Cloning of the *Nilaparvata lugens* reovirus genome: conserved terminal nucleotide sequences and nucleotide sequence of genome segment S10

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The segmented double-stranded RNA genome of *Nilaparvata lugens* reovirus (NLRV) was cloned, and the nucleotide sequence of genome segment S10 and terminal nucleotide sequences of the rest of the segments were determined. Genome segment S10 of NLRV consisted of 1430 nucleotides with a single open reading frame extending for 1293 nucleotides from nucleotide 46. It encoded a polypeptide of 431 amino acids with an M_r of 49·4K, which was a non-structural protein. The plus-strand RNA of all genome segments had the same conserved trinucleotide 5′ AGU- and hexanucleotide-GUUGUC 3′ in the 5′- and 3′-terminal regions, respectively. These conserved terminal sequences resembled those found in the segments of the members of the genus *Fijivirus*.

Nilaparvata lugens reovirus (NLRV) found in a healthy colony of the brown planthopper has double-shelled particles approximately 65 nm in diameter and 10 genome segments of dsRNA (Noda *et al.*, 1991*a*). This virus propagates in the planthopper, *N. lugens*, but not in rice, the only host plant of *N. lugens*, thus showing biological properties different from those of plant reoviruses that are transmitted by planthoppers. The family *Reoviridae* comprises nine genera: *Orthoreovirus*, *Aquareovirus*, *Coltivirus*, *Orbivirus*, *Rotavirus*, *Cypovirus*, *Fijivirus*, *Phytoreovirus* and *Oryzavirus* (Francki *et al.*, 1991; Holmes *et al.*, 1993). Members of the *Cypovirus* genus (cytoplasmic polyhedrosis virus; CPV) and some members of the *Aquareovirus* genus infect invertebrates, and those of the last three genera infect plants. Members of the genera *Coltivirus*, *Orbivirus*, *Fijivirus*, *Phytoreovirus* and *Oryzavirus* are transmitted by invertebrate vectors; the coltiviruses are transmitted by ticks, the orbiviruses by ticks, mosquitoes and *Culicoides* species, the phytoreoviruses by leafhoppers, and the fijiviruses and oryzaviruses by planthoppers.

Recent nucleotide sequence analyses have revealed the presence of common or similar structures in both nucleotide and amino acid sequences of the genome segments among viruses belonging to the same genera. In the *Phytoreovirus* genus, for example, wound tumour virus (WTV), rice dwarf virus (RDV) and rice gall dwarf virus (RGDV) have common sequences in both the 5′ and 3′ termini of all genome segments (Kudo *et al.*, 1991). They also show high similarity between amino acid sequences deduced from the corresponding genome segments (Suzuki *et al.*, 1990; Noda *et al.*, 1991*b*). The terminal nucleotide sequences are not always quite the same among the members of the same genus, for example in the cypoviruses and the orbiviruses; nevertheless nucleotide sequence analysis appears to be a reliable index for classification of genera in the family *Reoviridae*.

To classify NLRV and to clarify the relationship between it and other family members, we cloned the genome and determined the conserved terminal nucleotide sequences of all 10 dsRNA genome segments. The complete nucleotide sequence of genome segment S10 was also determined.

NLRV was purified from a naturally infected Izumo colony of the brown planthopper, *N. lugens* (Noda *et al.*, 1991*a*). Genomic dsRNA was extracted from the purified virus particles by 1% SDS and 0·01 M-EDTA at 37 °C for 10 min. The extracted dsRNA was passed through a mini-column (10 cm long) of Sepharose 2B or CL-2B (Pharmacia LKB) and approximately 7 μg of RNA was used for cloning. The 3′ termini of both plus and minus strands of the dsRNA were first polyadenylated with poly(A) polymerase at 37 °C for 8 min, according to the method of Cashdollar *et al.* (1982). For synthesis of cDNA, we employed the cDNA Synthesis System Plus kit (Amersham). The polyadenylated dsRNA was boiled for 3 min and immediately cooled on ice, then the first strand of cDNA was synthesized with oligo(dT) primer. The synthesized cDNA, which had blunt ends in both the 5′ and 3′ termini due to treatment with T4 DNA

The nucleotide sequence data for NLRV S10 reported in this paper will appear in the DDBJ, EMBL and GenBank databases under the accession number D14691.

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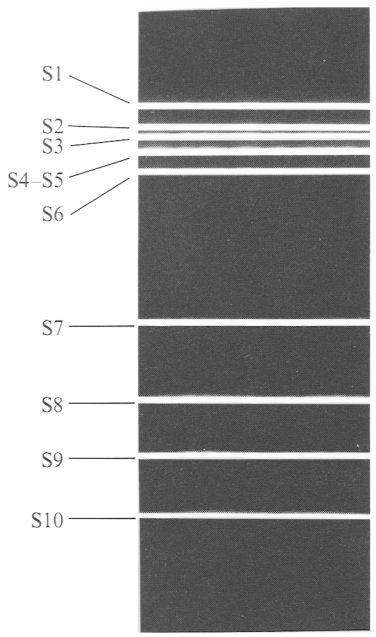

Fig. 1. Electrophoretic separation of genomic dsRNA segments of NLRV by 10% SDS–PAGE. Segments S4 and S5 comigrate.

Table 1. *The 5′- and 3′-terminal nucleotide sequences of the plus strands of NLRV genome segments*

S1	5′	AGUCUUUGAACAC-----GAGAAAAGUUGUC 3′
S2	5′	AGUUCGUGAACCA-----UUCCACAGUUGUC 3′
S3	5′	AGUUUGUUGUGAU-----CACACUUGUUGUC 3′
S4	5′	AGUUUGUAGUGAC-----ACUCGCAGUUGUC 3′
S5	5′	AGUAUGCUUUCCG-----ACAACAUGUUGUC 3′
S6	5′	AGUUCGAAACGAC-----CGUUGUUGUUGUC 3′
S7	5′	AGUCAAUACCAGA-----GGUACUAGUUGUC 3′
S8	5′	AGUUUGAUGACCA-----GUUACUAGUUGUC 3′
S9	5′	AGUUUCUAAACCA-----GGUUACUGUUGUC 3′
S10	5′	AGUUCGAAUCACA-----UGAUACUGUUGUC 3′

polymerase, was inserted into the *Sma*I site of pBluescript II (Stratagene). Transformants of *Escherichia coli* strain NM522 were isolated by standard techniques.

The 3′ ends of the two strands of the dsRNA are polyadenylated for cDNA synthesis, so that the terminal base of each strand following adenine addition cannot be distinguished from natural adenine. Poly(A) polymerase has the ability to synthesize RNA with ribonucleotides other than adenine though this activity is not as high as with adenine (Sano & Feix, 1976). Therefore, in another trial cDNA cloning, we first added cytosine to the 3′ ends of dsRNA in order to distinguish the terminal bases of the plus and minus strands. Denatured genomic RNA was incubated with cytosine at 37 °C for 5 min and was then polyadenylated, and cDNA was synthesized as above.

The cloned cDNAs were identified by hybridizing genomic RNA labelled with an enhanced chemiluminescence oligonucleotide labelling and detection system (Amersham). NLRV dsRNA was electrophoresed in a 10% polyacrylamide gel (Laemmli, 1970) and each genome segment of dsRNA was recovered from the gel. The segments were designated S10 to S1 in order of migration speed in PAGE (Fig. 1). Since genome segments S4 and S5 comigrated in the gel, clones corresponding to each one were distinguished by hybridizing their cDNA inserts. Plasmid DNA from each colony was prepared by a one-step miniprep method (Chowdhury, 1991), blotted onto a nylon membrane (Hybond N^+, Amersham), and hybridized with the labelled RNA probes. Insert sizes in plasmids were determined by agarose gel electrophoresis.

Sequences of the cloned cDNA in pBluescript II were determined by the *Taq* dye primer cycle sequencing method using a DNA Sequence System (Applied Biosystems Model 373A). The plasmid DNA was prepared by the alkaline lysis procedure (Sambrook *et al.*, 1989), and cDNA inserts were amplified by a thermal cycler using fluorescent T3 or T7 primers.

The 5′-terminal sequences of the plus and minus strands of the cDNA inserts were randomly examined using clones corresponding to S1 to S10 from the cDNA library. Two common sequences were recognized: 5′-TTTTTAGT-3′ and 5′-TTTTTGACAAC-3′. These sequences must include a sequence corresponding to the 5′-terminal region of plus and minus strands of dsRNA. Other cDNAs cloned from genome RNAs with cytosine added at the 3′ ends were then examined. Among 21 clones that contained the terminal sequences, nine had additional guanines in the 5′ end of cDNA inserts. Four clones of S3 or S5 were found to have guanine between poly(T) and AGT-3′ and five clones of S1, S7, S9 or S10 had guanines between poly(T) and GACAAC-3′. The other 12 clones did not have additional guanines between poly(T) and the inner sequences. This suggests that the activity of poly(A) polymerase when adding cytosine to the 3′ end of RNA is quite low in comparison to that with adenine as a substrate.

The above results indicate that the 5′-terminal sequences of segmented dsRNAs of NLRV are 5′ AGU- and 5′ GACAAC-, one belonging to the plus strand and the other to the minus strand. In order to determine the polarity of the strands of the dsRNA, the smallest segment, S10, was completely sequenced as mentioned later. An open reading frame was found on the strand including 5′ AGU- at the 5′ end, confirming that it was the plus strand. The other strand, which included 5′ GACAAC- at the 5′ end and which did not code for proteins of significant size, was the minus strand. Therefore, nucleotide sequences of the 5′ terminus and the 3′ terminus of the plus strand of genome segments were 5′ AGU- and -GUUGUC 3′, respectively. The

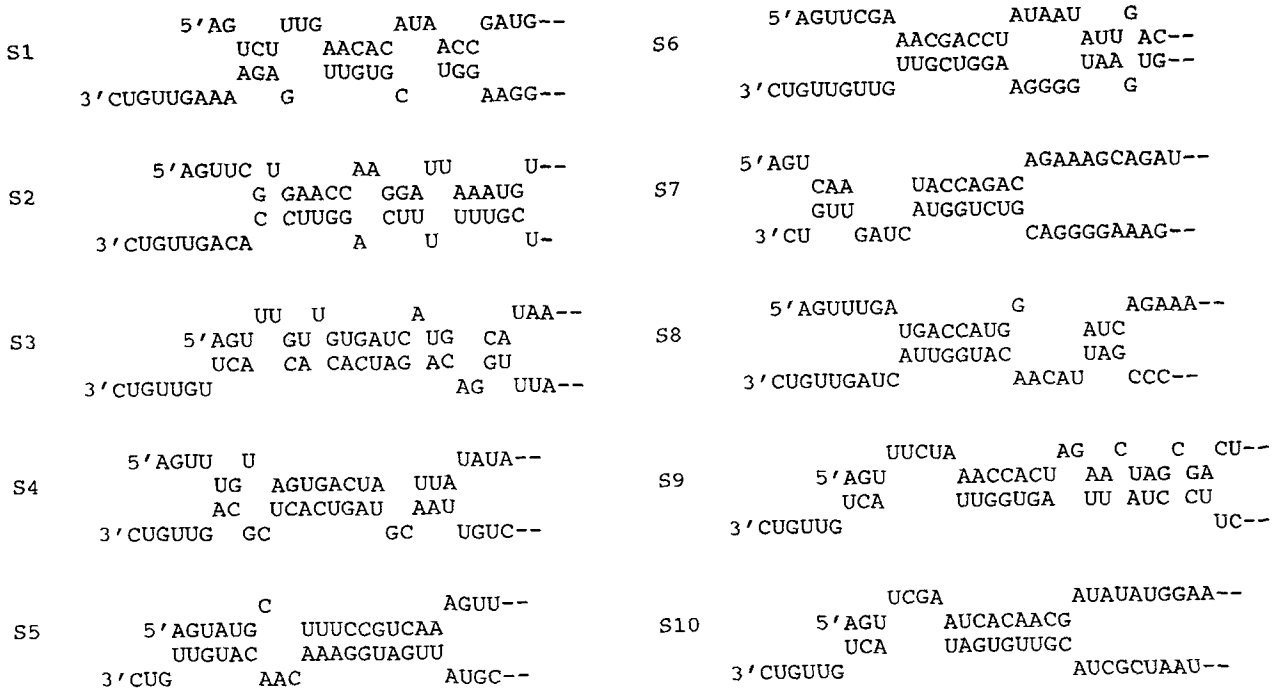

Fig. 2. Segment-specific inverted repeats in the terminal regions of the plus strands of all 10 genome segments of NLRV.

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AGTTCGAATCACAACGATATATGGAAGGTTAAGATTTGCACAAAAATGACTTTGCGACGAGCAACGAACAAATATAATGATTTTAGTAAAAAGTTTTATGACACTAGCTACGATGAACAA  120
                                                  M  T  L  R  R  A  T  N  K  Y  N  D  F  S  K  K  F  Y  D  T  S  Y  D  E  Q   25

CAGTACAGTATATTATATCAACCAATTAGCACCCATAAAGCACCGGTACGTTTAGAGAATTTCTCAAATTATTATTTGATTAGAACAGAACAAACGAAAATTATTGAAGAAATTGACCCC  240
 Q  Y  S  I  L  Y  Q  P  I  S  T  H  K  A  P  V  R  L  E  N  F  S  N  Y  Y  L  I  R  T  E  Q  T  K  I  I  E  E  I  D  P   65

CCTGTATTCATTGACGAGCAATACGGATGTGATCATTTTACTCAAAGCGTCGATTTGAACCAAATCTTGTGGGAAGTGTTCATGGGAGATGGTGATGTCGCCGCTTTGAGTTTAAACATG  360
 P  V  F  I  D  E  Q  Y  G  C  D  H  F  T  Q  S  V  D  L  N  Q  I  L  W  E  V  F  M  G  D  G  D  V  A  A  L  S  L  N  M  105

CTACGTATATATGATACTTACAAAAGTAAATTTGCAATAGTAAATAGATTTGGTATTTACGAATATTTACCTGAAGGTCATGACGACGATTTTGATGATGAAATGTTAGGTGTAATGAAC  480
 L  R  I  Y  D  T  Y  K  S  K  F  A  I  V  N  R  F  G  I  Y  E  Y  L  P  E  G  H  D  D  D  F  D  D  E  M  L  G  V  M  N  145

ATTTTTGGAATTATCGGTAAAGCTTTATTTAAAGATCAGTATATTACAGTCGACAGATTACGTATTACACATGAGAGATTTATCAATTGTTGGAAAGGACGTAGCTTTAGATCAGTGCAT  600
 I  F  G  I  I  G  K  A  L  F  K  D  Q  Y  I  T  V  D  R  L  R  I  T  H  E  R  F  I  N  C  W  K  G  R  S  F  R  S  V  H  185

GTCAATTGGGCTTCAAATGATGTTTTAGAGCGATCAAATGTTGTTACTCGAATGACTAATAATGTTGTAAAACAGTACATTGCTGAGCATCTTCATGATTTATTTTGTTGGCTTTTGGAA  720
 V  N  W  A  S  N  D  V  L  E  R  S  N  V  V  T  R  M  T  N  N  V  V  K  Q  Y  I  A  E  H  L  H  D  L  F  C  W  L  L  E  225

CCAGAACGATCGTATGGTGGTCAAGTTATGCACATTCAAAATTTATTGGGTTATTACTATATCTCACGGTTTGGATCGCCTTCTGGTGCTTATACTAATAAGATAGGTGGAGGGACGACT  840
 P  E  R  S  Y  G  G  Q  V  M  H  I  Q  N  L  L  G  Y  Y  Y  I  S  R  F  G  S  P  S  G  A  Y  T  N  K  I  G  G  G  T  T  265

TATACGTTGCCTAACACAAAAATCAGTTGTACTAACGTTTCTGGGTATAAAAATAACACTATTGGTAGATCATGGGCTTGTGCTGAATTATATATTGCATTACTCCGTAATTATGATGCA  960
 Y  T  L  P  N  T  K  I  S  C  T  N  V  S  G  Y  K  N  N  T  I  G  R  S  W  A  C  A  E  L  Y  I  A  L  L  R  N  Y  D  A  305

GGTCATAATATTGCTTCATTTTTCTTAAATCGTGAACTAATTGAGAAAGAATCTGAACAGTATTTACCATTCTCATCTGGAGGTTCAGGTATTAAAGCTGGGACATTAAGTAGAAGTCGT 1080
 G  H  N  I  A  S  F  F  L  N  R  E  L  I  E  K  E  S  E  Q  Y  L  P  F  S  S  G  G  S  G  I  K  A  G  T  L  S  R  S  R  345

AGCACAGTGGGGGCTACTCCTGACTTACGTAAGATTTTAAGGCCTAGGGACCAAGGACGTATTGTAAAAATTAAAACGAAAGATGGTAAAACTATAGAATTAGATGAAACTTTAGATATG 1200
 S  T  V  G  A  T  P  D  L  R  K  I  L  R  P  R  D  Q  G  R  I  V  K  I  K  T  K  D  G  K  T  I  E  L  D  E  T  L  D  M  385

GATAAACTACGTAAGTTGTGTGCTGATCCTTTGTCTGATGGTGAGGAAGATGCAACTAACGAGTTTAGTGCTGGCTATCCACCTAAAGACGGAGTTACAAAACAGAATGCTGAACCTATA 1320
 D  K  L  R  K  L  C  A  D  P  L  S  D  G  E  E  D  A  T  N  E  F  S  A  G  Y  P  P  K  D  G  V  T  K  Q  N  A  E  P  I  425

TATGAAATACCCAAATCTTAAGATGTTTGTTGTTCGCAGTATGTAATTGTTCATGCCAATCGGCTCTTAATGGCTTCTATATATAATCGCTACGTTGTGATACTGTTGTC.          1430
 Y  E  I  P  K  S  *                                                                                                    431
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Fig. 3. Nucleotide sequence of the plus-sense strand of segment S10 of NLRV and the amino acid sequence of its predicted translation product. The in-phase termination codon is indicated with an asterisk.

Table 2. *Conserved 5′- and 3′-terminal nucleotide sequences of the plus strands in* Reoviridae*

Orthoreovirus	5′ GCUA---------UCAUC 3′
Serotype 3	
Aquareovirus	
Coltivirus	
Orbivirus	5′ GUUAAA------ACUUAC 3′
BTV	
Rotavirus	5′ GGCAUU-----UGUGACC 3′
	U G
Cypovirus	5′ AGUAA------GUUAGCC 3′
BmCPV	
Fijivirus	5′ AAGUUUUUU-----UGUC 3′
RBSDV, MRDV	
Phytoreovirus	5′ GGUA----------UGAU 3′
WTV, RDV, RGDV	C C
Oryzavirus	5′ GAUAAA--------GUGC 3′
RRSV	
NLRV	5′ AGU-----GUUGUC 3′

* Modified from Kudo *et al.*, 1991. Classification is based on Holmes *et al.* (1993). Sequences are taken from: *Orthoreovirus* (McCrae, 1981; Antczak *et al.*, 1982); *Orbivirus* (Mertens & Sangar, 1985); *Rotavirus* (Imai *et al.*, 1983; Elleman *et al.*, 1983; Both *et al.*, 1984; Ward *et al.*, 1984); *Cypovirus* (Kuchino *et al.*, 1982); *Phytoreovirus* (Anzola *et al.*, 1987; Kudo *et al.*, 1991); *Fijivirus* (Marzachi *et al.*, 1991; Azuhata *et al.*, 1992); *Oryzavirus* (Yan *et al.*, 1992).

conserved nucleotide sequences of both 5′ and 3′ termini of all 10 genome segments in NLRV are shown in Table 1.

The conserved terminal sequences together with adjacent segment-specific domains of inverted complementarity (inverted repeats) are considered to play an important role in sorting and assembly of segmented RNA genomes (Anzola *et al.*, 1987). Both structures, the conserved terminal sequences and the inverted repeats, are found in WTV (Nuss & Dall, 1990), RDV and RGDV (Noda *et al.*, 1991*b*; Kudo *et al.*, 1991). In addition to the conserved terminal sequences, analysis by DNASIS (Hitachi Software Engineering) showed the inverted repeats in all genome segments of NLRV (Fig. 2).

The nucleotide sequence of segment S10 was determined using deletion mutants from two full-length cDNA clones with opposite orientations. cDNAs were unidirectionally deleted by exonuclease digestion (DNA Deletion Kit, Takara Shuzo), and contained 1430 bp. The entire sequence coincided in the two cDNA clones except for two bases, residues 963 and 1040. Therefore, the corresponding regions of two other cDNA clones of S10 were analysed and residues 963 and 1040 were finally determined as T and G, respectively (Fig. 3). GC content was 35·2%, and the sequence contained one open reading frame which started from residue 46 and extended for 1293 nucleotides. The open reading frame coded for a polypeptide composed of 431 amino acids with a calculated M_r of $4{\cdot}94 \times 10^4$. With an M_r of 49·4K, the polypeptide is smaller than the smallest structural protein of NLRV, which is 64K (Noda *et al.*, 1991*a*). The genome segment S10 of NLRV, therefore, codes for a non-structural protein. A search for a sequence homologous to that of the predicted 49·4K polypeptide in the PIR database library first pointed to the protein 1 of maize rough dwarf virus (MRDV) segment 6. These two polypeptides showed 19·1% identity in a 361 amino acid overlap; the homology was not as high as that seen among corresponding segments in the members of the genus *Phytoreovirus* (Nuss & Dall, 1990; Noda *et al.*, 1991*b*).

Analyses of the conserved terminal nucleotide sequences of dsRNA seem to indicate that NLRV is a member of the *Fijivirus* genus (Table 2). The common nucleotide sequence in the 5′ terminus of NLRV, 5′ AGU-, is recognized from the second to the fourth bases in the 5′ terminus of MRDV and rice black-streaked dwarf virus (RBSDV), and the nucleotide sequence in the 3′ terminus of NLRV is found in the 3′ terminus of RBSDV and MRDV as a tetranucleotide sequence, -UGUC 3′ (Marzachi *et al.*, 1991; Azuhata *et al.*, 1992), suggesting that NLRV and the members of the genus *Fijivirus* are evolutionarily related. NLRV, however, lacked the 5′-terminal base of RBSDV and MRDV, adenine. In this respect, terminal sequences are not always the same among members of the same genus. In the genus *Cypovirus*, for example, the terminal sequence of CPV of *Bombyx mori* (BmCPV) is 5′ AGUAA---GUUAGCC 3′, as shown in Table 2 (Kuchino *et al.*, 1982), whereas that of *Euxoa scandens* CPV (EsCPV) is 5′ AGUUU---GAGUUGC 3′ (Fossiez *et al.*, 1989). There is another example in the genus *Orbivirus*. Broadhaven virus has 5′ GUAAAA---AGAUAC 3′ as a terminal sequence of segment 5 (Moss *et al.*, 1990), whereas bluetongue virus (BTV) and Ibaraki virus have 5′ GUUAAA---ACUUAC 3′ (Mertens & Sangar, 1985). Lack of the 5′-terminal base, adenine, in NLRV, therefore, does not exclude NLRV from the genus *Fijivirus*. The fact that the predicted amino acid sequence of S10 showed a homology with protein 1 of MRDV segment 6 may also support the suggestion that NLRV is a member of the genus *Fijivirus*.

The genome analysis seems to contribute also to clarification of the evolution of the reoviruses. It is generally believed that reoviruses originate from invertebrates and that their association with insects is older than those with vertebrates and plants, because the reoviruses propagate in insects with no significant pathological effects except for members of *Cypovirus* genus, but they show a pathological effect on vertebrates and plants (Nault & Ammar, 1989). Sequence analyses of commensal viruses, such as NLRV, should provide invaluable information for evolutionary considerations of the reoviruses, because genome sequence similarities, ir-

respective of host, appear to be important from the point of view of the unity of the virus world (Van Regenmortel, 1990).

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