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Transcription and In Vitro Translation of the dsRNA Virus Isolated from Rhizoctonia solani.

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Abstract

A segmented double-stranded dsRNA virus has been isolated from virulent strains of *Rhizoctonia solani.* The dsRNA genome has mol. wts. of 1.45 and 1.32 × 10^6. Two full-size transcripts with mol. wts. of 0.74 and 0.66 × 10^6 (2.2 kb and 2 kb, respectively) were synthesized by the virus-associated RNA-dependent RNA polymerase and resolved by denaturing polyacrylamide gel electrophoresis. The transcripts cross-hybridized to the viral dsRNA isolated from a number of strains. The transcripts did not hybridize with the genomic DNA. An unencapsidated species of dsRNA with mol. wt. of 1.6 × 10^6 did not hybridize with the viral transcripts. No cross-hybridization between the two viral dsRNA segments was obtained. The viral-encoded proteins were studied by *in vitro* translation using the rabbit reticulocyte lysate system. The transcripts served as mRNA for the synthesis of the major capsid protein of 55 kD, and a number of other products. The viral coat protein was immunoprecipitated with antibodies against purified virus particles. Partial proteolysis of the major *in vitro* product and the authentic capsid protein using *Staphylococcus aureus* V8 protease produced similar peptide patterns. Denatured viral dsRNA also directed the synthesis of proteins identical to those translated from the transcripts *in vitro.*

Introduction

The fungal plant pathogen *Rhizoctonia solani* causes severe damage to a large number of hosts (1) and is a severe pathogen throughout the world. By screening many isolates of *R. solani* for virulence, a number of studies indicated the occurence of both virulent and avirulent strains in the natural population (2–4). A double-stranded dsRNA virus has been recently isolated from strains of *R. solani* (5, 6). The viral genome is composed of two segments of dsRNA with mol. wts. of 1.45 and 1.32 × 10^6 (2.2 kbp and 2 kbp, respectively). An additional dsRNA

molecule of 1.6×10^6 (2.4 kbp) is found in many strains by extraction of mycelia but has not been recovered from virions. The major capsid protein is 55 kD. An RNA-dependent RNA polymerase is associated with the viral capsids and is clearly a transcriptase (5).

Although initial studies reported that strains of the pathogen containing dsRNA are debilitated and hypovirulent (7), later studies indicated that a correlation between the presence of dsRNA and virulence cannot be established (2). Only recently, by stringent tests to clearly distinguish between virulent and hypovirulent strains of the pathogen, a clear correlation was established between the presence of viral information and virulence (4). Hypovirulent strains were found to be devoid of viruses and all the virulent strains examined contained dsRNA viruses. The relationship between the dsRNA viruses and virulence was further examined in attenuated strains and a correlation was established between the loss of virulence and either the total loss of dsRNA segments or the loss of specific segments (5). Furthermore, transmission of dsRNA from a virulent to an avirulent compatible strain by cytoplasmic exchange resulted in the transmission of virulence. These results suggested that dsRNA viruses may be associated with the regulation or production of virulence factors. The analysis of the virus-encoded proteins may therefore be significant for the elucidation of nonstructural proteins associated with the regulation of virulence.

In vitro translation of virus-specific mRNA has been extensively used in other dsRNA viral systems to determine the coding potential of the viral genome (8–12). *In vitro* translation of denatured dsRNA has also been reported in a number of dsRNA viruses (9, 13–16).

In this report we describe the proteins encoded by the *R. solani* viral genome. We have used both the viral-specific mRNA, synthesized *in vitro* by the virus-associated RNA-dependent RNA polymerase, and unfractionated dsRNA to direct the synthesis of proteins in rabbit reticulocyte lysate systems. In addition, the relationship between encapsidated and unencapsidated dsRNAs and the relatedeness of the viruses isolated from different strains is described.

Methods

Strains

R. solani cultures (No. 13, 82, 53, 56, and W) were isolated from various locations in Israel (4), and maintained on potato-dextrose agar at 27°C. The virulence of each strain was determined in a plate assay as described by Castanho & Bulter (17).

Purification of virus particles and dsRNA extraction

The techniques to purify virus particles and to extract dsRNA described by Finkler et al. (5) were followed.

Purification of dsRNA segments

Individual dsRNA segments were purified by at least 2 cycles of electrophoresis in 1.5% agarose and were finally electroeluted from the agarose using BND-cellulose columns as described by Silhavy et al. (18).

3' End-labelling

Segments of dsRNA were end-labelled with 5'-^{32}P-cytidine-5'-3'-diphosphate using T4 RNA ligase (Pharmacia) as described by Bruenn and Brennan (19). The specific activity of the [^{32}P]pCp was 3000 Ci/mmole. The labelled dsRNA was purified by phenol extraction, ethanol precipitation, and desalting was performed on Sephadex G100.

DNA isolation

Mycelia grown overnight at 27°C were incubated with 10 mg/ml Novozyme (Novo Industries, Sweden) in 0.6 M mannitol (pH 5.8) on a shaker for 1-2 hr at 30°C. The mycelia were centrifuged and treated with 50 µg/ml proteinase K and 1% NaDodSO$_4$ for 1 hr at 37°C in 10 mM Tris-HCl buffer (pH 7.5). The nucleic acids were extracted with phenol:chloroform (1:1).

Synthesis of viral transcripts

Full-size transcripts were synthesized basically as described by Ben-Zvi et al. (20) with few modifications. The reaction mixtures, containing 1 mM of each of the 4 nucleoside triphosphates and 1.6 mg/ml bentonite to inhibit RNase activity, were incubated with 3 mM Mg^{+2} at a pH optimum of 7 for 24 hr at 30°C. Labelled transcripts were synthesized in the presence of 10^7 cpm/ml of [^{32}P] UTP. After incubation, the reaction mixture was extracted with phenol-chlorophorm (1:1) and the nucleic acids were ethanol precipitated. The sRNA transcripts were purified on a CF11 column (21), ethanol precipitated, and washed with 70% cold ethanol several times to remove salts.

Analysis of viral transcripts

Size determination of viral transcripts by electrophoresis was performed in 3% polyacrylamide gel in the presence of 7M urea as described by Marzluff and Huang (22) with few modifications: ^{32}P-labelled transcripts with specific activity of 5×10^5 cpm/µg RNA were denatured by heating at 65°C for 6 min in sample buffer

containing 50 mM Tris, 50 mM boric acid, 50 uM EDTA (TBE buffer) to which 5M urea, 10% glycerol, 0.05% bromophenol blue and 0.05% xylene cyanol were added. The samples were quickly cooled and then electrophoresed for 24 hr in a vertical 12 × 23 cm gel at 110 volts using TBE buffer. The transcripts were detected by autoradiography of the dried gel.

RNA blot hybridization analysis

Double-stranded RNA samples were electrophoresed in horizontal 1% agarose gel in MOPS buffer containing 1mM EDTA, 5 mM sodium acetate and 20 mM Na-MOPS (pH 7.5) for 3 hr at 110 volts. Treatment of the gel prior to the transfer to nitrocellulose followed the procedure provided by J. Bruenn (personnal communication). The gel was incubated in 50% formamide and 6% formaldehyde in MOPS running buffer at 55°C. The RNA was denatured by treatment with 50 mM NaOH and 100 mM NaCl, and transferred from the gel to nitrocellulose filters in 20 × SSC (1 SSC = 0.15 mM NaCl, 0.015 mM sodium citrate as described by Southern (23). The filter was air dried and then baked at 80°C for 2 hr under vacuum.

The filters were prehybridized for 2–4 hr at 65°C in a hybridization mix containing 10 × Denhardt, 4 × SSC, 0.1% $NaDodSo_4$, 0.2% Ficoll, 0.2% polyvinyl pyrrolidone and 100 μg/ml denatured salmon sperm DNA.

Hybridization was performed at 65°C for 12–24 hr in hybridization mix with 1 × Denhardt. Transcripts with a specific activity of 5×10^5 cpm/μg were added. When ^{32}P-end-labelled dsRNA was used as a probe, it was denatured for 90 sec at 100°C. After hybridization, the blot was washed as described by Thomas (24).

Southern blot analysis

Hybridization of ^{32}P-labelled transcripts to DNA followed the procedure described by Southern (23).

In vitro translation

Double-stranded RNA (1 μg) was precipitated overnight with ethanol at −20°C and washed twice with 70% ethanol. To denature the dsRNA, the dried sample was resuspended in 2 μl distilled H_20 containing 1 mM EDTA, incubated 90 sec at 100°C and immediately cooled in ice water and subjected to *in vitro* translation. Dried samples of dsRNA (1 μg) were also denatured with 2 μl of 10 mM methyl mercury hydroxide for 10 min at room temperature prior to *in vitro* translation.

Purified transcripts or denatured dsRNA were translated in rabbit reticulocyte *in vitro* translation systems (Amersham, U.K. and Orgenics Ltd, Israel). Translation was carried out in proportions of 8 and 6 volumes of lysate, respectively, 1–2

volumes of RNA and 0.8–1.5 volume of [^{35}S]methionine (0.8–1.5 μCi/10 μl), and incubated at 30°C for 60–80 min.

Incorporation of the label into total proteins synthesized was determind by spotting 2 μl aliquots on GF filters and assaying for trichloroacetic acid precipitable radioactivity. The in vitro-synthesized proteins were electrophoresed in 10% NaDodSO$_4$-polyacrylamide gels using the buffer system of Laemmli (25). The proteins were detected by fluorography using an intensifying solution, Amplify (Amersham, U.K.), for 30 min. The gel was dried and autoradiographed.

Preparations of immune serum

Virus particles were purified on three successive 10–40% sucrose gradients, according to Finkler et al. (5) and fractions containing the viruses were identified by the polymerase activity. These fractions were pooled and dialyzed against buffer containing 50 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 40 mM NaCl, 10 mM $MgSO_4$, 4 mM 2-mercapthoethanol and 10% glycerol, and pelleted at 133000g for 4 hr. New Zealand white rabbits were immunized intradermally with 120 μg of purified virus preparation emulsified with an equal volume of Freund's complete adjuvant. Injections were repeated 21 days and 42 days after the initial immunization, with 60-100 μg of virus preparation. The rabbits were bled from the ear vein 10 days after each boost. Gammaglobulins were purified from the serum by ammonium sulfate precipitation and by dialysis against buffer containing 2 mM NaH_2PO_4, 10 mM Na_2HPO_4 and 150 mM NaCl (PBS), as described by Palmiter et al. (26). Finally, the gammaglobulin preparation was tested by double-diffusion Ouchterlony assays (27) against purified virus particles containing 1% NaDodSO$_4$.

Immunoprecipitation assay

The in vitro translation products of *R. solani* transcripts and denatured dsRNA were analyzed by immunoprecipitation as described by Segev et al. (28), using the rabbit antiserum and immunogammaglobulins prepared against purified viral particles.

Peptide mapping

Peptide mapping of proteins isolated from NaDodSO$_4$-polyacrylamide gels followed the procedure described by Cleveland (29). Both hydrated and dried gel slices were digested with *Staphylococcus aureus* V8 protease (1μg) and fractioned on 15% NaDodSO$_4$-polyacrylamide gels.

Results

Relatedness of dsRNA segments

The genome of the *R. solani* virus was shown to consist of two segments of dsRNA with mol. wts. of 1.45 and 1.32 × 10^6 (5). No additional dsRNA segments were obtained from viruses, as confirmed by 3′ end-labelling of the encapsidated dsRNA molecules. An additional nonencapsidated dsRNA segment with mol. wt. of 1.6 × 10^6 was purified from the mycelia of a number of virulent strains of *R. solani*. The relationship between the dsRNA segments was studied by hybridization of the total dsRNA isolated from the fungal mycelium to each purified ^{32}P-end-labelled dsRNA segment. The results in Fig. 1 indicate that the two encapsidated segments are unique since they do not cross-hybridize. The minor band in Fig. 1c is a result of cross-contamination between the dsRNA segment with mol. wt. of 1.45 × 10^6, used as a probe, with the 1.32 × 10^6 dalton segment. Purification of the heavier segment was hindered due to the proximity of the two bands. However, the dsRNA segment with mol. wt. of 1.6 × 10^6 did not hybridize to any viral dsRNA molecule. This further supports the earlier results suggesting the nonencapsidation of the latter dsRNA segment.

Analysis of viral transcripts

RNA-dependent RNA polymerase activity has been found in virus particles isolated from *R. solani*. The major product of the polymerase reaction is ssRNA and represents transcription activity (5).

In earlier studies of the virus-associated RNA polymerase activity, the viral transcripts were examined in 1.5% agarose gels after treatment with glyoxal. Only one transcript of 0.75 × 10^6 daltons was identified (5). In the current study, two viral transcripts were resolved using denaturing (7 M urea) polyacrylamide gel (Fig. 2). The second transcript is 0.66 × 10^6 daltons. Each of these transcripts corresponds to one half the molecular weight of one of the viral dsRNA segments. A transcript corresponding to the 1.6 × 10^6 dalton dsRNA segment, recovered from the mycelium, was not detected among the viral transcripts.

Relatedness of viral isolates

To determine the relatedness of the viruses obtained from different virulent strains of *R. solani,* the viral transcripts were used as probes in hybridization experiments with dsRNA extracted from the mycelia of a number of fungal isolates. All *Rhizoctonia* isolates contained two dsRNA segments with mol. wts. of 1.45 and 1.32 × 10^6 that are contained in virus particles. The mycelia of a number of strains (53 and 82) contained an additional dsRNA segment with mol. wt. of 1.6 × 10^6. The

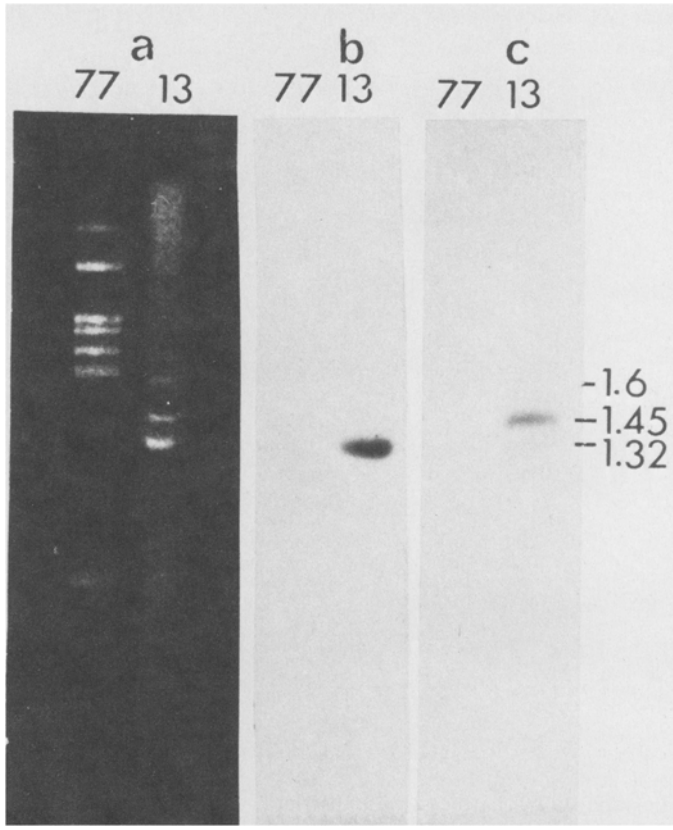

Fig. 1. Cross hybridization analysis of dsRNA segments. dsRNA isolated from *U. maydis* 77 and *R. solani* 13 were subjected to electrophoreis on a 1.5% agarose gel, stained with ethidium bromide (a), trasnferred to nitrocellulose as described in Methods and hybridized with pCp-labelled 1.32×10^6 dsRNA segment (b), pCp-labelled 1.45×10^6 dsRNA segment (c). Hybridizing bands were detected by autoradiography (b, c).

latter dsRNA band was never recovered from virions and appears to be an unencapsidated species. The viral transcripts, used as probes, served also to resolve the relationship of the heavy segment of dsRNA to that found encapsidated in viral particles.

As shown in Fig. 3a, the transcripts synthesized from strain 82 hybridized to the dsRNA segments with mol. wts. of 1.45 and 1.32×10^6, isolated from the *Rhizoctonia* strains W, 56, 82, 13, and 53. An identical hybridization pattern was obtained with the transcripts synthesized from strain 13 (Fig. 3b). No hybridization was obtained between either *Rhizoctonia* viral transcripts or the *U. maydis* dsRNA molecules. These results indicate that in every case the transcripts hybridized only to the segments that are known to be encapsidated in virions. Therefore, the

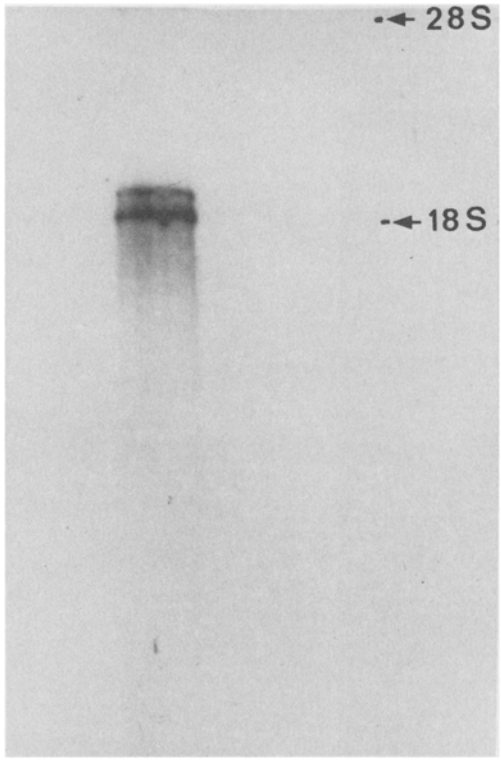

Fig. 2. Autoradiography of ^{32}P-labelled viral transcripts. ^{32}P-labelled transcripts were denatured as described in Methods and run on 3% polyacrylamide gel containing 7 M urea. Rabbit reticulocyte rRNA (28S and 18S) molecules, used as molecular weight markers, were treated as above and visualized by ethidium bromide staining.

viruses found in the differnt strains of *Rhizoctonia,* originating from different locations, are interrelated. The nonencapsidated dsRNA segment with a mol. wt. of 1.6×10^6 appears to be unrelated to the viral genome since there is no homology between this segment and the viral transcripts.

The viral transcripts were used also to test for the presence of a proviral state in the genome of the *Rhizoctonia* host. No homology was detected between genomic DNA of the host (strain 13) and the viral transcripts as determined by Southern blot hybridization of the restricted genomic DNA with the viral transcripts used as probes. Therefore, similarily to all other dsRNA viruses of fungi, the *R. solani* virus has no "provirus" state.

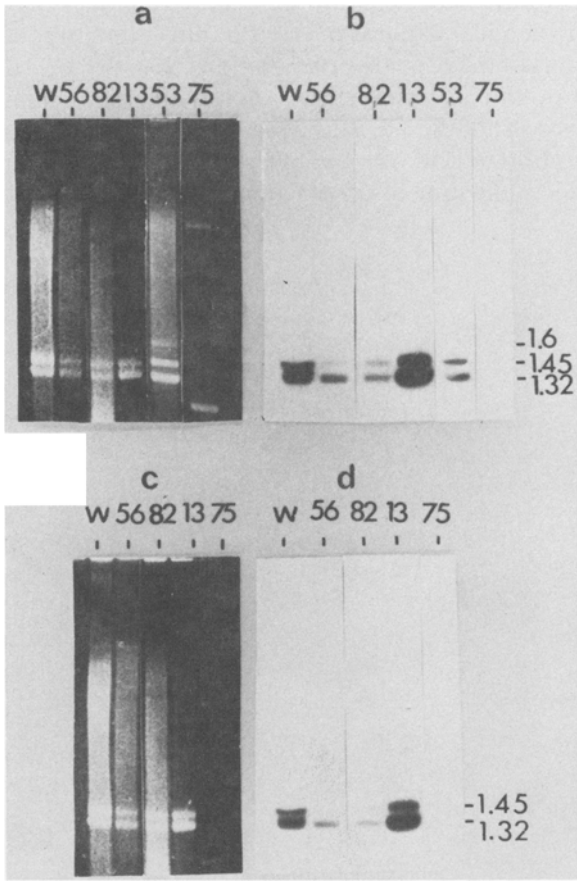

Fig. 3. Northern blot hybridization of dsRNAs. dsRNAs were separated on 1% agarose gels, stained with ethidium bromide (a, c) and transferred onto nitrocellulose. ^{32}P-labelled transcript from strain 82 was used as probe in hybridization with dsRNAs isolated from *R. solani* strains W, 56, 82, 13, and 53 and *U. maydis* 75 (b). ^{32}P-labelled transcript from strain 13 was used as probe in hybridization with dsRNAs isolated from *R solani* strains W, 56, 82, and 13 and *U. maydis* 75 (d).

In vitro translation of viral transcripts

To study the coding potential of the viral genome, in vitro translation experiments were performed with purified ssRNA transcripts. Maximum incorporation of [^{35}S]methionine into proteins was obtained with 100 μg/ml of purified transcripts obtained from viruses isolated from two strains, 82 and 13. The products of the in vitro translation assays were analyzed on NaDodSO$_4$-polyacrylamide gels (Fig.4). Identical patterns of in vitro translation products were obtained from the transcripts of the two virus preparations. A major protein of about 55 kD was synthesized in vitro. Occasionally, a closely migrating submajor product of 53 kD was observed in addition to a number of "minor" proteins of 47, 45, 43, 40, 38, and 35 kD. In an effort to determine whether some of these minor components are incomplete products, the time of the reaction was extended to 4 hr but the pattern

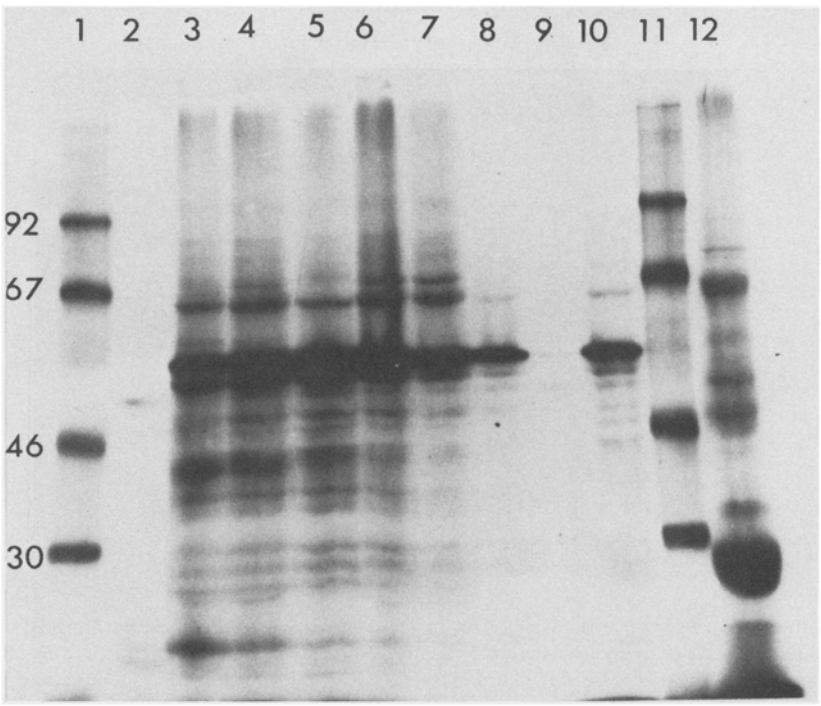

Fig. 4. In vitro translation of *R. solani* viral transcripts synthesized from strain 82. Each reaction mixture (10 μl) was diluted with 2 × Laemmli sample buffer, incubated 3 min at 100°C and analyzed on 10% NaDodSO$_4$-polyacrylamide gel. Lane 1, 11: [^{14}C]methyl-labelled protein molecular weight standards; lane 2, reticulocyte lysate reaction without added RNA; lanes 3–10, translation products of transcript RNA (50, 75, 100, 150, 200, 250, 300, 400 μg/ml respectively); lane 12, products of globin mRNA.

of these minor proteins remained unchanged. Whether these are pause products found often in in vitro translation or authentic nonstructural proteins is unclear and requires further characterization of virion coat proteins. A number of additional proteins of 10–30 kD, found among the products of the in vitro translation, were not virus encoded. Some of these proteins were found in control assays lacking RNA and others may represent short peptides wtill bound to tRNA.

Double-diffusion Outchterlony assays were used to test the identity of the major viral coat protein using rabbit antiserum and partially purified gammaglobulins prepared against purified virions. A single precipitin band was obtained between antiviral antibodies and the virus particles. These antibodies were used to immunoprecipitate the [^{35}S]methionine-labelled in vitro translation products derived from the viral transcripts (Fig. 5). It appears that the proteins of the viral capsids isolated from two different strains (13 and 82) are similar since both have the

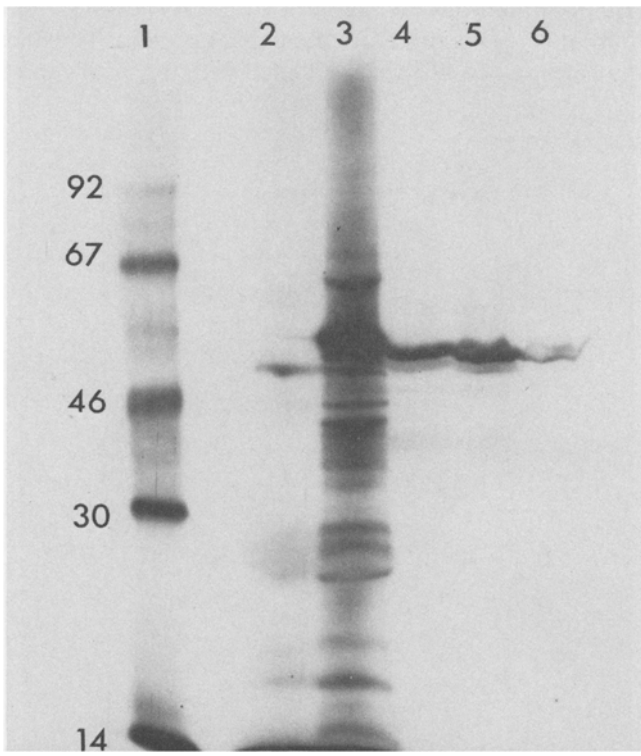

Fig. 5. Immunoprecipitation of in vitro translation products with antibodies against virions isolated from strain 13. Lane 1, [^{14}C] methyl-labelled protein standards; lane 2, reticulocyte lysate reaction with out added RNA; lane 3, in vitro translation products of viral transcript; lanes 4–6, translation products immunoprecipitated with 20, 15 and 10 μg/ml gammaglobulins, respectively. All reactions were previously treated with 5 μl normal rabbit serum and a 10% fixed suspension of *Staphylococcus aureus* to remove nonspecific binding antigens.

same mol. wts. and both are immunoprecipitated by antibodies prepared against the virus isolated from strain 13. A major protein of 55 kD was precipitated in all the experiments. Occasionally a closely migrating submajor product of 53 kD was synthesized. This protein was also precipitated by anticoat antibodies. Two other minor products of 47 and 45 kD were immunoprecipitated and are seen as faint bands on longer exposure; these may be due to nonspecific immunoprecipitation.

The identity of the major in vitro translation protein band was further studied by comparing the peptide map pattern of the authentic viral capsid and the 55 kD in vitro translation protein. The major in vitro translated protein band of strain 13, located by autoradiography of the $NaDodSO_4$-polyacrylamide gels, was excised and hydrolyzed with *Staphylococcus aureus* V8 protease. The authentic viral capsid was similarly treated. The resulting peptides were resolved on 15% $NaDodSO_4$-polyacrylamide gels and gave similar patterns (Fig. 6). Identical peptide map patterns were also obtained with the major in vitro translation protein of strain 82. The major protein of 55 kD is therefore the capsid protein. These results suggest that two proteins of 55 and 53 kD are associated with the viral capsids, and the 53

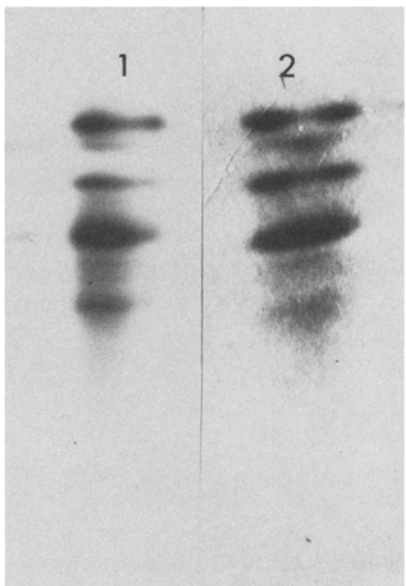

Fig. 6. Comparison of peptide map patterns of the major in vitro translation product and capsid protein. Gel slices were treated with 1 μg *Staphylococcus aureus* V8 protease and electrophoresed on 15% $NaDodSO_4$-polyacrylamide gel. Lane 1, autoradiograph representing peptide mapping of the major in vitro translation product of strain 13; lane 2, peptide mapping of authentic capsid protein (strain 13) visualized by Coomassie blue staining.

kD protein appears to be an incomplete product. The identity of the other products as nonstructural proteins will be discussed later.

In vitro translation of dsRNA

Total dsRNA extracted from the mycelium and purified on CF11 columns was used for in vitro translation. These samples contained the viral dsRNA segments (1.45 and 1.32 × 10^6 daltons) and in addition the unencapsidated dsRNA (1.6 × 10^6 daltons). The products of in vitro translation of the total dsRNA shown in Fig. 7 appear similar to those synthesized by the viral transcripts, but included an

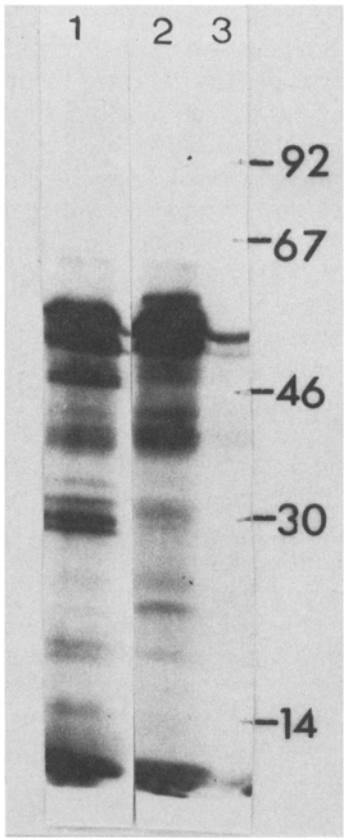

Fig. 7. Comparison between in vitro translation products of total denatured dsRNA and viral transcripts. Reaction mixtures (10µl) were run on 10% $NaDodSO_4$-polyacrylamide gels and analyzed by fluorography. Lane 1, in vitro translation of viral transcripts (strain 13); lane 2, translation of total dsRNA extracted from strain 13; lane 3, immunoprecipitations of protein, synthesized by total dsRNA.

additional protein of 60 kD. This protein was not immunoprecipitated by the antiviral antibodies. It is probably the product of the 1.6 × 10^6 dalton dsRNA segment that is not encapsidated.

Discussion

Based on purification of viral dsRNA and end-labelling of the segments it appears that the *R. solani* virus consists of two dsRNA segments. The two segments do not cross hybridize and they appear to be unique. Two unique transcripts are synthesized in vitro by the virus-associated RNA-dependent RNA polymerase. The full-size transcripts have a coding capacity of ca. 1300 amino acids assuming there are no overlapping open reading frames and unresolved overlapping segments.

The viral transcripts bear no homology to the host genomic DNA. Thus, as in all other dsRNA fungal viruses, the *R. solani* virus does not integrate into the host chromosomes and has no proviral state.

The viral isolates are interrelated, as indicated by hybridization of dsRNA extracted from a number of isolates with a probe of a viral transcript synthesized by one of the viral isolates. The relatedness of the viruses recovered from different sources is reflected also at the level of the proteins. In vitro translation of viral transcripts synthesized by two virulent strains resulted in the formation of a major protein of identical size, identical antigenicity and peptide maps. The major in vitro translation product, a 55 kD protein, corresponds clearly to the viral capsid protein by the following criteria: a) the viral capsid protein comigrated with the major in vitro translation product; b) this product was immunoprecipitated by antibodies prepared against virus particles; and c) identical peptide mapping patterns were obtained following limited proteolysis.

The polypeptides identified so far in the in vitro translation exceed the coding capacity of the viral information. Therefore, verification of the minor in vitro translation products must be further resolved. The 53 kD product that is occasionally synthesized closely with the major coat protein and immunoprecipitated by the antiviral antibodies may be an incomplete coat product. Other in vitro translation products are not immunoprecipitated with anticoat antibodies and therefore may be among the nonstructural proteins of the viral information. The identity of these proteins will have to be resolved by immunological tests and fingerprinting.

The coding assignments for the purified genome segments of the *Rhizoctonia* virus is under investigation and should distinguish the nonstructural components.

Acknowledgments

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