Hypovirulence-Associated Traits Induced by a Mycovirus of *Cryphonectria parasitica* Are Mimicked by Targeted Inactivation of a Host Gene

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Expression of the *Vir2* gene of *Cryphonectria parasitica* is down-regulated in strains of the fungus containing a double-stranded RNA genetic element that reduces fungal virulence (W. A. Powell and N. K. Van Alfen, Mol. Cell. Biol. 7:3688–3693, 1987). We have sequenced the *Vir2* gene and characterized its structure; the mRNA contains a short open reading frame whose product has structural similarities to several fungal pheromones. A null mutant was constructed by homologous recombination to determine the function of the *Vir2* gene and whether its disruption resulted in any of the altered phenotypes exhibited by many hypovirulent strains, such as reductions in virulence, pigmentation, and sporulation. The *Vir2* null mutant (18dm) exhibited a wild-type phenotype with respect to gross colony morphology, growth rate, pigmentation, asexual spore viability, and virulence in apple fruit and chestnut trees. However, numbers of asexual fruiting bodies (pycnidia) and conidia were reduced significantly in comparison with the wild-type strain EP155/2. In sexual crosses of 18dm with a wild-type strain of the opposite mating type, perithecia (sexual fruiting bodies) developed but were barren. Deletion of the *Vir2* gene results in a phenotype that mimics that of many double-stranded-RNA-containing hypovirulent strains; i.e., the null mutant exhibits significant reductions in asexual sporulation and pycnidium production as well as impaired sexual crossing ability. To our knowledge, this is the first report of the partial reproduction of a virus-induced phenotype by deletion of a virus-perturbed host gene.

The filamentous fungus *Cryphonectria* (*Endothia*) *parasitica* (Murr.) Barr is responsible for the chestnut blight disease, which destroyed American chestnut forests over a 40-year period during the early part of this century (reviewed in references 3, 4, and 42). Biological control of *C. parasitica* and recovery of infected trees has occurred in Europe by the cytoplasmic transmission of double-stranded RNA (dsRNA) genetic elements between fungal strains. These virus-like elements typically confer a reduction in virulence (hypovirulence) to the fungus and are transmitted from hypovirulent to virulent strains during hyphal anastomosis (fusion of hyphae). In addition to hypovirulence, fungal strains harboring these genetic elements may exhibit an altered colony morphology that includes reduced sporulation and changes in pigmentation (21; reviewed in references 4, 33, 42, and 52). Reductions in levels of enzymes such as laccase (9, 28, 40, 41) and cutinase (53) or of metabolites such as oxalate (27, 28) may also occur in hypovirulent strains. The phenomenon of cytoplasmically transmissible hypovirulence in *C. parasitica* is one of the best-documented examples of a naturally occurring form of biological control for plant disease. Our studies are directed towards understanding how dsRNA genetic elements in the fungus cause the attendant changes in host phenotype.

The dsRNA virus-like elements associated with different hypovirulent strains of *C. parasitica* exhibit considerable heterogeneity with respect to genome size, number of dsRNA species, genome concentration, and sequence homology (20, 31, 34). The largest dsRNA of a European hypovirulent strain and several smaller dsRNA species have been sequenced (45, 46). The largest dsRNA contains two open reading frames (ORFs) which encode polyproteins that proteolytically process themselves (12, 13, 46, 47). A structural resemblance between hypovirulence-associated dsRNAs and replicative forms of positive-strand RNA virus genomes has been noted (12, 22, 29, 46, 51). However, the hypovirulence-associated dsRNAs differ from typical plus-strand RNA viruses in that they lack a protein capsid and instead are associated with host-derived cytoplasmic membranous vesicles (22, 26).

Recently, a cDNA copy of ORF A of a dsRNA virus was transformed into a wild-type virulent strain of *C. parasitica* and shown to confer partial reductions in pigmentation, conidiation, and laccase accumulation without conferring hypovirulence (10). These results confirm a previous hypothesis (36, 37) that the altered phenotypes of dsRNA-containing strains are due to specific effects of the viral dsRNA on the fungus and not to a general debilitation caused by the physical presence of the virus. To firmly establish viral dsRNA as the causal agent of hypovirulence in *C. parasitica*, Choi and Nuss (11) have transformed virulent strains with a full-length cDNA copy of the dsRNA virus. The complete hypovirulence phenotype was conferred, and cytoplasmically replicating dsRNAs were generated from the integrated cDNA copy.

Previous reports have demonstrated that expression of specific poly(A)$^+$ RNAs and polypeptides of the fungus is altered in dsRNA-containing cells (8, 36, 37). To understand the mechanism by which dsRNA genetic elements perturb fungal gene expression, knowledge of the host genes that are affected and of their roles in fungal development and virulence is important. Differential plaque filter hybridization was used to identify *C. parasitica* genes whose expression is

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altered in the presence of a virus-encoded dsRNA (36). A genomic DNA fragment coding for one of the genes, *Vir2*, which is down-regulated in dsRNA-containing strains, was isolated as clone V-2A-1. It should be noted that the *Vir* designation for this gene was not used with the intent of implying knowledge of function but instead to identify those genes that are transcribed in virulent strains but less so or not at all in dsRNA-containing hypovirulent strains. V-2A-1 hybridizes intensely with total RNA of the wild-type, virulent strain EP155/2 but not with that of the isogenic, hypovirulent, dsRNA-containing strain UEP1 (36).

In this study, the *Vir2* gene of *C. parasitica* has been sequenced and its structure has been analyzed. Since *Vir2* gene expression is down-regulated in dsRNA-containing strains of the fungus, we were interested in determining if a specific function of the *Vir2* gene could be identified and linked to any aspects of the altered phenotype of hypovirulent strains. The transcribed region of *Vir2* was deleted by targeted gene replacement, and the altered phenotype of the resulting null mutant was found to mimic a portion of the virus-induced symptoms.

MATERIALS AND METHODS

Strains and culture conditions. EP155/2 (a single-spore isolate of EP155; ATCC 38755) and UEP1 are virulent and hypovirulent strains, respectively, of *C. parasitica*. They are isogenic except for the presence of dsRNA in UEP1, which was constructed as previously reported (36). 18dm is a *Vir2* null mutant constructed from EP155/2 as described in this paper. EP44 (mating type a; ATCC 22511) is a wild-type strain with the mating type opposite to that of EP155/2 (mating type A) and was utilized in sexual crosses.

Routine plate cultures were grown on PDAmb (1) at 26°C under continuous fluorescent light (250 to 500 lx). Liquid mycelial cultures were grown in EP complete medium (38) as modified by Day et al. (16) but without adjusting the pH. Nucleic acid isolations were done from liquid mycelial cultures. To inoculate liquid cultures, PDAmb plate cultures were grown until the mycelium was almost to the edge of each plate (6 to 8 days), and then each plate culture was homogenized in 100 ml of EP complete medium with a Waring blender and used as the inoculum for 1 liter of the same medium in a Fernbach flask. Cultures were incubated under a constant light intensity (1,700 lx) on a rotary shaker (100 rpm) at 26°C for 2 to 3 days (for DNA isolation) or for the times indicated (for RNA isolation). Cultures were harvested by vacuum filtration through Miracloth (Calbiochem), and the mycelial pad was lyophilized for 24 to 36 h.

Hygromycin-resistant strains were maintained on media containing 50 μg of hygromycin B per ml, and benomyl-resistant strains were maintained on media containing 0.5 μg of benomyl per ml. Tests for prototrophic growth were performed on minimal medium (38). Cultures analyzed for asexual sporulation were grown on either PDAmb or 8% malt extract agar (MEA) (80 g of Difco malt extract and 20 g of Difco agar per liter). Production of pycnidia (asexual fruiting bodies) was assessed on either 1.5 or 8% MEA (35 ml of agar per plate). Growth of *C. parasitica* on MEA reduces formation of aerial hyphae, thereby allowing quantitation of pycnidia.

Stock cultures were maintained either on PDAmb slants at 4°C or on silica gel as previously described (14). *Escherichia coli* XL1-Blue (Stratagene) was the recipient for bacterial transformation and routine propagation of plasmids.

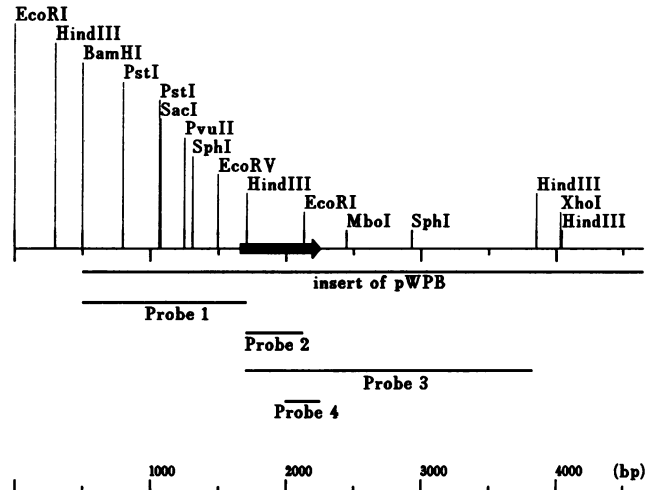**Fungal nucleic acid isolation.** Genomic DNA was isolated

FIG. 1. Partial restriction map of the *Vir2* transcribed region and adjacent sequences. The location and direction of *Vir2* transcription are indicated by the arrow. The four fragments labeled probe 1, probe 2, probe 3, and probe 4 were used for hybridization in Northern and Southern blot analyses. Probe 1 is a 1.2-kb *Bam*HI-*Hin*dIII fragment which contains 47 bp of the 5' region of the *Vir2* mRNA and upstream nontranscribed regions. Probe 2 is a 426-bp *Hin*dIII-*Eco*RI fragment containing a major portion of the transcribed region of *Vir2*. Probe 3 is a 2.1-kb *Hin*dIII fragment which includes the probe 2 and probe 4 regions as well as downstream nontranscribed regions. Probe 4 consists of 241 bp of the 3' end of the *Vir2* transcribed region (nt 472 to 713 in Fig. 3). The genomic DNA insert in pWPB is also indicated.

by a modification of the protocol of Yelton et al. (55) as previously described (57).

Total RNA was isolated as previously described (36). Poly(A)$^+$ RNA was isolated by using a Pharmacia mRNA purification kit according to the instructions of the manufacturer.

Southern and Northern (RNA) blot analyses. Southern and Northern blot analyses were performed as previously described (36). GeneScreen Plus (DuPont) was used as the hybridization membrane, and the formamide procedure suggested by the manufacturer was followed. [^{32}P]dCTP-labeled probes were prepared by using a random-primed DNA-labeling kit (Boehringer Mannheim Biochemicals).

Characterization of *Vir2* gene expression. A 4.2-kb genomic DNA fragment was subcloned from V-2A-1 (36) into pBluescript KS(+) (Stratagene) and designated pWPB. The location of the *Vir2* transcriptional region within this fragment was determined by digesting the 4.2-kb fragment into seven fragments ranging in size from 0.4 to 0.8 kb, using a variety of restriction enzymes. Each fragment was isolated from gel slices by using GENECLEAN (Bio 101), ^{32}P labeled by random priming, and hybridized to Northern blots of total RNA to determine which fragment(s) had homology to *Vir2* transcripts.

A time course of *Vir2* gene expression was conducted by isolating total RNA at 24-h intervals (beginning at day 2) from EP155/2 cultures grown in liquid medium for 8 days. Total RNA (7 μg) from each time point was separated by electrophoresis on a 1.5% agarose gel with glyoxal-dimethyl sulfoxide as the denaturing agent (5), Northern blotted, and hybridized with ^{32}P-labeled probe 4 (Fig. 1). Hybridization signals on the Northern blot were quantified with the aid of

an LKB densitometer (Ultroscan XL enhanced laser densitometer).

DNA sequencing and analysis. Genomic DNA was sequenced by using the M13 cloning/dideoxy sequencing kit (Bethesda Research Laboratories). *Vir2* cDNA clones were sequenced by using the Sequenase DNA-sequencing kit (version 2.0; U.S. Biochemical). Double-stranded plasmid template DNA was prepared by the methods of Del Sal et al. (18). The sequence reported was determined by sequencing both strands.

S1 nuclease mapping and primer extension studies. The location of the 5' end of *Vir2* was determined by S1 nuclease analysis, which was performed according to the methods of Ausubel et al. (5). A synthesized oligonucleotide complementary to nucleotides (nt) 153 to 173 of the *Vir2* sequence (see Fig. 3) was 5' end labeled with ^{32}P and annealed to a single-stranded M13mp19 vector containing the 215-bp *Eco*RV-*Hin*dIII fragment (Fig. 1). The oligonucleotide primer was extended with Klenow fragment, and the double-stranded molecule was digested with *Eco*RV. The single-stranded DNA probe was isolated, after separation on a 1.2% alkaline agarose gel, and used for S1 analysis. In the S1 analysis, 50 μg of total RNA or 0.5 μg of poly(A)$^{+}$ RNA, from 7-day-old liquid cultures, was used.

The location of the 3' end of *Vir2* was determined by S1 nuclease analysis (5). A uniformly ^{32}P-labeled antisense RNA was synthesized by using T7 RNA polymerase. A 313-bp *Eco*RI-*Mbo*I fragment (Fig. 1) was cloned into pBluescript KS(+) at the *Eco*RI-*Bam*HI site. The fragment containing the insert was excised with *Pvu*II and *Eco*RI, eluted from a 1.2% agarose gel, phenol-chloroform extracted, ethanol precipitated, and used as template in the RNA transcription reaction. The transcription reaction was performed according to the pBluescript II Exo/Mung DNA sequencing system instruction manual (Stratagene). The radiolabeled probe was further purified by electrophoresis in a 6% denaturing acrylamide gel.

Primer extension studies were performed according to the methods of Ausubel et al. (5). The same oligonucleotide that was used for the S1 nuclease analysis of the 5' end of *Vir2* was used for the primer extension experiment. RNA was isolated from 7-day-old liquid cultures.

Vir2 cDNA clone isolation. Single-stranded cDNA of EP155/2 was prepared as previously described (57).

The polymerase chain reaction (PCR) was used to isolate cDNA clones of the *Vir2* gene. Aliquots of 1 to 4 μl of single-stranded DNA were used in each PCR. The PCRs were performed by using the Repliprime DNA amplification system (DuPont) according to the recommendations of the manufacturer. Denaturation was at 94°C for 30 s, and annealing was at 55°C for 2 min with ramping to 72°C in 30 s and continuing at 72°C for 2 min for a total of 35 cycles. The final cycle of incubation at 72°C was for 10 min. The 3' primer used was the same as that used to make cDNA. Two 5' primers were synthesized. Primers 1 and 2 are nt 146 to 171 and 461 to 487, respectively (see Fig. 3). The PCR products of interest were excised from a 1.5% agarose gel and cloned into pBluescript KS(+).

Construction of plasmids for gene replacement and complementation. The gene replacement plasmid pDM18 was constructed as illustrated in Fig. 5. An 880-bp *Pvu*II-*Eco*RI fragment from pWPB was removed and replaced with the hygromycin phosphotransferase gene (*hph*) of *E. coli* (24) as a selectable marker. The *hph* gene, with *trpC* promoter and terminator sequences of *Aspergillus nidulans*, was retrieved from pDH25 (15) (provided by Genentech, Inc., San Fran-

cisco, Calif.) by restriction with the enzymes *Ssp*I and *Nru*I. The *hph* sequence was ligated to the *Vir2* gene flanking regions from pWPB and pBluescript KS(+) to produce the gene replacement plasmid pDM18. pDM18 was restricted with *Pvu*II, and the resulting 7.6-kb fragment, denoted fDM18, was eluted from an agarose gel with GENECLEAN. This linear fragment was used for fungal transformation of strain EP155/2. To construct a plasmid for complementation of *Vir2* in strain dm18, from which *Vir2* was deleted, a 2.6-kb *Sal*I fragment from pSV50 (54) containing the benomyl resistance gene was ligated into the *Xho*I site of pWPB. This plasmid (pCV2) was linearized with *Xba*I and transformed spheroplasts of dm18.

Fungal transformation. Spheroplast preparation, transformation, and selection for drug-resistant transformants were conducted under optimized conditions as previously described (14). Spheroplasts were transformed with 10 μg of linear gene or plasmid.

Phenotype of null mutant. In all assays used to characterize the phenotype of the *Vir2* null mutant, the wild-type strain EP155/2 and one to three pDH25 control transformants were included for comparison. Mitotic stability was initially examined by serial transfers of mycelial inoculum as previously described (14). Further examination of mitotic stability was by germination of conidia, isolated from cultures grown under nonselective conditions, on PDAmb with or without hygromycin B. The radial growth rate of colonies was measured after growth for 5 to 7 days on minimal medium, 1.5% MEA, or PDAmb with or without hygromycin B. Tests for virulence on apple fruit were performed as previously described (21). Virulence tests on chestnut trees were performed according to the procedures of Rigling et al. (40). The trees had a diameter at breast height of approximately 2.5 cm. They were grown and maintained after inoculation within a growth chamber at 30°C with a 16-h light/8-h dark photoperiod. The lesion area was measured at 7, 12, 19, 26, and 34 days after inoculation.

Asexual sporulation was assessed after growth of cultures on PDAmb for 2 or 3 weeks. Spores were harvested by flooding each petri dish culture (100 by 15 mm) with sterile distilled H_2O and dislodging conidia with a glass rod; each plate was rinsed once with an additional aliquot of H_2O. The spore solution was filtered through 20-μm nylon mesh (Spectrum Medical Industries, Inc.) to remove mycelial debris, and the spore concentration was determined by counting with a hemacytometer.

Pycnidium production was assessed on either 1.5 or 8% MEA. Isolates were compared by counting the pycnidia within a central circular 7-cm-diameter region of the petri dish (38.5-cm^2 area) with the aid of a dissecting microscope after growth for 2, 3, or 4 weeks. To estimate the average number of spores per pycnidium, the 38.5-cm^2 area of medium was cut from the petri dish and spores were harvested as described above. For all experiments, a minimum of three replicates were included for each treatment, and the data were analyzed by analysis of variance by the SAS GLM procedure (44) with Duncan's multiple range test.

Sexual crosses were performed on sterile chestnut wood according to the methods of Anagnostakis (2). The *Vir2* null mutant 18dm was crossed with the wild-type strain EP44 of opposite mating type. Control crosses included EP155/2 with EP44 and EP44 with 102pDH25 (a control transformant).

Nucleotide sequence accession number. The sequence data reported for the *Vir2* gene have been assigned GenBank accession number L11143.
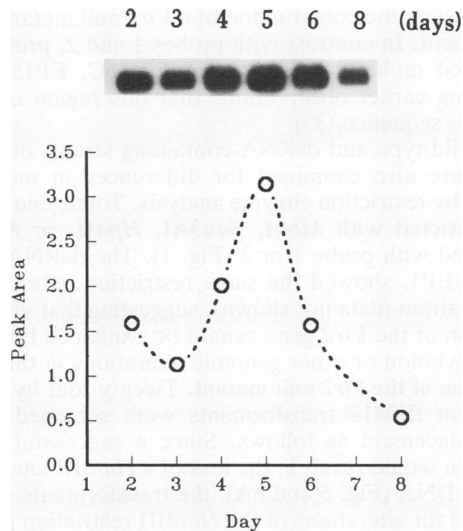
2　3　4　5　6　8　(days)

FIG. 2. Time course of *Vir2* gene expression by EP155/2 grown in liquid culture. Total RNA was extracted from mycelia at the indicated times. (Top) Autoradiogram of the Northern blot. Each lane contains 7 μg of total RNA. The blot was hybridized with ^{32}P-labeled probe 4 (Fig. 1), which represents 241 bp of the 3' transcriptional region of *Vir2*. Equal loading of RNA was confirmed in a parallel blot hybridized with an rDNA probe from *N. crassa* (data not shown). (Bottom) Densitometric scan of the autoradiogram in the top panel.

RESULTS

Location and direction of transcription of *Vir2*. A partial restriction map of the 4.7-kb genomic DNA fragment containing the *Vir2* transcribed region is shown in Fig. 1. By Northern blot analysis, the coding region of *Vir2* was localized to the 426-bp *Hind*III-*Eco*RI fragment and the adjacent 5' and 3' regions (data not shown). The direction of transcription was determined to be from the *Hind*III to the

*Eco*RI site (data not shown). These results were confirmed by the cDNA sequence data discussed below.

Time course of expression of *Vir2* mRNA. The time course of *Vir2* mRNA expression in the wild-type, virulent EP155/2 strain is shown in Fig. 2. *Vir2* mRNA was detected by Northern blot analysis at all points examined. mRNA levels were greatest at 5 days after inoculation, by which time the stationary growth phase had been reached. *Vir2* mRNA was not detected by Northern analysis in the dsRNA-containing strain UEP1 during the same time course (data not shown).

Vir2 gene sequence and structure analysis. The genomic DNA sequence of the *Vir2* transcribed and adjacent regions is shown in Fig. 3. Several sequences which may play important roles in gene expression and regulation were identified. A potential CCAAT sequence (CAAAT) is 97 bp upstream from the first transcription start point at position 123. A Goldberg-Hogness box, TATAAATA, at positions 75 to 82 is located 40 bp upstream from the first transcription start site. A putative initiator element (49), TATTCT, is located 13 bp upstream from the first transcription start site. A putative capping sequence, TCATCAG, is found at nt 121 around the first sites of transcription initiation. The region around the first ATG codon is TCAAAATG, a recognized sequence for protein translation start sites in some filamentous fungi (6, 25). Four small in-frame ORFs (corresponding to 23, 31, 13, and 11 amino acids, respectively), each starting with an ATG codon and followed by a TAA stop codon, are located at positions 197, 284, 479, and 539, respectively.

S1 nuclease analysis (Fig. 4) indicated the presence of multiple transcription start sites at positions 123 to 132. The predominant sites are A's located at positions 123, 126, and 129, which are 74, 71, and 68 bases, respectively, upstream of the first ATG codon (Fig. 3). A primer extension experiment, in which the same primer used for S1 nuclease analysis was utilized, confirmed these results (data not shown). Since the same results were obtained by primer extension and S1 nuclease analysis, it is unlikely that the multiple start sites are artifacts. In both experiments, total RNAs of the dsRNA-containing UEP1 strain and the *Vir2*

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  1 ACCAAAGTCC AAAATCAACT CAAATAATAC AGCATCATGG TCATGTCGCT GAGTTCTGTA GCAAGCATCA TAGATATAAA TATTCAGGTC GTTCCCCATT

                                                                                                       M  P
101 TGGTATTCTG TCTGTCCTCC TCATCAGCAT CACAAACCTC ACAAACACTC TACAGAAACA TCTTCTTAAG CTTCACGCTT CAAACCAACC ATCAAAATGC

     S  N  T  Q  T  S  N  S  S  M  G  V  N   G  Y  S  Y  C  V  V  M  *              M  P  L  C  S  C
201 CTTCCAACAC CCAGACCTCC AACTCCTCCA TGGGAGTCAA CGGCTACTCC TACTGCGTTG TCATGTAAAG GGGCTCCCAA CGGATGCCTC TTTGCAGTTG

     K  R  K  S  S  T  I  H  H  M  V  G  S  P  T  I  P  A  I  A  S  H  F  A  T  *
301 CAAGCGGAAG AGTTCTACAA TACACCACAT GGTTGGTAGT CCGACGATAC CAGCCATCGC TAGCCACTTC GCCACGTAAC AACACGCAAG AGCCTACCGA

                                                                          M  T  S  I   L  S  F  T
401 ACTTGCCTCG AAGAAACCCC AGGCTTGCCT TGTGACAGCC GCGCAACCAC CTCTCATCGA GCAGCAAGAT CACCAATAAT GACTTCGATT CTTTCATTTA

     C  R  L  S  H  *              M  R  T  D  C  G  G  F  A  M  L  *
501 CCTGCCGCCT TTCTCATTAA CCCTGCGGTG ACGACCGGAT GCGAACGGAC TGCGGCGGAT TTGCGATGTT GTAATCTATT GGCCATGTTT TCGGAATTCA

601 CACATGAGTG TGAAGAACGA GAGCAACGTT GATGGGAATT TTAAAGGAGG GAACGGTTTC ATTAGACATT ATCTGCTAGA CCTTGCCTAA AATGTGATTC

701 AGTCATCCTC CAG
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FIG. 3. Nucleotide sequence of the *Vir2* transcriptional region and the adjacent 5' nontranscribed regions. The first transcription start site is at position 123, and transcription ends at residue 713 (polyadenylation site). Underlined sequences are as follows: CCAAT motif (CAAAT, bases 21 to 25), TATA box motif (TATAAATA, bases 75 to 82), putative initiator sequence (TATTCT, bases 104 to 109), putative capping site (TCATCAG, bases 121 to 127) around the transcription start sites, and translational consensus sequence (TCAAAATG, bases 192 to 199) around the first ATG site. The protein sequences encoded by the four ORFs were deduced from the DNA sequence. A CAAX motif (CVVM), representing a possible site of prenylation, is located at the carboxyl-terminal end of ORF 1. Asterisks indicate the locations of stop codons. No introns were detected within the transcribed sequence.
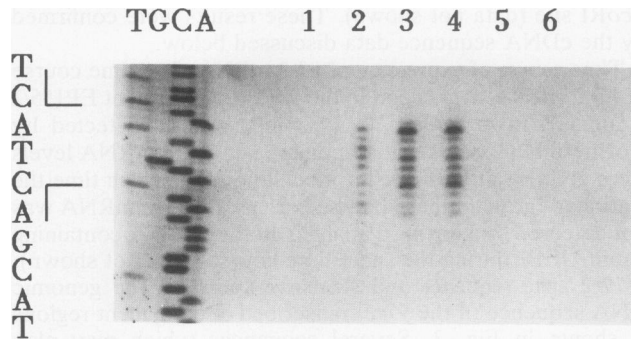
FIG. 4. S1 nuclease analysis of the 5' end of *Vir2*. A ^{32}P-5'-end-labeled 215-base single-stranded DNA complementary to the *Eco*RV-*Hin*dIII fragment (Fig. 1) was synthesized and used as a probe in S1 nuclease analysis. Lanes: 1, no RNA; 2, EP155/2 poly(A)$^+$ RNA (0.5 μg); 3 and 4, two separate preparations of EP155/2 total RNA (50 μg); 5, 18dm total RNA (50 μg); 6, UEP1 total RNA (50 μg); T, G, C, and A, DNA sequencing ladders. The same M13 DNA and primer were used in the sequencing reaction as in the single-stranded probe synthesis.

null mutant 18dm were used as negative controls to rule out any nonspecific signals.

To determine the location of the 3' end of the *Vir2* transcript, a ^{32}P-labeled antisense RNA complementary to the 313-bp *Eco*RI-*Mbo*I fragment (Fig. 1) was used to perform an S1 nuclease protection assay. A 120-base fragment was detected (data not shown), suggesting that the 3' end of *Vir2* is 120 bases downstream from the *Eco*RI site. The poly(A)$^+$ addition site was defined as nucleotide G at position 713 (Fig. 3) by sequencing seven independent cDNA clones. cDNAs were created and amplified by PCR using an oligo(dT)-containing 3' primer paired either with 5' primer 1 (nt 146 to 171) or 5' primer 2 (nt 461 to 487). A single 600-bp product was generated from amplifications by using the 5' primer 1-3' primer pair. The PCR product obtained from the 5' primer 2-3' primer pair showed a major band of 250 bp in length. Two independent clones from the primer 1 cDNA and five clones from the primer 2 cDNA were sequenced. All seven sequences showed the same poly(A)$^+$ addition site. No introns were found by comparing the cDNA sequence with the genomic DNA sequence. Although the cDNA clones were not full length, the fact that the primer 1 sequence (nt 146 to 171) overlapped with the primer used in the 5' end S1 nuclease analysis (nt 153 to 173) confirmed that there were no introns within the region from the transcription initiation site to the location of primer 1. These data are in agreement with the size of the *Vir2* transcript, which was determined to be approximately 650 bases by Northern analysis.

Copy number of the *Vir2* gene. Total genomic DNA of EP155/2 or UEP1 was restricted with *Bam*HI, *Eco*RI, *Hin*dIII, or both *Eco*RI and *Hin*dIII. Southern blots were hybridized with either probe 1 (Fig. 1), which contains 47 bp of the 5' transcribed sequence of *Vir2* as well as upstream regions, or probe 2 (Fig. 1), which contains the sequence representing the major transcribed region of *Vir2*. For each probe, identical banding patterns were observed for both strains (data not shown). A single band was identified for each digest at the position predicted by sequence analyses and genomic DNA clone restriction studies. These results suggested that the *Vir2* gene was present as a single copy in the genomes of both EP155/2 and UEP1 and, consequently,

made feasible the construction of a *Vir2* null mutant by gene replacement. In contrast with probes 1 and 2, probe 3 (Fig. 1) showed multiple bands (as in Fig. 6C, EP155/2 blot), supporting earlier observations that this region contains a repetitive sequence (35).

The wild-type and dsRNA-containing strains of *C. parasitica* were also examined for differences in methylation patterns by restriction enzyme analysis. Total genomic DNA was restricted with *Mbo*I, *Sau*3AI, *Hpa*II, or *Msp*I and hybridized with probe 1 or 2 (Fig. 1). The dsRNA-infected strain, UEP1, showed the same restriction patterns as the EP155/2 strain (data not shown), suggesting that viral down-regulation of the *Vir2* gene cannot be explained by differential methylation or other genomic alterations in this region.

Isolation of the *Vir2* null mutant. Twenty-four hygromycin B-resistant fDM18 transformants were screened for *Vir2* gene replacement as follows. Since a successful gene replacement would result in the loss of a *Hin*dIII site from the genomic DNA (Fig. 5 and 6A), the transformants were first examined for alterations of the *Hin*dIII restriction pattern in Southern blots of total genomic DNA (data not shown). One transformant, 18dm, was identified as a putative *Vir2* null mutant and further studied by detailed Southern blot analysis. Figure 6B shows the Southern blots of 18dm genomic DNA when hybridized with either probe 1 or the *hph* gene probe (Fig. 6A). The restriction patterns on the Southern blots fit the predicted band sizes, although the *Eco*RI digestion apparently was not complete.

Since the 0.42-kb region corresponding to probe 2 (Fig. 1) was deleted from the genomic DNA of 18dm, no *Vir2* bands were detected in Southern blots of 18dm DNA hybridized to this probe (Fig. 6C). The faint bands visible on the EP155/2 and 18dm blots hybridized with probe 2 represent homology of the probe to a second distinct gene, *Vir1* (56). To prove that the absence of *Vir2* bands in the 18dm-probe 2 Southern blot was not due to an experimental artifact, probe 3, containing a repeat sequence distributed among several chromosomes (35), was hybridized with an identical blot (Fig. 6C). Probe 3 detected several bands in 18dm total DNA that represent repetitive DNA sequences also found in EP155/2. Other bands detected by probe 3 on the 18dm blot changed mobility relative to the EP155/2 bands because of the replacement of the 880-bp *Pvu*II-*Eco*RI fragment with the *hph* gene. The restriction mapping studies indicated that the 880-bp fragment of the *Vir2* gene was deleted in strain 18dm by homologous recombination and replaced with one copy of the *hph* gene with a *trpC* promoter and terminator.

Northern blot analysis showed that *Vir2* mRNA was not expressed in 18dm. However, two independent pDH25 transformants which showed stable hygromycin B resistance exhibited *Vir2* mRNA expression comparable to that of the wild-type strain EP155/2 (data not shown).

Phenotype of the *Vir2* null mutant. 18dm and the pDH25 control transformants were mitotically stable and maintained resistance to hygromycin B in the absence of the drug for up to at least 3 weeks of active growth. Growth rate and pigmentation of the *Vir2* null mutant on minimal and complete media, as well as colony morphology, were comparable to those of EP155/2 and the pDH25 control transformants. Viability of 18dm conidia was not significantly different from that of EP155/2. In both the apple fruit and chestnut tree virulence tests, lesion size caused by the *Vir2* null mutant was comparable to or greater than that caused by EP155/2 and always significantly greater than that caused by UEP1, indicating that deletion of the *Vir2* transcribed region had no measurable effect on virulence (data not shown). Inocula of
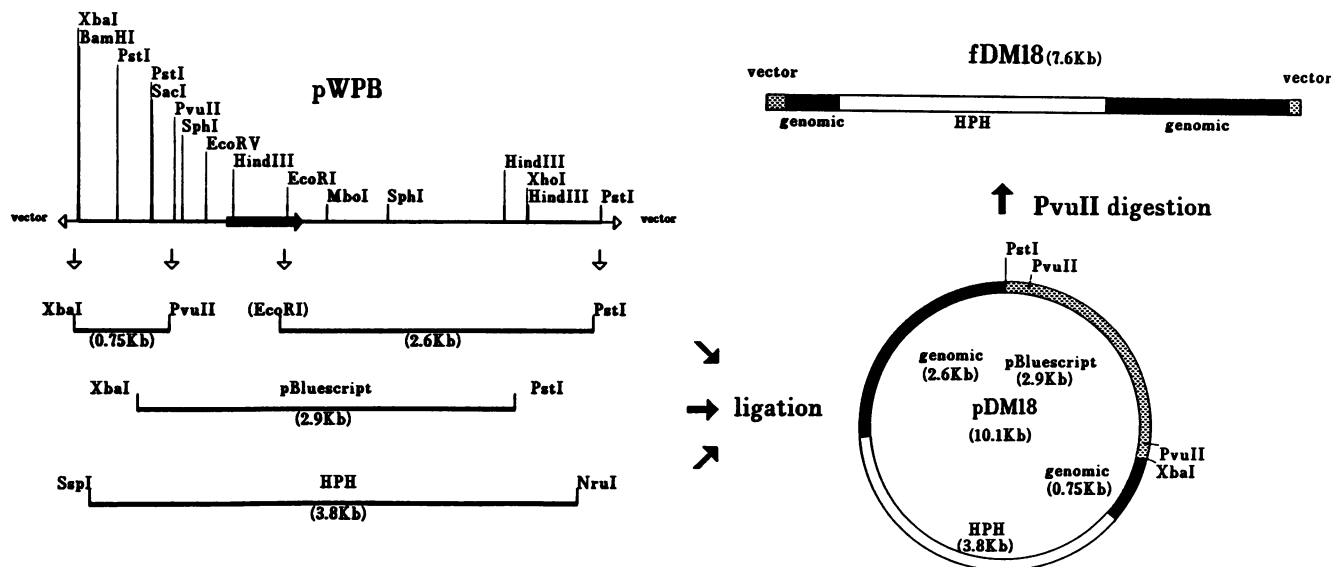
FIG. 5. Construction of pDM18 for the *Vir2* gene replacement. The *Xba*I-*Pvu*II (0.75-kb) and the *Eco*RI-*Pst*I (2.6-kb) genomic DNA fragments from pWPB were used as the 5' and 3' flanking regions, respectively, in this construction. After restriction enzyme digestion, the fragments were purified by gel electrophoresis and retrieved by GENECLEAN. The *Eco*RI site was blunted by the fill-in activity of Klenow. The *E. coli hph* gene, with an *A. nidulans trpC* promoter and terminator, was retrieved from pDH25 by restriction with *Ssp*I and *Nru*I. The two genomic DNA fragments and the *hph* gene were ligated to pBluescript KS(+) that had been restricted with *Xba*I and *Pst*I, to construct pDM18. pDM18 was digested with *Pvu*II, and the 7.6-kb linear DNA fragment fDM18, containing the *hph* gene and *Vir2* gene flanking regions, was used for transformation of *C. parasitica*.

18dm and a control transformant reisolated from chestnut tree cankers at the completion of the experiment showed stable resistance to hygromycin B.

Quantitative analysis of sporulation and pycnidium production by 18dm revealed statistically significant reductions in both functions when compared with EP155/2 and the three pDH25 transformants. In the first set of four independent experiments conducted, sporulation was assessed by growth of each fungal isolate on PDAmb. Under these conditions, sporulation was reduced in the *Vir2* null mutant to 8.7, 0.2, 0.1, and 0.4% of the wild-type level represented by EP155/2 and the pDH25 transformants. Figure 7A shows data from experiment 3 that are representative of the effect of deletion of the *Vir2* gene on sporulation.

Pycnidium production was assessed in four independent experiments by the growth of fungal isolates on 1.5% MEA. Under these conditions, pycnidium formation was reduced in 18dm to 69.5, 22.1, 46.0, and 32.1% of that in control isolates. Figure 7B shows data from experiment 2 that are representative of the effect of deletion of the *Vir2* gene on pycnidium production. For determination of both spore and pycnidium numbers, similar results were obtained when single-spore isolates were tested in comparison with the primary transformant. The morphology of the pycnidia produced by 18dm appeared normal by gross comparison, with the aid of a dissecting microscope, with wild-type and pDH25 transformant control strains.

In an attempt to determine if reduced pycnidium formation was the sole factor causing reduced sporulation in 18dm, two additional experiments were conducted. The average number of spores per pycnidium was calculated by assessing both sporulation and pycnidium production on a single medium, 8% MEA, to determine if pycnidia from 18dm produced fewer spores on average than wild-type and control strains. In one experiment, pycnidium production and the average number of spores per pycnidium were both

reduced in the *Vir2* null mutant by 3.1- and 3.3-fold, respectively, almost completely accounting for the 11.3-fold reduction in sporulation of 18dm compared with controls. However, when the experiment was repeated, the 71.2% reduction in sporulation by 18dm corresponded to an approximately equivalent reduction in pycnidium production. No effect on the average number of spores per pycnidium was detected in the second experiment. Therefore, it is unclear whether deletion of the *Vir2* gene causes a reduction in sporulation solely by reducing the numbers of pycnidia formed or whether other processes that affect spore number are perturbed as well.

Sexual crosses were performed to confirm the linkage between reduced sporulation and pycnidium number in 18dm with *Vir2* gene inactivation. The perithecia that formed from the crosses of 18dm with the wild-type strain EP44, however, were barren; i.e., perithecia were present, but few to no ascospores were produced. In contrast, control crosses utilizing two wild-type strains (EP44 with EP155/2) or a control transformant and a wild-type strain (102pDH25 with EP44) resulted in the production of normal perithecia and ascospores.

Complementation of *Vir2* by transformation of this gene into strain 18dm was done to determine if the wild-type level of asexual sporulation could be restored. A total of 16 random benomyl-resistant transformants of 18dm by pCV2 were selected and separately analyzed for asexual sporulation levels, presence of *Vir2* mRNA by Northern blots, and integration of pCV2 into the genome by Southern analysis. Each of the transformants showed a different fragment pattern when their DNA was hybridized with the 426-bp probe 2 described in Fig. 1, indicating random integrations of pCV2 into each transformant. Expression of *Vir2* could be detected by Northern blots in 14 of the 16 transformants. Asexual sporulation was restored to wild-type levels in 5 of these 14 transformants. The two transformants which did not
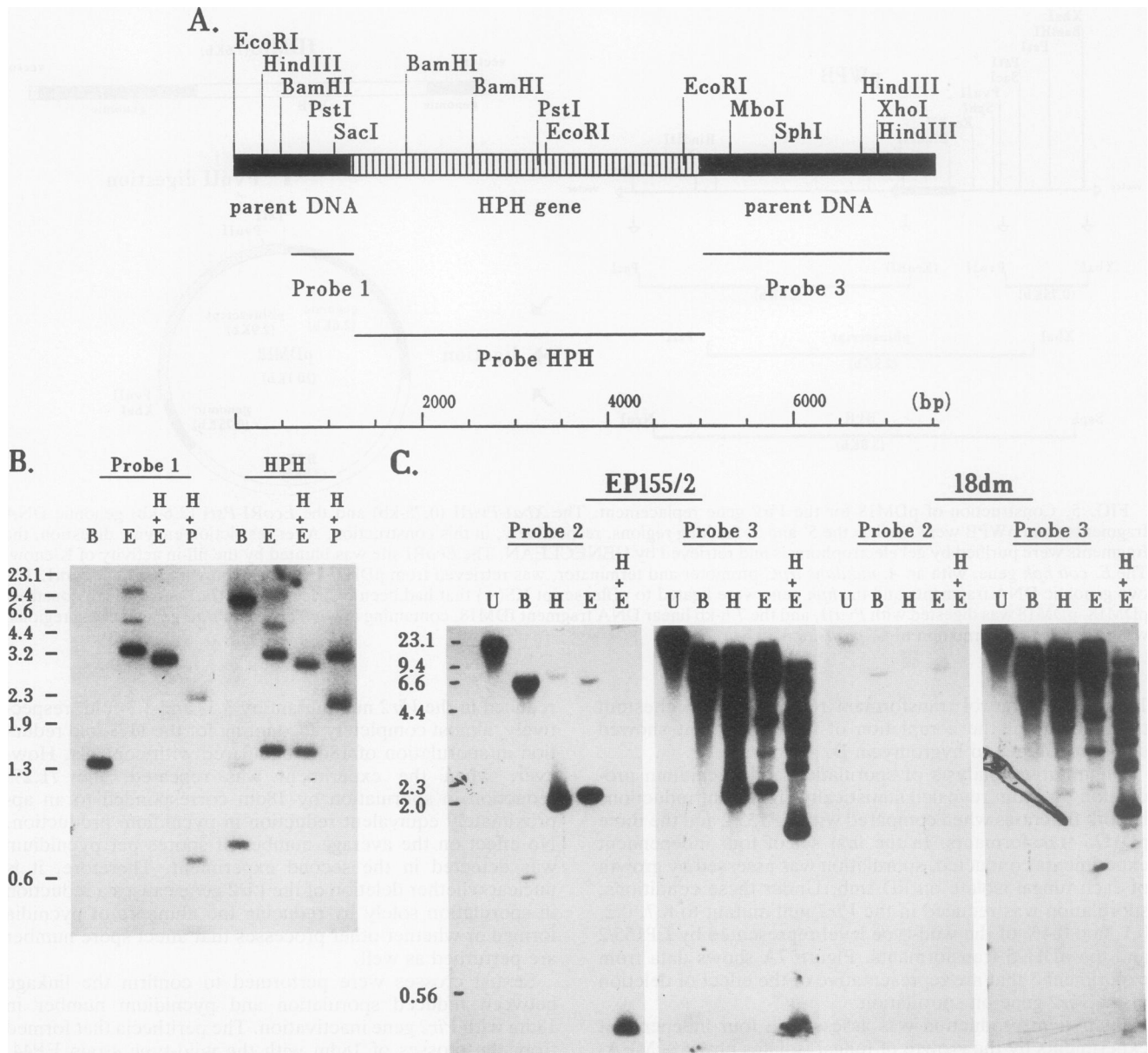
FIG. 6. Southern blot analyses of 18dm genomic DNA. (A) Partial restriction map of the gene replacement site of 18dm. Probes 1, 2, and 3 used in this set of experiments are the same as those shown in Fig. 1. However, here probes 1 and 3 are shown truncated to indicate the existing regions of homology with the parental DNA after deletion and replacement of the Vir2 transcriptional region with the hph gene. (B) Southern blot analysis of the null mutant 18dm when hybridized with either probe 1 or the hph probe. (C) Southern blot analyses of EP155/2 and the null mutant 18dm hybridized with either probe 2 (Fig. 1) or probe 3, which contains a repetitive sequence. Abbreviations: U, uncut DNA; B, BamHI; H, HindIII; E, EcoRI; P, PstI.

express Vir2 sporulated at the reduced levels typical of 18dm.

DISCUSSION

We report here the sequence, structure, and possible biological function of a fungal gene of C. parasitica, Vir2, expression of which is down-regulated in the presence of a virus-encoded dsRNA (36). No detectable differences were found in the hybridization patterns of EP155/2 and UEP1 DNAs restricted with methylation-sensitive and -insensitive restriction enzymes. Therefore, we have no evidence for differential methylation or other genomic alterations at sites within or near the Vir2 gene of UEP1 as an explanation for how viral dsRNA might down-regulate this gene.

Vir2 gene structure. Sequences resembling CCAAT and TATA boxes are present in the 5' nontranscribed region of Vir2 (TATAAATA). These sequences show similarities with the promoter region of another highly expressed C. parasitica gene, that for cryparin (TATAAA) (57). The C. parasitica laccase gene also contains a similar TATA box sequence, TATATAAA (9).

The regions around the transcription initiation sites of both the Vir2 and cryparin genes are also very similar
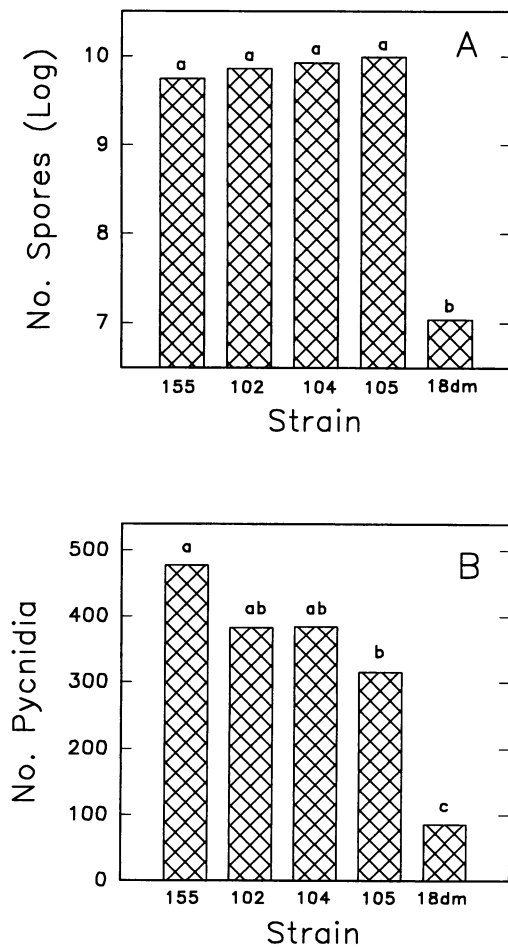
FIG. 7. Effect of deletion of the *Vir2* gene on sporulation and pycnidium production by *C. parasitica*. (A) Log of the average number of spores produced per petri dish (100 by 15 mm) for each isolate tested. Each bar represents the average of three replicates. Similar results were observed in five additional experiments, each showing a statistically significant reduction in spore number by the *Vir2* null mutant 18dm in comparison with the wild-type and pDH25 controls ($P < 0.002$). (B) Average number of pycnidia produced within a 38.5-cm^2 area for each isolate tested. Each bar represents the average of three replicates. This experiment is representative of the results observed in five additional experiments, each showing a significant reduction in pycnidium number by the *Vir2* null mutant 18dm in comparison with the wild-type and pDH25 controls ($P < 0.03$). In most experiments, the number of pycnidia produced by strain 105pDH25 was not significantly different from that produced by strain EP155/2. Data were analyzed by analysis of variance with Duncan's multiple range test. For each histogram, bars labeled with the same letters are not significantly different from one another (P values are listed above). Isolate abbreviations: 155, EP155/2 (wild-type control); 102, 102pDH25; 104, 104pDH25; 105, 105pDH25 (pDH25 control transformants); 18dm, *Vir2* null mutant.

(TCCTCCTCATCAG for *Vir2* and TCCTCCTTCTTCATCA for the cryparin gene). For the *Vir2* gene, 70% of the sequence between the TATA box and the first transcription start site is pyrimidine rich (28 of 40 residues). In many instances, such pyrimidine-rich regions are a common feature of sites of transcription initiation in filamentous fungi (6, 25). The sequence around the first transcription initiation site of *Vir2*, TCATCAG, is equivalent to the putative *Neurospora crassa* capping consensus sequence PyCATCAPu (43).

With only a few exceptions, translation of most eukaryotic genes begins at the first ATG site after transcription initiation (30). In filamentous fungi, as in higher eukaryotes, the sequence immediately upstream of the ATG codon is instrumental for correct translation initiation (25). The consensus sequence for this region is TCAC/AC/A (6). Of the four ORFs present in the *Vir2* transcribed sequence, the region 5' to the first ATG codon, TCAAA, fits most closely with this consensus sequence. The cryparin gene also contains a TCAAA sequence immediately upstream of the first ATG site, which has been shown to be the translation initiation site by protein N-terminal analysis (57). It is likely that this consensus sequence is also the site of translation initiation for the *Vir2* gene.

Although the sequence around the first ATG site appears to be a strong translation start signal on the basis of its sequence similarity to the fungal translational consensus sequence, the ORF following it encodes only 23 amino acids. Furthermore, the three in-frame ORFs following ORF 1 are also very short (31, 13, and 11 amino acids, in length). No significant homologies were found in the EMBL data library for any of these ORFs. Interestingly, the C-terminal sequence encoded by ORF 1 contains a CAAX box (CVVM), where C is cysteine, A is an aliphatic amino acid, and X is any amino acid. In many eukaryotic systems, including fungi, the CAAX motif predicts the site of posttranslational prenylation (48). Several fungal mating pheromones have been identified as prenylated lipopeptides (reviewed in references 48 and 50). Additionally, many *ras* oncogene proteins, *ras*-related small G proteins, heterotrimeric G proteins, and nuclear lamin proteins contain C-terminal CAAX motifs (50). The sequence encoded by the *Vir2* ORF 1 also contains an asparagine at a site conserved in most known fungal propheromones (32). This asparagine in the ORF 1 product, amino acid 9, likely predicts the N terminus of the processed pheromone, as was found in the *Saccharomyces cerevisiae* a-propheromone.

The codons used in the first ORF of *Vir2*, in comparison with those in the remaining ORFs, are in greatest agreement with the preferred codon usage of filamentous fungi (25). For example, serine is a common amino acid in the deduced sequence encoded by the first three ORFs of *Vir2*. Of 54 genes sequenced from 12 filamentous fungi, the codons used for serine are UCC (29.7%), UCU (20.8%), AGC (19.7%), UCG (16.0%), AGU (7.2%), and UCA (6.8%) (compiled from reference 25). In the first ORF of *Vir2*, all five serine residues are encoded by UCC, the most common serine codon in fungal genes. In contrast, less frequently occurring serine codons are utilized in the second and third ORFs. Additionally, a putative N-glycosylation site consisting of Asn-Ser-Ser (19) is present in ORF 1.

In summary, several structural features of ORF 1 suggest that it is translated: the presence of the first ATG codon, the common codon usage, a canonical translation initiation sequence, a potential N-glycosylation site, and a CAAX box. However, it is possible that some other mechanism of gene regulation involving an RNA intermediate could account for the altered phenotype of the null mutant. For example, both the mouse *H19* gene (7) and the *Drosophila* heat shock response gene *hsrw* (23) are transcribed into RNA molecules that contain only very small ORFs; they are polyadenylated and spliced but not translated into detectable protein. Fini et al. (23) suggest that the cytoplasmic RNA *w3* is translated but does not lead to detectable accumulation of the protein product. Instead, the act of translation itself may play a role in the regulation of cellular activities. At this

point, it is unclear whether the final gene product of *Vir2* is a protein or an RNA molecule.

The *Vir2* null mutant partially mimics some of the hypovirulence-associated traits of dsRNA-containing strains. Sporulation and pycnidium production by the pDH25 control transformants were not significantly different from those of EP155/2. In contrast, spore and pycnidium numbers (assayed on two separate media) were reduced to as little as 0.1 and 22.1%, respectively, of wild-type levels in 18dm, in which the *Vir2* gene was deleted by a site-specific integration event. Comparable reductions were observed at all time points examined, including 2, 3, and 4 weeks after plate inoculations (data not shown), suggesting that the effect was not due merely to a delay in development of 18dm. Under the same growth conditions, the dsRNA-containing strain UEP1, in which *Vir2* is down-regulated, failed to produce any detectable spores or pycnidia. Therefore, deletion of the *Vir2* gene results in a phenotype that partially mimics one aspect of many dsRNA-containing strains of *C. parasitica*, i.e., a reduction in asexual sporulation. This reduction in the *Vir2* null mutant appears to be caused primarily by a reduction in pycnidium number. However, it is unclear whether other processes that affect spore number, e.g., number of spores per pycnidium, are perturbed as well. Deletion of the *Vir2* gene had no observable effect on pigmentation or virulence, two traits commonly altered by the presence of dsRNA.

The association of the reduction of asexual sporulation with disruption of the *Vir2* gene was confirmed by restoration of the wild-type phenotype in a significant number of transformants (5 of 14) into which the *Vir2* gene was introduced and in which its expression was detectable. Complementation of the *Vir2* null mutation by pCV2 did not restore wild-type sporulation in all transformants, but it would be unrealistic to expect the randomly inserted gene with variable copy numbers to function perfectly in a developmental process. Southern blot analysis of the transformants showed that the linearized plasmid integrated into different locations in each of the transformants. The two transformants in which *Vir2* mRNA was not detectable exhibited the low level of sporulation typical of 18dm. In these transformants, *Vir1* mRNA (see the last paragraph below) was expressed normally, indicating that lack of detection of *Vir2* mRNA was not the result of RNA extraction or gel loading problems. These results confirm the role of *Vir2* in asexual sporulation and suggest that normal function of the gene is affected by its location within the genome. A variety of genetic abnormalities causing barren perithecia have been characterized by cytological examination of *N. crassa* mutants (39). From these studies, barrenness generally indicates an impairment in meiosis or ascus development. That inactivation of *Vir2* affects both sexual and asexual sporulation is also of interest. A number of genes in *A. nidulans* have been implicated as playing roles in both asexual and sexual development (reviewed in reference 17). We are particularly intrigued by the presence of a putative prenylation site in ORF 1 of *Vir2*. The small size of ORF 1 and the presence of a CAAX motif at the C terminus of the predicted polypeptide are suggestive of the precursors of prenylated mating factors that have been described for several fungal species (see references cited in reference 32). That disruption of the *Vir2* gene alters mating ability and outcome is supportive of this possibility. Studies are under way to further characterize the possible role of the *Vir2* gene as a mating factor in *C. parasitica*.

The effect of deletion of *Vir2* on both asexual sporulation and sexual mating is unique, particularly if *Vir2* is found to

encode a pheromone. The biology of sporulation of *C. parasitica* is not inconsistent, however, with a single signal molecule being involved in both asexual and sexual sporulation. Where perithecia form, they are always found in the stroma below and after maturation of the pycnidia (42).

Reductions in pycnidium production and sporulation, and altered mating ability, are common traits associated with the hypovirulent phenotype of dsRNA-containing strains of *C. parasitica*. That deletion of the *Vir2* gene resulted in only a partial loss of asexual sporulation and pycnidium production, in contrast to the near total suppression exhibited by the isogenic dsRNA-containing strain UEP1, suggests that the virus perturbs other genes that are redundant in function or are otherwise involved in these processes. To our knowledge, this is the first example of the partial reproduction of a virus-induced phenotype by deletion of a virus-perturbed host gene. The specific role of the *Vir2* gene of *C. parasitica* in asexual and sexual sporulation is unknown at this time.

We have determined by Northern blots, S1 nuclease protection analyses, and DNA sequencing data that the *Vir2* gene has homology with another *C. parasitica* gene, *Vir1* (56). Deletion of the *Vir2* gene does not affect the expression of *Vir1*. Both genes are down-regulated by virus-encoded dsRNA, and *Vir1* shows a time course of expression similar to that of *Vir2*. We have cloned the *Vir1* gene and are in the process of characterizing it further by targeted gene disruption. We do not yet know the significance of the homology between *Vir1* and *Vir2*, although it may suggest a similar function. Comparison of the structures of fungal genes regulated by virus-encoded dsRNA may offer clues as to the mechanism of dsRNA regulation of *C. parasitica* gene expression.

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