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Genetics

A small mitochondrial double-stranded (ds) RNA element associated with a hypovirulent strain of the chestnut blight fungus and ancestrally related to yeast cytoplasmic T and W dsRNAs

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ABSTRACT **A small double-stranded (ds) RNA element was isolated from a moderately hypovirulent strain of the chestnut blight fungus *Cryphonectria parasitica* (Murr.) Barr. from eastern New Jersey. Virulence was somewhat lower in the dsRNA-containing strain than in a virulent dsRNA-free control strain, but colony morphology and sporulation levels were comparable. A library of cDNA clones was constructed, and overlapping clones representing the entire genome were sequenced. The 2728-bp dsRNA was considerably smaller than previously characterized *C. parasitica* dsRNAs, which are 12–13 kb and ancestrally related to the Potyviridae family of plant viruses. Sequence analysis revealed one large open reading frame, but only if mitochondrial codon usage (UGA = Trp) was invoked. Nuclease assays of purified mitochondria confirmed that the dsRNA was localized within mitochondria. Assuming mitochondrial translation, the deduced amino acid sequence had landmarks typical of RNA-dependent RNA polymerases. Alignments of the conserved regions indicate that this dsRNA is more closely related to yeast T and W dsRNAs and single-stranded RNA bacteriophages such as Qβ than to other hypovirulence-associated dsRNAs.**

Cryphonectria parasitica, the filamentous ascomycete that causes chestnut blight, was responsible for the decline of the chestnut [*Castanea dentata* (Marsh.) Borkh.] in North America. Virulence of *C. parasitica* can be reduced to various degrees by viral infection, a phenomenon referred to as hypovirulence (1). Considerable progress on the characterization of double-stranded (ds) RNA elements has been made in recent years (for reviews, see refs. 2–4). The most common of these have been assigned to a newly described family of viruses, the Hypoviridae (5). Members of this family have dsRNA genomes of 12–13 kb (6, 7) that are encapsulated in host-derived lipid vesicles with a viral-encoded RNA-dependent RNA polymerase (8, 9). A full-length cDNA clone of the type member of the Hypoviridae, CHV1-713 [previously called HAV (6)] has been shown to be infectious through stable transformation of *C. parasitica* spheroplasts (10).

Besides reducing virulence, the presence of viral dsRNAs may have other effects on *C. parasitica*. These include reductions in growth rate, pigmentation, and sporulation (11); reduced laccase and cutinase production (12–14); and general down-regulation of fungal proteins and mRNAs (15, 16). Changes in culture morphology are often associated with dsRNA-containing strains such as EP713 or NB58, and such strains can easily be identified as infected.

The isolation from a *C. parasitica* culture of a very small dsRNA molecule not associated with any other dsRNAs suggested that it would be fundamentally different from members of the Hypoviridae. Results of this preliminary characterization indicate that it is different not only in its ancestry but also in its localization within mitochondria of infected fungi.

MATERIALS AND METHODS

Fungal Culture and dsRNA Isolation. *C. parasitica* strain NB631 was isolated from a recovering American chestnut tree in Howell Township, New Jersey, by P. J. Bedker. Other dsRNA-containing, hypovirulent strains described are NB58 (ATCC no. 76220), EP713 (ATCC no. 52571), and 9B-2-1 from West Virginia (17). dsRNA-free, virulent strains described are EP155 (ATCC no. 38755) and NB58-19 (ATCC no. 76221). Maintenance of strains, dsRNA extraction, and analysis were as described (18).

Virulence and Sporulation. Virulence studies were performed on Granny Smith apples (19, 20). Each apple was inoculated with the test strains as well as EP155 as a virulent standard. After inoculation, apples were incubated at 25°C in plastic boxes with moist towels. Necrotic lesions were measured after 3 weeks.

Sporulation under high light intensity was determined essentially as described (11). Conidia were washed from each plate after 5 days with 10 ml of distilled water, diluted appropriately, and counted with a hemacytometer.

Cloning and Sequencing of NB631 dsRNA. A randomly primed cDNA library of purified, denatured dsRNA was constructed in the plasmid vector pUC9 as described by Hillman *et al.* (18). Clones representing the ends of the dsRNA were obtained by using the 5′ RACE (rapid amplification of cDNA ends) procedure (21, 22) on 2.5-μg template dsRNA. Primers and reagents from BRL were used essentially according to the manufacturer's recommendations. Representative clones were mapped and sequenced using the Sequenase kit (United States Biochemical) and specific primers where required. The entire genome was sequenced from overlapping clones on both strands.* Ninety-five percent of the genome was sequenced on multiple clones. To verify the sequence at the ends, three independent 5′ RACE clones were analyzed. Sequences of all oligonucleotides used in this study are available on request.

Isolation and Analysis of Mitochondria. Strain NB631 was grown for 5 days in liquid medium (250 ml/1-liter flask). This complete medium was made essentially as described by Puhalla and Anagnostakis (23), except that the salt solution was replaced with Murashige and Skoog basal salt mixture (24). Flasks were inoculated with $\approx 5 \times 10^5$ conidia. Tissue was prepared and mitochondria were purified by the method of Cramer *et al.* (25) as described by Taylor and Natvig (26).

Nuclease assays were performed on aliquots of purified mitochondria that were lysed and extracted once with an

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Abbreviations: ds, double stranded; ss, single stranded; RACE, rapid amplification of cDNA ends.

*The sequence reported in this paper has been deposited in the GenBank data base (accession no. L31849).

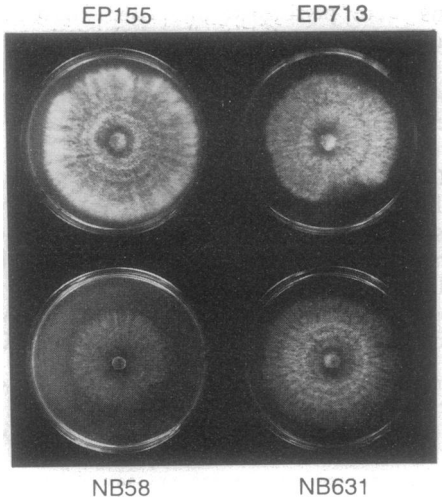

FIG. 1. Colony morphologies of virulent and hypovirulent *C. parasitica* isolates. EP155 is a standard, orange, dsRNA-free virulent culture. EP713 and NB58 are, respectively, white and brown hypovirulent cultures with large (>12 kb) cytoplasmic dsRNAs. NB631 is orange and hypovirulent, and it contains a 2.7-kb mitochondrial dsRNA.

equal volume of phenol/chloroform/isoamyl alcohol (25:24:1). Restriction digestions were performed with buffers supplied by the manufacturer. Nuclease digestions were as described by Hillman *et al.* (11), except that high-salt RNase digestions were performed in 0.6 M rather than 0.3 M NaCl.

Northern Blot Analysis. dsRNA was separated on a 1% agarose gel and alkaline blotted to Zeta-Probe nylon membrane (Bio-Rad) according to the manufacturer's protocol. Single-stranded (ss) RNA was isolated and separated on a 1.2% formaldehyde gel as described (27) and blotted as described above. Blots were probed with cDNA clones that were ^{32}P-labeled by random hexamer priming as recommended by the manufacturer (BRL).

RESULTS AND DISCUSSION

Strain Characteristics. Many hypovirulent strains of *C. parasitica* exhibit grossly altered morphology compared to virulent strains (3, 4). NB631 did not appear abnormal in culture but looked similar to virulent, dsRNA-free strain EP155 (Fig. 1). One of the screens we use to identify

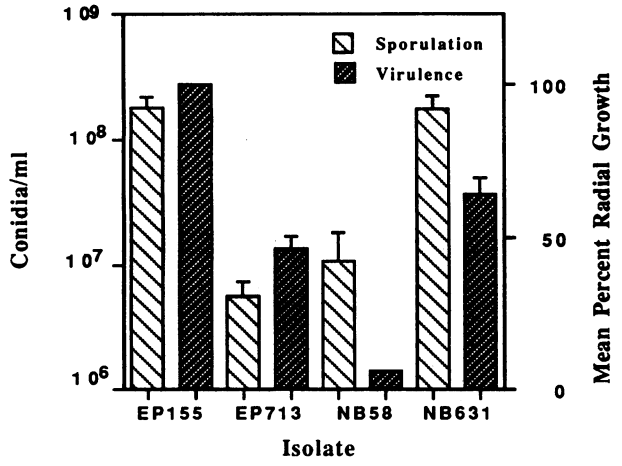

FIG. 2. Sporulation and virulence of dsRNA-containing and dsRNA-free *C. parasitica* isolates. Sporulation was determined by conidial counts from colonies grown on potato dextrose agar. Estimation of virulence was determined by growth on apple fruits as described in the text and is presented as a percentage of virulent strain EP155.

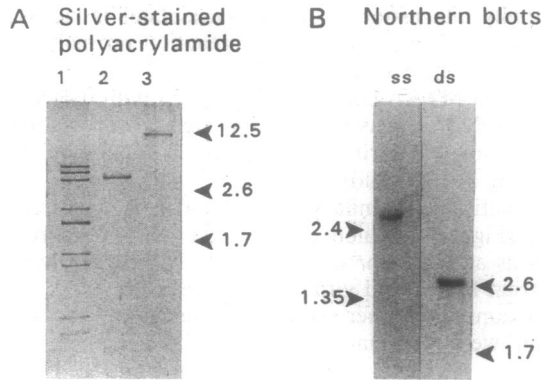

FIG. 3. Gel electrophoresis and Northern blot analysis of ds- and ssRNAs from NB631 tissue. (*A*) Silver-stained 8% polyacrylamide gel of dsRNAs from three hypovirulent strains of *C. parasitica*. Lanes: 1, strain 9B-2-1 [a hypovirulent strain from West Virginia (17)]; 2, strain NB631; 3, strain NB58. Size standards are NB58 dsRNA (12,507 bp; GenBank accession no. L29010) and wound tumor reovirus segments 4 (2565 bp) and 7 (1726 bp) (29). (*B*) Northern blots of 2 M LiCl-precipitated RNA (lane ss) and purified dsRNA (lane ds) fractions from NB631 tissue separated on 1.2% agarose, formaldehyde-permeated denaturing gel or 1% nondenaturing gel, respectively. Both gels were probed with ^{32}P-labeled clone 631-9 (see Fig. 4). Size standards are from BRL ssRNA ladder (left) or wound tumor virus dsRNA segments 4 and 7 (right).

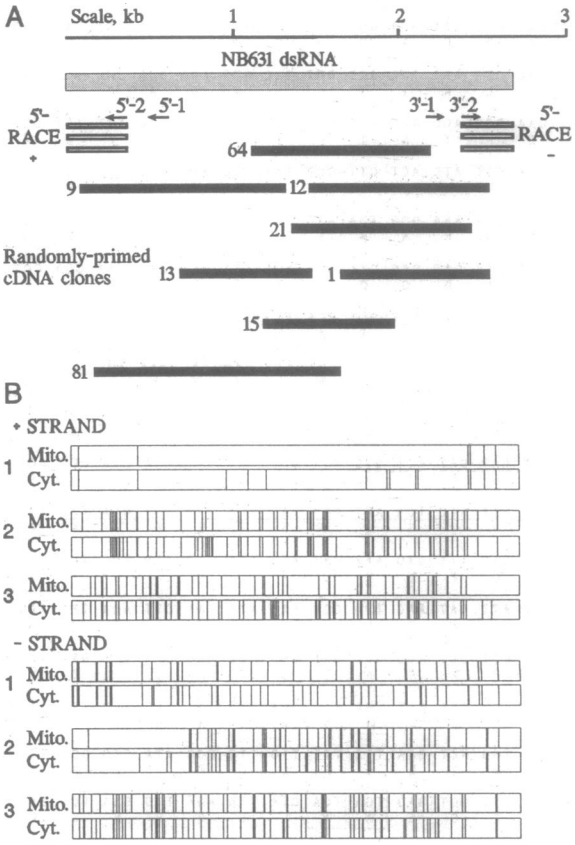

FIG. 4. Map of NB631 cDNA clones and potential deduced translation frames. (*A*) Randomly primed clones (solid boxes) were mapped by restriction digest and sequence analysis. cDNA reaction mixtures for 5′ RACE clones were primed with oligonucleotides 5′-1 (upper strand) or 3′-1 (lower strand) and amplified with primers 5′-2 or 3′-2, in conjunction with external, dC-tail-specific primers (BRL). (*B*) Possible translation frames deduced from the sequence in Fig. 5. Mitochondrial translation (Mito) assumes UGA = Trp; cytoplasmic codon usage (Cyt) assumes UGA = Stop. Each line represents a translation terminator, with some appearing heavier than others because two or more lines are immediately adjacent to each other.

potentially interesting *C. parasitica* isolates is laccase production, as determined by color reaction on malt extract/tannic acid agar medium (MTAA) (28). Lighter color of colonies on MTAA correlates quite well with dsRNA presence (12). NB631 was identified as significantly lighter than virulent isolates during such a screen (data not shown), prompting further study.

Sporulation was similar in NB631 and in dsRNA-free strain EP155 (Fig. 2). Virulence of NB631 using Granny Smith apples as an indicator was intermediate between hypovirulent strain EP713 and virulent strain EP155.

To examine whether reduced virulence and laccase production were associated with dsRNA presence, dsRNA extractions were performed on NB631 tissue. A single dsRNA substantially smaller than CHV2-NB58 dsRNA (12.5 kb) and slightly larger than wound tumor phytoreovirus segment 4 (2.6 kb) was identified (Fig. 3*A*). Even on greatly overloaded gels, no other dsRNA species were evident (data not shown).

cDNA Cloning and Nucleotide Sequence Analysis. To characterize the dsRNA associated with NB631, a cDNA library encompassing the entire genome was generated. Clones were demonstrated to represent dsRNA sequences by hybridization to Northern blots (Fig. 3*B*). A single dsRNA species hybridized, confirming that the 2.7-kb dsRNA was not a deletion of a larger species as is the case with CHV1-713

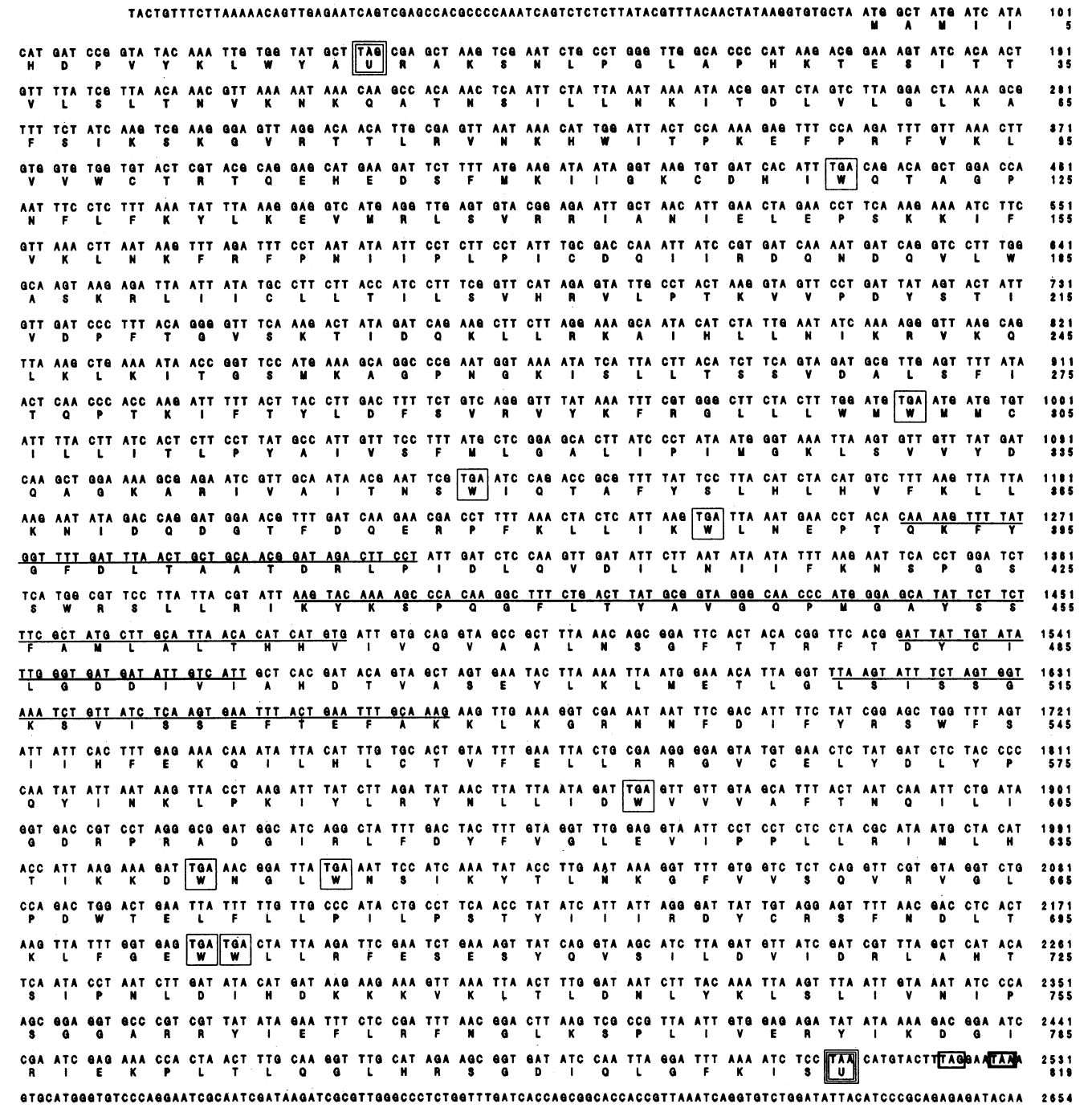

FIG. 5. Nucleotide sequence (presented as cDNA) and deduced translation of NB631 dsRNA. Translation is shown in the frame deduced from the first AUG. Single boxes surround UGA codons, assumed to encode Trp. Double and triple boxes denote UAG and UAA codons, respectively. Underlined sequence represents core RNA-dependent RNA polymerase motifs A–D in Fig. 6.

dsRNA (4). Northern blots of the 2 M LiCl-precipitated ssRNA fraction were used to examine whether a single transcript or multiple transcripts were associated with NB631. Only one species of ssRNA hybridized, suggesting that a single transcript is associated with NB631 expression.

Overlapping randomly primed cDNA clones were sequenced, and dsRNA terminal sequences were determined from 5′ RACE clones (Fig. 4*A*). At each terminus, all three clones sequenced were identical. Sequences and termini of the 5′ RACE clones were confirmed by direct dideoxynucleotide sequencing of dimethyl sulfoxide-denatured dsRNA templates (data not shown). Neither terminus of NB631 dsRNA was polyadenylylated. In this respect, NB631 dsRNA differs from dsRNA genomes of the Hypoviridae, which have poly(A) sequences at the 3′ ends of the coding strands and complementary poly(U) sequences on the minus-sense strands (6). The size of the sequence was 2728 residues (Fig. 5).

Analysis of the sequence revealed no long open reading frames on either strand when normal cytoplasmic codon usage was invoked (Fig. 4*B*). A reading frame on one strand, however, contained nine UGA codons, as well as a single UAG codon shortly after the first AUG. The role of the UAG codon is unclear; however, the UGA codons may be more easily explained. Mitochondria in other fungi use UGA to encode tryptophan rather than as a translation terminator (reviewed in ref. 30), although this has not been formally demonstrated for *C. parasitica*. Allowing for mitochondrial translation opens this reading frame to a size that could encode a functional RNA-dependent RNA polymerase (RDRP). Analysis of the deduced amino acid sequence revealed all four domains identified by Poch *et al.* (31) as conserved among RDRPs (Fig. 6*A*).

Alignments of the conserved core polymerase region of NB631 dsRNA with other viral RDRPs suggested an ancestral relationship not with previously sequenced *C. parasitica* dsRNAs (6, 7) but with two small dsRNA elements identified in the yeast *Saccharomyces cerevisiae* (37, 38, 40). These elements, named T and W, are similar in size to NB631 dsRNA (2.5–2.9 kb) and also appear to be autonomous. They are in the same lineage as plus-sense RNA bacteriophages such as Qβ (38, 41). Our alignments indicate that NB631 and the yeast elements form a separate phylogenetic cluster (Fig. 6*B*). No evidence has been presented for a mitochondrial association of T or W dsRNAs, and neither has been demonstrated to cause disease in yeast (42).

Mitochondrial Association of NB631 dsRNA. The possibility of mitochondrial association was further pursued by nuclease digestion analysis of purified mitochondria from strain NB631. Total mitochondrial nucleic acid preparations included a band that comigrated with column-purified NB631 dsRNA (Fig. 7). The sharp banding pattern evident when the mitochondrial nucleic acid was digested with *Eco*RI suggests that the preparation was virtually free of contaminating genomic DNA (43). Nuclease sensitivity assays indicate that the 2.7-kb element associated with the mitochondrial fraction is resistant to DNase and high-salt RNase digestion, confirming its identity as dsRNA.

If NB631 dsRNA is in fact intimately associated with mitochondria, we would predict maternal inheritance through ascospores, which is not normally a characteristic of *C. parasitica* dsRNAs (4), and a high rate of transmission through conidia. Both of these characteristics have been observed (J.J.P., P. J. Bedker, and B.I.H., unpublished results).

Conclusions. We have described an unusual dsRNA element that appears to be localized for at least part of its life cycle in mitochondria of *C. parasitica* and is not related to previously described hypovirulence-associated dsRNAs. In addition to the relationship between NB631 dsRNA and yeast T and W dsRNAs, several other intriguing connections can be made. A small dsRNA was identified in *C. parasitica* isolate RC-1 from Michigan by Fulbright (19), but this has not been characterized. Of particular interest, a mitochondrial association was demonstrated for one of the dsRNA elements from *Ophiostoma ulmi*, the Dutch elm disease pathogen (44). The *O. ulmi* system is somewhat more complicated and less well-characterized at the molecular level than *C. parasitica*. Finally, intriguing parallels may be drawn from similar-sized autonomously replicating RNAs from mitochondria of plants

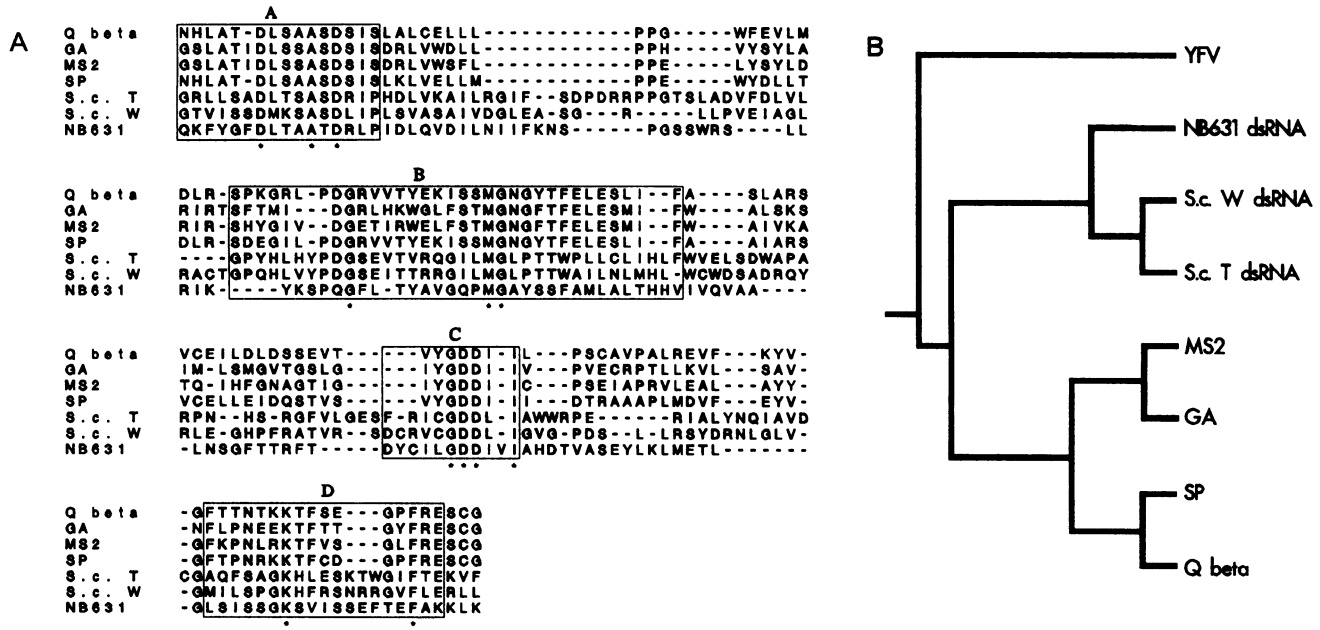

FIG. 6. Alignment and phenogram of putative RNA-dependent RNA polymerase domains [A–D from Poch *et al.* (31)] of NB631 dsRNA and several other ss- and dsRNA elements. Alignments in *A* were performed with the program CLUSTAL V (32). The phenogram in *B* was drawn with the DRAWGRAM program of PHYLIP (33). Sequences came from the following sources: MS2 phage (34), GA phage (35), SP phage (36), Qβ (GenBank accession no. X14764), *S. cerevisiae* (S.C.) dsRNA W (37), *S. cerevisiae* dsRNA T (38), and yellow fever virus [YFV; used as an outgroup in *B* (39)].

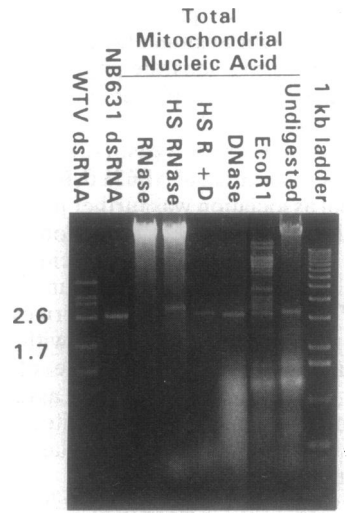

FIG. 7. Nuclease assays of mitochondria isolated from strain NB631. Total mitochondrial nucleic acid extracts were treated with RNase A in water (RNase), RNase A in 0.6 M NaCl (HS RNase), RNase A in 0.6 M NaCl + DNase I in 20 mM $MgCl_2$ (HS R + D), DNase I in 20 mM $MgCl_2$ (DNase), or EcoRI as described in the text, electrophoresed through a 0.8% agarose gel, and stained with ethidium bromide. Size standards (kb) are wound tumor virus dsRNA and 1-kb dsDNA ladder (BRL). Column-purified NB631 dsRNA is shown for reference.

with S-type male-sterile cytoplasm (45). Since there are currently no available sequence data for the RNAs described above, it is unclear whether there is a direct relationship between these elements and NB631.

Properties of NB631 dsRNA and its relationships with other viruses and virus-like elements bring up interesting evolutionary questions. Koonin and Dolja (41) have speculated that RNA bacteriophages may have originated by horizontal transfer of a eukaryotic progenitor virus common also to *S. cerevisiae* T and W dsRNAs. Since mitochondrial dsRNA elements such as NB631 are rare, it seems likely that they too evolved from such a cytoplasmic progenitor by transfer into mitochondria and subsequent specialized adaptation. The apparent absence of a coat protein encapsidating NB631 dsRNA (and the *S. cerevisiae* dsRNAs) is a property these virus-like dsRNAs share with the more common Potyvirus-related dsRNAs that cause hypovirulence in *C. parasitica* (46). This supports the idea that loss of coat protein function may be a common adaptation of viruses to fungi (46).

Since NB631 dsRNA is small, it is an attractive candidate for manipulation and reintroduction into *C. parasitica*. Incorporation of full-length cDNA clones into mitochondria by biolistic particle bombardment and mutagenesis of a full-length NB631 clone for possible cytoplasmic, nonmitochondrial replication remain to be investigated.

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