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# Terminal structure of hypovirulence-associated dsRNAs in the chestnut blight fungus *Endothia parasitica*

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ABSTRACT

The 3'- and 5'-terminal sequences of the five large double-stranded RNA species (L-dsRNA; 4.5-6.0 X $10^6$ daltons) of EP713, a hypovirulent strain of Endothia parasitica, were determined by mobility-shift and enzymatic methods. All the L-dsRNAs appeared to have identical terminal sequences. A heteropolymer sequence was found at one 3'-terminus and a poly(A) sequence of variable length at the other. It was possible to label only one 5'-terminus using polynucleotide kinase and [$\gamma$-$^{32}$P]ATP, and this was shown to be a poly(U) sequence of variable length. We propose that the dsRNAs have the following structure, where X represents a blocking group:

5'-A(U)$_{\overline{20}}$NNN....NNNGAGCUCACUAUGUAGAUCACCCAUAAGA-3'
3'-(A)$_{\overline{35}}$NNN....NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNX-5'

A recombinant plasmid containing dsRNA-related sequences was constructed. Hybridization analysis using the recombinant probe indicated that the sequence homology among the L-dsRNAs extended beyond these terminal regions and was also shared by small dsRNAs (0.3-0.45 X $10^6$ daltons).

## INTRODUCTION

The discovery of hypovirulent (H) strains of the chestnut blight fungus Endothia parasitica has rekindled hopes of controlling the blight and permitting the return of the American chestnut as a major forest species (1). The potential of using the H strains for biological control has been well demonstrated (2). Hypovirulence is cytoplasmically controlled, and evidence strongly suggests that dsRNAs are its primary, if not sole, determinants. These dsRNAs could potentially be of viral origin, since the vast majority of fungal viruses have dsRNA genomes (3). However, no viral particles, typical of those found in other dsRNA-containing fungi, have been detected to date. All H strains contain dsRNA, which occurs in a multiplicity of size classes but usually includes at least one major component of 5-7 X $10^6$ daltons (4).

The French-derived H strain, EP713, was used as the source of dsRNA in the work reported here since its dsRNA is perhaps the best studied of all H agents to date (5-10). This strain was originally produced by converting the American virulent (V) strain, EP155, with the French H strain, EP113 (5).

Electrophoretic examination of the dsRNA pattern has revealed considerable variation in the size and relative abundance of the different dsRNA components (4,5,8,9,11). However, there is always a group of components in the size range of 4.5-5.8 X $10^6$ daltons, referred to as major bands (8,9) or, in the present study, L-dsRNAs. Minor components of smaller size have also been observed (4,9).

In spite of the evidence suggesting that dsRNAs are the cytoplasmic determinants of hypovirulence, very little is known about their structure. The various dsRNA components may represent different viruses, different components of the same virus, or intact viruses together with satellites or defective interfering particles. Information of the structure of and relationships among dsRNA components of a given isolate as well as the homology among dsRNAs of different isolates should be helpful in understanding the biochemical basis of hypovirulence. We describe here structural information concerning the dsRNAs of EP713, determined by direct RNA sequencing and hybridization to a recombinant probe.

## MATERIALS AND METHODS

### Isolation of nucleic acids.

Fungal mycelium was suspended in STE buffer (50 mM Tris-HCl, pH 7.0, 0.1 M NaCl, and 1 mM EDTA) containing 1 mg/ml sodium bentonite and homogenized using a Braun model MSK cell homogenizer. Alternatively, the tissue was ground to a fine powder in presence of liquid nitrogen and then suspended in STE buffer. Total nucleic acid (TNA) was isolated by repeated extraction with phenol-chloroform-isoamyl alcohol followed by precipitaion with ethanol (11). Double-stranded RNA was enriched from TNA by CF11 cellulose chromatography (12). In some cases, L-dsRNA was further purified by subjecting total dsRNA to electrophoresis in either agarose or polyacrylamide gels followed by elution from the gels.

### Labelling of 3'- and 5'-termini of dsRNAs.

The dsRNAs were purified by agarose gel electrophoresis and used directly for labelling at the 3'-termini with [5'-$^{32}$P]pCp as described previously (13). For labelling at the 5'-termini, the purified dsRNAs were first dephosphorylated using calf intestine alkaline phosphatase (14), extracted with phenol:chloroform and precipitated with ethanol. They were then labelled with [$\gamma$-$^{32}$P]ATP using T4-induced polynucleotide kinase (15). The labelled dsRNAs were further purified by electrophoresis on agarose gels. To isolate individual 3'-labelled dsRNA components, labelled dsRNAs were subjected to

electrophoresis in a 5% polyacrylamide gel for 36 h at 17 mamp. The individual components, visualized by staining with ethidium bromide, were excised, electrophoretically eluted, and precipitated with ethanol using 50 µg of carrier tRNA.

RNase T1 analysis.

The 3'-labelled dsRNAs were denatured at 100°C for 10 min. Enzyme digestions were carried out in a reaction mixture (10 µl) containing denatured dsRNA, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 25 µg carrier RNA. Partial digestions were done by adding 0.01 units of RNase T1 (Pharmacia) followed by incubation at 37°C for 1 h. For complete digestion, 10 units of enzyme were added, and incubation was at 37°C for 2 h.

RNA sequencing by mobility-shift analysis.

The labelled dsRNAs were dissolved in 50 mM $NaHCO_3$, pH 9.0, and incubated at 100°C for 15 min. The partial digestion products were subjected to two-dimensional polyacrylamide gel electrophoresis as described previously (14,16,17), except that a 25% polyacrylamide gel was employed in the second dimension, and detected by autoradiography.

RNA sequence analysis by partial enzymatic digestion.

The 3'-labelled dsRNAs were subjected to partial digestion with RNase T1 and the oligonucleotides separated on polyacrylamide gels as described above. Individual oligonucleotides were detected by autoradiography, extracted from acrylamide gel pieces, and precipitated with ethanol using carrier tRNA. Sequencing of these oligonucletides using base-specific nucleases was performed as described previously (17,18). For sequencing at the 5'-terminus, the 5'-labelled dsRNAs were digested with the nucleases without prior isolation of oligonucleotides.

Terminal nucleotide analysis.

5'- and 3'-labelled dsRNAs were denatured as described above. The 3'-labelled RNA was digested with 0.5 units RNase T2 (Pharmacia) at 37°C for 3 h in a reaction mixture (5 µl) containing 20 mM ammonium acetate, pH 4.5. The 5'-labelled RNA was incubated at 37°C for 2 h in a 3 µl reaction mixture containing 30 mM Tris-HCl, pH 7.5, and 10 µg nuclease P1 (Calbiochem-Behring). The terminal residues were identified by chromatography on polyethyleneimine-cellulose sheets developed in 1 M LiCl (19).

Construction of a recombinant plasmid containing dsRNA-related cDNA.

The dsRNA purified by electrophoresis in polyacrylamide gels was denatured by heating and polyadenylated using calf thymus poly(A) polymerase (20) as described previously (21). The polyadenylated dsRNA was isolated by

oligo(dT)-cellulose chromatography (22), denatured at 100°C, and transcribed into cDNA with avian myeloblastosis virus RNA-dependent DNA polymerase using oligo(dT) as primer (21). The second strand was synthesized with E. coli DNA polymerase I, and the product was digested with S1 nuclease to produce blunt ends. HindIII linkers containing eight nucleotide residues (Pharmacia P-L Biochemicals) were then ligated to the cDNA using T4 DNA ligase. After digestion with HindIII, the DNA was ligated into the HindIII site of pBR322 and the recombinant plasmid used to transform E. coli strain DG-75. Ampicillin-resistant colonies were screened by colony hybridization (23) using a probe consisting of partially hydrolyzed L-dsRNA from EP713 which had been end-labelled with $^{32}P$ using T4-induced polynucleotide kinase (24). Plasmid DNA was isolated from positive colonies (25). One recombinant plasmid, termed pEP9, was further characterized by digestion with the restriction endonuclease HindIII. The DNA fragments were separated by electrophoresis on 1% agarose gels and transferred to nitrocellulose (26). Hybridization with $^{32}P$-labelled dsRNA fragments confirmed that the plasmid contained an insert in the HindIII site (data not shown).

M13 cloning and sequencing.

The cDNA insert from pEP9 was excised with HindIII and subcloned into the HindIII site of M13mp18 (27). Single-stranded phage DNA containing the cDNA insert in both orientations was isolated and sequenced by the dideoxy chain termination procedure (28,29).

Northern hybridization.

Double-stranded RNA was subjected to electrophoresis on either 3% or 5% polyacrylamide gels. The nucleic acid was transferred to diazophenylthioether (DPT) paper (Bio-Rad), essentially as described previously (30) except that the transfer was done electrophoretically for 12-16 h at 20 V in Bio-Rad Trans-blot apparatus in 25 mM sodium phosphate buffer, pH 5.5. Tne transferred nucleic acid was subjected to hybridization (21) using as probe pEP9 labelled with $^{32}P$ by nick translation (31).

## RESULTS

### Labelling of L-dsRNAs at the 3'-terminus and isolation of individual dsRNAs.

The resolution of the L-dsRNA components of EP713 into characteristic patterns depended on the conditions used for electrophoresis. On 3% polyacrylamide gels run for a short time, the L-dsRNAs did not resolve well and could be seen as a doublet (Fig. 1A). When electrophoresis was performed on 5% gels, they resolved into a characteristic pattern of five components

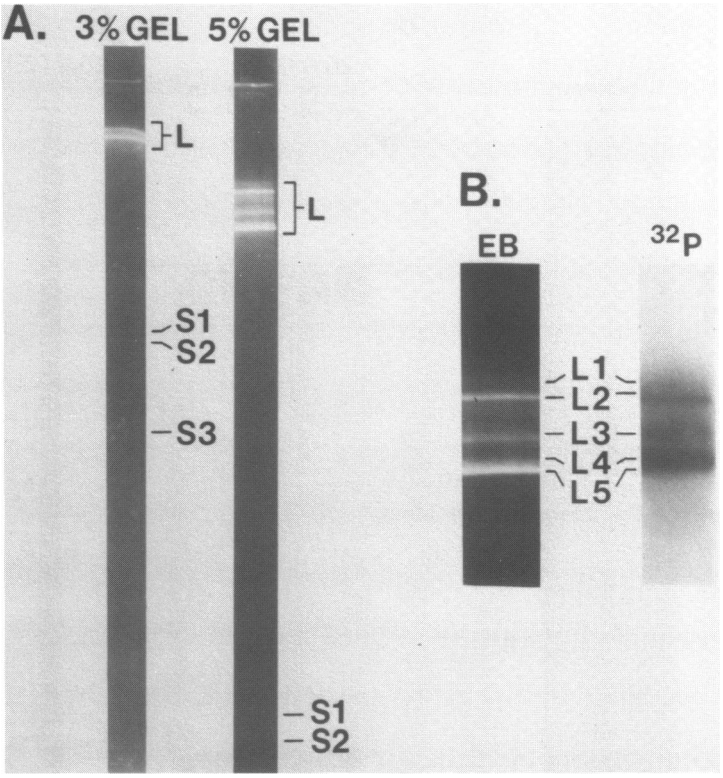



Fig. 1. Labelling L-dsRNAs of EP713 at the 3'-termini and separation of individual dsRNA components. (A) Double-stranded RNA was isolated as described in Materials and Methods and 3 µg were subjected to electrophoresis in either 3% or 5% polyacrylamide gels in 0.1 M Tris-borate, pH 8.3, 2.5 mM EDTA. Electrophoresis was for 3.5 h (3% gel) or 22 h (5% gel) at 100V. The symbols L, S1, S2, and S3 refer to different dsRNA components visualised after staining with ethidium bromide. (B) The L-dsRNAs (L) purified by electrophoresis on 1% agarose gel, were labelled at the 3'-termini as described in Materials and Methods. The labelled dsRNAs were resolved by electrophoresis on 5% polyacrylamide gel as in (A) except that electrophoresis was for 36 h at 17 mamp. The dsRNAs were detected by staining with ethidium bromide (EB) and by subjecting the gel to autoradiography ($^{32}P$). The symbols L1, L2, L3, L4, and L5 refer to different L-dsRNA components of EP713.

(L1-L5), components L2, L3, and L5 being relatively more abundant (Fig. 1A). The molecular weights of these components range from 4.6-6.0 X $10^6$ (11). Three other dsRNAs (S1, S2, and S3) were also detected, two of which migrated at the position of about 0.45 X $10^6$ daltons and one, at 0.3 X $10^6$ daltons. These smaller dsRNAs represented a minor proportion of dsRNA in

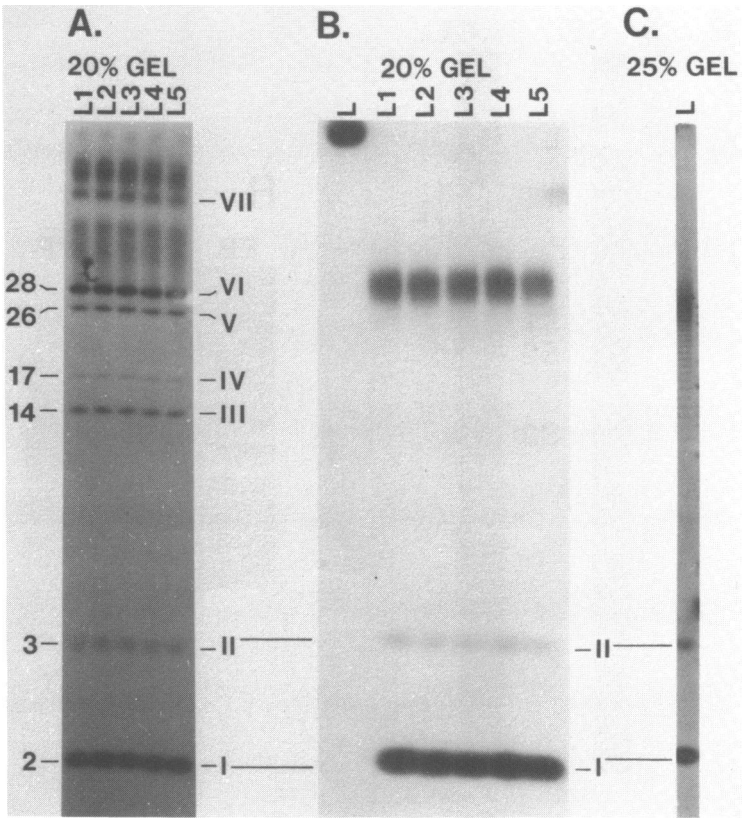



Fig. 2. Analysis of 3'-labelled L-dsRNA components of EP713 by digestion with RNase T1 and polyacrylamide gel electrophoresis. The 3'-labelled dsRNA components L1, L2, L3, L4, and L5 were isolated and subjected to either partial (A) or complete (B and C) digestion with RNase T1. The products were analyzed on either 20% (A and B) or 25% (C) polyacrylamide sequencing gels (18). Lanes marked L contained a mixture of L1-L5, either not treated with the enzyme (B), or treated with the enzyme (C). The symbols I-VII refer to different 3'-terminal oligonucleotides generated by digestion with RNase T1. The sizes of these oligonucleotides are indicated on the left of the figure.

EP713 and could be detected only when the gel was overloaded. All of them were shown to be dsRNAs by their resistance to digestion by S1 nuclease, although the removal of a small number of nucleotide residues from the termini would not have produced a shift in electrophoretic mobility.

Electrophoresis on 1% agarose gels was routinely employed to purify L-dsRNAs collectively from other nucleic acids present in the CF11 cellulose-purified preparations of dsRNAs. Agarose gel-purified L-dsRNAs were

labelled at their 3'-termini and individual labelled components separated by electrophoresis on 5% polyacrylamide gel (Fig. 1B). The dsRNAs were detected by staining with ethidium bromide as well as by autoradiography. The similarity of Fig. 1A "5% Gel" to Fig. 1B "EB" indicates that the agarose gel purification and labelling procedures did not degrade the dsRNA.

RNase T1 analysis.

Each 3'-labelled component of L-dsRNA was subjected to partial and complete digestion by RNase T1. Partial digestion produced seven oligonucleotides (I-VII) varying in size from 2 to about 50 residues (Fig. 2A). A diffuse band was observed in the 35-residue range. The pattern of oligonucleotides was identical for all five components, suggesting that their respective 3'-termini were homologous. Complete digestion (Fig. 2B) resulted in two bands (I and II), corresponding to a dinucleotide and a trinucleotide, respectively, as well as the diffuse band seen in Fig. 2A. The relative intensities of the bands suggested that oligonucleotide I and the diffuse band represented the two 3'-termini of each L-dsRNA component and that oligonucleotide II was a minor constituent.

Better resolution of the diffuse band was achieved by electrophoresis on 25% polyacrylamide gels and indicated that it actually consisted of a set of oligonucleotides with a Guassian size distribution (Fig. 2C). Most of the radioactivity was found in bands corresponding to 31-39 nucleotide residues. A similar set of oligonucleotides in the range of 36-44 nucleotide residues was observed by digestion with RNase B. cereus (data not shown). This suggests heterogeneity of length at one 3'-terminus of all L-dsRNA components.

Mobility-shift analysis.

Since RNase T1 digestion analyses suggested that the individual L-dsRNA components were identical at their 3'-termini, a mixture of L1-L5 was employed for mobility-shift analysis to determine the actual sequence. L-dsRNAs labelled at their 3'-termini were subjected to partial alkaline hydrolysis and the products analyzed by two-dimensional polyacrylamide gel electrophoresis. Two arrays of labelled oligonucleotides were observed, each presumably corresponding to one 3'-terminus (Fig. 3A). When the analysis was performed with only one dsRNA component (L5), an identical pattern was obtained (data not shown). These results further confirm that all the L-dsRNA components share a common sequence at their respective 3'-termini.

The 3'-termini of the two strands were not equally labelled. The more strongly labelled strand contained a heteropolymer sequence. The other strand appeared to consist of only poly(A) for at least 15 residues from the

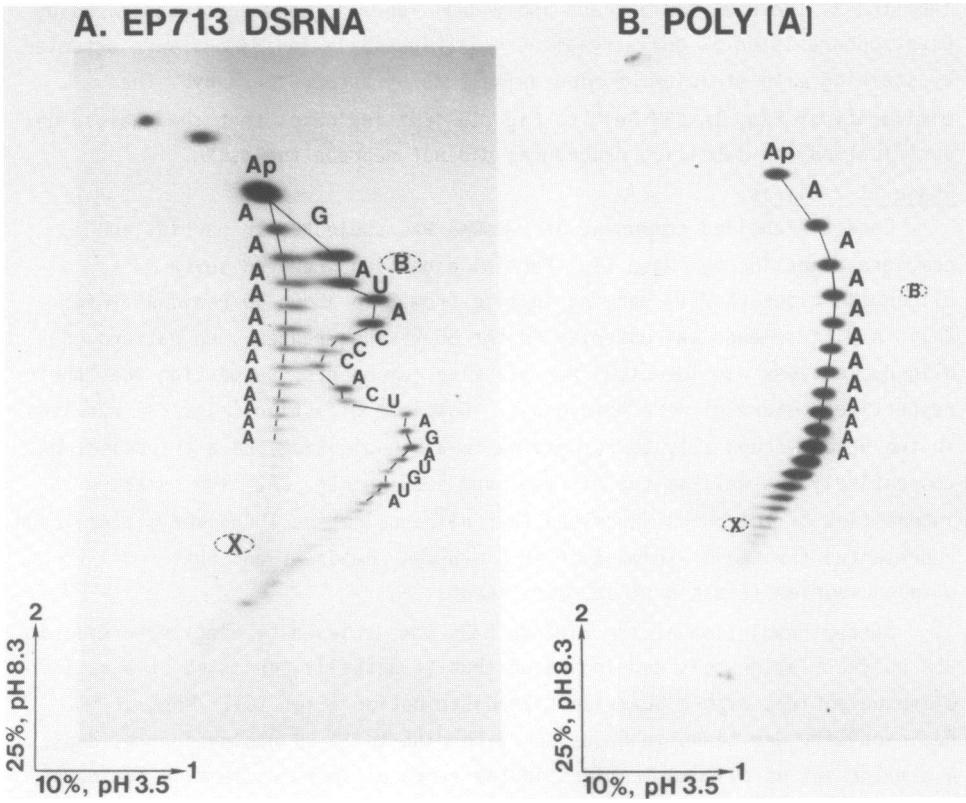



Fig. 3. Mobility-shift analysis of 3'-labelled L-dsRNAs of EP713. (A) L-dsRNAs from EP713 labelled at their 3'-termini were subjected to partial alkaline hydrolysis as described in Materails and Methods. The products were analyzed by two-dimensional polyacrylamide gel electrophoresis followed by autoradiography. Electrophoresis in the first dimension was at pH 3.5 in a 10% gel, while a 25% gel at pH 8.3 was employed for the second dimension. Circled B and X indicate the locations of bromophenol blue and xylene cyanole dyes, respectively. (B) Autoradiogram of the pattern obtained when synthetic poly(A), labelled at the 3'-terminus with [5'-$^{32}$P]pCp, was subjected to analysis as in (A).

3'-terminus. To confirm the identification of poly(A), synthetic poly(A) (Sigma) was labelled with [5'-$^{32}$P]pCp and subjected to mobility-shift analysis (Fig. 3B). The pattern indicated that the homopolymer stretch shown in Fig. 3A was indeed poly(A).

A similar analysis was performed with 5'-labelled L-dsRNAs (Fig. 4A). The same pattern was obtained, irrespective of whether a single component (L5) or a mixture of L1-L5 was used (data not shown). A single major array of

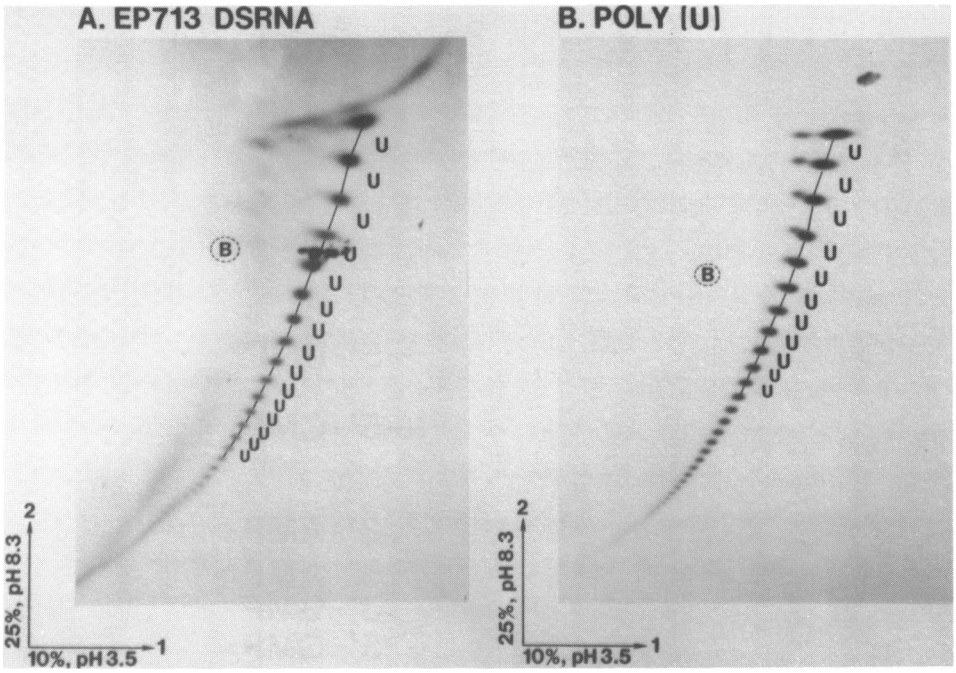



Fig. 4. Mobilty-shift analysis of 5'-labelled L-dsRNAs of EP713. (A) Large dsRNAs of EP713 were labelled at the 5'-terminus using T4-induced polynucleotide kinase and subjected to mobility-shift analysis as in Fig. 3. The pattern obtained by similar analysis on 5'-labelled poly(U) is shown in (B).

labelled oligonucleotides was observed, indicating that only one of the 5'-termini had been labelled under the conditions employed. There was a suggestion of a second strand, labelled to a considerably lower extent, but the pattern of labelled spots was diffuse, indicating a heterogeneous collection of RNA sequences. The major pattern of oligonucleotides obtained suggested a poly(U) sequence at the 5'-terminus. To confirm this, synthetic poly(U) (Sigma) was labelled at the 5'-terminus and subjected to mobility-shift analysis. The pattern obtained was identical to that of 5'-labelled dsRNAs up until approximately 20 residues (Fig. 4B). One reason for the resistance of the other terminus to labelling could be the presence of a recessed 5'-terminus. However, prior digestion with S1 nuclease did not result in labelling of the other 5'-terminus (data not shown). Another possiblity is the presence of a 5'-blocking structure such as a cap or genome-linked protein. Digestion with tobacco acid pyrophosphatase (14) did

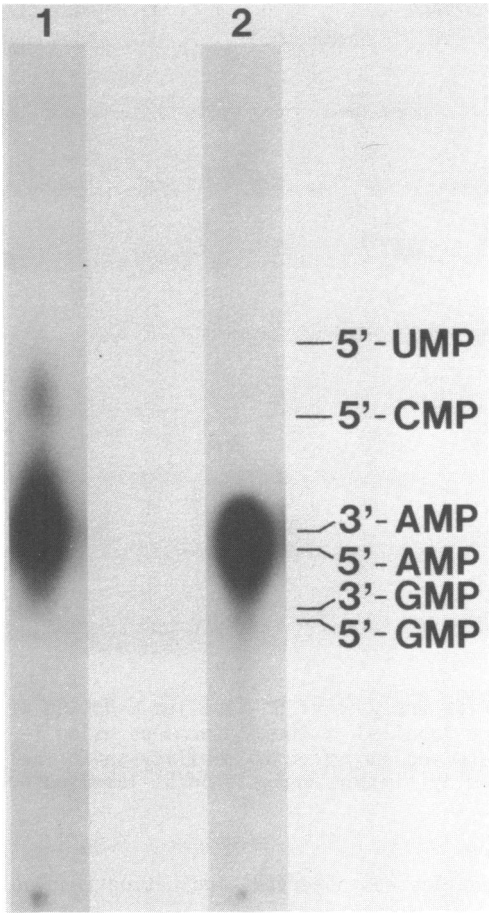



Fig. 5. Terminal nucleotide analysis of L-dsRNAs of EP713. L-dsRNAs labelled at the termini were digested with enzymes and analyzed by thin layer chromatography as described in Materials and Methods. Lane 1, 3'-labelled dsRNAs treated with RNase T2; lane 2, 5'-labelled dsRNAs treated with nuclease P1. Mobilities of the standard 3'- and 5'-mononucleotides are indicated at the right of the figure.

not permit labeling of the second strand, however, arguing against a cap.

Identification of the terminal nucleotides.

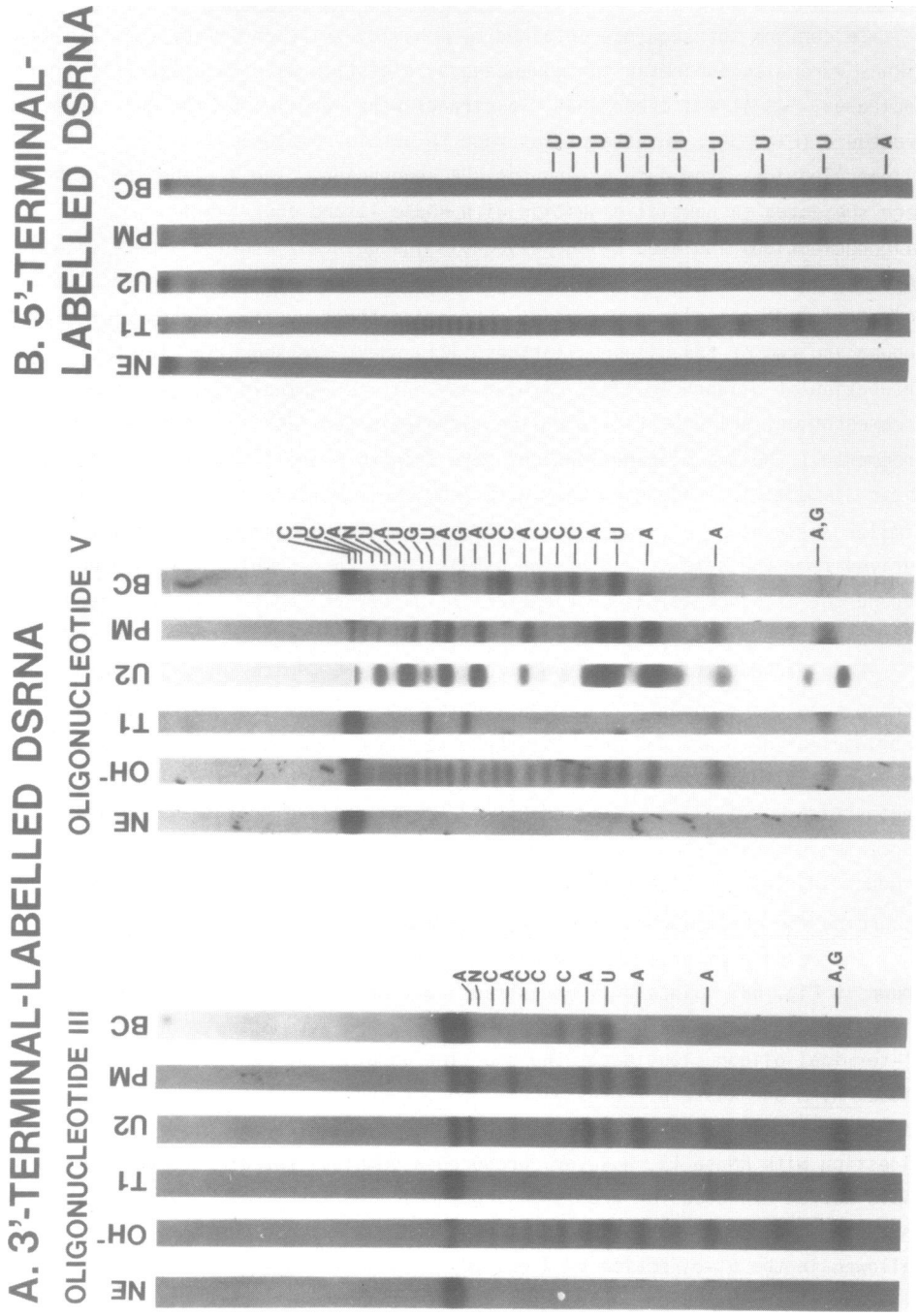
Analysis of RNase T2-treated, 3'-labelled dsRNA by thin layer chromatography revealed the presence of [3'-$^{32}$P]AMP (Fig. 5, lane 1). The terminal nucleotide of 5'-labelled dsRNA (of the strand that could be labelled) was found to be [5'-$^{32}$P]AMP (Fig. 5, lane 2).

Sequence analysis by partial digestion with base-specific enzymes.

To confirm the sequence obtained by mobility-shift analyses, the labelled dsRNAs were also subjected to sequencing by digestion with base-specific nucleases. As it was clear that two strands were labelled by the RNA ligase procedure (Fig. 3A), an attempt was made to obtain homogeneous oligonucleotides from each strand for RNA sequencing. The 3'-labelled dsRNAs were subjected to partial digestion with RNase T1 and individual oligonucleotides isolated using polyacrylamide gels as described above (Fig. 2A). Each of them migrated as a single spot upon two-dimensional polyacrylamide gel electrophoresis, confirming their purity. Results of sequencing two of the oligonucleotides, III and V, are shown in Fig. 6A. Incubation of oligonucleotides without any enzymes (NE) did not cause fragmentation, while partial alkaline hydrolysis generated a complete set of fragments. The two oligonucleotides were seen to share a common sequence at their 3'-termini, indicating they were from the same strand of the dsRNAs. Similar analyses of oligonucleotides IV, VI and VII indicated that all were derived from the same strand of the dsRNAs (data not shown). The absence of a second set of discrete oligonucleotides of different sequence is consistent with one 3'-terminus being a homopolymer of variable length.

Complete digestion of oligonucleotides III - VII with RNase T1 yielded a strong band corresponding to a dinucleotide and a faint band corresponding to a trinucleotide (data not shown). These were the same as bands I and II, respectively, observed with total 3'-labelled L-dsRNAs (Fig 2). This demonstrates that oligonucleotides I and II are derived from the same strand. Since the dinucleotide is the major band, the trinucleotide may be an artifact produced during isolation or labelling of the dsRNAs, or may reflect naturally occurring microheterogeneity in the dsRNA population.

The results of enzymatic sequence analysis of the 5'-labelled dsRNAs are shown in Fig. 6B. Since only one strand was labelled, the dsRNAs were used directly, without either separating the strands or attempting to isolate 5'-terminal oligonucleotides. The profiles obtained by partial digestion with RNase Phy M and RNase B. cereus indicated the presence of a poly(U) sequence, in agreement with the results obtained by mobility-shift analysis (Fig. 4A). Digestion with RNase T1, however, produced a complete set of fragments, which would not be expected for a poly(U) sequence. One must therefore postulate that the 5'-proximal G residue (of the strand which can be labelled) is followed in the 5'-direction by a poly(U) sequence of heterogeneous length. Cleavage at the G residue produces a set of oligonucleotides of the form


A. 3'-TERMINAL-LABELLED DSRNA
OLIGONUCLEOTIDE III
NE
OH-
T1
U2
PM
BC
A
N
C
A
C
C
C
A
U
A
A
A,G
OLIGONUCLEOTIDE V
NE
OH-
T1
U2
PM
BC
C
U
A
N
C
A
C
G
C
A
G
A
C
C
A
C
C
C
C
A
U
A
A
A
A,G
B. 5'-TERMINAL-LABELLED DSRNA
NE
T1
U2
PM
BC
U
U
U
U
U
U
U
U
U
U
A

5'-$(U)_n$Gp-3', where n varies from 1 to 25. Based on the distribution of T1-oligonucleotides, the average size of the poly(U) stretch is 20 residues.

Construction of a recombinant DNA probe.

Having established that the individual dsRNA components shared a common sequence at their termini, we wanted to determine whether the homology extended further into the dsRNA molecules. For this purpose a recombinant probe complementary to the dsRNAs was constructed. Attempts to utilize denatured dsRNAs directly as a template to synthesize cDNA using oligo(dT) as primer were unsuccessful. Therefore, the dsRNA was denatured and a poly(A) tail enzymatically added. The polyadenylated RNA, by contrast, was an excellent template for reverse transcriptase in the presence of oligo(dT). The single-stranded cDNA was converted to double-stranded form and cloned into a bacterial plasmid. The insert portion of one such recombinant, termed pEP9, was subcloned into bacteriophage M13 and sequenced (Fig. 7). The entire insert was 194 bp, including the HindIII linkers used for cloning and a poly(A) tract of 24 residues at one 3'-terminus of the insert. The sequence did not correspond to that of the heteropolymer region determined by direct RNA sequencing.

Homology among dsRNAs by northern hybridization analysis.

In order to detect which of the dsRNA components hybridized to the probe, dsRNA preparations from EP713 were separated on 3% and 5% polyacrylamide gels, transferred to DPT paper, and hybridized to $^{32}$P-labelled pEP9 (Fig. 8). No hybridization was observed with TNA from the progenitor V strain, EP155, but the probe hybridized with both L- and S-dsRNA components in EP713. In experiments where 5% polyacrylamide gel electrophoresis was employed, hybridization occurred with all L-dsRNA components, roughly in proportion to their ethidium bromide staining (cf. Fig 1).

The cloned probe also hybridized to the smaller dsRNAs S1, S2, and S3. The amount of hybridization to these was stronger, which is surprising since EP713 dsRNA consists mainly of L-dsRNAs. However, part of this may have been due to varying efficiencies of electrophoretic transfer of different RNA species to DPT paper.

Fig. 6. Sequence analysis of L-dsRNA of EP713 by partial digestion with base-specific nucleases. (A) Oligonucleotides III and V produced by partial RNase T1 digestion of 3'-labelled dsRNA (Fig. 2A) were isolated and subjected to partial digestions with RNase T1 (T1), RNase U2 (U2), RNase Phy M (PM), and RNase B.cereus (BC). The digests were analyzed on 25% polyacrylamide-7 M urea sequencing gels. Lane NE, no enzyme added; lane $OH^-$, subjected to partial alkaline hydrolysis (see Materials and Methods). (B) 5'-labelled dsRNA was digested with nucleases and analyzed as in (A).

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AAGCTTGGAAGATGAGAACTATACTTTGACAACAAAGGGTATAGGGATCG
          10        20        30        40        50
HindIII

GAAAGGCCGCTGCAGTAGGATTCAGACAAATAAATTTTCTCTTAAAATGT
        60        70        80        90        100
          Pst I

CCGCCGTTTTCTTTTGTTGGCTATTCCCTTTCACCGTGCGTACGGTGGGA
       110       120       130       140       150

AGAGAACAACAAGAAAAAAAAAAAAAAAAAAAAAAAAACAAGCTT
       160       170       180       190
                 Poly(A) Tail      HindIII
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Fig. 7. Nucleotide sequence of the cDNA insert of the recombinant plasmid pEP9 . The insert was subcloned into M13mp18 and sequenced in both directions by the dideoxy chain termination method (28,29). "HindIII" indicates the linkers used in cloning and "PstI", a restriction site.

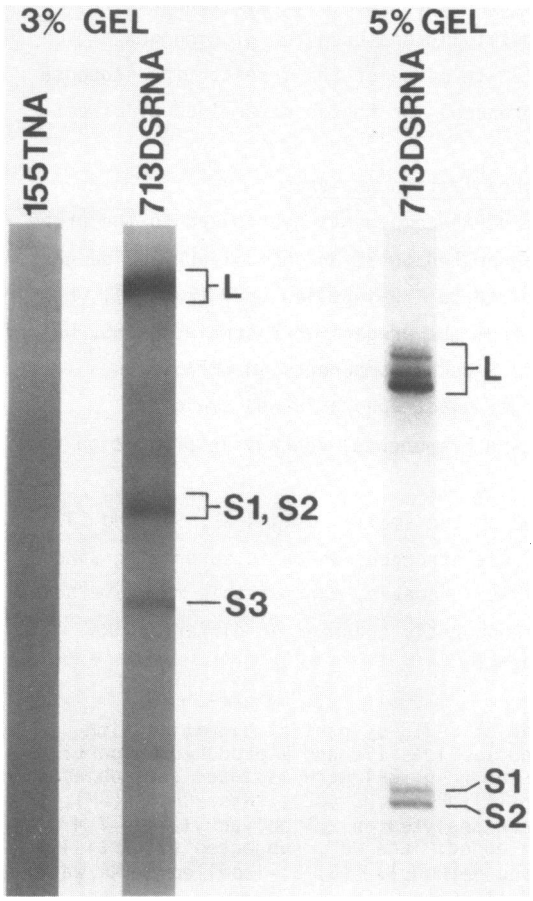


Fig. 8. Northern-blot analysis of RNAs from virulent (EP155) and hypovirulent (EP713) strains. Total nucleic acid (TNA; 20 µg) from EP155 and dsRNA (3 µg) from EP713 were subjected to electrophoresis on polyacrylamide gels as in Fig.1A and transferred to DPT paper. Hybridization was to nick-translated pEP9.

It is possible that the observed hybridization was only due to interaction of the poly(dA):poly(dT) tract of pEP9 with the poly(U):poly(A) regions of the L-dsRNAs. To rule this out, pEP9 was digested with PstI to generate two fragments, only one of which contained the poly(dA):poly(dT) tract (Fig. 7). Northern hybridization analysis using either fragment as probe produced the same results as with intact pEP9 (data not shown).

## DISCUSSION

The results of various methods used for sequence determination are summarized in Table 1, together with an overall consensus structure. The two termini of a dsRNA molecule are characterized as containing either heteropolymer or homopolymer sequences. Because both strands were labelled at their respective 3'-termini (Fig. 3), it was necessary to isolate individual oligonucleotides for enzymatic sequencing (Fig. 2). The sequence obtained by this method was unambiguous with the exception of the 3'-terminal few bases. A dinucleotide product was obtained with RNAses T1, Phy M, and U2 (Fig. 6). This apparent contradiction may be resolved with the additional information that the 3'-terminal residue is A (Fig. 5). All three enzymes would generate a labelled dinucleotide from the sequence ...ApGpA[$^{32}$P]Cp, the final C being added by RNA ligase. The bands at the trinucleotide position in the RNAse T1 lanes (Fig. 6A) are apparently the minor oligonucleotide II (Fig. 2) and do not indicate a G three residues from the 3'-terminus. The sequence derived by mobility shift analysis was in general agreement with that of the enzymatic analysis (Fig. 3A), except that it predicted the terminal sequence ...UAGA-3' while enzymatic sequence predicted ...UAAGA-3'. The latter sequence was chosen because it is very clear that RNase B. cereus generated a penta- and not a tetranucleotide (Fig. 6A). The mobility-shift data were also consistent with the presence of a 3'-terminal A.

The other strand at the heteropolymer terminus was not labelled under the conditions used (Fig. 4A), and thus no confirming heteropolymer sequence data are available. There are two possible explanations for this. The 5'-termini in dsRNAs may be identical, giving a single pattern of digestion products. This seems unlikely, since the 3'-termini, which are presumably complementary, are not identical. The other possibility is that one of the 5'-termini is blocked and unavailable for labelling under the conditions employed. This could be due to the presence of a cap, although the experiment with tobacco acid pyrophosphatase would argue against it, or a genome-linked protein such as that found in certain plant and animal viruses (32).

Table 1. Summary of sequence analyses of the termini of EP713 L-dsRNAs.

I. 3'-Termini

A. Heteropolymer terminus

| | |
|---|---|
| | 30 20 10 |
| 1. Mobility-shift analysis | 5'....GAGCUCACUAUGUAGAUCACCCAUAGA-3' |
| 2. Enzymatic analysis | |
| a. Oligonucleotide III | 5'-ANCACCCAUAAGA-3' |
| b. Oligonucleotide IV | 5'-UAGANCACCCAUAAGA-3' |
| c. Oligonucleotide V | 5'-CUCANUAUGUAGACCACCCAUAAGA-3' |
| d. Oligonucleotide VI | 5'-AGCNCANUAUGUAGANCANNNAUAAGA-3' |
| e. Oligonucleotide VII | 5'....AGNNNANUAUGUAGACCANNCAUAAGA-3' |
| 3. Consensus Sequence | 5'-...GAGCUCACUAUGUAGAUCACCCAUAAGA-3' |

B. Homopolymer terminus

| | | |
|---|---|---|
| 1. Mobility-shift analysis | 5'-...NNN$(A)_n$-3' | n = ~25 |
| 2. Enzymatic analysis | 5'-...NNN$(A)_n$-3' | n = 31-39, av. 35 |

II. 5'-Terminus

| | | |
|---|---|---|
| A. Mobility-shift analysis | 5'-A$(U)_n$NNN...-3' | n = ~20 |
| B. Enzymatic analysis | 5'-A$(U)_n$NNN...-3' | n = 1-25 |

III. Terminal nucleotide analysis

| | |
|---|---|
| A. 3'-Termini | A |
| B. 5'-Terminus | A |

IV. Overall Terminal Structure

5'-A$(U)_{\overline{20}}$NNN.....NNNGAGCUCACUAUGUAGAUCACCCAUAAGA-3'
3'-$(A)_{\overline{35}}$NNN.....NNNNNNNNNNNNNNNNNNNNNNNNNNNNNX-5'

where X is a blocking structure.

The homopolymer end of the dsRNA molecule is characterized by the presence of a 3'-terminal poly(A) tract of variable length. The average size of the T1-resistant product was 35 residues (Fig. 2C) and that of the RNase B. cereus-resistant product, approximately 40 (data not shown). This is presumably because the 3'-proximal pyrimidine is five residues further from the homopolymer tract than the 3'-proximal G residue. These two results set the size for the poly(A) tract at an average of 35 residues. Mobility-shift analysis confirmed the existence of a poly(A) sequence, although the presence

of strands of variable length cannot be detected by this method (Fig. 3A). The other homopolymer structure, poly(U), was detected with 5'-labelled dsRNA by enzymatic digestion (Fig. 6B). The fact that it was variable in length is indicated by the T1 digestion products, which consisted of a set of oligonucleotides centering around 20 as well as mono- through trinucleotides. The latter result may indicate that some molecules lack the poly(U) tract. Mobility-shift analysis confirmed the presence of a homogeneous poly(U) tract for approximately 20 residues, after which there was evidence for the beginning of a heteropolymer sequence (Fig. 4A).

The most logical structure for the intact dsRNA is one which places the poly(A) tract complementary to the poly(U) tract. The proposed structure in Table 1 would place the blocking structure at the 5'-terminus of one strand and the variable-length poly(A) tract at the 3'-terminus. The apparent difference in the lengths of the homopolymer tracts may indicate that the poly(A) overhangs the poly(U) by approximately 15 residues. The only problem with this relative orientation of the two strands is the presence of an A residue at the homopolymer 5'-terminus (Fig. 5, lane 2), since it would not be complementary to the A residues in the other strand. A dsRNA structure with a terminal poly(A):poly(U) does not, to our knowledge, have a precedent. The most nearly similar structure is that of an internal poly(A):poly(U) region of variable length, found in the M-dsRNA from the type 1 killer strain of Saccharomyces cerevisiae (33). The presence of the poly(A) in these transcripts has been hypothesized to be template-encoded.

Additional sequence information from the dsRNAs of EP713 is provided by the recombinant plasmid derived from L-dsRNAs. The sequence did not, however, correspond to that of the heteropolymer terminus determined by direct RNA sequencing. Thus, the region of the dsRNA represented by the recombinbant plasmid insert cannot be determined from the available data. There are two possible origins for the poly(dA) tract observed (Fig. 7). It may represent the natural poly(A) found at the 3'-terminus of the homopolymer region of the dsRNA. Alternatively, it may be derived from poly(A) added enzymatically to an internal nick in the dsRNA.

One of the principal findings of this study is that most or all of the dsRNA components of the H strain under investigation are structurally related. Nuclease digestion analysis of the various L-dsRNA components indicated similar or identical 3'-termini for at least 50 nucleotide residues (Figs. 2 and 6). This sequence identity was confirmed by mobility-shift analyses, since the same patterns were obtained with single isolated L-dsRNAs

or the mixture of L1-L5 (Figs. 3 and 4). Hybridization to the recombinant probe occured with all L-dsRNAs in proportion to their amounts in the preparation (Fig. 8). This indicates that homology extends beyond the terminal regions to that represented by the probe. If the recombinant probe is indeed adjacent to the 3'-poly(A) tract (see above), then the homology extends at least 100 residues inward from the homopolymer end. This conclusion is based on the fact that the PstI-digested pEP9 fragment which contains no poly(dA):poly(dT) tract hybridized to all L-dsRNA components.

These homology relationships support a viral origin for the dsRNA associated with hypovirulence in E. parasitica. The occurrence of homologous terminal sequences at the respective ends of genome segments is a common phenomenon for viruses with multipartite single- or double-stranded RNA genomes (34,35). The recent demonstration that the club shaped particles (vesicles) which enclose dsRNA in vivo in E. parasitica possess RNA polymerase activity further strengthens the hypothesis of a viral origin (4). The homologies observed rule out the possibility that some of the L-dsRNA represent mixed virus infections. It is also unlikely that they are satellites, since satellites have been found to possess little or no sequence homology with their associated viral genomes (36-39). The question of whether all the five L-dsRNAs contain necessary genomic information, assuming they are viral in origin, cannot be directly answered in the absence of infectivity assays. This seems unlikely, though, because several other H strains of European origin have been found which contain only one or two dsRNA molecules of comparable size (L1 and L2, Fig. 1B) with sequences homologous to EP713-dsRNA (11). It is possible that components L4 and L5, for example, are variant RNAs containing terminally conserved genomic segments (34). This is comparable to such remnants or defective interfering RNA associated with dsRNA fungal, plant, and animal viruses. Such variant dsRNAs would arise by internal deletions of genomic dsRNA and would contain extensive homology with parental RNA. An extreme case of such deletions may be the S-dsRNAs which, though present in very small quantities in comparison to the L-dsRNAs (Fig. 1A), show strong hybridization to the recombinant probe (Fig. 8). Thus, they may contain terminal sequences, to which pEP9 is complementary, but little of the internal portion of the L-dsRNAs.

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