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**Complete nucleotide sequence of alfalfa mosaic virus RNA 2**

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**ABSTRACT**

Double-stranded cDNA of *in vitro* polyadenylated alfalfa mosaic virus (AIMV) RNA 2 has been cloned and sequenced. The use of an oligodeoxyribonucleotide corresponding to the known sequence of the 5'-end of RNA 2 to prime second-strand DNA synthesis, enabled us to construct the complete primary structure of AIMV RNA 2. The sequence of 2,593 nucleotides contains a long open reading frame for a protein of Mr 89,753 starting at the first AUG codon from the 5'-end. This coding region is flanked by a 5'-terminal sequence of 54 nucleotides and a 3'-noncoding region of 166 nucleotides which includes the sequence of 145 nucleotides the three genomic RNAs of AIMV have in common.

**INTRODUCTION**

The coat protein dependent genome of alfalfa mosaic virus (AIMV) consists of three single-stranded RNAs, designated RNAs 1, 2, and 3 in order of decreasing molecular weights (1). The coat protein is translated from a sub-genomic messenger, RNA 4 (1), the sequence of which is located at the 3'-end of RNA 3 (2). *In vitro* translation of the dicistronic RNA 3 results in a protein of Mr 35,000, while the coat protein cistron remains silent (3, 4). RNAs 1 and 2 code *in vitro* for a Mr 115,000 and a Mr 100,000 protein, respectively (3-5).

Knowledge of the primary structure of the AIMV genome might be of great value in studying the replication and expression of the RNAs, and the functions of the proteins they are coding for. Previously, we have reported information on the 5'- and 3'-terminal sequences of RNAs 1, 2, and 3 (6, 7) and the complete nucleotide sequences of RNA 1 (8) and RNA 4 (9). Here we present the primary structure of AIMV RNA 2.

**MATERIALS AND METHODS**

*Enzymes and nucleotides.* ATP:RNA adenylyltransferase was isolated from *E. coli* Q13 as described (10). DNA polymerase I (Klenow fragment) was obtain-

ed from BRL. Restriction enzymes were purchased from New England Biolabs, Boehringer and Amersham. T4 polynucleotide kinase and ( $\gamma$ - $^{32}\text{P}$ )ATP were from New England Nuclear.

*Isolation of RNA and polyadenylation.* AIMV (strain 425) was isolated and RNA 2 was purified as described previously (11). To the 3'-end of RNA 2 a poly(A) chain was attached with ATP:RNA adenytransferase by the procedure of Devos *et al.* (12).

*Synthesis and cloning of cDNA.* Double-stranded cDNA to polyadenylated RNA 2 was synthesized and cloned into pBR322 at the *Pst*I site as described for RNA 1 (8).

*DNA sequencing.* DNA of recombinant plasmids was isolated as described (8). After cutting DNA with an appropriate restriction enzyme, fragments were separated on and subsequently eluted from 5% polyacrylamide gels (13). Labeling of the DNA fragments was followed by digesting with a second restriction enzyme. Base specific cleavage reactions (G, G + A, A > C, C + T, C) on single-end labeled DNA fragments were carried out according to Maxam and Gilbert (13).

*Synthesis of the oligodeoxyribonucleotide.* The 11 bases long oligodeoxyribonucleotide, d-(GTTTTATCTT<sub>OH</sub>), corresponding to the 5'-end sequence of AIMV RNA 2 (6), was prepared by a recently developed solid-phase phosphotriester approach (14).

*Sequence determination at the 5'-end of AIMV RNA 2.* Single-stranded cDNA to polyadenylated RNA 2 was synthesized as described (8). A 40 molar excess of kinase-labeled 11-mer homologous to the 5'-end of RNA 2 was annealed to 4  $\mu\text{g}$  unfractionated cDNA in a 20  $\mu\text{l}$  reaction mixture, containing 6.7 mM Tris-HCl pH 7.5, 6.7 mM  $\text{MgCl}_2$ , 6.7 mM  $\beta$ -mercaptoethanol and 50 mM NaCl, by heating the mixture for 3 min at 100°C and cooling down quickly on ice. After incubation for 15 min at 15°C, the primer was extended with DNA polymerase I (Klenow fragment). The reaction was carried out in 6.7 mM Tris-HCl pH 7.5, 6.7 mM  $\text{MgCl}_2$ , 6.7 mM  $\beta$ -mercaptoethanol, 50 mM NaCl and 1 mM dNTPs using 15 units DNA polymerase I. Incubation was for 3 hr at 15°C in a total volume of 80  $\mu\text{l}$ . The reaction was stopped by two successive 1 : 1 phenol/chloroform extractions. Upon fractionation on a Sephadex G-50 column, the DNA was digested with the endonuclease *Taq*I and sized on a 5% polyacrylamide gel. Autoradiography showed a single band which was eluted and sequenced according to Maxam and Gilbert (13).

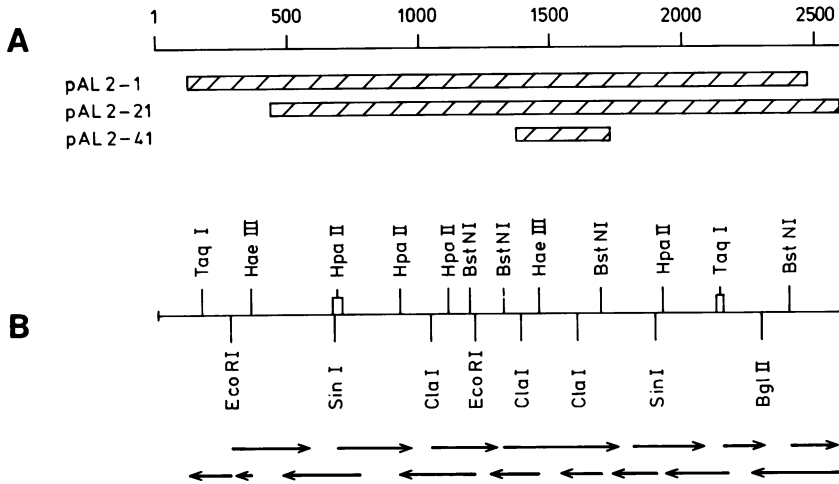


Figure 1. (A) Alignment of inserts of hybrid plasmids that were used to construct the complete nucleotide sequence of AIMV RNA 2. (B) Restriction endonuclease map and strategy used to sequence the DNA of the inserts. Only the position of three out of the sixteen *TaqI* sites is given.

## RESULTS AND DISCUSSION

### *Construction of the sequence*

Previously, we have reported the sequences of the 5'-terminal 13 nucleotides and the 3'-terminal 169 nucleotides of AIMV RNA 2 (6, 7). In order to complete our sequence studies on this RNA, we cloned (nearly) full length double-stranded cDNA copies of RNA 2 into the *Pst*I site of pBR322 vector DNA by the dC/dG-tailing technique. Analysis of plasmids from tetracycline-resistant clones revealed only two hybrid plasmids with inserts longer than 2,000 bp, notably pAL2-1 and pAL2-21. Although the ds cDNA was selected on size before tailing and cloning, the inserts of all the other hybrid plasmids were much shorter than expected.

Figure 1A shows an alignment of inserts from hybrid plasmids we used in our studies. Figure 1B shows a restriction map of these inserts and the strategy, used to sequence the DNA by the method of Maxam and Gilbert. Plasmid pAL2-21 appeared to contain the 3'-terminal sequence of AIMV RNA 2, except for the last six bases; pAL2-1 lacked the last 120 residues of the 3'-terminus.

In 30 clones investigated the 5'-terminal sequence of RNA 2 was not present. To deduce this sequence, we took advantage of our knowledge of the primary structure at the 3'-end. An oligodeoxyribonucleotide corresponding to the 5'-terminal 11 nucleotides of RNA 2 was synthesized and used to prime DNA syn-

thesis on cDNA, transcribed from polyadenylated RNA 2. After synthesis of the second strand, primed by the kinase labeled 11-mer, double-stranded cDNA was cut by the endonuclease *TaqI* and electrophoresed on a 5% polyacrylamide gel. As expected, only one band was visible after autoradiography. Analysis of this DNA fragment by the Maxam and Gilbert technique revealed the sequence of the 5'-terminal 170 nucleotides of RNA 2. The last 50 bases of this DNA fragment were found to overlap with the terminal sequence of the insert of pAL2-1. This allowed us to construct the complete primary structure of AIMV RNA 2.

Corresponding sequences obtained from different DNA inserts were in complete agreement with each other except in one case. Nucleotide 1,431 was read as C in clone pAL2-1, whereas a T residue was found in this position in pAL2-21 and in a third clone, pAL2-41. This base conversion, however, does not lead to a change in the amino acid sequence of the protein encoded by the long open reading frame in RNA 2 (see below). Sequence variability is also observed in AIMV RNA 1 (8) and in other plant viral RNAs like tobacco mosaic virus RNA (15) and cucumber mosaic virus RNA 3 (16). It is not known whether those divergencies reflect errors made during the reverse transcription of those RNAs or sequence heterogeneities in the RNA populations used.

### *Primary structure of RNA 2*

Figure 2 gives the complete sequence of 2,593 nucleotides of AIMV RNA 2. The first AUG codon from the 5'-end (nucleotides 55 to 57) is the beginning of a long open reading frame of 2,370 nucleotides encoding a Mr 89,753 protein of 790 amino acids. This amino acid sequence is also given in figure 2. The open reading frame terminates some 20 nucleotides ahead of the 3'-terminal sequence of 145 nucleotides that is homologous in all four AIMV RNAs (7).

The Mr 89,753 protein probably corresponds to the Mr 100,000 protein that is obtained by *in vitro* translation of RNA 2 (3, 4). The observation that in protoplasts RNAs 1 and 2 can replicate independently of RNA 3 (17) indicates that the Mr 125,685 protein encoded by RNA 1 (8) and the Mr 89,753 protein encoded by RNA 2 are both involved in viral RNA synthesis. From the amino acid compositions of these proteins it can be calculated that at pH 7.0 the Mr 125,685 protein is slightly basic (charge + 22) whereas the Mr 89,753 protein is acidic (charge - 24). Inspection of the distribution of basic, acidic, polar and hydrophobic amino acids in the two proteins does not reveal notable features, except for a clustering of basic amino acids at the C-terminal region of the Mr 89,753 protein (11 basic amino acids in the C-terminal 21 residues). Possibly, this region interacts with viral RNA during replication.

In RNAs 1 and 3 of our AIMV strain 425 the 5'-proximal AUG codon is

1  
m7GpppGUUUUUUUCUUUUCCGGAUUGAAAGAUAGUUUUUCAGUUUUUUUUUCAAU

55 Met Phe Thr Leu Leu Arg Cys Leu Gly Phe Gly Val Asn Glu Pro Thr Asn Thr Ser Ser Ser Glu Tyr Val Pro Glu Tyr Ser Val Glu  
AUG UUC ACU CUU UUG AGA UGU CUC GUA UUC GGU GUU AAU GAA CCU ACU AAC ACU UCC UCA UCA GAG UAU GUU CCC GAG UAU UCC GUU GAA  
149  
Glu Ile Ser Asn Gaa Val Ala Glu Glu Leu Asp Ser Uca Asp Gaa Pro Leu Phe Gln Cys Tyr Lys His Val Phe Val Ser Leu Met Leu Val Arg  
GAG AUU UCC ACC GAA GGC GCU GAA CUC UUC UUC UCA UUA UUC CAA UGU UAC AAA CAU GUU UUU GUA UCA UUA UUG UCC GUA UCC GUA AGA  
235 Lys Met Thr Gln Ala Ala Glu Asp Phe Leu Glu Ser Phe Gly Gly Glu Phe Asp Ser Pro Cys Cys Arg Val Tyr Arg Leu Tyr Arg His  
AAG AUG ACU CAA GCU GCC GAA GAC UUC CUC GAG AGU UUU GGG GGA GAA UUC GAU AGC CCU UGU UGU AGG GUU UAC CGU UUA UUA AGA CUA  
325 Phe Val Asn Glu Asp Asp Ala Pro Ala Trp Ala Ile Pro Asn Val Val Asn Glu Asp Ser Tyr Asp Asp Tyr Ala Tyr Leu Arg Glu Glu  
UUU GUU AAU GAA GAC GAU GCA CCC GCU UGG GCC AUA CCG AAU GUC GUG AAU GAA GAU UCU UAC GAG GAU UAU GCC UAC CUC CGA GAG GAG  
415 Leu Asp Ala Ile Asp Ser Ser Phe Glu Leu Leu Asn Glu Glu Arg Glu Leu Ser Glu Ile Thr Asp Arg Leu Asn Ala Leu Arg Phe Phe  
UUA GAU GCC AUA GAC AGC UCU UUU GAG UUG CUA AAC GAA GAG CGU GAG UUA UCG GAA AUU ACG GAC AGA CUC AAC GCU UUA AGA UUU UUC  
505 Pro Val Ser Lys Thr Glu Ala Leu Pro Val Ala Asn Val Gln Glu Val Lys Leu Ile Ser Glu Thr Tyr Gln Leu Met Thr Phe Ile  
CCU GUU UCU AAA ACA GAA GCG CUA CCA GUG GCG AAU GUC CAA GAG GUC AAA CUC AUU UCU GAG ACA UAC CAG UUA UUG AUG ACC UUU AUU  
595 Asn Tyr Ser Asp Glu Asn Ile Pro Ser Glu Met Pro Ala Pro Leu Leu Asp Glu Leu Gly Met Leu Pro Glu Glu Leu Gly Pro Leu Asn  
AAC UAC UCU GAC GAG AAU AUU CCG UCU GAA AUG CCC GCA CCA UUA CUG GAU GAG UUG GGG UUA CCG GAG GAA UUC GGA CUC GUU Asn  
685 Glu Ile Glu Asp Ile Lys Pro Val Ala Glu Pro Ile Thr Leu Leu Ser Glu Phe Arg Ala Ser Asp Asn Ala Lys Pro Leu Asp Ile Val  
GAA AUG GAA GAC AAU AAG CCG GUG ACG GCG CCA ACA UCU UUA UCU GAG UUU AGA GGC CCA UUA GCU AAG CCA CUC GAC AUA GUC  
775 Glu Ile Ile Pro Asp Val Ser Pro Thr Lys Pro Tyr Glu Ala Val Ile Ser Gly Asn Asp Trp Met Thr Leu Gly Arg Ile Ile Pro Thr  
GAA AUG AUU CCA GAC GUA AGU CCG ACC GAA CCU UAU GAA GCC GUC AUA UCA GGU AAU GAU UGG AUG ACG UUG GGG AGG AUU ACA UCU ACC  
865 Thr Pro Val Pro Thr Arg Asp Val Phe Ser Gly Phe Ser Arg His Gly Ser Pro Asp Phe Ile Ser Glu Val Ile Gln Asn Ala Leu Asp Glu  
ACU CCC GUU CCU ACC AUA AGG GAU GUC UUC UCU UCU GGU CUU UCU CGG CAC GGA UCG CCG GAA GUG AUC CAU AAU GCU CUU GAU GAA UUU  
955 Leu Pro Leu His His Ser Ile Asp Asp Lys Tyr Phe Gln Glu Trp Val Glu Thr Ser Asp Lys Ser Leu Asp Val Asp Pro Lys Arg Ile  
CUU CCG CUC CAU CAU UCA AUU GAU GAU AAG UAU UUU CAA GAA UGG GUU GAA ACC UCA GAU AAA UCU CUC GAU GUC GAU CCA UGU CUA AUG  
1045 Asp Leu Ser Val Phe Asn Asn Trp Gln Ser Ser Glu Asn Cys Tyr Glu Pro Arg Phe Lys Thr Gly Ala Leu Ser Thr Arg Lys Gly Thr  
GAU CUG AGU GUU UAC AAC ACN Trp Gln Ser UCU UCG GAA AAC UGC UAU GAA CCU CGG UUU AAA ACC GGU GCA UUA UCC ACA CGU AAG CCG ACU  
1135 Gln Thr Glu Ala Leu Leu Ala Ile Lys Lys Arg Asn Met Asn Val Pro Asn Leu Gly Gln Ile Tyr Asp Val Asn Ser Val Ala Asn Ser  
CAA ACU GAA GCC CUA UUA GCG AUA AAG AAA CCU AAU AUG AAU GUG CCU AAC CUG GGG CAG AAU UAU GAC GUG AAU UCU GUU GCU AAU UCC  
1225 Val Val Asn Lys Leu Thr Thr Val Ile Asp Pro Asp Lys Leu Cys Met Phe Pro Asp Phe Ile Ser Glu Glu Val Ser Tyr Phe  
GUG GUU AAU AAG CUC UUA ACA ACU AUU GAU GAA CCU AAU AAG CUG UGC AUG UUU CCA GAU UUC AUA UCU GAG GGU GAA GUU UCG UAU UUC  
1315 Gln Asp Tyr Ile Val Gly Lys Asn Pro Asp Pro Glu Leu Tyr Ser Asp Pro Leu Gly Val Arg Leu Thr Ser Lys Thr Asn Ala His Arg Lys Ser  
CAG GAC UAU AUA GUU GGG AAG AAU CCC GAC CCU GAA UUA UAU UCA GAU CCU CUA GGU GUU CGU UCC AUG GCU AGC UAU AAA CAC MET Ile  
1405 Lys Ser Val Leu Lys Pro Val Glu Asp Asn Ser Leu His Leu Glu Arg Pro Met Pro Ala Thr Ile Thr Tyr His Asp Lys Asp Ile Val  
AAA AUG GUA UUA AAG CCC GUU GAA GAU AAU UCU CUG CAC CUA GAA CCG CCG AUG CCA GCA ACC AUC ACA UAC CAU GAU AAA GAU AUG GUG  
1495 Met Ser Ser Ser Pro Ile Phe Ule Ala Ala Ala Arg Leu Met Leu Ile Leu Arg Asp Lys Ile Thr Ile Pro Ser Gly Lys Phe His  
AUG UCA UCU UCA CCA AAU UUU UUG GCU GCU GCG GCC UUG AUG UUA UUA AGA GAU AAG AUA ACC AUA CCA AGC GUA AAA UUC LAU  
1585 Gln Leu Phe Ser Ile Asp Ala Glu Ala Phe Asp Ala Ser Phe His Phe Lys Glu Ile Asp Phe Ser Lys Phe Asp Lys Ser Gln Asn Glu  
CAA UUG UUU UCC AUC GAU GCU GAA GCC UUU GAU GCA AGU UUC CAU UUU AAA GAG AUA GAC UUU UCG AAG UUU GAC AAA AGU CAA AAU GAU  
1675 Leu His His Leu Ile Gln Glu Arg Phe Leu Thr Val Ile Asp Pro Asp Lys Tyr Leu Gly Ile Pro Asn Glu Phe Leu Thr Leu Trp Thr Asn Ala His Arg Lys Ser  
UUG CAU CAC UUG AUC CAG GAA AGG UUU CUG AAA UAC UUA GGU AUA CCC AAC GAA UUU CUA ACC UUA UGG UUU AAU GGC CAU AGA AAA UCC  
1765 Arg Ile Ser Asp Ser Lys Asn Gly Val Phe Phe Asn Val Asp Phe Gln Arg Arg Thr Gly Asp Ala Leu Thr Tyr Leu Gly Asn Thr Ile  
CGA AUC UCA GAU UCG AAG AAC GGC GUU UUU UUU AUC GUC CAU UUC CAA CGU ACU GGA GAU GCU CUC ACU UUG GAA AAC ACA AAU  
1855 Val Thr Leu Ala Cys Leu Cys His Val Tyr Asp Leu Met Asp Pro Asn Val Lys Phe Val Val Ala Ser Gly Asp Asp Ser Leu Ile Gly  
GUG ACA UUA GCU UGU CUG UGU GAC GUG UAU GAC UUG AUG GAC CCA AAU GUG AAA UUC GUU GUU GCU UCC GGU GAU UCA UUG AUA GGC  
1945 Thr Val Glu Glu Leu Pro Arg Asp Gln Glu Phe Leu Phe Thr Thr Leu Phe Asn Leu Glu Ala Lys Phe Pro His Asn Gln Pro Phe Ile  
ACU GUG GAG GAA UUA CCA AGA GAU CAA GAG UUU CUU UUC ACG ACU CUU UUU AAU CUU GAA GCA AAG UUU CCU CAU AAC CAG CCU UUC AUA  
2035 Cys Ser Lys Phe Leu Ile Thr Met Pro Thr Thr Ser Gly Gly Lys Val Val Leu Pro Ile Pro Asn Pro Leu Lys Leu Leu Ile Arg Leu  
UGC AGU AAG UUU UUG AUU ACU AUG CCC ACU ACA AGU GGA GGC AAA GUU GUC CUG CCG AUA CCG AAU CCA UUG AAA CUC CUC AUA CGC UUU  
2125 Gly Ser Lys Lys Val Asn Ala Asp Ile Phe Asp Glu Trp Tyr Gln Ser Trp Ile Asp Ile Ile Gly Gly Phe Asn Asp His His Val Ile  
GGU UCG AAG AAA CUC AAU GCC GAU AUA UUC GAU GAA UGG UAU CAA UCU UGG AUU GAU AUA AUU GGU GGU UUU AAC GAC CAC CAU GUC AUC  
2215 Arg Cys Val Ala Ala Met Thr Ala His Arg Tyr Leu Arg Arg Pro Ser Leu Tyr Leu Glu Ala Ala Leu Glu Ser Leu Gly Lys Ile Phe  
CGA UGC GUU GCC GCG AUG ACA GCA CAU AGG UAU CUC AGA AGA CCG AGU UUA UAC CUA GAA GCU UUG GAA UCC CUA GGU AAG AUC UUC  
2305 Ala Gly Lys Thr Leu Cys Lys Glu Cys Leu Phe Asn Glu Lys His Glu Ser Asn Val Lys Ile Lys Pro Arg Arg Val Lys Lys Ser His  
GCU CGU AAG ACC UUG UGU AAG GAA GGC CUC UUU AAU GAG AAG CAC GAG UCU AAU GUA AAA AUA AAG CCU CGU AGA GUG AUA AAA UCL CAC  
2395 Ser Asp Ala Arg Ser Arg Ala Arg Arg Ala \*\*\*  
UCG GAU GCC AGG UCA AGG GCA CGC GCA GCU UGA UGUUUUCUGACUAAGUCAAAUUGCCAAACCCUCCACUGGGUGGUCAGGUGUAGGUUAUGAAUCCUAUUCUCUC  
2503 CUGAUGGAGAAAUUCUAUUGUCUAUUAUUGUCUUCACGCCACAUAUAUUAUUGUCUAUGCCAAACUGCAUGAAGUCCCUAAGGUAUGC

Figure 2. Nucleotide sequence of A1MV RNA 2 (strain 425) and amino acid sequence corresponding to the long open reading frame.

closely followed by a termination codon (8, 6), whereas in RNA 2 and RNA 4 (9) the first AUG codon from the 5'-end is the beginning of a long open reading frame. In addition to the coding region for the Mr 89,753 protein, RNA 2

Table 1. Base composition of AIMV RNAs 1, 2, and 4. Base composition of RNAs 1 and 4 are taken from (8, 9).

	RNA 1	RNA 2	RNA 4
A	28.5%	28.4%	24.4%
U	29.0%	30.0%	27.7%
C	20.4%	20.5%	24.1%
G	22.1%	21.1%	23.8%

contains several open reading frames of 100 to 150 bases. The longest frame starting with an AUG triplet encodes a sequence of 42 amino acids (nucleotides 92 to 223). The 5'-proximal AUG codon in minus-strand RNA 2 is followed by a reading frame for 60 amino acids (nucleotides 2,548 to 2,366 of the plus-strand). The longest open frames in minus-strand RNA 2 encode potential polypeptides of 91 amino acids (nucleotides 2,441 to 2,166) and 94 amino acids (nucleotides 2,341 to 1,957). When sequences of other AIMV strains become available, it will be interesting to see if any of these reading frames is conserved.

Table 1 shows a comparison of the base compositions of AIMV RNAs 1, 2, and 4. RNAs 1 and 2 are notably rich in U and A residues whereas in RNA 4 only the percentage of U residues is above average. The observation that leader sequences of plant virus RNAs are rich in U (8, 18) can account only

Table 2. Codon utilization of the Mr 89,753 protein encoded by AIMV RNA 2. The frequency of use of each codon is indicated.

Phe	UUU 28		UCU 17		Tyr	UAU 15		Cys	UGU 8
	UUC 19	Ser	UCC 12			UAC 10			UGC 5
Leu	UUA 20		UCA 14		End	UAA 0		End	UGA 1
	UUG 19		UCG 8			UAG 0		Trp	UGG 7
	CUU 9		CCU 15		His	CAU 12			CGU 8
Leu	CUC 16	Pro	CCC 8			CAC 8		Arg	CGC 3
	CUA 9		CCA 14		Gln	GAA 10			CGA 5
	CUG 9		CCG 11			CAG 7			CGG 3
	AUU 16		ACU 13		Asn	AAU 26		Ser	AGU 8
Ile	AUC 13	Thr	ACC 9			AAC 14			AGC 4
	AUA 22		ACA 10		Lys	AAA 22		Arg	AGA 12
Met	AUG 16		ACG 5			AAG 21			AGG 7
	GUU 22		GCU 18		Asp	GAU 39			GGU 13
Val	GUC 12	Ala	GCC 12			GAC 17		Gly	GGC 4
	GUA 4		GCA 8		Glu	GAA 37			GGA 8
	GUG 15		GCG 7			GAG 22			GGG 5

in part for this phenomenon. Probably, the base composition of RNA 2 has some effect on the codon usage. Table 2 shows that in the codon families used in the cistron for the Mr 89,753 protein, in many cases codons with a U or A in the third position are preferred over those with C or G. Whether this phenomenon has any functions either in translation or in replication of viral RNA remains to be demonstrated.

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