Virology 258, 118–127 (1999)
Article ID viro.1999.9691, available online at http://www.idealibrary.com on IDEAL®

Multiple Mitochondrial Viruses in an Isolate of the Dutch Elm Disease Fungus *Ophiostoma novo-ulmi*

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Received December 16, 1998; returned to author for revision January 26, 1999; accepted March 3, 1999

The nucleotide sequences of three mitochondrial virus double-stranded (ds) RNAs, RNA-4 (2599 nucleotides), RNA-5 (2474 nucleotides), and RNA-6 (2343 nucleotides), in a diseased isolate Log1/3–8d[2] (Ld) of the Dutch elm disease fungus *Ophiostoma novo-ulmi* have been determined. All these RNAs are A-U-rich (71–73% A + U residues). Using the fungal mitochondrial genetic code in which UGA codes for tryptophan, the positive-strand of each of RNAs 4, 5, and 6 contains a single open reading frame (ORF) with the potential to encode a protein of 783, 729, and 695 amino acids, respectively, all of which contain conserved motifs characteristic of RNA-dependent RNA polymerases (RdRps). Sequence comparisons showed that these RNAs are related to each other and to a previously characterized RNA, RNA-3a, from the same *O. novo-ulmi* isolate, especially within the RdRp-like motifs. However, the overall RNA nucleotide and RdRp amino acid sequence identities were relatively low (43–55% and 20–32%, respectively). The 5′- and 3′-terminal sequences of these RNAs are different, but they can all be folded into potentially stable stem-loop structures. Those of RNA-4 and RNA-6 have inverted complementarity, potentially forming panhandle structures. Their molecular and biological properties indicate that RNAs 3a, 4, 5, and 6 are the genomes of four different viruses, which replicate independently in the same cell. These four viruses are also related to a mitochondrial RNA virus from another fungus, *Cryphonectria parasitica*, recently designated the type species of the *Mitovirus* genus of the Narnaviridae family, and to a virus from the fungus *Rhizoctonia solani*. It is proposed that the four *O. novo-ulmi* mitochondrial viruses are assigned to the *Mitovirus* genus and designated *O. novo-ulmi* mitovirus (OnuMV) 3a-Ld, 4-Ld, 5-Ld, and 6-Ld, respectively. Northern blot analysis indicated that *O. novo-ulmi* Ld nucleic acid extracts contain more single-stranded (ss, positive-stranded) RNA than dsRNA for all three newly described mitoviruses. *O. novo-ulmi* RNA-7, previously believed to be a satellite-like RNA, is shown to be a defective RNA, derived from OnuMV4-Ld RNA by multiple internal deletions. OnuMV4-Ld is therefore the helper virus for the replication of both RNA-7 and another defective RNA, RNA-10. Sequence comparisons indicate that RNA-10 could be derived from RNA-7, as previously suggested, or derived directly from RNA-4.

Key Words: Ophiostoma novo-ulmi; RNA-dependent RNA polymerase; mitovirus; defective RNA.

INTRODUCTION

A diseased isolate Log1/3–8d[2] (abbreviated Ld) of the Dutch elm disease fungus *Ophiostoma novo-ulmi* was previously reported to contain 12 unencapsidated mitochondrial virus-like double-stranded (ds) RNAs (1a, 1b, 2, 3a, 3b, 4, 5, 6, 7, 8, 9, and 10) with sizes in the range of 3.5–0.33 kb (Cole *et al.,* 1998; Rogers *et al.,* 1986, 1987). Transmission of the dsRNAs from Ld to healthy *O. novo-ulmi* isolates causes disease development, characterized by slow growth, formation of abnormal "ameboid" colonies, and reduction in numbers of viable asexual spores (Brasier, 1983), reductions in the levels of mitochondrial cytochrome oxidase (Rogers *et al.,* 1987), and the formation of mitochondrial DNA plasmids (Abu-Amero *et al.,* 1995; Charter *et al.,* 1993). Analysis of dsRNA profiles from single-conidial isolates derived from Ld has shown that the dsRNAs represent several different groups of replicons and that one or more of dsRNAs 4, 7, and 10 may constitute the genetic determinant of disease in *O. novo-ulmi* isolate Ld (Cole *et al.,* 1998; Rogers *et al.,* 1986, 1987). To exploit the potential of the Ld dsRNAs for the development of biological control agents for Dutch elm disease, molecular characterization of Ld dsRNAs has been undertaken. The nucleotide sequences of three Ld dsRNAs have been reported (Hong *et al.,* 1998a, 1998b). RNA-3a was shown to encode an RNA-dependent RNA polymerase (RdRp)-like protein, whereas RNAs 7 and 10 probably do not encode a protein. The sequence of RNA-10 was composed largely of rearranged sequences of RNA-7 and was considered to be a defective RNA.

In this report, we determined and analyzed the nucleotide sequences of *O. novo-ulmi* Ld RNAs 4, 5, and 6, each of which exists predominantly in a single-stranded (ss, positive-stranded) form and encodes a distinct but

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0042-6822/99 $30.00

TABLE 1

Nucleotide Sequence Identities of Mitochondrial Virus and Related RNAs

	OnuMV3a-Ld	OnuMV4-Ld	OnuMV5-Ld	OnuMV6-Ld	CpMV1-NB631
OnuMV4-Ld	46.2				
OnuMV5-Ld	42.9	54.8			
OnuMV6-Ld	44.9	49.6	51.8		
CpMV1-NB631	43.8	44.6	43.0	45.3	
RsM2-1A1	43.7	42.6	41.0	41.4	42.7

Note. Nucleotide sequence identities between pairs of sequences were determined using the GAP program. O. *novo-ulmi* Ld mitochondrial viruses: OnuMV4-Ld (RNA-4, present study), OnuMV5-Ld (RNA-5, present study), OnuMV6-Ld (RNA-6, present study), OnuMV3a-Ld (RNA-3a, Hong *et al.,* 1998b); *C. parasitica* NB631 mitochondrial dsRNA (CpMV1-NB631, Polashock and Hillman, 1994); and *R. solani* 1A1 M2 dsRNA (RsM2-1A1, Lakshman *et al.,* 1998).

related RdRp-like protein. We also analyzed evolutionary relationships among putative RdRps encoded by *O. novo-ulmi* Ld RNAs 4, 5, and 6, by *O. novo-ulmi* Ld RNA-3a, and by other fungal mitochondrial RNAs. RNA-7 was shown to be derived from RNA-4.

RESULTS AND DISCUSSION

Nucleotide sequences of RNAs 4, 5, and 6

To characterize the dsRNAs associated with *O. novo-ulmi* isolate Ld, cDNA libraries corresponding to RNAs 4, 5, and 6, respectively, were constructed using gel-purified individual dsRNA species. cDNA clones specific for each RNA were identified by Northern analysis and RT-PCR detection of dsRNAs from Ld and single conidial isolates containing subsets of Ld dsRNAs (Cole *et al.,* 1998; Rogers *et al.,* 1986) (data not shown). Sequence analysis of overlapping cDNA and 5′-RACE clones showed that RNAs 4, 5, and 6 contain 2599, 2474, and 2343 nucleotides, respectively. All three RNAs are enriched in A + U residues (73.3%, RNA-4; 73.2%, RNA-5; and 70.7%, RNA-6), which are higher than the 61.9% of A + U residues in RNA-3a (Hong *et al.,* 1998b). The A-U-rich character of *O. novo-ulmi* RNAs is similar to that of fungal and plant mitochondrial genomes (Cummings *et al.,* 1990; Paquin *et al.,* 1997; Unseld *et al.,* 1997) and is consistent with the mitochondrial localization of these RNAs.

Nucleotide sequence identities between pairwise combinations of RNAs 4, 5, and 6, and the previously characterized RNA-3a (Hong *et al.,* 1998b), are shown in Table 1. Considering the A-U-rich nature of the RNAs, the sequence identities are relatively low (43–55%). The significance of the sequence relationships was tested by comparing the quality of the alignments for each RNA pair with those in which the sequence of the second RNA was randomized (10 randomizations) using the GAP program. For all RNA pairs, the quality of the alignments exceeded those of the average alignments with randomized second RNA sequences by 16–31 standard deviations, indicating that the sequence relationships for all RNA pairs are highly significant. Similar tests showed that the *O. novo-ulmi* Ld dsRNAs were also significantly related to a mitochondrial dsRNA from the fungus *Cryphonectria parasitica* (Polashock and Hillman, 1994) and to the M2 dsRNA from the fungus *Rhizoctonia solani* (Lakshman *et al.,* 1998) with sequence identities in the range of 41–45%.

RNAs 4, 5, and 6 encode distinct RdRp-like proteins

The nucleotide sequences of RNAs 4, 5, and 6 were examined for the presence of ORFs on both strands. Because these RNAs are located in the mitochondria (Rogers *et al.,* 1987), UGA was used as a tryptophan codon as in the mitochondria of most ascomycete fungi (Osawa *et al.,* 1992; Paquin *et al.,* 1997). The genome organizations of RNAs 4, 5, and 6 are similar (Fig. 1), with each having a long ORF in the positive strand. The long ORF of RNA-4 contains 11 UGA codons and has the potential to encode a protein of 783 amino acids (molecular mass 92,244 kDa); that of RNA-5 contain 8 UGA codons and may encode a protein of 729 amino acids (molecular mass 86,181 kDa); and that of RNA-6 contains 7 UGA codons and may encode a protein of 695 amino acids (molecular mass 80,087 kDa). These ORFs have a codon preference of either A or U in the third wobble position, which is characteristic of mitochondrial codons (Cummings *et al.,* 1990; Paquin *et al.,* 1997; Unseld *et al.,* 1997). Analysis of these three proteins revealed that they all have amino acid sequence motifs typical of RdRps (Fig. 2). Motifs II–VI are similar to RdRp motifs described by others (Bruenn, 1991, 1993; Habili and Symons, 1989; Koonin, 1991; Poch *et al.,* 1989) and designated A–E by Hong *et al.* (1998b). Motif I is a combination of motifs X and Y described by Hong *et al.* (1998b).

Comparisons of amino acid sequences of pairwise combinations of RdRps encoded by RNAs 4, 5, and 6, and that encoded by RNA-3a (Hong *et al.,* 1998b), indicated that they are all related (Table 2). Sequence identities were much higher within the RdRp motifs I-VI (52–72%) than within the remainder of the protein, and overall

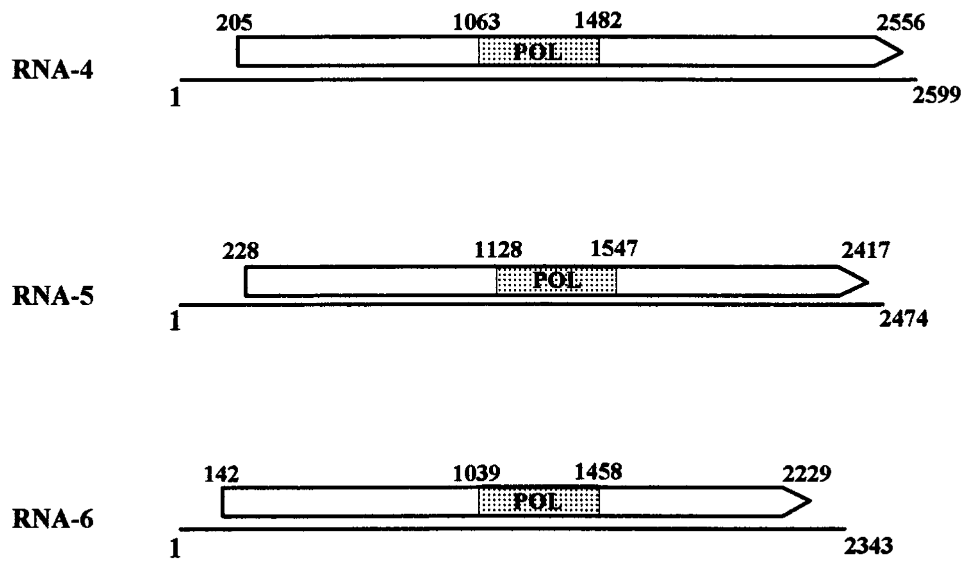

FIG. 1. Genome organizations of *O. novo-ulmi* Ld RNAs 4, 5, and 6. A single ORF appears in the positive strand of each of RNAs 4, 5, and 6, assuming UGA codons specify tryptophan. The shaded region in each ORF, designated POL, contains the RdRp motifs I–VI. The sequences of RNAs 4, 5, and 6 are available from the EMBL, GenBank, and DDBJ databases with the accession nos. AJ132754, AJ132755, and AJ132756, respectively.

sequence identities were relatively low (20–33%). Significant relationships were also found between these four *O. novo-ulmi* RdRps and RdRps encoded by a mitochondrial dsRNA of *C. parasitica* and the M2 dsRNA of *R. solani*, with amino acid sequence identities in the range of 46–64% for motifs I–VI and 19–26% for the complete proteins (Table 2). Within motif I, there are nine positions at which amino acids are identical in all six RdRps and an additional 17 positions at which the amino acids are all chemically similar. This strengthens the proposal of Hong *et al.* (1998b) that this motif, which is not found in RdRps of other RNA viruses, is characteristic of RdRps encoded by mitochondrial dsRNAs.

Only relatively short ORFs were found in the negative strands of each RNA with maximum coding capacities of 40, 60, and 100 amino acids for RNAs 4, 5, and 6, respectively.

Detection of the ss (positive-stranded) form of viral dsRNAs 4, 5, and 6

The ss (2 M LiCl-fraction) and ds (5 M LiCl-fraction) forms of RNAs 4, 5, and 6 from Ld were individually detectable by Northern blotting using riboprobes specific to each of the three RNAs (Fig. 3). The ssRNA and dsRNA were distinguished by their capability to hybridize with

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                                               I                                    II
OnuMV4-Ld    196 LGKLSIVHDPELKERVIAMVDYTTQFALRPIHNILLNNLSKLPCDRTFTQD(11)RYHSLDLSAATDRFP(38)
OnuMV5-Ld    241 FGKISIVKDPELKMRVIAMVDYHSQFVLKKIHNSLFNKLKLIKSDRTFTQD(10)RFWSMDLSAATDRFP(38)
OnuMV6-Ld    239 IRRLSIVHDPECKERVIAIFDYGSQMVLKPIADVLFDLLRNIPSDRTFTQS(12)KFWSIDLSSATDRFP(38)
OnuMV3a-Ld   260 LGKLAIKEEAAGKARVFAMADSITQSVMAPLNSWVFSKLKDLPMDGTFNQQ(18)EFYSYDLSSATDRLP(37)
RsM2-1A1     335 LSQFALKEEAAGKIRLFALMDSITQSVMSPLHDYMFAILRNIPNDGTFDQE(14)KAFSYDLTAATDRLP(48)
CpMV1-NB631  327 MGKLSVVYDQAGKARIVAITNSWIQTAFYSLHLHVFKLLKNIDQDGTFDQE(15)KFYGFDLTAATDRLP(36)
                 : ::::  :    * *:.*: :    * .:  :   ::  *  :  * ** *.     .  . **::****:*
                                .

                           III                        IV                   V        VI
OnuMV4-Ld    311 YSVGQPMGAYTSWAAFTLTHHLVVHWAA(7)FKDYIILGDDIVIKNNKVAQIYINLM(2)WGVDISLSKTHVS(4)EFAKR
OnuMV5-Ld    355 YKVGQPMGAYSSWAAFTLTHHLVVFYSA(7)FTNYILLGDDIVINNDKVAKYYIRTM(2)LGVELSMNKTHVS(4)EFAKR
OnuMV6-Ld    354 YNCGQPMGAQSSWPMFTLAHHVIVRVAA(7)FDKYIILGDDIVINNDNVALKYMEIM(2)FKVEISRNKTHVS(4)EFAKR
OnuMV3a-Ld   381 YSVGQPMGALSSWAMLALSHHVIVQIAA(7)FTNYALLGDDIVIADKAVATSYHMIM(3)LGVEINLSKSLVS(4)EFAKR
RsM2-1A1     463 YEVGQPMGALSSWPGLALTHHWIVQVAA(9)NTEYEILGDDIVIFNELIAQEYLNIM(2)IGCEINLNKSISS(6)EFAKR
CpMV1-NB631  444 YAVGQPMGAYSSFAMLALTHHVIVQVAA(8)FTDYCILGDDIVIAHDTVASEYLKLM(2)LGLSISSGKSVIS(4)EFAKK
                 *  ****** :*:. ::*:** :*  :*     .* :******* .. :*  *   *      .:. .*:  *    ****:
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FIG. 2. Conserved amino acid sequence motifs in the RdRp-like proteins encoded by mitochondrial virus and related RNAs. Virus notations are as in Table 1. The alignment was made with the CLUSTAL W program. Symbols below the sequence alignment: * indicates identical amino acids residues; : and . indicate higher and lower chemically similar residues, as defined in the CLUSTAL W program. Numbers in parentheses are numbers of amino acid residues between motifs.

TABLE 2

Amino Acid Sequence Identities of Mitochondrial Virus and Related RdRp-like Proteins

	OnuMV3a-Ld	OnuMV4-Ld	OnuMV5-Ld	OnuMV6-Ld	CpMV1-NB631	RsM2-1A1
OnuMV3a-Ld		22.4	20.3	24.0	22.8	26.2
OnuMV4-Ld	53.6		33.3	27.2	20.8	19.5
OnuMV5-Ld	53.6	71.7		32.0	21.1	19.2
OnuMV6-Ld	52.2	61.6	65.2		21.0	20.4
CpMV1-NB631	57.2	49.3	50.0	50.0		20.6
RsM2-1A1	63.8	47.8	45.7	50.7	55.8	

Note. Amino acid sequence identities between pairs of sequences were determined using the GAP program. Virus notations are as in Table 1. Top right triangle: amino acid sequence identities between complete RdRp-like protein sequences. Absent amino acids in gaps, including terminal gaps, were treated as mismatches. Bottom left triangle: amino acid sequence identities between conserved RdRp sequence motifs I to VI (Fig. 2). There were no gaps in these alignments.

only the positive strand-specific probes or both positive strand- and negative strand-specific probes and their resistance or sensitivity to pretreatment with DNase I alone (Fig. 3, lanes 2 and 5) or with DNase I and S1 nuclease (Fig. 3, lanes 3 and 6). The results indicated that nucleic acid extracts from Ld contain, for all three RNAs 4, 5, and 6, both dsRNA and positive-stranded ssRNA encoding the RdRp-like protein. Because the amounts of ssRNA and dsRNA analyzed were derived from the same amount of total nucleic acids, the relatively strong signals of ssRNA and weak signals of dsRNA from blotting indicated that each of RNAs 4, 5, and 6 exists predominantly in the positive-stranded ssRNA form in Ld mitochondria, which is similar to our previous reports for RNA-7 and RNA-3a from Ld (Hong, *et al.,* 1998a, 1998b). No significant amounts of negative-stranded ssRNAs were found, suggesting that the short ORFs found on the negative strands of RNAs 4, 5, and 6 probably are not expressed.

Predicted secondary structures in the 5′- and 3′-terminal regions of the three viral RNA positive strands

The lengths of the 5′- and 3′-untranslated regions (UTRs) in the positive strands of RNAs 4, 5, and 6 differed (RNA-4, 5′-UTR 204 nucleotides, 3′-UTR 43 nucleotides; RNA-5, 5′-UTR 227 nucleotides, 3′-UTR 57 nucleotides; RNA-6, 5′-UTR 141 nucleotides, 3′-UTR 114 nucleotides). All three RNAs can be folded into potentially stable

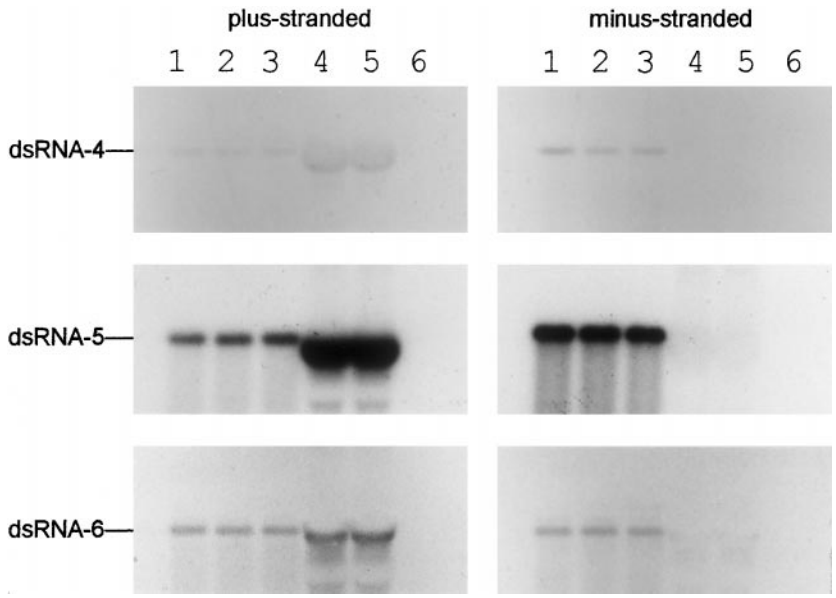

FIG. 3. Northern detection of ssRNA and dsRNA from *O. novo-ulmi* isolate Ld. Amounts of viral ssRNA and dsRNA, equivalent to 10 μg of total nucleic acids before LiCl fractionation, were electrophoresed in 1.4% nondenaturing agarose gels and blotted onto nylon membranes. Hybridization was carried out using positive strand-specific and negative strand-specific riboprobes for each viral RNA: top, RNA-4; middle, RNA-5; and bottom, RNA-6. Lanes 1–3, dsRNA; lanes 4–6, ssRNA. RNA samples were treated with 1 unit of DNase I (lanes 2 and 5), and 1 unit of DNase I and 1 unit of S1 nuclease (lanes 3 and 6). The positions of the ds forms of each RNA are indicated on the left side of the blots.

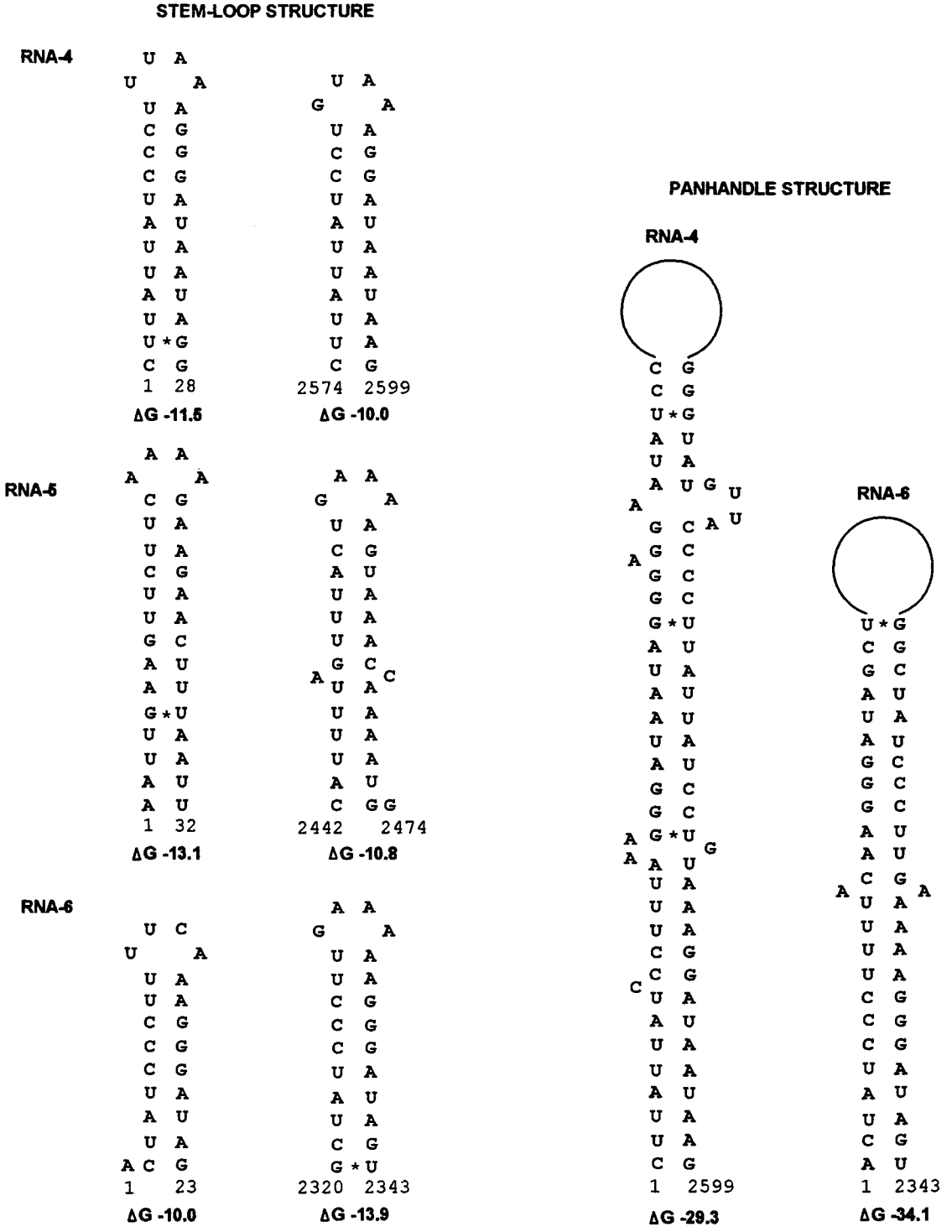

FIG. 4. Potential secondary structures of the 5′ and 3′ termini of the positive strands of RNAs 4, 5, and 6. ΔG values (kcal/mol) were calculated with the MFOLD program. Values for the RNA-4 and RNA-6 panhandle structures are averages of two values calculated by adding, to the stem, a loop with the same sequence as the loop of the 5′ or 3′ stem-loop for each RNA.

stem-loop structures at both the 5′ and 3′ termini (Fig. 4). These structures were all maintained when terminal sequences up to 300 nucleotides were folded with the MFOLD program. There is no significant sequence similarity in the primary structures of these stem-loops between RNA-5 and either RNA-4 or RNA-6. The stem-loops of RNA-4 and RNA-6, although of different lengths, are partially related. Nucleotides 6–14 of RNA-4 are identical to nucleotides 3–11 of RNA-6 on one side of the stem-loops, and the complementary nucleotides 16–23 of

RNA-4 are identical to nucleotides 15–22 of RNA-6 on the other side of the stem-loops. The 5′- and 3′-terminal sequences of both RNA-4 and RNA-6 have inverted complementarity with the potential to form panhandle structures (Fig. 4).

The presence of both 5′- and 3′-terminal stem-loop structures may be a characteristic feature of the fungal mitochondrial virus RNAs. The 5′- and 3′-terminal sequences of *O. novo-ulmi* Ld RNA-3a can also be folded into stem-loop structures (Hong *et al.,* 1998b). Stem-loop structures near the 3′ terminus of the positive strand, and a possible panhandle, formed by 10 complementary bases at the 5′ and 3′ termini, of the M2 dsRNA from *R. solani* have been reported (Lakshman *et al.,* 1998). No secondary structure has been reported for the 5′ terminus of the *R. solani* M2 RNA or for the 5′ and 3′ termini of the *C. parasitica* mitochondrial RNA. However, analysis with the MFOLD program indicates that the *R. solani* M2 RNA 5′-terminal 93 nucleotides, and the *C. parasitica* mitochondrial RNA 5′-terminal 20 nucleotides and 3′-terminal 26 nucleotides can all be folded into stable stem-loop structures with ΔG values of −29.8, −5.6, and −12.9 kcal/mol, respectively.

O. novo-ulmi RNAs 3a, 4, 5, and 6 constitute four different viruses that may be assigned to the *Mitovirus* genus of the Narnaviridae family

Two lineages of RdRps of fungal RNA viruses and virus-like RNAs have recently been described (Hong *et al.,* 1998b). One lineage includes RdRps of fungal viruses belonging to the Partitiviridae, Totiviridae, Barnaviridae, and Hypoviridae families. The second lineage contains three clusters, the RdRps of fungal mitochondrial virus-like RNAs plus a putative RdRp encoded by an *Arabidopsis* mitochondrial gene, the RdRps encoded by yeast cytoplasmic virus-like RNA replicons, and RdRps of RNA bacteriophages of the Leviviridae family, from which the first two clusters may have evolved (Hong *et al.,* 1998b; Polashock *et al.,* 1994). A phylogenetic tree, derived from an alignment of amino acid sequences of the RdRps encoded by *O. novo-ulmi* Ld RNAs 4, 5, and 6 with those of RdRps of the second lineage (Hong *et al.,* 1998b), is shown in Fig. 5. It is clear that the Ld RdRps cluster with the RdRps encoded by the *C. parasitica* mitochondrial dsRNA, by the *R. solani* M2 dsRNA, and by the *Arabidopsis* mitochondrial gene. Recently, the International Committee on Taxonomy of Viruses established a new family of naked RNA viruses: the *Narnaviridae*, composed of two genera, *Narnavirus* and *Mitovirus* (Wickner *et al.,* 1999). The *Narnavirus* genus contains the yeast 20S and 23S ssRNA replicons (corresponding to W and T dsRNAs), which are termed *Saccharomyces cerevisiae* 20S RNA narnavirus (ScNV-20S) and *S. cerevisiae* 23S RNA narnavirus (ScNV-23S), respectively. The *Mitovirus* genus contains the mitochondrial dsRNA (which also has a ssRNA form) from *C. parasitica* isolate NB631, which is termed *C. parasitica* mitovirus 1-NB631 (CpMV1-NB631). We propose that *O. novo-ulmi* Ld RNAs 3a, 4, 5, and 6 likewise be classified as viruses, assigned to the *Mitovirus* genus, and designated *O. novo-ulmi* mitoviruses 3a-Ld, 4-Ld, 5-Ld, and 6-Ld (OnuMV3a-Ld, OnuMV4-Ld, OnuMV5-Ld, and OnuMV6-Ld, respectively). Based on sequence similarities and predicted 5′- and 3′-terminal structures, the *R. solani* M2 dsRNA (from *R. solani* isolate 1A1; Lakshman *et al.,* 1998) is a possible candidate for a mitovirus. However, only a small fraction of *R. solani* M2 dsRNA copurifies with the mitochondria, and its RdRp contains no UGA tryptophan codons (Lakshman *et al.,* 1998). It is possible, therefore, that this virus replicates in the cytoplasm or in both the cytoplasm and the mitochondria. Because site of replication and mode of replication could be important taxonomic features, it is premature to assign the *R. solani* M2 dsRNA to the *Mitovirus* genus at this time.

There are several lines of evidence that indicate that *O. novo-ulmi* mitoviruses 3a-Ld, 4-Ld, 5-Ld, and 6-Ld should be regarded as separate virus species rather than strains of a single virus. (1) They each encode a distinct RdRp with limited sequence identity (20–33%). This range overlaps the range for amino sequence identity between the *O. novo-ulmi* mitovirus RdRps and those of *C. parasitica* and *R. solani* (19–26%). In fact, the RdRp of OnuMV3a-Ld is more closely related to the RdRp of *R. solani* M2 dsRNA than to those of other *O. novo-ulmi* mitoviruses (Table 2). (2) Single conidial isolates, derived from *O. novo-ulmi* Ld, have been obtained that contain only dsRNA-6 (Cole *et al.,* 1998). OnuMV6-Ld can therefore replicate independently of the other Ld mitoviruses. Similarly, it has been shown that OnuMV3a-Ld and OnuMV5-Ld can replicate independently of each other. (3) Stem-loop structures are common RdRp recognition structures (Buck, 1996). Generally, the secondary structure of the stem is more important than its primary sequence (Havelda and Burgyan, 1995; Song and Simon, 1995; Turner and Buck, 1999), although the sequence of the loop can be important for some viruses (Turner and Buck, 1999). The predicted secondary structures of the 5′ and 3′ termini of the four *O. novo-ulmi* mitoviruses are sufficiently different to suggest that they are specifically recognized by the different RdRps. The number of nucleotides in the predicted 5′- and 3′-terminal stem-loop structures are 20 and 116 for OnuMV3a-Ld, 28 and 26 for OnuMV4-Ld, 32 and 33 for OnuMV5-Ld, and 23 and 24 for OnuMV6-Ld, respectively, and the 3′-terminal structures of OnuMV3a-Ld and OnuMV4-Ld RNAs have mismatched bases in their stems (Fig. 4; Hong *et al.,* 1998b). The loop sequences are all different, except for the 3′-terminal loops of OnuMV5-Ld and OnuMV6-Ld. Only OnuMV4-Ld and OnuMV6-Ld RNAs have the potential to produce panhandle structures, and the lengths and structures of these are different.

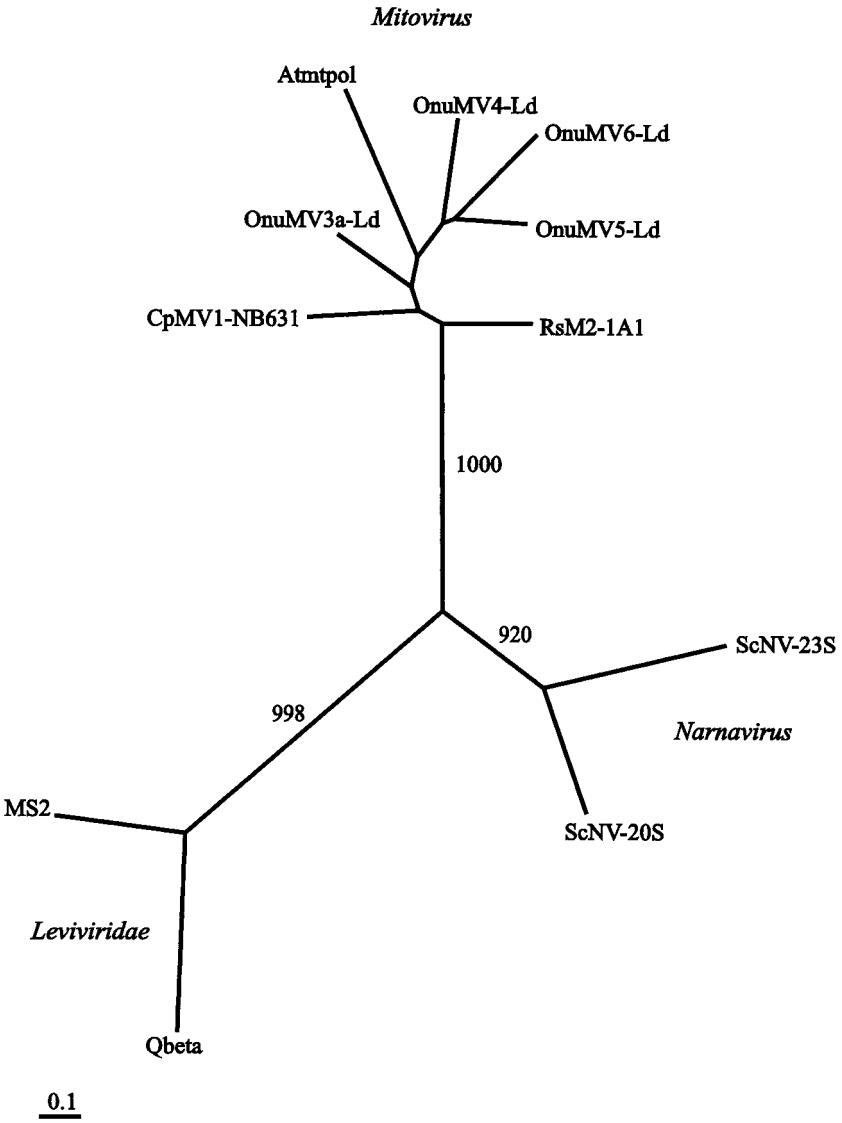

FIG. 5. Evolutionary relationships between the RdRps of viruses of the *Mitovirus* and *Narnavirus* genera of the Narnaviridae family and of bacteriophages of the Leviviridae family. Alignments using the conserved motifs A–E (Hong *et al.,* 1998b), construction of the phenogram, and bootstrapping analysis were done with the CLUSTAL W program. The tree was displayed using the TREEVIEW program. The numbers on the branches of the tree are the results of the bootstrapping analysis (1000 replicates). Sequence sources are as described here and in Hong *et al.* (1998b). Mitovirus notations are as in Table 1. Atmtpol, RdRp-like gene in *Arabidopsis thaliana* mitochondrial DNA; ScNV-20S, *S. cerevisiae* 20S narnavirus; ScNV-23S, *S. cerevisiae* 23S narnavirus; Qbeta, bacteriophage Qβ; MS2, bacteriophage MS2.

Evolution and coexistence of mitoviruses

It is noteworthy that an ascospore isolate derived from *O. novo-ulmi* Ld contains only dsRNA-2 (Rogers *et al.,* 1986). This RNA is therefore also able to replicate independently of all the other Ld dsRNAs. Based on analysis of dsRNA profiles of Ld single conidial isolates, which contain subsets of the 12 Ld dsRNAs, it was suggested that 8 of these dsRNAs (1a, 1b, 2, 3a, 3b, 4, 5, and 6) may all replicate independently (Cole *et al.,* 1998) and therefore may constitute the genomes of 8 different mitoviruses. The failure of OnuMV3a-Ld, 4-Ld, 5-Ld, and 6-Ld riboprobes to hybridize detectably with RNAs 1a, 1b, 2, and 3b in Northern blots is consistent with this suggestion. The presence of so many independently replicating viruses in the mitochondria of one fungal isolate raises questions about their evolution. Because mycoviruses appear to be transmitted only by intracellular routes (Buck, 1986), it has been suggested that they may coevolve with their hosts (Buck, 1998, Ghabrial, 1998). Coevolution of a virus with its host may occur if virus replication requires specific interactions between host proteins and viral proteins or RNA. Bacteriophages of the *Leviviridae* family, from which mitoviruses may have evolved (Hong *et al.,* 1998b; Polashock and Hillman, 1994), contain four host proteins, as well as the phage-encoded RdRp, in their replication complexes (Blumen-

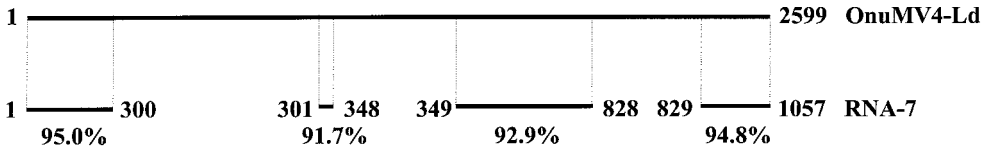

FIG. 6. Scheme for the derivation of RNA-7 from OnuMV4-Ld RNA and percent nucleotide sequence identities between corresponding regions of these two RNAs.

thal and Carmichael, 1979). A mutation in a host protein may need a compensatory mutation in a viral protein or RNA to maintain an interaction. If long-term coevolution of mitoviruses with their fungal hosts had occurred, it would be expected that sequence divergence of different viruses would reflect the divergence of their fungal hosts, with viruses of ascomycete fungi (*O. novo-ulmi* and *C. parasitica*) being more closely related to each other than to a virus of a basidiomycete fungus (*R. solani* is the anamorph or asexual form of *Thanatephorus cucumeris*, a basidiomycete fungus). This is clearly not the case (Tables 1 and 2, and *Discussion* above). Indeed, the wide divergence of mitoviruses in one *O. novo-ulmi* isolate suggests that evolution of these viruses is driven by factors other than virus–host interactions. One possibility is that the ability of different mitoviruses to replicate in the same cell could require a minimum sequence divergence. Mitovirus RNAs that are closely related and can be replicated by the same polymerase may be incompatible because replication of one RNA outcompetes the other. In mixed viral infections, mutants may be selected on the basis of replication compatibility (i.e., the inability of the RdRp of one virus to replicate the RNA of other viruses). Because mutation rates of RNA viruses are 10^6 to 10^7 times higher than those of fungi due to the lack of RdRp proofreading activity and high copy number (Li, 1997), such selection could result in rapid mitovirus sequence divergence within one host. The idea that closely related mitoviruses may be incompatible in the same cell is similar to that proposed for the basis of DNA plasmid incompatibility in bacteria (Kornberg and Baker, 1992). Bacterial plasmids are only compatible (i.e., two or more plasmids can only replicate stably together in the same cell) if they are all replicated by different proteins.

Another possible explanation for sequence divergence of mitoviruses within one host is rare horizontal virus transmission between different fungal species (Hong *et al.,* 1998b) or even between fungi and plants (Marienfeld *et al.,* 1997). Although the sequences so far determined are too diverse to provide evidence for or against this hypothesis, it seems unlikely in *O. novo-ulmi* Ld because of the number of such events that would be needed to generate the multiplicity of mitoviruses in this isolate. A further possibility is that different mitoviruses in *O. novo-ulmi* Ld might replicate in different mitochondria because cells contain multiple mitochondria. This again seems unlikely because mitovirus RNA is known to be able to move from one mitochondrion to another (Charter *et al.,* 1993) and fusion of mitochondria is a common event. Evolution of the different viruses in different *O. novo-ulmi* genotypes followed by horizontal transmission into a common genotype is also possible, but neither evolution in different mitochondria or in different *O. novo-ulmi* genotypes would provide any obvious mechanism of selecting for sequence divergence.

OnuMV4-Ld is the helper virus of RNA-7 and RNA-10

It has been shown that single-conidial isolates that lack OnuMV4-Ld RNA also lack RNAs 7 and 10 (Cole *et al.,* 1998; Rogers *et al.,* 1986). Recently, the nucleotide sequences of RNAs 7 and 10 were determined. Neither RNA-7 nor RNA-10 could encode proteins of more than 41 amino acids, so their replication must rely on an RNA polymerase encoded by a helper virus. RNA-10 was found to be a defective-like RNA that could be derived from RNA-7. It was suggested that RNA-7 could be a satellite-like RNA on the basis that no hybridization with the other viral RNAs in *O. novo-ulmi* Ld could be detected (Hong *et al.,* 1998a). However, direct sequence comparison of OnuMV4-Ld RNA and RNA-7 now clearly shows that RNA-7 can be derived from OnuMV4-Ld RNA by multiple internal sequence deletions. The corresponding sequences of RNA-7 and OnuM4V RNA have 91.7–95% nucleotide identities (Fig. 6). The failure to detect hybridization of OnuMV4-Ld RNA and RNA-7 in our previous analysis could be due to the use of an RNA-7 riboprobe consisting of nucleotides 71–902 (Hong *et al.,* 1998a). A hybrid between the RNA-7 probe and OnuMV4-Ld RNA would contain four loops in the OnuMV4-Ld RNA sequence, which would make the hybrids unstable, resulting in stripping of the probe during posthybridization washing. It is clear that like RNA-10, RNA-7 should also be regarded as a defective RNA. Moreover, RNA-10 could be derived from RNA-7 as previously shown (Hong *et al.,* 1998a), directly from OnuMV4-Ld RNA, or both. Therefore, OnuMV4-Ld is the helper virus of both RNA-7 and RNA-10. OnuMV4-Ld dsRNA is the least abundant of the *O. novo-ulmi* Ld dsRNAs (Cole *et al.,* 1998; Rogers *et al.,* 1987), suggesting that RNA-7 and RNA-10 may act as defective-interfering RNAs.

MATERIALS AND METHODS

Fungal isolates

O. novo-ulmi isolate Ld and an Ld-derived single conidial isolate, sci31, have been described previously

(Brasier, 1986, Cole *et al.,* 1998). Fungal isolates were maintained on malt extract agar (Oxoid) (Charter *et al.,* 1993).

RNA isolation

Total nucleic acids were isolated as described (Covey & Hull, 1981) from mycelia after 14 days of growth on cellophane membranes overlaying malt extract agar at 22°C. Fractionation of ssRNA and dsRNA with LiCl was performed as described previously (Hong *et al.,* 1998a). dsRNAs were separated by polyacrylamide gel electrophoresis (Cole *et al.,* 1998) and extracted from the gel using an RNaid kit (Bio 101, Vista, CA).

cDNA synthesis, cloning, and sequence analysis

cDNA libraries were constructed using gel-purified dsRNA-4 and dsRNA-5 from isolate Ld and dsRNA-6 from sci31. cDNA synthesis, cloning, and screening of cDNA clones were performed as described previously (Hong *et al.,* 1998a). Clones for each of the three dsRNAs were further confirmed by Northern blotting. Determination of the ends of each dsRNA was done using a GIBCO BRL (Gaithersburg, MD) 5′ RACE System (Frohman *et al.,* 1988), and the 5′ RACE PCR products were cloned into the vector pGEM-T Easy (Promega, Madison, WI). Sequences of cDNA clones and subclones were obtained by the Sanger chain termination method (Sanger *et al.,* 1977) using Dye-Terminator Cycle Sequencing with AmpliTaq DNA polymerase FS (ABI PRISM 377). Sequence analysis was performed using the University of Wisconsin Genetics Computer Group (GCG) programs (version 8.1) (Devereux *et al.,* 1984), made available by the Daresbury Laboratory SEQNET service. MFOLD is a GCG adaptation of the mfold package (Zucker, 1989). Multiple sequence alignments, construction of phenograms, and bootstrapping analysis were accomplished with the CLUSTAL W programs (Thompson *et al.,* 1994). Phylogenetic trees were displayed using the program TREEVIEW (Page, 1996).

Northern blotting

Gel separation, denaturation, neutralization, and blotting of dsRNA and ssRNA fractions, pretreated with RNase-free DNase with or without S1 nuclease, were as described by Hong *et al.* (1998a). Blots were hybridized with strand-specific riboprobes prepared by *in vitro* transcription using T7 RNA polymerase (NBL) and either α-^{32}P-UTP (Amersham) or DIG-11-UTP (Boehringer) (Sambrook *et al.,* 1989), followed by either autoradiography or immunological detection using a DIG detection kit (Boehringer), respectively.

ACKNOWLEDGMENTS

We thank the Gatsby Charitable Foundation for support of this work, the Biotechnology and Biological Sciences Research Council for the award of CASE studentships to Sharon L. Dover and Thomas E. Cole, and Claude M. Fauquet and Bradley I. Hillman for copies of the 7th ICTV Report relating to the Narnaviridae family and *Mitovirus* genus, before publication.

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