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Deletions in the 3′-terminal tRNA-like structure of brome mosaic virus RNA differentially affect aminoacylation and replication *in vitro*

(replicase template activity/tyrosyl-tRNA)

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ABSTRACT **Deletions in cDNA clones covering the 3′ 201 nucleotides of brome mosaic virus RNA 3 were produced by S1 nuclease treatment of cloned DNA linearized at several different restriction sites. Transcription of these clones yielded RNAs containing structural alterations in the 3′-terminal tRNA-like structure that is involved in aminoacylation and replication. Replicase template activity, but not aminoacylation activity, was especially sensitive to deletions in arm C, which contains a tyrosyl anticodon. Deletions in arm B were detrimental to aminoacylation, but the proportion of replicase template activity lost depended on the site of the deletion. Removal of arm D had little effect on aminoacylation and, in some instances, resulted in a 2-fold stimulation of replicase template activity.**

The genomic RNAs of many RNA plant viruses possess at their 3′ ends a highly conserved region responsible for several tRNA-specific activities (1, 2). The tRNA-like structure formed by this region represents an integral part of the three genomic and single subgenomic RNAs of brome mosaic virus (BMV) and is capable of being stoichiometrically aminoacylated *in vitro* with tyrosine (3). Tyrosylation has also been observed *in vivo* during infection of barley protoplasts with BMV (4), indicating that the viral RNAs are able to compete with tRNAs for the cognate synthetases. Although such observations suggest a significant role in infection processes, no function for the tRNA-like properties of any of the plant viral RNAs has as yet been defined. The failure to detect any involvement of charged viral RNA in translational functions (2, 5, 6) has focused attention on a possible role in replication (1, 7).

The strongest evidence to date supporting an interaction between the tRNA-like properties and replication comes from our recent discovery that the 3′-terminal 134-nucleotide region of BMV RNA responsible for aminoacylation (8) also contains the entire information needed for specific recognition and for initiation of (−) strand synthesis by BMV replicase *in vitro* (ref. 9; unpublished data). To explore the dual role of this region in aminoacylation and replication, we have studied the effects of deletions of segments of the tRNA-like structure on both functions. The production of substantial amounts of such modified RNAs was made possible by the development of a system for transcribing correctly terminated, biologically active 3′-terminal fragments of BMV RNA *in vitro* from cDNA (10). Transcripts may be readily assayed *in vitro* for template activity with the template-specific, template-dependent BMV replicase (11, 12) and for aminoacylation with wheat germ tyrosyl-tRNA synthetase (3, 13).

In this paper we localize and differentiate between several areas of the tRNA-like structure involved in replication and aminoacylation by analyzing the effects of deletions obtained in four separate regions. The selection of deletions studied and the interpretation of their effects on substrate activities have been made in reference to a proposed structural model for BMV RNA (Fig. 1; refs. 8, 14, 15).

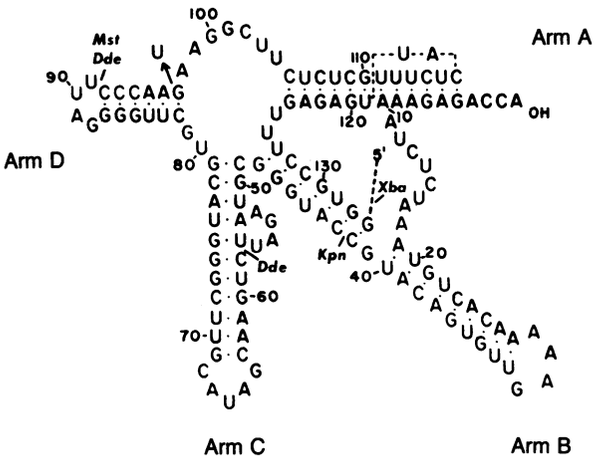

FIG. 1. tRNA-like structure at the 3′ terminus of BMV RNA 3. Nucleotides in the recently proposed conformation (8, 15) are numbered from the 3′ end. The G to U substitution at position 97 does not affect aminoacylation and replication activities (10) and is the difference in sequence between "wild-type" transcripts and viral RNA 3. The locations of restriction sites within the cDNA used to generate deletions are also indicated. Arm C contains a tyrosyl -AUA- anticodon.

MATERIALS AND METHODS

Chemicals and Enzymes. All chemicals used were of reagent grade. Restriction enzymes were obtained from Bethesda Research Laboratories, New England Biolabs, and Promega Biotec (Madison, WI). T4 DNA ligase and T4 polynucleotide kinase were from Bethesda Research Laboratories; T4 RNA ligase, from Pharmacia P-L Biochemicals; SP6 RNA polymerase, from Promega Biotec; and *Escherichia coli* DNA polymerase I, large (Klenow) fragment, from Boehringer Mannheim. Radiochemicals: deoxyadenosine 5′-(α-[^{35}S]thio)triphosphate (900 Ci/mmol), [γ-^{32}P]ATP (5000 Ci/mmol), and [α-^{32}P]UTP (800 Ci/mmol) were obtained from Amersham, whereas [5,6-^{3}H]UTP (38 Ci/mmol) was from New England Nuclear (1 Ci = 37 GBq).

Creation of *Xba* I Restriction Site by Oligonucleotide-Directed Mutagenesis. The *Xba* I restriction site was created

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Abbreviations: BMV, brome mosaic virus; bp, base pair(s).

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in clone mpTD11 (see text and Fig. 2 *Upper*), by a single base substitution of G by A at position 132 (Fig. 1). The 20-mer deoxyoligonucleotide d(CTCAAAGGCATCTAGACACT) was synthesized by a phosphite triester procedure (16), using the New England Biolabs kit, and purified on a 20% polyacrylamide/7 M urea gel, followed by DEAE-cellulose (DE-52, Whatman) chromatography (17). The sequence was verified by the Maxam–Gilbert procedure after 5′-^{32}P-labeling with T4 polynucleotide kinase and [γ-^{32}P]ATP (18). The single-stranded mpTD11 DNA and the oligomer (molar ratio 1:10) were annealed (50°C for 10 min and room temperature for 30 min) and subsequent second-strand synthesis, ligation, separation of the ligated DNA, and transfection were according to the procedures of Zoller and Smith (19). Clones were screened for the presence of a new *Xba* I restriction site.

Establishment of Deletions in BMV cDNA by Using S1 Nuclease Digestion. Replicative form (RF) DNA from phage M13-based clone mpTD11 (10) (3 μg) was linearized at the desired restriction site and treated at room temperature with 12 units of S1 nuclease in 50 mM sodium acetate, pH 4.0/6 mM $ZnSO_4$/250 mM NaCl for 10, 25, or 40 min. After extraction with phenol/chloroform, the DNAs in each reaction mixture were recircularized with T4 DNA ligase before transfection. RF DNA was prepared from the resultant plaques, using a NaOH/sodium dodecyl sulfate minilysate procedure (20), and screened for the absence of the restriction site. The size of the deletion was determined by dideoxy chain-termination sequencing with DNA polymerase I (Klenow fragment) (21) and (α-[^{35}S]thio)dATP (22). Clones harboring deletions were inserted as *Hin*dIII/*Eco*RI fragments into one or both of the transcriptional plasmids used in this work: pSP64, a pUC12 derivative containing an SP6 promoter fragment (23) or pSP62, a pBR322 derivative with the SP6 promoter (24). Plasmids were purified by cesium chloride gradient ultracentrifugation and linearized with *Tth*111 I restriction enzyme prior to preparative transcription (10).

Deletion of a 33-Base-Pair (bp) Fragment Between Two *Dde* I Restriction Sites. Since several *Dde* I restriction sites exist in clone mpTD11, the *Hin*dIII/*Eco*RI insert was isolated by electrophoresis in low-melting point agarose and subsequently digested with *Dde* I restriction enzyme (see Fig. 1). The *Eco*RI/*Dde* I fragment was inserted into *Mst* II/*Eco*RI sites of pSP62 or pSP64 transcriptional plasmids.

Preparative Transcription *in Vitro* with SP6 RNA Polymerase. Transcripts were synthesized from *Tth*111 I-linearized DNA as described (10), except that incubations were at 40°C for 4 hr. After incubation, unincorporated nucleotides were removed on a DEAE-Sephacel (Pharmacia) column followed by ethanol precipitation. The amount of RNA synthesized was estimated from the incorporated radioactivity. Transcripts used for template activity assays were labeled with [5,6-^{3}H]UTP, whereas transcripts for aminoacylation assays were labeled with [α-^{32}P]UTP.

Assays *in Vitro* for Template and Aminoacylation Activities. Transcript RNAs were assayed for template activity by incubation with micrococcal nuclease-treated BMV replicase, prepared from BMV-infected barley leaves (12). The replication products were extracted and electrophoresed on a denaturing 6% polyacrylamide/7 M urea gel, as described (10). Since product and template comigrate, bands were cut out of the gel and digested in Soluene 350 (Packard, 30°C overnight), and the ^{32}P-to-^{3}H ratio was determined by liquid scintillation counting. Since ^{32}P counts reflected the amount of UMP incorporated after replication, whereas ^{3}H counts corresponded to the amount of the synthetic RNA used as a template, comparison of ^{32}P-to-^{3}H ratio for each mutated RNA with that for the wild-type RNA gave the relative template activity. The aminoacylation assay, described previously (10), utilized transcripts labeled with [α-^{32}P]UTP and a partially purified preparation of aminoacyl-tRNA synthetase from wheat germ (3). The incorporated radioactivity was determined by ^{32}P/^{3}H liquid scintillation double counting.

Replication and aminoacylation assays were performed in duplicate on at least two groups of independently synthesized transcripts.

RESULTS

Synthesis of 3′-Terminal Fragments of BMV RNA Containing Various Deletions. Deletions in four separate regions of the tRNA-like structure of BMV RNA 3 (Fig. 1) were generated from clone mpTD11, containing a cDNA insert of the 3′-terminal 201 nucleotides of BMV RNA 3 (10). In all but one case the deletions were produced by a controlled removal with S1 nuclease of nucleotides surrounding selected restriction sites (Fig. 2 *Upper* and *Materials and Methods*) that either occurred naturally in the BMV cDNA sequence (*Dde* I, *Kpn* I, and *Mst* II) or were created by oligonucleotide-directed mutagenesis (*Xba* I). A further deletion was produced by excision of a 35-bp *Dde* I/*Dde* I segment from the BMV cDNA sequence (*Materials and Methods*). After placement of the deletion-bearing insert under the control of an SP6 promoter (Fig. 2 *Upper*), correctly terminated RNAs (10) were synthesized by transcription of *Tth*111 I-linearized DNA (Fig. 2 *Lower*).

Two types of transcriptional plasmids (pSP62 and pSP64) were used in this work (Fig. 2 *Upper*). Clones in pSP62 (*Mst* II deletions) produced transcripts with 42 nucleotides of heterologous, SP6 gene-derived nucleotides at the 5′ end, whereas pSP64-cloned transcripts (all other deletions) contained only 6 heterologous nucleotides. These heterologous sequences do not appear to interfere with the activities of the tRNA-like structure, because wild-type transcripts from both vectors had the expected activity with reference to virion RNA (10). Also, transcripts containing the same deletion but produced from either vector had similar activities (Table 1, D1 and D2).

Deletions in the Region of Arms B and C. Five clones (K1–K5) with deletions of different length in the region of arms B and C were obtained by using the *Kpn* I restriction site (compare Fig. 1 and Table 1) and RNAs were synthesized from the pSP64 vector (Fig. 2 *Upper*). Table 1 shows the relative template activities for BMV replicase quantified after analysis of replication products on denaturing gels (Fig. 3, *Kpn* I deletions). Within this region, the extent of reduction in activity depends on the length of sequence removed. The 1-nucleotide (clone K1), 5-nucleotide (clone K2), and 14-nucleotide (clone K3) deletions reduced template activity to 60%, 10%, and 5%, respectively, of the wild-type RNA. The removal of the larger fragments (49 and 53 nucleotides in clones K4 and K5) abolished the template activity. Aminoacylation results (Table 1) paralleled those obtained for replication.

Deletion of the Anticodon Arm (Arm C). A 34-nucleotide sequence, between two *Dde* I restriction sites, involved in the formation of arms C and D (region 57–92), was deleted (Fig. 1) and the resultant clone was subcloned in both transcriptional vectors (D1 in pSP62 and D2 in pSP64). RNAs obtained from either vector were totally inactive in replication (Fig. 3 and Table 1). However, both D1 and D2 RNAs showed about 60% of wild-type activity for aminoacylation, indicating that the presence of this entire region is not obligatory for this function (see *Discussion*).

Deletions in the Arm D Region. Five nuclease S1-derived deletions were obtained in this region by using the *Mst* II restriction site (Fig. 1). Because of the presence of an *Mst* II site within the M13 sequence, S1 nuclease treatment was performed in the vector pSP62. Four deletions, of 5, 7, 14, and 20 nucleotides (clones M1–M4) which, to various ex-

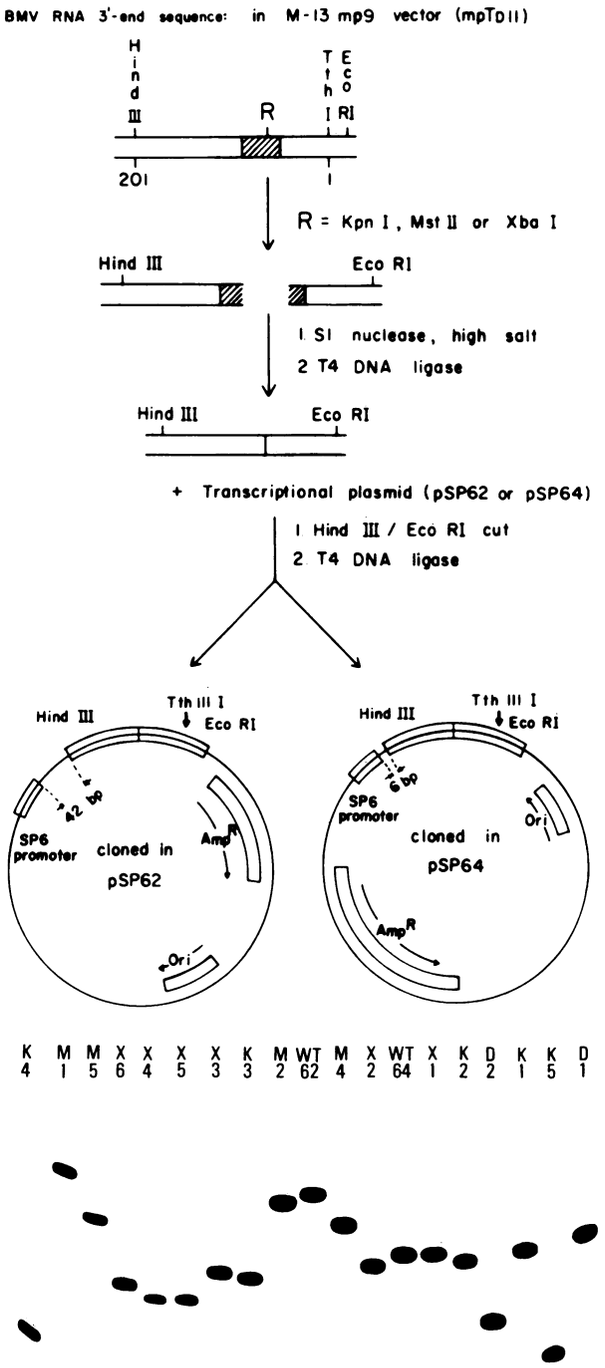

FIG. 2. Synthesis of 3'-end BMV RNA transcripts with deletions. (*Upper*) General strategy used for generation of deletions. Plasmid mpTD11 was linearized at a restriction site R and the hatched region was removed by S1 nuclease digestion; after recircularization and sequencing, the *Hin*dIII/*Eco*RI fragment was inserted into transcriptional plasmids pSP62 or pSP64 (not to scale). Amp^R, ampicillin resistance; Ori, origin of replication. (*Lower*) Electrophoretic analysis of RNAs synthesized with SP6 RNA polymerase. Transcripts labeled with [^{3}H]UTP were synthesized from *Tth*111 I-linearized pPS62 or pSP64 transcriptional plasmids containing individual deletion clones (indicated on top; refer to Table 1), electrophoresed on a 6% polyacrylamide/7 M urea sequencing gel, and detected by fluorography with 1 M sodium salicylate (25). Markers indicating the migration of wild-type transcripts (WT62, 243 nucleotides, and WT64, 207 nucleotides) were synthesized from the wild-type clone in pSP62 and pSP64, respectively.

tents, remove arm D, did not cause loss of the replicase template activity of the transcribed RNAs (Fig. 3, Table 1).

Table 1. Aminoacylation and replication activities of 3'-end BMV RNA transcripts with deletions

Transcript and corresponding clone	Deletion*	Replication, %†	Aminoacylation, %‡
K1	41–43	60	69
K2	42–48	10	22
K3	36–51	5	31
K4	6–60	0	3
K5	23–73	0	4
D1	57–92	0	65
D2	57–92	0	55
M1	87–93	70	85
M2	87–95	220	79
M3	86–101	160	99
M4	80–101	200	100
M5	75–105	5	72
X1	132G→A	80	51
X2	131–138	70	8
X3	131–144	50	11
X4	130–158	20	12
X5	124–151	55	4
X6	126–149	70	5

*Numbers show the positions of nucleotides connected after deletion and refer to the numbering system shown on Fig. 1. The portion that was deleted was determined by dideoxy chain-termination sequencing of the cDNA clones.

†Template activity of individual transcript RNAs as a percentage of the activity of the wild-type transcript. Transcript RNA (0.2–1 μg) was assayed with BMV replicase, and the double-stranded products were extracted as described in ref. 10.

‡Tyrosylation as a percentage of the wild-type transcript RNA activity; 0.5–0.7 μg of transcript RNA was assayed (10).

Indeed, three RNAs (from clones M2–M4) showed increased activity, to about twice that of the wild type (Table 1). In contrast, removal of arm D as well as its 5' and 3' flanking regions (clone M5, deletion between nucleotides 75 and 105) resulted in almost total loss of replication activity (Fig. 3 and Table 1).

Aminoacylation data (Table 1) show that RNAs synthesized from all M clones retained between 70% and 100% of the wild-type RNA level of tyrosine esterification. The kinetic data show a slight decrease in the rate of tyrosylation for M1 and M4 deletions, and a substantial decrease for M5, the largest deletion (Fig. 4).

Deletions at the 5' Side of tRNA-Like Structure. Five nuclease S1-derived deletions (clones X2–X6) in the sequence complementary to that modified by the K deletions (Fig. 1) were nested around the newly created *Xba* I restriction site, which cleaves the BMV cDNA sequence between positions 135 and 136. Deletions in this region can be considered as substitution mutations, in which 5' sequences are brought within the 133-nucleotide tRNA-like structure. Both sequence alteration and the extent to which stable base-pairing in the base of arm B (Fig. 1) is disrupted may be important. The relative template activities of clones X1, X2, X3, and X4 (80%, 70%, 50%, and 20%) correlated with the number of mismatches in this region: one (G-132 in clone X1; G-133 in clone X2), two (GG in clone X3), and three (UGG in clone X4) nucleotides were replaced by A, U, AC, or CAC mismatching sequences, respectively. The aminoacylation activities of these RNAs were drastically reduced to 51%, 8%, 11%, and 12%, respectively. However, two other RNAs, with substitutions starting at positions 125 and 127 (from clones X5 and X6, respectively), and that might on the basis of base-mismatches have been expected to be less active as replicase templates than the transcript of clone X4, were found to have 55% and 70% of the wild-type activity. Aminoacylation was reduced to 4% and 5%, respectively, by

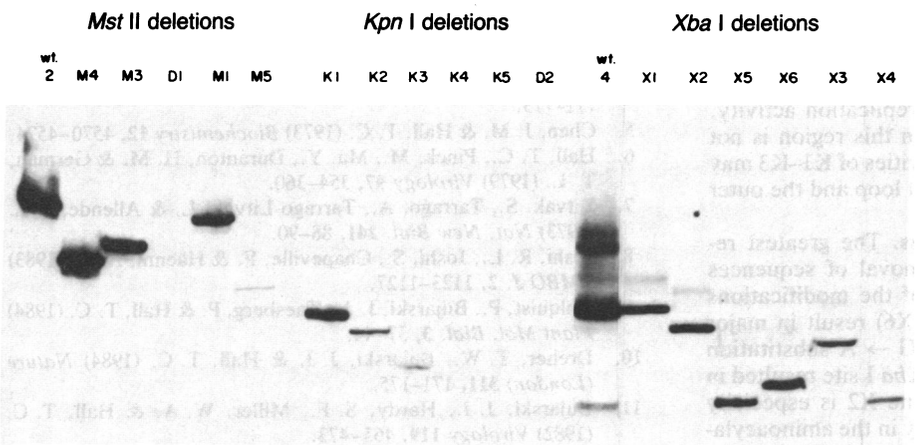

FIG. 3. Double-stranded replication products of transcribed RNAs. Autoradiogram after electrophoresis on a 6% polyacrylamide/7 M urea denaturing gel of [α-^{32}P]UTP-labeled double-stranded products of replication of transcripts with deletions (indicated on top). Equimolar amounts of transcript RNA (equivalent to 0.5 μg of the wild-type control transcripts wt.2 and wt.4) were used as templates for BMV replicase.

these modifications. Apparently, mismatches in the region 5′ to nucleotide 124 and the resultant disturbance of the postulated tRNA-like structure (Fig. 1) were not as disruptive for replicase template activity as they were for tyrosylation.

DISCUSSION

The deletions described above have resulted in a wide spectrum of altered aminoacylation and replication activities. Significant portions of the tRNA-like structure can be deleted without total loss of biological activity. The deletions of greatest interest are those selectively affecting one of the two activities studied; as with all of the modifications made thus far (10), investigation of the effects on other infection properties such as virulence, lesion phenotype, and host range awaits their insertion into clones permitting the transcription of full-length viral RNAs (26).

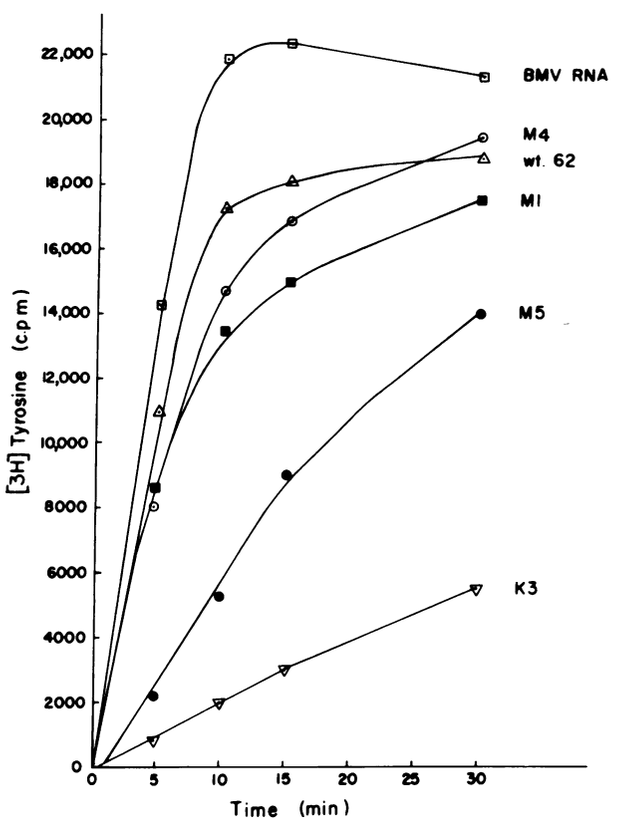

FIG. 4. Effects of deletions on the rate of aminoacylation of synthetic 3′-terminal BMV RNA fragments. Equimolar amounts of synthetic RNAs (equivalent to 0.5 μg of the wild-type wt. 62 control RNA) were aminoacylated, and levels of incorporated [^{3}H]tyrosine were determined.

Enhancement of Replicase Template Activity. One of the more surprising results was the 2-fold enhancement of replicase template activity of transcript M4, without decrease in aminoacylation activity (Table 1). Indeed, various deletions removing sequences from arm D (M2–M4) and hence making the 3′ region of BMV RNA structurally analogous to the RNAs from broad bean mottle virus (BBMV) had similar activities. Despite lacking arm D, which is conserved in the RNAs of all other bromo- and cucumoviruses (14, 27), the RNAs of BBMV are tyrosylated very efficiently (13).

Transcript M1, the smallest M deletion, is atypical in this group, since the replication activity is lower than that of the wild type. This may reflect the formation of a new stem and loop, which involves the 104-UUCG-101 sequence in base pairing (data not shown). This sequence is present and is single stranded in the native and M2–M4 RNAs. It corresponds to the TΨCG(A) which is conserved in cellular tRNAs (28), and the predicted locations of these bases in three-dimensional models are analogous among tRNA and the tRNA-like structures of bromo-, cucumo- and tobamoviruses (29). The postulated interaction of these tRNA bases with 5S ribosomal RNA may not be as important during translation as previously thought (30), but the sequence is part of the consensus promoter sequence for genes transcribed by RNA polymerase III (31). The authors of ref. 31 suggest that the DNA of tRNA genes form intrastrand stems and loops reminiscent of tRNA folding patterns and that key regions are recognized by a transcription factor. Since it is conceivable that BMV replicase has evolved from a cellular RNA polymerase, it would be of interest to determine whether BMV replicase recognizes the -UUCG- locus in its interaction with BMV RNA templates. Because the only deletion studied here (M5) in which UUCG was absent also lacked part of the anticodon loop, no conclusions can be drawn on this question. However, the aminoacylation activity of M5 indicates that the sequence UUCG is not obligatory for aminoacylation. The same is true for the entire arm D.

Reduction or Elimination of Replicase Template Activity. Deletions in two regions have resulted in severe loss of replicase template activity. All deletions that completely or partially removed sequences responsible for forming arm C (transcripts D1, D2, K4, K5, and M5) showed very low template activity. In contrast, removal of arm C (transcripts D1 and D2) had a relatively minor effect on the aminoacylation activity. These observations extend our earlier results (10) indicating that base substitutions in this loop, which cause loss of template activity, leave the aminoacylation rate unaffected. It is clear that the anticodon region is very important in the recognition of BMV RNA by the replicase.

Deletions surrounding the *Kpn* I site (K1–K3) also severely reduce replicase template activity. In contrast, deletions in the other strand involved in forming the basal part of arm B (X deletions) have much less effect on replication activity. Apparently an intact base-paired helix in this region is not necessary, and the reduced template activities of K1–K3 may be due to the inability to form the normal loop and the outer part of arm B.

Reduction in Aminoacylation Functions. The greatest reduction in aminoacylation involved removal of sequences from arm B (K and X deletions). All of the modifications surrounding the *Xba* I site (clones X1–X6) result in major decreases in aminoacylation. Even the G → A substitution introduced at position 132 to create the *Xba* I site resulted in a 49% decrease in aminoacylation. Clone X2 is especially interesting in that it has very poor activity in the aminoacylation reaction (8% of the wild type) while retaining 70% of the replicase template function. This observation suggests that discrimination between these functions *in vitro* can be obtained, and it will be of importance to determine the infectivity of full-length transcripts of clones containing deletions and replacements in this region. These data, which show great sensitivity of the aminoacylation activity to changes in the region around bases 130–131, correlate well with experiments showing that the shortest 3′ fragments of BMV RNA capable of aminoacylation are 134 nucleotides long (8).

The results reported here have emphasized our previous discovery that the so-called anticodon arm of BMV RNA is vitally involved in replicase template recognition. By analogy with phage Qβ replicase (32), this recognition may involve the binding of a tRNA-specific host factor in assembling an active replicase complex. Our construction of BMV RNA variants incapable of being aminoacylated, but yet able to act as efficient replicase templates *in vitro*, means that tyrosyl-tRNA synthetase cannot be filling such a role. However, we do not believe that this precludes a role for aminoacylation during an infection. The BMV replicase preparation we employ has the qualitative properties one would expect of a viral replicase [template specificity and dependency, and the ability to utilize both (+) and (−) strands as template (see ref. 33)], but the enzyme activity observed *in vitro* is far lower than must be the case *in vivo*. It is thus possible that aminoacylation has a role in modulating replicase template activity, which we are not in a position to study currently. Further studies are required to identify a host factor whose interaction with BMV RNA facilitates replication.

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