Partial Sequence Analysis of the dsRNA Associated with Hypovirulence in a Michigan Strain of the Chestnut Blight Fungus, *Cryphonectria parasitica*

C. Durbahn Smart,[1] Donald L. Nuss[2] and Dennis W. Fulbright[1]

[1] Department of Botany and Plant Pathology, Michigan State University, East Lansing, MI 48824; [2]Department of Molecular Oncology and Virology, Roche Institute of Molecular Biology, Roche Research Center, Nutley, NJ 07110, USA

ABSTRACT. To analyze the dsRNA associated with hypovirulence in *Cryphonectria parasitica* at a molecular level, a complimentary DNA (cDNA) library of the largest dsRNA molecule from Michigan strain GH2 was constructed. To date, sequence analysis of the cDNA clones has revealed an open reading frame of at least 6,540 nucleotides. The deduced amino acid sequence contains helicase and polymerase motifs similar to those reported for the dsRNA in the hypovirulent C. *parasitica* strain EP713. These two motifs appear to be in the same genomic organization as seen in EP713. With the exception of these motifs, the deduced amino acid sequence of the Michigan dsRNA molecule thus far shows low identity to sequences derived from the geographically distinct strain EP713. The identification of common domains suggests that there may be an evolutionary relationship between these virus-like dsRNA molecules.

INTRODUCTION

Cryphonectria parasitica (Murr.) Barr strain GH2 was first isolated from a recovering American chestnut grove in the early 1980's in Grand Haven, Mich. (4). Strain GH2 is hypovirulent and has been used successfully as a biological control of chestnut blight in Michigan (5). Strain GH2 differs from many other hypovirulent strains in that it is orange pigmented and sporulates abundantly. Strain GH2 ;ontains three double-stranded RNA (dsRNA) molecules approximately 9.0 kilobases (kb), 3.5 kb and 0.8 kb in size 2, 12). The 9.0 kb and 3.5 kb molecules hybridize, but either hybridize with the 0.8 kb molecule (8, 12). However, le three different sized molecules are all 3'-polyadenyled (12).

Recent studies on the hypovirulent European *C. parasitica* strain EP713 have shown that dsRNA molecules within this strain contain two large open reading frames (ORFs) encoding polyproteins that appear to be processed during translation (1, 11). Putative helicase and RNA polymerase motifs have been identified within the second large ORF (6). Interestingly, dsRNA molecules from strains EP713 and GH2 did not cross hybridize in northern analyses (8). In order to study the molecular action of the GH2 dsRNA, as well as to make further comparisons between GH2 and EP713, cDNA clones of the GH2 dsRNA were constructed. In this study, we report sequence analysis of cDNA clones of dsRNA isolated from strain GH2 and comparison of the GH2 sequence to that of dsRNA isolated from strain EP713.

MATERIALS AND METHODS

Cultures and growth conditions. *C. parasitica* strains EP155 and EP713 were obtained from D.L. Nuss. All other strains of *C. parasitica* were isolated from natural cankers on American chestnut trees *(Castanea dentata* [Marsh.] Borkh.) in Michigan (4, 5). C. *parasitica* cultures were grown on potato-dextrose agar (PDA; Difco, Detroit, Mich.) at room temperature under cool white fluorescent lights with a 16-hr photoperiod (5). Cultures were stored on PDA slants at 4 C. Cultures used for dsRNA isolation were grown on stationary culture in Endothia complete broth without glucose (9) for 14-21 days or on cellophane-covered PDA plates.

Double-stranded RNA isolation and cDNA cloning. Double-stranded RNA was isolated as described by Morris and Dodds (7). Electrophoresis was performed using 5% polyacrylamide gels, that were stained with ethidium bromide following electrophoresis. The largest (9.0 kb) dsRNA band of strain GH2 was cut from polyacrylamide gels and eluted using the Elutrap system, as described by the manufacturer (Schleicher and Schuell, Keene, N.H.). A cDNA library was generated from the electroeluted dsRNA segment as described by Rae et al. (10). DNA sequence was obtained from double-stranded DNA plasmid templates containing cDNA inserts using the Sequenase dideoxy sequencing system (United States Biochemical, Cleveland, Ohio). Deduced amino acid sequence comparisons were made using the programs of the University of Wisconsin Genetics Computer Group.

RESULTS AND DISCUSSION

Cloning and sequence analysis of strain GH2. A partial cDNA library of the GH2 9.0 kb dsRNA molecule was constructed in the plasmid vector pUC9 (Figure 1). Complementary DNA clones were confirmed by dot-blot analysis using ^{32}P-labeled GH2 dsRNA. Cloned inserts were sequenced and were found to span 7,360 nucleotides from the 3' end of the GH2 9.0 kb dsRNA molecule. Sequence analysis revealed a large ORF of at least 6,540 nucleotides, which ends 857 base pairs (bp) from the 3' terminus of the

dsRNA molecule. We have not yet isolated cDNA clones representing the 5' end of the GH2 9.0 kb dsRNA molecule, but are continuing to make new cDNA clones that should give us a complete cDNA copy of the GH2 9.0 kb dsRNA molecule. Clones of the 5' end of the molecule probably were not obtained in the first attempt since we used a polythymidine primer that bound to the polyadenylated 3' end of the GH2 dsRNA molecule. Reverse transcription probably did not continue through to the 5' terminus.

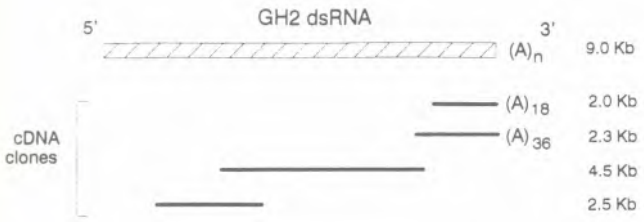

Figure 1. Map of representative cDNA clones of the 9.0 GH2 dsRNA molecule. The hatched box represents GH2 dsRNA. The bars represent cDNA clones.

Computer alignments of deduced amino acid sequences. The deduced amino acid sequence of dsRNA from EP713, including helicase and RNA polymerase motifs, was aligned with the deduced amino acid sequence of dsRNA from GH2. The results of these comparisons are shown in Figures 2 and 3. Both the helicase and RNA polymerase motifs can be identified within the deduced amino acid sequence of dsRNA from GH2. Interestingly, the motifs are in similar positions within a large ORF in each of the two dsRNAs. It appears that these two dsRNA molecules with very low sequence identity have a similar genomic organization, suggesting that there is an evolutionary relationship between these two geographically distinct hypovirulence-associated dsRNA molecules.

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     <---I---->              <------IA------>
   2 GVPGAGKSTDFI.........ISLKKYETVIVACPRQIL.........VK 33
     : .:.|||| |           |::|. .|:   ||.||          ::
2664 AKTASGKSTFFPAAVWAERRNIGIKKLWIVM..PRKILRDNWEIPFDIR 2710

                                                  <-----II--
  34 NNPVAQTKLYSGCEDNLIRGYINFGTAGYLRRTLADLPESTILCLDEFHE 83
     .. | ..| ...:.| .: .| :| |     |. : :| ..:: :|||||
2711 SQIVKRGKTLDPSADIYVTTYGHFRT.....RIGGLVPRDNLVFFDEFHE 2755

     -->
  84 MDEDSLWLLDRYRGQCVVITATP...DFYGSQRFSEVRLSKGRN.SAWTI 129
     ||:  |  :: ::|..:.:.|||   .  :: .| |. |.|  | ..:.:
2756 MDGFMLQDVEDWKGPTIFMSATPVALHGMAGIPFLEPTLPKRFNLTVYKV 2805

 130 QDDFRDTPGKLEDGWNCLMESAKTNDRVLMIVPSIQDVETCKRHAAQLVT 179
     :.|  |. :.:: ::| : : :.  .|.::|||...::...    ..|
2806 DSD..DVLEMWNRARNQFADQPELLARPMIIVPTYNELKKTIAGLENLDR 2853

           <--III--><----------V---------->
 180 NKRVCGLYRGQNTVTEADWYFATSIVDAGFDY.PGLTKIIDLGWSLGYKH 228
     .    :: ..   |...: ...|..|:.|:|. |: . :|| |:.:  ..
2854 SITWHEVSSNSPLVPKTGGLVCTPYVQTGIDIKPAPSILIDSGRDVIVHK 2903

                <---VI---->
 229 GKFIKRPS..SRNISAQRRGRTGRT 251
     |:::....  . ....|| .|.|||
2904 GRLVTPHPYTDEKTNEQRVNRVGRT 2928
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Figure 2. Alignment of deduced amino acid sequence from cDNA clones of GH2 dsRNA with the ORF B helicase domain from cDNA clones of dsRNA from *C. parasitica* strain EP713 (11). The putative GH2 open reading frame is on the top line and a portion of the ORF B amino acid sequence is on the bottom line. Conserved helicase domains are indicated with arrows. Identical matches are shown with a vertical dash. Conservative residue substitutions are shown with a colon. Semi-conservative substitutions are shown with a period. The ORF B helicase domain was originally aligned with that of the cylindrical inclusion protein of the potyvirus tobacco vein mottling virus (3).

Figure 3. Alignment of deduced amino acid sequence from cDNA clones of strain GH2 dsRNA with the RNA-dependent RNA polymerase domain of cDNA clones from strain EP713. The GH2 sequence is on the top line, and the EP713 sequence is on the bottom line. Alignment symbols are as in Figure 2.

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