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The characterization of double-stranded RNA from Michigan hypovirulent isolates of *Endothia parasitica*

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Michigan State University, 1988

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THE CHARACTERIZATION OF DOUBLE-STRANDED RNA FROM MICHIGAN HYPOVIRULENT ISOLATES OF *ENDOTHIA PARASITICA*

By

Cynthia Poorman Paul

A DISSERTATION

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ABSTRACT

THE CHARACTERIZATION OF DOUBLE-STRANDED RNA
FROM MICHIGAN HYPOVIRULENT ISOLATES OF ENDOTHIA PARASITICA

By

Cynthia Poorman Paul

The fungal pathogen, Endothia parasitica causes chestnut blight on American and European chestnut trees. Hypovirulent isolates of E. parasitica have been found in the United States and Europe. Hypovirulent isolates are reduced in virulence relative to normal virulent isolates and harbor double-stranded RNA (dsRNA). The isolates also often exhibit changes in culture morphology. These characteristics are transmitted from hypovirulent to virulent isolates through hyphal anastomosis.

The dsRNA banding patterns of representative hypovirulent isolates from Michigan were compared on polyacrylamide gels. The dsRNA molecules found in these isolates varied in size, number, and relative concentrations. The number of dsRNA segments in a single isolate varied from one to four. The segments ranged in size from 0.82 to 9.0 kbp. No single size of dsRNA segment was found in all hypovirulent isolates. All segments examined by electron microscopy were linear.

Using Northern blot hybridization, dsRNA from GH2, a Michigan

hypovirulent isolate, was found to share homology with dsRNA from all other Michigan isolates tested, with the exception of one, isolate RC1. RC1 dsRNA had no detectable homology with dsRNA from any other Michigan isolates examined. The dsRNA from isolates GH2 and RC1 did not have detectable homology with dsRNA from any of the isolates from other states or Europe that were included in this study.

If the dsRNA is transcribed to yield a single-stranded mRNA, the mRNA is probably full length since no smaller than full length mRNAs were detected when poly(A) RNAs were isolated. The full length single-stranded RNAs detected may represent denatured dsRNA as well as mRNA. No dsRNA-specific proteins were detected in in vitro translations using rabbit reticulocyte or wheat germ systems.

Portions of cDNA corresponding to the dsRNA from isolate GH2 were cloned into an M13 vector. These small cloned sequences were useful in showing that the hybridization observed between GH2 dsRNA and fungal DNA is due to contamination of the dsRNA with host nucleic acids. No GH2 dsRNA sequences were detected in DNA from the isolates GH2, CL1-16, or Ess6.

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Introduction

Hypovirulence in Endothia parasitica (Murr.) Anderson [=Cryphonectria parasitica (Murr.) Barr], the causal organism of chestnut blight, holds a special place in plant pathology and the study of biological control mechanisms. Conventional methods for controlling fungal plant diseases failed to control chestnut blight, but the fungal disease has succumbed to a "disease" of its own in some areas. The natural spread of hypovirulent isolates of E. parasitica has allowed the survival of chestnut trees in Europe and North America. This type of hypovirulence has been termed cytoplasmic (30), transmissible (89), or exclusive (48) hypovirulence. Hypovirulent isolates are reduced in virulence relative to normal isolates, they often exhibit abnormal morphology in culture, and they harbor double-stranded RNA (dsRNA). They can transmit these characteristics to virulent isolates through hyphal anastomosis. Because of these correlations, the study of the dsRNA may lead to a better understanding of hypovirulence and virulence in E. parasitica.

The History of Chestnut Blight

Chestnut blight is among the most devastating plant diseases known. It has eliminated the American chestnut [Castanea dentata (Marsh) Borkh.] as a major species in eastern North America, where

these trees once made up 25% of the forest. The tall straight form of the main stem of the tree and the decay resistance of the wood made chestnut wood valuable for use in telephone poles, masts of ships, and in fences. Furniture, paneling, and caskets were often made from chestnut lumber. The tannins in the bark were used in the leather tanning industry and the nuts were eaten by people, their livestock, and wildlife. Chestnuts were also popular shade trees in the nineteeth and early twentieth centuries.

Chestnut blight was first reported in North America in 1904 in New York (2). The causal organism, E. parasitica, is thought to have been imported from the Orient on Japanese (C. crenata Sieb. and Zucc.) or Chinese chestnut (C. mollissima Bl.). These species exhibit a high level of tolerance to the blight, but can be infected with E. parasitica. Thus, they can act as carriers of the fungus. The American chestnut is highly susceptible and the fungus spread quickly throughout the natural range of the chestnut. This area extends from Maine to Ontario and eastern Michigan, south along the Appalachian Mountains, and into Mississippi. Within the native range few large trees remain. E. parasitica causes cankers which kill branches and trunks of trees by girdling them. However, the root systems remain alive and sprouts grow from the base of stumps of infected trees. These sprouts are usually also girdled within a few years, before they reach the flowering stage.

E. parasitica was introduced into Europe and European chestnut (C. sativa Mill.) proved to be extremely susceptible. The blight was first observed in Italy in 1938, where chestnuts were grown primarily

for the nut crop. The recovery of chestnut stands was also first observed in Italy. In 1950, signs of improvement were noticed in some of the sites of earliest infection (69,88). This recovery was found to be due to an altered form of E. parasitica (48). This form of the fungus was white in culture, lacking the orange pigmentation of normal colonies. Pycnidia were not produced under conditions which resulted in their formation by normal cultures. These isolates were also reduced in virulence, producing "healing" cankers in chestnut trees.

The characteristics of the new form of the fungus could be transmitted to normal isolates of E. parasitica through hyphal anastomosis. Grente termed these forms of E. parasitica "hypovirulent". Hypovirulent forms of the fungus established themselves by natural spread in Italy (69,88) and have been used successfully to control the blight in French chestnut orchards (48).

Native hypovirulent forms of E. parasitica have also been found in North America. They were first found in 1977 in areas of Michigan that are outside of the natural range of the chestnut (36,61). Unlike the European hypovirulent isolates, these hypovirulent isolates are pigmented. However, their morphology in culture is characterized by slower growth and sectoring. Like the European hypovirulent isolates, they exhibit reduced sporulation and can transmit the hypovirulent phenotype to virulent isolates. These native Michigan hypovirulent isolates have allowed the survival of many large chestnut trees in western Michigan (44). Hypovirulent isolates have subsequently been found within the natural range (51), although widespread recovery of chestnut trees from the blight has not yet occurred there.

The Biology of Endothia parasitica

Hypovirulent isolates of E. parasitica were discovered through their curative properties and the lack of pigment in most European hypovirulent isolates. Subsequently, pigmented European and American hypovirulent isolates have been found. All hypovirulant isolates are debilited in some way. Most show changes in morphology relative to virulent isolates in culture. They may grow more slowly, exhibit sectoring, lack aerial hyphae, or lack pigment. They commonly exhibit reduced capacity to produce conidia and ascospores. In chestnut, they produce cankers that are superficial or slower to expand, allowing the survival of trees or branches for a longer period of time.

The ability of hypovirulent isolates to transfer their curative property to virulent isolates had been noted during the recovery of chestnuts from blight in Italy (48). The transmissibility of hypovirulence is central to its ability to control blight. The transfer of hypovirulence to virulent isolates occurs between compatible isolates of the fungus through hyphal anastomosis. In a compatible reaction, the hyphae of the two isolates fuse and cytoplasm is exchanged. The transfer of the hypovirulent phenotype is evident by the change in morphology along the margin of the virulent colony. Compatible reactions occur when the two isolates belong to the same vegetative compatibility group (2). Vegetative compatibility is controlled by a number of loci in E. parasitica. The larger the number of the alleles shared by two isolates the more likely it is that hyphae will fuse and transfer will occur (2). Therefore,

transfer can occur between incompatible isolates, but with less frequency (4). Death of the fused cells and development of pycnidia along the line where the two cultures meet characterize an incompatible reaction. This is called a "barrage" and it is clearly visible in culture as a dark line between the two isolates. The hypovirulence trait may be transferred occasionally before cell death results. So, although vegetative compatibility presents a barrier to the spread of hypovirulence, the barrier is not insurmountable.

Transfer via hyphal anastomosis suggested the possibility that the hypovirulence trait was carried by a cytoplasmic element. Van Alfen et al. (91) tested this hypothesis using genetically marked isolates. A virulent isolate which was auxotrophic for methionine was coinoculated with a hypovirulent lysine auxotroph in chestnut trees. Hypovirulent methionine-requiring isolates were recovered from the resultant cankers. Conidia collected from a heterokaryon forced in culture between a hypovirulent methionine auxotroph and a virulent arginine auxotroph produced hypovirulent colonies which were either methionine- or arginine-requiring. Thus, transmission of the hypovirulent phenotype is consistent with a cytoplasmic pattern of inheritance.

Due to the cytoplasmic mode of inheritance, it was suspected that a mycovirus was the cytoplasmic factor responsible for hypovirulence. Mycoviral genomes are often made up of dsRNA. As suspected, dsRNA was found in hypovirulent, but not in virulent isolates of E. parasitica (25). When the hypovirulence trait is transmitted to a virulent isolate, the dsRNA is also transmitted (4,25). Curing of a

hypovirulent E. parasitica isolate of dsRNA by treatment with cyclohexamide results in the concomitant loss of hypovirulence (40). Cured isolates converted back to hypovirulence through hyphal anastomosis with a hypovirulent isolate also regained dsRNA. Colonies grown from single ascospores of hypovirulent isolates are virulent and lack dsRNA (25). The direct transfer of purified dsRNA in completion of Koch's postulates has not yet been accomplished, but the cause and effect relationship between dsRNA and hypovirulence in E. parasitica is generally accepted.

More extensive studies have shown that a continuum of virulence, from avirulent to highly virulent, exists among different E. parasitica isolates (33). One isolate harboring dsRNA was found that was as virulent as some isolates lacking dsRNA. This is not surprising, as reduced fitness in any way affects the ability of the fungus to grow on chestnut and virulence of individual isolates can vary depending on the method of measurement and the definition of hypovirulence (33). Many factors could affect the level of virulence in a particular isolate. For example, methionine auxotrophs derived from virulent isolates registered as hypovirulent in some of Elliston's tests. As discussed in the next section, the quality and quantity of dsRNA segments among isolates is highly variable and evidence exists for more than one type of dsRNA in an isolate (multiple "infections"). For any given fungal background in Elliston's study, isolates with dsRNA were reduced in virulence, but the level of reduction varied with different sources of dsRNA.

Although there is a correlation between dsRNA and hypovirulence, this does not rule out the possibility of other kinds of hypovirulence caused by nuclear or other cytoplasmic factors in certain isolates. There are two lines of evidence for nuclear-encoded hypovirulence. The first comes from the discovery of some slow growing white hypovirulent isolates that produced four types of single-conidial isolates (49,88). These isolates formed normal virulent single-conidial isolates and white hypovirulent single-conidial isolates as did other hypovirulent isolates. Unlike other hypovirulent isolates, their conida also produced white hypovirulent isolates with intermediate cultural characteristics and deeply pigmented hypovirulent isolates. These deeply pigmented isolates were termed "JR" types. The proportion of single conidial isolates of each type recovered can be varied by altering cultural conditions. Anagnostakis (3) combined JR and normal virulent isolates in sexual crosses. Single ascospore isolates exhibited approximately a 1:1 ratio of JR:normal, indicating that this phenotype is controlled by a single nuclear gene. Transferring dsRNA to JR isolates which lack dsRNA through hyphal anastomosis does not change their culture morphology or virulence. However, JR isolates can support dsRNA replication and dsRNA-containing JR isolates can transfer the dsRNA to virulent isolates which then become white and hypovirulent. JR progeny are produced only by isolates carrying dsRNA. Isolates of identical nuclear genotype never produce JR progeny if they have not carried dsRNA. Anagnostakis speculated that the dsRNA induces instability at the JR locus or that the dsRNA integrates into the fungal genome.

The second type of nuclear-encoded hypovirulence is found in UV-induced mutants that resemble the European hypovirulent phenotype. These mutants are white in culture, have decreased virulence relative to normal isolates, and produce fewer conidia. In all of the cases studied the mutation occurred at a single nuclear locus (90). The frequency of white mutants is higher when spores from virulent single conidial isolates of hypovirulent isolates are irradiated (16%) than when spores from virulent isolates that have never been vegetatively paired with hypovirulent isolates are exposed to UV light (0.1%) (92). The possible relationship between dsRNA and these nuclear mutants is not presently understood. Both examples of nuclear-encoded hypovirulence involve the presence of dsRNA in the history of the isolate. However, the nuclear mutations are stable and inherited in a pattern indicating that they are a nuclear trait, while dsRNA-containing hypovirulent isolates can be unstable and do not transfer the hypovirulent phenotype to progeny through ascospores (25).

Cytoplasmic factors other than dsRNA may be found which also decrease the virulence of E. parasitica. A Michigan hypovirulent isolate has been found which lacks detectable dsRNA (41). However, the hypovirulence trait is cytoplasmically inherited.

Cases of hypovirulence in E. parasitica lacking dsRNA are exceptional, although they may prove important in elucidating the mechanism of hypovirulence. The correlation between dsRNA and hypovirulence in the vast majority of isolates examined supported the hypothesis that a mycovirus was involved. However, the dsRNA was found to be unencapsidated. Using polyethylene glycol precipitation

and equilibrium density or rate-zonal centrifugation, Dodds found pleiomorphic club-shaped vesicles in European hypovirulent isolates (29). The fractions containing the particles had the greatest concentration of dsRNA. The particles were not found in extracts of a virulent isolate which also lacked dsRNA. These club-shaped particles also could not be found in extracts from American hypovirulent isolates (27). Using the same techniques, Chmelo and Kaczmarczyk reported similar particles in an isolate with an American fungal background and dsRNA from a European source (18). These particles do not resemble typical virus particles, but similar structures have been described in a few other virus-infected fungi (9,57).

The particles were isolated by Hansen et al. (57) using Dodds' methods. Unlike Dodds, they also found particles in a virulent isolate. The particles from the hypovirulent isolate contained dsRNA, but those from the virulent isolate did not. The particles consisted of lipid, protein, and carbohydrate. The relative amounts of each varied between particles from the hypovirulent and virulent isolates, the greatest difference being the larger amount of protein in particles from the virulent isolate. Particles from the hypovirulent isolate did not contain protein in sufficient amounts to allow for the presence of a viral capsid. The constituents of the particles resembled those of the fungal cell wall. The same sugars were present (arabinose, mannose, galactose, and glucose), although the relative amounts were different. This suggests that these particles are of fungal origin and may be fungal vesicles involved in packaging of cell wall components. It has been suggested that they serve to protect the

cell from the dsRNA, to protect the dsRNA from cellular ribonucleases, or they may serve as sites of replication for the dsRNA (57). Hansen et al. (57) have also found a dsRNA-dependent RNA polymerase activity associated with the dsRNA-containing particles from the hypovirulent isolate. The particles from the virulent isolate apparently were not checked for this activity.

Newhouse et al. (71) found spherical membrane-bound virus-like particles in a hypovirulent isolate, but not in a virulent isolate in electronmicrographs of thin sections of hyphae. They resembled particles found in other virus-infected fungi and were associated with changes in the host rough endoplasmic reticulum.

Double-stranded RNA

The correlation between cytoplasmic dsRNA and the hypovirulent phenotype in E. parasitica was first suggested by Day et al. (25). All of the hypovirulent isolates and none of the virulent isolates examined in this study carried dsRNA. In a long-term study, Elliston found that although the hypovirulent isolates carried dsRNA, they varied greatly in the degree to which they were debilitated (33). One dsRNA-containing isolate was as virulent as some of the isolates which lack dsRNA. When isolates of the same genetic background were compared, with and without dsRNA, those with dsRNA were reduced in virulence. The dsRNA of the relatively more virulent dsRNA-containing E. parasitica isolate may be lacking the functional region of the dsRNA in less virulent isolates. Alternatively, the dsRNA in this isolate may be that of a latent mycovirus which is unrelated to the

hypovirulence-associated dsRNA. Although Elliston found more variability than previously described, the correlation between dsRNA and hypovirulence held up with one exception.

Although hypovirulent isolates of E. parasitica carry dsRNA, the dsRNA varies between isolates. Dodds (28) described three types of dsRNA banding patterns on gels, two from European isolates and one from North American isolates. Subsequent studies have shown that the dsRNA of all hypovirulent isolates does not belong to one of the three originally described types (44). Banding patterns described by Dodds included from 1-6 readily visible bands on gels. The molecular weights of the dsRNA segments were between 4.3 and 6.2 X 10^6. By photographing overloaded gels and using long exposure times smaller bands were also detected (28,29).

Certain dsRNA banding patterns on gels are associated with specific culture morphologies which are easy to distinguish from one another (32,42). When the dsRNA is transferred into a virulent isolate of another nuclear genotype, the distinct morphology of the hypovirulent isolate is transferred as well. However, the dsRNA banding patterns of the hypovirulent isolates are sometimes unstable. They have been observed to change through subculture or transfer to a different genetic background (1,4,25,29,40,46,89). These changes involve the loss or gain of segments or changes in the relative concentration of segments. Some of these changes may be due to interactions with the fungal host, to differences in the efficiency of various segments in replication, or to a change in environmental conditions which may favor one segment over another.

Changes in the dsRNA banding pattern of a particular isolate (loss or decrease in concentration of segments) often do not result in detectable changes in phenotype of the fungus. In most cases, there has not been a correlation of a particular segment with a specific trait. However, Fulbright (40) recovered dsRNA-containing cultures with normal morphology after treatment with cyclohexamide. The isolates were found to carry dsRNA but, in each case, one segment was decreased in concentration relative to other dsRNA segments. This suggests that this segment may be important in causing the changes in culture morphology associated with hypovirulence. This study provides evidence that dsRNA segments may not be alike, even when similar in size.

Elliston (34,35) has provided evidence that there is more than one cytoplasmic determinant of hypovirulence in a Michigan hypovirulent isolate. Based on segregation of the culture morphology and virulence of serial single-conidial isolates (SCI), the isolate carried two hypovirulence agents. SCI of the original isolate segregated into those like the original isolate in morphology and virulence (Type A), those that were less debilitated (Type B), and those that resembled virulent E. parasitica (Type C). Conidial isolates of some Type A SCI segregated like the original (Type A) isolate. These SCI carry both agents. Some Type A (Type A') and all Type B SCI produced two types of conidial isolates: those resembling the parent and those resembling virulent isolates. These isolates each carry one of the agents. Conidial isolates grown from Type C SCI always resembled virulent isolates (Type C). These isolates lacked a hypovirulence agent. The

dsRNA banding patterns of types A' and B were different from each other. When Type A' and B dsRNAs were run in the same lane in a gel, the resulting pattern matched that of type A SCI. Type C single conidial isolates lacked dsRNA. This resembles infection with multiple viruses. It is further evidence that certain phenotypes are associated with particular dsRNA segments.

The relationships between the dsRNA segments within an isolate has also been examined using biochemical techniques. The Michigan isolate, GH2, carries three segments that are easily separated by agarose gel electrophoresis. The large segment (L) and the middle segment (M) were found to share sequence homology as determined by dot blot analysis (85). The small segment (S) did not hybridize with L and M. The termini of each segment were examined by partial and complete digestion of denatured end-labeled dsRNA using RNase T1, a ribonuclease which cleaves specifically at guanosine residues. The digestion products were analyzed by polyacrylamide gel electrophoresis. The termini of L and M exhibited the same patterns, while the patterns of S were different. This supports the hybridization results and also suggests that M was generated from L by an internal deletion event. In this respect, M resembles the variant dsRNAs described in wound tumor virus (WTV) (73). However, the WTV variant dsRNAs were associated with a change in phenotype and either the loss or decrease in relative concentration of the segment from which they were generated. No change in the GH2 phenotype is observed when the middle segment undergoes further reduction in size or is deleted (46). The S segment resembles the satellite RNAs described in

some plant viruses (38). The small segment has been cloned in a DNA plasmid and sequenced and has been found to have no open reading frames (D.L. Nuss, personal communication).

The dsRNA of the European isolate, EP713, has also been examined in detail (58). The five major segments all hybridized to a cloned 194 bp probe generated from EP713 dsRNA, indicating that all detectable segments in EP713 share homology. The major segments also shared common termini as determined by analysis with base-specific ribonucleases. The cloned probe also hybridized to small dsRNA segments which were only detected on ethidium bromide-stained gels when the gel was overloaded. Two open reading frames have been found in an EP713 segment (D.L. Nuss, personal communication).

The analyses of the dsRNA in GH2 and EP713 suggest that many of the dsRNA segments in hypovirulent E. parasitica isolates may be related, but that unrelated segments can be maintained within an isolate. Although GH2 and EP713 dsRNAs do not share sequence homology (D.L. Nuss, personal communication), all segments of both isolates have a poly(adenylic):poly(uridylic acid) (poly(A:U)) terminus (58,85). The significance of this is not known; it may be important for dsRNA replication or expression. Yeast killer factor dsRNA was found to have a similar internal poly(A:U) sequence (39,56), which is thought to function similarly to post-transcriptionally added poly(A) tails on eukaryotic mRNAs (87).

Mode of Action

The variety found among the dsRNAs of hypovirulent E. parasitica would seem to indicate that the presence of dsRNA in the fungus is sufficient to cause a reduction in virulence. The dsRNA may compete with normal cellular RNAs for factors needed for replication or expression. However, the dsRNA genomes of mycoviruses are replicated and maintained in most cases with no adverse affect on the fungus (47). In fact, many have only been detected by electron microscopy of fungal cells (9). This suggests that the dsRNA of E. parasitica has a more specific effect. Mycovirus genomes encode protein products (16) and the recent finding of open reading frames in one E. parasitica isolate suggests that this may be found to be the case in this system as well. The observation that different phenotypes are associated with different segments (34,35,40) suggests that different products or products of varying efficiency are encoded by separate dsRNA segments. The nuclear hypovirulence in some isolates which have been exposed to dsRNA (3,90,92), suggests the possibility of an interaction between dsRNA and the fungal genome.

The small segment in the GH2 isolate may be unusual or other segments lacking coding capacity may be found in other isolates. This segment may function in regulation of cellular functions or dsRNA expression. The known number of ways in which RNA functions in cellular metabolism is increasing (7,13,21,54,65,75).

Several effects of dsRNA on E. parasitica at the molecular level are known. Vesicles were isolated from virulent and hypovirulent isolates (57), but the components of the dsRNA-containing vesicles

were present in different relative amounts than in vesicles from virulent isolates. Powell and Van Alfen found the level of certain mRNAs was decreased in hypovirulent isolates relative to virulent isolates, while the level of other mRNAs was greater in hypovirulent isolates (76). Protein profiles from virulent and hypovirulent E. parasitica have revealed eight proteins which are present in virulent isolates but absent or present in reduced amounts in hypovirulent isolates (77). The two hypovirulent isolates examined produced the same changes in proteins produced by the fungus. Furthermore, one of the proteins is excreted by the fungus into the medium when the fungus is grown in culture. This suggests that the dsRNA or its products regulate the expression of specific virulence-associated mRNAs and proteins.

Different dsRNAs could interfere with fungal metabolism in different ways. Specific changes in culture morphology occur upon transfer of certain "strains" of dsRNA to E. parasitica with different nuclear backgrounds (32,42). The factors involved in E. parasitica pathogenesis of chestnut are not well characterized. Roles for diaporthine, a toxin produced by E. parasitica, and oxalic acid have been suggested (49,50). The ability of the fungus to utilize tannins as a nutrient source may play a role in the ability of E. parasitica to grow on chestnut wood (50). By studying the disruption of the disease process by hypovirulence, knowledge may be gained about the E. parasitica-chestnut interaction.

Origin

The relatively sudden appearance of hypovirulence in populations of virulent E. parasitica both in Europe and the United States is puzzling (36,51,69,88). Initially, a mycovirus was suspected due to the ability of hypovirulent isolates to spread the trait to virulent isolates. This, in fact, led to the discovery of the dsRNA in hypovirulent isolates. The dsRNA in E. parasitica resembles mycoviral genomes in several ways. It is double-stranded and segmented (9). The segments are larger than most mycoviral dsRNA segments which are generally of molecular weights smaller than $4.5X10^6$. However, one mycovirus from Helminthosporium maydis (Cochliobolus heterostrophus) has an unsegmented genome with a molecular weight of $6.3X10^6$. The dsRNA of E. parasitica is not encapsidated as are most fungal viruses. Vesicles similar to those associated with dsRNA in E. parasitica have been described in virus-infected mushrooms (Agaricus bisporus) (16). However, analysis of the vesicles from hypovirulent E. parasitica suggests they are of fungal origin (57).

Structural analysis of the dsRNA from two hypovirulent isolates has revealed similarity to single-stranded and double-stranded RNA viral genomes (58,85). These similarities include a poly(A:U) terminus, homologous termini between segments within an isolate, and the presence of segments which may be the result of internal deletions of larger segments. Dodds (29) did not detect any ssRNAs of the appropriate size for a single-stranded RNA genome in ethidium bromide-stained gels. The association of dsRNA and RNA polymerase activity with membranous structures also supports a viral origin (29,57,58,85).

Like mycoviruses, the dsRNA of hypovirulent E. parasitica does not undergo extracelluar transmission. For this reason, it has been suggested that fungal viruses resemble plasmids as well as viruses (87,95). Extrachromosomal elements are common in eukaryotes. There are several examples of dsRNAs that are not viral-associated in plants (37,52) and they may also occur in fungi. Although they may share some features with mycoviruses, the dsRNAs of E. parasitica may not be defective mycoviruses in the sense of having evolved from a virus. The dsRNAs of hypovirulent E. parasitica may represent genetic elements which have developed strategies similar to those of mycoviruses for maintenance in the cell.

The similarity between the hypovirulence-associated dsRNA and viral genomes does not explain separate appearances in widely separated geographical areas. It has been postulated that another fungus, native to these areas, which is capable of anastomosing with E. parasitica may transfer viruses to E. parasitica. No such fungus is known, but E. radicalis has been suggested as a possibility (31). Alternatively, there may be latent dsRNA present in all E. parasitica at undetectable levels which multiplies to detectable levels in response to some environmental stimulus (31). The dsRNA has not been found to be passed to progeny through ascospores (25), indicating it is not encoded by nuclear genes.

Two types of nuclear-encoded hypovirulence have been described (3,90) but in these cases exposure to dsRNA was required for or increased the frequency of these mutations. This suggests a proviral mechanism involving insertion of dsRNA sequences into the genome (89)

or some unknown regulatory mechanism involving the dsRNA. The transposable elements of yeast provide a model for this type of event in fungi (16,45). However, the structural data available on E. parasitica dsRNAs does not support this theory (58,85). Currently, all possibilities concerning the origin of the dsRNA in E. parasitica must be considered.

Prospects for Biological Control

Naturally occurring hypovirulence has allowed the recovery of chestnut populations in Italy and Michigan (44,69,88). Hypovirulent isolates have been used successfully to control E. parasitica in the orchards of France (48). Although hypovirulent isolates have been found within the natural range of the chestnut in North America, widespread recovery has not occurred. Many reasons for this have been suggested (50,89): high virulent inoculum density, many vegetative compatibility groups, the absence of a necessary vector to spread fungal spores, the dilution of the chestnut population in North America with other tree species, and the high blight susceptibility of American chestnut. Due to these factors, a longer period of time may be required for hypovirulence to become established in the natural range of the chestnut in North America.

The hypovirulent isolates to be used in the field must not be so debilitated that they can not spread (43). They must also be able to transfer the hypovirulent phenotype to virulent strains of the fungus already present in the infected area, therefore a cytoplasmic agent is ideal. This must be considered when genetically engineering

hypovirulent fungal isolates. Vegetative compatibility groups must be matched with those already present in the affected area.

To be effective in controlling other plant pathogenic fungi, the newly hypovirulent strains must be able to spread and convert the naturally occurring virulent fungi already present to hypovirulence. The hypovirulent strains must not be too severely debilitated. The cytoplasmic nature of the hypovirulence agent must be maintained, whether the agent is the dsRNA itself or a cDNA copy of the dsRNA.

The potential of dsRNA as a control of virulence in other plant pathogenic fungi will depend on the mechanisms by which dsRNA exerts its effect on the fungus and the mechanisms by which E. parasitica and the other fungi cause disease in their hosts. If the dsRNA interferes with specific processes involved in the virulence of E. parasitica on chestnut, the virulence of fungi which invade their hosts by other mechanisms may be unaffected. However, if the dsRNA interferes with processes which are required for virulence and shared by many fungi, these fungi may react similarly to the presence of the dsRNA. Since the factor(s) involved in virulence of E. parasitica on chestnut and the mechanism by which dsRNA may cause hypovirulence are unknown, it is premature to speculate on the potential of dsRNA in reducing the virulence of other fungi.

CHAPTER 1

BANDING PATTERNS OF dsRNA FROM MICHIGAN ISOLATES

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BANDING PATTERNS OF dsRNA FROM MICHIGAN ISOLATES

Introduction

In 1977 a native American hypovirulent isolate was recovered from a chestnut tree in Rockford, Michigan (36). Although European hypovirulent isolates had been successfully used to control chestnut blight in Europe, this was the first report of hypovirulence in the United States. Hypovirulent isolates have more recently been found in the natural range of the chestnut, but widespread recovery has not occurred. Attempts to use European hypovirulent isolates to control chestnut blight in the United States have not been successful (2). The discovery of native American hypovirulence opens up new possibilities of reducing chestnut blight on this continent. Unlike the European hypovirulent isolates, American hypovirulent isolates are pigmented (44,61). North American hypovirulent isolates often show other morphological changes in culture, such as reduced growth and sectoring. Like the European isolates, they are reduced in virulence, carry dsRNA, and can transfer these traits to virulent isolates through hyphal anasotmosis. Cankers which appeared to be recovering were observed throughout Michigan (44). These cankers are swollen in appearance due to the formation of callus and do not result in death of the branch. Isolates were recovered from a number of these

cankers. Many isolates were assayed for the presence of dsRNA and a variety of banding patterns were observed on polyacrylamide gels. A survey of the banding patterns from different sites in Michigan was conducted.

Materials and Methods

Cultures. All E. parasitica isolates were maintained on potato dextrose agar (Difco, Detroit, MI) at 20 C under fluorescent lights with a 16 hour photoperiod. For dsRNA isolation, cultures were grown in stationary liquid culture in Endothia complete medium (78) modified by the omission of glucose. Cultures were usually grown for 10-14 days, although older cultures were occasionally used. The locations from which Michigan isolates were recovered are shown in Figure 1.

dsRNA isolation. The dsRNA was isolated as described by Morris and Dodds (70) and Fulbright et al. (44). Mycelia were washed with 1 X STE buffer [0.05 M Tris pH 6.8, 0.1 M sodium chloride, 0.001 M (ethylenediamine) tetraacetic acid (EDTA)] and pressed dry. Samples were then ground in a mortar and pestle using glass beads (0.12-0.18 mm) and liquid nitrogen. The ground mycelia were suspended in 10 ml 2 X STE per 2-3 g sample. One tenth volume of 100 mg bentonite/ml, 0.15 volume of 10% sodium dodecyl sulfate (w/v) (SDS), and 1.5 volumes of STE-saturated phenol were added. The mixture was shaken on ice for 30 minutes and then centrifuged to separate the phases. The aqueous phase was adjusted to 15% ethanol and subjected to CF-11 chromatography. Ten ml CF-11 columns (Whatman, Clifton, NJ) were washed with 80 ml STE:15% ethanol prior to the elution of the dsRNA

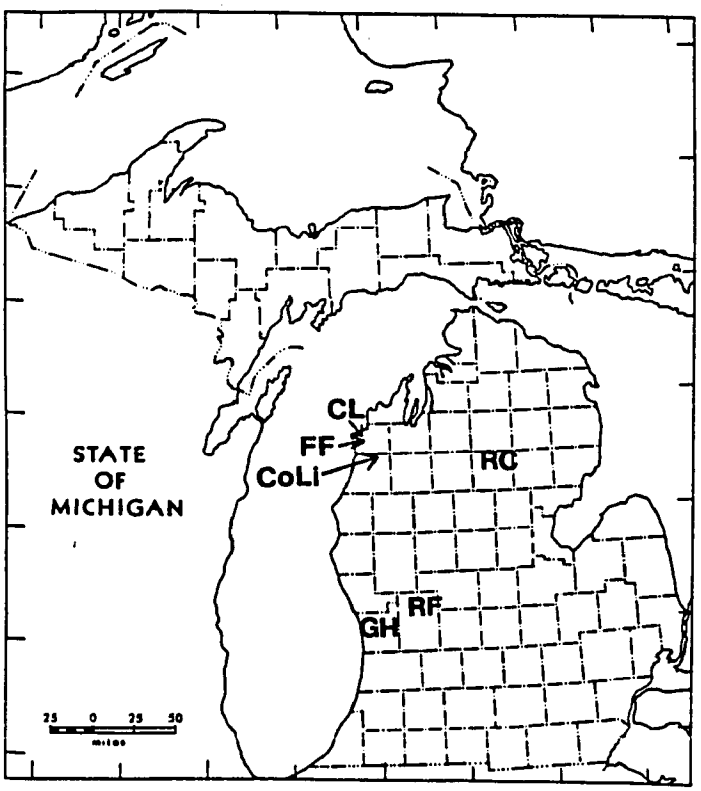

Figure 1. Location of sites from which Michigan isolates of E. parasitica were collected. GH: Grand Haven, RF: Rockford, FF: Frankfort, CoLi: County Line, CL: Crystal Lake, RC: Roscommon.

with 14 ml 1 X STE. Column chromatography was repeated and the dsRNA was concentrated by ethanol precipitation.

Equivalent amounts of fungal mycelia from two virulent isolates (CL1 and CL1-16), which do not contain dsRNA, were carried through the extraction procedure. The resulting solution was handled in the same way as the dsRNA isolated from hypovirulent isolates.

Polyacrylamide gels. The dsRNA was separated in 5% polyacrylamide gels run for 12 hours at 40 milliamperes in 0.04 M Tris pH 7.8, 0.02 M sodium acetate, 0.001 M EDTA. Gels were stained with an ethidium bromide solution (0.25 μg/ml) for one hour, followed by destaining in distilled water for 15 minutes prior to photography. Reovirus serotype 3 dsRNA, phage ϕ6 dsRNA, and dsRNA from viruses of Cochliobolus heterostrophus, Penicillium stoloniferum, and P. chrysogenum were included on the gels as molecular weight markers (10,11,81,82). Reovirus dsRNA segments have molecular weights of 2.5, 2.4, 2.3, 1.6, 1.6, 1.4, 0.92, 0.76, 0.64, 0.61 X 10^6 (82). The molecular weights of the phage ϕ6 dsRNA are 4.78, 2.87, 2.0 X 10^6 (81). C. heterostrophus virus dsRNA has a molecular weight of 5.7 X 10^6 (10,11). The molecular weights of the P. stoloniferum virus dsRNAs average 1.0 X 10^6 with a smaller segment of 0.46 X 10^6 , while the dsRNAs of P. chrysogenum dsRNA average 2.0 X 10^6 (10,11). Molecular weights were estimated by the method described by Bozarth and Harley (10).

Results

A variety of dsRNA banding patterns from Michigan isolates of E. parasitica were observed in polyacrylamide gels. The patterns varied in size, number, and relative intensity of dsRNA bands. Six isolates were selected as having representative dsRNA banding patterns (Figure 2). Most isolates consistently yielded one to three brightly stained dsRNA segments. The largest segment, which is often considered to be one element, resolves into two bands on polyacrylamide gels (Figure 2, lanes A 2-4, B 2 and 3). Many dsRNA segments that stained less intensely were not consistently detected during repeated assays. A continuum of bands was present below the largest segment in gels from many extractions. The most intensely stained segments and the continuum of bands were resistant to RNase A (5 mg/ml) in a solution of 0.3 M sodium chloride, but were degraded by RNase A in distilled water, confirming their identities as dsRNA (70).

The sizes of the brightest and most consistent dsRNA bands in the six representative isolates were estimated by comparing their migration in polyacrylamide gels with that of dsRNA molecular weight markers (Table 1). The molecular weights of the dsRNA segments in the hypovirulent isolates varied from 0.56 to 6.2 X 10^6. No one size segment was common to all isolates, but five of the six isolates analyzed consistently contained high molecular weight segments (5.4-6.2 X 10^6). The RC1 isolate was an exception; the only segment consistently present had a molecular weight of 1.1 X 10^6. Segments with molecular weights of 1.9 X 10^6 and 0.92 X 10^6 were often, but not always, detected. Isolates GH2, GHU4, and RF also consistently

Figure 2. Banding patterns of dsRNA from Michigan isolates of E. parasitica in 5% polyacrylamide gels stained with ethidium bromide. Lanes: A) 1, Cochliobolus heterostrophus, Penicillium stoloniferum, and P. chrysogenum viruses; 2, GH2; 3, GHU4; 4, RF; 5, RC1; 6, reovirus; 7, phage ϕ6. B) 1, C. heterostrophus, P. stoloniferum, and P. chrysogenum virus; 2, CoLi 5'; 3, FF; 4, CL1; 5, reovirus; 6, phage ϕ6. Arrows indicate bands which are often present, but in reduced amounts. The molecular weights of selected size markers are indicated ($X\ 10^6$).

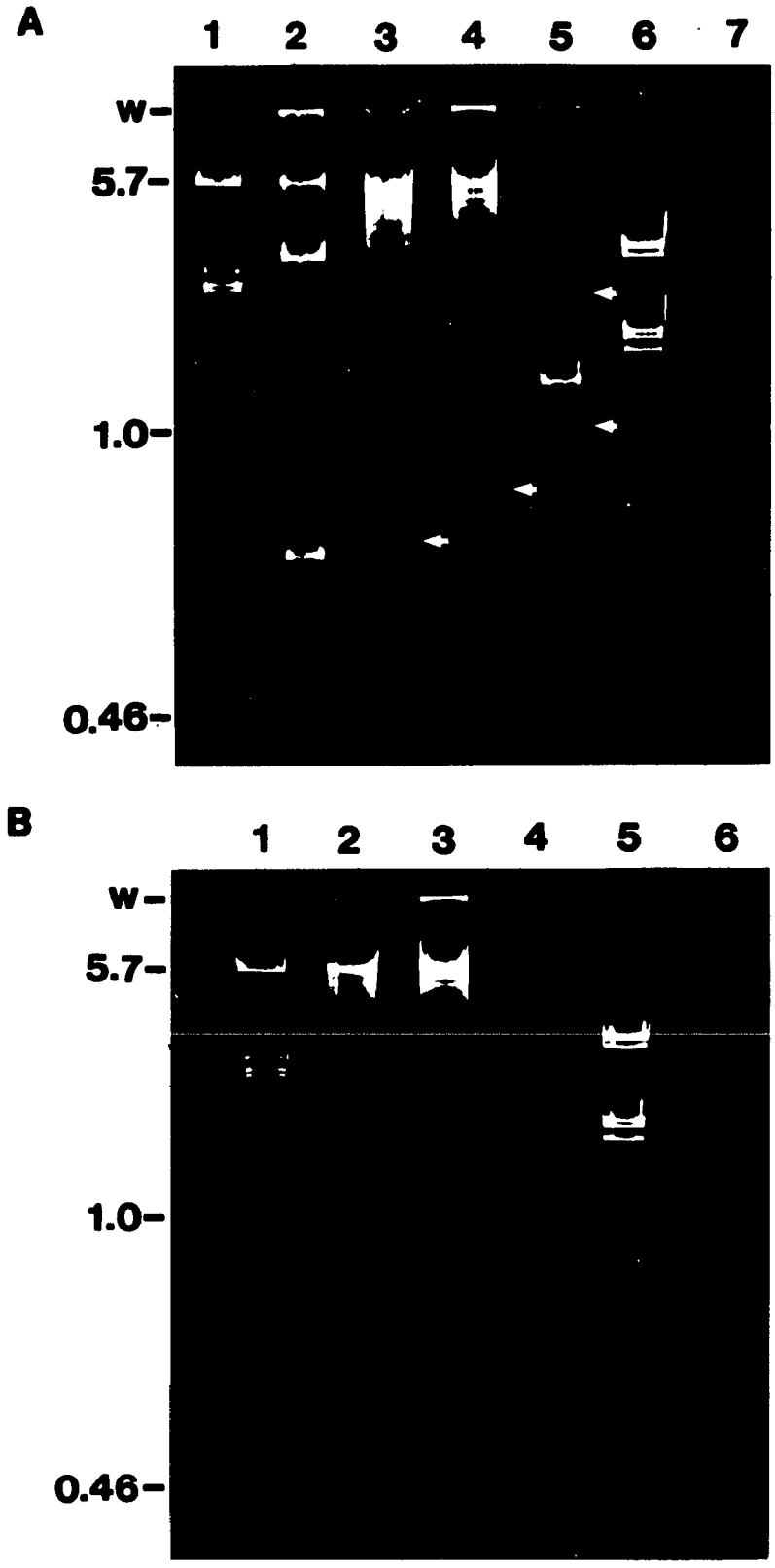

Figure 2.

Table 1. Sizes of dsRNA molecules from Michigan isolates of E. parasitica.

Size of dsRNA molecules present		Michigan Hypovirulent Strains					
Molecular weight X10^6	kilobase pairs	GH2	GHU4	RF	RC1	CoLi 5'	FF
6.2	9.0			X			
6.1	8.9	X				X	
5.9	8.6						X
5.7	8.3					X	
5.4	7.9	X	X	X			
5.2	7.6						X
4.4	6.4		X	X			
2.4	3.5	X					
1.9	2.8				X		
1.1	1.6				X		
0.92	1.3				X		
0.68	0.99			X			
0.58	0.84		X				
0.56	0.82	X					

exhibited smaller segments with molecular weights between 0.56 X 10^6 and 2.4 X 10^6. All size comparison studies were performed several times.

Discussion

Hypovirulence is exhibited by Michigan isolates of E. parasitica with varying numbers, sizes, and relative amounts of dsRNA segments. No one size dsRNA segment was found to correlate with hypovirulence. Many dsRNA banding patterns in gels are found in Michigan other that the three reported by Dodds (28). Two of the banding patterns described by Dodds were found in European isolates and the other in North American isolates. Molecular weights of the dsRNA segments reported by Dodds ranged from 4.3-6.2 X 10^6. All but one of the Michigan hypovirulent isolates included in this study harbored dsRNA corresponding to these large molecular weight segments. One isolate, RC1, was exceptional in that it did not carry detectable large molecular weight segments, but carried three segments of molecular weight between 0.92 and 1.9 X 10^6. Dodds also described minor bands, with molecular weights of less than 4.0 X 10^6. These bands required overloading of the gel and long photographic exposures to be visualized (28,29). The smaller segments (0.65-0.68 X 10^6) found in the Michigan isolates GH2, GHU4, and RF were easily detected, unlike the low molecular weight segments found by Dodds. The molecular weights assigned to these segments should be regarded as estimates (10,28), but are useful for comparitive purposes.

The dsRNA banding patterns in E. parasitica have been shown to change when transferred to another genetic background or upon repeated subculturing (1,4,25,29,40,46,89). The banding patterns described include segments which were consistently detected in many separate dsRNA extractions. Other segments were detected in some isolates with variable degrees of consistency. One segment with a molecular weight of approximately 1.5 X 10^6 was sometimes present in dsRNA extractions from the GH2 isolate, although it appeared less frequently than the 0.92 and 1.9 X 10^6 MW segments found in RC1 dsRNA. Due to their occasional instability and the fact that no one size of band is common to the hypovirulent isolates, it is difficult to categorize E. parasitica isolates based on banding patterns. The variability in banding patterns may be a consequence of the lack of extracellular transmission in the dsRNA "life cycle". This may allow the maintenance of defective dsRNA segments. The large and middle-sized GH2 dsRNA segments share sequence homology as determined by dot blot analysis (85). They also share common termini (85). The middle segment in isolate GH2 may be the result of an internal deletion of the large segment. Internal deletion has also been suggested as an explanation for the presence of smaller segments in a European isolate (58).

Changes in banding patterns of the dsRNA genomes of wound tumor virus also have been observed. The "variant RNAs" (73) are usually associated with a change in phenotype. The segment from which they were derived is absent or reduced in relative concentration. The variant RNAs have common termini with the other dsRNA segments and are

thought to be the result of internal deletion of one of the original segments. Smaller than genome length sized dsRNAs also have been extracted from plants infected with ssRNA viruses (23,24,98). These "subgenomic RNAs" are thought to serve as replicative forms of mRNAs which function in the expression of open reading frames that are not adjacent to the 5'-terminus of the viral RNA. The middle segment of the GH2 isolate resembles variant RNAs in that it shares common termini with a larger segment. However, no change in phenotype has been observed when this segment is deleted or changes in size (46). The middle segment in the GH2 isolate does not interfere with maintenance of the large segment. Upon close examination, the continuum of bands below the largest bands is found to maintain a consistent pattern in all extractions. These bands may be similar to either variant RNAs or subgenomic RNAs, but their structure, function, and relationship to other segments have not been investigated.

A 3'-poly(adenylic acid):5'-poly(uridylic acid) sequence is found at one terminus in all segments of both the American and the European isolate examined, indicating dsRNAs from E. parasitica share common features which may be important for dsRNA replication or gene expression. Defective dsRNA segments retaining the necessary structural features would be maintained in the fungal isolate resulting in the complex and changing banding patterns observed. In spite of some common terminal structural features, the small segment of the GH2 isolate does not share extensive sequence homology with the two larger GH2 segments (85), suggesting an independent origin for

this segment. This segment resembles the satellite RNAs found in many plant viruses (38).

The isolates used in this study were collected from six different stands of trees in the western half of Michigan's lower peninsula (Figure 1). Hypovirulent isolates were found at all sites. The Crystal Lake isolates, represented here by the virulent isolate CL1-16, did not contain detectable dsRNA. Different dsRNA banding patterns were found within the same recovering stand of trees, as shown by the GH2 and GHU4 banding patterns (Figure 2), while similar banding patterns were found at the County Line and Frankfort sites (data not shown). These two sites are located approximately 10 miles apart. However, the RC1 isolate has the most distinct banding pattern and this isolate was collected from a site that is farther east in Michigan than any of the other sites: approximately 70 miles from County Line and approximately 125 miles from Grand Haven. There was no consistent relationship between dsRNA banding pattern and the locality from which Michigan isolates were recovered.

CHAPTER 2

HOMOLOGY BETWEEN dsRNAs FROM DIFFERENT ISOLATES

CHAPTER 2

HOMOLOGY BETWEEN dsRNAs FROM DIFFERENT ISOLATES

Introduction

Although hypovirulent isolates of E. parasitica harbor dsRNA, the number, size, and relative amounts of the dsRNA segments vary between isolates. Knowledge of the similarities and differences between dsRNA molecules found in different isolates may provide clues to the origin of the dsRNA and its ability to spread geographically. It may also provide insights into the mechanism by which dsRNA results in the hypovirulent phenotype. The dsRNA from Michigan hypovirulent isolates, hypovirulent isolates from other states, and hypovirulent isolates from Europe were tested for sequence homology using Northern blot hybridization.

Materials and Methods

Cultures. Virulent and hypovirulent isolates used in this study are described in Table 2 and the locations from which Michigan isolates were recovered are shown in Figure 1 (Chapter 1). Strains 780 and 422 are derived from American virulent isolates into which dsRNA of Italian (32) and French origin, respectively, has been transferred through hyphal anastomosis.

Table 2. E. parasitica isolates used in homology studies.

Strain	Description	Origin
CL1	virulent, bark isolate	Crystal Lake, MI
CL1-16	virulent, single spore isolate of CL1	Crystal Lake, MI
GH2	hypovirulent[a], bark isolate	Grand Haven, MI
GHU4	hypovirulent, bark isolate	Grand Haven, MI
RF	hypovirulent, bark isolate	Rockford, MI
RC1	hypovirulent, bark isolate	Roscommon, MI
Coli 5'	hypovirulent, bark isolate	County Line site, MI
MSU 12#1	hypovirulent, bark isolate	County Line site, MI
FF	hypovirulent, bark isolate	Frankfort, MI
BF5	hypovirulent	West Virginia
9-B-2-1	hypovirulent	West Virginia
905	hypovirulent	Tennessee
780	hypovirulent, ATCC No. 38755	Italian dsRNA in American background
422	hypovirulent, ATCC No. 48537	French dsRNA in American background

[a]Hypovirulent isolates are reduced in virulence as determined by rate of growth in apple and contain dsRNA. They are able to transmit these characteristics through hyphal anastomosis.

All cultures were maintained and grown for dsRNA isolation as described in Chapter 1.

dsRNA isolation. The dsRNA was isolated as described in Chapter 1, except that the STE buffer used in the initial stages of the isolation contained 5 mM ethylene-glycol-bis (β-aminoethyl ether) N,N tetraacetic acid (EGTA), 5 mM N-ethylmaleimide, 100 µg heparin/ml, 0.5% 2-mercaptoethanol (v/v), and 50 µg spermine/ml (8). The aqueous phase was precipitated with ethanol after extraction with phenol and nucleic acids were resuspended in 1 X STE without additives. CF-11 chromatography was as described in Chapter 1. The concentration of dsRNA was determined spectrophotometrically. As in Chapter 1, equivalent amounts of fungal mycelia from the virulent isolates were carried through the extraction procedure.

Agarose gels. After addition of 0.25 volumes of loading buffer (0.15% bromphenol blue, 50% glycerol) to each sample, dsRNA (2 µg) was loaded onto 1% agarose minisub gels. Electrophoresis was performed for 1-1.5 hrs at 60 V in buffer containing 0.04 M Tris pH 7.8, 0.02 M sodium acetate, and 0.001 M EDTA. Gels were stained with 0.5 µg ethidium bromide/ml for 10 minutes, followed by brief destaining in distilled water prior to photography.

Denaturing gels and transfer to nitrocellulose. The dsRNA (4 µg) was denatured by heating at 65 C for 5 minutes in a solution of 50% formamide (v/v), 6% formaldehyde (v/v), 20 mM sodium borate pH 8.3, 0.2 mM EDTA. The samples were then adjusted to 0.03% bromphenol blue (w/v) and 10% glycerol (v/v) and loaded onto denaturing gels. The 1.2% agarose, 3% formaldehyde gels were then run for approximately 17

hours at 40 milliamperes in 20 mM sodium borate pH 8.3, 0.2 mM EDTA, 3% formaldehyde buffer (26). Lanes containing size standards were cut from the gel and stained with ethidium bromide (67).

The dsRNA was transferred from the gel to nitrocellulose filters (Schleicher and Schuell, Keene, NH) by blotting in 10 X SSC (1 X SSC = 0.15 M sodium chloride, 0.015 M sodium citrate pH 7.0) (26,67). Nitrocellulose filters were baked under a vacuum at 80 C for two hours.

End-labeling of dsRNA. T4 polynucleotide kinase was used to end-label dsRNA by the procedure of Jordan and Dodds (62). Two to ten micrograms of dsRNA was subjected to alkaline hydrolysis in 25 mM glycine, five mM magnesium chloride pH 9.0 for 30-60 minutes at 60 C. The dsRNA was precipitated with ethanol and resuspended in distilled water. Reaction mixtures contained 70 mM Tris-HCl pH 7.0, 100 mM potassium chloride, 10 mM magnesium chloride, 5 mM dithiothreitol, 7-10 units T4 polynucleotide kinase (Bethesda Research Laboratories, Gaithersburg, MD), and 25-60 µCi of 5'-(γ-^{32}P) ATP (3000-5000 Ci/mmol, Amersham, Arlington Heights, IL). Reactions were allowed to proceed overnight at 37 C. Sonicated calf thymus DNA (0.4 volumes of a 0.5 mg/ml solution) was added to each sample. This was followed by the addition of one volume of 4 M ammonium acetate. The reaction mixture was extracted with phenol:chloroform:isoamyl alcohol (25:24:1). The aqueous phase was subjected to Sephadex G-50 chromatography and fractions were monitored for radioactivity by absorbtion to DE-81 filters (Whatman, Clifton, NJ) which were then counted in a liquid scintillation counter (67). Fractions showing the highest levels of

radioactivity were precipitated with ethanol. Specific activities of 10^5-10^7 cpm/µg were obtained.

Hybridization. Nitrocellulose filters were prehybridized in a solution containing 50% formamide, 5 X SSC, 50 mM sodium phosphate pH 6.5, 250 µg sonicated denatured calf thymus DNA/ml, 0.02% bovine serum albumin (w/v), 0.02% ficoll (MW=400,000) (w/v), 0.02% polyvinylpyrrolidone (MW=40,000) (w/v) for a minimum of two hours. ^{32}P-labeled dsRNA was denatured by heating at 100 C for 5-10 minutes. Filters were incubated at 42 C in the above hybridization solution containing 10^4-10^6 cpm/ml ^{32}P-labeled RNA for 24 hours (86). The filters were washed four times with 2 X SSC, 0.1% SDS for 5 minutes at room temperature and then twice in 0.1 X SSC, 0.1% SDS at 50 C for 15 minutes. Kodak XAR-5 film was exposed to the filter at -75 C with a DuPont Cronex Lightning Plus intensifying screen. Sizes of hybridized segments were determined by comparison with size markers in lanes cut from the gel prior to transfer of dsRNA to nitrocellulose.

Selected isolate combinations were examined under lower stringency conditions. GH2 dsRNA was hybridized to dsRNA from isolates RC1, 780, 422, and BF5 as above with the omission of the two final washes at 50 C. GH2 dsRNA was also hybridized to dsRNA from the same isolates for 30 hours during which the temperature was allowed to drop from 80 C to 32 C. The filter was then washed as usual.

Results

The ability of dsRNA from two Michigan isolates, GH2 and RC1, to hybridize to dsRNA from other Michigan isolates as well as to isolates from Tennessee, West Virginia, France, and Italy was examined. These isolates exhibited a wide range of culture morphologies (Figure 3). Many different banding patterns were found among the isolates used in this study (Figure 4). The isolates from West Virginia (Figure 4, lanes 9 and 10) exhibited many more bands than did the other isolates.

Double-stranded RNA from GH2 hybridized to all dsRNAs of Michigan origin which were tested (GHU4, RF, FF, CoLi 5', MSU 12#1), except to dsRNA from isolate RC1 (Figures 5-7, Table 3). Hybridization to the $0.58X10^6$ molecular weight GHU4 segment was observed in some autoradiograms (Figure 8). GH2 dsRNA did not hybridize to dsRNA from other states (905, BF5, 9-B-2-1) or Europe (422, 780). RC1 dsRNA, when used as a probe, did not share homology with dsRNA from any of the isolates tested except itself (Figures 9 and 10, Table 3). No hybridization was ever observed in lanes containing extracts from CL1-16, a virulent isolate lacking detectable dsRNA. Reducing the stringency of hybridization did not result in hybridization of GH2 dsRNA to RC1 dsRNA or to dsRNA from outside of Michigan (Figure 11). No hybridization between GH2 dsRNA and RC1 dsRNA or dsRNA from other states or Europe was found when the hybridization was started at 80C and the temperature was allowed to slowly fall to 32 C (Figure 12). This indicates that the lack of hybridization was not due to the renaturation of the probe dsRNA.

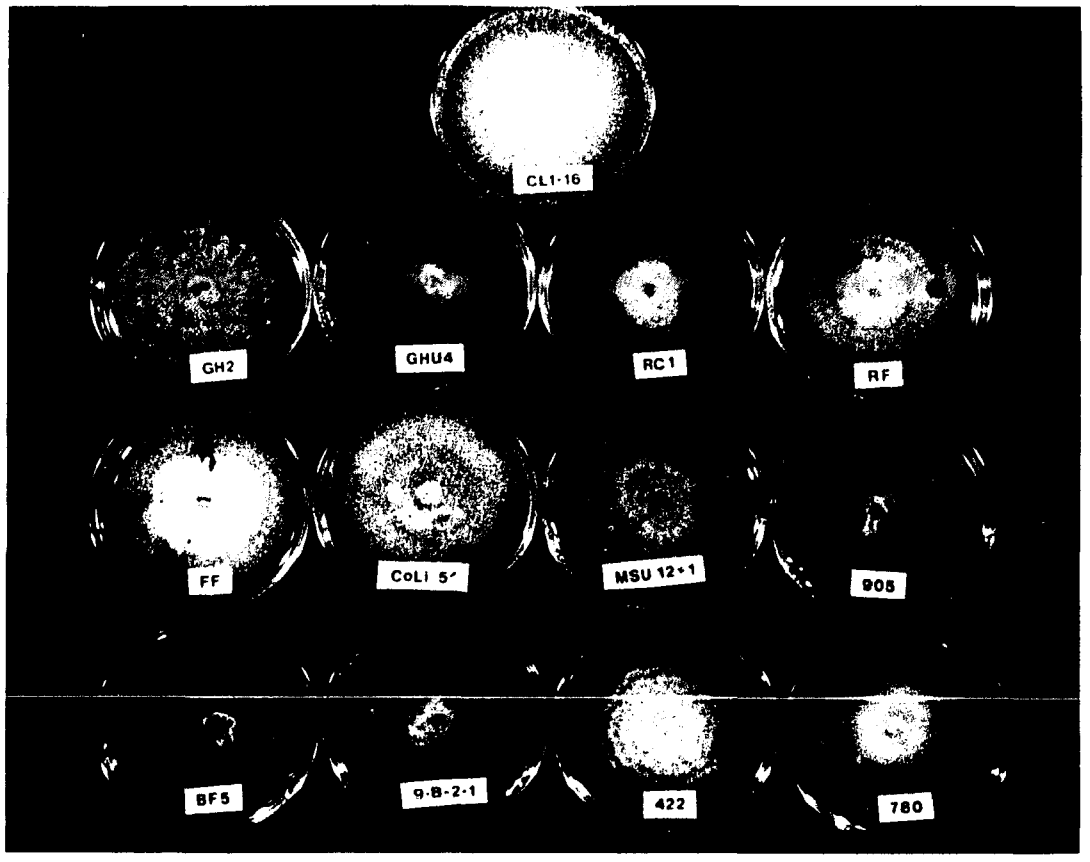

Figure 3. Culture morphologies of E. parasitica isolates used in hybridization studies.

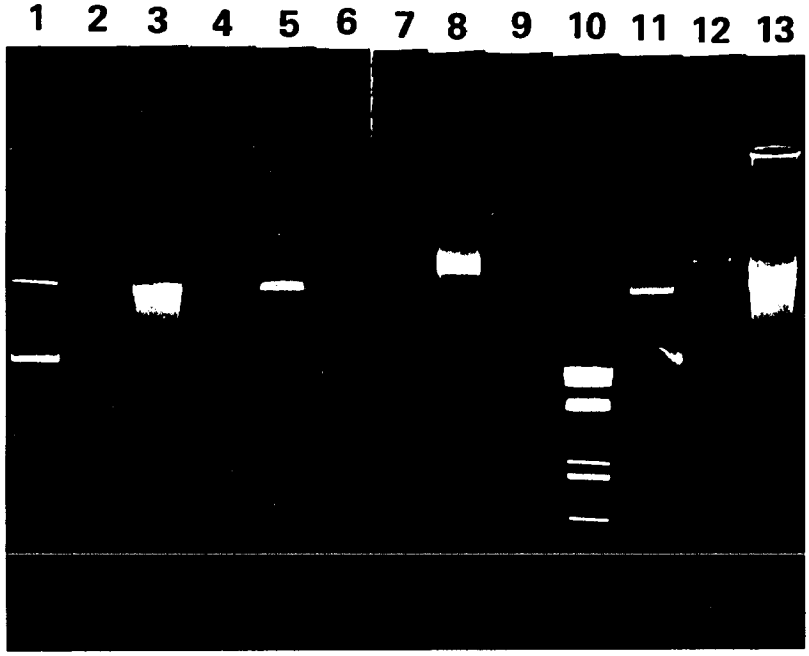

Figure 4. Banding patterns of dsRNA from E. parasitica isolates used for hybridization studies in 1% agarose gels stained with ethidium bromide. Lanes: 1, GH2; 2, CL1-16; 3, GHU4; 4, RC1; 5, FF; 6, RF; 7, CoLi 5'; 8, MSU 12#1; 9, BF5; 10, 9-B-2-1; 11, 905; 12, 780; 13, 422.

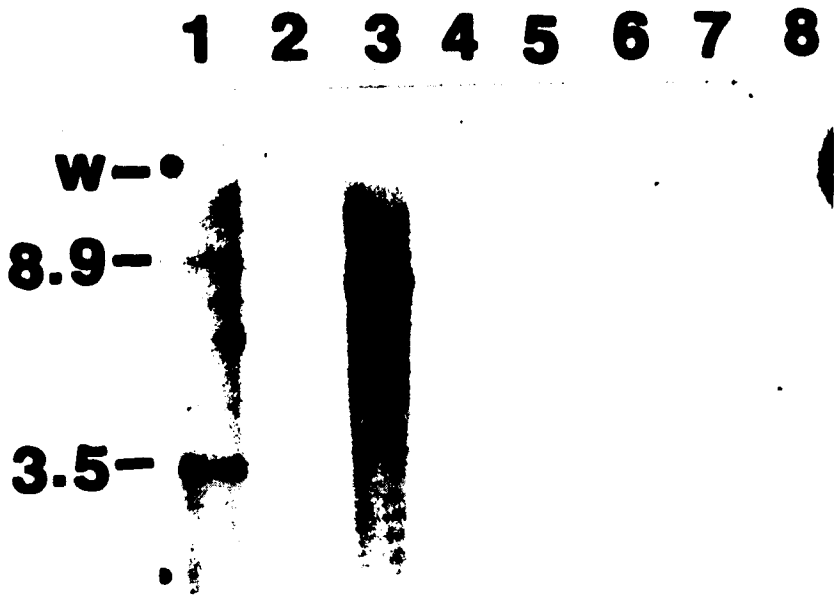

Figure 5. Hybridization of GH2 dsRNA to dsRNA from North American and European E. parasitica isolates. Lanes: 1, GH2; 2, CL1-16; 3, GHU4; 4, RC1; 5, 780; 6, 422; 7, 9-B-2-1; 8, reovirus. The sizes of the GH2 dsRNA segments are indicated in kilobase pairs.

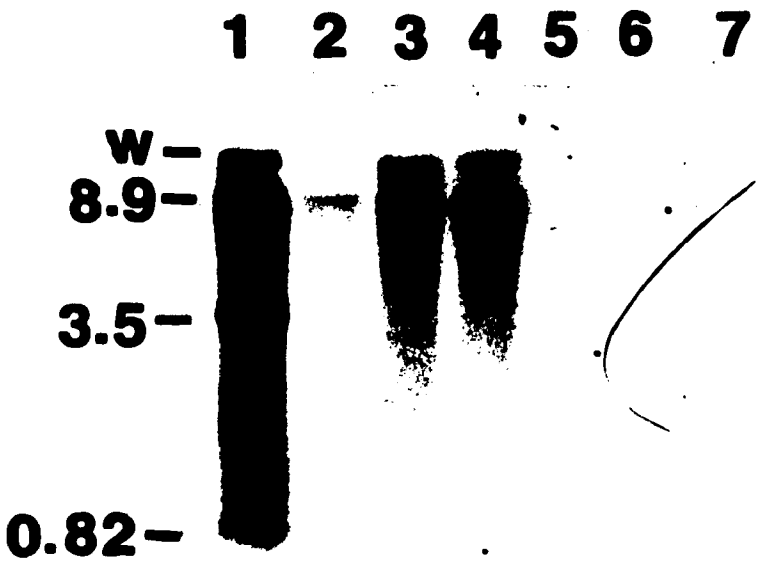

Figure 6. Hybridization of GH2 dsRNA to dsRNA from a selection of North American E. parasitica isolates. Lanes: 1, GH2; 2, RF; 3, FF; 4, MSU 12#1; 5, BF5; 6, 905; 7, reovirus. Although four micrograms dsRNA was loaded in most lanes, only two nanograms RF dsRNA and three micrograms MSU 12#1 dsRNA were loaded on to this gel. The sizes of the GH2 dsRNA segments are indicated in kilobase pairs.

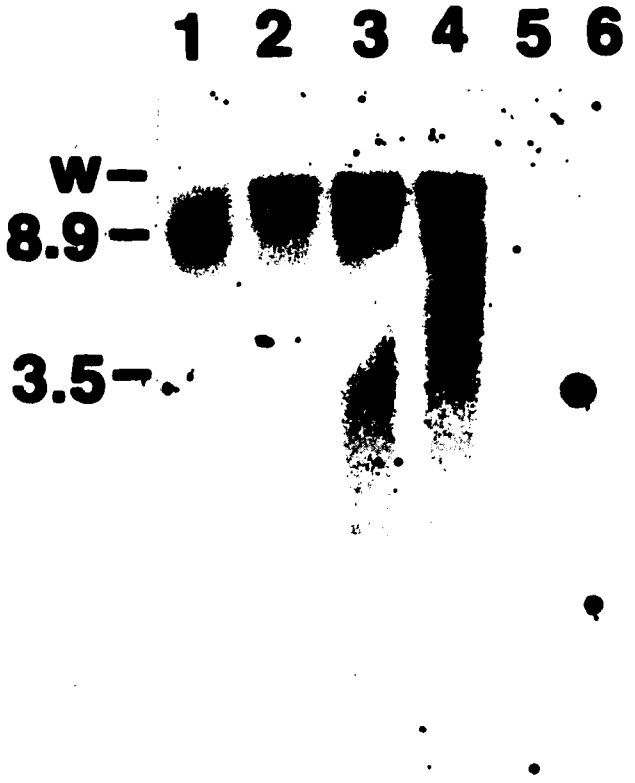

Figure 7. Hybridization of GH2 dsRNA to dsRNA from a set of North American E. parasitica isolates. Lanes: 1, RF; 2, FF; 3, CoLi 5'; 4, MSU 12#1; 5, 9-B-2-1; 6, reovirus. The positions of the GH2 segments are indicated and the sizes are given in kilobase pairs.

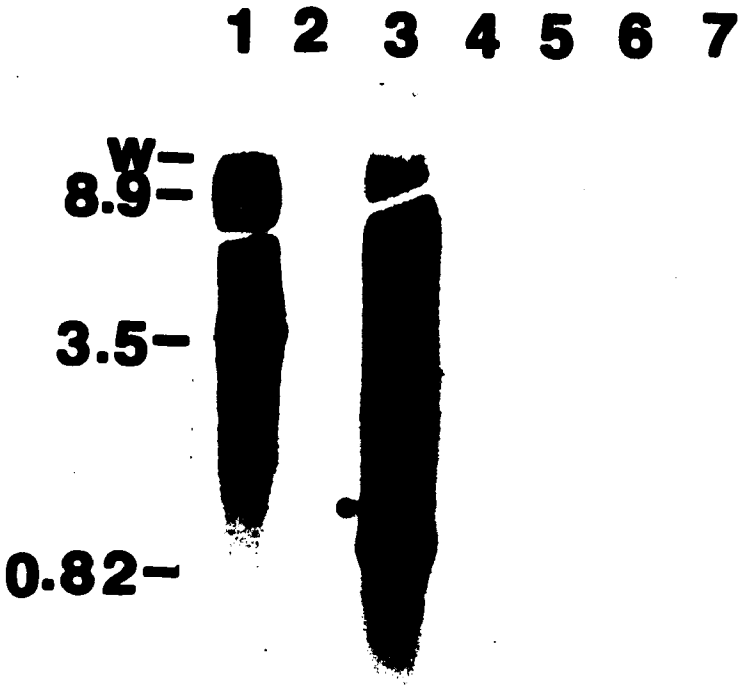

Figure 8. Hybridization of GH2 dsRNA to the small dsRNA segment in the GHU4 isolate. Lanes: 1, GH2; 2, CL1-16; 3, GHU4; 4, RC1; 5, 780; 6, 905; 7, 422. The sizes of the GH2 dsRNA segments are indicated in kilobase pairs.

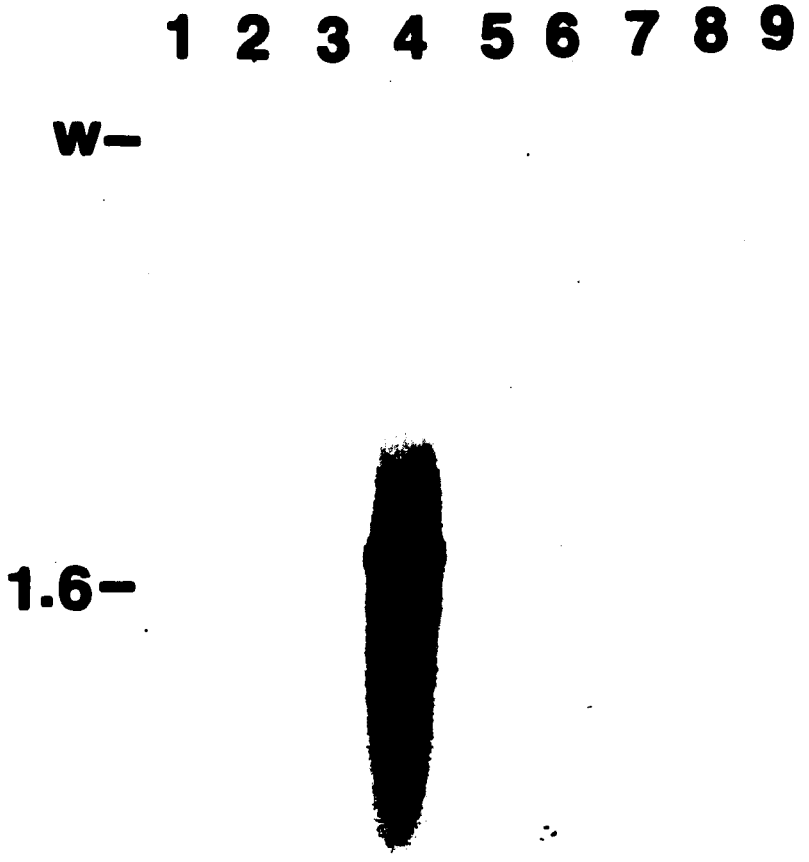

Figure 9. Hybridization of RC1 dsRNA to dsRNA from North American and European E. parasitica isolates. Lanes: 1, GH2; 2, CL1-16; 3, GHU4; 4, RC1; 5, 780; 6, 422; 7, BF5; 8, 905; 9, reovirus. The size of the RC1 dsRNA segment is indicated in kilobase pairs.

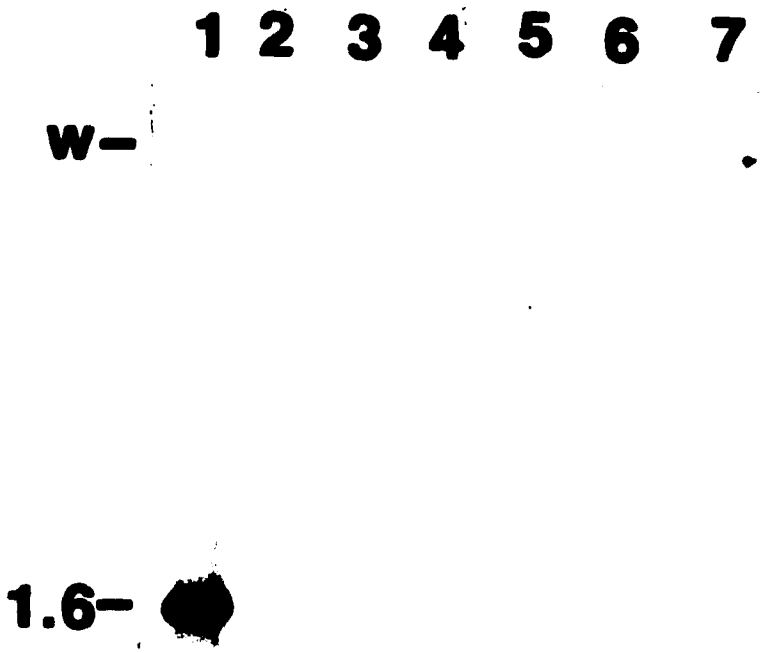

Figure 10. Hybridization of RC1 dsRNA to dsRNA from a set of North American E. parasitica isolates. Lanes: 1, RC1; 2, RF; 3, FF; 4, CoLi 5'; 5, MSU 12#1; 6, 9-B-2-1; 7, reovirus. The size of the RC1 dsRNA segment is indicated in kilobase pairs.

Table 3. Summary of hybridization results between dsRNA from isolates GH2 and RC1 and dsRNA from other isolates from Michigan, other states, and Europe.

Source of dsRNA used in hybridizations	Source of probe dsRNA	
	GH2	RC1
Michigan		
GH2	+	-
RC1	-	+
GHU4	+	-
RF	+	-
FF	+	-
CoLi 5'	+	-
MSU 12#1	+	-
CL1-16[a]	-	-
Other states		
905	-	-
BF5	-	-
9-B-2-1	-	-
Europe		
422	-	-
780	-	-

[a]CL1-16 is a virulent isolate which does not carry dsRNA.

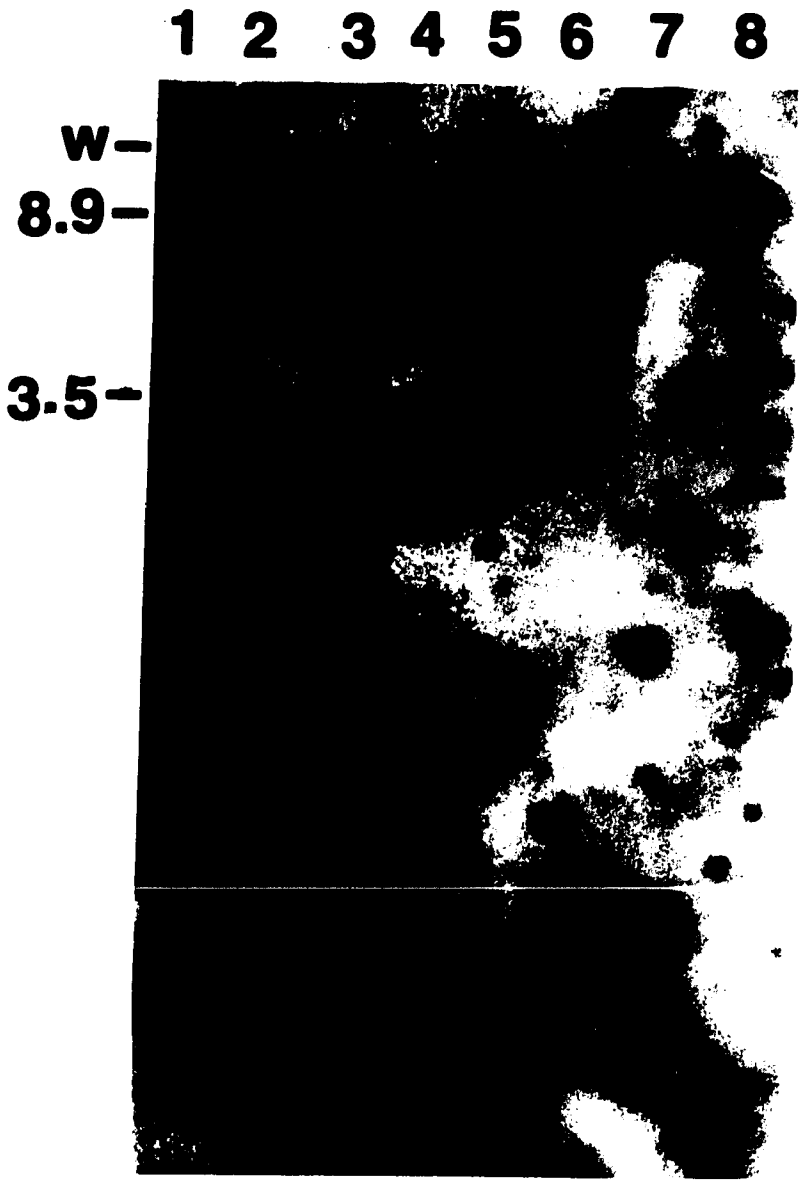

Figure 11. Hybridization of GH2 dsRNA to dsRNA from North American and European E. parasitica isolates under lower stringency conditions. Lanes: 1, GH2; 2, CL1-16; 3, GHU4; 4, RC1; 5, 780; 6, 422; 7, BF5; 8, reovirus. The sizes of the GH2 dsRNA segments are indicated in kilobase pairs.

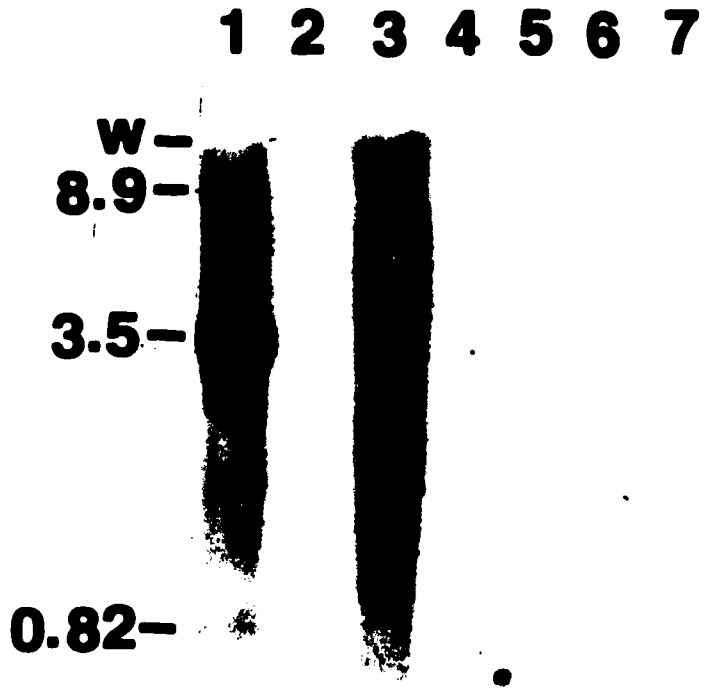

Figure 12. Hybridization of GH2 dsRNA to dsRNA from North American and European E. parasitica isolates under conditions of slowly decreasing temperature. Lanes: 1, GH2; 2, CL1-16; 3, GHU4; 4, RC1; 5, 780; 6, 422; 7, BF5. The sizes of the GH2 dsRNA segments are indicated in kilobase pairs.

Discussion

Hybridization results indicate that the dsRNA in populations of E. parasitica in North America is varied, with at least three types of dsRNA based on sequence homology. Even the dsRNAs from the isolates from Michigan did not share common sequences at a detectable level (Table 3). The dsRNA sharing homology with GH2 dsRNA constitutes one type, while RC1 dsRNA is in its own class. Based on this work, North American dsRNA from outside Michigan makes up a third category of dsRNA based on sequence homology. However, L'Hostis et al. have reported finding sequence homology between dsRNA from a Michigan and a Virginia isolate (66). Thus, homology relationships between dsRNAs from different geographical areas are more complex than previously reported. The relationships between dsRNAs from other states (i.e. Tennessee and West Virginia) have not been examined. European dsRNA constitutes a fourth type of dsRNA. Using different isolates than those in this study, L'Hostis et al. also have found that North American and European dsRNA lack homology (66). As is the case with North American dsRNA, the relationships among different European dsRNAs have not been studied. It is possible that there is more than one type of dsRNA in Europe as well as in North America. The lack of homology suggests different origins for the dsRNA on the two continents. More than one independent origin for dsRNA in E. parasitica may have occurred even within the state of Michigan.

The RC1 isolate is unique among the Michigan hypovirulent isolates examined. RC1 dsRNA did not hybridize to the dsRNA from any of the other Michigan isolates tested and it also has the most distinct

banding pattern (40). Additionally, the RC1 isolate has a unique culture morphology, differing from other hypovirulent isolates recovered from Michigan in that it has a fibrous appearance, smooth colony margins, and lacks aerial hyphae. The two homology groups from Michigan were recovered from two different geographical areas. As discussed in Chapter 1, the RC1 isolate was collected from a site that is farther east in Michigan than any of the other sites. It is approximately 70 miles from County Line, the nearest site, and approximately 125 miles from Grand Haven.

The presence of isolates within Michigan carrying dsRNAs that share homology suggests that these dsRNAs share a common origin and that the natural spread of dsRNA within the E. parasitica population has occurred. The sequence of the dsRNA apparently has been more highly conserved than the size of the segments (Chapter 1). Isolates with dsRNA sharing homology with GH2 dsRNA were collected from the western side of the state near Lake Michigan (Figure 1, Chapter 1). GH2-type dsRNA was collected up to 105 miles from the Grand Haven site. Within a localized area, dsRNA sequences are related, indicating that the dsRNA may have spread from one original source.

This also suggests that dsRNA which is introduced into the E. parasitica population may be able to spread throughout and become established in the population. The results of Garrod et al. (46) indicate that dsRNA which has been introduced in a hypovirulent isolate to a stand of chestnuts infected with virulent E. parasitica can be recovered from trees which were not inoculated with the hypovirulent isolate. These findings support the potential use of

dsRNA as a biological control of E. parasitica and possibly other fungal plant pathogens.

The lack of detectable sequence homology found between dsRNAs from hypovirulent E. parasitica isolates adds emphasis to the question of how dsRNA may cause hypovirulence. It would seem to suggest that any dsRNA will cause a reduction of virulence in E. parasitica. Many other fungi support the replication of the dsRNA genomes of mycoviruses without exhibiting any physical effects (9,47), but E. parasitica may be particularly sensitive to the presence of dsRNA, perhaps due to a relatively low level of some cellular component.

Evidence for a specific interaction between dsRNA and its fungal host is accumulating. It is interesting that GH2 and GHU4 dsRNAs share homology, despite very different levels in debilitation of the fungus. There is evidence suggesting that particular dsRNA segments are responsible for particular phenotypes. All of the dsRNA segments examined from both an American and a European isolate have a poly(A):(U) terminus (58,85). In spite of this, the dsRNAs from these two isolates do not share detectable sequence homology and not all GH2 dsRNA segments share detectable homology. It is possible that there are small similarities in sequence or tertiary structure of the RNA or RNA products which are not detectable by the methods used in this study, but are critical in causing the hypovirulent phenotype. Hypovirulence is associated with changes in the amounts of certain mRNAs and proteins in E. parasitica (76,77), suggesting that the dsRNA or its products affect the expression of the corresponding host genes.

CHAPTER 3

SEARCH FOR dsRNA SEQUENCES IN FUNGAL DNA

CHAPTER 3

SEARCH FOR dsRNA SEQUENCES IN FUNGAL DNA

Introduction

Among the suggested possibilities concerning the origin of the dsRNA in E. parasitica is that of a provirus state in which a copy of the dsRNA sequence is present in the fungal DNA. This theory would account for the multiple origins of dsRNA in E. parasitica populations. There is also genetic evidence to support this model. UV-irradiated spores of some isolates produce hypovirulent cultures resembling the European white hypovirulent isolates (90). Hypovirulence in these isolates is inherited as a single nuclear gene. The frequency of mutation is higher among spores from virulent single-conidial isolates of dsRNA-containing cultures than among isolates that have never been hypovirulent. Hypovirulence is also inherited as a single nuclear gene in sexual crosses involving JR isolates (3). These isolates have been found only among laboratory cultures which at one time carried dsRNA.

Recently, another line of evidence has suggested that dsRNA sequences may be able to integrate into the fungal DNA (C. Durbahn and D.W. Fulbright, personal communication). When the dsRNA from the Michigan hypovirulent isolates, GH2 and RC1, are combined in one strain, both the GH2 and RC1 dsRNA banding patterns are found on

gels. Single-conidial isolates of this isolate may carry both GH2 and RC1 dsRNA, dsRNA from either isolate, or no dsRNA. One single conidial isolate harboring RC1 dsRNA, Ess6, is of particular interest. When single conidial cultures from this isolate are analyzed for dsRNA content, the four dsRNA patterns observed for isolates carrying both GH2 and RC1 dsRNA are found. Although GH2 dsRNA is not detectable in the Ess6 isolate, it is found in single conidial isolates of Ess6. The sequences for GH2 dsRNA must be maintained in Ess6, although they are not expressed. The possibility that sequences sharing homology with E. parasitica dsRNA are present in the fungal DNA was investigated.

Materials and Methods

E. parasitica cultures. Isolates GH2 and CL1-16 are described in Chapters 1 and 2. Ess6 is a single conidial isolate of E, an isolate formed by hyphal anastomosis between isolates made up of the CL1-16 nuclear background into which GH2 and RC1 dsRNA had been transferred (Figure 13). Although Ess6 exhibits the RC1 dsRNA banding pattern on gels, single conidial isolates of Ess6 may lack dsRNA or have dsRNA of the GH2, RC1, or both GH2 and RC1 isolates.

Fungal cultures were grown as described in Chapter 1.

E. parasitica DNA isolation. DNA was extracted from E. parasitica by a procedure similar to that described by Yelton et al. (96). The mycelia, grown in liquid stationary culture, were separated from the broth by filtration and washed with cold deionized water. The fungal tissue was pressed dry, frozen in liquid nitrogen, and ground in a

Figure 13. The dsRNA banding patterns of hypovirulent E. parasitica isolates used to form isolate E and of single-spore isolates of E and Ess6.

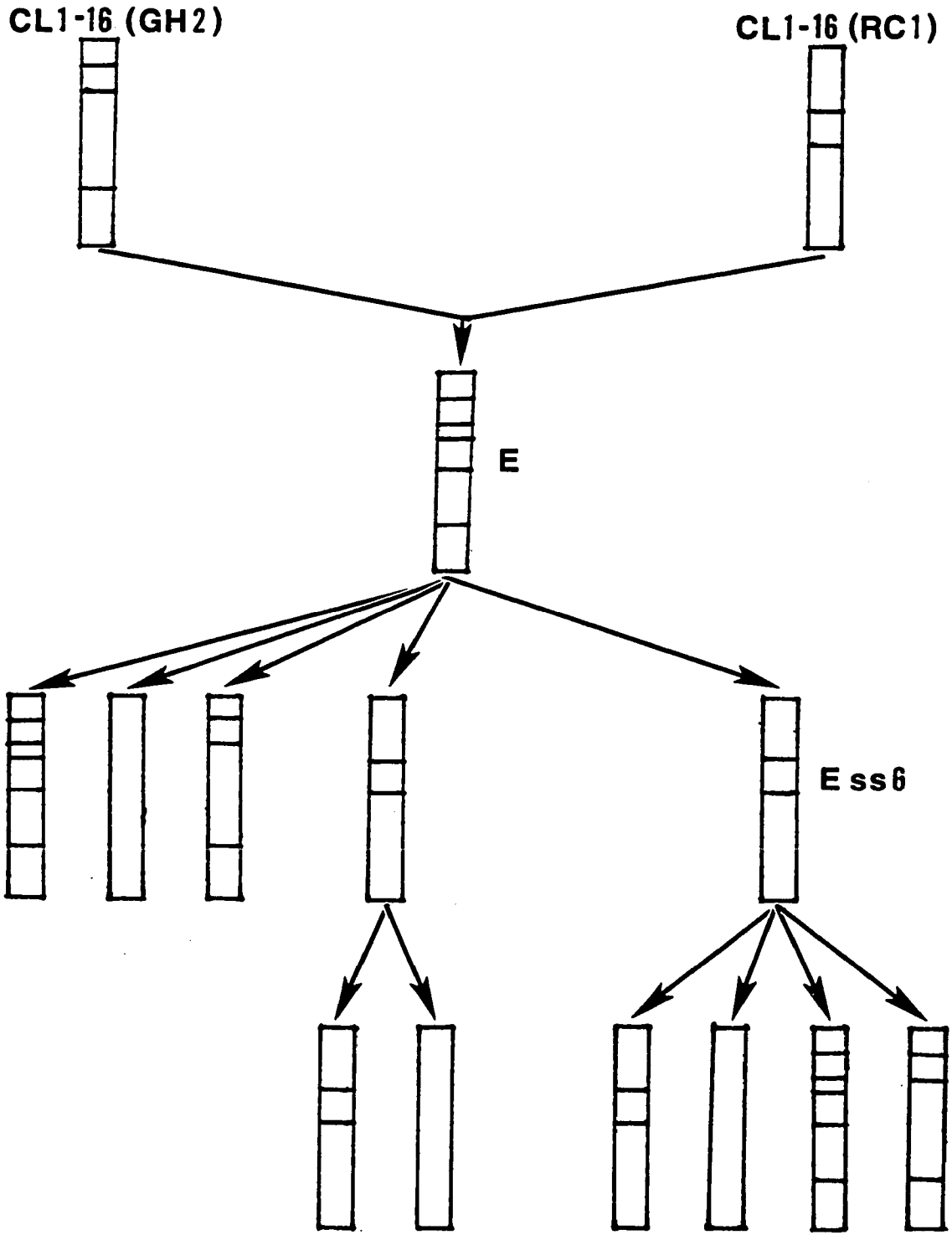

Figure 13.

mortar and pestle using glass beads (0.12-0.18 mm). The tissue was then suspended in a solution of 50 mM EDTA pH 8.5, 0.2% SDS in a ratio of 1:6 (w/v). Diethyl pyrocarbonate (0.001 volume) was added and the mixture was shaken for one minute at room temperature. It was then heated to 68 C for 15 minutes and allowed to cool to room temperature. The solution was centrifuged in an SS-34 rotor at 11,500 rpm for 15 minutes. The supernantant was transferred to a new tube, being careful to avoid contamination from the pellet. The supernatant was adjusted to 0.5 M potassium acetate using a 5 M stock solution (67). After mixing thoroughly, the sample was kept on ice for one hour. It was then centrifuged in an SS-34 rotor at 16,500 rpm for 15 minutes at 4 C. The supernatant was transferred to a new tube, being careful not to disturb the pellet. Nucleic acids were precipitated by adding an equal volume of cold isopropanol and collected by centrifugation in an SS-34 rotor at 11,500 rpm for 15 minutes. The pellets were dried under a stream of nitrogen and resuspended in TER buffer (10 mM Tris-HCl pH 7.5, one mM EDTA, and 10 µg RNase A/ml). The sample was incubated at 37 C for a minimum of one hour. DNA was precipitated by addition of an equal volume of cold isopropanol followed by centrifugation as above. Pellets were dried and resuspended in 1.5 ml of buffer containing 10 mM Tris pH 7.5, one mM EDTA.

Agarose gels, as described in Chapter 2, were used to estimate the concentration of DNA from E. parasitica using known quantities of HindIII digested lambda DNA (Bethesda Research Laboratories, Gaithersburg, MD) as standards. These gels were also used to check

for any remaining RNA. If there was evidence of RNA in any preparations, the RNase treatment was repeated for several hours.

Cloning of GH2 dsRNA cDNA. GH2 dsRNA was extracted as described in Chapter 2. The synthesis of GH2 cDNA involved extension of an oligo(dT) primer by reverse transcriptase, followed by digestion of the template with RNase H and second strand synthesis using the large fragment of DNA polymerase I (5,17,53,74). Materials used in cDNA synthesis and cloning were purchased as follows: poly(A) polymerase, cloned M-MLV reverse transcriptase, and RNase H from Bethesda Research Laboratories, Gaithersburg, MD; oligo(dT) primers, terminal transferase, and some of the M13 vector from P-L, Piscataway, NJ; large fragment of DNA polymerase I and T4 DNA polymerase from New England Biolabs, Beverly, MA; RNAsin from Promega, Madison, WI; and restriction enzyme buffers from International Biotechnologies, Inc., New Haven, CT.

The dsRNA was polyadenylated using poly(A) polymerase. Reaction mixtures contained 50 mM Tris pH 8.0, 10 mM magnesium chloride, 1 mM manganese chloride, 50 µg bovine serum albumin (BSA)/ml, 0.2 M sodium chloride, 0.1 mM ATP, 200 µCi ^{3}H-ATP/ml (1 mCi/ml stock solution), 80 U poly(A) polymerase/ml, 250 U RNAsin, 1 mM dithiothreitol (DTT), and 120 µg dsRNA/ml. Samples were incubated for 40 minutes at 37 C. The reaction mixture was extracted twice with phenol:chloroform (1:1,v/v) and subjected to Sephadex G-50 chromatography. Fractions with the greatest number of counts were reduced in volume with N-butanol (67) and the dsRNA was precipitated with ethanol.

Oligo(dT)12-18 primers and poly(A)-tailed dsRNA were incubated at 65 C for 20 minutes in 90% dimethyl sulfoxide (DMSO) to denature the dsRNA. After precipitation with ethanol, M-MLV reverse transcriptase was used to synthesize cDNA. Reaction mixtures were incubated at 37 C for one hour in buffer containing 50 mM Tris pH 7.5, 75 mM potassium chloride, 10 mM DTT, 3 mM magnesium chloride, 0.5 mM deoxynucleoside triphosphates, 50 µg actinomycin D/ml, 20 µCi ^{32}P-dTTP (10 mCi/ml), and 1600 U reverse transcriptase/ml in a 50 ml reaction volume. Reactions were terminated by the addition of 0.25 M EDTA (four µl) and the sample was extracted with phenol:chloroform (1:1,v/v). Nucleic acids were precipitated twice by the addition of 0.5 volumes 7.5 M ammonium acetate and three volumes of ethanol. The sample was placed in a dry ice-ethanol bath for 10 minutes, allowed to sit at room temperature for 10 minutes, and centrifuged to collect the pellet.

The synthesis of single-stranded cDNA was confirmed by the use of alkaline agarose gels (67). Agarose gels formed in a solution of 50 mM sodium chloride, 1 mM EDTA were soaked in alkaline electrophoresis buffer (30 mM sodium hydroxide, 1 mM EDTA) for at least 30 minutes. The buffer was replaced with fresh alkaline electrophoresis buffer prior to electorphoresis. Gels were prepared for autoradiography by soaking them in 7% trichloro acetic acid (TCA) for 30 minutes and then drying them.

The RNA template was replaced by cDNA using RNase H and the large fragment of DNA polymerase I. Reaction mixtures (100 µl volume) contained 10 µg cDNA:RNA hybrid/ml, 20 mM Tris pH 7.5, 5 mM magnesium chloride, 10 mM ammonium sulfate, 100 mM potassium chloride, 50 mg

BSA/ml, 40 μM nucleoside triphosphates, 18 U RNase H/ml, and 230 U DNA polymerase I/ml. Incubation was for one hour at 12 C, followed by one hour at 22 C. The reaction was terminated by the addition of 8 μl of a solution of 0.25 M EDTA, the sample was extracted with phenol:chloroform (1:1,v/v), and the nucleic acids were precipitated twice by the addition of ammonium acetate and ethanol as described above.

The cDNA was then treated with T4 DNA polymerase. Reaction mixtures (50 μl volume) contained 50 mM Tris pH 8.3, 10 mM magnesium chloride, 10 mM DTT, 50 mM sodium chloride, 50 μM deoxynucleoside triphosphates, approximately one μg cDNA, and 1.5 U T4 DNA polymerase. The reaction was conducted at 37 C for 30 minutes. EDTA (four μl of a 0.25 M solution) was added and the samples were extracted with phenol:chloroform (1:1,v/v). Nucleic acids were collected by precipitation using ammonium acetate and ethanol as described above.

Poly(C) tails were added to the cDNA using terminal transferase followed by Sephacryl chromatography or the cDNA was treated with S1 nuclease and subjected to chromatography on a Sephacryl column prior to digestion with restriction endonucleases. Reaction mixtures for the addition of poly(C) tails contained 100 ng cDNA, 4 μl of a solution containing 9 μl of 5 mM dCTP and 1 μl 50 mM cobalt chloride which had been preincubated at 20 C for five minutes, 8 μl 0.5 M potassium-cacodylate pH 6.9, and 4 μl 25 mg BSA/ml in a 20 μl reaction volume. Reaction mixtures were incubated at 22 C for five minutes before the addition of 10 U terminal transferase. Reactions were

allowed to proceed at 22 C for times varying from five to 20 minutes and then were heat inactivated by treatment at 65 C for 10 minutes. Reaction mixtures from all time points were combined and subjected to Sephacryl S300 chromatography. Fractions showing the highest levels of radioactivity were precipitated with ethanol.

Double-stranded cDNA which was not treated with T4 DNA polymerase or tailed with poly(C) was treated with S1 nuclease prior to digestion with restriction enzymes. S1 nuclease digestions were performed in a reaction buffer containing 200 mM sodium chloride, 50 mM sodium acetate pH 4.5, 1 mM zinc chloride, and 0.5% glycerol. Reaction mixtures were incubated at 37 C for 30 minutes and then 0.1 volume of a solution of EDTA (0.1 M) was added to terminate the reaction. The sample was extracted with phenol:chloroform (1:1,v/v) and subjected to chromatography on a Sephacryl S300 column. Fractions containing the highest levels of radioactivity were reduced in volume using N-butanol and precipitated with ethanol.

Partial restriction digests were performed on the cDNA, which was then inserted into M13mp8 replicative form (RF) using T4 DNA ligase. The cDNA was incubated with Sau3A or AluI in buffer containing 25 mM Tris-HCl pH 7.8, 50 mM sodium chloride, 10 mM magnesium chloride, 100 µg BSA/ml, and 2 mM 2-mercaptoethanol for 40-60 minutes at 37 C. After extraction with phenol:chloroform (1:1,v/v) and precipitation with ethanol, 100 ng of digested cDNA was combined with 20 ng of BamHI- or SmaI-treated M13mp8 vector and incubated with T4 DNA ligase at 4 C overnight.

Ligation mixtures (five µl) were combined with competent E. coli JM103 cells (25 µl) (Δlacpro, supE, thi, strA, sbcB15, endA, hspR4, F' traD36, proAB, $lacI^{q}Z\Delta M15$) and placed on ice for 15 to 40 minutes. They were then incubated at 37 C for 5 minutes, followed by 15 minutes at room temperature. These cells were then combined with 500 µl of cells from a culture in the exponential growth phase, 10 µl 100 mM isopropylthiogalactoside (IPTG), 50 µl 2% 5-dibromo4-chloro3-indolylgalactoside (X-Gal) in dimethyl formamide, and 3 ml of H top agar (10 g bactotryptone/1, 8 g sodium chloride/1, 8 g agar/1) at 55 C. This mixture was plated on to H plates (10 g bactotryptone/1, 8 g sodium chloride/1, 12 g agar/1) and incubated overnight at 37 C. Colorless plaques (those caused by phage with interrupted β-galactosidase fragments) were chosen. These plaques were purified by streaking them on to an H plate which was then overlaid with H top agar containing IPTG, X-Gal, and JM103 cells as described above. The plates were incubated at 37 C overnight. If any blue colonies were found, a colorless plaque was chosen and the purification step repeated.

The supernatants of E. coli cultures which were infected with recombinant M13 phage were prepared for storage and analysis of M13 clones. 2XTY medium (16 g bactotryptone/1, 10 g yeast extract/1, five g sodium chloride/1) was inoculated with E. coli cells from an overnight culture and the cells were grown to an OD of 0.1. A plaque was dispersed in two ml of this culture and incubation was continued for five hours at 37 C with shaking at 230-250 rpm. The cells were

pelleted in a centrifuge and the supernatants, which contain the phage, were stored at -20 C.

The relative sizes of the inserts in the phage DNA were checked on 0.7% agarose gels using tris-acetate buffer as described in Chapter 1 or tris-borate buffer (0.089 M Tris-base pH 7.5, 0.089 M boric acid, and 0.002 M EDTA) (67). One µl 2% SDS and 6 µl of loading buffer (Chapter 2) were added to 20 µl supernatant from each clone. Samples were heated at 65 C for five minutes and then loaded on to the gels. Gels were run for 16-18 hours, stained in ethidium bromide solution (0.5 µg/ml) for 45 minutes, destained in distilled water for 20 minutes, and photographed.

M13 clones were checked for homology with the three major segments of the GH2 dsRNA (68). The M13 supernatants were prepared as for gel electrophoresis and 20 µl were spotted on to a nitrocellulose or nylon (Amersham, Arlington heights, IL) filter using a dot blot apparatus (BioRad, Rockville Centre, NY). GH2 dsRNA (0.25 µg) was spotted on to each filter as a positive control. Filters were floated on a solution of 0.1 N sodium hydroxide, 1.5 M sodium chloride for five minutes and blotted dry. They were then floated on a solution of 3 M sodium chloride, 0.5 M Tris pH 7.0 for five minutes and air dried. Nitrocellulose filters were baked as described in Chapter 2. Nylon filters were wrapped in plastic film and exposed to UV light on a transilluminator for five minutes.

The GH2 dsRNA segments were separated by agarose gel electrophoresis (0.8% agarose gels) as described in Chapter 2. The three major segments were cut from the gel individually after staining

with ethidium bromide. The largest band, which appears as a doublet on polyacrylamide gels (Chapter 1), was treated as one segment in this study. The dsRNA was extracted from the agarose by passing the gel slices through a one ml syringe fitted with a small pipetman tip. When the agarose was sufficiently homogenized, 200 µl Tris-EDTA buffer was added and the suspension was mixed thoroughly on a vortex mixer. Buffer-saturated phenol was added. The suspension was mixed thoroughly on a vortex mixer again, placed at -70 C for 30 minutes, and centrifuged in a microfuge for 15 to 30 minutes. The aqueous phase was extracted with phenol, then with phenol:chloroform:isoamyl alcohol (25:24:1,v/v/v), and then choloform:isoamyl alcohol (24:1,v/v). The dsRNA was concentrated by precipitation of the aqueous phase with ethanol. The large, middle-sized, and small GH2 segments were end-labeled with T4 polynucleotide kinase and hybridized to the filters as described in Chapter 2.

Isolation of M13GH2-9 RF. The replicative form of one of the recombinant M13 phage (M13GH2-9) was isolated by the procedure of Kahn et al. (63) which was modified by the addition of a centrifugation step to reduce the amount of RNA in the sample prior to ultracentrifugation (67). 2XTY medium (four ml) was inoculated with two M13GH2-9 plaques and 40 µl E. coli overnight culture. This culture was grown to an OD of approximately 1.0 and then combined with one liter 2XTY medium and ten ml of E. coli overnight culture. This was incubated for five hours at 37 C, with shaking at 300 rpm. The cells were then pelleted in a GS3 rotor at 3000 rpm for 15 minutes. They were resuspended in six ml of a solution containing 50 mM Tris pH

8.0 and 10% sucrose and chilled on ice for five minutes. After the addition of 0.5 ml lysozyme solution (10 mg/ml), the sample was chilled on ice for another five minutes and 0.6 ml of a solution of EDTA (0.25 M, pH 8.0) was added. The cells were lysed by adding three ml of buffer containing 50 mM Tris, 25 mM EDTA, 0.4% triton X-100 and gently mixing on ice. The sample was centrifuged in a SS-34 rotor at 13,000 rpm for 20 minutes. The supernatant was removed and three ml phenol was added. After mixing, six ml chloroform was added and the sample was mixed again. The phases were separated by centrifugation in a bench top centrifuge. The supernatant was extracted again using six ml chloroform and the centrifugation was repeated. The DNA was precipitated from the aqueous phase by the addition of an equal volume of isopropanol. The sample was placed at -20 C for approximately one hour and then centrifuged in a SS-34 rotor at 11,500 rpm for 15 minutes. The pellet was dried under a stream of nitrogen and resuspended in 28 ml of buffer containing 10 mM Tris pH 7.5, 1 mM EDTA.

Cesium chloride (28 g) was added to the sample and it was centrifuged in an SS-34 rotor at 20,000 rpm for 15 minutes. Ethidium bromide was added (2.8 ml of a 10 mg/ml stock) and the refractive index was adjusted to 1.3950 using solid cesium chloride. Ultracentrifugation was in a TV850 rotor at 40,000 rpm for 14-18 hours. The lower band was collected and the volume and refractive index adjusted as before. The sample was centrifuged in a TV865 rotor at 42,000 rpm for 12 hours. Ethidium bromide was removed by repeated extractions with water-saturated sec-butanol (67). DNA was recovered

from the cesium chloride solution by increasing the volume of the sample ten times with distilled water and precipitating the DNA by the addition of 0.1 volume of 3 M sodium acetate and one volume of isopropanol. This was placed at -20 C for a minimum of two hours and centrifuged in an SS-34 rotor at 11,500 rpm for 15 minutes. The pellet was washed with 70% ethanol, dried, and resuspended in 100 µl of buffer containing 10 mM Tris pH 7.5, 1 mM EDTA. The concentration of the DNA was estimated on an agarose gel as described above.

Isolation of single-stranded M13GH2-9 DNA. Single-stranded M13GH2-9 DNA was isolated from the supernatant from cells grown for M13 RF preparation (68). Any remaining cells were pelleted by centrifugation in a microfuge for five minutes. Supernatant was added to 1.5 ml microfuge tubes containing 200 µl of a solution containing 27% polyethylene glycol (PEG-8000), 3.3 M sodium chloride and left at 4 C for one hour. The sample was then centrifuged in a microfuge for five minutes. The pellet was resuspended in 0.65 ml low-Tris buffer (10 mM Tris pH 7.5, 0.1 mM EDTA). After the addition of 40 µl 40% PEG-8000 and 80 µl 5 M sodium chloride, the sample was left at room temperature for 30 minutes and then centrifuged in microfuge. The pellet was resuspended in 300 µl low-Tris buffer. The sample was then extracted once with phenol saturated with 10 mM Tris pH 7.5, 1 mM EDTA and twice with phenol:chloroform:isoamyl alcohol (25:24:1,v/v/v). The DNA was precipitated at -20 C by the addition of 0.1 volume 3 M sodium acetate and 2.5 volumes 95% ethanol. After pelleting the DNA in a microfuge and drying the pellet, it was resuspended in low-Tris buffer.

Restriction digests. DNA from the GH2, Ess6, and CL1-16 isolates of E. parasitica, the M13GH2-9 RF, and salmon sperm DNA (Sigma, St. Louis, Mo.) were digested with the restriction enzymes HindIII (New England Biolabs, Beverly, MA, 20 U/µl), BamHI (Bethesda Research Laboratories, Gaithersburg, MD, 10 U/µl), or EcoRI (New England Biolabs, 20 U/µl or Bethesda Research Laboratories, 10 U/µl). Buffer for HindIII and BamHI reactions contained 50 mM sodium chloride, 10 mM Tris-HCl pH 7.5, 10 mM magnesium chloride, and 1 mM DTT, while EcoRI buffer contained 100 mM sodium chloride, 50 mM Tris-HCl pH 7.5, 10 mM magnesium chloride, and 1 mM DTT (67). Digestions were performed in 100 µl volumes containing 20 U of enzyme. All reactions were incubated for seven hours at 37 C and terminated by the addition of a solution of 0.5 M EDTA pH 8.0 to a final concentration of 10 mM. Samples were then precipitated at -20 C by the addition of 0.1 volume 2.5 M sodium acetate pH 5.2 and two volumes 95% ethanol.

Agarose gels and blotting. DNA from the GH2, Ess6, and CL1-16 isolates of E. parasitica, M13GH2-9 RF, and salmon sperm DNA were run on 1% agarose gels with tris-acetate or tris-borate buffer. Four µg E. parasitica or salmon sperm DNA were run in each lane. Gels to be blotted and hybridized to labeled dsRNA were loaded with one µg M13GH2-9 RF per lane, while those to be hybridized to labeled M13GH2-9 were loaded with 0.02 µg per lane. Gels were run for 17 hours, stained with an ethidium bromide solution (0.5 µg/ml) for 45 minutes, destained for 20 minutes, and photographed.

Gels were soaked for one hour in several changes of a solution containing 1.5 M sodium chloride, 0.5 M sodium hydroxide. They were then neutralized by soaking in several changes of buffer containing 1 M Tris-base pH 8.0, 1.5 M sodium chloride for one hour (67). They were then blotted as described in Chapter 2 to nitrocellulose or nylon filters and DNA was affixed to the filters as described above. Gels to be probed with labeled M13GH2-9 usually were cut in half, so that positive controls and unknown samples could be hybridized separately.

Labeling of probes and hybridization. The dsRNA probes were labeled with T4 polynucleotide kinase as described in Chapter 2 with two minor changes: the spun-column method was used for Sephadex G-50 chromatography (67) and yeast tRNA was substituted for calf thymus DNA as a carrier nucleic acid for the labeled RNA. Ribosomal RNA (28S and 18S) (rRNA) from Saccharomyces cerevisiae (ATCC 9763) was purchased from Pharmacia (Piscataway, NJ) and labeled by the procedure used to label dsRNA.

M13GH2-9 single-stranded DNA was labeled by the procedure of Hu and Messing (59). The M13 hybridization primer was purchased from Amersham (Arlington Heights, IL). The primer was annealed to M13GH2-9 in reaction mixtures containing two ng primer, 50 ng M13GH2-9 single-stranded DNA, 1.5 µl H buffer (100 mM Tris-HCl pH 7.9, 600 mM sodium chloride, 66 mM magnesium chloride), and one µl 0.1 M dithiothreitol. The reaction volume was adjusted to nine µl and the mixture was heated at 55 C for five minutes and then allowed to cool at room temperature.

The template-primer reaction mixture was then added to 10 μCi dried (α-^{32}P) dATP (2000-3000 Ci/mmol, Amersham, Arlington Heights, IL). One μl of a solution containing 500 μM each dGTP, dCTP, and dTTP and one μl DNA polymerase (Klenow) (6 U/μl) were added to the reaction mixture, which was then incubated at room temperature for one hour. The reaction was terminated by the addition of one μl of a solution of EDTA (0.25 M, pH 8.3). The spun-column method was used for Sephadex G-50 columns. Incorporation was monitored using DE-81 filters as described in Chapter 2. Specific activities of 1-5 X 10^8 were obtained.

Hybridizations and autoradiography were performed as described in Chapter 2, except that yeast tRNA was substituted for calf thymus DNA in the hybridization buffer used with dsRNA and rRNA probes. The concentration of the M13 label was 10^5-10^7 cpm/ml. Longer prehybridizations (over night) reduced the background in hybridizations using M13GH2-9.

Results

Sixty two M13 cDNA clones of dsRNA from the GH2 isolate were recovered based on lack of functional β-galactosidase activity. The cDNA inserts interrupted the β-galactosidase gene in the phage, eliminating the ability to cleave the X-Gal in the medium to form a blue dye. Thus, these phage produced clear plaques.

Attempts to obtain full length clones were hampered by the inability to achieve full length second-strand synthesis. Additional RNase H digestion combined with S1 nuclease digestion of the labeled cDNA after the second strand synthesis resulted in a greater reduction

of counts in the labeled DNA than when incubation was with S1 nuclease alone; therefore the inability to get full length second strands was due to incomplete digestion of the RNA template by RNase H. The cDNA obtained was digested with restriction enzymes and ligated into M13mp8 RF, resulting in small (approximately 200 bp) cDNA clones which are useful hybridization probes. These cDNA clones are easily labeled to high specific activity (59,68).

From the M13 clones, M13GH2-9 was chosen for further studies because it contained one of the largest inserts and hybridized consistently to the large and middle segments of GH2 dsRNA, but not to the small segment. Since this clone did not hybridize to all GH2 dsRNA segments, it does not correspond to the poly(A):(U) terminus shared by all of the segments.

DNA from three E. parasitica isolates was tested for homology to both labeled GH2 dsRNA and M13GH2-9 using Southern blotting and hybridization techniques. The total digested DNA from E. parasitica, salmon sperm, and M13GH2-9 is visible on ethidium bromide-stained gels (Figure 14). Autoradiograms show that GH2 dsRNA preparations hybridized to DNA from all three E. parasitica isolates (Figures 15-17). Different patterns of hybridization were obtained with each of the three restriction enzymes used, indicating that the hybridization was not caused by contamination of the DNA preparations with dsRNA. The hybridization observed in the CL1-16 DNA lane also supports this, since the CL1-16 isolate does not harbor detectable dsRNA. The hybridization patterns were the same for GH2, Ess6, and CL1-16 DNA when they were cut with the enzymes HindIII or EcoRI. The

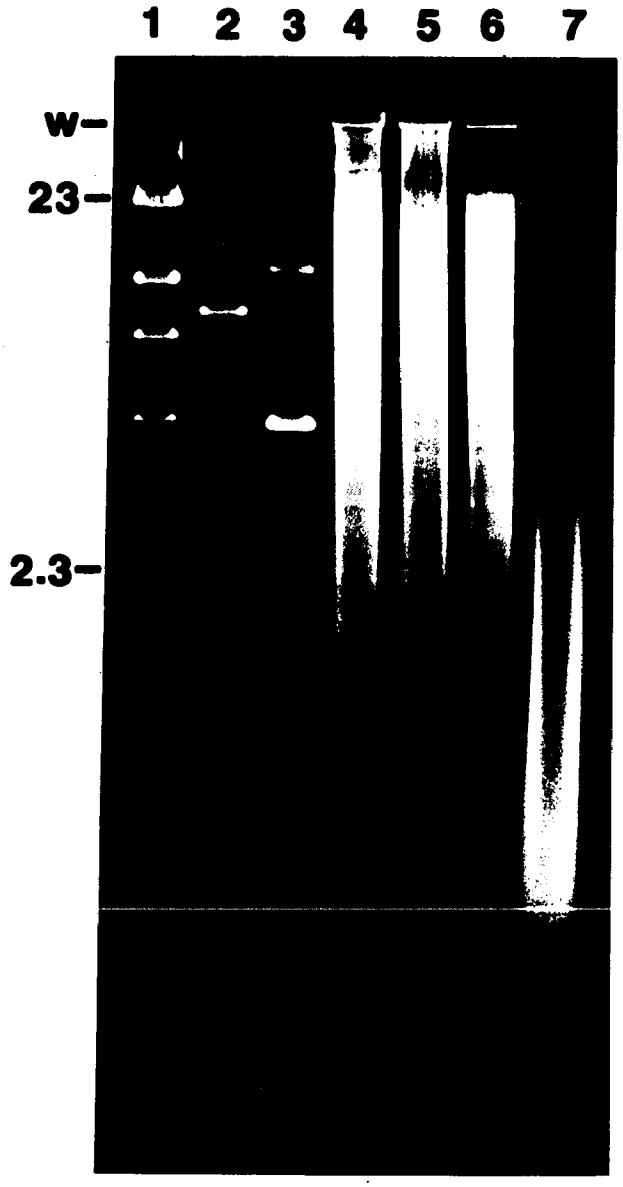

Figure 14. Ethidium bromide-stained gel of E. parasitica DNA prior to blotting. Lanes: 1, lambda HindIII fragments; 2, HindIII-cut M13GH2-9 RF; 3, uncut M13GH2-9 RF; 4, HindIII-cut GH2 DNA; 5, HindIII-cut Ess6 DNA; 6, HindIII-cut CL1-16 DNA; 7, HindIII-cut salmon sperm DNA. Sizes of selected molecular weight markers are indicated in kilobase pairs.

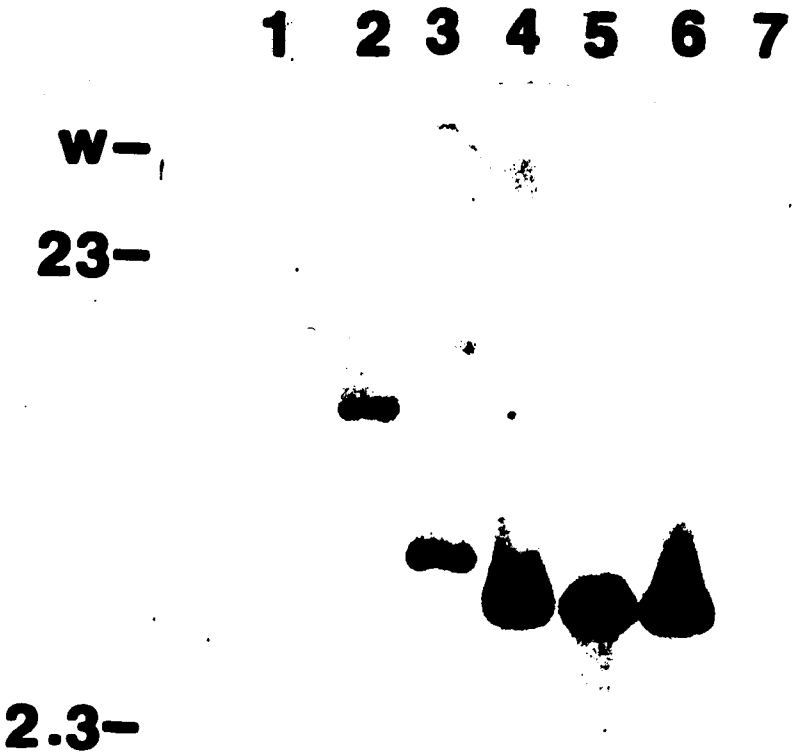

Figure 15. Hybridization of GH2 dsRNA probe to HindIII-cut E. parasitica DNA. Lanes: 1, lambda HindIII fragments; 2, HindIII-cut M13GH2-9 RF; 3, uncut M13GH2-9 RF; 4, HindIII-cut GH2 DNA; 5, HindIII-cut Ess6 DNA; 6, HindIII-cut CL1-16 DNA; 7, HindIII-cut salmon sperm DNA. Sizes of selected molecular weight markers are indicated in kilobase pairs.

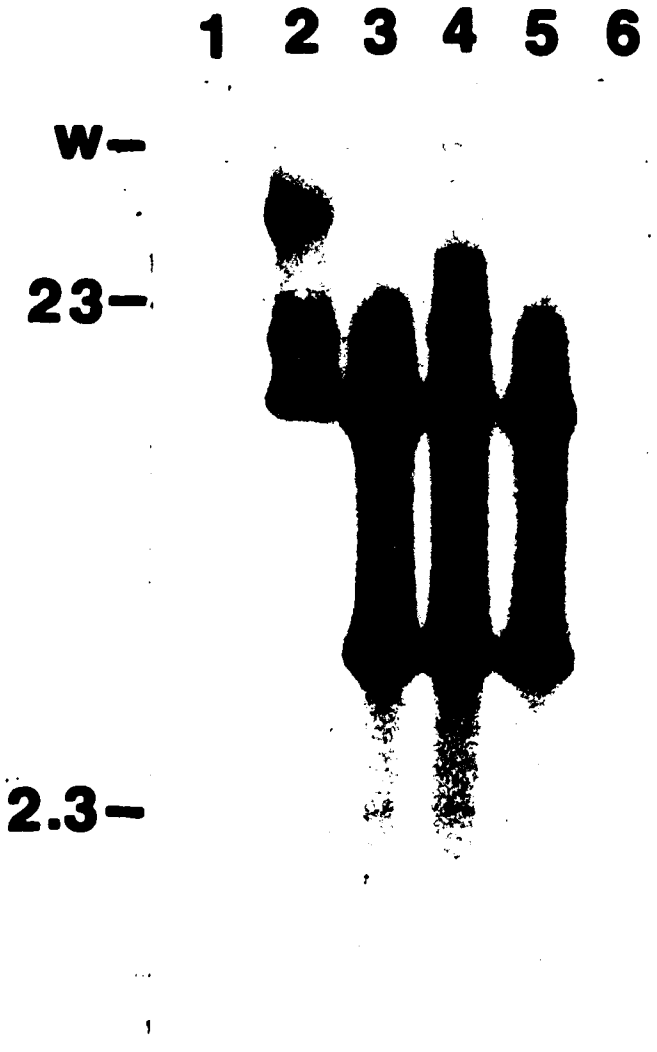

Figure 16. Hybridization of GH2 dsRNA probe to EcoRI-cut E. parasitica DNA. Lanes: 1, lambda HindIII fragments; 2, EcoRI-cut M13GH2-9 RF; 3, EcoRI-cut GH2 DNA; 4, EcoRI-cut Ess6 DNA; 5, EcoRI-cut CL1-16 DNA; 6, EcoRI-cut salmon sperm DNA. Sizes of selected molecular weight markers are indicated in kilobase pairs.

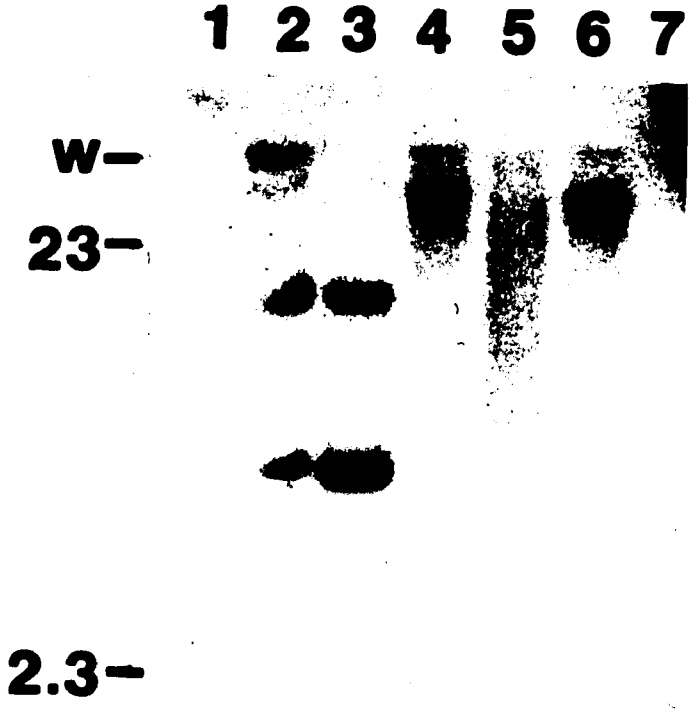

Figure 17. Hybridization of GH2 dsRNA probe to BamHI-cut E. parasitica DNA. Lanes: 1, lambda HindIII fragments; 2, BamHI treated M13GH2-9 RF; 3, M13GH2-9 RF (not treated with BamHI); 4, BamHI-cut GH2 DNA; 5, BamHI-cut Ess6 DNA; 6, BamHI-cut CL1-16 DNA; 7, BamHI-cut salmon sperm DNA. The sizes of selected molecular weight markers are indicated in kilobase pairs.

hybridization observed with HindIII-cut DNA did not resemble the GH2 dsRNA banding pattern (Figure 15). A major HindIII band of approximately 3800 bp hybridized to GH2 dsRNA. Some gels showed that this band is actually a doublet. Long exposures of autoradiograms revealed two bands of approximately 7600 and 5600 bp which hybridized less intensely. The EcoRI digested DNA revealed three hybridizing bands of approximately 9500, 6500, and 3300 bp (Figure 16). These bands were not the same size as the GH2 dsRNA segments (Chapter 1). This is additional evidence that the hybridization observed was not due to dsRNA contamination of DNA preparations.

The dsRNA probe hybridized to a large molecular weight band in lanes containing GH2 or CL1-16 DNA when the DNA was cut with BamHI (Figure 17). This band ran at the approximate position in the gels corresponding to the position where uncut DNA would be expected to run. On ethidium bromide-stained gels, DNA cut with BamHI appeared as it did for other enzymes, indicating that the BamHI enzyme was cutting the DNA. The M13GH2-9 DNA was not cut with the BamHI enzyme because the BamHI site in M13 was destroyed by insertion of the cDNA. Although bands were evident in the DNA from GH2 and CL1-16, a well defined band was not observed in autoradiograms in the Ess6 DNA lane when the DNA was cut with BamHI. However, a diffuse smear was observed. The results with HindIII and EcoRI digested DNA indicate that the hybridizing sequences are present in Ess6 DNA, suggesting that the result with BamHI-digested Ess6 DNA is an artifact. The lack of a band similar to that in the lanes containing GH2 and CL1-16 DNA may be due to degradation of the Ess6 DNA sample. Although the

quantity of DNA loaded in each lane had been estimated to be the same, there may be slightly less DNA in the Ess6 samples as indicated by less intense ethidium bromide staining and hybridization (Figures 14-17).

When labeled M13GH2-9 was used as a probe against E. parasitica DNA, no hybridization was observed (Figure 18). However, the probe hybridized strongly to M13GH2-9 RF. Some filters were cut in half and the two halves were hybridized separately so that the positive controls, which share M13 as well as insert sequences with the probe, would not interfere with hybridization to the E. parasitica DNA. This did not result in hybridization of M13GH2-9 to the fungal DNA. Background was a problem with this probe, but could be reduced by increasing the length of the prehybridization.

Labeled rRNA from yeast hybridized to the same size fragments of HindIII digested E. parasitica DNA as did the dsRNA preparations (Figure 19).

Discussion

Hybridization results between the GH2 dsRNA and the fungal DNA suggested that there are homologous sequences to the dsRNA in the E. parasitica genome. The results rule out the possibility that dsRNA contamination of the DNA samples is responsible for the observed hybridization. However, the lack of hybridization found between a cDNA clone of the dsRNA and the fungal DNA suggests that some other nucleic acid is responsible for the observed hybridization of the dsRNA probe. Ribosomal RNA is abundant in cells and is a common

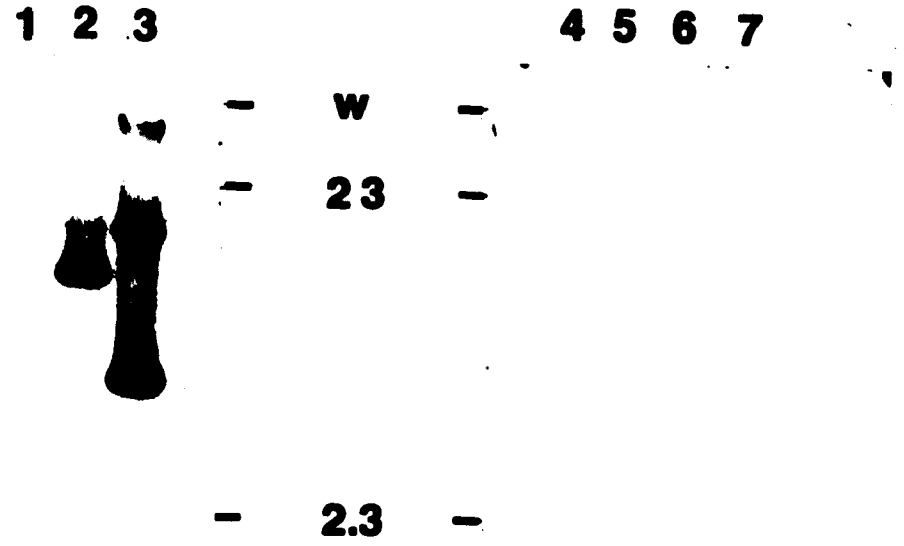

Figure 18. Hybridization of M13GH2-9 to HindIII-cut E. parasitica DNA. Lanes: 1, lambda HindIII fragments; 2, HindIII-cut M13GH2-9 RF; 3, uncut M13GH2-9 RF; 4, HindIII-cut GH2 DNA; 5, HindIII-cut Ess6 DNA; 6, HindIII-cut CL1-16 DNA; 7, HindIII-cut salmon sperm DNA. The sizes of selected molecular weight markers are indicated in kilobase pairs.

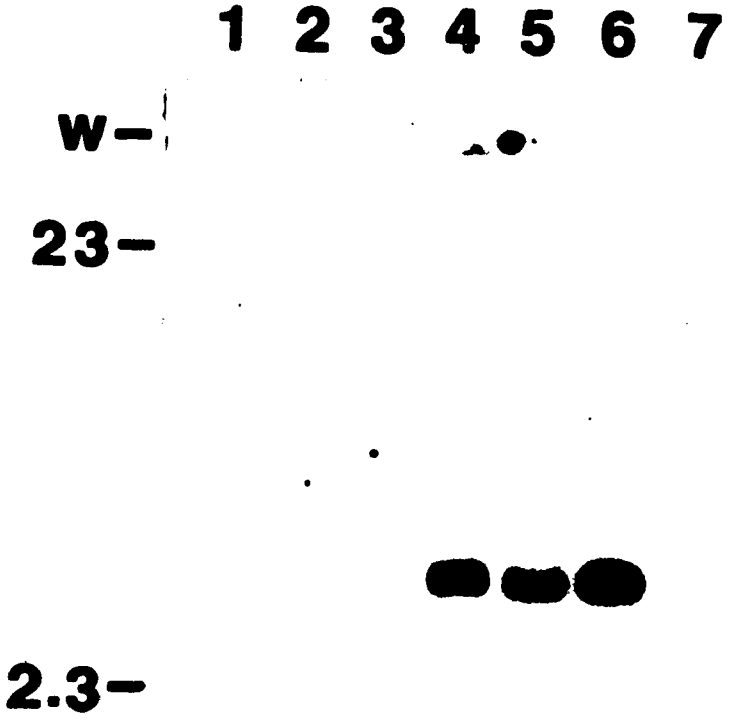

Figure 19. Hybridization of yeast ribosomal RNA to HindIII-cut E. parasitica DNA. Lanes: 1, lambda HindIII fragments; 2, HindIII-cut M13GH2-9 RF; 3, uncut M13GH2-9 RF; 4, HindIII-cut GH2 DNA; 5, HindIII-cut Ess6 DNA; 6, HindIII-cut CL1-16 DNA; 7, HindIII-cut salmon sperm DNA. Sizes of selected molecular weight markers are indicated in kilobase pairs.

contaminant when other cellular RNAs are isolated (12,14,94,97). The possibility that the hybridization observed when dsRNA was hybridized to E. parasitica DNA was caused by contaminating rRNA was investigated by probing the fungal DNA with labeled yeast rRNA. The same hybridization pattern was observed when yeast rRNA and dsRNA were hybridized to HindIII-cut E. parasitica DNA. This indicates that the dsRNA is contaminated with host rRNA and that the homology found between dsRNA preparations and host DNA is a result of the rRNA present in dsRNA preparations. Results with the cDNA probe must be interpreted with caution because this probe does not contain all of the dsRNA sequences. Although no homology was found between E. parasitica dsRNA and DNA in this study, there may be homology that was not detected by the techniques used.

There are reports of hypovirulent isolates in which hypovirulence is inherited as a nuclear trait (3,90). These isolates carried dsRNA at one time. The dsRNA may interact with the host DNA, causing a stable change in the host phenotype. The Ess6 isolate provides another example which suggests an interaction between the dsRNA and the fungal genome. Integration of the dsRNA sequences into the host genome is one way in which this could be accomplished. This mechanism is utilized by retroviruses. Plant viruses with RNA genomes may replicate via a DNA intermediate or DNA viruses may have an RNA intermediate, even though there is no evidence for insertion of the DNA into the genome (60). All of the dsRNA genomes of the mycoviruses that have been studied replicate through RNA intermediates (15,72),

but yeast transposable elements provide a model for reverse-transcribing systems in fungi (16,45). The Ty element in yeast is associated with a virus-like particle and the RNA structure is similar to that of a retrovirus.

Reports of homology between the RNA of virus-like agents and the DNA of their hosts have been published (55,84,93). However, further work often does not support these results (12,14,83,97). Even when dsRNA has been highly purified, it is often found to include other cellular RNAs, including ribosomal RNA and tRNA (12,14,94,97). If homology was found between dsRNA and the E. parasitica DNA, it would represent a novel mechanism of dsRNA replication, but in view of the many strategies adopted by viruses to propagate themselves, it is conceivable.

Further work is needed on the E. parasitica system before claims of homology between fungal DNA and dsRNA can be made. The presence of sequences homologous to E. parasitica dsRNA in the fungal genomes would explain how dsRNA could appear in fungal populations that are widely separated geographically. The question of whether the dsRNA represents a mobile host genetic element or a virus capable of insertion into the host genome would remain open. The possibility of insertion of sequences homologous to dsRNA into the fungal DNA leads to speculation concerning how this may result in the hypovirulent phenotype. The insertion of the sequences homologous to the dsRNA may interrupt a virulence gene or these sequences could encode products which are expressed only upon insertion of the sequences in the

genome. Alternatively, the dsRNA may carry regulatory sequences which exert their effect on host genes upon integration into the genome.

If the integration of dsRNA sequences into the host genome does not occur, there must be another explanation for the white nuclear hypovirulent mutants, the JR isolates, and the Ess6 isolate. In the cases of the white nuclear mutants and the JR isolates, the dsRNA or its products may interact with the host genome to regulate host virulence genes. However, this regulation would have to occur in a way that allowed stable maintenance of the trait. In the case of Ess6, there may be regulation of dsRNA replication. GH2 dsRNA may be present in Ess6, but at an undetectable level. The interactions between the host genome and yeast killer factor dsRNA have been extensively studied and are very complex (95). Similar interactions may be involved in E. parasitica, but isolate CL1-16 is a single-conidial isolate of a virulent isolate, so the fungal nuclear background is the same in all isolates diagrammed in Figure 13. Therefore, differences in nuclear background can not explain all of the changes in the dsRNA. The isolates do not change banding patterns after repeated subculture; Ess6 does not develop GH2 dsRNA segments with time. The mechanism by which the dsRNA replication in E. parasitica may be regulated is not currently understood.

The possibility of undetectable levels of dsRNA within E. parasitica isolates may indicate that dsRNA is present within populations of the fungus at low levels as an extrachromasomal genetic element. An unknown stimulus, possibly environmental, may cause it to replicate to levels that cause the hypovirulent phenotype, explaining

the multiple appearances of dsRNA in E. parasitica in different parts of the world. Hypovirulence may result from the over-produced dsRNA monopolizing a cellular component. However, recent evidence indicates that dsRNA caused specific changes in the expression of fungal genes (76,77). A threshold level of dsRNA or its products may be required to cause these changes. The available structural data supports a viral origin for the dsRNA in E. parasitica (58,85). If this is the case it may cause the hypovirulent phenotype by any of the same mechanisms as an extrachromosomal element.

The results presented here provide evidence suggesting that there are no homologous sequences to dsRNA in the E. parasitica DNA. However, highly purified dsRNA probes or well characterized cDNA probes of high specific activity would provide more conclusive results. Until further evidence is available, none of the possibilities concerning origin or mode of action of the dsRNA can be ruled out.

APPENDICES

APPENDIX 1

ELECTRON MICROSCOPY

APPENDIX 1

ELECTRON MICROSCOPY

The vast majority of dsRNAs described in the literature are linear double helices, but several exceptions have been reported. Circular dsRNA molecules have been observed in preparations of mengovirus replicative forms (80). However, the authors considered these to consist of linear duplexes whose ends had annealed with each other. Viroids and virusoids are small circular single-stranded RNA molecules which are extensively base-paired (64,79). The structure of the dsRNA molecules from hypovirulent isolates of E. parasitica was examined by electron microscopy.

The dsRNA used in these studies was isolated from the Michigan hypovirulent E. parasitica isolate, RF. The growth of the fungal cultures and the dsRNA isolation procedure were as described in Chapter 1, except that the bentonite and SDS were omitted during the phenol extraction step. Copper electron microscope grids were coated with 3% parlodian in amyl acetate. Samples were prepared by the spreading technique described by Davis et al. (22) and Chow et al. (19). The hypophase consisted of 0.01 M Tris pH 8.5, 0.001 M EDTA, and 50% formamide. The hyperphase was made up of 0.1 M Tris pH 8.5, 0.01 M EDTA, 0.05 mg cytochrome c/ml, 80% formamide, and one ng dsRNA/ µl. The hypophase was spread over the hyperphase by running it down

the surface of a heat-sealed Pasteur pipet. Coated grids were touched to the surface of the hyperphase and then dipped in 5 X 10^{-5}M uranyl acetate in 90% ethanol. The grids were then dipped in 90% ethanol, allowed to dry, and rotary shadowed with platinum and palladium. They were viewed on a Phillips 201 transmission electron microscope and photographed.

All dsRNA molecules observed were linear (Figure 20). Although samples were not run under denaturing conditions, no indications that any of the dsRNA segments in E. parasitica are single-stranded circles were apparent. Photographs resembled those of linear dsRNA molecules (9). The ability to label dsRNA molecules with enzymes that require free 3'- or 5'-ends supports these results. When the alkaline hydrolysis step was omitted, the 5'-termini of the intact E. parasitica dsRNA segments can be labeled with ^{32}P using T4 polynucleotide kinase as described in Chapter 2. The 3'-ends of dsRNA segments can be labeled using RNA ligase (85) and polyadenylated using poly(A) polymerase (Chapter 3). These results do not rule out random breakage of the molecules during their extraction. However, enzymatic RNA sequencing of E. parasitica dsRNA from the GH2 isolate indicated that all three major segments had distinct ends (85).

Rough measurements were made of photographed molecules and their sizes were calculated, accounting for the magnification and using the conversion for dsRNA (1 micron = 2.15 X 10^6 daltons = 3100 bp) (28). The sizes of 15 measured molecules ranged from 1 to 9.6 kbp. These sizes are in agreement with those determined for the RF isolate by comparative gel electrophoresis using linear dsRNA molecular weight

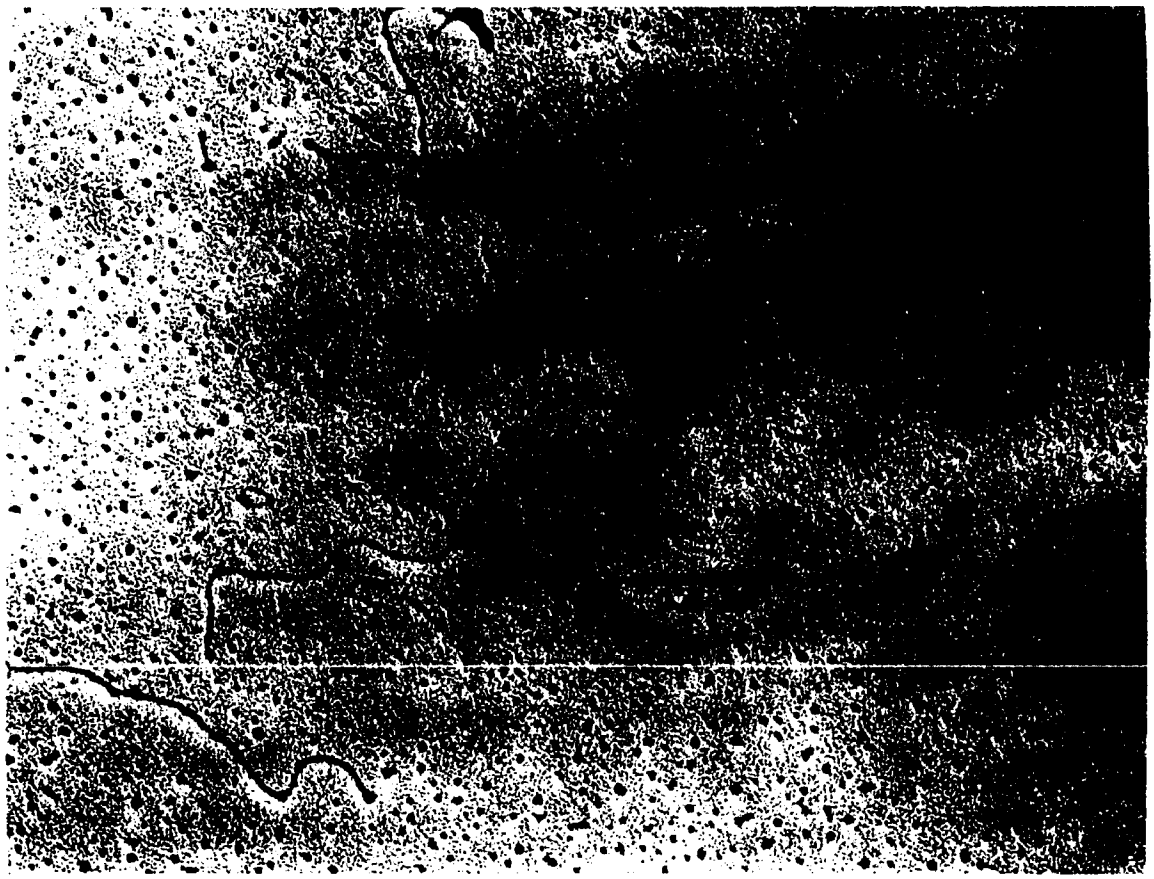

Figure 20. Electron micrograph of dsRNA from the RF E. parasitica isolate.

size standards (Chapter 1). Molecules approximately the same size as the largest segment were observed suggesting that breakage was not extensive.

Although these studies do not rule out the possibility of circular dsRNAs in some hypovirulent E. parasitica isolates, the available evidence suggests that the dsRNA segments in E. parasitica are linear.

APPENDIX 2

PRELIMINARY STUDIES ON THE EXPRESSION OF THE dsRNA GENOME

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PRELIMINARY STUDIES ON THE EXPRESSION OF THE dsRNA GENOME

Although there is a correlation between the presence of dsRNA in the cytoplasm and the hypovirulent phenotype in E. parasitica, the mechanism by which dsRNA may cause hypovirulence is unknown. Available evidence indicates that dsRNA exerts specific effects on the fungus (34,35,40,76,77). Studies on expression of the dsRNA genome were initiated to elucidate how the dsRNA may affect fungal metabolism. A search for dsRNA-specific mRNAs was begun to determine whether dsRNA in fact produced complimentary single-stranded RNAs (ssRNAs). Most eukaryotic mRNAs are polyadenylated and can be isolated based on their affinity for oligo(dT) cellulose. Nucleic acid preparations (total nucleic acids or nucleic acids from which dsRNA was removed by CF-11 chromotography) from the dsRNA-carrying isolate, GH2, were examined for the presence of polyadenylated mRNAs with homology to GH2 dsRNA. If mRNAs were found, they would be helpful in making cDNA to dsRNA sequences and in in vitro translation experiments. In vitro translations were performed using denatured dsRNA in an attempt to find a dsRNA-encoded protein.

E. parasitica cultures were grown as described in Chapter 1. The GH2 isolate was used in mRNA and in vitro translation studies. The procedure used for isolating the total nucleic acid preparations used

in mRNA studies was as described in Chapter 2 except that CF-11 chromatography was omitted. The dsRNA (CF-11-bound fraction) used in denaturing gels was isolated as described in Chapter 2. Nucleic acids that bound to an oligo(dT) column [oligo(dT)-bound fraction] were selected either from total nucleic acids or from the nucleic acids which had failed to bind to a CF-11 column in STE:15% ethanol (CF-11-unbound). The concentration of all nucleic acid preparations was determined by measuring the UV absorbance at 260 nm.

Total nucleic acids or the CF-11-unbound fraction was applied to an oligo(dT) cellulose column at a concentration of one mg/ml in binding buffer (0.5 M sodium chloride, 10 mM Tris pH 7.5, 1 mM EDTA, 0.5% SDS) (6). The concentration of dsRNA subjected to oligo(dT) chromatography was 0.5 mg/ml. Columns were washed with binding buffer until the elution of nonbinding nucleic acids was complete as monitored by UV absorbance. The oligo(dT)-bound fraction was then eluted from columns with buffer made up of 10 mM Tris pH 7.5, 1 mM EDTA, 0.2% SDS. The bound and unbound fractions were concentrated by ethanol precipitation.

Denaturing gels, transfer of nucleic acids to nitrocellulose filters, end-labeling of dsRNA using T4 polynucleotide kinase, hybridization of labeled dsRNA to filters, and autoradiography were as described in Chapter 2. The dsRNA used as a probe in hybridizations was extracted as described in Chapter 1 or 2. Electrophoresis on agarose gels was as described in Chapter 2.

In initial studies total nucleic acids were run through an oligo(dT) cellulose column to collect polyadenylated nucleic acids and then the unbound fraction was run through a CF-11 cellulose column to isolate the dsRNA fraction. However, no dsRNA was recovered from the CF-11 column as determined by ethidium bromide staining of gels and autoradiography and genome length RNAs were found in the fraction binding to the oligo(dT) column on autoradiograms. These observations led to the suspicion that the dsRNA was adhering to the oligo(dT) column. This was confirmed when GH2 dsRNA was subjected to oligo(dT) chromatography (Figure 21). An estimated 10-20% of the GH2 dsRNA bound to the oligo(dT) column. The smallest GH2 segment did not exhibit as great an affinity for the oligo(dT) cellulose as the large and middle-sized segments. The binding of dsRNA to the oligo(dT) column suggests that at least some of the GH2 dsRNA is poly(A)-tailed or contains poly(adenylic acid):poly(uridylic acid) rich regions [poly (A:U)]. Internal poly(A:U) rich regions have been found in one segment of yeast killer factor dsRNA (39,56,87). These regions result in an affinity of that segment for oligo(dT) cellulose and poly(U) sepharose. Subsequent results show that all three major segments of the GH2 isolate have a poly(A:U) terminus (85). These termini are probably responsible for the behavior of GH2 dsRNA on oligo(dT) columns.

Even when nucleic acid preparations were run through CF-11 columns twice to remove dsRNA prior to oligo(dT) chromatography, bands the same size as the denatured dsRNA bands were pronounced in the lane in which oligo(dT)-bound fraction was run on autoradiograms of denaturing

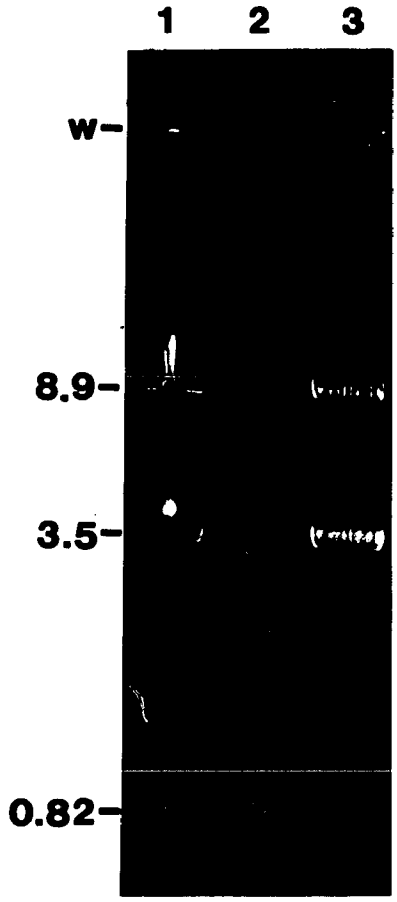

Figure 21. Affinity of GH2 dsRNA for oligo(dT) cellulose. Lanes: 1, GH2 dsRNA; 2, the fraction of GH2 dsRNA that bound to an oligo(dT) column; 3, the fraction of GH2 dsRNA that did not bind to an oligo(dT) column. Sizes of the GH2 dsRNA segments are indicated in kilobase pairs.

gels (Figure 22). No bands other than those corresponding in size to those of the denatured genome were found in the lane with oligo(dT)-bound nucleic acids, but a band which ran between the middle and small bands was sometimes present. A dsRNA segment of this approximate size was present in some preparations of GH2 dsRNA (Chapter 1). Interpretation of these results is complicated by the affinity of dsRNA for oligo(dT) cellulose since oligo(dT)-bound fractions would contain dsRNA that had escaped the CF-11 column. The possibility that the genome-size RNAs present in autoradiograms are actually denatured dsRNA cannot be ruled out. The largest band was disproportionally less intense on autoradiograms in the lane with the fraction which did not bind to the CF-11 column, but did bind to the oligo(dT) column, while the smallest GH2 band gave a strong signal. This contrasts with the results when dsRNA was subjected to oligo(dT) chromatography, suggesting that not all of the hybridization of labeled dsRNA to genome-sized bands is due to the incomplete removal of dsRNA from CF-11-unbound preparations. Available evidence indicates that if the dsRNA makes polyadenylated single-stranded mRNAs they are probably full length transcripts. However, mRNAs of a different size may be made in amounts that were not detectable by these techniques. The genome-length ssRNAs may represent a step in dsRNA replication as well as mRNA transcripts.

The preliminary evidence from transcription studies indicates that if the dsRNA of hypovirulent isolates of E. parasitica encodes protein products, it probably does so by the translation of either full length

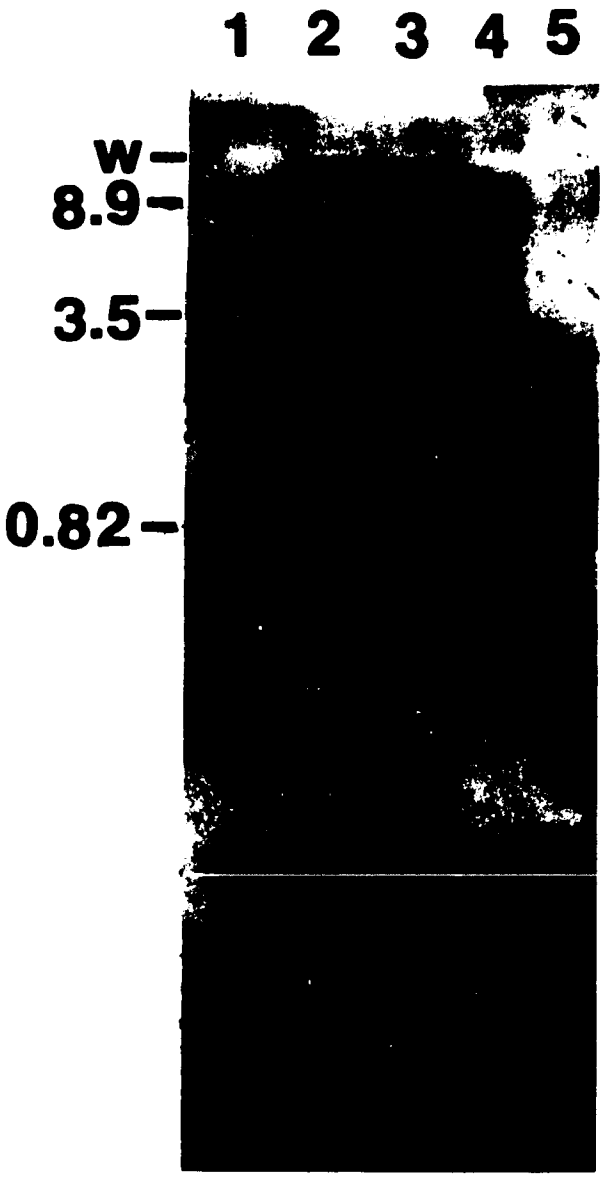

Figure 22. Hybridization of GH2 dsRNA to GH2 nucleic acids fractionated by CF-11 and oligo(dT) chromatography. Lanes: 1, total nucleic acids; 2, nucleic acids that bound to CF-11 columns; 3, nucleic acids that bound to oligo(dT) column; 4, nucleic acids that did not bind to CF-11 columns, but did bind to oligo(dT) column; 5, nucleic acids that did not bind to CF-11 or oligo(dT) columns. Sizes of the GH2 dsRNA segments are indicated in kilobase pairs.

mRNAs or of the positive strand of the genome. A search for dsRNA-encoded proteins was conducted using denatured dsRNA as the template. The dsRNA used for the in vitro translations was from the GH2 isolate. Cultures were grown and the dsRNA isolated as described in Chapter 1. The rabbit reticulocyte and wheat germ in vitro translation systems were used. Kits were supplied by BRL (Bethesda Research Laboratories, Gaithersburg, MD). Globin mRNA (50 µg/ml) was provided as a control with both systems. The dsRNA was denatured by heating at 100 C for 2 or 4 minutes or was suspended in 90% DMSO followed by heating at 50 C for 45 minutes.

Reaction mixtures for the rabbit reticulocyte system contained 1.3 µl 2 M potassium acetate pH 7.2, 1.5 µl 20 mM magnesium acetate pH 7.2, 5 µl ^{35}S-methionine (11.61 µCi/µl; Amersham, Arlington Heights, IL), 3 µl 10X BRL reaction mixture (250 mM HEPES, 400 mM potassium chloride, 100 mM creatine phosphate, and 500 µM each of the essential amino acids with the exception of methionine), 10 µl reticulocyte lysate, and 0-11 µl denatured 0.5 µg dsRNA/µl. Reaction volumes were adjusted to approximately 30 µl with 10 mM HEPES pH 7.2, 160 mM potassium acetate. Incubation was for one hour at 30 C.

Reaction mixtures for the wheat germ system contained two µl 500 mM potassium acetate pH 7.5, 0.9 µl 20 mM magnesium acetate pH 7.5, four µl ^{35}S-methionine (11.61 µCi/µl), three µl 10X BRL reaction mixture (20 mM HEPES, 300 mM potassium acetate, one mM magnesium acetate, 12 mM ATP, one mM GTP, 55 mM creatine phosphate, two mg creatine kinase/ml, 800 µM spermidine phosphate, and 500 µM each of the essential amino acids with the exception of methionine), 10 µl

wheat germ extract, and zero or six µl denatured 0.5 µg dsRNA/µl. Reaction volumes were adjusted to 30 µl with 10 mM HEPES pH 7.2, 160 mM potassium acetate. One µl 520 µM D,L-methionine was added to some samples using the wheat germ system and the volume adjusted so that the final volume was 30 µl. Incubation was for one to three hours at 25 C.

Protein from both systems was usually precipitated by the addition of acetone and collected by centrifugation. Protein in some samples from the wheat germ system was collected by TCA precipitation. After the addition of 10% TCA, samples were left on ice for 10 minutes and then collected by centrifugation.

Samples from both systems were resuspended in 30 µl 25 mM Tris pH 8.0, 20 µl loading buffer (5% SDS, 30% glycerol, 0.1% bromphenol blue), and five µl 10% 2-mercaptoethanol. Samples were heated at 100 C for two minutes before loading on to SDS-polyacrylamide gels (20). Stacking gels were 6% polyacrylamide, 0.16% bis-acrylamide, while resolving gels were 10% polyacrylamide, 0.27% bis-acrylamide. Gels were run at eight milliamperes until the tracking dye had run through the stacking gel. The amperage was then increased to 25 milliamperes for the duration of the resolving gel. Gels were run until the tracking dye reached the bottom of the gel. Gels were fixed in 40% methanol, 10% acetic acid for a minimum of 30 minutes, followed by a minimum of 30 minutes in 25% methanol. They were then treated with Fluoro-Hance (Research Products International, Mount Prospect, IL) for a minimum of 30 minutes and dried. Autoradiography was performed using Kodak XAR-5 film.

No dsRNA-specific proteins were observed on polyacrylamide gels using either the rabbit reticulocyte or the wheat germ in vitro translation systems. The rabbit reticulocyte system efficiently translated globin mRNA whether the mRNA was boiled or treated with DMSO (Figure 23). The addition of dsRNA and globin to the reticulocyte system together did not result in the inhibition of globin mRNA translation indicating that the dsRNA was not inhibiting protein synthesis and the dsRNA preparations are not contaminated with any other translation inhibitor.

Neither globin nor dsRNA-specific proteins were detected on polyacrylamide gels using the wheat germ in vitro translation system. However, the addition of globin mRNA resulted in an increase in one of the endogenous bands produced by all reactions even when no RNA was added (Figure 24). Both heat and DMSO denaturation were used as well as precipitation of the proteins with either acetone or TCA following the reaction. The addition of nonradioactive methionine to reactions with the wheat germ system did not result in the production of globin or dsRNA-specific proteins (Figure 24). Samples containing globin mRNA often showed an increase in an endogenous band on gels. This band ran just behind the bromphenol blue marker, and thus should consist of small polypeptides. The increase in this band in lanes containing globin mRNA may be the result of early termination of globin mRNA translation. The increase in this endogenous band was not inhibited by the addition of dsRNA to the reaction mixture containing globin mRNA. As in the reticulocyte system, the dsRNA or impurities in the preparation do not inhibit in vitro translation. The increase

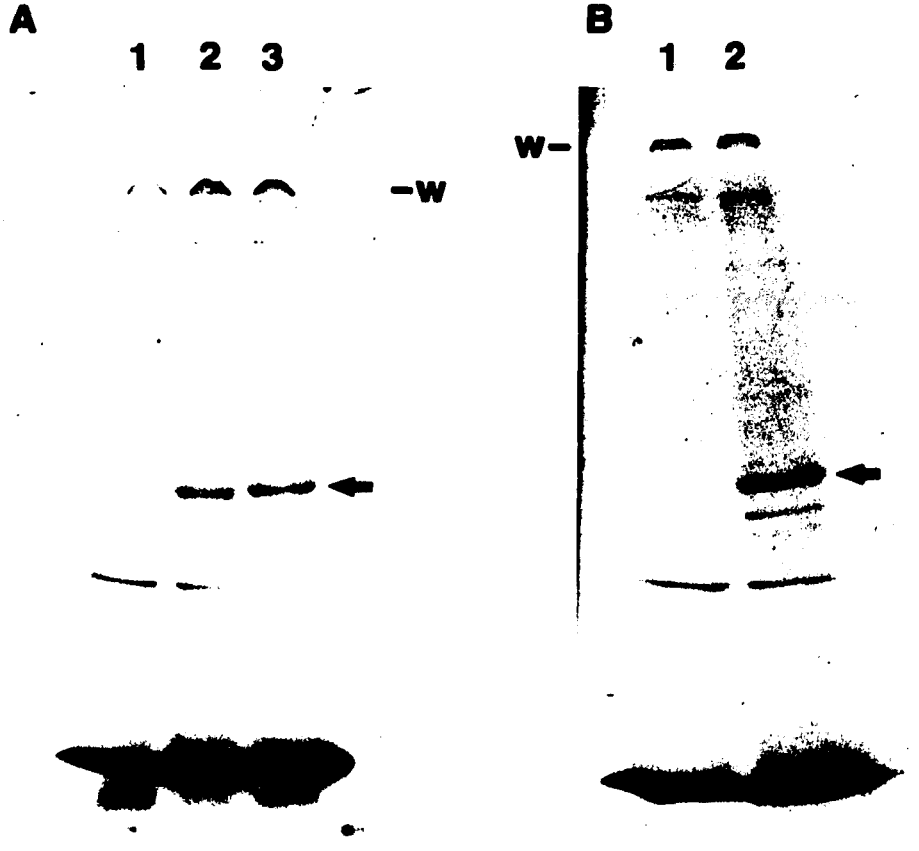

Figure 23. SDS-polyacrylamide gels of products of in vitro translation of GH2 dsRNA in the rabbit reticulocyte system. Lanes: A) RNA was denatured by heating at 100 C for four minutes. 1, GH2 dsRNA; 2, globin mRNA; 3, GH2 dsRNA and globin mRNA. B) RNA was denatured by heating for 45 minutes at 50 C in 90% DMSO. 1, GH2 dsRNA; 2, globin mRNA. Arrows indicate position of the globin band.

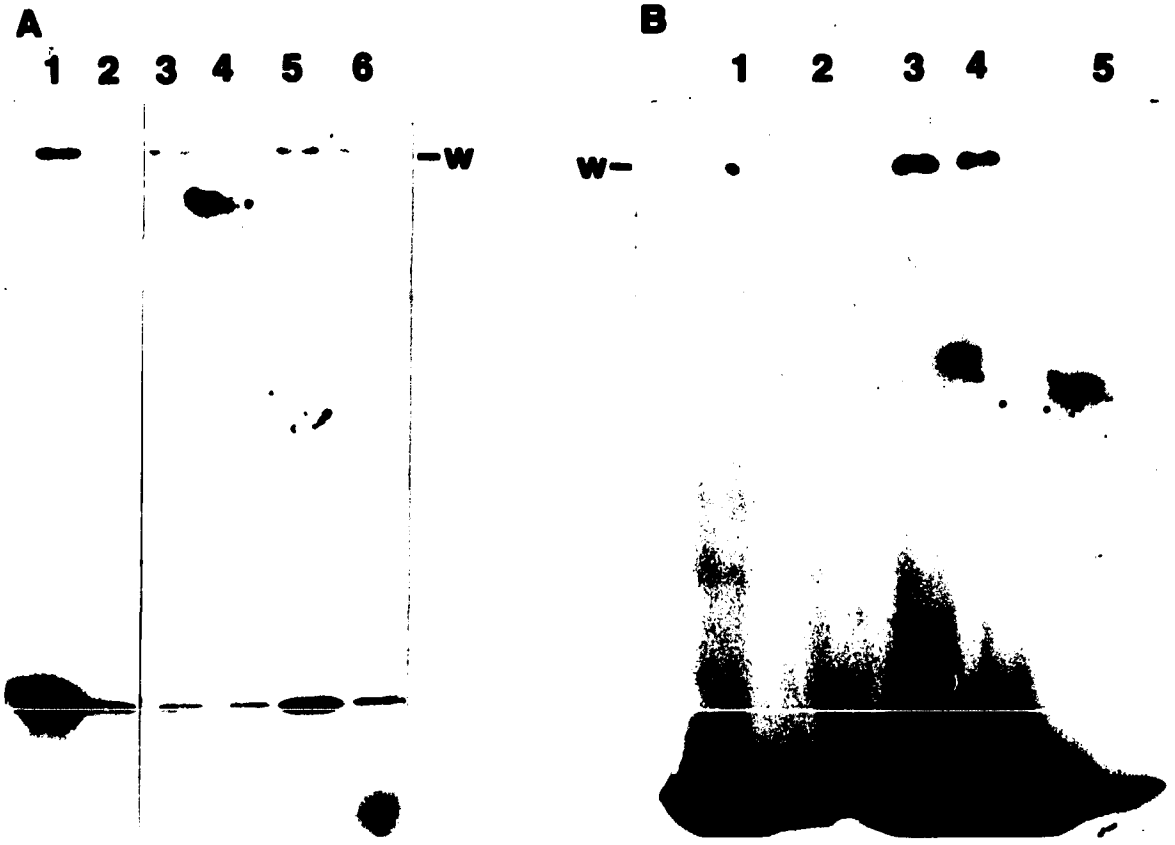

Figure 24. SDS-polyacrylamide gels of products of <u>in vitro</u> translation of GH2 dsRNA in the wheat germ system. Lanes: A) Protein was collected by precipitation with acetone. 1, globin mRNA; 2, no RNA; 3, heat denatured GH2 dsRNA; 4, DMSO denatured GH2 dsRNA; 5, heat denatured globin mRNA and GH2 dsRNA; 6, DMSO denatured globin mRNA and GH2 dsRNA. B) Protein was collected by precipitation with TCA, one microliter 520 μM methionine was added to each reaction. 1, globin mRNA and GH2 dsRNA; 2, GH2 dsRNA; 3, 3X concentration globin mRNA; 4, globin mRNA; 5, no RNA.

in the endogenous band occurred when RNA was denatured with DMSO as well as by heat treatment, but the increase was not as great when DMSO was present (Figure 24). DMSO may have an inhibitory effect on this system.

Although no dsRNA-specific proteins were identified in this study, this may be due to technical problems rather than a lack of coding capacity by the dsRNA. The dsRNA used in these experiments was not degraded based on nondenaturing gels, but single-stranded nicks in the RNA would result in an unfit template for translation. Results with the wheat germ kit were unsatisfactory; globin mRNA was not translated and the background of endogenous bands was high.

It is not known whether GH2 dsRNA contains any open reading frames. Only the small GH2 segment has been completely cloned and sequenced and it lacks open reading frames (D.L. Nuss, personal communication). The small segment of GH2 does not share homology with the two major larger segments (85), but satellite RNAs often do not encode proteins and rely on other segments for their replication (38). However, a dsRNA segment from a European isolate (EP713) contains two open reading frames (D.L. Nuss, personal communication). Caution must be used in extrapolating between these isolates or between segments in the GH2 isolate since European and North American dsRNA do not share homology (Chapter 2, 66). The GH2 and EP713 isolates have been tested for homology and they did not hybridize (D.L. Nuss, personal communication). Cloning and sequencing of the GH2 large segments would indicate whether this line of research should be pursued with dsRNA from this isolate.

Available evidence suggests that the dsRNA of E. parasitica acts in precise ways to cause specific changes in phenotype. This could be accomplished through a dsRNA-encoded protein or through an interaction of the dsRNA itself with cellular components.

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