THREE DOUBLE-STRANDED RNA GENOME SEGMENTS OF BACTERIOPHAGE ϕ6 HAVE HOMOLOGOUS TERMINAL SEQUENCES

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1. Introduction

Bacteriophage ϕ6 [1] has a double-stranded (ds) RNA genome consisting of 3 segments with M_r 4.5 X 10^6 (L segment), 2.8 X 10^6 (M segment) and 2.2 X 10^6 (S segment) [2]. The largest segment L codes for the viral early proteins, while M and S segments code for the late proteins [3–5]. In infected cells, the virion-associated ϕ6 RNA polymerase transcribes these L, M and S segments to produce 3 species of mRNA (*l, m, s,* respectively) [6] and the synthetic rates of these mRNAs are temporally controlled for the sequential production of viral proteins [6,7].

Biochemical studies on the ϕ6 genome have shown that the 3 dsRNA segments have similar base compositions and suggested that they might have ssRNA tails [8]. More detailed biochemical analysis of the genome is, however, essential to understand the regulation of transcription and replication of such a divided genome. Here, we determine the 5′-terminal sequences of the plus and minus strands of each ϕ6 RNA segment and show that 3 dsRNA segments have homologous terminal sequences at both ends. From the sequence analysis of 3′-end labeled dsRNA segments, we further show that each segment has flush ends at both termini, contrary to [8].

2. Materials and methods

2.1. *Reagents*

RNase T1, T2 and U2 were purchased from Sankyo, nuclease P1 fromYamasa Shoyu, RNase PhyM, *Bacillus cereus* RNase and T4 RNA ligase from PL Biochemicals, T4 polynucleotide kinase from Takara Shuzo and bacterial alkaline phosphatase from Millipore. [γ-^{32}P]ATP (3000 Ci/mmol) was purchased from Amersham and [5′-^{32}P]cytidine-5′,3′-diphosphate ([^{32}P]pCp) was prepared from [γ-^{32}P]ATP as in [9].

2.2. *Purification of dsRNA genome and ssRNA transcript*

Double-stranded RNA was extracted from ϕ6 particles as in [10] and further purified by collecting the dsRNA fraction of CF 11 column chromatography as in [11]. Single stranded RNA transcript was synthesized by an in vitro RNA polymerase reaction and extracted by phenol essentially as in [4] and further passed through a Sephadex G-100 column to remove substrates. The RNA fraction was chromatographed on CF 11 cellulose to separate ssRNA fraction from dsRNA template and replicative-intermediates. Single stranded RNA fraction was collected by ethanol precipitation.

2.3. *Post-labeling of genome dsRNA segments*

For the 5′-end labeling, dsRNA segments were dephosphorylated by bacterial alkaline phosphatase and phosphorylated by [γ-^{32}P]ATP and T4 polynucleotide kinase on the condition used for dsDNA having protruding 5′-ends [12]. ϕ6 dsRNA segments were labeled at their 3′-termini using [^{32}P]pCp and T4 RNA ligase as in [13]. The labeled L, M and S segments were separated by 3.0% polyacrylamide gel electrophoresis (5% cross-linking) and recovered from the individual radioactive gel band [12] detected by autoradiography. Re-electrophoresis of the recovered dsRNA samples confirmed their purity.

2.4. *End base analysis*

5′-end labeled RNA and 3′-end labeled RNA were hydrolyzed by nuclease P1 and RNase T2, respectively. The complete digests (5′- or 3′-nucleotide monophosphates) were analyzed by thin-layer chromatography

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as in [14]. The radioactive spots were detected by autoradiography, and quantitated by liquid scintillation counting.

2.5. *Separation of plus and minus strands of each 5′-end labeled dsRNA segment*

5′-End labeled dsRNA segment was denatured and hybridized to an excess amount (90-fold excess for M or S segment, 15-fold excess for L segment) of unlabeled unfractionated mRNA synthesized in vitro as in [15] except that RNA was annealed at 78°C. The ssRNA and dsRNA were then separated by chromatography on CF 11 cellulose. The labeled plus strand was recovered from the ssRNA fraction and the labeled minus strand from the dsRNA fraction.

2.6. *Sequence techniques and autoradiography*

5′- or 3′-End labeled RNA was partially digested in 0.1 N NaOH for 4–30 min and the product was subjected to two-dimensional (2-D) polyacrylamide gel electrophoresis as in [16]. 5′-End labeled plus or minus strands was also partially digested by RNase T1, U2, [17] PhyM [18] or *B. sereus* RNase [19]. The products were separated on a 7 M urea 20% polyacrylamide gel (5% cross-linking) [12] together with their partial alkaline digests. Double-stranded RNA was denatured in dimethylsulfoxide (90%) and precipitated with ethanol before the digestion. Autoradiographs were obtained after 1–14 days exposure to Kodak XAR film at −70°C with intensifying screens (Dupont High Plus).

3. Results

3.1. *5′-End labeling of genome RNA and strand separation*

After 5′-end labeling of 3 dsRNA segments as in section 2.3., 3 segments were electrophoretically separated on a polyacrylamide gel. The 3 segments were equally labeled and each segment had A and G residues at its 5′-ends (table 1). Since none of the segments were labeled without the alkaline phosphatase treatment, 5′-ends of the plus and the minus strands of each dsRNA segment have 5′-terminal phosphate(s).

Each labeled dsRNA segment was then separated into its constituent plus and minus strands by annealing with excess unlabeled ϕ6 mRNA transcript as in section 2.5. The ssRNA fraction and the dsRNA fraction of the CF 11 column chromatography were collected and 5′-end base of each RNA fraction was analyzed (table 1). The result indicates that in all 3 segments 5′-terminal base of the plus strand is G and that of the minus strand is A. The contamination of one strand with the other one was very low (<4%) in M and S segments. As for L segment, however, the dsRNA fraction contained a little higher amount of the plus strand (~17%). This is because only a limited non-radioactive *l* mRNA was available in the experiment owing to the low production of *l* mRNA by ϕ6 RNA polymerase in vitro [10].

3.2. *5′-Terminal sequence analyses of each plus and minus strand*

For the analysis of 5′-terminal sequence of the

Table 1
Terminal base analyses of 5′- or 3′-end labeled dsRNA segments

Segment	5′-End labeling				3′-End labeling	
	Radioactivity[a] (10^6 cpm)	Terminal base[b] (%)			Radioactivity[a] (10^5 cpm)	Terminal base[c] (%), dsRNA
		dsRNA	+ Strand[d]	− Strand[e]		
L	1.30	A (59) C (41)	A (10) G (90)	A (83) G (17)	5.15	C (58) U (42)
M	1.20	A (71) G (29)	A (4) G (96)	A (97) G (3)	4.24	C (62) U (38)
S	1.12	A (72) G (28)	A (4) G (96)	A (96) G (4)	5.68	C (60) U (40)

[a] Double-stranded RNA genome (10 μg) was end labeled and each segment was separated on a gel and recovered from the gel as in section 2.3
[b] C and U were not detected; [c] A and G were not detected
[d] The ssRNA fraction of CF 11 column prepared as in section 2.5
[e] The dsRNA fraction of CF 11 column prepared as in section 2.5

plus strand, partial alkaline digests of each ssRNA strand fraction prepared above were subjected to 2-D polyacrylamide gel electrophoresis (fig.1A). The pattern of wandering spots clearly indicates that 5′-terminal sequences of the plus strands are extensively homologous among 3 segments. Another aliquot of each ssRNA fraction was partially digested by 4 kinds of RNases and analyzed on sequence gels (not shown). The result of the sequence gels completely agreed with that of wandering spot analysis and the first 20 nucleotide sequences were determined (fig.2). It shows that 5′-terminal sequences of plus strands of L, M and S are identical for the first 18 bases, with one exception, that residue 2 of the L segment is U while that of the other segments is G (fig.1A, fig.2). Within the sequences determined, the initiation codon AUG was not found in the 5′-terminal region of plus strands.

For the analysis of 5′-terminal sequence of each minus strand, dsRNA fraction of each segment prepared as in section 3.1 was partially hydrolyzed by alkaline, and analyzed by 2-D polyacrylamide gel electrophoresis (fig.1B). From these wandering spot analyses together with the sequence gel electrophoreses of partial digests by RNases (not shown), the terminal 18 nucleotide sequences were determined and shown in fig.2. The first 17 nucleotide sequences are completely identical among the 3 segments. Two-dimensional gel analysis further suggests that the sequence homology between the minus strands of S and M segments continues rather long beyond residue 17 (fig.1B). It is also obvious that the 5′-terminal sequence of the plus strand is non-homologous to that of the

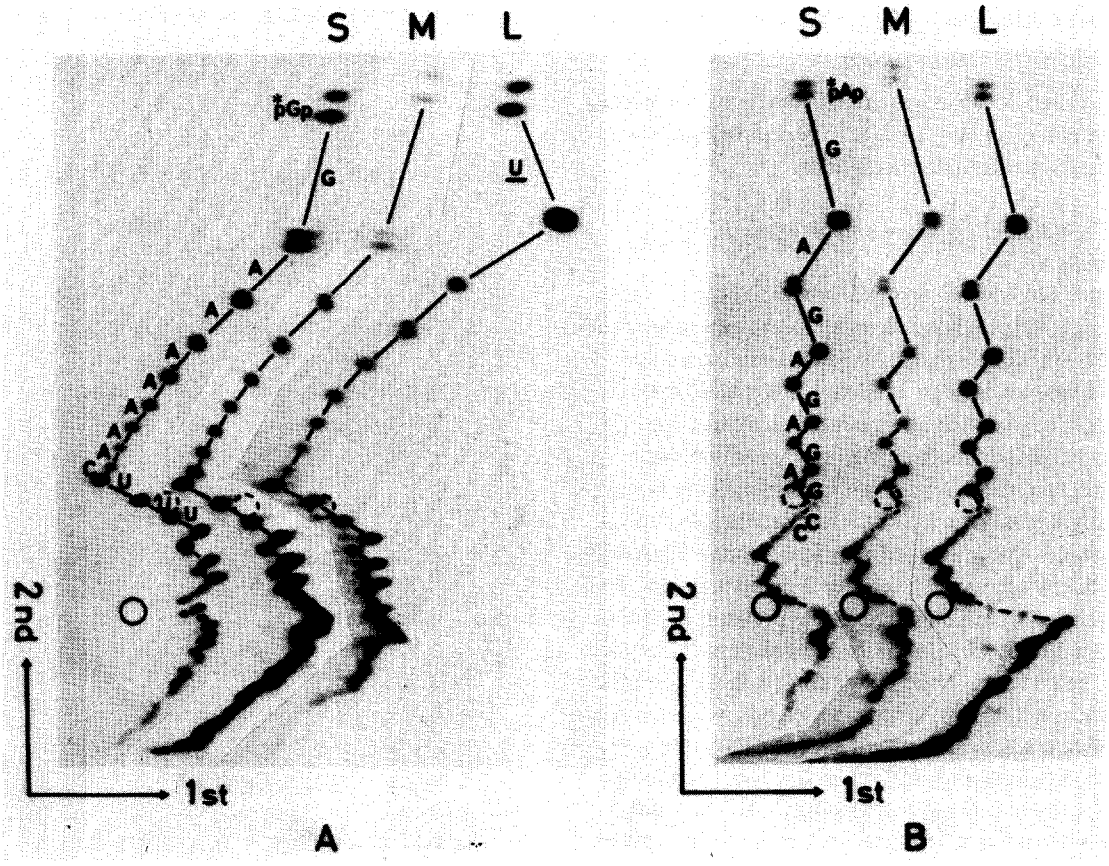

Fig.1. Two-dimensional gel electrophoresis of 5′-end labeled plus strand RNA (A) and minus strand RNA (B) of L, M and S segments after partial alkaline digestion. The solid and broken circles show the positions of the xylene cyanol and bromophenol blue markers, respectively. The xylene cyanol marker of the plus strand of M and L are not shown. The faint spots detected in the minus strand of L segment, are derived from the plus strand owing to incomplete strand separation.

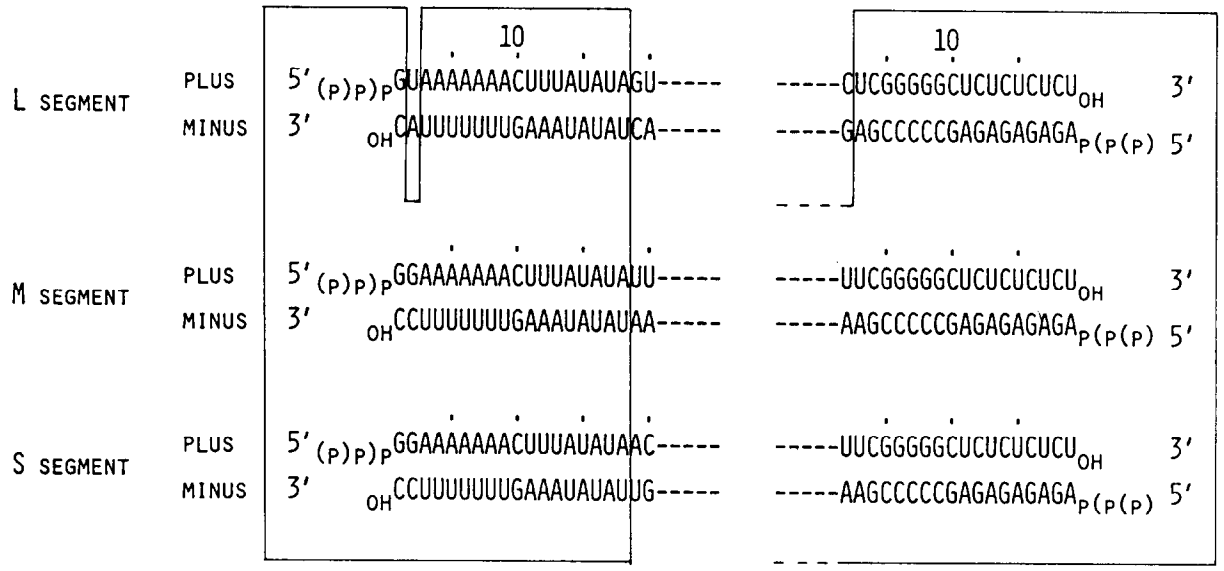

Fig.2. Terminal sequences of 3 dsRNA segments of bacteriophage ϕ6. The 3′-OH is deduced from the specificity of T4 RNA ligase.

minus strand in each segment, although both sequences share a common feature that they are very purine-rich up to the first 10 nucleotides.

3.3. *3′-End labeling of genome RNA and 2-D gel analysis*

Three ϕ6 dsRNA segments labeled at their 3′-termini by [^{32}P]pCp were electrophoretically separated. Three segments are equally labeled, as shown in table 1. 3′-End base analysis of L, M and S segments showed that each segment has the same 3′-termini; C and U (table 1). Without separating the plus and minus strands, each dsRNA segment labeled at 3′-ends was partially hydrolyzed by alkali and separated on 2-D gel system. As shown for the representative RNA segment S, the digest resolved into 2 distinct tracks. The 2 top spots, which migrated at the position of dinucleotide diphosphate in the 2nd-dimension, correspond to U(*pCp) and C(*pCp). We refer to the 3′-terminal sequences without their *pCp added by T4 RNA ligase. When we traced one series of spots as shown by the solid line (which ends in U) and the other as shown by the broken line (which ends in C), the mobility shifts of the solid and broken lines could be interpreted as GGCUCUCUCUCU and AAGUUUUUUUCC, respectively. These 2 sequences agreed with the complementary sequences of 5′-terminal regions of the minus and plus strands of S segment determined in section 3.2, respectively. The same explanation is

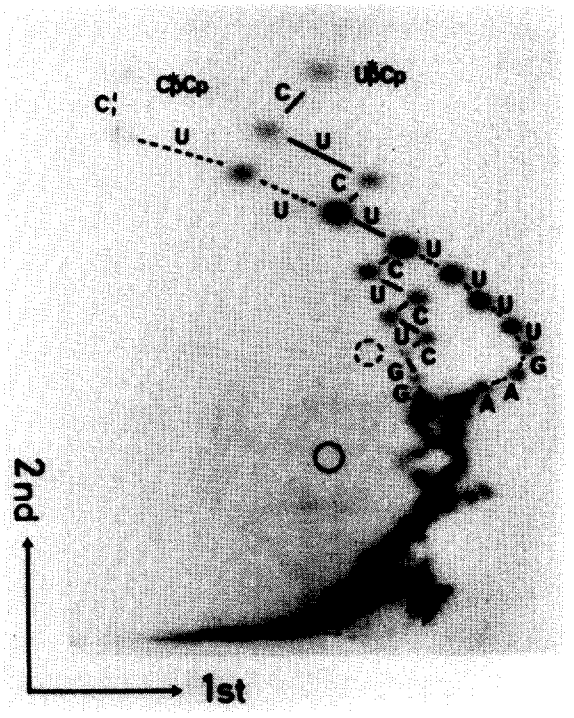

Fig.3. Two-dimensional gel electrophoresis of 3′-end labeled S segment of dsRNA after partial alkaline digestion. The solid and broken circle show the positions of the xylene cyanol and bromophenol blue markers, respectively.

applied to the other 2 segments (M and S) (not shown). These results are summarized in fig.2.

4. Discussion

We determined 5′-terminal sequences of both the plus and minus strands of all three ϕ6 dsRNA genome segments. These results, together with the 3′-terminal sequence analysis, eliminate the possibility that dsRNA segments have ssRNA tails as reported in [8] and we conclude that each segment is base-paired to the end (fig.2). Three ϕ6 dsRNA segments (L,M,S) have extensively homologous sequences at both ends (fig.2). Reovirus, a dsRNA animal virus having 10 genome segments, has also common sequences at the terminal regions of their segmented genomes [20,15] but the homology is less extensive than that of bacteriophage ϕ6.

The left regions of 3 segments in fig.2 which contain the 5′-terminal sequences of the plus strands have a long common sequence (residue 1,3–18) and the sequence probably contains the recognition site of the virion-associated RNA transcriptase. This region has a very low G–C content (2 and 3 G–C pair in the first 18 basepairs of L segment and the others, respectively). Interestingly, the dsRNA genome of *Saccharomyces cerevisiae* virus (ScV) also has a terminus of low G–C content for its transcriptase recognition site [21].

The penetrated ϕ6 subviral particle [22,23] transcribes L, M and S to produce almost similar amounts of *l*, *m* and *s* in the early infection step [6,7] while the previrion II formed in the late infection step produces exclusively *m* and *s* mRNA [7]. The difference in residue 2 between L segment and the others may have some roles to regulate the temporal expression among the segmented genome.

On the other hand, 3′-terminal sequences of the plus strands (right end of each segment shown in fig.2) are expected to function as the initiation site of replication (ssRNA → dsRNA step). The identical sequence (residues 1–17) among 3 segments is expected to contain the recognition site of replicase. Our studies on ϕ6 morphogenesis [7] have suggested that this replication step is coupled with the formation of previrion II which contains 3 dsRNA segments. So the sequence homology in this region may be also necessary for the correct assembly of 3 segments into 1 virion.

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