Volume 14 Number 21 1986 Nucleic Acids Research

In vitro mutagenesis of the putative replicase genes of tobacco mosaic virus

Masayuki Ishikawa*, Tetsuo Meshi, Fusao Motoyoshi+, Nobuhiko Takamatsu and Yoshimi Okada

Department of Biophysics and Biochemistry, Faculty of Science, University of Tokyo, Tokyo 113 and +National Institute of Agrobiological Resources, Tsukuba Science City, Ibaraki 305, Japan

Received 5 September 1986; Accepted 6 October 1986

ABSTRACT

We have established an in vitro transcription system to produce infectious tobacco mosaic virus (TMV) RNA from a cloned cDNA copy. Using this system, several TMV mutants were transcribed in vitro from cDNA clones mutagenized at or near the leaky amber termination codon of the 130K protein gene, and their infectivity was assayed on tobacco plants. Three (two frame-shift and one non-sense) mutants with an intact 130K but a defective 180K protein gene were not infectious, while two mutants with a one-amino-acid insertion in the 180K protein gene were infectious. When the amber codon of the 130K protein gene was deleted, infectivity was lost. However, when the amber termination codon was replaced with ochre or tyrosine codon, infectivity was retained. Sequence analyses revealed that introduced mutations were retained in progeny viral sequences except in the progeny of the amber-to-tyrosine mutant, which was a mixture of the parental mutagenized virus and a pseudo-revertant with ochre codon.

INTRODUCTION

Tobacco mosaic virus (TMV) is a plant virus whose genome is a messenger-sense, single-stranded RNA of about 6,400 nucleotides (1). The complete nucleotide sequences of the genomic RNAs of common strain vulgare (2) and tomato strain L (3,4) have been determined and it has been proved that the genomic RNA encodes three non-structural proteins (130K, 180K, 30K proteins) and the coat protein. The 130K and 180K proteins have a common N-terminus (5). The latter is synthesized by read-through over an amber termination codon of the 130K protein gene (5), incorporating tyrosine (6). The non-structural proteins are actually synthesized in vivo (7,8,9) but their functions are not yet fully resolved at the molecular level.

The 130K and 180K proteins are suggested to be involved in viral RNA replication, from the observations that they are the only proteins translated from the genomic RNA directly (10) and have considerable amino acid sequence homology both to other viral RNA dependent polymerases (11)

and to certain proteins needed for viral RNA replication (12). To understand the functions of the 130K and 180K proteins (putative TMV replicase or its subunits), more precisely and directly, a genetic approach is thought to be very useful. Recently, we succeeded in constructing an experimental system of *in vitro* transcription by which infectious TMV RNA can be regenerated from a full-length cDNA clone (13). It has become possible to introduce desired mutations at any site of the TMV sequence at the cDNA level by ordinary recombinant DNA techniques, and to obtain a homogeneous preparation of TMV RNA with the introduced mutations by *in vitro* transcription.

In this paper, we introduce several kinds of mutation on TMV RNA at or near the amber termination codon of the 130K protein gene, and reveal basic characters of these artificially constructed mutants by testing their infectivity on tobacco plants and by analyzing their progenies.

MATERIALS AND METHODS

Enzymes

Restriction endonucleases were purchased from Takara Shuzo Co., Nippon Gene and Toyobo Co. *Escherichia coli* DNA polymerase I (Klenow fragment), *E. coli* alkaline phosphatase, T4 DNA ligase, T4 polynucleotide kinase and reverse transcriptase (RAV-2) were from Takara Shuzo Co. *E. coli* RNA polymerase was purified from *E. coli* A19 by the method of Burgess and Jendrisak (14) with slight modification.

Construction of mutant TMV cDNA clones

The plasmid pLFW3 (Fig. 1(a)) carries full-length double-stranded cDNA of TMV L (tomato strain) just downstream of the Pm promoter (15,16) and has a unique MluI site at the 3' terminus of the TMV sequence; infectious TMV RNA is transcribed *in vitro* from MluI-cut pLFW3 (13). We constructed various mutants of TMV cDNA from the pLFW3 by site-directed mutagenesis.

(i) Construction of pLFR0, pLFR1 and pLFR2 These plasmids were constructed by oligonucleotide-directed mutagenesis; an outline of the strategy is shown in Fig. 1(b). The 492 bp SalI fragment of pLFW3 (residues 2941-3432 (3)) was cloned into the SalI site of M13mp18. The recombinant phage carrying TMV-plus strand sequence on its viral strand was called mL1P. The genomic DNA of mL1P (40 pmole) was annealed with 10-fold molar excess of 5'-phosphorylated oligonucleotide (dCCTGTAATTGT-TATTGAGTACC for mLR0, dCCTGTAATTGTTGAGTACCTGC for mLR1, and dCCTGTAATTG-

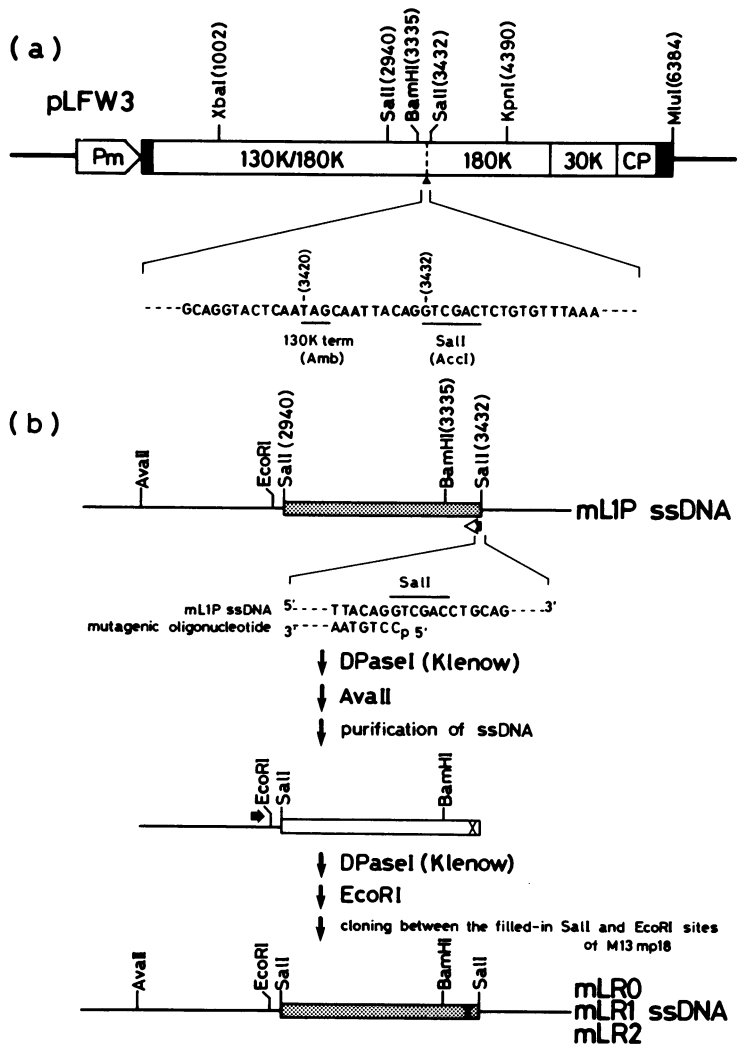

Fig. 1 (a) The structure of pLFW3 and the sequence of TMV-L RNA around the amber termination codon of the 130K protein gene. Numbers in parentheses indicate the residues of the TMV-L sequence. Locations of restriction endonuclease recognition sites are expressed as the residue numbers of the first bases within the recognition sequences.
(b) Schematic representation of oligonucleotide directed mutagenesis of the amber termination codon. Numbers in parentheses refer to the residues of the TMV-L sequence (3). The shadowed and open boxes show the TMV plus and minus sequences, respectively. Lines show the M13mp18 sequence. Open and solid arrows show the synthetic oligonucleotides and M13 reverse sequence primer, respectively. Cross (X) indicates the mutagenized point. The details are given in the text.

ATATTGAGTACC for mLR2) in a mixture (50 μl) containing 14 mM Tris-HCl pH 7.5 at 25°C, 14 mM $MgCl_2$ and 100 mM NaCl by sequential incubation at 90°C for 3 min, at 55°C for 3 min, at 30°C for 30 min and at 0°C for 30 min. Primer directed DNA synthesis was performed in a mixture containing 7 mM Tris-HCl pH 7.5 at 25°C, 7 mM $MgCl_2$, 50 mM NaCl, 5 mM DTT, 0.2 mM each 4 dNTPs, 0.4 μM mL1P genomic DNA annealed with the oligonucleotide and 100 U/ml E. coli DNA polymerase I (Klenow fragment) at 16°C for 20-24 hr. The resulting ds-DNA was digested with AvaII, unique in mL1P (Fig. 1(b)), denatured by 0.1 N NaOH, and fractionated by 4% polyacrylamide-8.3 M urea gel electrophoresis (17). The 839 base (for mLR0 and mLR2) or 836 base (for mLR1) ss-DNA was purified from gel and annealed with 10-fold molar excess of M13 reverse sequence primer (P-L Biochemicals Inc.). The second strands were synthesized as described (13) and digested with EcoRI. The generated 0.5 Kbp DNA fragment was cloned between the filled-in SalI and EcoRI sites of M13mp18 RF DNA to create mLR0, mLR1 and mLR2 (Fig. 1(b)). Recombinant phages with the desired sequences were selected by DNA sequencing using dideoxy chain terminator (18). The 97 bp (for mLR0 and mLR2) or 94 bp (for mLR1) BamHI/SalI fragment of RF DNA corresponding to residues 3336-3432 of TMV-L RNA was ligated with three fragments derived from pLFW3 of the 2.3 kbp XbaI/BamHI (residues 1003-3335), 1.0 kbp SalI/KpnI (residues 3433-4394) and 6.5 kbp KpnI/XbaI (residues 4395-6384 + vector sequence + residues 1-1002) fragments (Fig. 1(a)) and used to transform E. coli HB101 to generate pLFR0, pLFR1 and pLFR2. The sequence of the mutagenized region of each plasmid was confirmed by the chemical method (17).

(ii) Construction of pLFR3, pLFR4, pLFR6 and pLFR7 These plasmids were constructed by manipulating the SalI (AccI) site (residue 3432) 10 nucleotides downstream of the amber termination codon (Fig. 1(a)).

Ligations of the two filled-in SalI ends and of the filled-in AccI ends resulted in 4-bp (pLFR3) and 2-bp (pLFR4) insertions, respectively. Three-bp insertion was obtained by ligating the filled-in SalI end to the filled-in AccI end (pLFR6) or inversely (pLFR7). The full-length clones were constructed by ligating four DNA fragments derived from pLFW3 of the 2.3 kbp XbaI/BamHI, 0.1 kbp BamHI/filled-in SalI or AccI, 1.0 kbp filled-in SalI or AccI/KpnI and 6.5 kbp KpnI/XbaI fragments as above. Mutations in the constructed plasmids were confirmed by dideoxy sequencing (19) with 5'-^{32}P-labeled synthetic primer, dCCAGTCTTTGGAGCTGC, complementary to residues 3468-3484 of TMV-L sequence.

(iii) Construction of pLFR5 pLta1-28 is a cDNA clone carrying the 1.6 kbp BglII/BglII fragment of TMV-Lta1 ds-cDNA (corresponding to residues 2625-4187 of L RNA) at the BamHI site of pBR322. TMV-Lta1 was isolated from TMV-L strain as a mutant which overcomes the resistance controlled by Tm-1 gene of tomato (20,21, Motoyoshi, unpublished data). pLta1-28 had a point mutation at residue 3427 (thymidine to adenine; Meshi et al., unpublished data), which is thought to be the artefact in the cDNA cloning. By this base change, the second codon after the amber codon changes from leucine codon (TTA) to ochre codon (TAA). pLFR5 was constructed by replacing the 0.1 kbp BamHI/SalI (residues 3336-3432) fragment of pLFW3 with the corresponding fragment of pLta1-28, whose sequence was identical to that of pLFW3 except residue 3427. We confirmed that pLFR5 has the expected mutation by sequencing (19).

In vitro translation

RNA transcripts synthesized in vitro from MluI-cut pLF-plasmids under the uncapped condition (13,15) were translated in a rabbit reticulocyte lysate (Amersham) in the presence of ^{35}S-methionine. We used unfractionated, uncapped transcripts as templates since they gave the same products as the purified, full-sized, capped transcripts did (our unpublished observation). Translation products were separated on SDS-8.5% polyacrylamide gel (22) and detected by fluorography(23).

Infectivity test

In vitro transcription, reconstitution and inoculation were performed as described previously (13). Nicotiana tabacum L. cv. Xanthi nc and cv. Samsun were used as local lesion and systemic hosts, respectively. Inoculum contained reconstituted transcripts derived from 3.8 μg template plasmid per ml and 17 mM Na-phosphate pH 7.0 unless otherwise specified. Sixty microliters and 50-80 μl of inoculum were used for a half leaf of local lesion host and a leaf of systemic host, respectively. Virus multiplication in Samsun tobacco leaves was monitored by both ELISA (24) and local lesion assay of leaf homogenate in 10 mM Na-phosphate buffer pH 7.0, as well as by symptom developments.

Sequence analysis of progeny virus

Virus particles were extracted from the inoculated leaves or the upper uninoculated leaves of Samsun tobacco as described (25). Viral RNA was extracted by phenol-bentonite method (26). RNA sequence was determined by dideoxy method (27) using 5'-^{32}P-labeled synthetic oligonucleotide, complementary to residue 3468-3484, as a primer.

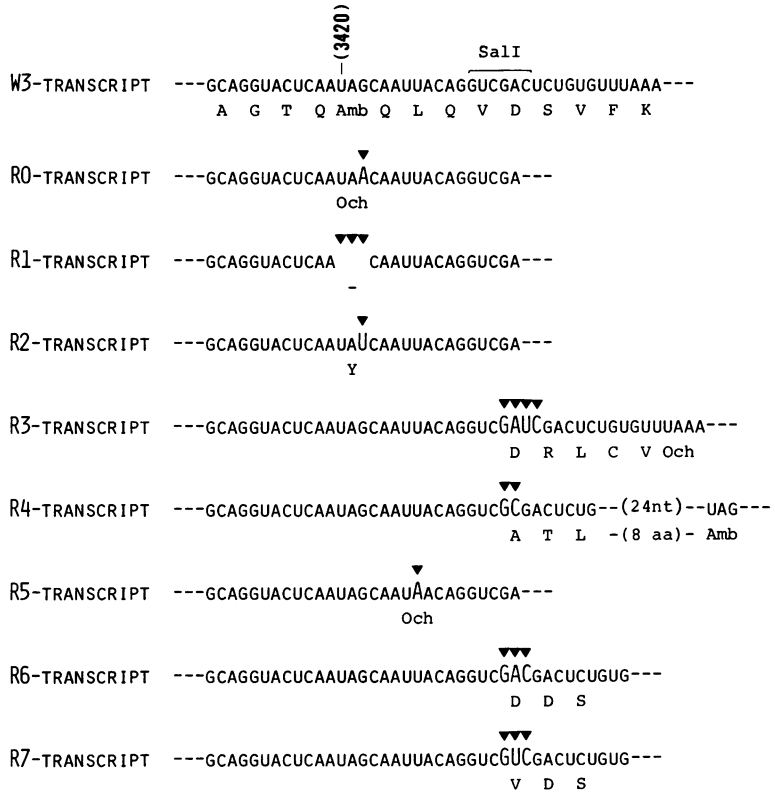

Fig. 2 The nucleotide sequences and amino acid sequences around the mutagenized region. The mutagenized bases are indicated by ▼. Only the altered amino acid sequences are shown in other than the W3 transcript. Numbers in parentheses show the residues of the TMV-L sequence.

RESULTS AND DISCUSSION

Site-directed mutagenesis of TMV cDNA

As the first step to investigate the functions of the 130K and 180K proteins of TMV, we intended to generate TMV mutants *in vitro* from which only either the 130K or 180K protein was produced. We constructed eight mutant clones by manipulating the leaky termination codon of the 130K protein gene or its vicinity. In Fig. 2 are shown the RNA sequence around the amber termination codon of the wild type virus and the corresponding regions of the constructed mutants with amino acid sequences deduced. Hereafter, *in vitro* synthesized transcript derived from a pLFRx plasmid is referred to as Rx transcript (x=0,1,2,⋯ and 7). In the same manner, W3 transcript means the *in vitro* transcript from pLFW3 (a wild type clone).

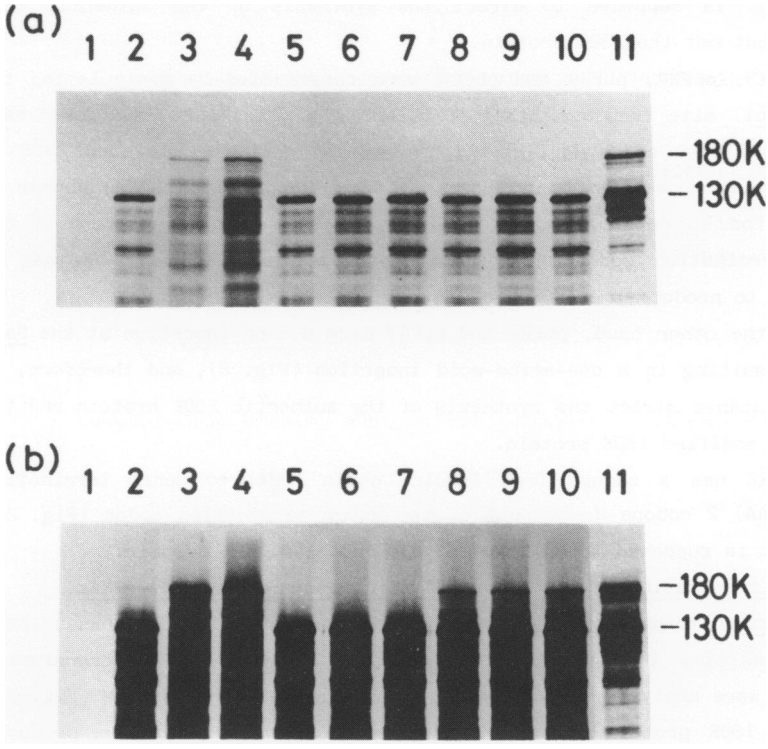

Fig. 3 Analysis of translation products in rabbit reticulocyte lysate of TMV-L genomic RNA (lane 11) and in vitro transcripts from MluI-cut pLFR0 (Lane2), pLFR1 (lane 3), pLFR2 (lane 4), pLFR3 (lane 5), pLFR4 (lane 6), pLFR5 (lane 7), pLFR6 (lane 8), pLFR7 (lane 9), pLFW3 (lane 10), and minus RNA (lane 1). Longer exposure was used for panel (b) than (a).

The term, Rx mutant, will be used for an imaged virus that carries the genomic sequence precisely reflecting the cloned cDNA in a pLFRx plasmid.

The first three mutants derived from pLFR0, pLFR1 and pLFR2 have modifications at the amber termination codon of the 130K protein gene (Fig. 2). pLFR0 has a change from amber codon (UAG) to ochre codon (UAA). Since ochre termination codon is commonly found in the other genes of the 180K, 30K and coat proteins of TMV-L (3), we first supposed that R0 mutant could not produce the 180K protein. pLFR1 has a 3-bp deletion of the amber termination codon. R1 mutant is supposed to be unable to produce the 130K protein because it lacks the termination codon, and the 180K protein synthesized has a modification of a one-amino-acid deletion. pLFR2 has a change from amber codon (UAG) to tyrosine codon (UAU), so that

R2 mutant is supposed to direct the synthesis of the authentic 180K protein but not the 130K protein.

pLFR3, pLFR4, pLFR6 and pLFR7 were constructed by manipulating the SalI (AccI) site (residue 3432) of pLFW3 (Fig. 2). pLFR3 and pLFR4 have respectively 4- and 2-bp insertions between residues 3434 and 3435 of TMV-L sequence, resulting in frame shifts. Termination codons appear 10 codons (for R3 mutant) and 16 codons (for R4 mutant) downstream of the amber termination codon of the 130K protein gene. Neither mutant is supposed to produce the 180K protein.

On the other hand, pLFR6 and pLFR7 have a 3-bp insertion at the SalI site, resulting in a one-amino-acid insertion (Fig. 2), and therefore, R6 and R7 mutants direct the synthesis of the authentic 130K protein and the slightly modified 180K protein.

pLFR5 has a change from leucine codon (UUA) to ochre termination codon (UAA) 2 codons downstream of the amber termination codon (Fig. 2). R5 mutant is supposed to be unable to produce the 180K protein.

In vitro translation of RNA transcripts from mutagenized TMV-cDNAs

In vitro transcripts from the MluI-cut pLFR plasmids (Rx transcripts) were translated in the rabbit reticulocyte lysate, and the translation products were analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 3).

The 180K protein could not be detected in the translation products from either R3, R4 or R5 transcript, while the 130K protein was synthesized (Fig. 3, lanes 5, 6 and 7). R1 and R2 transcripts directed much of the production of the 180K protein (Fig. 3, lanes 3 and 4). However, it was not clear whether any protein identical to 130K protein was synthesized by premature translation termination because of the high background around the 130K region on the gel. R6 and R7 transcripts synthesized both the 130K and 180K proteins, and the proportion of the 180K protein to the 130K protein seemed to be identical to that of the wild type transcript (Fig. 3, lanes 8, 9 and 10). Both the 130K and the 180K proteins were synthesized from R0 transcript in this *in vitro* translation system, although the proportion of the 180K protein to the 130K decreased compared with the products from the W3 transcript (Fig. 3, lanes 2 and 10). This indicates the existence of UAA suppressor tRNA in rabbit reticulocyte cells as well as UAG suppressor tRNA.

Infectivity of in vitro transcripts from pLFR clones

The infectivity of *in vitro* transcripts from pLFR plasmids was assayed on both local lesion host (*Nicotiana tabacum* L. cv. Xanthi nc) and

TABLE 1. Infectivity assay of TMV mutants constructed in vitro

No.	Plasmid	Mutation	Inocula	Day[a]	Infectivity[b]			
1.	pLFW3	wild type	virus[c]	3	102/168,	131/122,	116/141,	30/54
2.	pLFW3	wild type	RNA[d]	3	33/115,	55/40		
3.	pLFR0	Amb to Och	virus	3	130/110,	69/68,	100/94,	51/41
4.	pLFR1	Amb deletion	virus	7	0/21,	0/35,	0/39,	0/71
5.	pLFR2	Amb to Tyr	virus	3	0/86,	0/142,	0/58,	0/45
				7	166/90,	290/144,	175/65,	111/49
6.	pLFR3	frame shift	virus	7	0/68,	0/50,	0/95,	0/54
7.	pLFR3	frame shift	RNA	7	0/77,	0/63		
8.	pLFR4	frame shift	virus	7	0/98,	0/50,	0/26,	0/28
9.	pLFR5	Leu to Och	virus	7	0/135,	0/77,	0/61,	0/39
10.	pLFR5	Leu to Och	RNA	7	0/46,	0/40		
11.	pLFR6	Asp insertion	virus	3	100/156,	62/124,	42/56,	133/56
12.	pLFR7	Val insertion	virus	3	34/58,	72/32,	37/57,	133/49
13.	mock[e]	-	-	7	0/38,	0/73,	0/30,	0/25

a: Days after inoculation when the number of lesions were counted.
b: Infectivity is expressed as the ratio of the number of local lesions produced by each inoculum on one half of a leaf of Nicotiana tabacum L. cv. Xanthi nc to that produced by the standard TMV-L virion (0.03 μg/ml) in 10 mM Na-phosphate pH 7 on the other half of the leaf.
c: Virus denotes that the inoculum was reconstituted capped transcript derived from MluI-cut plasmid. The concentration of template DNA in the inoculum was 3.8 μg/ml.
d: RNA denotes that the inoculum was naked capped transcript. The concentration of template DNA in the inoculum was 180 μg/ml.
e: Buffer (10 mM Na-phosphate pH 7) inoculation.

systemic host (Nicotiana tabacum L. cv. Samsun). Viral multiplication in the systemic host was monitored by both ELISA and reinoculation of leaf homogenate on the local lesion host and also by the symptoms. We used a reconstituted transcript as inoculum unless otherwise specified and so we continue to use the term, Rx transcript, for a reconstituted transcript. If a transcript had infectivity, its progeny virus was purified from the inoculated leaves 7 days after inoculation and, in some cases, from the upper uninoculated leaves 30 days after inoculation. Progeny RNA sequence around the mutagenized region was determined by dideoxy method using 5'-^{32}P-labeled synthetic primer and reverse transcriptase. These experiments were repeated using transcripts derived from independently isolated clones, and results were confirmed.

Typical results with the local lesion host are summarized in Table 1.

(i) R6 and R7 mutants with a one-amino-acid insertion in the 180K protein R6 and R7 transcripts were infectious (Table 1, Nos. 11 and 12). Local lesions appeared 2 days after inoculation on Xanthi nc leaves, at the same interval as in the wild type (W3) transcript, although the size of local lesions derived from the R6 and R7 transcripts were smaller (data not shown). Disease symptoms of the upper uninoculated Samsun leaves caused by these mutants were typical mosaic symptoms indistinguishable from those of the W3 transcript and appeared at the same interval, 5 to 6 days after inoculation. The progenies of the R6 and R7 transcripts were purified with a similar yield to the progeny of the W3 transcript and had the same sequence as their parental transcripts around the mutagenized region as far as we sequenced (Fig. 5, panels R6-I and R7-I). These observations indicate that introduced mutations did not destroy the infectivity.

(ii) Frame shift (R3 and R4) and nonsense (R5) mutants R3, R4 and R5 transcript did not produce any local lesions up to at least 7 days after inoculation on Xanthi nc leaves (Table 1 Nos. 6, 8 and 9), nor was any viral multiplication detected in Samsun tobacco (systemic host) inoculated with these transcripts. The lack of infectivity of these transcripts was not due to their inability to uncoat, because naked transcripts (without reconstitution) were not infectious (Table 1, Nos. 7 and 10, compared with No. 2). We conclude that the 180K protein is necessary for the multiplication on tobacco plants.

(iii) Amber to ochre (RO) mutant RO transcript produced local lesions with the same characteristics as the wild type (W3) transcript on Xanthi nc leaves (Table 1, No.3), although in some assays slightly smaller lesions were observed (data not shown). Progeny virus was purified from Samsun tobacco leaves inoculated with the RO transcript with the similar yield to that of the W3 transcript. Typical mosaic symptoms could be seen in the upper uninoculated Samsun leaves 5 to 6 days after inoculation. The progeny of the RO transcript retained the introduced point mutation (Fig. 5, panel RO-I).

Considering the necessity of the 180K protein for infection, it is deduced that the ochre codon of the RO mutant can be suppressed in tobacco plants and, as a result, the active 180K protein can be produced. In fact, we detected the 180K protein in tobacco protoplasts inoculated with the progeny RNA of the RO transcript (data not shown), whose nucleotide sequence around the ochre termination codon was homogeneous (Fig. 5, panel

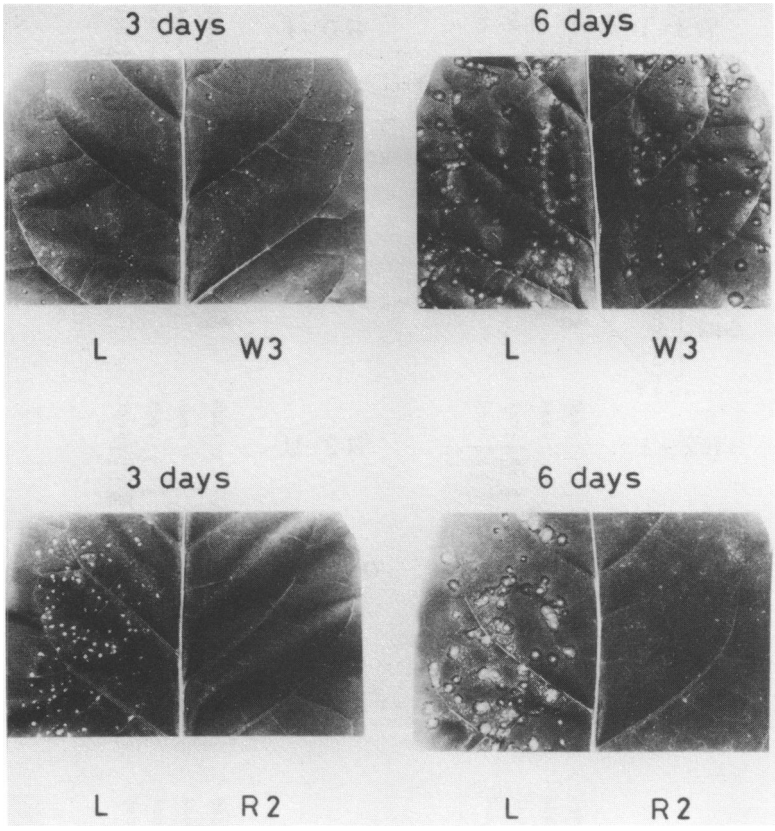

Fig. 4 Local lesions on Nicotiana tabacum L. cv. Xanthi nc leaves inoculated with TMV-L virus and reconstituted capped transcripts from MluI-cut pLFW3 and pLFR2, 3 days and 6 days after inoculation. The concentration of template DNA in inoculum was 3.8 μg/ml for the W3 and the R2 virus, and that of control TMV-L was 0.03 μg/ml.

RO-I). These findings on the RO mutant present some novel questions: which tRNA works as the suppressor?; which amino acid is incorporated at the ochre codon of the RO mutant, also tyrosine or another amino acid?; are there any read-through events over the other ochre termination codons of the 180K, 30K and coat proteins?; and so on.

(iv) Amber-to-tyrosine (R2) mutant When R2 transcript was inoculated on Xanthi nc, no local lesion was observed 3 days after inoculation, but tiny lesions appeared 4 to 6 days after inoculation (Table 1, No. 5 and Fig. 4). Progeny virus was purified at a decreased level from the inoculated Samsun leaves 7 days after inoculation (about 1/10 of the yield of the wild type (W3) progeny). Typical mosaic symptoms

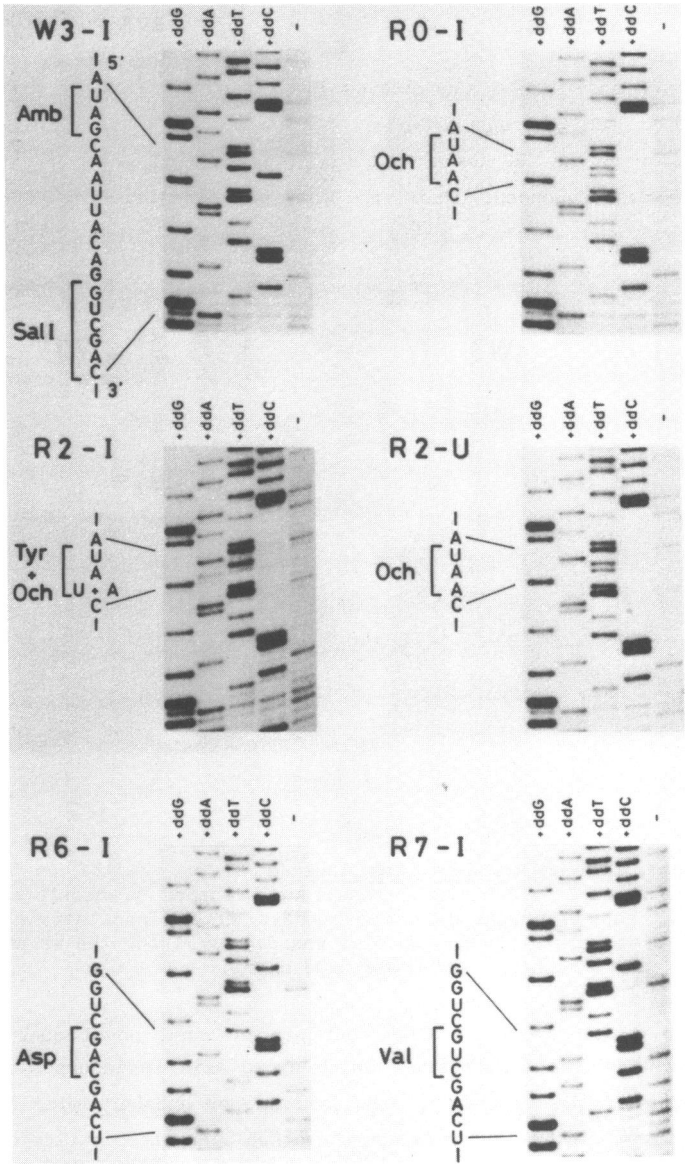

Fig. 5 Sequence around the amber termination codon of progeny of wild type (W3) transcript and corresponding regions of progenies of transcripts with mutations. Progeny viral RNA sequences were determined directly using 5'-^{32}P-labeled primer and dideoxy chain terminator. W3-I, R0-I, R2-I, R6-I, and R7-I identify the progeny purified from inoculated leaves of Nicotiana tabacum L. cv. Samsun 7 days after inoculation with W3, R0, R2, R6 and R7 transcripts, respectively. R2-U indicates the progeny from the upper uninoculated leaves of a Samsun tobacco plant inoculated with R2 transcript 30 days after inoculation.

in the upper uninoculated Samsun leaves appeared about 2 weeks after inoculation. The accumulation of the progeny virus of the R2 transcript in the upper uninoculated Samsun leaves 30 days after inoculation was the same level as in the W3 transcript.

The progeny of the R2 transcript in the inoculated Samsun leaves was a mixture of two sequences, uracil and adenine at residue 3422 (Fig. 5, panel R2-I). The former is the same as the parental (R2) transcript, corresponding to tyrosine codon (UAU); the latter corresponds to ochre termination codon (UAA). Reversion to amber codon (wild type sequence) was below the background level, if present at all (Fig. 5, panel R2-I). Progeny virus purified from the upper uninoculated Samsun leaves homogeneously had adenine at residue 3422 (Fig.5, panel R2-U), corresponding to ochre codon (UAA). The same results were obtained with the R2 transcript derived from the other clone independently isolated.

Observations of considerable numbers of local lesions and the finding of progeny virus having a parental sequence strongly suggest that the R2 transcript can replicate by itself. The 130K protein may not be essential for replication of TMV-RNA. Since the 180K protein has the whole amino acid sequence of the 130K protein in its molecule, the 180K protein might also function as the 130K protein although the activity would be very low. We cannot exclude the possibility, however, that the premature translation product(s) or the degradation product(s) of the 180K protein generated *in vivo* might have the activity of the 130K protein.

In any case, the R2 mutant propagated slowly in the inoculated Samsun leaves. Possibly, the pseudo-revertant with ochre codon appeared in the course of multiplication, propagated much faster than the R2 mutant and became exclusively detectable progeny in the upper uninoculated Samsun leaves. We do not know why the tyrosine codon changed to ochre termination codon but not to amber codon.

(v) R1 mutant with a deletion of the amber termination codon R1 transcript did not produce any local lesions on Xanthi nc leaves up to at least 7 days after inoculation (Table 1, No.4). In the leaves of Samsun tobacco inoculated with the R1 transcript, no viral multiplication was detected. One possibile explanation for the lack of infectivity is that the 180K protein with a one-amino-acid deletion produced from the R1 mutant is inactive.

We have described the basic characters of eight TMV mutants synthesized *in vitro*. Results show that the expression of both 130K and

180K proteins is necessary for normal multiplication in tobacco plants. Among the eight mutants, the amber-to-tyrosine (R2) mutant offers special interest for further analyses. The R2 mutant could multiply but only a low yield of progeny was isolated. Assuming the involvement of the 130K and 180K proteins in replication (10,11,12), it is reasonable to suspect that this is the result of the low replicability of the R2 mutant, possibly due to the unbalanced synthesis of the 130K (no or little production) and 180K (abundant production) proteins. Detailed analysis of the replication process of the R2 mutant, for example, kinetics of plus and minus strand RNA synthesis at the early stage (before the appearance of pseudo-revertant), is in progress using a protoplast system and will offer novel information on the functions of the 130K and 180K proteins.

ACKNOWLEDGEMENTS

We thank Dr. P. Ahlquist and Agrigenetics Research Associates for the use of the Pm promoter, and Drs. Y. Watanabe, T. Saito and M. Nishiguchi for their advice and discussions. This work was supported in part by Grants-in-Aid from the Ministry of Education, Science and Culture, and from the Ministry of Agriculture, Forestry and Fisheries, Japan.

*To whom correspondence should be addressed

REFERENCES

1. Hirth, L and Richards, K.E. (1981) Adv. Virus Res. **26**, 145-199.
2. Goelet, P., Lomonossoff, G.P., Butler, P.J.G., Akam, M.E., Gait, M.J. and Karn, J. (1982) Proc. Natl. Acad. Sci. USA **79**, 5818-5822.
3. Ohno, T., Aoyagi, M., Yamanashi, Y., Saito, H., Ikawa, S., Meshi, T., and Okada, Y. (1984) J. Biochem. **96**, 1915-1923.
4. Takamatsu, N., Ohno, T., Meshi, T. and Okada, Y. (1983) Nucl. Acids Res. **11**, 3767-3778.
5. Pelham, H.R.B. (1978) Nature **272**, 469-471.
6. Beier, H., Barciszewska, M., Krupp, G., Mitnacht, R. and Gross, H.J. (1984) EMBO J. **3**, 351-356.
7. Kiberstis, P.A., Pessi, A., Atherton, E., Jackson, R., Hunter, T. and Zimmern, D. (1983) FEBS Lett. **164**, 355-360.
8. Ooshika, I., Watanabe, Y., Meshi, T., Okada, Y., Igano, K., Inouye, K. and Yoshida, N. (1984) Virology **132**, 71-78.
9. Saito, T., Watanabe, Y., Meshi, T., and Okada, Y. (1986) Molec. Gen. Genet., in press.
10. Hunter, T., Hunt, T., Knowland, J. and Zimmern, D. (1976) Nature **260**, 759-764.
11. Kamer, G. and Argos, P. (1984) Nucl. Acids Res. **12**, 7269-7282.
12. Haseloff, J., Goelet, P., Zimmern, D., Ahlquist, P., Dasgupta, R. and Kaesberg, P. (1984) Proc. Natl. Acad. Sci. USA **81**, 4358-4362.
13. Meshi, T., Ishikawa, M., Motoyoshi, F., Semba, K. and Okada, Y. (1986) Proc. Natl. Acad. Sci. USA **83**, 5043-5047.
14. Burgess, R.R. and Jendrisak, J.J. (1975) Biochemistry **14**, 4634-4638.
15. Ahlquist, P. and Janda, M. (1984) Mol. Cell. Biol. **4**, 2876-2882.

16. Ahlquist, P., French, R., Janda, M. and Loesch-Fries, L.S. (1984) Proc. Natl. Acad. Sci. USA **81**, 7066-7070.
17. Maxam, A.M. and Gilbert, W. (1980) Methods Enzymol. **65**, 499-560.
18. Sanger, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA **74**, 5463-5467.
19. Zagursky, R.J., Baumeister, K., Lomax, N. and Berman, N.L. (1985) Gene Anal. Techn. **2**, 89-94.
20. Pelham, J. (1972) Annals of Applied Biology **71**, 219-228.
21. Motoyoshi, F. and Oshima, N. (1979) J. Gen. Virol. **44**, 801-806.
22. Laemmli, U.K. (1970) Nature **227**, 680-685.
23. Laskey, R.A. and Mills, A.D. (1975) Eur. J. Biochem. **56**, 335-341.
24. Clark, M.F. and Adams, A.N. (1977) J. Gen. virol. **34**, 475-483.
25. Otsuki, Y., Takebe, I., Ohno, T., Fukuda, M. and Okada, Y. (1977) Proc. Natl. Acad. Sci. USA **75**,1727-1730.
26. Frankel-Conrat, H., Singer, B. and Tsugita, A. (1961) Virology **14**, 54-58.
27. Meshi, T., Ishikawa, M., Takamatsu, N., Ohno, T. and Okada, Y. (1983) FEBS Lett. **162**, 282-285.