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Complete nucleotide sequences of the coat protein messenger RNAs of brome mosaic virus and cowpea chlorotic mottle virus

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ABSTRACT

The nucleotide sequences of the subgenomic coat protein messengers (RNA4's) of two related bromoviruses, brome mosaic virus (BMV) and cowpea chlorotic mottle virus (CCMV), have been determined by direct RNA and cDNA sequencing without cloning. BMV RNA4 is 876 b long including a 5' noncoding region of nine nucleotides and a 3' noncoding region of 300 nucleotides. CCMV RNA 4 is 824 b long, including a 5' noncoding region of 10 nucleotides and a 3' noncoding region of 244 nucleotides. The encoded coat proteins are similar in length (188 amino acids for BMV and 189 amino acids for CCMV) and display about 70% homology in their amino acid sequences. Length difference between the two RNAs is due mostly to a single deletion, in CCMV with respect to BMV, of about 57 b immediately following the coding region. Allowing for this deletion the RNAs are about 65% homologous. Analysis of the sites of sequence divergence indicate that mutations leading to divergence were constrained in the coding region primarily by the requirement of maintaining a favorable coat protein structure and in the 3' noncoding region primarily by the requirement of maintaining a favorable RNA spatial configuration.

INTRODUCTION

BMV and CCMV are members of the bromovirus group of spherical plant viruses. Although related serologically, they differ widely in host range (1). The genetic information of bromoviruses is divided among three genomic RNAs, designated RNAs 1, 2 and 3, encapsidated in separate virions. In addition, the RNA3-containing virion encapsidates a subgenomic RNA (RNA4) which is the virion coat protein mRNA (1). We are investigating the primary and secondary structure of the RNAs from these viruses to elucidate their function in translation, replication and encapsidation as well as to help understand their evolutionary relationship.

We have previously reported the 5' and 3' untranslated sequences of BMV RNA4 (2,3) and have shown that bromovirus RNAs as well as the RNAs of cucumber mosaic virus share extensive sequence homology and nearly identical secondary structure at their 3' termini (4). We here present

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and compare the complete nucleotide sequences of BMV RNA4 and CCMV RNA4 and compare, also, the encoded coat protein sequences.

MATERIALS AND METHODS

Materials

Enzymes and isotopes used for direct and dideoxy RNA sequencing were as described (3-5). Tobacco acid pyrophosphatase was from Bethesda Research Laboratories, Inc. *E. coli* poly(A) polymerase was a gift of J. Bol and E. Koper-Zwarthoff or was from Bethesda Research Laboratories, Inc. AMV reverse transcriptase was provided by J. Beard through the Office of Program Resources and Logistics, National Cancer Institute. Restriction enzymes HaeIII and AluI were from New England Biolabs. BMV and CCMV RNAs were isolated as described (3,4,6).

Enzymatic sequencing of RNA

Intact RNA was 5' labeled with T4 polynucleotide kinase(3) after chemical (7) or enzymatic (8) decapping and phosphatase treatment or was 3' labeled with T4 RNA ligase (9). RNase T1 fragments were isolated as described (3) and were labeled as above. These end-labeled RNAs were sequenced by the enzymatic cleavage method of Donis-Keller (10) as described (3).

Dideoxy sequencing

Sequences at the 3' ends of both BMV and CCMV RNA4 were obtained by the dideoxy chain termination method (4,11,12).

Chemical Sequencing of cDNA

BMV and CCMV cDNAs were prepared, fragmented with restriction enzymes and sequenced as follows.

(i) Preparation of single stranded cDNA.

BMV and CCMV RNA4 were polyadenylated, reverse transcribed and template RNA hydrolyzed as described (13). One unit of poly(A) polymerase per 10 μg (36 pmole) of RNA was used. A typical reverse transcription reaction (250 μl) contained 100 μg (360 pmole) polyadenylated RNA4; 50 μg p(dT)12; 250 units of reverse transcriptase; 1 mM each of dCTP, dGTP, dTTP and 0.5 mM dATP containing 5 μCi of [α-^{32}P]dATP. The reaction was carried out for 2 hours at 42°C in a buffer containing 50 mM Tris-HCl (pH 8.3), 50 ml KCl, 8 mM $MgCl_2$ and 20 mM DTT. The resultant specific activity of uniform label in the cDNA was too low to interfere with the sequencing.

(ii) Preparation of end-labeled cDNA restriction fragments.

BMV4 and CCMV4 cDNAs were digested at 37°C for 20 hours with HaeIII

(14 units/μg of DNA in 6 mM Tris-HCl, pH 7.5, 6 mM $MgCl_2$, 6 mM NaCl, 6 mM 2-mercaptoethanol and 100 μg/ml BSA) or AluI (20 units/μg of DNA in 6 mM Tris-HCl, pH 7.5, 6 mM $MgCl_2$, 50 mM NaCl, 1 mM DTT and 100 μg/ml BSA). After phenol extraction and ethanol precipitation the DNA fragments were 3' end-labeled (5) or dephosphorylated (8) and 5' end-labeled (3). Labeled fragments were fractionated by electrophoresis on 8% polyacrylamide, 7 M urea gels (14) and were eluted (5).

(iii) Sequence analysis.

Fragments of cDNA were sequenced by the method of Maxam and Gilbert (5) except that formic acid instead of piperidinium formate was used in the A+G reactions (15). To read the first 200 bases of a fragment, samples were run on thin (0.4 mm) 20% and 6% or 8% polyacrylamide gels 40 cm long. Sequences over 500 bases long were analyzed on 6% polyacrylamide gels 85 cm long. Occasionally 0.25 mm thick gels were used in which case the gel was bonded onto one glass plate, fixed in 10% acetic acid and dried (16). Autoradiography was at -70°C with or without intensifying screens. Bands were substantially sharper when screens were omitted (3,5).

RESULTS

Construction of the BMV and CCMV RNA4 Sequences

Some years ago we determined the sequence of 54 nucleotides from the 5' end of BMV RNA4 by the original radiochemical techniques of Sanger et al. (2). We reported the sequence of the 3' untranslated region of 300 bases obtained by direct enzyme-specific cleavage of RNA and by dideoxy RNA sequencing (3,4). We used direct enzyme-specific cleavage to determine about 95% of the remaining sequence. However, nucleotides in positions 174-189 were not accessible to ribonucleases even in 8.3 M urea at 75°C, and nucleotides in positions 350-400 were only marginally accessible to ribonucleases.

These uncertainties were resolved by sequencing restriction fragments of BMV4 cDNA. Our approach was similar to that recently used by Rice and Strauss (17) to determine the sequence of 26S mRNA of Sindbis virus. Starting with approximately 50 μg of RNA4 we obtained cDNA in 10-15% the amount of the input RNA. More than 20% of such cDNA showed a single gel electrophoresis band that corresponded to full-length cDNA. HaeIII digestion of unfractionated cDNAs yielded eight bands, one a partial digestion product. These were labeled at their 5' or 3' terminus and all but one of the 5' labeled fragments were sequenced completely. No significant band compressions were encountered. HaeIII fragments were ordered by comparison with the

RNA sequencing data. The assembled fragments spanned the entire RNA, yielding the sequence of the previously inaccessible regions and completely confirming the remainder. Fig. 1 gives the BMV RNA4 sequence.

Experience gained in sequencing BMV RNA4 impressed us with the reliability of sequencing RNA via end-labeled cDNA restriction fragments. We therefore relied principally on this method to obtain the complete sequence of CCMV RNA4. Two restriction enzymes, HaeIII and AluI, were used to provide overlapping fragments. To provide more extensive sequence overlap we also analyzed approximately 430 bases from the 5' end of the RNA by the direct enzymatic method. We also verified the first 15 bases at the 5' end by the wandering spot method (18). Previously we had reported the sequence of 230 bases at the 3' end, obtained by direct enzymatic and dideoxy sequencing (4). These analyses produced the complete sequence of CCMV RNA4 as shown in Fig. 2.

Fig. 3 shows maps of BMV and CCMV RNA4 and indicates the portions of the sequence data obtained by the various methods.

Amino Acid Sequence of the Virion Coat Proteins

BMV and CCMV RNA4 each have a long open reading frame that translates into the known coat protein sequences. The BMV coat protein sequence was determined by Moosic (19) in our laboratory with some ambiguities left between ser/ala, glu/gln, and asp/asn. The sequence of the first 25 amino acids of CCMV coat protein was reported by Tremaine *et al*. (20) and the complete CCMV coat protein sequence, with some ambiguity in tryptic peptide placement, has been reported recently by Rees and Short of the John Innes Institute (personal communication). Our nucleotide sequences agree with the reported amino acid sequences and resolve their ambiguities. The amino acid sequences, aligned with the nucleotide sequences are shown in Figs. 1 and 2 for BMV and CCMV, respectively.

BMV coat protein is 188 amino acids long and CCMV coat protein is 189 amino acids long (excluding, in both cases, the initial methionine residue which is absent in the mature coat proteins). The two proteins have substantial homology in their amino acid sequences. If from the CCMV sequence we exclude one of the two contiguous valine residues in positions 26 and 27 and one of the two contiguous alanine residues in positions 150 and 151 and if we exclude the C-terminal arg of BMV, which has no counterpart in CCMV, the remaining amino acid residues align so that 70% (i.e. 132 out of 187) match. The amino acid residues of CCMV coat protein that are not homologous to those of BMV are shown in capital letters in Fig. 2. It may

10
(met)ser thr ser gly thr gly lys met thr arg
m^7GpppGUAUUAAUA *AUG* UCG ACU UCA GGA ACU GGU AAG AUG ACU CGC
10 20 30 40

25
ala gln arg arg ala ala ala arg arg asn arg trp thr ala arg
GCG CAG CGU CGU GCU GCC GCU CGC AGA AAU CGU UGG ACC GCU AGG
50 60 70 80

40
val gln pro val ile val glu pro leu ala ala gly gln gly lys
GUC CAA CCA GUA AUU GUC GAA CCA CUC GCU GCU GGC CAA GGC AAG
90 100 110 120 130

55
ala ile lys ala ile ala gly tyr ser ile ser lys trp glu ala
GCC AUU AAA GCG AUU GCA GGA UAC AGC AUA UCA AAG UGG GAG GCG
140 150 160 170

70
ser ser asp ala ile thr ala lys ala thr asn ala met ser ile
UCU UCG GAC GCG AUU ACA GCG AAA GCC ACC AAU GCC AUG AGU AUC
180 190 200 210 220

85
thr leu pro his glu leu ser ser glu lys asn lys glu leu lys
ACU CUG CCC CAU GAG CUC UCU UCU GAA AAG AAU AAG GAG CUU AAG
230 240 250 260

100
val gly arg val leu leu trp leu gly leu leu pro ser val ala
GUC GGC AGG GUG CUG CUU UGG UUG GGA CUU CUU CCU AGC GUU GCU
270 280 290 300 310

115
gly arg ile lys ala cys val ala glu lys gln ala gln ala glu
GGG AGG AUU AAG GCU UGU GUU GCU GAG AAA CAG GCA CAG GCC GAG
320 330 340 350

130
ala ala phe gln val ala leu ala val ala asp ser ser lys glu
GCU GCU UUU CAA GUA GCC UUG GCG GUU GCA GAC UCC UCG AAA GAG
360 370 380 390 400

145
val val ala ala met tyr thr asp ala phe arg gly ala thr leu
GUG GUC GCG GCC AUG UAU ACG GAC GCC UUU CGA GGG GCG ACU CUG
410 420 430 440

160
gly asp leu leu asn leu gln ile tyr leu tyr ala ser glu ala
GGG GAU UUG CUU AAU CUC CAG AUU UAU CUG UAU GCA UCU GAA GCA
450 460 470 480 490

175
val pro ala lys ala val val val his leu glu val glu his val
GUG CCU GCU AAG GCG GUC GUU GUA CAU CUA GAA GUU GAG CAC GUA
500 510 520 530

188
arg pro thr phe asp asp phe phe thr pro val tyr arg *
AGG CCU ACG UUC GAU GAC UUC UUC ACC CCG GUU UAU AGG *UAG* UGC
540 550 560 570 580

CCCUGCUCGGAGAGCCCCUGACUGGGUUAAAGUCACAGGCCCCUUGUCUCAGGUAGAGA
590 600 610 620 630 640

CCCUGUCCAGGUAGGACACUUUGGCUAAGGUUAAAAGCUUGUUGAAUCAGUACAAUAAC
650 660 670 680 690 700

UGAUAGUCGUGGUUGACACGCAGACCUCUUACAAGAGUGUCUAGGUGCCUUUGAGAGUU
710 720 730 740 750

ACUCUUUGCUCUCUUCGGAAGAACCCUUAGGGGUUCGUGCAUGGGCUUGCAUAGCAAGU
760 770 780 790 800 810

CUUAGAAUGCGGGUACCGUACAGUGUUGAAAAACACUGUAAAUCUCUAAAAGAGACCA$_{OH}$
820 830 840 850 860 870

Figure 1. Nucleotide sequence of BMV RNA4 and amino acid sequence of the viral coat protein.

(met)ser thr VAL gly thr gly lys LEU thr arg 10
m^7GpppGUAAUUUAUC *AUG* UCU ACA GUC GGA ACA GGG AAG UUA ACU CGU
10 20 30 40

ala gln arg arg ala ala ala arg LYS asn LYS ARG ASN THR arg 25
GCA CAA CGA AGG GCU GCG GCC CGU AAG AAC AAG CGG AAC ACU CGU
50 60 70 80

val VAL gln pro val ile val glu pro ILE ala SER gly gln gly 40
GUG GUC CAA CCU GUU AUU GUA GAA CCC AUC GCU UCA GGC CAA GGC
90 100 110 120 130

lys ala ile lys ala TRP THR gly tyr ser VAL ser lys trp THR 55
AAG GCU AUU AAA GCA UGG ACC GGU UAC AGC GUA UCG AAG UGG ACC
140 150 160 170

ala ser CYS ALA ala ALA GLU ala lys VAL thr SER ala ILE THR 70
GCC UCU UGU GCG GCU GCC GAA GCU AAA GUA ACC UCG GCU AUA ACU
180 190 200 210 220

ile SER leu pro ASN glu leu ser ser glu ARG asn lys GLN leu 85
AUC UCU CUC CCU AAU GAG CUA UCG UCC GAA AGG AAC AAG CAG CUC
230 240 250 260

lys val gly arg val leu leu trp leu gly leu leu pro ser val 100
AAG GUA GGU AGA GUU UUA UUA UGG CUU GGG UUG CUU CCC AGU GUU
270 280 290 300 310

SER gly THR VAL lys SER cys val THR glu THR gln THR THR ala 115
AGU GGC ACA GUG AAA UCC UGU GUU ACA GAG ACG CAG ACU ACU GCU
320 330 340 350

ALA ala SER phe gln val ala leu ala val ala asp ASN ser lys 130
GCU GCC UCC UUU CAG GUG GCA UUA GCU GUG GCC GAC AAC UCG AAA
360 370 380 390 400

ASP val val ala ala met tyr PRO GLU ala phe LYS gly ILE thr 145
GAU GUU GUC GCU GCU AUG UAC CCC GAG GCG UUU AAG GGU AUA ACC
410 420 430 440

leu GLU GLN leu ALA ALA ASP LEU THR ile tyr leu tyr SER ser 160
CUU GAA CAA CUC GCC GCG GAU UUA ACG AUC UAC UUG UAC AGC AGU
450 460 470 480 490

ALA ala LEU THR GLU GLY ASP val ILE val his leu glu val glu 175
GCG GCU CUC ACU GAG GGC GAC GUC AUC GUG CAU UUG GAG GUU GAG
500 510 520 530

his val arg pro thr phe asp asp SER phe thr pro val tyr * 189
CAU GUC AGA CCU ACG UUU GAC GAC UCU UUC ACU CCG GUG UAU *UAG*
540 550 560 570 580

UGCCCGCUGAAGAGCGUUACACUAGUGUGGCCUACUUGAAGGCUAGUUAUAACCGUUUC
590 600 610 620 630 640

UUUAAACGGUAAUCGUUGUUGAAACGUCUUCCUUUUACAAGAGGAUUGAGCUGCCCUUG
650 660 670 680 690 700

GGUUUUACUCCUUGAACCCUUCGGAAGAACUCUUUGGAGUUCGUACCAGUACCUCACAU
710 720 730 740 750 760

AGUGAGGUAAUAAGACUGGUGGGCAGCGCCUAGUCGAAAGACUAGGUGAUCUCUAAGGA
770 780 790 800 810

$GACCA_{OH}$
820

Figure 2. Nucleotide sequence of CCMV RNA4 and amino acid sequence of the viral coat protein. Amino acids differing from the BMV coat protein sequence are indicated by capital letters.

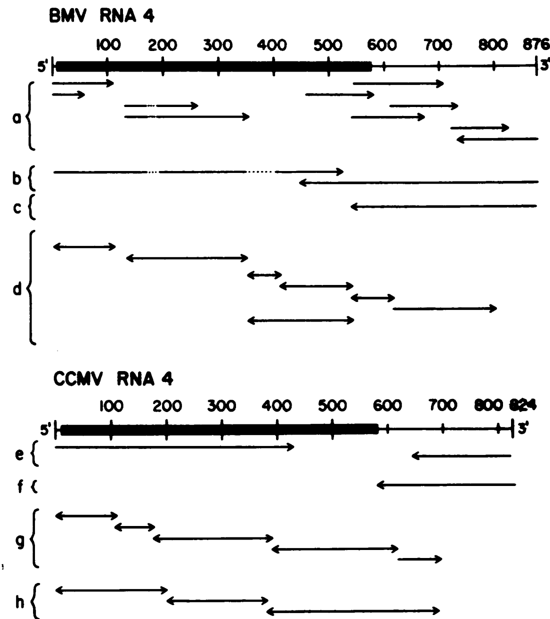

Figure 3. Schematic diagram summarizing the BMV and CCMV RNA4 sequence data. The scale at the top indicates nucleotide position starting from the 5' end. The darkened bar indicates the location of the coat protein cistron. The arrows, which are aligned with the scale, show the direction and extent of the sequence determined in each experiment. (a) Direct enzymatic sequencing of the 5'-end or 3'-end labeled BMV RNA4 fragments produced by partial T1 RNase digestion. Dotted portions indicate parts of the sequence that could not be determined unambiguously because of secondary structure effects (see text). (b) and (e) Direct enzymatic sequencing of 5'- and 3'-end labeled intact BMV and CCMV RNA4. (c) and (f) Dideoxy sequencing of BMV and CCMV RNA4. The synthetic primer p(dT)$_8$dG was used on in vitro 3' polyadenylated RNAs. (d) and (g) Sequencing of HaeIII fragments from BMV4 and CCMV4 cDNA by Maxam and Gilbert's chemical cleavage method. Arrows on both sides of the fragment indicate that it was sequenced from both ends after terminal labeling. (h) Sequencing AluI fragments of CCMV RNA4 cDNA.

be noted that a stretch of 16 amino acids corresponding to positions 84-99 in BMV and to positions 85-100 in CCMV coat protein are identical. Also there is only one change (phe at position 182 in BMV replaced by ser at position 184 in CCMV) in the 20 amino acids at the C-terminus (excluding the C-terminal arg of BMV).

Base Sequence Homology in the Coding Region

If we exclude from consideration CCMV RNA4 bases 89-91 (corresponding

to val 26) and bases 461-463 (corresponding to ala 150) and BMV RNA4 bases 574-576 (corresponding to arg 188) the remaining 564 bases in the coding region of the RNAs align so that 65% of them are homologous. There is a 75% homology in the first codon position, an 83% homology in the second position and a 37% homology in the third position.

Although a detailed analysis of spatial configuration of the two RNAs is beyond the scope of this study we have calculated the minimum energy secondary structure of each molecule according to the procedure of Zuker and Stiegler (21). Broad common features of the two RNAs are recognizable but their detailed structures are much different. This is to be expected in view of the high conservation of bases in the first and second positions coupled with the high base divergence in the third position.

Codon Utilization

The high base homology in the first and second coding positions and the low homology in the third position reflects different codon usage in the two messages. In over 60% of the amino acid sequence matches between the two coding regions, the codons used are different. For example, four out of five tyrosines residues are coded by UAU in BMV while four of five are coded by UAC in CCMV. The leucine codon, UUA, is not used at all in BMV, whereas it codes for five leucine residues in CCMV. The leucine codon, CUG, is not used in CCMV but it codes for four leucine residues in BMV. AAC codes for four asparagine residues in CCMV but is not used in BMV. Fig. 4 shows a particularly clear example of the use of different codons to generate the same amino acid sequence. Although there are obvious differences in codon usage there is no strong overall preference for particular bases in the third position of either protein. Fig. 5 summarizes the codon utilization for the two viruses.

Comparison of the Nucleotide Sequences in the Non-coding Regions

The 5' non-coding sequences of both BMV and CCMV RNA4 are short,

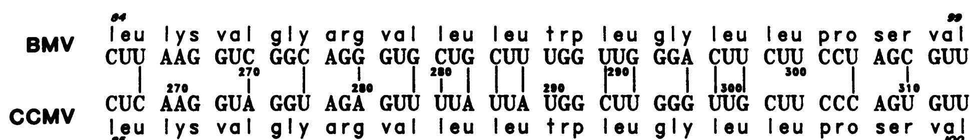

Figure 4. Nucleotide sequence of a part of BMV and CCMV RNA4 showing silent substitutions (indicated by vertical lines) in codons for conserved amino acids.

	U	C	A	G
U	UUU 2;3 Phe UUC 3;1 Phe UUA 0;5 Leu UUG 3;2 Leu	UCU 4;4 Ser UCC 1;3 Ser UCA 2;1 Ser UCG 3;4 Ser	UAU 4;1 Tyr UAC 1;4 Tyr UAA 0;0 End UAG 1;1 End	UGU 1;2 Cys UGC 0;0 Cys UGA 0;0 End UGG 3;3 Trp
C	CUU 5;4 Leu CUC 3;4 Leu CUA 1;1 Leu CUG 4;0 Leu	CCU 3;3 Pro CCC 1;3 Pro CCA 2;0 Pro CCG 1;1 Pro	CAU 2;2 His CAC 1;0 His CAA 3;4 Gln CAG 4;3 Gln	CGU 3;3 Arg CGC 2;0 Arg CGA 1;1 Arg CGG 0;1 Arg
A	AUU 6;2 Ile AUC 1;4 Ile AUA 1;2 Ile AUG 3;1 Met	ACU 5;7 Thr ACC 3;4 Thr ACA 1;4 Thr ACG 2;3 Thr	AAU 4;1 Asn AAC 0;4 Asn AAA 2;4 Lys AAG10;8 Lys	AGU 1;3 Ser AGC 2;2 Ser AGA 1;2 Arg AGG 5;2 Arg
G	GUU 6;6 Val GUC 5;5 Val GUA 4;4 Val GUG 3;6 Val	GCU 11;12 Ala GCC 8;6 Ala GCA 5;3 Ala GCG 9;5 Ala	GAU 2;2 Asp GAC 4;4 Asp GAA 4;4 Glu GAG 7;6 Glu	GGU 1;3 Gly GGC 3;4 Gly GGA 3;1 Gly GGG 3;2 Gly

Figure 5. Codon utilization of the BMV and CCMV coat protein cistron. The numbers refer to the frequency with which each triplet is used. The first number indicates the frequency in the case of BMV and the second number indicates that of CCMV.

containing only nine and ten bases, respectively, following the 5' terminal cap structure. Both sequences are rich in A's and U's and lack G.

Assessment of base sequence homology requires an assumption of how the comparable sequences are to be aligned to take into account deletions and insertions. In the coding region we aligned so as to get optimal fit of the corresponding amino acid sequences. The 3' terminal 200 or so bases of all bromovirus RNAs can be arranged into nearly identical loop and base-paired stem configurations even though the base pairs within the stems vary and deletions between adjacent stem regions vary (4). Thus in the 3' terminal non-coding regions we have aligned on the basis of this remarkably similar secondary structure.

In approximately the 200 3' terminal bases, if we exclude bases 633, 746, 770, 772 and 780 of CCMV RNA4 and bases 837, 838, 858, and 869 of BMV RNA4 (these bases are primarily between contiguous stem regions and do not affect the secondary structure; please see Fig. 2, Ref. 4), the remaining bases of the two RNAs align so that 64% of the 200 3' proximal bases are homologous.

The region between the coding region and the 3' region of homologous

secondary structure shows no obvious similarity. There are about 35 and 92 bases, respectively, in CCMV and BMV RNA4's between the coding region and the 3' matching regions of secondary structure. The best alignment of the 35 CCMV RNA bases to the 92 BMV RNA bases results in only 15 matches. This deletion in CCMV RNA4, with respect to BMV RNA4, accounts for most of the difference in length between the two RNAs.

Comparison With Other Plant Viral mRNA Sequences

Complete sequences of the coat protein mRNAs from several plant viruses are known. These are turnip yellow mosaic virus (22), tobacco mosaic virus (23) and alfalfa mosaic virus (24), the latter having a tripartite genome similar to that of BMV. We compared the primary sequences of these RNAs with those of BMV and CCMV with a simple computer procedure described previously (25). Except for a few short stretches, to be expected on a random basis with RNA molecules of these sizes, we found no obvious sequence relationships.

DISCUSSION

In the coat protein cistrons of the BMV and CCMV the preponderence of mutational changes from the putative common ancestor did not result in coat protein differences. This fact and the observed differences in minimal energy RNA secondary structure suggest that the most important constraint on nucleotide sequence changes in the coding region is preservation of the encoded protein sequence. Furthermore, many of the silent nucleotide differences which do occur may not be random but may be dictated by other translational constraints. This marked divergence between the two messages in codon usage for some amino acids indicates that there may have been differential pressure on the two viruses to provide particular codons, probably to facilitate interaction with the tRNAs of their hosts.

In the 3' non-coding region the proportion of sequence differences is about the same as in the coding region. However, in this case, the homology in secondary structure was preserved in the 200 3' proximal bases. This suggests that in this region mutational events were constrained to maintain a common spatial configuration of the two RNAs.

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Abbreviations: BMV, brome mosaic virus; CCMV, cowpea chlorotic mottle virus.

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