A North American hypovirulent isolate of the chestnut blight fungus with European isolate-related dsRNA

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We have synthesized and mapped a cDNA library representing the one major dsRNA element associated with hypovirulence in strain NB58 of the chestnut blight fungus, *Cryphonectria (=Endothia) parasitica*, which was isolated from recovering chestnut trees in New Jersey, U.S.A. The linear dsRNA has a size of approximately 12.5 kbp and is polyadenylated at the 3'-terminus of one strand. Molecular hybridization experiments indicate that there is sequence similarity between the NB58 dsRNA and dsRNAs from European isolates of *C. parasitica*, but not among dsRNAs of NB58 and those associated with other North American isolates. Hybridization experiments with mapped cDNA clones representing different regions of the 12.5 kbp dsRNA indicate that the termini and the 3'-proximal two-thirds (relative to the plus strand) are more conserved among NB58 and the European isolates than the rest of the 5'-proximal one-third. Nucleotide sequence analysis of the termini of NB58 dsRNA suggests common organizational features between it and the dsRNA from French-derived strain EP713.

Introduction

The chestnut blight fungus, *Cryphonectria (=Endothia) parasitica*, was responsible for considerable damage to European chestnut forests and virtually eliminated the American chestnut in the first half of this century (for reviews see Van Alfen, 1982; Anagnostakis, 1987). The term hypovirulence generally refers to infections of the fungus by cytoplasmic agents that can be transmitted via hyphal anastomosis, often resulting in altered phenotypes as well as reduced fungal virulence. Because of the unrestrained nature of this pandemic and the aesthetic and economic importance of the chestnut tree, a number of debilitated, hypovirulent *C. parasitica* isolates of European origin were released in the 1970s in various parts of northeastern U.S.A., including New Jersey, in attempts at biological control of the fungus by transmission of the hypovirulence-inducing agents. Unfortunately, records identifying exactly which strains were released are incomplete, and the investigators involved are not available (S. L. Anagnostakis, personal communication). Previous studies regarding the interrelatedness of dsRNAs from *C. parasitica* indicated that dsRNAs from European strains are related to one another, and dsRNAs from several North American strains are related to one another, but that dsRNAs from European strains are not closely related to those from North American strains (L’Hostis et al., 1985; Paul et al., 1988).

The apparent absence of a protein coat surrounding the dsRNA molecules and the inability of researchers to infect a dsRNA-free strain of *C. parasitica* with any purified form of a hypovirulence-inducing agent have greatly hindered their study from a virological perspective. However, the weight of evidence clearly indicates that many such agents are sufficient to cause hypovirulence in the fungal host and are of viral origin (Day et al., 1977; Anagnostakis & Day, 1979; Fulbright, 1984; Hansen et al., 1985; Hiremath et al., 1986; Rae et al., 1989; Choi et al., 1991a, b; Shapira et al., 1991a, b). In the French-derived strain EP713, the easily isolated dsRNA form of the genome is encapsulated within lipid vesicles that appear to be devoid of a major structural protein component, but demonstrate RNA-dependent RNA polymerase activity (Dodds, 1980; Hansen et al., 1985). Besides dsRNA, the cytoplasmic fraction contains virus-specific ssRNA which is polyadenylated and hybridizes to probes representing only one strand of the dsRNA (Hiremath et al., 1986; Tartaglia et al., 1986). This poly(A)-containing strand is referred to as the plus strand, and utilizes two open reading frames (ORFs) to express at least four polypeptides (Choi et al., 1991b; Shapira et al., 1991b).

We are interested in comparing hypovirulence-associated viruses of *C. parasitica* with the aim of understanding the mechanisms of hypovirulence itself and differentiating those from the hypovirulence-associated changes in fungal morphology (Hillman et al., 1990). Strain NB58, isolated from recovering chestnut trees in New
Jersey (P. J. Bedker & B. I. Hillman, unpublished results), was a particularly good candidate for further study for several reasons. Virulence of strain NB58 is reduced by virus infection to a level at or below the virulence of EP713, a French-derived strain. In contrast to EP713, strain NB58 is pigmented and sporulates when cultures are grown in normal laboratory conditions without a growth chamber (22 to 26 °C, 1000 to 1500 lux). Furthermore, the single dsRNA species in NB58, compared to multiple dsRNAs associated with strain EP713, suggested that the NB58 virus would be simpler to study in terms of its genetic organization and expression, and provides a good contrasting system for studying the effects of defective RNAs such as those in EP713 (Shapira et al., 1991a).

Methods

Fungal strains, growth and dsRNA extraction. Hypovirulent C. parasitica strain EP713 (ATCC no. 52571) and its isogenic, virulent counterpart, strain EP155 (ATCC no. 38755), were cultured and maintained as described (Rae et al., 1989). The virulent counterpart of strain EP747 (ATCC no. 52575) is also EP155, but the dsRNA is of Italian rather than French origin (Anagnostakis, 1981). Strain NB58 is a hypovirulent, dsRNA-containing field isolate from New Jersey (ATCC no. 76220). NB58-19 (ATCC no. 76221) is a virulent, single conidial isolate from strain NB58 that does not contain dsRNA. Growth and maintenance of these two strains was the same as for EP713, EP747 and EP155. Details of cultural and pathogenicity characteristics of these two strains will be given elsewhere (P. J. Bedker & B. I. Hillman, unpublished results).

Strains EP60 (ATCC no. 38765) and EP90 (ATCC no. 38764), both from Michigan, and strains FSL514 (ATCC no. 48539) and FSL548 (ATCC no. 48551), from Virginia and Tennessee, respectively, were obtained from the ATCC. Strain GH2 from Grand Haven, Michigan (Fulbright, 1984) was a gift from D. W. Fulbright, Michigan State University. Strains D2 and SR2 from West Virginia were kindly provided by W. L. MacDonald, West Virginia University, Morgantown.

Extraction of nucleic acids from fungal mycelium and purification of the dsRNA fraction by cellulose chromatography were performed essentially as described by Morris & Dodds (1979). Two cycles of cellulose chromatography were used to purify the dsRNA.

cDNA cloning and analysis of clones. Two cDNA libraries were synthesized, each starting with 4 µg of purified dsRNA. First-strand synthesis for one library was primed with oligo (dT)₁₂₁₈ and the other library was randomly primed with calf thymus DNA fragments at 0.5 µg/µl. Primers were mixed with dsRNA in water, dried and resuspended in 5 µl of 90% DMSO, 1 mM-Tris-HCl pH 8.0 and 0.1 mM-EDTA. After incubating for 30 min at 60 °C, an ice-cold mix containing reverse transcriptase buffer (250 mM-Tris–HCl pH 8.3, 375 mM-KCl, 15 mM-MgCl₂, 50 mM-DTT, final concentrations) and 500 µM of each dNTP was added, followed by 200 units of Moloney murine leukemia virus reverse transcriptase (BRL). First-strand synthesis reactions were incubated for 1 h at 37 °C. The remainder of the cDNA cloning procedure was essentially that of Gubler & Hoffman (1983), described in Hillman et al. (1989a), except that the cloning vector was pUC9 and recombinant plasmids were transformed to Escherichia coli strain DH5-α (BRL).

General recombinant DNA methods are described in Sambrook et al. (1989). Recombinant plasmids were extracted from ampicillin-resistant, white colonies by a slight modification of the mini-lysis procedure of Holmes & Quigley (1981). To confirm that they were clones of NB58 dsRNA, several plasmids were ³²P-labelled by random hexamer priming (BRL) and used to probe 20 ng of homologous dsRNA which had been denatured in DMSO/Tris/EDTA as described above and spotted onto Zeta-Probe nylon filters (Bio-Rad). This spot-hybridization procedure was also used to examine the relatedness of mapped cDNA clones of NB58 dsRNA to other hypovirulence-associated dsRNAs of different geographical origins.

Southern blots were performed as described in Hillman et al. (1989a) using Zeta-Probe nylon filters. After successful exposure to autoradiographic film, the filters were stripped by boiling for 30 min in 1 M NaOH and re-processed. Filters were stripped and re-probed in this manner 10 times without appreciable loss of signal.

Nucleotide sequence analysis. Dideoxynucleotide sequencing reactions (Sanger et al., 1977) were performed on NaOH-denatured plasmid preparations using Sequenase (U.S. Biochemicals) and [³²S]dATP (Dupont). Reactions designed to read across the homopolymer tail that was added for cloning were primed with the −40 forward primer (U.S. Biochemicals) or the pUC/M13 17-mer reverse primer (Promega). Oligonucleotide primers specific for NB58 dsRNA sequences were synthesized on a Biotix model 102 synthesizer using β-cyanoethyl chemistry. Sequence analysis was performed with the aid of the Microgenie programs (Queen & Korn, 1984).
Analysis of chestnut blight dsRNA

Results

Mapping of cDNA clones of NB58 dsRNA

Double-stranded RNA extracted from strain NB58 consisted of a single major component with a mobility slightly faster than the largest component of EP713 (Fig. 1). Since the largest dsRNA component of EP713 is approximately 12.7 kbp in length (Shapira et al., 1991b), we estimate the size of the NB58 dsRNA to be approximately 12 to 12.5 kbp. This estimate agrees well with results presented below for the mapping of cDNA clones. The yield of dsRNA from strain NB58 was significantly less than that from EP713 or EP747: the lanes in Fig. 1 represent 0.05 g fresh weight fungal tissue, and relative amounts of dsRNA are representative of many other extractions. Major, shorter-than-full-length dsRNA components from NB58 have not been observed even by overloading polyacrylamide gels and silver staining (not shown).

Because of cloning artefacts encountered during analysis of EP713 dsRNA (Hiremath et al., 1988; Hillman et al., 1989b; Rae et al., 1989), we mapped by Southern blot analysis the linkages of 100 cDNA clones with inserts averaging 1.0 kbp. Surprisingly, none of the original 100 clones bridged the gap between pNB58-16 and pNB58-19 (Fig. 2a). We selected clones pNB58-17 and pNB58-18 by hybridization of an additional 300 recombinant colonies with 32P-labelled inserts from pNB58-16 and pNB58-19. A total of 46 clones were chosen for more detailed restriction and sequence analysis. Three clones had apparent defects: two had deletions and one had a snapback repeat. The other 43 clones mapped without any logical inconsistency. In Fig. 2 (a), the original clones are re-numbered to progress 5' to 3' with respect to the plus strand.

The structures of the termini of NB58 dsRNA have not been examined in detail, but we can make some inferences by analogy with EP713 and the absence of clones mapping outside the region depicted in Fig. 2(a).
Like the 3' terminus of the positive strand of the EP713 dsRNA (Hiremath et al., 1986; Hillman et al., 1989b), the 3' terminus of the analogous strand of strain NB58 dsRNA appears to contain a short poly(A) tail. Also consistent with the results of Hiremath et al. (1986), initiation of cDNA synthesis from this short tail was very inefficient. Only one oligo(dT)-primed clone for which sequence analysis was performed, pNB58-43, contained a poly(A) moiety. The poly(A) of pNB58-43 was comparable in its relative location to the poly(A) of EP713 dsRNA (Rae et al., 1989). Based on these analyses and on the absence of clones mapping 3'-proximal of pNB58-43, it is likely that pNB58-43 represents the 3' terminus of the plus strand of NB58 dsRNA.

The 5' terminus of NB58 dsRNA is also similar to that of EP713. Two clones, pNB58-1 and pNB58-2, contained identical sequences for 500 residues that aligned with the 5' end of the plus strand of EP713 dsRNA. Only one oligo(dT)-primed clone for which sequence analysis was performed, pNB58-43, contained a poly(A) aligned with residues upstream from the poly(A) of EP713. Two clones, pNB58-1 and pNB58-2, contained identical sequences for 500 residues that aligned with the 5' end of the plus strand of EP713 dsRNA. Two clones, pNB58-1 and pNB58-2, contained identical sequences for 500 residues that aligned with the 5' end of the plus strand of EP713 dsRNA. Only one oligo(dT)-primed clone for which sequence analysis was performed, pNB58-43, contained a poly(A) aligned with residues upstream from the poly(A) of EP713. Two clones, pNB58-1 and pNB58-2, contained identical sequences for 500 residues that aligned with the 5' end of the plus strand of EP713 dsRNA. pNB58-43 contained a poly(A) moiety. The poly(A) of pNB58-43 was comparable in its relative location to the poly(A) of EP713 dsRNA (Rae et al., 1989). Based on these analyses and on the absence of clones mapping 3'-proximal of pNB58-43, it is likely that pNB58-43 represents the 3' terminus of the plus strand of NB58 dsRNA.

Preliminary spot hybridizations to confirm the veracity of the cDNA library of NB58 dsRNA indicated some similarity between NB58 dsRNA and that of EP713. We performed further experiments to map the regions of similarity, including dsRNAs from one other European isolate, the Italian-derived dsRNA of strain EP747, and from seven North American isolates. Eleven cDNA clones that represented the entire length of NB58 dsRNA were used to probe dsRNAs that had been denatured and spotted to nylon membranes. A summary of these results is presented in Fig. 2(b). Overall, we observed cross-hybridization between cDNA clones of NB58 dsRNA and dsRNAs of both European-derived strains, but no cross-hybridization between the same cDNA clones and dsRNAs of any other North American strains. These results are consistent with the general results of L'Hostis et al. (1985) and Paul et al. (1988) in their studies utilizing slightly different strains. Clones representing the 5' terminus and the 3'-proximal two-thirds of NB58 dsRNA hybridized to EP713 and EP747 dsRNA, but no cross-hybridization was observed with clones 0.5 to 3.0 kbp from the 5' terminus of the plus strand.

**Terminal nucleotide sequences of NB58 dsRNA**

We determined the nucleotide sequences of cDNA clones representing the termini of NB58 dsRNA and compared them to those of EP713 (Rae et al., 1989). Sequences and alignments are provided in Fig. 3. Like the dsRNA associated with EP713, which has a leader of 495 nucleotides prior to the first long (>120 nucleotide residues) ORF, the 5'-terminal 487 residues of the plus strand of NB58 dsRNA are devoid of long ORFs. Whereas the AUG codon that initiates the first large ORF is double-underlined. Both sequences in (b) are terminated with poly(A) tails of variable lengths.
ORF of EP713 dsRNA is preceded by seven other AUG codons (Rae et al., 1989), nine AUG triplets are found in the analogous region of NB58 (Fig. 3a; Fig. 4). Analysis of nucleotide sequences for the 3'-proximal 760 residues of the plus strand of NB58 dsRNA (Fig. 3b) predicted no long ORFs within this region, similar to the homologous region of EP713 (Rae et al., 1989). Optimal alignments of the two 5'-proximal sequences revealed an overall similarity of 60 to 65%, and 65 to 70% for the two 3'-proximal sequences. No direct or inverted terminal repeats were discerned.

Discussion

We have begun a molecular examination of the dsRNA associated with a virus that greatly reduces virulence of C. parasitica without eliminating pigment and spore production. Hybridization experiments indicate that the virus of NB58 is related to those of European origin, which cause an almost total elimination of pigment and spore production, but is not related to those of North American origin. Unlike viruses from the European isolates, we have not observed more than one major dsRNA species associated with single conidial isolates of NB58 examined to date (P. J. Bedker & B. I. Hillman, unpublished results). This observation strengthens the argument that the largest dsRNA of strain EP713 represents the viral genome (Rae et al., 1989; Shapira et al., 1991a, b).

The similar structures of the 5' leader sequences of EP713 and NB58 dsRNAs lead to speculation that similar translational strategies are used by the two agents. Preliminary experiments indicate that the 5' leader of NB58 dsRNA, like the 5' leader of EP713 (Rae et al., 1989), inhibits downstream gene expression in vitro. Like the AUG codon at residues 496 to 498 of EP713 (Rae et al., 1989), the AUG at positions 488 to 490 of NB58 initiates a long ORF (B. I. Hillman, unpublished results). Seven of the nine AUG codons preceding the one at nucleotides 488 to 490 have pyrimidine residues in the –3 position and would be predicted to be rather poor for translation initiation. The other two (nucleotides 146 to 148 and 452 to 454) have purine residues at the –3 and +4 positions and would be predicted to be more efficient as initiation codons (Kozak, 1989). We have no information as to whether any of the small upstream ORFs are translationally functional. One argument against this is the relative randomness of reading frames in the leaders of NB58 and EP713. For example, the positions and structures of the two small ORFs between residues 110 and 220 (Fig. 4) are sufficiently similar to suggest functional similarity. However, the two small ORFs of NB58 are not homologous with the two ORFs of EP713. Thus, if the small ORFs in the leaders of NB58 and EP713 are translated, the function of their products would appear not to require conservation of amino acid sequences.

Our spot hybridizations and limited nucleotide sequence analysis indicate that the regions of similarity between the dsRNAs of NB58 and EP713 are not distributed evenly throughout the viral genomes, but are clustered at the termini and in a large internal region. The divergence of these sequences in the 5'-proximal coding regions of the viral genomes is at a position that encodes several polypeptides including a viral proteinase, p29, in EP713 (Choi et al., 1991a, b). The suggestion that this region is not conserved between the two viruses has been supported recently by sequence and translation data for NB58 (M. P. Brown & B. I. Hillman, unpublished results).

The 65 to 70% sequence similarity between the 3' termini of NB58 and EP713 is greater than in any other region that has been compared (B. I. Hillman, unpublished results). The strong hybridization observed with pNB58-43 in the spot hybridization experiments is consistent with these alignments. There are three stretches of greater than 90% identity spanning more than 50 residues. Taken together, alignments of the 5' and 3' termini of NB58 and EP713 suggest that some sequences near the ends of these dsRNAs are strongly conserved, probably for replication.

No formal taxonomy currently includes the chestnut blight fungal viruses, but it has become clear that taxonomic groupings are as applicable to these agents as to any other viruses. Construction of a proper taxonomic framework that can accommodate the many varied viruses of C. parasitica will be more easily accomplished upon completion of dsRNA sequences in addition to EP713, including the NB58 dsRNA sequence.
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References


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