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NOTE

Terminal Nucleotide Sequences in the Double-stranded RNA Genome Segments of Infectious Pancreatic Necrosis Virus DRT Strain

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The terminal regions of the double-stranded RNA (dsRNA) genome segments of infectious pancreatic necrosis virus (IPNV) DRT strain were sequenced. The dsRNAs, which were ^{32}P-labelled at their 3'-termini by incubation with [^{32}P]pCp and T4 RNA ligase, were separated by 5% polyacrylamide gel electrophoresis, and the segments A and B of IPNV-DRT were sequenced by two-dimensional gel electrophoresis. The 5'-terminal sequences of the IPNV-DRT plus strand from two genome segments were found to have the same conserved nucleotide (5'-CGG(C/A)A-), but the 3'-terminal sequences -CCCCAGGCG-3' and -CGGACCCCG-3' were found in the plus strand from segments A and B, respectively. The inverted oligonucleotide sequences of 3'-terminal of between segments A and B were found and they differ from those of other IPNVs.

Infectious pancreatic necrosis virus (IPNV) is a member of the family *Birnaviridae*. It possesses a bisegmented double-stranded (ds) RNA genome. Genome segment A (approximately 3,100 bp) encodes three proteins as a polyprotein in a single large open reading frame, which is cleaved by a virus-coded protease (1-3). The small ORF (444 bases) on genome segment A, in a different reading frame, could encode for a 17 kDa arginine-rich protein, tentatively designated VP5 (1, 3, 6), but its function is still unknown. Genome segment B (approximately 2,800 bp) encodes a single gene product (VP1), the putative RNA dependent RNA polymerase. Until now, only three strains of IPNV have been totally sequenced over segment A: Jasper (4), N1 strain (6), DRT strain (2).

However, the terminal nucleotide sequences of the genome segments of these strains of IPNV have not been determined. The terminal sequences of the dsRNA segments have been of significant interest in relation to understanding mechanisms of transcription initiation and possibly genome packaging has been recognized. We now report the terminal sequences of genome segments of IPNV-DRT by direct RNA sequencing using 3'-pCp labeled RNA, by enzymatic method and by wandering spot analysis.

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Extraction and purification of dsRNAs from the IPNV particles were done as previously described (1). The 5'and 3'-terminal regions were directly sequenced by a dideoxynucleotide chain termination method using reverse transcriptase and synthetic oligonucleotide (Table 1).

Purified dsRNAs were labelled at their 3' termini using 3', 5' bis-cytidine [^{32}P] diphosphate (pCp; 3000 ci/mmol, Amersham Co.) and T4 RNA ligase (Takara Shuzo Co.) as described by England & Uhlenbeck (5) and Kuchino *et al.* (7). The labelled dsRNA genome segments were separated by 5% PAGE in Tris-boric acid-EDTA. Individual genome segments were localized by autoradiography and eluted from the crushed gel as described by Smith (9).

The 3'-terminally labelled RNAs were digested by using RNase T2 (Yamasa Shoyu Co). The complete digests were analysed on a polyethyleneimine (PEI)-cellulose sheet developed in 0.4 M-LiCl. The terminal nucleotides were identified by autoradiography of the chromatograph. The radioactive phosphate was predominantly incorporated into GMP. Incorporation into AMP was less 50% of that into GMP. Therefore, it is possible that the 3'-termini of plus and minus strands of genome segments A and B are G (Fig. 1). The 5' terminal labelled RNAs were also analyzed. Incorporation of radioactivity of the 5'-termini of plus and minus strands of genome segments A and B were detected predominantly

Table 1. Primers for direct sequencing of the genomic dsRNA of DRT strain.

Primers	Base position*	Base sequences (5'→3')
A5'	70 to 86	5' TAGAAAGGGCTTTCGCC 3'
A3'	3053 to 3069	5' CCCGACTGATACCCTGG 3'
B5'	87 to 103	5' CATATCATGAGTTGTGT 3'
B3'	2671 to 2687	5' TCAGCTGGACTCATATG 3'

* The positions of the primers correspond to the sequences of the DRT genome (Chung *et al.*, 1994ab).

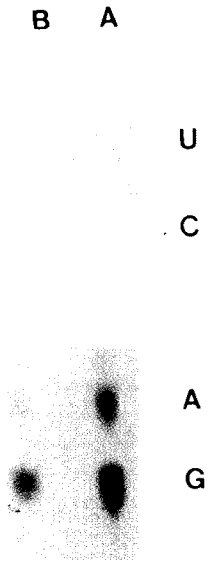

Fig. 1. PEI-cellulose chromatograph of 3'-terminal-labelled genomic dsRNA segments A and B.
The dsRNA segments were completely digested with nuclease T2 and chromatographed.

in CMP (data not shown).

Without separating the plus and minus strands, two 3'-terminal labelled dsRNA segments were partially hydrolyzed by alkaline in 100 mM $NaHCO_3$ for 1 h at 1000°C, and the product was subjected to 2D PAGE system as described by Kudo *et al.* (8). The sequence was read after exposing the gel at Bioimmaging analyzer (BAS 1000 MacBAS, FuJIX) for 1 h.

Fig. 2 shows the terminal nucleotide sequence analyses of segments A and B of IPNV-DRT. Although two tracks were expected, only one wandering spot could be resolved up to 21 nucleotides from the 3'-terminus of segment A. This indicated that only one strand of the genomic dsRNA has been labelled by T4 RNA ligase. The sequence obtained from 2D PAGE was interpreted to be -UGAAUUGAAACUCUCUGUCCG-3'. This sequence was that of a minus strand because it was complementary to that obtained from the analysis of the cDNA of segment A containing the plus sense 5'-terminal portion. The 3'-terminal sequence of plus strand obtained from dideoxynucleotide chain termination method using the viral dsRNA as template was interpreted to be -CCCCAGGCG-3' (Fig. 3, Table 2). When the 3' terminus of segment A of the genomic dsRNA was labelled with [^{32}P]pCp using RNA ligase, incorporation of radioactivity of plus strand was not possible. This may be due to no work of T4 RNA ligase. Another possibility is that some of the molecules of the plus strand of genome segment A are blocked or modified, so that they are not accessible to RNA ligase. The reason for this deficiency is unknown.

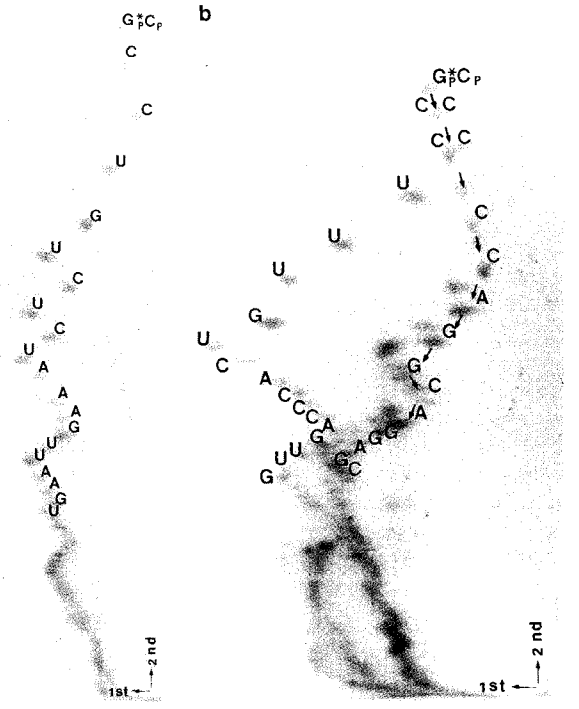

Fig. 2. Wandering spot analysis of 3'-terminal-labelled genomic dsRNA segments A(a) and B(b).
Partial alkaline digest of the RNA was fractionated by two-dimensional polyacrylamide gel electrophoresis with 7.4% polyacrylamide-6M urea gel (pH 3.3) as the first dimension and 20% polyacrylamide (pH 8.2) as the second dimension.

As shown by analysis of genome segment B, the digest resolved into two distinct tracks. There are unexpected spots on the plus strand. It is possible that the genome segment B were contaminated with defective plus strands during the experiment. When the nucleotides resolved into two distinct tracts, the sequences could be interpreted as -CGAGGACGGACCCCG-3' and -GUUGACCCACUGUUUCCG-

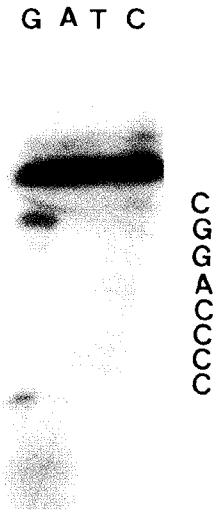

Fig. 3. RNA-dependant DNA sequence analysis of 3'-terminal sequence of plus strand of segment A by dideoxynucleotide chain termination method using reverse transcriptase.

Table 2. Summary of 3'-terminal sequences of the dsRNA segments of DRT strain.

Genome segments	Mid terminals*	Terminal sequences
A segment	5' CGGACAGA ·····	CCCCAGGCG 3'
	3' GCCUGUCU ·····	GGGGUCCGC 5'
B segment	5' CGGAAACA ·····	ACGGACCCCG 3'
	3' GCCUUUGU ·····	UGCCUGGGGC 5'

* The data of the mid terminal sequeces originated from Chung *et al.* (1994ab).

3', respectively. The former agreed with the result of the cDNA containing the 3'-terminal region of the plus strand sequencing analyses. The latter agreed with the complementary sequences of 5'-terminal regions of the plus strand of segment B determined by analyses of 5'-terminal cDNA. Therefore, these sequences represented those of the 3'-terminal regions of the plus and minus strands, respectively.

The 3'-terminal sequences of plus strands obtained from genome segments between A and B showed inverted oligonucleotide pattern each other. The 3'-terminal sequences of plus strand is expected to play a significant role in the initiation of minus strand synthesis and/or the packaging of the genome segment. Thus, it was surprising to find the inverted terminal sequences of segment A and B.

On the other hand, the 5'-terminal sequence of two genome segments is conserved, 5'-CGGAAA- and 5'-CGGACA - according to complementary sequences of 3'-terminal sequence (Fig. 3, Table 2). Duncan *et al.* (4) reported tha the cDNA sequences of segments A and B of IPNV-Jasper both start with a conserved pentanucleotide sequence (GGAAA) and terminate with a conserved tetranucleotide sequence (CCCC). Analyses of the terminal sequecnes of other members of the birnavirus will be of interest for the understanding of transcription initiation and genome packaging. The precise nature of the 3'-terminus of plus strand of IPNVs awaits further analysis.

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