Genetic Organization of the dsRNA Element Associated with Strain NB58 of the Chestnut Blight Fungus and Preliminary Examination of a dsRNA-Free Mutant

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ABSTRACT. Strain NB58 of the chestnut blight fungus, *Cryphonectria parasitica, is* a highly debilitated strain that contains one major double-stranded (ds) RNA molecule. We have completed the primary characterization of this dsRNA, including its cDNA cloning and nucleotide sequence analysis. NB58 dsRNA has a size of 12,507 base pairs, excluding the poly (A) tail at the 3' end of the plus strand. The organization of the NB58 genome is similar to that of the largest EP713 dsRNA. On the poly (A)-containing strand, a long non-translated region preceded two open reading frames, designated ORF A and ORF B. The two open reading frames are connected by a UAAUG pentanucleotide that terminates ORF A and initiates ORF B. NB58 and EP 713 share approximately 60 percent nucleotide sequence identity. In contrast to EP713, the NB58 ORF A product does not appear to undergo autoproteolysis. In a separate but related study, a stable, dsRNA-free sector of NB58, designated NB58F, was isolated and partially characterized. NB58F was intermediate between NB58 and its isogenic, dsRNA-free parent strain, NB58-19, in terms of virulence and cultural characteristics. In repeated attempts, we have not been able to introduce dsRNA into strain NB58F.

Hypovirulence of the chestnut blight fungus, *Cryphonectria parasitica* (Murr.) Barr, may result from infection of the fungus by any of numerous viruses or virus-like agents, which have been characterized to varying degrees. Partial characterization of several of these viruses has provided strong evidence that they fall into at least three distinct families. The virus family that appears to be the most commonly represented in fungal isolates exhibiting hypovirulence characteristics is also the most thoroughly characterized in biological and molecular terms. These viruses have a single large (> 9 kbp) dsRNA that contains all the information necessary for their multiplication and transmission (3). A proposal to recognize this group as a virus family, designated Hypoviridae, has been submitted to the International Committee for the Taxonomy of Viruses (B.I.H., D.W. Fulbright, D.L. Nuss and N.K. Van Alfen, unpublished). The first member of the proposed Hypoviridae to be completely characterized with respect to primary sequence was the virus associated with strain EP713 (21).

Hypovirulent strain NB58 was isolated from New Jersey, USA in 1988 (2). Characteristics of strain NB58 include a brown, relatively flat, slow growing phenotype in culture. Sporulation of NB58 is not as high under normal lab bench lighting conditions as it is for single conidial isolates that are virus-free, but is considerably higher than in "white" strains such as the French-derived strain EP713 or the Italian-derived EP747. Virulence of NB58 is at least as low as EP713, which is widely regarded as one of the least virulent *C. parasitica* strains. Unlike EP713 and many other hypovirulence-associated viruses (20), the virus responsible for the hypovirulence of NB58 contains as its genome a single large dsRNA segment, with no detectable shorter-than-full-length segments. We previously reported the cloning of NB58 dsRNA and comparison of some of its features to those of EP713 dsRNA (12). In this paper, we extend comparisons of some of those features.

An important element in virus gene expression is the involvement of the 5' leader sequence, also referred to as the 5' untranslated region (UTR), on translation efficiency. The 5' UTR of NB58 is relatively long, consisting of almost 500 nucleotide residues, and contains numerous start codons (12). In these respects the NB58 5' UTR is similar to those described for picornaviruses (e.g., 14), and caulimoviruses (e.g., 10). In each of those systems, cellular proteins have been implicated in the relatively higher levels of expression observed *in vivo* than would be predicted from cell-free translation results (9, 10, 16, 17).

The length of the leader and number of start codons suggest that translation initiation in this system does not proceed by the ribosome scanning model proposed for most mRNAs (15). Among the other models advanced to explain translation with these types of leaders are the ribosome shunt, in which ribosomes begin scanning but bypass the bulk of the leader and begin translation further downstream, and internal initiation, in which ribosomes initiate translation downstream of the leader (for discussion, see 8). We have begun experiments to address the role of the leader in translation regulation of the NB58 genome.

Like many filamentous fungi, sectors that are phenotypically different from the isolate that was originally plated arise fairly regularly with *C. parasitica.* Although some of these sectors prove to be unstable upon subculture, others are stable. One such phenotypically stable sector was observed in virus-containing strain NB58. This sector was notable because it had more aerial mycelium than the parental strain, and was designated NB58F (fluffy). Because strain NB58 is itself more stable phenotypically than its dsRNA-free single conidial isolate, NB58-19, we pro-

ceeded to examine NB58F for dsRNA content, cultural characteristics and virulence.

METHODS

Fungal strains, culture and dsRNA analysis. Strain NB58 (ATCC# 76220) was isolated by P.J. Bedker (2) and partially described by Hillman et al. (5). NB58-19 is a dsRNA-free single conidial isolate of NB58 (ATCC# 76221). Strains EP713 and EP747 were described by Anagnostakis (1). Methods for fungal culture, dsRNA extraction and analysis were described previously (11, 12). Strain NB58F was isolated as a sector of NB58 that had a distinct phenotype. Methods for its subculture and maintenance were the same as for other strains.

Methods for the cloning, mapping and sequence analysis of NB58 dsRNA were described previously (12). For cell-free translation experiments, inserts of portions of inserts representing NB58 dsRNA sequences were subcloned from the original pUC9-based library or from the original randomly primed cDNA reaction products using specific primers and polymerase chain reactions into pGEM3Zf + or pGEM4 (Promega). Transcripts for cell-free translation experiments were generated using T7 or SP6 RNA polymerase, following the manufacturers protocols. Cell-free translations incorporating ^{35}S methionine were performed using rabbit reticulocyte lysate or wheat germ extracts, essentially as recommended by the manufacturer (Promega). Computer predictions of 5' UTR folding structures were performed using the program of Zuker and Steigler (23) on a VAX computer.

RESULTS AND DISCUSSION

Genome structure of NB58 dsRNA. Cloning and mapping of NB58 dsRNA indicated that the genome had a size of approximately 12.5 kbp (12). The sequence at the 5' end of the positive strand is very similar to the homologous region of EP713 dsRNA, and, like EP713 dsRNA, the 3' end of the plus strand terminates with a poly(a) moiety. The complete nucleotide sequence of NB58 dsRNA has an overall size of 12,507 bp, excluding the poly(A). Details of some results discussed here in general terms will be presented elsewhere (B.I.H., B.T.H., and M.P.B., in preparation). Many features of NB58 dsRNA (Figure 1) are similar to those reported for EP713 dsRNA. (21).

5' leader region. Between the 5' terminus and the AUG colon that initiates the first long open reading frame is a leader of 487 nt, which contains 9 UAG codons that have in-frame terminators within 100 nucleotide residues (Figure 2). To examine which of the models discussed above is the most likely for NB58 translation and to identify the important regions of the 5' UTR, we have begun a series of translation studies. To synthesize large amounts of transcript, the T7 bacteriophage promoter was fused to the NB58 5' UTR. Quantification of translation was facilitated by fusing the 5' UTR to the *E. coli* beta-glucuronidase (GUS) reporter gene, the product of which is easily and sensitively assayed (13). A control construct was made as described above, but without the NB58 5' UTR. Since most eukaryotic messenger RNAs have a m7G cap at the 5' terminus, transcripts were synthesized and tested with and without such a cap structure.

Preliminary studies using both rabbit reticulocyte and wheat germ *in vitro* translation systems indicated that the leader greatly depressed translation compared to the leaderless construct (Figure 3). These results are similar to those reported by Rae et al. (18) for the EP713 leader. In our experiments, capped RNAs were required for efficient translations. These data suggest that a cellular factor from *C. parasitica* or a viral gene product may be involved in translation efficiency of the NB58 genome *in vivo*. To

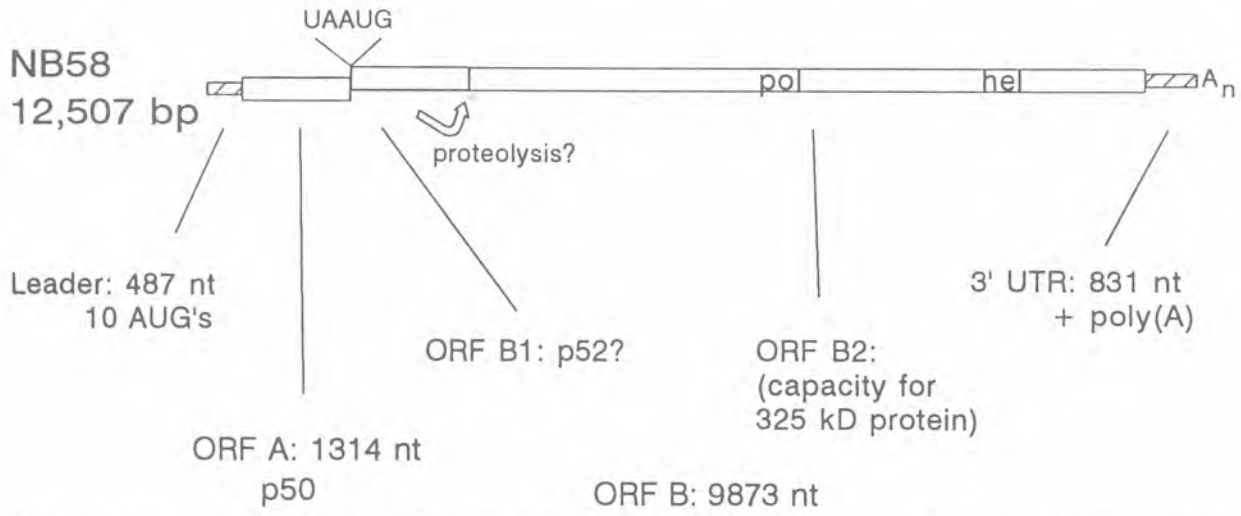

Figure 1. Genetic organization of NB58 dsRNA. The positive (polyadenylated, coding) strand is represented, with sizes in nucleotide residues (nt). Details of the sequence will be presented elsewhere (B.I. Hillman, B.T. Halpern and M.P. Brown, manuscript in preparation). The 50 kd ORF A product has been demonstrated by *in vitro* translations. Proteolytic processing of the N-terminal 52 kd of ORF B has not been demonstrated, but is theorized based on homology of this region with p48 of EP713 dsRNA and the presence of similar residues in NB58 that have been shown to represent catalytic and cleavage sites of EP713 ORF B.

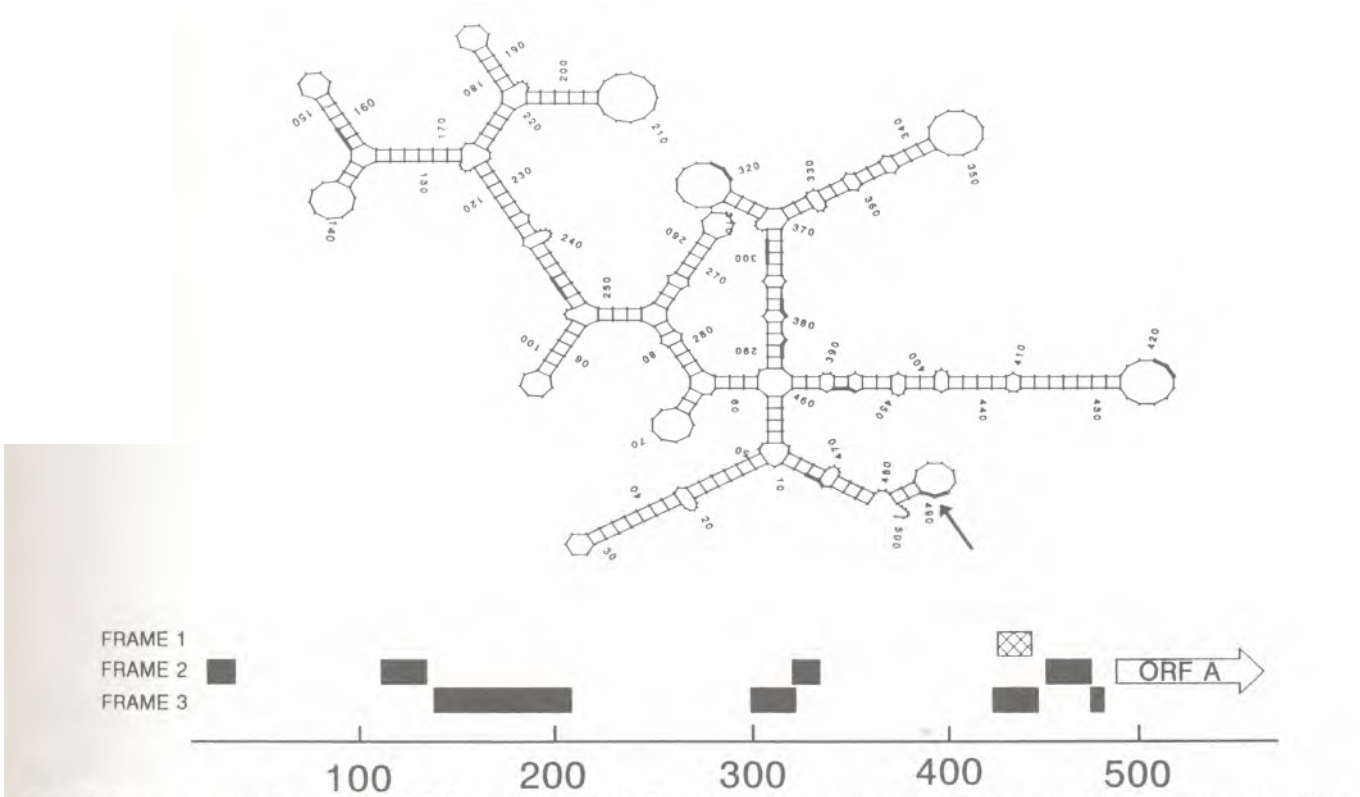

Figure 2. Computer generated fold of the NB58 5′ untranslated region (UTR). This fold represents the first 500 nucleotides of the NB58 genome, which includes the entire 5′ UTR. All ten AUG's are shown in bold, the last of which (indicated by an arrow) is the start codon that is used to initiate translation of ORF A. Possible coding regions represented in the 5′ UTR are represented in the lower portion of the figure, adapted from reference 5. Sizes are in nucleotide residues.

determine the important parts of the 5' UTR, deletion constructs will be made and used in both *in vitro* and *in vivo* studies. We have initiated experiments to identify any *C. parasitica* factors that interact with the 5' UTR using fractionated cellular extracts *in vitro*.

Coding regions. The coding region of NB58 dsRNA, like that of EP713, is divided into two open reading frames, designated ORF A and ORF B (Figure 2). NB58 ORF A has the potential to encode a protein of 50 kd. Unlike ORF A coding region of EP713 dsRNA (5, 6), NB58 ORF A appears to have no cysteine protease motif, based on its deduced amino acid sequence. Cell-free translation of *in vitro* generated transcripts from cDNA clones consistently result in a single major product of approximately 50 kd, suggesting that processing of the ORF A product does not occur. The apparent lack of a homologue of the EP713 p29 product, which has been demonstrated to down-regulated pigment production and sporulation even when expressed from the fungal genome (3), may be significant in view of the higher levels of pigment and spore production in NB58 compared to EP713. To determine whether the ORF A product of NB58 dsRNA has a discernable effect on fungal morphology, we have initiated experiments to express the first open reading frame of NB58 from the fungal genome. The ORF A coding region was cloned into the same fungal transformation vector used in the EP713 studies (3), in collaboration with G.H. Choi and D.L. Nuss. Protoplasts of *C. parasitica* strain EP155 and NB58-19 were transformed with the completed construct following the general method outlined by Churchill et al. (7). Putative transformants were selected by plating on PDA plates amended with hygromycin B, and the integration of ORF A into the *C. parasitica* genome was confirmed by southern blots. In contrast with the expression of EP713 ORF A in EP155, which resulted in a white phenotype and in the reduction of specific fungal products, NB58 ORF A transformants examined thus far have shown neither alteration in cultural morphology nor pigmentation.

NB58 ORF A terminates with a UAAUG pentanucleotide, which also contains the initiation colon for ORF B. This is the same sequence that was shown to separate the EP713 ORF A and ORF B coding regions (21), so it is reasonable to assume that a similar mechanism for regulation of this portion of the NB58 genome is in effect.

EP713 ORF B encodes a polyprotein, the N-terminal portion of which has been shown to be a cysteine protease of 48 kd (19). The N-terminal 52 kd of the NB58 ORF B product is homologous to p48 of EP713. We have not yet addressed the question of proteolytic processing of the NB58 ORF B product, but it is notable that it contains possible catalytic cysteine and histidine residues at similar positions and in similar environments to those in EP713 ORF B.

NB58F: a stable, dsRNA-free sector of strain NB58. A phenotypically distinct sector from the virus-containing strain NB58 was found to be dsRNA free. The new isolate was intermediate between the parental strain NB58 and the representative virus-free single conidial isolate NB58-19,

in growth rate, pigmentation and conidiation. The mutant isolate, named NB58F, was found to be stable on subculture, and all single conidial progeny of this isolate had identical phenotypes and characteristics. Repeated attempts to infect NB58F by anastomosis with the parent strain, NB58, or other virus-containing isolates have thus far been unsuccessful. There are several possible explanations for the appearance of this isolate: 1) a contaminant appeared on the plate that can not be infected with the tested viruses; 2) a vegetative compatibility (VC) group switch had occurred, preventing virus transfer into this sector; or, 3) a mutation occurred that prevented initial viral infection and/or maintenance of the virus in this isolate.

To address the first possibility, DNA fingerprint analysis was performed by Michael Milgroom (Cornell University) on the parental strain NB58, NB58F, and single conidial isolate NB58-19. Among the controls was the common tester strain EP155. Under the probe conditions used, no differences in banding patterns were observed between NB58, NB58F, and NB58-19. In contrast, all of the controls including EP155 were distinctly different (J.J.P., B.I.H., S.L Anagnostakis and M.G. Milgroom, in prep). These data suggested that the NB58F isolate was not a contaminant and in fact, had the same nuclear background as the parental strain.

If a VC group switch were responsible for the NB58F phenotype, a simultaneous loss of the virus from NB58 at the time of sectoring also would be required to explain the lack of dsRNA in this sector. Pairings were set up with several tester strains to determine if a VC group switch had occurred. The control for all pairings was the virus-free single conidial isolate NB58-19. Although striking phenotypic differences between the strains in some pairings made compatibility/incompatibility determinations difficult, NB58F and NB58-19 were judged to be in the same VC group.

The experiments described above suggested the third explanation (a mutation resulting in a virus-resistant strain of the fungus) was the most likely for the appearance of the NB58F sector. As a first step in addressing this possibility, matings were set up to determine if the NB58F phenotype and virus resistance segregate as a cytoplasmic factor or a nuclear gene. These studies arc in progress.

Further characterization of the NB58F isolate will include additional conversion attempts with different types of *C. parasitica* viruses. These studies will help determine if virus resistance is limited to a particular type of dsRNA virus. If the mating study suggests nuclear gene involvement in the NB58F phenotype, attempts will be made to convert single ascospore progeny to determine whether virus resistance segregate with the phenomenon. Subsequent experiments would include attempts to complement the mutation with a cDNA or cosmid library from *C. parasitica.* Identification of any complementing sequences could be facilitated by hybridization and sequence comparisons to known yeast viral maintenance genes, as several of these have already been described (for examples, see ref. 23).

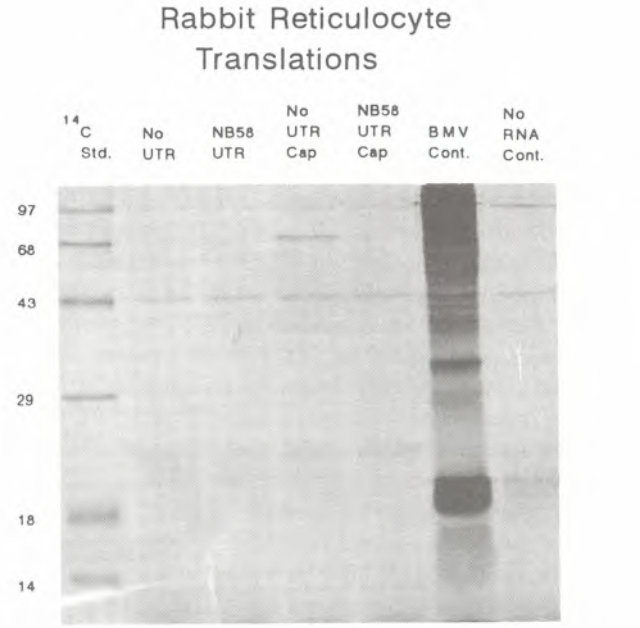

Figure 3. Rabbit reticulocyte translations. A autoradiogram blot of cell-free translation assays using rabbit reticulocyte lysates is shown, with ^{14}C standard size markers shown in kilodaltons. Other lanes include constructs with and without the NB58 5' untranslated region (UTR), both capped and uncapped, as described in the text. Brome mosaic virus (BMV) and no added RNA were used as positive and negative controls, respectively. Note the presence of a distinct band of approximately 70 kd in the leaderless (no UTR), capped construct, the correct for the GUS reporter gene. A faint band of the same size also is present in the leaderless, uncapped lane.

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