ブロムモザイクウイルスATCC 66株由来のcDNAクローンの塩基配列およびロシア株ゲノムとの比較

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Nucleotide Sequence of a Set of cDNA Clones Derived from the Brome Mosaic Virus ATCC66 Strain† and Comparison with the Russian Strain Genome

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Abstract

The complete nucleotide sequence of a set of cDNA clones designated KU1 strain, from which infectious *in vitro* transcripts can be transcribed, of the ATCC66 strain of brome mosaic virus (BMV) was determined and compared with the Russian strain of BMV in both nucleotide and the deduced amino acid sequence. Each corresponding genomic RNA in the KU1 strain and the Russian strain was homologous by more than 97% in nucleotide sequence. Base substitution of the transition type was more frequent than that of the transversion type in all three genomic RNAs. Amino acid changes in three regions where significant amino acid sequence homology is shown among the Sindbis-like plant RNA viruses were mostly substitutions for amino acids categorized to similar groups. The nucleotide sequence of the 5′ noncoding region in RNA1 and RNA3 was identical in the corresponding RNAs of the two strains while seven base changes were observed in RNA2. Any base change in the 3′ noncoding region did not disrupt a predicted secondary structure. Two adjacent adenine residues were absent in the noncoding leader sequence of the coat protein gene of both KU1 strain and ATCC66 strain. The intercistronic oligo(A) tract in RNA3 of the ATCC66 strain was heterogeneous in length in which more than two guanine residues were observed by direct RNA sequencing, while one guanine residue was present in the RNA3 of the cDNA clone of the KU1 strain.

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Key words: brome mosaic virus, nucleotide sequence, genome structure.

INTRODUCTION

Brome mosaic virus (BMV) is a positive-sense RNA plant virus with a tripartite genome consisting of single-stranded RNAs, designated RNAs 1, 2 and 3[11]. Monocistronic RNA1 and RNA2 encode nonstructural 1a (104 kDa) and 2a (94 kDa) proteins, respectively[3]. RNA3 encodes nonstructural 3a protein (32 kDa) and the coat protein (20 kDa), which is translated from subgenomic RNA4[2,6,23]. We have noticed that the ATCC66 strain of BMV (BMV-66) has two types of coat protein with different electrophoretic mobility despite the Russian strain of BMV (BMV-R) mainly contains a single type of coat protein[21]. Recently we have reported that the lack of two adjacent adenine residues in the 5′

† The nucleotide sequence data are in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number X58456, X58457 and X58458.

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noncoding region of RNA4 of BMV-66 is responsible for the synthesis of the two types of coat protein[15]. In addition, BMV-66 has several other properties different from BMV-R. These include the reduced amount of the RNA1 in purified virions compared to BMV-R and differences in response of some host plants upon infection[19]. BMV-66 induced necrotic lesions on the inoculated leaves of *Chenopodium quinoa*, while BMV-R induced chlorotic spots on the inoculated leaves, systemically spread and caused stunting (Okuno *et al.*, unpublished results).

The complete nucleotide sequence of BMV was determined in BMV-R[2,3]. We have constructed a set of cDNA clones for BMV-66 from which infectious virus RNA can be transcribed *in vitro* by T7 RNA polymerase[18]. Progeny viruses derived from the cDNA clones of BMV-66 consisting of three plasmids, pBTF1, pBTF2 and pBTF3[18] and all *in vitro* or *in vivo* products arising from them are referred to as a KU1 strain to distinguish them from the original BMV-66 stock. No difference has been observed between BMV-66 and BMV-KU1 in their properties described above. To delimit and analyze biological properties associated with BMV-66 and BMV-R at the nucleotide and amino acid sequence level, we have first determined the complete nucleotide sequence of BMV-KU1, a set of cDNA clones of BMV-66, and compared it with that of BMV-R. For some parts of the noncoding region, nucleotide sequence of BMV-66 was also determined by direct RNA sequencing.

MATERIALS AND METHODS

BMV-66 was kindly gifted from Dr. C. Hiruki, University of Alberta, Canada in 1973 and has been propagated through barley plants at Kyoto University. The 3′ and 5′ terminal sequence of the genomic RNA of BMV-66 had been determined by direct RNA sequencing[14,21] prior to the cDNA cloning to generate BMV-KU1[18]. To determine the 3′ terminus of the genomic RNAs, virion RNAs were fractionated, polyadenylated and sequenced using d(TTTTTTTTTTTTG) as a primer[2]. To determine the 5′ terminus of the genomic RNAs, RNA1, RNA2 and RNA3 of unfractionated virion RNAs were sequenced using d(GATAGAACTTGACAT), d(GGTTTTCGAAGACAT) and d(AACTATGTTAGACAT) as primers, respectively.

The cDNA inserts of RNA1, RNA2 and RNA3 of BMV-KU1 in plasmids pBTF1, pBTF2 and pBTF3[18], respectively, were sequenced by the dideoxy chain termination method[17,22] using "Sequenase" (United States Biochemical Corp.) according to the manufacturer's specifications. Each plasmid was digested with various restriction enzymes which were chosen according to the nucleotide sequence data of BMV-R[2,3]. The DNA fragments were subcloned into pUC118 or pUC119[25] and all sequences were determined on both strands. The contiguity of adjacent restriction fragments was verified by sequencing across the relevant restriction site on one or more overlapping clones. Sequences were assembled and analyzed with an NEC Personal Computer (PC9801-m) equipped with the nucleotide sequence analysis program (GENETYX ver. 6.04, SDC, Japan). To determine the nucleotide sequence of the 3′ noncoding region of RNA2 and the intercistronic region of RNA3, dideoxy sequencing was performed as described[14,21] using purified, unfractionated viral RNA or alkali-denatured pBTF3. Oligonucleotide primers for dideoxy sequencing and the nucleotide positions of their annealing sites within each RNA were d(TAACCTTAACCAAAG) (bases 2613 to 2627; primer P1) for RNA2, and d(CGCTGCGCGCGAGTC) (bases 1274 to 1288; primer P2) for RNA3.

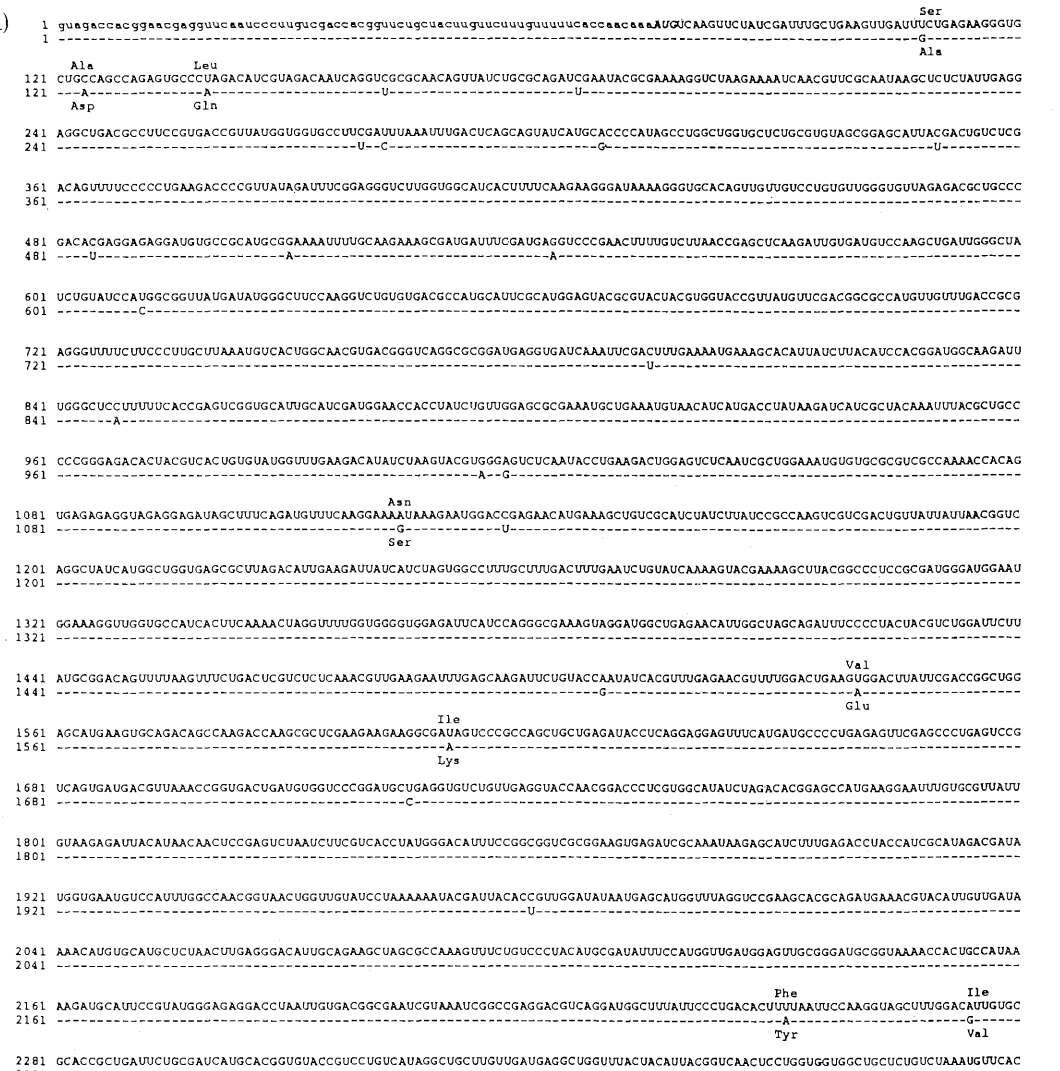
RESULTS AND DISCUSSION

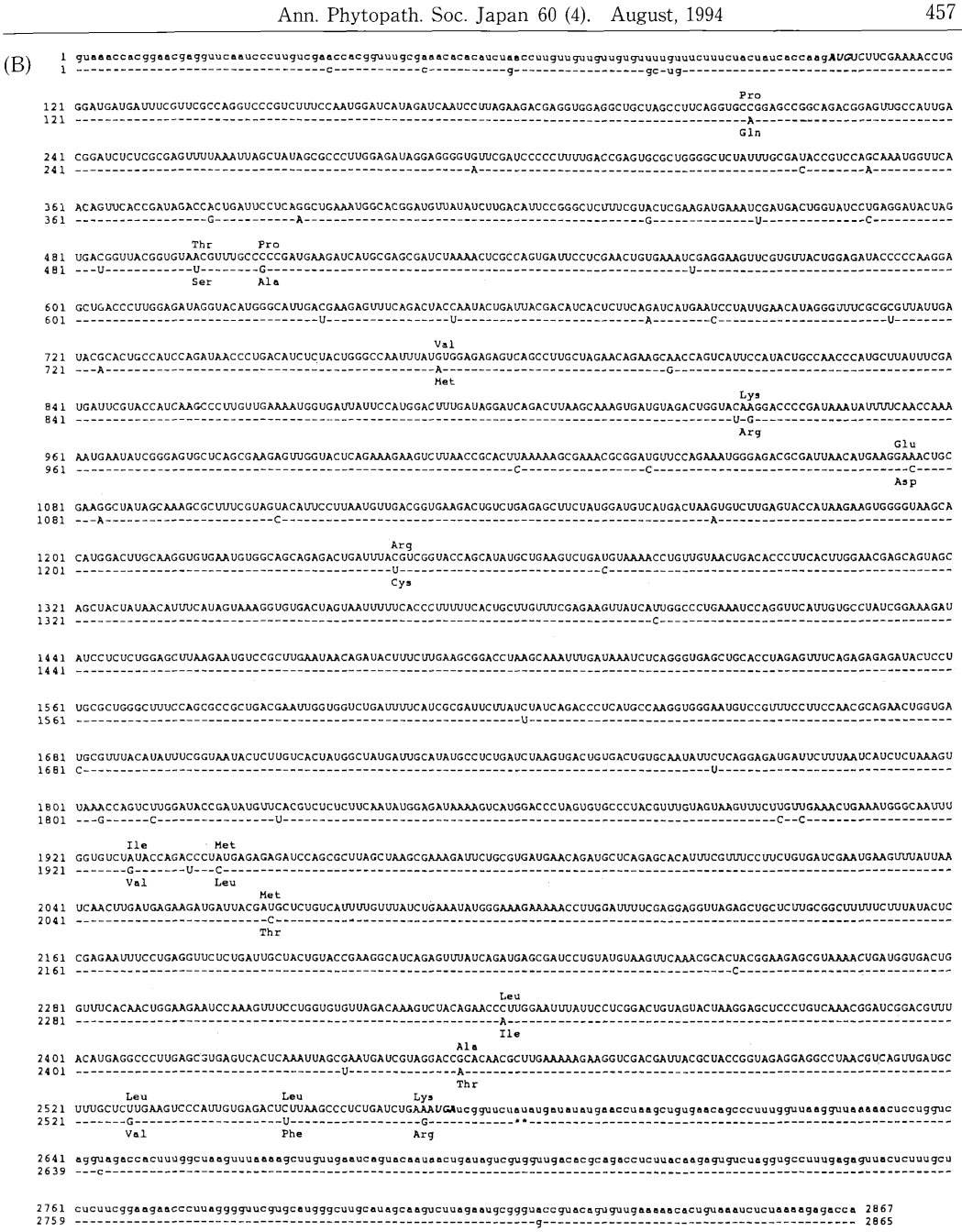
Complete nucleotide sequence of RNAs 1, 2 and 3 of the BMV-KU1 was determined by sequencing cDNA inserts of pBTF1, pBTF2 and pBTF3 and is presented in Fig. 1 (upper line).

Comparison of nucleotide and the deduced amino acid sequence of genomic RNAs between BMV-KU1 and BMV-R

The complete nucleotide sequence of RNAs 1, 2 and 3 of BMV-KU1 was compared with that of BMV-R in each corresponding RNA (Fig. 1). RNA1 of both strains was 3234 residues in length (Fig. 1A). RNA2 of BMV-KU1 and BMV-R was 2867 and 2865 residues in length, respectively, as RNA2 of

(A)





BMV-KU1 contained two additional bases at positions 2581 and 2582 in the 3′ noncoding region just downstream of the 2a protein gene as described below (Fig. 1B). RNA3 of BMV-KU1 was shorter in length by two nucleotides than RNA3 of BMV-R except for the oligo(A) tract and was 2114 residues in length. This was due to the absence of two adjacent adenine residues in the noncoding leader sequence of the KU1 coat gene (Fig. 1C). Nucleotide sequence homology between two strains was 98.9%, 97.8% and 98.7% in RNAs 1, 2 and 3, respectively. Of total base substitutions, 67% was at the third position

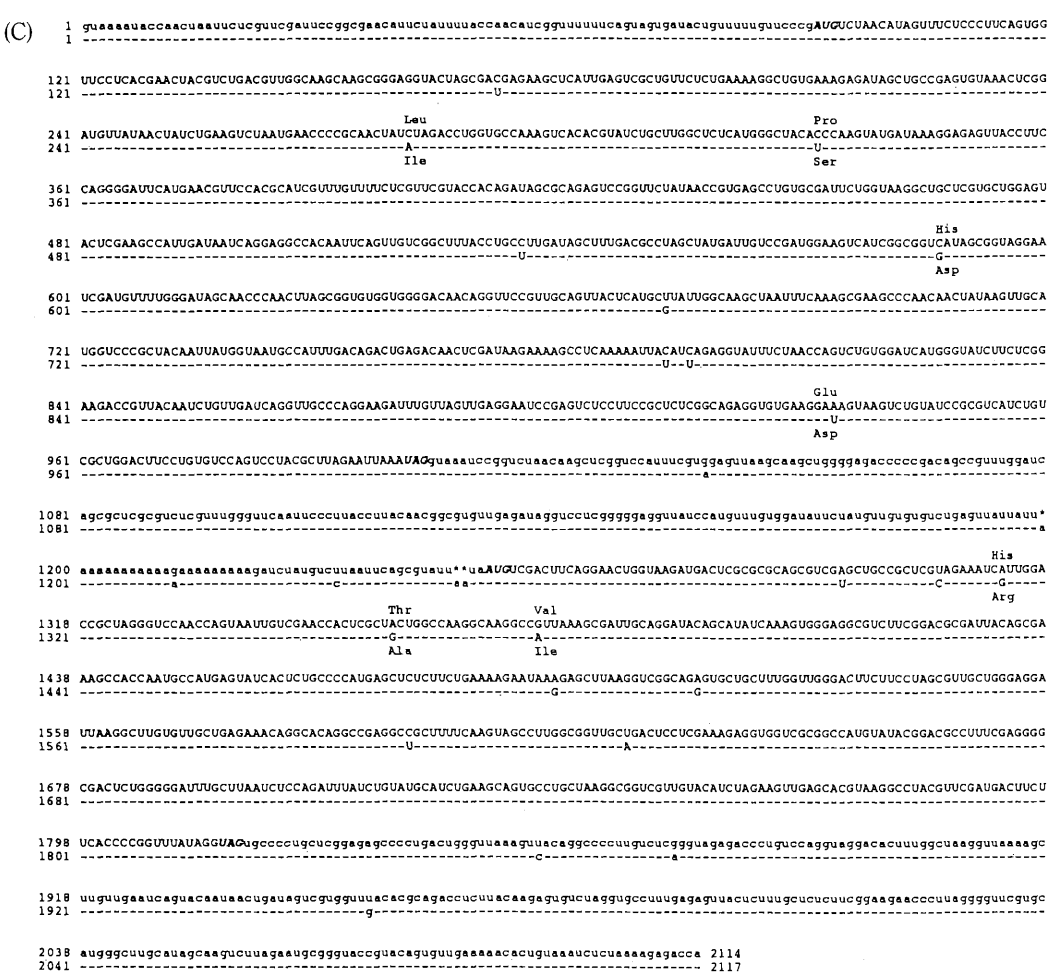


Fig. 1. Comparison of the complete nucleotide sequence of RNA1 (A), RNA2 (B) and RNA3 (C) between BMV-KU1 (upper line) and BMV-R (lower line). BMV-KU1 is a set of cDNA plasmids, pBTF1, pBTF2 and pBTF3[18], derived from BMV-66 and all *in vitro* and *in vivo* products arising from them. Initiation and termination codons are indicated by bold-italic letters. The noncoding region sequences of each RNA are shown by small letters. BMV-R nucleotides identical to those of BMV-KU1 are indicated by (−). Deletions are indicated by (*). Only the amino acids of BMV-KU1 different from those of BMV-R and the corresponding amino acids of BMV-R are denoted over and below the codon sequences, respectively.

of codons in all four BMV genes. According to the codon table, most of the base changes at the third position do not lead to concomitant amino acid substitutions relative to those at the other positions, so that such base changes seem to accumulate in the genomic sequence. In base substitutions, the transition type was more frequent than the transversion type in all four virus genes (Fig. 1). In the transition type, base substitutions between pyrimidines were more frequent than those between purines. This may reflect favored usage of "U" in the third position of each codon in all BMV genes[3].

Gene organization in all genomic RNAs 1, 2 and 3 of BMV-KU1 was almost the same as that of BMV-R in size and in the initiation and termination positions in each open reading frame (ORF)[2,3]. Dissimilarity of amino acids[16] in corresponding ORF between BMV-KU1 and BMV-R is shown in Fig.

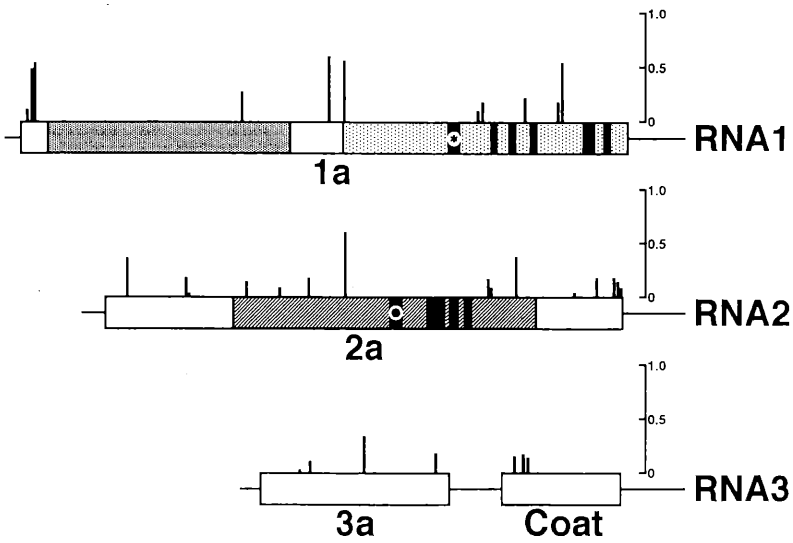


Fig. 2. Dissimilarity of BMV-KU1 amino acids different from those of BMV-R. Extents of dissimilarity are based on the values proposed by Miyata *et al.*[16] and indicated by the length of perpendicular lines on open bars. Shading regions are conserved among polypeptides encoded on the genomic RNAs of the Sindbis-like plant RNA viruses. Closed black regions in RNA1, conserved motifs in helicase reported by Hodgman[9]; Closed black regions in RNA2, conserved motifs in RNA polymerase reported by Poch *et al.*[20] (*) in RNA1, nucleotide binding domain; (●) in RNA2, polymerase domain.

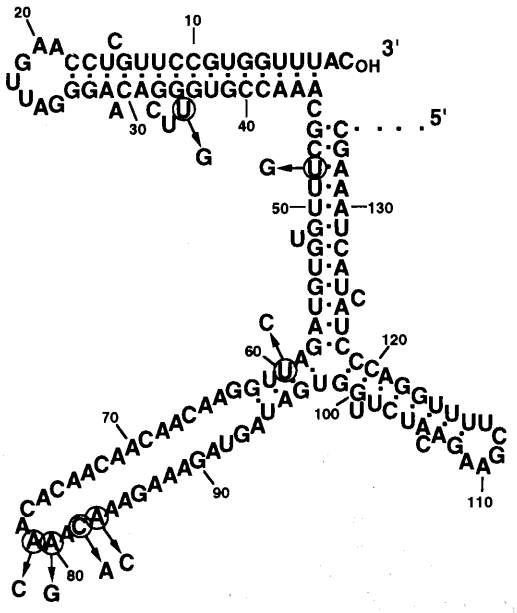


Fig. 3. A possible secondary structure of the (−) strand 3′ terminus of BMV-KU1 RNA2, which is predicted based on the model proposed for the corresponding region in RNA1 of BMV-R by Marsh *et al.*[13] Nucleotide numbers are from the 3′ end of (−) strand RNA2. Nucleotide substitutions that occur in BMV-KU1 relative to BMV-R are marked by circles, accompanying with the substitutive nucleotides.

2. The amino acid changes were mostly substitutions to amino acids categorized to similar groups, when compared in three conserved regions[4,7-10,20]; the C-terminal helicase-like domain of the 1a protein, the second N-terminal domain of the 1a protein and the central polymerase-like domain of the 2a protein. No amino acid changes were observed in the six conserved motifs of the 1a protein[9] and the four conserved motifs of the 2a protein[20] (Fig. 2). In contrast, the frequency of amino acid change was relatively high in the N-terminal region of the 1a protein and in the C-terminal region of the 2a protein where no amino acid sequence homology is observed among the Sindbis-like plant viruses[7], supporting the recent results that the last 125 amino acids of the 2a protein are dispensable for its function in viral RNA replication[24]. Amino acid change between two dissimilar ones, histidine (BMV-KU1) and aspartic acid (BMV-R), was observed in the central region of the 3a protein where extra three amino acid residues are present in BMV relative to cowpea chlorotic mottle virus, a related bromovirus[5] (Fig. 1C and 2).

5′ terminal sequence of the BMV genomic RNAs

The nucleotide sequence of the 5′ noncoding regions of RNA1 and RNA3 was identical in the corresponding RNAs of BMV-KU1 and BMV-R (Fig. 1A and 1C), which was consistent with the previous results obtained by direct RNA sequencing of BMV-66. In the 5′ noncoding region of RNA2, BMV-KU1 had seven nucleotides different from BMV-R (Fig. 1B). These changes, except for one nucleotide change at position 48, did not disrupt a secondary structure of the (−) strand 3′ terminus of RNA2, which was predicted based on the model proposed for the corresponding region in the RNA1 of BMV-R by Marsh *et al.*[13] (Fig. 3). The model contained a large loop structure with a number of adenine residues. The mechanism by which the mutation occurred in the region and the function of the region

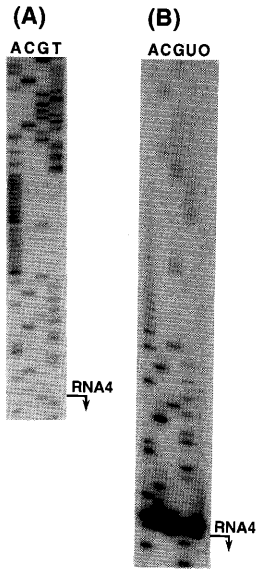


Fig. 4. Sequences flanking the internal oligo(A) in BMV RNA3. (A) Autoradiogram of a 6% polyacrylamide sequencing gel showing the intercistronic region of RNA3 of BMV-KU1. Oligonucleotide P2 (see MATERIALS AND METHODS) was used to prime DNA synthesis using alkali denatured pBTF3, an RNA3 cDNA clone of BMV-KU1. Lanes are labeled at top to reflect the template DNA sequence; each reaction contained the complementary dideoxynucleotide. Sequences below the arrow correspond to the 5′ end of BMV RNA4. (B) Autoradiogram of a 7.5% polyacrylamide sequencing gel showing the intercistronic region of BMV-66 RNA3. 5′ ^{32}P-labeled oligonucleotide P2 was used to prime cDNA synthesis using unfractionated BMV-66 RNAs which had been used as templates for the cDNA cloning. Lanes are labeled at top to reflect the template (plus-sense) RNA sequence; each reaction contained the complementary dideoxynucleotide or no dideoxynucleotide (O). Sequences below the arrow correspond to the 5′ end of BMV RNA4.

are open question.

3′ noncoding region of the BMV genomic RNAs

BMV-KU1 and BMV-R had identical nucleotide sequence in thirty bases from the 3′ end of RNAs 1, 2 and 3 (Fig. 1). This was consistent with the result obtained by direct RNA sequencing of BMV-66. Eight nucleotide substitutions were observed in the 3′ noncoding regions of genomic RNAs in BMV-KU1 relative to BMV-R; three, two and three substitutions in RNAs 1, 2 and 3, respectively (Fig. 1). Two additional bases were observed in RNA2 of BMV-KU1 at positions 2581 and 2582 (Fig. 1). Six out of the eight nucleotide substitutions were in a secondary structure proposed for the 3′ noncoding region of the genomic RNAs of BMV-R[1] and two of them were involved in base-pairing, although these changes did not disrupt the secondary structure. The two base addition was also found by direct RNA sequencing of BMV-66 (data not shown). The two nucleotides in RNA2 might have been added in BMV-KU1 or deleted in BMV-R at the loop structure region flanked by the sequences from position 2575 to 2579 and from position 2592 to 2596 which were complementary and could form the stem region (data not shown).

Intercistronic region of RNA3

Two adjacent adenine residues were absent in the noncoding leader sequence of the coat gene of BMV-KU1 compared to BMV-R. The position was in the subgenomic promoter region in the (−) strand of RNA3[12]. Either the two nucleotide deletion or two base substitutions observed in the intercistronic region of RNA3 of BMV-KU1 did not disrupt a secondary structure proposed for the (−) strand in this region[12]. The DNA sequencing of pBTF3 of BMV-KU1 revealed that one guanine residue was present within the oligo(A) tract (Fig. 4A). The direct sequencing of RNA3 of the original BMV-66 stock, however, showed the oligo(A) tract contained more than two guanine residues (Fig. 4B) and the register in the band pattern was not observed above the oligo(A) tract, which was also reported in RNA3 of BMV-R and considered to be due to heterogeneity in the length of the oligo(A)[2]. This difference could be explained as follows; one is that BMV-66 is composed of heterogeneous virus population whose RNA3s have various numbers of guanine residues within the oligo(A) tract. Another explanation is that RNA3 of BMV-66 has only one guanine residue as well as pBTF3 and the number of oligo(A) tract downstream of the guanine residue is heterogeneous in length among RNA3 population. The guanine residue(s) in the oligo(A) tract [present as cytosine residue(s) in oligo(U) tract of the (−) strand of RNA3] did not affect the secondary structure, as the oligo(U) tract forms a large loop structure[12]. The presence of guanine residue within the oligo(A) tract as well as the natural heterogeneity in the length of the oligo(A) tract[2] suggested that the oligo(A) tract may serve primarily as an unstructured spacer between the two core sequences of the subgenomic promoter[12].

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和　文　摘　要

三瀬和之・森　正之・中屋敷均・小山　毅・奥野哲郎・古澤　巌：ブロムモザイクウイルスATCC 66 株由来のcDNAクローンの塩基配列およびロシア株ゲノムとの比較

ブロムモザイクウイルス（BMV）ATCC 66 株の感染性 *in vitro* RNA を転写できる cDNA クローン（KU 1 株）の全塩基配列を決定し，BMV ロシア株と比較した。BMV RNA 1, 2, 3 それぞれにおける KU 1 株とロシア株の間での相同性は 97 %以上で，transition タイプの置換が transversion タイプの置換よりも多かった。Sindbis 様植物 RNA ウイルスに保存されている 3 つの領域でのアミノ酸変異のほとんどは類似アミノ酸への変換であった。RNA 1 と 3 の 5′ 端非翻訳領域の塩基配列は両株間で完全に同じであったが，RNA 2 では 7 つの塩基が異なっていた。いずれの RNA の 3′ 端の塩基配列の変異も想定される 2 次構造に影響を与えないものであった。KU 1 株，ATCC 66 株ともに外被タンパク質遺伝子の 5′端非翻訳領域において 2 つの隣接したアデニン塩基が欠失していた。RNA 3 のシストロン間領域に存在するオリゴ（A）配列のアデニンの数は ATCC 66 株では一定ではなく，KU 1 株の RNA 3 の cDNA でグアニン塩基が 1 個含まれていたのに対して，ATCC 66 株の RNA 3 では 2 個以上のグアニン塩基が認められた。