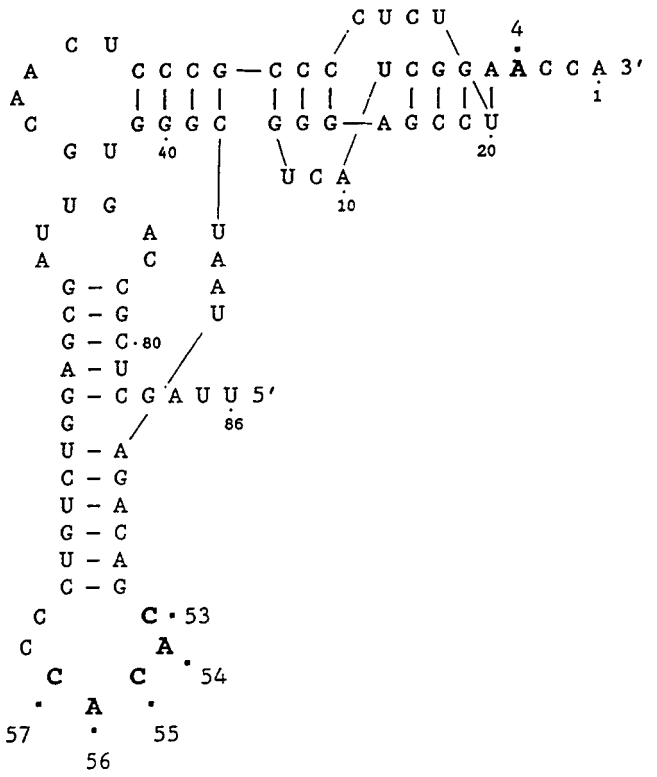

FIGURE 1: Proposed L-conformation of the tRNA-like structure of TYMV RNA. The nucleotides substituted in the present studies are shown in boldface type and include the anticodon 57-CAC-55 and the discriminator base adjacent to the 3′-CCA. Note that nucleotides are numbered from the 3′ end, and only the 86 3′ nucleotides of the 264-nucleotide-long RNAs studied are shown. The 3′ 42 nucleotides are proposed to form the amino acid acceptor stem, which includes a pseudoknot formed by the 3′ 27 nucleotides. Nucleotides $^{5'}82$–$47^{3'}$ form the anticodon stem.

and clearly contains a major determinant recognized by that enzyme (Florentz et al., 1991). In *E. coli* $tRNA^{Val}$, the anticodon also contains major identity determinants (Schulman & Pelka, 1988; Tamura et al., 1991), suggesting that valine identity elements have, like alanine determinants (Hou & Schimmel, 1989), been evolutionarily conserved. Indeed, the anticodon loop is the site of greatest sequence similarity among valylatable viral and transfer RNAs: all eukaryotic $tRNA^{Val}$ species (Sprinzl et al., 1991) and tymoviral tRNA-like structures (van Belkum et al., 1987) share the sequence 5′-NACAC-3′ (anticodon nucleotides underlined). Finally, identity is less likely to reside in those parts of the TYMV tRNA-like structure whose conformations deviate significantly from that of $tRNA^{Val}$, notably the acceptor stem containing the pseudoknot.

Our experimental design has been to deduce valine identity determinants by a subtractive approach. The goal has been to determine the minimal nucleotide substitutions (not involving universally conserved nucleotides) needed to reduce valylation to very low or undetectable levels. Our analysis permits a quantitation of the contribution provided by three nucleotides in the anticodon loop that are key members of the valine identity set. As expected from the fact that TYMV RNA can be valylated with *E. coli*, yeast, and wheat germ ValRS, similar identity elements are perceived in the anticodon by the eukaryotic and prokaryotic enzymes.

MATERIALS AND METHODS

Materials. *BstN*I restriction enzyme and T4 RNA ligase were obtained from New England Biolabs, T7 RNA polymerase and T7 DNA polymerase (Sequenase) were from U.S. Biochemicals, and Taq DNA polymerase was from Promega. Deoxyoligonucleotides were synthesized by automated phosphoramidite chemistry and purified where necessary on 20% polyacrylamide/7 M urea gels. All enzymes were used as recommended by the manufacturer. [^{3}H]L-Valine, [α-^{32}P]-UTP, [α-^{32}P]dATP, and [5′-^{32}P]cytidine 3′,5′-bisphosphate were obtained from New England Nuclear.

A partially purified cytoplasmic extract from wheat germ, enriched by ammonium sulfate precipitation and DEAE-cellulose chromatography (Steinmetz & Weil, 1986), was used as the source of the ValRS activity. One unit was defined as the amount of enzyme that catalyzes the addition of 1 pmol of [^{3}H]valine to TYMV RNA per minute at 30 °C (0.6 μM wild-type transcript RNA; experimental conditions as listed below).

Wild-Type and Variant TYMV RNAs. Most of the RNAs studied were transcripts arising from clones related to mpT7YSma, an M13 clone derived from pT7YSma (Dreher et al., 1988). Replicative form DNA was purified on CsCl gradients and treated with ribonuclease followed by proteinase K to ensure the absence of bacterial tRNA. The DNA was linearized to completion with *BstN*I restriction endonuclease prior to transcription [essentially as described by Dreher et al. (1988)] with T7 RNA polymerase (15 units/μg of DNA) in the presence of 100 μg/mL DNA, 2 mM each nucleoside triphosphate, and 20 Ci/mol [α-^{32}P]UTP. The 264-nucleotide-long RNA transcripts were purified by electrophoresis on 5% polyacrylamide gels run in Tris–borate buffer, followed by electroelution, dialysis against water, and ethanol precipitation in the presence of sodium acetate. The RNAs were redissolved in water and their concentrations were calculated after the incorporated [^{32}P]UMP was determined by liquid scintillation counting.

Mutant variants of mpT7YSma were generated by standard deoxyoligonucleotide-directed mutagenesis using dUMP-containing single-stranded DNA templates (Kunkel et al., 1987). The entire inserts of all mutant clones were sequenced using T7 DNA polymerase.

In a few instances, substrate RNAs were transcribed with T7 RNA polymerase directly from DNA templates made in a polymerase chain reaction, using the opposing deoxyoligonucleotides, d(TAATACGACTCACTATAGGGAGA-GGGTCAAAGATTCG) and d(TGGTTCCGATGACCC-TCG), and Taq DNA polymerase (Tsai & Dreher, 1992). The former oligomer comprises the T7 promoter adjacent to TYMV RNA sequences, while the latter oligomer is complementary to the 3′ end of TYMV RNA. The amplified DNA corresponds exactly to the TYMV sequences present in the mpT7YSma-related clones and ends precisely at the 3′-CCA.

Analysis of 3′ Termini of RNA Transcripts. RNAs were labeled at the 3′ end by the addition of [5′-^{32}P]cytidine 3′,5′-bisphosphate with T4 RNA ligase (Dreher et al., 1984). After purification by gel electrophoresis to remove degraded molecules, the labeled RNA was analyzed in two ways. The identity of 3′ bases was determined by complete digestion with a mixture of ribonucleases T1, A, and T2, followed by two-dimensional thin-layer chromatography on cellulose plates [first dimension, isobutyric acid/0.5 M NH_4OH, 5:3 (v/v); second dimension, 2-propanol/concentrated HCl/water, 70:15:14 (v/v/v)] and counting with a β-emission scanner (Ambis Systems, San Diego, CA). Complete digestion with ribonuclease T1, followed by separation on 20% polyacrylamide/7 M urea gels and densitometry of autoradiographs, determined

Table I: Valylation of Anticodon Loop and Discriminator Base Mutants of TYMV RNA with Wheat Germ ValRS

mutant	anticodon loop sequence	mol of Val/ mol of RNA[a]	K_M (nM)[b]	rel V_{max}[c]	rel V_{max}/K_M	loss of specificity (x-fold)[d]
wild type	59-CCCACAC-53	1.01	31	1.00	1.00	
TY-G57	..G...	0.98	38	0.95	0.78	1.3
TY-A57	..A...	0.98	49	1.08	0.68	1.5
TY-U57[f]	..U...	1.02	29	1.06	1.14	0.9
TY-G56	...G..	<0.001	3200	0.024	0.00020[e]	5000
TY-U56	...U..	<0.001	3300	0.016	0.00011[e]	9100
TY-U55	....U.	0.45	1600	1.05	0.017[e]	59
TY-A55	....A.	0.13	2300	0.39	0.0046[e]	220
TY-G55	....G.	0.04	2200	0.12	0.0015[e]	670
TY-G54	.....G	0.99	38	0.99	0.81	1.2
TY-C54	C	1.05	105	0.76	0.22	4.5
TY-U54[f]	.....U	0.97	68	1.48	0.67	1.5
TY-A53	..... A	0.92	157	0.47	0.093	11
TY-U53	..... U	1.06	183	1.88	0.32	3.1
TY-C4		0.97	38	0.64	0.52	1.9
TY-G4		1.01	190	1.16	0.19	5.3

[a] Extent of valylation after 60 min at 0.6 μM RNA and 20 units of ValRS. [b] Apparent K_M. Concentrations refer to the population of RNAs with 3'-CCA termini. Standard error, ~20%. [c] Standard error, ~20%. [d] Loss of valine charging specificity equals the reciprocal of relative V_{max}/K_M. [e] Estimate derived both by computation from the measured V_{max} and K_M values and by direct determination of the slope of the linear section of the plot of [RNA] vs initial rate of valylation [after Schulman and Pelka (1988)]. [f] Transcript made from a DNA template generated by polymerase chain reaction.

the proportion of transcripts yielding the hexamer expected from 3'-labeled wild-type RNA. Results from the two techniques were combined to determine the proportion of RNAs with 3'-CCA termini as 75 ± 5% for transcripts made from *BstN*I-linearized DNA and 63 ± 5% for transcripts made from DNA templates generated by the polymerase chain reaction.

Valylation Assays. Valylation assays were performed in 25 mM Tris–HCl (pH 8.0), 2 mM $MgCl_2$, 1 mM ATP, 0.1 mM spermine, and 10 μM [^{3}H]valine (12 Ci/mmol) at 30 °C. Kinetic parameters were determined by estimating initial valylation rates over RNA concentrations between 10 and 5000 nM, as appropriate, and analyzing data on double-reciprocal Lineweaver–Burk plots. For the less active mutants, valylations were carried out at twice the above specific activity of [^{3}H]valine, with increased levels of enzyme, and over longer time periods, in order to reliably measure initial valylation rates. The valylation rate was shown to be directly proportional to enzyme concentration over the range of enzyme levels used. Valylation was determined in TCA precipitation assays using Whatman 3MM filters counted in a liquid scintillation spectrometer set to detect both [^{3}H]valine and [^{32}P]RNA.

RESULTS

Synthesis of tRNA-like Substrates for ValRS. RNA comprising the 3' region of TYMV genomic RNA can be transcribed in vitro from *BstN*I-linearized mpT7YSma DNA with T7 RNA polymerase. The majority of these 264-nucleotide-long transcripts have 3'-CCA termini and are as efficiently valylated as natural viral RNA (Dreher et al., 1988). Via mutagenesis of the mpT7YSma DNA, a family of mutant RNAs with substitutions at five nucleotides in the anticodon loop (nucleotides 57-CACAC-53, Figure 1) was generated (Table I). These nucleotides include the anticodon (57-CAC-55) and nucleotides conserved among eukaryotic $tRNA^{Val}$ (Sprinzl et al., 1991) and valylatable tymoviral RNA species (van Belkum et al., 1987). Substitutions of the discriminator base adjacent to the 3'-CCA were also studied.

Transcripts used in analyses of valylation kinetics were purified by gel electrophoresis. Because of the length of these RNAs (264 nucleotides), it was not possible to obtain single base resolution, but this purification served to remove degraded molecules and aborted transcription products. The purified RNAs were a small family of related transcripts, the most abundant being the 3'-CCA species. The 3' termini of all transcript preparations were analyzed as described in Materials and Methods, permitting the proportion of the 3'-CCA species in the total population to be deduced; within narrow limits, this proportion was the same for wild type and all mutants (75 ± 5%). The contaminating RNAs possessed one, or less frequently two, additional nucleotides at the 3' end and most commonly terminated in guanosine. All RNA concentrations given in this paper refer to the 3'-CCA species. As shown by the similar valylation characteristics of the wild-type transcripts and a 3' fragment prepared directly from virion RNA (Dreher et al., 1988), the minor RNA species do not interfere noticeably with the determination of accurate kinetic parameters.

In some cases (indicated in Tables I and II), transcripts were made from DNA templates arising from a polymerase chain reaction (PCR). A lower proportion of 3'-CCA termini was present in these transcripts (63 ± 5%), but after adjustment to equal concentrations of the 3'-CCA RNAs, transcripts from PCR-generated or *BstN*I-linearized DNA yielded essentially the same kinetic parameters characterizing their valylation by wheat germ ValRS. Wild-type transcripts could be completely valylated (Figure 2, Table I). Lineweaver–Burk analysis of initial reaction rates yielded a K_M of 31 nM for the wild-type transcripts (Table I), substantially lower than that previously determined in a different buffer [550 nM, determined in 0.1 M HEPES (pH 7.6), 48 mM KCl, 5 mM $MgCl_2$, 0.64 mM ATP; Dreher et al. (1988)]. These results are in line with the influence of different electrolyte environments on the kinetic parameters of tRNAs [K_M values are lower at low ionic strength; Bonnet and Ebel (1972)]. We have compared the two aminoacylation conditions, finding similar valylation rates at RNA concentrations well above either K_M, and similar discrimination between mutants (not shown). The present conditions, based on "accurate" aminoacylation conditions used by Bare and Uhlenbeck (1986), were used in order to permit the determination of K_M and V_{max} values for inactive mutants with greatly elevated K_M's.

Valylation of Anticodon Mutants. Mutants with substitutions in two anticodon positions, the middle position (wild type, A56) and the 3' position (wild type, C55), had severely impaired valylation properties (Figure 2, Table I). Mutants

Table II: Effects of Combined Substitutions in the Anticodon Loop of TYMV RNA

mutant	anticodon loop sequence	mol of Val/ mol of RNA[a]	K_M (nM)[b]	rel V_{max}[c]	rel V_{max}/K_M	loss of specificity (x-fold)[d]
wild type	59-CCCACAC-53	1.01	31	1.00	1.00	
TY-U56/A55	. . .UA. .	<0.001	2800	0.0071	0.000055[e]	18000
TY-C56/A55	. . .CA. .	<0.001	ND[f]	ND[f]	$<10^{-3}$	>1000
TY-G57/U56/G55	. . GUG. .	<0.001	1300	0.0074	0.00013[e]	7700
TY-U55	. . . .U. .	0.45	1600	1.05	0.017[e]	59
TY-A53	. . . . .A	0.92	157	0.47	0.093	11
TY-U55/A53	. . . .U.A	<0.001	2900	0.051	0.00042[e]	2400
TY-C54	. . . . .C.	1.05	105	0.76	0.22	4.5
TY-U55/C54	. . . .UC.	0.04	1590	0.12	0.0020[e]	500
TY-U55/C54/A53	. . . .UCA	<0.001	4100	0.021	0.00013[e]	7700
TY-ΔC58/U55/C54/A53	. . .UCA	0.00		0.00	0.00	∞
TY-U57[g]	. .U. . . .	1.02	29	1.06	1.14	0.9
TY-A55	. . . .A. .	0.13	2300	0.39	0.0046[e]	2200
TY-U57/A55[g]	. .U.A. .	1.03	520	1.28	0.075	13

[a] Extent of valylation after 60 min at 0.6 μM RNA and 20 units of ValRS. [b] Apparent K_M. Concentrations refer to the population of RNAs with 3′-CCA termini. Standard error, ~20%. [c] Standard error, ~20%. [d] Loss of valine charging specificity equals the reciprocal of relative V_{max}/K_M. [e] Estimate derived both by computation from the measured V_{max} and K_M values and by direct determination of the slope of the linear section of the plot of [RNA] vs initial rate of valylation [after Schulman and Pelka (1988)]. [f] Not determined. [g] Transcript made from a DNA template generated by polymerase chain reaction.

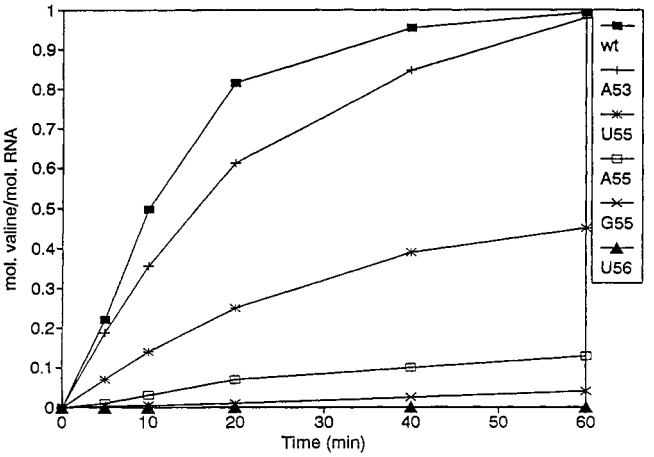

FIGURE 2: Valylation curves for selected TYMV RNA transcripts (0.6 μM) charged with wheat germ ValRS. The transcripts represented are identified in the key: wt, wild type.

TY-G56 and TY-U56 could only be valylated at measurable rates with ValRS concentrations 10-fold higher than those used in the valylation of wild-type RNAs. The K_M for both transcripts was about 100-fold higher than for the wild type, while V_{max} values were about 50-fold lower than for wild-type transcripts. The specificity of valylation of these RNAs, reflected in their V_{max}/K_M relative to wild type, was about 10^{-4}, indicating the clear importance of A56 as a valine identity element.

Mutants with the 3′ anticodon nucleotide replaced by each of the alternative bases were studied. The valylation of each mutant was impaired, and none could be completely charged under normal conditions (Figure 2). As for the nucleotide 56 mutants, substitution of C55 resulted in greatly elevated K_M values, 50–75-fold higher than for wild-type RNA (Table I). The effect of the nucleotide 55 mutations on V_{max} values was much smaller and varied depending on the sequence change: TY-U55 had a V_{max} similar to wild type, but the transversion substitution mutants TY-A55 and TY-G55 had V_{max} values decreased by factors of 3 and 8, respectively, relative to wild type. With relative V_{max}/K_M values ranging from 1.7×10^{-2} to 1.5×10^{-3} for these mutants, C55 is clearly an important valine identity element recognized by wheat germ ValRS.

With four codons in the genetic code specifying valine, ValRS must accept $tRNA^{Val}$ species with at least some variations in the wobble position; inosine and cytidine are common (Sprinzl et al., 1991). We studied mutants with the TYMV wobble nucleotide C57 changed to each of the unmodified alternatives: TY-G57, TY-A57, and TY-U57. Both K_M and V_{max} values for these mutants were similar to those of wild-type RNA (Table I), indicating that nucleotide 57 is not used by wheat germ ValRS in substrate discrimination.

Valylation of Mutants with Substitutions in the 3′ Part of the Anticodon Loop. The partial valylatability of TY-U55 made it clear that significant valine identity resides outside the anticodon nucleotides of TYMV RNA. Because the nucleotides 54-AC-53 flanking the anticodon on the 3′ side are conserved among all higher eukaryotic valylatable RNAs, the contribution of these nucleotides to the valine identity of TYMV RNA was studied. Among eukaryotic elongator tRNAs, the analogous nucleotides, 37 (adjacent to the anticodon) and 38, are only purines and never G (Grosjean et al., 1982; Sprinzl et al., 1991). We have studied all possible substitutions of unmodified bases replacing A54, and all but G replacing C53 (Figure 1). Mutants TY-G54, TY-C54, TY-U54, TY-A53, and TY-U53 could all be completely valylated under normal conditions (Figure 2, Table I).

Substitution of A54 with a purine (TY-G54) yielded an RNA with kinetic parameters not significantly different from those of wild-type RNA (Table I). The mutants with pyrimidine substitutions (TY-C54 and TY-U54) also were excellent substrates for ValRS, although their V_{max}/K_M values were slightly decreased relative to wild type. Especially considering the exclusion of pyrimidines from eukaryotic tRNA molecules at this position, A54 cannot be considered as a valine identity element used in the recognition of TYMV RNA by wheat germ ValRS.

Replacement of C53 with a pyrimidine (TY-U53) likewise resulted in an excellent substrate for ValRS. Although the K_M was 6-fold higher than for the wild-type RNA, the V_{max} was also increased, resulting in a relative V_{max}/K_M of 0.32 (Table I). In contrast, substitution with a purine (TY-A53), resulted in a V_{max}/K_M decreased relative to wild type by a factor of 11. These results suggested that C53 contributes to the valine identity of TYMV RNA, but less strongly than nucleotides A56 and C55.

Effects of Combined Substitutions in Identity Elements. In order to further explore the role of C53 as an identity element, mutant TY-U55/A53, combining substitutions of C55 and C53, was prepared and studied. The valylation rates

of this RNA could only be measured at elevated ValRS concentrations and had a K_M 94-fold higher than that of wild-type RNA (Table II). Its V_{max} was decreased 20-fold relative to wild type, resulting in a relative V_{max}/K_m of 4.2×10^{-4}. The combined substitutions thus resulted in an RNA retaining only a low residual valylation capacity, with a valine specificity almost as low as those of mutants TY-G56 and TY-U56. Other combinations of substitutions at C55 and C53 are likely to result in RNAs at least as inactive for valylation as TY-U55/A53, since the presence of a purine at nucleotide 55 resulted in low V_{max}/K_M values (Table I). These results demonstrate the importance of both C53 and C55 as valine identity elements.

The three valine identity nucleotides that we have found with the preceding experiments all appear to be directly recognized by ValRS in its binding of the TYMV RNA substrate, since substitution of these nucleotides resulted in large increases in K_M. To investigate whether identity at these three nucleotides is independently discriminated, we studied the effect on valylation of multiple changes in the anticodon loop. The effect of multiple mutations on aminoacylation can be predicted from the kinetic data obtained for single mutations. If the effects are additive, the relative specificity constant (relative V_{max}/K_M) of a double mutant equals the product of the relative specificity constants of the single mutants (Carter et al., 1984; Wells, 1990). In cases of cooperative or anticooperative effects, the calculated values of the relative specificity constants will deviate from the experimental determinations. Mutant TY-U55/A53, discussed above, had a relative V_{max}/K_M 3.8 times lower than expected if the individual mutations were additive (expected V_{max}/K_M, $0.017 \times 0.093 = 0.0016$; cf. experimentally determined V_{max}/K_M, 0.000 42; Table II). Similarly, TY-U55/C54 and TY-U55/C54/A53 RNAs had relative V_{max}/K_M values 1.9 and 2.6 times lower than expected, respectively, for independently detected, additive mutations (Table II). For these three mutants, there appears to be a mild cooperative effect (cf. 14-fold deviation between expected and determined V_{max}/K_M for TY-U57/A55; see below) between the anticodon and flanking substitutions in degrading the valine identity as detected by wheat germ ValRS. The 3′ flanking substitutions A53 and C54 may well alter the presentation of the anticodon, enhancing the detection of missing identity elements by ValRS. Mutant TY-ΔC58/U55/C54/A53, a fortuitously isolated mutant, is probably a more extreme case of such structural perturbation: the deletion of C58 contributes to the complete absence of valylation for this RNA (cf. TY-U55/C54/A53), even in assays using [^{3}H]valine at increased specific activity and high levels of ValRS. All other mutants studied retained a measurable valylation capacity. Apparently, the combination of anticodon substitutions and altered anticodon loop structure resulting from the deletion produced a molecule totally lacking valine identity. This emphasizes the localization of valine identity elements to the anticodon loop of TYMV RNA.

If combined mutations in the anticodon loop are able to potentiate the discrimination by the wheat germ ValRS of identity elements, the opposite effect would also seem possible. Indeed, the combination of substitutions at nucleotides 56 and 55 in mutants TY-U56/A55 and TY-G57/U56/G55 (Table II) resulted in V_{max}/K_M values not much lower than those of mutants with substitutions at nucleotide 56 alone (Table I). A surprising example was observed, however, with RNA TY-U57/A55 (Table II). This mutant was obtained after inoculation of Chinese cabbage plants with a TYMV genome carrying the A55 mutation. The A55 mutant did not give rise to systemic infections, but the spontaneously acquired second mutation C57 → U permitted systemic spread and improved valine charging (Tsai & Dreher, 1991). TY-U57/A55 could be stoichiometrically valylated and had a V_{max}/K_M only 13-fold reduced relative to wild type. The addition of the C57 → U substitution to TY-A55 increased the V_{max}/K_M by a factor of 16.3 (TY-U57/A55 vs TY-A55), with improvements in both K_M and V_{max} (Table II); the experimentally determined V_{max}/K_M deviates from that expected if the mutations are additive by a factor of 14, representing a marked anticooperative effect. The compensating effects of the U57 and A55 mutations suggest that the anticodon loop is recognized as a structural unit and that the phosphate backbone as well as functional groups on the purine or pyrimidine rings of identity nucleotides could be recognized in this system. We are aware of no similar example of such a marked effect of nucleotide context in decreasing the potency of an identity element. It will be interesting to determine whether this phenomenon is restricted to TYMV RNA, a molecule whose optimal charging requires conditions somewhat favoring mischarging. Certainly, a similar situation with tRNAs could result in decreased translational fidelity.

Valylation of Mutants with Substitutions Adjacent to the 3′-CCA. The residual valine identity of mutants with substitutions at both nucleotides 56 and 55 suggested the possibility that weak valine identity elements exist outside the anticodon loop (although this suggestion is not supported by the fact that TY-ΔC58/U55/C54/A53 totally lacks valine identity). The discriminator base adjacent to the 3′-CCA is an identity element in several systems (Schulman, 1991), and substitutions at this nucleotide in TYMV RNA were found to decrease valylation by yeast ValRS (Florentz et al., 1991) and in $tRNA^{Val}$ by *E. coli* ValRS (Tamura et al., 1991). Mutants TY-C4 and TY-G4 were both excellent substrates for the wheat germ ValRS (Table I). Both RNAs could be valylated to completion under normal conditions and had V_{max} values similar to wild type. The K_M for TY-C4 did not differ significantly from that of the wild-type RNA, while the K_M for TY-G4 was elevated 6-fold. In contrast to the *E. coli* (Tamura et al., 1991) and yeast (Florentz et al., 1991) ValRS enzymes, the wheat germ enzyme discriminates only slightly against discriminator base mutants. Nevertheless, A4 could be considered a weak valine identity element.

DISCUSSION

Principal Residence of Valine Identity of TYMV RNA in Three Residues of the Anticodon Loop. We have studied the effects of substitutions in the anticodon loop of TYMV RNA in diminishing the viral RNA's ability to be valylated by wheat germ ValRS. A systematic approach involving single and multiple substitutions has yielded a quantitative picture of the contribution toward valine identity made by the three major identity determinants recognized by wheat germ ValRS in TYMV RNA: A56, C55, and C53. Changes involving only these three nucleotides are able to reduce the V_{max}/K_M for valylation at least 10^3-fold relative to wild-type RNA (Tables I and II).

The most important nucleotide conferring valine identity is A56. Single substitutions (TY-G56, TY-U56) resulted in RNA molecules with V_{max}/K_M about 10^{-4} relative to wild type. Nucleotide C55 is the second important element of valine identity, with substitutions decreasing V_{max}/K_M values by 60–700-fold. Mutants with a purine nucleotide at this site were discriminated better than the uridine mutant. The least

potent of the three major identity nucleotides is C53. Substitution with adenosine decreased V_{max}/K_M 11-fold, although the uridine substitution had only a 3-fold effect. The importance of C53 as an identity determinant is better demonstrated by the low valylation of TY-U55/A53 relative to TY-U55 (Table II). The involvement of nucleotides in the anticodon as well as the 3′ of the anticodon loop in identity determination has also been seen in the *E. coli* glutamine system (Rould et al., 1991; Jahn et al., 1991).

The magnitudes of the V_{max}/K_M effects of substitutions in A56 and C55 are similar to those observed in tRNAs whose aminoacylation identities reside mostly in localized, rather than distributed, sites on the molecule. Thus, for *E. coli* $tRNA^{Val}$ and $tRNA^{Met}$, substitution of two nucleotides within the anticodon decreased V_{max}/K_M for the cognate synthetase by about 10^4-fold (Schulman & Pelka, 1988), and the alteration of a G·U base pair in *E. coli* $tRNA^{Ala}$ decreased V_{max}/K_M by a similar amount (Shi et al., 1990). By contrast, the identity elements of yeast $tRNA^{Asp}$ and $tRNA^{Phe}$ that are recognized by their cognate synthetases are scattered among three far-flung sites on the RNA, the anticodon, the D-arm, and nucleotide 73, and substitutions at these sites independently result in decreases of V_{max}/K_M of only 10–15-fold in the phenylalanine system (Bruce & Uhlenbeck, 1982; Sampson et al., 1989) and 10–530-fold in the aspartic acid system (Pütz et al., 1991).

All the mutants studied retained residual valylatability, except TY-ΔC58/U55/C54/A53, which was totally unable to accept valine. It is thus possible that features outside the anticodon loop contribute to valine identity in a minor way, perhaps in maintaining a conformation compatible with ValRS recognition. Nucleotide A4, whose substitution slightly decreased valylation by wheat germ ValRS (Table I), may be a relevant example, by affecting the conformation of the pseudoknot and 3′ region of the amino acid acceptor arm (Florentz et al., 1991). We consider nucleotide 54 also to serve such a structural purpose, although in conferring generic aminoacylation rather than specific valylation. Either of the purines adenosine or guanosine is acceptable at this position (wild type, TY-G54), but a pyrimidine mutant (TY-C54) had a 5-fold decreased V_{max}/K_M (Table I). This is probably the result of poorer base stacking of nucleotides 57–53, influencing the disposition of the anticodon. It is noteworthy that the analogous position in tRNAs (nucleotide 37) is always occupied by a purine (Sprinzl et al., 1991).

Involvement of Both K_M and V_{max} Discrimination in Selection of Cognate RNA by Wheat Germ ValRS. All mutants with impaired valine identity had elevated K_M values, suggesting that significant discrimination by wheat germ ValRS occurs on the basis of substrate recognition and binding. The primary identity elements we have described in this paper (A56, C55, C53) are thus almost certainly nucleotides directly contacted by wheat germ ValRS. This is probably also true for the *E. coli* methionyl-tRNA synthetase/$tRNA^{Met}$ system, where substitution of the wobble base results in very high K_M values that can be suppressed by compensating amino acid substitutions in the synthetase (Ghosh et al., 1990). In the two systems studied in most detail to date, the X-ray structures of both *E. coli* $tRNA^{Gln}$ (Rould et al., 1991) and yeast $tRNA^{Asp}$ (Ruff et al., 1991) bound to their cognate synthetases have revealed direct contacts between the synthetase and functional groups of anticodon bases of the tRNA.

Our observation that mutant TY-U57/A55 exhibited an unexpectedly high level of valine identity (Table II) suggests that the wheat germ ValRS may recognize the anticodon nucleotides in a different fashion, perhaps detecting a specific configuration of the phosphate backbone in combination with functional groups of bases at identity positions. Certain substitutions adjacent to the most significant identity element in TYMV RNA (A56) may modulate the efficiency of recognition of this nucleotide by the ValRS by altering the conformation of the anticodon loop in a subtle way. It will be interesting to see if this is more generally true for canonical tRNA substrates of ValRS and other synthetases. Clearly, the significant valine identity of TY-U57/A55 raises the possibility that $tRNA^{Leu}$ with the same UAA anticodon might be valylated in vivo. The presence of one or more antideterminant elements (e.g., the long variable loop) in this tRNA would be necessary to preclude valylation.

Most mutants with diminished valine identity had decreased V_{max} values in addition to elevated K_M's. This was especially so for mutants with substitutions of A56. Nevertheless, the great importance of K_M discrimination for the wheat germ ValRS suggests a somewhat different substrate discrimination mechanism from that employed by the yeast enzyme. Competition studies and enzyme/RNA binding studies with TY-U56 and TY-G56 demonstrated only minor differences in ValRS interaction between wild-type and mutant RNAs (Florentz et al., 1991), indicating that mutants were discriminated predominantly by a kinetic mechanism. We have performed similar competition experiments between the above mutants and wild-type viral RNA using wheat germ ValRS and found no influence on the valylation rate of wild-type TYMV RNA in the presence of mutant RNAs (not shown). This is consistent with the mutants having elevated K_M's and with differential substrate binding being a significant discrimination mechanism for wheat germ ValRS. In preliminary experiments (not shown), the mutants studied in this paper with wheat germ ValRS have similarly ranked valylation activities with the yeast enzyme, indicating that the two enzymes detect similar identity determinants in the TYMV RNA. One significant difference between the two synthetases is the recognition of mutations at A4. TY-C4 and TY-G4 were excellent substrates for wheat germ ValRS (Table I), but could only be partially charged by yeast ValRS (Florentz et al., 1991). Kinetic studies of the full set of mutants with yeast ValRS should elucidate the apparent mechanistic differences the two synthetases use in discriminating similar identity determinants. An understanding of ValRS structure, and a comparison of the sequences of the synthetase, may also shed light on this problem.

Anticodon Nucleotides as the Dominant Valine Identity Determinants across the Phyla. In mapping the valine identity determinants recognized by wheat germ ValRS to the anticodon, our studies show that ValRS enzymes from bacterial (*E. coli*), lower eukaryotic (yeast), and higher eukaryotic (wheat germ) sources detect valine identity determinants in or near the anticodon of their substrates. In the *E. coli* system, there are 10^4-fold differences in V_{max}/K_M between $tRNA^{Val}$ with UAC (wild type) and CAU anticodons (Schulman & Pelka, 1988) and between $tRNA^{Val}$ with UAC and UGC anticodons (Tamura et al., 1991). Yeast ValRS, which recognizes TYMV RNA as an efficient substrate, discriminates the substitution of the middle position of the anticodon (mutants TY-G56, TY-U56) with a 20 000-fold decrease in V_{max}/K_M (Florentz et al., 1991). These same mutants were valylated by wheat germ ValRS with a 5000–9000-fold decrease in V_{max}/K_M (Table I). Thus, the anticodon is an important identity element recognized by ValRS from diverse sources, but the precise spectrum of identity determinants

recognized by eukaryotic ValRS may differ from those recognized by the *E. coli* enzyme. In particular, the results of Schulman and Pelka (1988) suggest that the 3′ nucleotide of the anticodon is a stronger determinant in *E. coli*. Knowledge of the roles of other anticodon loop nucleotides in determining the identity of *E. coli* $tRNA^{Val}$ awaits further analysis of that system.

Analysis of the charging of discriminator base mutants of TYMV RNA (TY-C4 and TY-G4; Table I) indicated only a weak role for this nucleotide in the specificity of TYMV RNA valylation by wheat germ ValRS. In contrast, ValRS enzymes from *E. coli* and yeast appear to utilize the discriminator base as a significant identity element: while the A → G substitution at nucleotide 4 of TYMV RNA resulted in only a 5-fold decrease in V_{max}/K_M with the wheat germ enzyme (Table I), the same substitution resulted in a 300-fold decrease with yeast ValRS (Florentz et al., 1991), and the A73 → G substitution in *E. coli* $tRNA^{Val}$ resulted in a 55-fold difference with *E. coli* ValRS (Tamura et al., 1991). Thus, while the anticodon has been conserved across the phyla as the primary identity element, the importance of the discriminator base varies. Comparison of the phenylalanine identity sets recognized in the *E. coli*, yeast, and human systems (Nazarenko et al., 1992) likewise reveals a general conservation of identity elements, but with significant deviations especially between prokaryotes and eukaryotes. A stronger conservation of identity determinants, focused on a G·U base pair in the acceptor arm, appears to exist in the alanine system, as deduced from studies with $tRNAs^{Ala}$ and alanyl-tRNA synthetases from bacterial, insect, and mammalian sources (Hou & Schimmel, 1989).

Future Prospects. Work is in progress to extend the results presented in this paper to determine whether an identical set of nucleotides specifies the valylation of higher plant $tRNA^{Val}$ by wheat germ ValRS. It will also be of interest to test whether transplantation of the valine identity set into non-valine-accepting tRNAs and tRNA-like structures is able to convert those RNAs into efficient valine acceptors.

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