Molecular Cloning of Hypovirulence-associated Double-stranded RNA in Endothia parasitica and Detection of Sequence-related Single-stranded RNA

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SUMMARY

Recombinant plasmids containing cDNA copies of the dsRNAs present in one hypovirulent strain of Endothia (Cryphonectria) parasitica, EP713, were constructed and analysed by restriction endonuclease mapping and Southern hybridization. Overlapping inserts of four plasmids were found to represent most of the large dsRNA (L-dsRNA) sequences. Inserts which represented the two termini of the L-dsRNAs, designated ‘homopolymer’ and ‘heteropolymer’, were identified. These plasmids were used as probes in Northern hybridization experiments in an attempt to detect other RNAs having sequences related to those of the L-dsRNAs. No additional RNAs were detected that hybridized to plasmids representing the middle region of L-dsRNAs. However, plasmids representing the termini of L-dsRNAs hybridized to several RNAs in EP713 ranging in size from 300 to 1300 nucleotides. These RNAs were absent from EP155, the isogenic virulent strain of E. parasitica. RNAs related to the homopolymer terminus were more abundant than those related to the heteropolymer terminus. All were sensitive to digestion by S1 nuclease but resistant to RNase III, indicating that they were single-stranded. Only those ssRNAs related to the homopolymer terminus of L-dsRNAs were retained on oligo(dT)-cellulose. Single-stranded M13 phage DNAs containing the insert of one of the plasmids, in each orientation, were used as probes in Northern hybridization experiments. Hybridization to the ssRNAs was observed with only one of these probes, indicating that the transcripts are derived from only one strand of the L-dsRNAs. These results establish the existence of a set of poly(A)-containing ssRNAs that are Y-coterminal with the homopolymer terminus of L-dsRNAs and have the same polarity as the poly(A)-containing strand of L-dsRNAs.

INTRODUCTION

Endothia (Cryphonectria) parasitica, the chestnut blight fungus, has virtually eliminated the American chestnut (Castanea dentata) from its natural range in eastern hardwood forests (Anagnostakis, 1982). The chestnut still survives, however, as sprouts from the roots of diseased trees. The European chestnut C. sativa, which suffered from a similar blight epidemic, appears to have recovered as a result of the appearance and dissemination of hypovirulent (H) strains of the fungus (Biraghi, 1953; Grente & Sauret, 1969a, b). Hypovirulence is cytoplasmically inherited, and transmission of cytoplasmic determinants from H strains to compatible virulent (V) strains following hyphal anastomosis is responsible for conversion of V strains to H forms. Present evidence indicates that dsRNAs are the cytoplasmic determinants of hypovirulence (Day & Dodds, 1979). The dsRNAs have been presumed to be of viral origin because the vast majority of mycoviruses have dsRNA genomes (Ghabrial, 1980). However, no virus particles...
typical of dsRNA mycoviruses (Day & Dodds, 1979) have been isolated to date. Day & Dodds (1979) reported the association of dsRNA with spherical or club-shaped membranous particles which were referred to as virus-like particles (VLPs). Chemical and physicochemical analyses of these VLPs did not support assignment as capsids but suggested that the naked dsRNAs are packaged within vesicles formed by the fungal host (Hansen et al., 1985). Analysis of hypovirulence-associated large dsRNAs (L-dsRNAs) of European and American origin (strains EP713 and GH2, respectively) has revealed that one terminus in each case consists of a poly(A): poly(U) tract of variable length, referred to as the 'homopolymer terminus' (Hiremath et al., 1986; Tartaglia et al., 1986). The other terminus, referred to as the 'heteropolymer terminus', has an identical sequence in all five L-dsRNA segments of EP713 (Hiremath et al., 1986). Likewise the L- and medium (M) dsRNA segments in GH2 have identical sequences at the heteropolymer terminus (Tartaglia et al., 1986). Appearance of homologous terminal sequences at the respective ends of dsRNA segments is reminiscent of ss- and dsRNA viruses with segmented genomes (Nuss & Summers, 1984; Szekeres et al., 1985; Stoeckle et al., 1987).

Thus, despite its lack of capsid and its unusual packaging in vesicles, the dsRNA in E. parasitica has many features in common with known virus genomes. The recent demonstration of RNA polymerase activity in dsRNA-containing vesicles further supports the concept of a viral origin for dsRNA (Hansen et al., 1985). It is possible that these vesicles function as transcriptional units and/or sites for dsRNA replication. However, before it can be determined whether the polymerase is a transcriptase or a replicase, or both, the characteristics of the polymerase activity need to be examined in detail and its reaction products analysed.

In this report, we provide evidence for the presence, in vivo, of non-complementary ssRNA species with sequences corresponding to portions of the L-dsRNAs of EP713 which may be the products of such a polymerase.

**METHODS**

The strains of E. parasitica, isolation of nucleic acids, labelling of dsRNA at the 3' terminus, and Northern hybridization protocol have been described previously (Hiremath et al., 1986).

**Construction of recombinant plasmids.** A cDNA library of EP713 L-dsRNAs was constructed as described for the dsRNA genome of wound tumour virus (Asamizu et al., 1985). Transformants were screened using either 3' (Asamizu et al., 1985) or 5' (Hiremath et al., 1986) end-labelled fragments of EP713 L-dsRNA.

**Southern analysis.** The procedure was identical to that of the Northern hybridization experiments except that DNA samples were separated by electrophoresis in 1% agarose gels and transferred to nitrocellulose paper (Southern, 1975).

**Oligodeoxyribonucleotides.** Two oligodeoxyribonucleotides, designated a and b, were synthesized using either an Applied Biosystems Model 380A synthesizer or manually by the triester procedure (McBride & Caruthers, 1983). Oligonucleotide a (5'-TCTTATGGTGATCTACATAGTGAGCTC-3') had a sequence complementary to the denatured by heating at 100 °C for 10 min, in the presence or absence of a 100-fold molar excess of oligonucleotide (5'-CCCACCGTACGCACGG-3') was complementary to residues 134 to 149 of the insert from pEP9 (Hiremath et al., 1986). For use as probes, oligonucleotides were labelled at the 5' terminus using [γ-32P]ATP and T4 polynucleotide kinase (Maniatis et al., 1982).

**RNase H analysis.** L-dsRNAs were labelled at the 3' termini and purified as described previously (Hiremath et al., 1986). They were suspended in 10 μl of buffer (20 mM-HEPES pH 7.0, 100 mM-KCl, 1 mM-EDTA) and denatured by heating at 100 °C for 10 min, in the presence or absence of a 100-fold molar excess of oligonucleotide b. The reaction mixture was cooled gradually to room temperature. To this, 5 μl of a solution containing 100 mM-KCl, 3 mM-MgCl2, 3 mM-dithiothreitol (DTT), 150 μg/ml bovine serum albumin, 60 mM-Tris-HCl pH 7.5, was added, followed by either 1 unit of RNase H or 10 units of RNase T1. The reaction mixtures were incubated for 1 h at 37 °C and analysed on 20% polyacrylamide sequencing gels (Donis-Keller et al., 1977).

**S1 nuclease and RNase III analysis.** Twenty μg of total nucleic acid, 10 μg total RNA or 3 μg of purified dsRNA were treated either with 300 units of S1 nuclease (Pharmacia) in a 10 μl reaction containing 0.5 mM-NaCl, 50 mM-sodium acetate pH 4.5, 1 mM-ZnSO4 and 0.5%-glycerol or with Escherichia coli RNase III (kindly provided by Dr Hugh Robertson, Rockefeller University, New York, N.Y., U.S.A.) in a 10 μl reaction containing 20 mM-Tris-HCl pH 7.9, 1 mM-DTT, 5 mM-MgCl2, 75 mM-KCl, 0.5 mM-EDTA and 1.5 units of RNase III; incubations were at 37 °C for 1 h.

**Preparation of DNA probes.** The plasmid DNAs were labelled by nick translation using [α-32P]dCTP (Maniatis et al., 1982). Single-stranded M13mp8 phage DNAs containing cDNA inserts were labelled and used as described by Hu & Messing (1982).
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Oligo(dT)-cellulose chromatography. Total RNA from EP713 was prepared from total nucleic acid by precipitation with 4 M-LiCl (Diaz-Ruiz & Kaper, 1978) and subjected to oligo(dT)-cellulose chromatography as described by Aviv & Leder (1972).

**RESULTS**

**Characterization of recombinant plasmids**

Preliminary Northern analyses of EP713 RNA using pEP9, a previously described recombinant plasmid (Hiremath *et al.*, 1986), showed the presence of several RNAs, tentatively identified as being single-stranded, which contained L-dsRNA-related sequences ranging from 300 to 1300 nt (Hiremath *et al.*, 1985). From their size and hybridization properties, it was clear that these were partial length copies of the L-dsRNAs in the region represented by pEP9. To identify ssRNAs corresponding to other portions of L-dsRNAs, it was necessary to construct additional recombinant probes. A mixture of all the L-dsRNAs was used as template to obtain recombinant plasmids. Plasmids with L-dsRNA-related inserts ranging from 100 bp to 3.5 kbp were isolated. Four plasmids, pEP15-6, pEP1-9, pEP1-28 and pEPI-16, which contained inserts ranging from 2.5 kbp to 3.5 kbp, were selected for further analysis. Also included in these analyses was the plasmid pEP9, which contains a 194 nt insert (Hiremath *et al.*, 1986). Fig. 1(a) shows the electrophoretic pattern of the plasmids on a 1% agarose gel. Each of the recombinant plasmids contained at least one PstI site within the insert; the sizes of the PstI fragments are given in the legend to Fig. 1. In the blots, all of the inserts hybridized to the 5' end-labelled L-dsRNA fragments (data not shown) confirming that they contained L-dsRNA-related sequences.

In Southern hybridization experiments using each of the plasmids as a probe against each of the others (Fig. 1b to f), each probe hybridized to the linear form of pBR322 (4.35 kbp) and to the homologous insert fragments (e.g. in Fig. 1b, the pEP15-6 probe hybridized to the 1.95 and 0.55 kbp fragments of pEP15-6). In addition, however, each probe hybridized to certain fragments of other plasmids. In particular, plasmid pEP15-6 hybridized to the 1.85 and 0.65 kbp fragments of pEP1-9 (Fig. 1b). Plasmid pEP1-9 hybridized to the 1.95 and 0.55 kbp fragments of pEP15-6 as well as the 2.75 kbp fragment (but not the 0.74 kbp fragment) of pEP1-28 (Fig. 1c). Plasmid pEP1-28 hybridized to the 1.8 kbp (but not the 0.65 kbp) fragment of pEP1-9 as well as the 2.3 and 0.69 kbp fragments of pEP1-16 (Fig. 1d). pEP1-16 hybridized to the 2.75 and 0.74 kbp fragments of pEP1-28 as well as the 194 bp fragment of pEP9 (Fig. 1e). Finally, pEP9 hybridized to the 2.3 kbp (but not the 0.69 kbp) fragment of pEP1-16 (Fig. 1f). Based on these results a map showing the relative positions of these plasmids was constructed (Fig. 2).

In order to provide more detailed alignment of the inserts, restriction mapping was performed using the enzymes indicated in Fig. 2. All the regions of overlap were located except for that between plasmids pEP1-9 and pEP1-28, for which no appropriate restriction site has yet been found. Nonetheless, these two inserts presumably have an overlap of 500 bp or less, since the HindIII site situated approximately 500 bp from one end of the 2.75 kbp fragment of pEP1-28 was not present in pEP1-9.

To examine whether any of these plasmids had sequences specific to a particular L-dsRNA component, the L-dsRNAs were resolved on a 5% polyacrylamide gel and subjected to Northern hybridization analysis using each plasmid as a probe (Fig. 3). The results showed that all the probes hybridized equally to each of the L-dsRNA components, indicating that all plasmids have sequences common to all L-dsRNAs.

**Identification of plasmids representing the termini of dsRNAs**

The recombinant plasmids shown in Fig. 2 span over 8000 nt. This is within the range of 7 to 10 kbp estimated for the L-dsRNAs of EP713 (Dodds, 1980a, b; Hansen *et al.*, 1985) and suggested that pEP9 and pEP15-6 may contain the two termini of the L-dsRNAs. To test this, an oligonucleotide complementary to the heteroplymer terminus of the L-dsRNAs (Hiremath *et al.*, 1986; oligonucleotide a) was synthesized and used as a probe in Southern hybridization experiments similar to those described in Fig. 1(b to f). The probe hybridized only to the
Fig. 1. Characterization of recombinant plasmids. The plasmids pEP15-6 (lanes 1), pEP1-9 (lanes 2), pEP1-28 (lanes 3) and pEP1-16 (lanes 4) were digested with PstI and plasmid pEP9 (lanes 5) was digested with HindIII. The products were subjected to electrophoresis in a 1% agarose gel and visualized with ethidium bromide (a). The calculated sizes of the various insert fragments were: pEP15-6, 1.95 kbp and 0.55 kbp; pEP1-9, 1.82 and 0.65 kbp; pEP1-28, 2.75 and 0.74 kbp; pEP1-16, 2.30 and 0.69 kbp. The nucleic acids resolved in an agarose gel as shown in (a) were transferred to nitrocellulose paper and subjected to hybridization using the following labelled plasmids as probes: (b) pEP15-6; (c) pEP1-9; (d) pEP1-28; (e) pEP1-16; (f) pEP9. The mobilities and the sizes (kbp) of DNA size markers are indicated on the left of each panel.
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Fig. 2. Restriction map of the recombinant probes. Based on the Southern analyses (Fig. 1), a map showing the relative positions of the probes was constructed. The overlap of pEP1-9 and pEP1-28 is not known accurately but is 500 bp or less. Restriction enzyme sites are *ApaI*, A; *BamHI*, B; *EcoRI*, E; *HindIII*, H; *Ncol*, N; *PstI*, P; *SmaI*, Sm; *SstI*, Ss; *XhoI*, X.

Fig. 3. Northern analysis of L-dsRNAs using each plasmid as a probe. Three µg of L-dsRNA was subjected to 5% PAGE for 40 h at 17 mA, transferred to diazophenylthioether paper and hybridized to the labelled plasmids: pEP15-6, lane 2; pEP1-9, lane 3; pEP1-28, lane 4; pEP1-16, lane 5; pEP9, lane 6. The ethidium bromide-stained pattern of L-dsRNAs resolved under the same conditions is shown in lane 1.

1.95 kbp *PstI* fragment of pEP15-6 (Fig. 4a). Similar analyses, in which *BamHI* and *SmaI* were used instead of *PstI*, demonstrated that the site of oligonucleotide hybridization was at the end of the 1.95 kbp *PstI* fragment which was distal to the internal *PstI* site (data not shown).

A different strategy was employed to test for a recombinant plasmid containing sequences from the homopolymer terminus of the L-dsRNAs. Since the L-dsRNAs of EP713 contain homopolymer tails of varying lengths (Hiremath *et al.*, 1986), direct RNA sequencing could not be used to determine the sequences adjacent to the homopolymer tails. Instead, the hypothesis was made that the poly(dA):poly(dT) tract observed in pEP9 was at least partially derived from the poly(A):poly(U) homopolymer terminus of the L-dsRNA, which had been determined by direct RNA sequencing. This hypothesis is supported by the terminal location of pEP9 deduced from Southern blotting (Fig. 1f, Fig. 2) and was confirmed by the following experiment. An oligonucleotide complementary to the region 14 to 29 nt upstream from the poly(A) tail in pEP9 (oligonucleotide *b*) was used in combination with RNase H to cleave 3'-labelled L-dsRNAs, and the products were analysed by PAGE (Fig. 4b). Hybrids not treated with the enzyme remained
Fig. 4. Identification of plasmids representing the termini of the dsRNAs. (a) Heteropolymer terminus. Blots (as in Fig. 1) were hybridized with 5'-terminal-labelled oligonucleotide a. The plasmids used in the blots are lane 1, pEP15-6; lane 2, pEP1-9; lane 3, pEP1-28; lane 4, pEP1-16; lane 5, pEP9. (b) Homopolymer terminus. L-dsRNAs were labelled at their 3' termini, purified, denatured and annealed in the presence (lane 2 and lane 3) or absence (lane 4) of a 100-fold molar excess of oligonucleotide b. Aliquots were analysed on 20% polyacrylamide sequencing gels directly (lane 1), after digestion with RNase T1 (lane 2), or after digestion with RNase H in the presence (lane 3) or absence (lane 4) of oligonucleotide b. The gel was subjected to autoradiography. The mobilities and the sizes (a, kbp; b, nt) of the markers are indicated on the left.

at the top of the gel whereas those treated with RNase T1 produced a band corresponding to a dinucleotide and a diffuse band of about 35 nt. These correspond to the heteropolymer and the homopolymer termini of the dsRNAs, respectively (Hiremath et al., 1986). RNase H treatment of the hybrids yielded a diffuse band at about 49 nt (lane 3), which is the size predicted if cleavage occurred 14 nt upstream of the poly(A) tail of the L-dsRNAs. (The label at the top of the gel in this lane corresponds to the 3'-labelled heteropolymer terminus of the dsRNAs, which would not be cleaved by this treatment.) The band of approximately 49 nt was not observed when the oligonucleotide was omitted (lane 4). These results confirm that pEP9 represents the homopolymer terminus of the L-dsRNAs and suggest that at least some of the poly(dA) tract of pEP9 originated by reverse transcription of the natural homopolymer tail of the dsRNA, as opposed to enzymic addition.

In vivo transcripts of dsRNA

The five recombinant plasmids were labelled and used as probes in Northern hybridization experiments to examine nucleic acid preparations from EP713 and EP155 (Fig. 5). No probe hybridized to any nucleic acid present in total nucleic acid from the virulent strain EP155 (lanes...
Fig. 5. Single-stranded RNAs with sequence relationships to the L-dsRNAs of EP713. Total nucleic acid (20 µg) from EP713 (lanes 2 and 3) was either not treated (lanes 1 and 2) or treated (lanes 3 and 4) with S1 nuclease. These and 20 µg samples of total nucleic acid from EP155 (lane 1) and 3 µg of EP713 dsRNA treated with S1 nuclease (lane 4) were electrophoresed for 4.5 h in 4% polyacrylamide gels, transferred to diazophenylthioether paper and hybridized to pEP15-6, (a); pEP1-9, (b); pEP1-28, (c); pEP1-16, (d); pEP9, (e). The sizes of the ssRNAs were calculated from the mobilities of ssRNA markers (RNA ladder, Bethesda Research Laboratories) which were electrophoresed on the same gels (not shown). Band a is L-dsRNA; bands b, c, d, x and y are ssRNAs; bands S1, S2 and S3 are S-dsRNAs.
Fig. 6. Northern blot hybridization using 5% polyacrylamide gels. Total nucleic acid (20 µg) (lanes 1) and 0.5 µg EP713 dsRNA treated with S1 nuclease (lanes 2) were subjected to electrophoresis in 5% polyacrylamide gels for 3.5 h (a) or 22 h (b). Transfer and hybridization to pEP9, labelled by nick translation were as described in the legend to Fig. 5. The symbols a and S1 to $3 refer to the L-dsRNAs (7 to 10 kbp) and S-dsRNA (500 to 750 bp), respectively. The symbols b, c and d refer to various ssRNAs (1-0 to 1.3 kb).

1), whereas all probes hybridized to L-dsRNAs of the hypovirulent strain EP713 (band a in lanes 2, 3 and 4). Plasmids pEP9 and pEP1-16 also hybridized to small dsRNAs (S-dsRNAs) reported earlier (Hiremath et al., 1986; designated S1 to S3 in Fig. 5).

An unexpected finding was that, of the five plasmids, three hybridized to RNA species other than L- and S-dsRNAs. Plasmid pEP15-6, which contains sequences present at the heteropolymer terminus of the L-dsRNAs, hybridized to two RNA species of 1000 and 800 nt (Fig. 5a, bands x and y). These RNA species were less abundant than the L-dsRNAs. Digestion with the ss-specific nuclease S1 removed the 1000 and 800 nt bands but did not affect the L-dsRNAs (lane 3), indicating that the former are ssRNAs.

A different pattern of RNA species was observed when pEP9 and pEP1-16 were used as hybridization probes. These RNAs were more abundant than those detected with pEP15-6 as probe and ranged in size from 300 to 1300 nt (bands b, c, d, etc., in Fig. 5d, e; lanes 2). Digestion with nuclease S1 demonstrated that they were ssRNAs, whereas the S- and L-dsRNAs were stable to this treatment (Fig. 5d, e; lanes 3). When oligonucleotide b was used as probe, a hybridization pattern identical to those obtained with pEP9 and pEP1-16 was observed (data not shown), indicating that all of these ssRNAs contain the homopolymer terminus. Some of these bands represented more than one RNA species of similar size, as shown by longer electrophoresis on 5% polyacrylamide gels (e.g., Fig. 6, bands b and c).

It was possible that the RNAs represented by bands b to d in Fig. 5 and 6 were actually poly(A)-containing fungal mRNAs and that hybridization was due solely to the poly(dT) tract.
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Fig. 7. Oligo(dT)-cellulose chromatography of ssRNAs from EP713. Unfractionated RNA (lanes 1, 2) and oligo(dT)-cellulose-bound RNA (lanes 3, 4) were subjected to electrophoresis in a 3% polyacrylamide gel followed by Northern hybridization with pEP9 (a) and pEP15-6 (b) as in the legend to Fig. 5. The samples were either untreated (lanes 1 and 3) or treated with S1 nuclease (lanes 2 and 4) before electrophoresis. The amounts of nucleic acids used were (a) 20 μg in lanes 1 and 2, 0.25 μg in lane 3 and 1.5 μg in lane 4, (b) 40 μg in lanes 1 and 2, 1.5 μg in lane 3 and 0.5 μg in lane 4.

Present in pEP9 and, presumably, pEP1-16. In order to rule this out, pEP9 was cut with *PstI* to generate a new probe consisting of a fragment of pBR322 containing 58 nt of the insert (residues 1 to 64; Hiremath *et al.*, 1986) but lacking the poly(dA):poly(dT) region. Hybridization with this probe produced the same pattern as with intact pEP9 (data not shown). This, together with the hybridization pattern obtained with oligonucleotide b, cited above, indicates that RNAs b to d contain dsRNA-related heteropolymer sequences and are not fungal mRNAs. Also, lack of hybridization with total nucleic acid from EP155 (Fig. 5, lanes 1) supports this argument.

Since the PAGE used in these experiments did not resolve ssRNAs of large size, it was not possible to know whether any ssRNA corresponding to full-length transcripts of the L-dsRNAs was present in EP713. Therefore total nucleic acid and total RNA were subjected to electrophoresis on 1% agarose gels (20 cm long), and the nucleic acids were transferred to nitrocellulose and hybridized to labelled pEP15-6 and pEP9. The results indicated that none of the RNAs hybridizing to the probes was larger than approximately 1300 nt (data not shown).
Fig. 8. Polarity of the ssRNAs. Total nucleic acid (20 μg; lanes 1) and 0.5 μg EP713 dsRNA treated with S1 nuclease (lanes 2) were subjected to electrophoresis in 3% polyacrylamide gels for 3.5 h. Transfer and hybridization were as described in the legend to Fig. 5. The probes used were S15 (a) and S2 (b), which are M13 phage ssDNAs containing the insert from pEP9 in two different orientations.

Fig. 9. Resistance of ssRNAs to RNase III. Ten μg of total RNA (a) and 0.5 μg of oligo(dT)-cellulose-bound RNA (b) were subjected to electrophoresis for 7 h in 3% polyacrylamide gel either directly (lanes 1) or after treatment with 1.5 units of Escherichia coli RNase III (lanes 2). Transfer and hybridization to pEP9 were as described in the legend to Fig. 5.

To determine whether the ssRNAs contained poly(A), their ability to bind to oligo(dT)-cellulose was examined. Approximately 1.5% of total RNA, obtained from total nucleic acid, bound to the column. Both the bound and unbound RNAs were recovered and analysed by Northern hybridization using pEP9 or pEP15-6 as probes. All of the ssRNA species that hybridized to pEP9 were enriched by oligo(dT)-cellulose chromatography (Fig. 7a, lane 3). Comparison of hybridization signals from total RNA (10 μg) and oligo(dT)-cellulose-bound
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RNA (0.25 μg) indicated that the pEP9-related RNAs were quantitatively bound to the column (compare Fig. 7a lane 1 and lane 3). Additional evidence that the ssRNAs were quantitatively bound is that no hybridization was observed when 10 μg of RNA that did not bind to oligo(dT)-cellulose was probed with pEP9 (data not shown). That these were ssRNAs was further confirmed by their sensitivity to S1 nuclease (compare Fig. 7a lanes 2 and 4 with lanes 1 and 3). The band in lanes 2 and 4 having the mobility similar to that of band d is due to the S-dsRNAs S1 and S2. On 3% gels these RNAs have similar mobilities but can be resolved on 4 or 5% gels (see Fig. 5 and 6). By contrast to the results with pEP9, none of the ssRNAs hybridizing to pEP15-6 bound to oligo(dT)-cellulose (compare Fig. 7b lane 1 with lane 3).

These results indicated that the RNA species designated b, c and d plus a number of smaller RNAs (Fig. 5, 6, 7) were single-stranded, collinear with the homopolymer terminus of the L-dsRNAs, and contained a poly(A) tract. To determine the polarity of the RNAs, M13mp8 ssDNAs containing the pEP9 sequence in either orientation were used as probes in Northern hybridization experiments (Fig. 8). Probe S2 (Fig. 8b) had an insert sequence of the same polarity as the poly(dA)-containing strand of pEP9, while probe S15 (Fig. 8a) was of the same polarity as the poly(dT)-containing strand (Hiremath *et al.*, 1986). Both of the probes hybridized to the L-dsRNAs (band a) in dsRNA preparations (lanes 2), but only S15 hybridized to the ssRNA species in total nucleic acid preparations (lanes 1). (The concentration of L-dsRNAs in total nucleic acid was so much lower than that of ssRNAs that proper exposure of band a in lanes 1 would have resulted in overexposure of bands b to d). Thus, in agreement with the binding to oligo(dT)-cellulose, these data indicate that the ssRNAs are 3′-coterminal copies having the same polarity as the poly(A)-containing strand of the L-dsRNAs.

It was possible that the RNA species we have designated as being ssRNAs actually had unusual structures such as a double-stranded form with gaps in one of the strands; such structures would be susceptible to S1 digestion. To test this possibility, the RNAs were treated with RNase III, which specifically digests dsRNA, and the products were analysed by Northern hybridization (Fig. 9). Total RNA and oligo(dT)-cellulose-bound RNA were digested with RNase III in the presence of dsRNAs from *Penicillium chrysogenum* virus used as internal markers to follow the activity of RNase III. The enzyme digested the dsRNAs (data not shown), but RNA that hybridized with pEP9 was unaffected, suggesting that these RNAs are completely single-stranded.

**DISCUSSION**

A set of overlapping cDNA clones of the L-dsRNAs associated with a hypovirulent strain of *E. parasitica*, EP713, has been constructed; the cloned region corresponds to 8850 to 9350 nt. The largest of the L-dsRNA components in EP713, L1, has been reported to be about 10000 nt long (Dodds, 1980a, b). This suggests that the set of probes reported here could represent as much as 94% of L1. However, since total EP713 dsRNA rather than a homogeneous preparation of L1 was used as a template for cDNA synthesis, the library may reflect the heterogeneity of the EP713 L-dsRNAs. Consequently, the map shown in Fig. 2 cannot be considered an accurate restriction map of the cDNA for L1. It does, however, provide an indication of the relatedness of the five cDNA clones and is, therefore, useful for further characterization of the L-dsRNAs.

The results presented here confirm and extend the findings of Hiremath *et al.* (1986) which suggested that the L-dsRNAs are structurally related. By direct RNA sequencing of the termini and hybridization to the probe pEP9, it was shown that all components had identical termini. However, it was not possible in that study to determine whether the sequence homology extended into the internal regions. Northern analyses using the probes described here clearly indicate that the homology among the L-dsRNAs is extensive and not limited to the termini. This suggests that the different components may be produced by internal deletions of a single large dsRNA. This would be similar to the several S-dsRNAs associated with the killer virus of yeast (Fried & Fink, 1978; Kane *et al.*, 1979; Thiele *et al.*, 1984) and the variant dsRNAs found in the transmission-defective isolates of wound tumour virus (Nuss & Summers, 1984; Anzola *et al.*, 1987).
Although a mixture of all L-dsRNA components was used for constructing the probes, none of the probes had sequences unique to a particular L-dsRNA. This is probably because the probes used, except for pEP9, contained large inserts of dsRNA-related sequences (2.5 to 3.5 kbp). Plasmid pEP9 itself contained sequences corresponding to one of the common termini of the L-dsRNAs. If it is possible to obtain probes specific to a particular component it may be necessary to use smaller restriction fragments of these large probes, especially those of pEP1-9, pEP1-28 and pEP1-16.

We have presented several lines of evidence to support the conclusion that ssRNA species containing L-dsRNA-related sequences occur in vivo and are presumably transcription products of dsRNAs. The RNA species that hybridized to our probes were sensitive to S1 nuclease, resistant to RNase III and partitioned with ssRNAs when processed by CF-11 cellulose chromatography. The bulk of the ssRNAs hybridized to pEP9 and were homologous to only one of the dsRNA strands, the one having a 3' poly(A) terminus. In the subsequent discussion we refer to this strand as the (+) strand and the one with sequences complementary to the ssRNAs as the (-) strand. The complexity of these ssRNA transcripts may be due to the multiplicity of the dsRNAs (five L-dsRNAs and three S-RNAs). The ssRNAs that hybridized to pEP15-6 comprised a minor fraction of the total ssRNA population. Because we were unable to construct M13 phage subclones representing pEP15-6, the polarity of the RNAs that hybridize to this clone is not known.

The ssRNAs ranged from 300 to 1300 nt, but none were as long as the L-dsRNAs. Most of these partial transcripts were homologous to the 3' terminus of the (+) strand of the L-dsRNAs and contained poly(A) tails. Thus they resemble eukaryotic mRNAs, but translation of these transcripts in a cell-free system will be required to determine whether they indeed have messenger activity. No major translation products could be detected when denatured dsRNA was used as template in a cell-free system (S. Ghabrial, unpublished data). Conceivably, generation of partial transcripts could result in subgenomic RNA more favourable for initiation of translation.

It is not known how dsRNA determines the H phenotype. Two mechanisms may be proposed. First, the dsRNA may exert its effect directly by inhibiting the binding of host mRNA to ribosomes and thus inhibiting the synthesis of proteins that may be essential for virulence. The second possibility is that the dsRNA acts indirectly through the generation of monocistronic mRNAs. These would be translated into polypeptides which act to weaken or alter virulence. Our detection of partial transcripts favours the second possibility that hypovirulence may be mediated by gene products encoded by the dsRNAs.

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