In vitro Transcription of Double-stranded RNA by Virion-associated RNA Polymerases of Viruses from *Gaeumannomyces graminis*

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SUMMARY

Several double-stranded (ds) RNA *Gaeumannomyces graminis* viruses in groups I and II, but none in group III, have been shown to possess virion-associated RNA polymerase activity. The products of polymerase reaction were full-length single-stranded (ss) RNA transcripts of one of the strands of each of the dsRNA genome segments. Synthesis of ssRNA *in vitro* continued for up to 48 h during which, on average, up to eight full-length transcripts were produced per dsRNA molecule, i.e. reinitiation of transcription occurred in the *in vitro* system. In reactions containing [³H]UTP, label was also incorporated into all the dsRNA genome segments, reaching a maximum after 8 h. Examination of transcribing particles by electron microscopy revealed the presence of particles showing the release of looped ssRNA molecules, both ends of which were attached to the particle, as well as particles with one or two linear RNA strands attached. The modal length of the single linear RNA strands was within the range expected for full-length transcripts of the genome dsRNA segments.

INTRODUCTION

Isometric virus particles with genomes of double-stranded RNA (dsRNA) occur in a high proportion of field isolates of the wheat take-all fungus, *Gaeumannomyces graminis* var. *tritici* (hereafter called *G. graminis*) (Rawlinson & Buck, 1981). The viruses, even those taken from fungal isolates in a single field, are characterized by a considerable degree of serological variability (Frick & Lister, 1978), which may explain conflicting reports on the association of viruses with hypovirulence in the take-all fungus (Lapierre *et al.*, 1970; Lemaire *et al.*, 1970; Rawlinson *et al.*, 1973). Recently, thirteen viruses obtained from eight isolates of *G. graminis* have been characterized and divided into three groups based on their serological and physical properties (Buck *et al.*, 1981). Viruses in groups I and II had particles of 35 nm diam. with genomes of two to four dsRNA components in the mol. wt. range 1×10^6 to $1 \cdot 3 \times 10^6$ and $1 \cdot 4 \times 10^6$ to $1 \cdot 6 \times 10^6$, and capsids constructed from polypeptides in the mol. wt. range 54×10^3 to 60×10^3 and 68×10^3 to 72×10^3 respectively. Group III viruses were 40 nm in diam. and had two dsRNA components in the range $3 \cdot 2 \times 10^6$ to $4 \cdot 2 \times 10^6$ daltons and three capsid polypeptide species in the range 78×10^3 to 87×10^3 daltons respectively. Serological relationships were found between viruses within a group, but not between viruses of different groups.

In the present paper it is shown that *G. graminis* viruses from groups I and II have virion-associated RNA polymerase activity, but no polymerase activity could be detected in

virions of either of the two group III viruses. The properties of the RNA polymerase of a group I virus (38-4-A) are reported and briefly compared with those of the polymerases of other group I and group II viruses.

METHODS

Preparation of viruses. Fungi were cultured and viruses were isolated, purified and separated as described by Buck *et al.* (1981).

RNA polymerase reactions. Unless otherwise stated, reaction mixtures contained: 0·05 M-tris–HCl buffer pH 7·9, 0·15 M-NaCl, 0·1 mM-EDTA, 0·15 mM-ATP, 0·15 mM-GTP, 0·15 mM-CTP, 0·15 mM-[^{3}H]UTP (sp. act. 53 to 83 mCi/mmol; Amersham International); virus (25 to 250 μg/ml); actinomycin D (125 μg/ml); concentrations of Mg^{2+} optimal for each virus polymerase (see Table 1) and bentonite (800 μg/ml), prepared according to Fraenkel-Conrat *et al.* (1961). Incubation was at 30 °C, unless otherwise stated. Incorporation of [^{3}H]UMP into acid-insoluble product was determined by mixing 10 to 100 μl of reaction mixture with 3 ml of 10% (w/v) trichloroacetic acid (TCA) and allowing precipitation to occur at 0 °C for 30 min. The precipitate was collected on glass fibre filters (Whatman GF/F) and washed with 4 × 20 ml of 2% TCA and then with ethanol. Filters were dried and radioactivity was determined by liquid scintillation counting using 0·6% butyl PBD and 5% naphthalene in toluene.

Preparations of RNA. Virus suspensions or RNA polymerization reaction mixtures were made 1% in SDS and heated to 60 °C for 20 min. After cooling, the mixture was extracted with phenol and the RNA was concentrated by ethanol precipitation. For separating ssRNA and dsRNA, selective precipitation of ssRNA in 2 M-LiCl (Baltimore, 1966) was employed.

Analysis of RNA by electrophoresis. This was carried out in 4% polyacrylamide gels containing 8 M-urea and 0·1% SDS as described by Buck & Ratti (1977). For analysis of ^{3}H-labelled RNA, electrophoresis was carried out in slab gels (20 × 20 × 0·15 cm) at 100 V for 16 h and bands were detected by fluorography (Laskey & Mills, 1975). For spectrophotometric analysis of RNA, samples were electrophoresed in 10 × 0·5 cm tube gels at 60 V for 16 h. Gels were then stained in 0·01% aqueous toluidine blue and scanned at 550 nm with a Gilford Model 240 spectrophotometer equipped with a Model 2410-S linear transport. Denaturation of dsRNA, for use as an ssRNA electrophoresis marker, was carried out by glyoxalation, by a modification of the procedure of McMaster & Carmichael (1977), in which 50% (v/v) dimethyl sulphoxide was replaced by 60% (v/v) dimethyl sulphoxide. For distinguishing dsRNA and ssRNA, RNA samples in 0·3 M-NaCl + 0·03 M-sodium citrate pH 7, were incubated with ribonuclease A (0·2 μg/ml) at 37 °C for 2 h prior to electrophoresis; under these conditions dsRNA was completely stable whereas ssRNA was degraded to small oligonucleotides (Bellamy *et al.*, 1967).

Hybridization analysis. Samples of ^{3}H-labelled ssRNA (1000 to 10000 ct/min, 0·1 to 1 μg) and 0 to 25 μg dsRNA in 150 μl 0·005 M-tris–HCl buffer pH 7·9, containing 0·015 M-NaCl and 0·01 mM-EDTA, were heated to 100 °C for 5 min then cooled rapidly on ice. To this denatured RNA was added 50 μl of 0·8 M-tris–HCl buffer pH 7, containing 0·8 M-NaCl and 0·08 M-EDTA pH 7, and the mixture was heated to 70 °C for 4 h to allow re-annealing. After cooling, 25 μl of ribonuclease A (10 μg/ml in 0·3 M-NaCl + 0·03 M-sodium citrate pH 7) was added and the solution was incubated at 30 °C for 2 h. Ribonuclease-resistant dsRNA molecules were then precipitated with TCA and assayed as described for the RNA polymerase reactions above.

Electron microscopy. For examinations of transcribing virus particles, standard RNA polymerase reaction mixtures (without bentonite) were incubated for 5 or 15 min. Samples were then spread using a modification of the Kleinschmidt cytochrome *c* technique, mounted on Parlodion-coated grids, stained with uranyl acetate and shadowed with platinum (all as

described by Bartlett *et al.*, 1974) prior to examination in a Hitachi H-600 electron microscope, operating at 50 kV. Micrographs were recorded on Kodak technical pan 2415 film and negatives were projected to a final magnification of ×75 000 for contour length measurement with a Hewlett-Packard 9821A calculator, coupled to a 9864A digitizer. For calibration a Polaron cross-grating replica with 2160 lines/min was used.

Sucrose density-gradient centrifugation. A sample of 38-4-A virus (60 ml; A_{260} = 4) was loaded on to a linear density gradient (500 ml) of 20 to 50% (w/w) sucrose in 0·03 M-sodium phosphate buffer pH 7·6, containing 0·15 M-KCl, in an M.S.E. B1V zonal rotor and centrifuged at 40000 rev/min for 3·5 h. Fractions of 20 ml were then collected and dialysed against 0·05 M-tris–HCl buffer pH 7·9, containing 0·15 M-NaCl and 0·1 mM-EDTA. Fractions containing virus particles were located by electron microscopy and their A_{260} values were measured. A 50 µl sample of each fraction was assayed for RNA polymerase activity in an 18 h incubation.

RESULTS

The RNA polymerase of G. graminis virus 38-4-A

Location of RNA polymerase activity

Virus 38-4-A is a group I virus with three dsRNA components (Table 1). When a purified virus preparation was incubated in an RNA polymerase reaction mixture containing ATP, CTP, GTP and [³H]UTP, radioactivity was incorporated into an acid-insoluble product. In 18 h incubations the amount of radioactive product was linearly dependent on the virus concentration in the range 2·5 to 250 µg/ml. The activity was dependent on the presence of all four nucleoside triphosphates and further studies, described below, showed that the radioactive product was RNA. 38-4-A virus was sedimented in a sucrose density gradient (see Methods) and fractions from the gradient were analysed for RNA polymerase activity. Only those fractions containing virus particles exhibited activity; [³H]UMP incorporation into the acid-insoluble product by each fraction was proportional to the virus concentration. This is good evidence that the polymerase is associated with the virions and is not a contaminating activity.

Optimization of reaction conditions

The polymerase reaction showed a sharp dependence on the concentration of Mg^{2+} with maximum activity at 5 mM. Activities at other Mg^{2+} concentrations, expressed as a percentage of the optimal activity were: 1 mM, 12·5; 2 mM, 40; 3 mM, 70; 4 mM, 85; 6 mM, 80; 8 mM, 55; 10 mM, 40. The time course for a typical polymerase reaction is shown in Fig. 1. The [³H]UMP incorporation continued for up to 4 days but after this period no significant further reaction took place, even after addition of further nucleoside triphosphates. This is probably due to disruption of the virus particles which occurs after prolonged incubation at 30 °C.

Bentonite is often added to RNA polymerase reaction mixtures to inhibit contaminating or endogenous ribonuclease. In the case of the virus 38-4-A polymerase reaction, addition of bentonite at a concentration of 2 mg/ml had no effect on the [³H]UMP incorporation after an 18 h incubation indicating absence of ribonuclease activity in purified preparations of virus 38-4-A. Nevertheless, bentonite was included in standard incubations as a precaution against adventitious nuclease contamination.

Reovirion-associated RNA polymerase is activated by treatment of virions with various proteinases (Joklik, 1974). Therefore, proteinase k was added at several concentrations to reaction mixtures containing the virus 38-4-A, in the absence of bentonite and, after
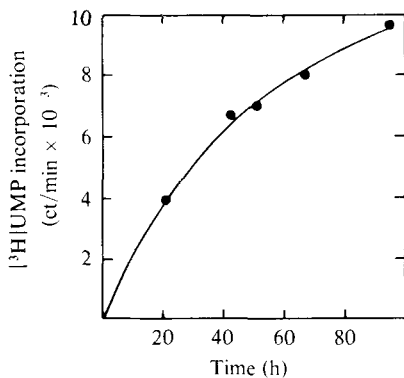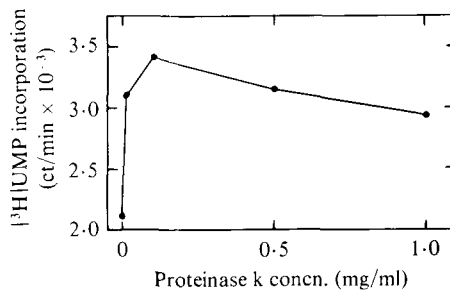
Fig. 1

Fig. 2

Fig. 1. Time-course of 38-4-A virus RNA polymerase reaction. A 200 μl RNA polymerase reaction mixture, containing 5 mM Mg^{2+}, [^{3}H]UTP (sp. act. 67 mCi/mmol) and 38-4-A virus at a final concn. corresponding to $A_{260} = 1$, was incubated at 30 °C; 10 μl samples were withdrawn at the times indicated and assayed for acid-insoluble radioactivity as described in Methods.

Fig. 2. Effect of proteinase k on 38-4-A virus RNA polymerase activity. The 25 μl RNA polymerase reaction mixtures, containing 5 mM Mg^{2+}, [^{3}H]UTP (sp. act. 83 mCi/mmol) and 38-4-A virus at a final concn. corresponding to $A_{260} = 1$, were incubated at 30 °C for 18 h and assayed for acid-insoluble radioactivity as described in Methods.

Table 1. *Properties of virion-associated RNA polymerases of* G. graminis *viruses*

Virus	Group*	Mol. wt. of dsRNA components* ($\times 10^{-6}$)	Mg^{2+} optimum† (mM)	[^{3}H]UMP incorporation‡ (ct/min $\times 10^{-3}$)
38-4-A	I	1·27, 1·19, 1·09	5	30
019/6-A	I	1·27, 1·19	6	140
01-1-4-A	I	1·22, 1·14	2	45
F6-C§	I	1·27, 1·19	5	3
3b1a-C	I	1·27, 1·19, 1·11 ⎫	5‖	63‖
3b1a-B	II	1·60, 1·54, 1·49, 1·45, 1·43 ⎬		
F6-B§	II	1·56, 1·45	5	22

* Data from Buck *et al.* (1981). A small amount of an additional dsRNA component (mol. wt. 1·49 $\times 10^6$) not previously detected, was found in 3b1a-B virus.

† Determined for 18 h polymerase reactions in a buffer containing 0·1 mM-EDTA (see Methods).

‡ Determined at the optimal Mg^{2+} concentration for 18 h, polymerase reactions with final virus concentration corresponding to $A_{260} = 1$ as described in the legend to Fig. 1. The specific activity of [^{3}H]UTP was 83 Ci/mmol.

§ Obtained from *G. graminis* F6-SM8 isolate.

‖ Determined with a mixture of the two viruses.

incubation for 18 h at 30 °C, [^{3}H]UMP incorporation into acid-insoluble product was measured by filter assay. The results (Fig. 2) showed that proteinase k could stimulate the reaction with a maximum increase in the amount of product formed of almost twofold at a proteinase k concentration of 0·1 mg/ml.

The rate of RNA synthesis catalysed by the virion-associated RNA polymerase of cytoplasmic polyhedrosis virus is considerably increased in the presence of *S*-adenosyl-methionine (Furuichi, 1974); a similar, but smaller effect has been observed with the reovirion-associated RNA polymerase (Faust & Millward, 1974). To examine the effect of *S*-adenosyl-methionine on the polymerase of 38-4-A virus, standard reaction mixtures containing 1 μM-, 10 μM-, 100 μM- and 1 mM-*S*-adenosyl-methionine were incubated at 30 °C for 16 h. In all cases the amount of labelled product synthesized was the same as that in similar reaction mixtures without *S*-adenosyl-methionine.
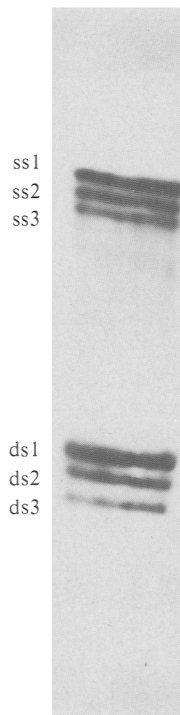
Fig. 3. Electrophoresis of 38-4-A virus RNA reaction products. 20000 ct/min of ^{3}H-labelled RNA extracted from an 18 h RNA polymerase reaction were subjected to electrophoresis in a 4% polyacrylamide–8 M-urea–0·1% SDS gel for 16 h at 5 V/cm. Radioactive bands were detected by fluorography. Abbreviations ss1, ss2 and ss3 denote the three ssRNA transcripts; ds1, ds2 and ds3 denote the three dsRNA template components. Electrophoresis was from top to bottom.

Analysis of reaction products

The RNA products of an 18 h standard polymerase reaction were isolated by phenol/SDS extraction and examined by electrophoresis in a 4% polyacrylamide–8 M-urea–0·1% SDS slab gel alongside virus dsRNA and glyoxalated denatured virus dsRNA markers. After staining of gels with toluidine blue, three new bands were detected which had migrated about half the distance of the three virus dsRNA bands. These new bands were shown to consist of ssRNA by their susceptibility to ribonuclease A in annealing buffer (see Methods) under conditions where virus dsRNA was stable. The three newly synthesized ssRNA components co-migrated with, and therefore have approx. the same length as, the three ssRNA markers, derived from virus dsRNA by denaturation and glyoxalation. This analysis makes the assumption that, in the 8 M-urea buffer employed for electrophoresis, the ssRNA transcripts had little secondary structure which might affect their mobilities. Fluorography of the gel (Fig. 3) showed that radioactivity had been incorporated into the three virus dsRNA components as well as into the ssRNA components.

In order to prove that the ssRNA product arises by transcription of the virus template dsRNA, the ^{3}H-labelled ssRNA product from a 4-day polymerase reaction was isolated by precipitation with 2 M-LiCl and shown to be free of dsRNA by electrophoresis in urea–SDS–polyacrylamide gels followed by fluorography. Aliquots of ^{3}H-labelled ssRNA were annealed with increasing amounts of denatured unlabelled virus dsRNA in hybridization buffer and the extent of hybridization was measured by the amount of label which remained insoluble after treatment with ribonuclease A. The proportion of product capable of
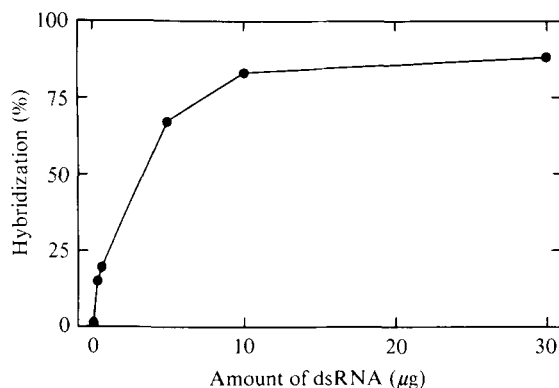
Fig. 4. Hybridization of 38-4-A virus RNA polymerase product to virus template RNA. [3H]-labelled, ssRNA product (2400 ct/min) was hybridized with increasing amounts of unlabelled virus dsRNA. Hybridization is expressed as the percentage of input ssRNA which becomes ribonuclease-resistant in the hybridization conditions described in Methods.

hybridizing to the virus template RNA increased with the amount of virus dsRNA and added up to about 90% (Fig. 4).

The annealing procedure was also used to determine whether one or both strands of the template dsRNA are transcribed *in vitro*. The [3H]-labelled ssRNA product was incubated in annealing conditions at a concn. of 50 μg/ml. Control experiments with denatured dsRNA showed that at this concentration, duplex formation would be expected to increase the ribonuclease resistance of the single-stranded product to 85%, if both strands were equally represented. However, only 1·6% of the incubated RNA remained acid-precipitable after ribonuclease digestion. Such a low value might be expected because of the secondary/tertiary structure of ssRNA and the results indicate that transcription occurs asymmetrically.

The amount of ssRNA product synthesized after a 4-day RNA polymerase reaction was estimated by electrophoresis of total product RNA in urea–SDS–polyacrylamide tube gels. After staining the gels with toluidine blue, six bands (three corresponding to virus dsRNA and three of newly synthesized ssRNA) were clearly resolved. A scan of a typical gel is shown in Fig. 5. Measurement of peak areas, in comparison with similar gels containing ssRNA and dsRNA standards of known concentration, or measurement of the [3H]UMP incorporation into ssRNA (assuming a UMP content of 25%) indicated that a weight of ssRNA approx. equal to that of the template dsRNA had been synthesized, i.e. on average, two rounds of transcription per dsRNA molecule had occurred. The ratios of the areas of the three ssRNA bands (ss1:ss2:ss3) and those of the three dsRNA bands (ds1:ds2:ds3) were similar, implying that each of the three component virion dsRNAs was transcribed with approx. equal efficiency. Scans of gels following electrophoresis of unreacted virus dsRNA also gave similar ratios of the three bands, indicating that the relative amounts of the three virus dsRNA components had not changed during the reaction.

In order to estimate the [3H]UMP incorporation into virus dsRNA during a polymerase reaction, total RNA from a 4-day reaction was isolated by phenol extraction and ssRNA was precipitated with 2 M-LiCl. The dsRNA was recovered from the supernatant by ethanol precipitation and shown to be free from ssRNA by gel electrophoresis and by its resistance to ribonuclease A in hybridization buffer. The amount of [3H]UMP incorporated into the total virus dsRNA was, on average, approx. 180 pmol per pmol of dsRNA. This is equivalent to the synthesis of 40% of one strand per dsRNA molecule, making the assumption that the newly synthesized strand contains 25% UMP.
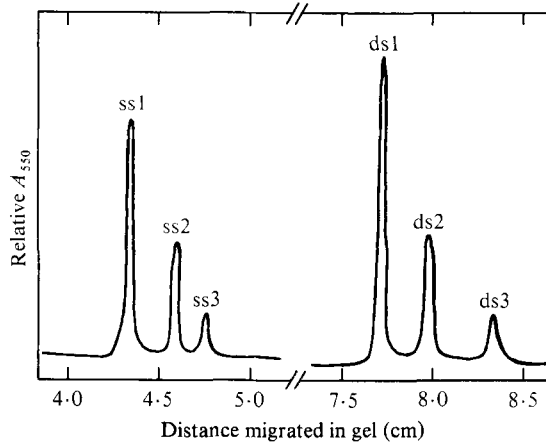
Fig. 5. Gel scan of 38-4-A virus RNA polymerase products. RNA products from a 4-day RNA polymerase incubation were subject to electrophoresis in a 4% polyacrylamide–8 M-urea–0·1% SDS gel at 6 V/cm for 16 h. The gel was then stained with toluidine blue and scanned at 550 nm. The direction of electrophoresis was from left to right. The ss1, ss2 and ss3 denote the three ssRNA transcripts and ds1, ds2 and ds3 denote the three dsRNA template components.

RNA polymerases of other G. graminis viruses

Several other purified *G. graminis* viruses, belonging to both groups I and II, were found to have RNA polymerase activities similar to that of virus 38-4-A when incubated at 30 °C in polymerase reaction mixtures containing [³H]UTP. All the reactions were totally dependent on the presence of Mg ions. The optimal Mg^{2+} concentrations for these reactions and the amounts of RNA synthesis as measured by [³H]UMP incorporation into acid-soluble product in 18 h incubations are given in Table 1. Included in this table, for reference, are the mol. wt. of the dsRNA components of each virus. In all cases the RNA polymerase reactions continued for at least 4 days with time courses similar to that of the 38-4-A virus polymerase reaction (Fig. 1).

For each virus the product RNA was extracted from a 4-day polymerase reaction and analysed by electrophoresis in urea–SDS–polyacrylamide gels. In each case fluorography of the gel indicated that new bands of RNA, one for each virus dsRNA molecule but with lower mobilities than the dsRNA components, had been formed. The newly synthesized RNA components were shown to be single-stranded and to have mol. wt. of one-half of those of the corresponding dsRNA components by using the methods described for 38-4-A virus transcripts. The ³H-labelled ssRNA products were isolated by precipitation with 2 M-LiCl and aliquots were annealed with increasing amounts of virus dsRNA, as described in the legend to Fig. 3. In each case curves similar to that shown in Fig. 3 were obtained with more than 90% of the ³H-labelled ssRNA product hybridizing with its template dsRNA at saturation. Little self-annealing could be detected in the absence of added denatured dsRNA. It was concluded that for each virus the products of polymerase reaction are full-length ssRNA transcripts of one of the strands of each of the virus dsRNA components.

Fluorography of gels following electrophoresis of the RNA product of the virus RNA polymerase reactions showed that, as with 38-4-A virus, all the dsRNA bands of each of the viruses listed in Table 1 had become labelled with tritium. When the RNA samples were digested with ribonuclease A prior to electrophoresis, under conditions where the ssRNA products were completely degraded (see Methods), the label in the dsRNA bands was unchanged, showing that [³H]UMP had become incorporated into the double-stranded structure.
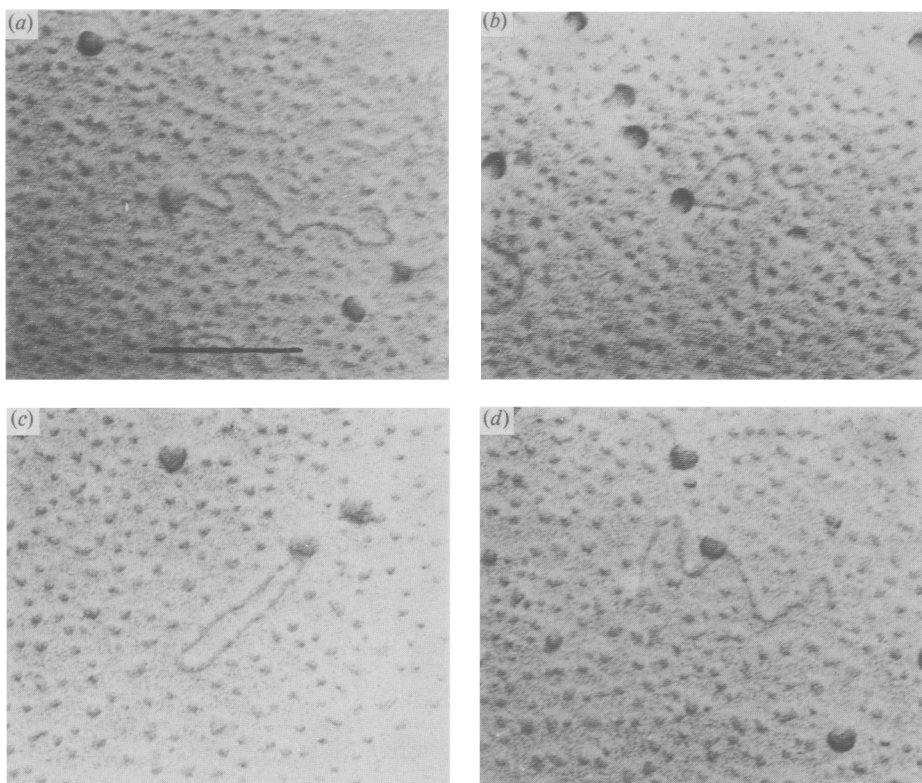
Fig. 6. Electron microscopy of transcribing particles of viruses 3b1a-B and 3b1a-C. Particles showed (*a*) one linear RNA strand; (*b*) a short RNA loop; (*c*) a long RNA loop; (*d*) two linear RNA strands. The bar marker represents 200 nm in all cases.

In order to compare the time course of [³H]UMP incorporation into virus dsRNA and ssRNA, an RNA polymerase reaction mixture containing viruses 3b1a-B and 3b1a-C and [³H]UTP was incubated at 30 °C and aliquots were withdrawn after 30 s, 2·5 h, 8 h, 24 h, 72 h and 144 h. RNA was prepared from each aliquot and analysed by electrophoresis in a urea–SDS–polyacrylamide slab gel, followed by fluorography. Incorporation of [³H]UMP could be detected in the dsRNA components of both viruses after 30 s reaction and was maximal after 8 h, showing no further increase in the 24 h, 72 h and 144 h samples. On the other hand, bands of ssRNA were just detectable in the 2·5 h sample and their densities showed a steady increase in amount with time up to 144 h, becoming approx. equal to those of the dsRNA after 24 h, and greater than those of the dsRNA in the 72 h and 144 h samples.

Electron microscopy of transcribing virus particles

Nothing is known concerning the mode of release of ssRNA transcripts from virions of any dsRNA mycovirus. In order to obtain some information on this aspect, transcribing particles of viruses 3b1a-B and 3b1a-C were examined by electron microscopy after a 5 min RNA polymerase reaction. At this stage of the reaction only 101 particles out of 1106 particles examined (9%) appeared to be releasing transcripts. This could indicate that initiation of transcription occurred asynchronously, or that only a small proportion of the particles released their transcripts or carried an active polymerase. No transcripts were observed if particles were incubated in the absence of nucleoside triphosphates. At a later stage of the reaction (15 min) the presence of many released transcripts mixed with virions made it

impossible to determine if this proportion had altered. The ssRNA nature of the transcripts being released was confirmed by their absence if reaction mixtures, after 5 min or 15 min, were treated with RNase A under conditions where dsRNA is stable (see Methods) prior to electron microscopy.

Of the 101 particles showing release of transcripts after a 5 min polymerase reaction, 35 particles showed the release of a single linear ssRNA molecule (Fig. 6a), which varied in length between 20 nm and 720 nm, but with a distinct mode around 560 nm. A significant number (28) of particles showed the release of looped ssRNA molecules, both ends of which remained attached to the particle (Fig. 6b, c); the loops varied in length from about 20 nm to 500 nm with a fairly even distribution and no obvious modes. Additionally, 38 particles showed what appeared to be two ssRNA molecules attached or else one molecule attached at the middle (Fig. 6d). The lengths of the two arms were similar in some cases and different in others; the combined lengths ranged from about 30 nm to 720 nm with a continuous distribution of lengths. The length of complete transcripts from template dsRNA molecules of 3b1a viruses B and C (mol. wt. 1×10^6 to $1 \cdot 6 \times 10^6$) would be expected to be in the range 490 nm to 780 nm, assuming the value of $1 \cdot 05 \times 10^6$ mol. wt. ssRNA/μm found for reovirus transcripts spread under the same conditions (Bartlett *et al.*, 1974). The modal length of the single linear strands indicates that these correspond to complete or nearly complete transcripts.

Absence of detectable RNA polymerase activity in G. graminis group III viruses

When F6-A virus, a group III virus with two dsRNA components, mol. wt. $4 \cdot 3 \times 10^6$ and $3 \cdot 2 \times 10^6$, was incubated in an RNA polymerase reaction mixture containing [³H]UTP for 72 h, no incorporation of [³H]UMP into acid-soluble material could be detected. Moreover, after analysis of RNA extracted from such an incubation by electrophoresis in a urea–SDS–polyacrylamide slab gel and fluorography, neither any new bands of RNA, nor incorporation of ³H-label into the two virus dsRNA bands, could be detected.

Purified samples of 3b1a-A virus, the only other known group III virus, which has two dsRNA components, mol. wt. $4 \cdot 1 \times 10^6$ and $3 \cdot 5 \times 10^6$, contained traces of 3b1a-B virus. When such samples were incubated in RNA polymerase reaction mixtures at 30 °C and analysed as above, low levels of [³H]UMP incorporation into the ssRNA transcripts and dsRNA components of 3b1a-B virus were detected, but no labelled transcripts or dsRNA components of 3b1a-A virus could be detected. Various attempts to activate the 3b1a-A virus polymerase were made by addition or subtraction of substances from the reaction mixtures. Table 2 shows the effects of these alterations on the incorporation of [³H]UMP into acid-soluble product. Many of these alterations reduced the [³H]UMP incorporation, presumably by inhibiting the low level of reaction due to 3b1a-B virus. In the few cases where a small increase in ³H incorporation was observed (+chymotrypsin at 200 μg/ml; −actinomycin D; 10 × normal NTP concn.), the RNA products were analysed by gel electrophoresis and fluorography but no labelled RNA bands, other than those produced from 3b1a-B virus, could be detected. In another experiment, mixtures of viruses 3b1a-A, 3b1a-B and 3b1a-C in approx. equal amounts were incubated for 6 h in RNA polymerase reaction mixtures to which had been added 1 μM-, 10 μM-, 100 μM- and 1 mM-*S*-adenosyl-methionine. No change in the ³H-incorporation of any of these incubations, compared with controls without *S*-adenosyl-methionine, was observed.

It was considered possible that the absence of detectable polymerase activity in the group III viruses could be due to their instability at the temperature of the RNA polymerase incubation (30 °C). Some support for this hypothesis was obtained by the observation that after 2·5 h incubation in an RNA polymerase reaction at 30 °C free dsRNA molecules of virus 3b1a-A were released into the supernatant, after removal of bentonite by centrifugation

Table 2. *Attempts to activate the putative RNA polymerase of virus 3b1a-A by alterations to the standard reaction mixture*

Alteration	^{3}H-incorporation* (ct/min)
+ 0·5% Nonidet	128
+ 0·5% Nonidet + 2 mM-DTT	75
+ 2 mM-DTT	173
+ Proteinase k (50 μg/ml)	759
+ Proteinase k (200 μg/ml)	870
+ Chymotrypsin (50 μg/ml)	950
+ Chymotrypsin (200 μg/ml)	1320
+ 10% (v/v) Glycerol	120
+ 10% (w/v) Bovine serum albumin	550
− Actinomycin D	1530
− Bentonite	639
10 × NTP concn.†	1580
2 mM-Magnesium acetate‡	1044
8 mM-Magnesium acetate‡	830
12 mM-Magnesium acetate‡	178
Standard reaction mixture §	1126

* Acid-insoluble product after a 72 h incubation (sp. act. of [^{3}H]UTP in Ci/mmol).
† Final specific activity of [^{3}H]UTP was the same as in all the other incubations.
‡ Final concn.
§ Mg^{2+} concn. was 5 mM.

(intact virions bind strongly to bentonite). Since the virus is known to be stable at 4 °C, an RNA polymerase reaction containing a mixture of viruses 3b1a-A, 3b1a-B and 3b1a-C and [^{3}H]UTP was incubated at 4 °C for 21 days and then the RNA products were analysed by gel electrophoresis and fluorography. Although labelled ssRNA transcripts and dsRNA molecules of viruses 3b1a-B and 3b1a-C could be detected after this prolonged cold incubation, no new products corresponding to virus 3b1a-A were found.

DISCUSSION

The results show that the RNA polymerases associated with virions of *G. graminis* viruses from both groups I and II are transcriptases, which are able to catalyse the synthesis of ssRNA copies of one of the strands of each of the virus template dsRNA molecules. In the case of 38-4-A virus two rounds of transcription occurred, on average, per dsRNA molecule after a 4-day incubation, indicating that re-initiation of the reaction can occur in the *in vitro* system. Comparison of the [^{3}H]UMP incorporation into product RNA by 38-4-A virus with that of the other *G. graminis* viruses in Table 1 indicated that this was true for most of the other viruses examined. The most active polymerase was that of 019/6-A virus with [^{3}H]UMP incorporation corresponding to about eight rounds of transcription after a 4-day incubation. The rate of transcription in individual particles may be much greater however. Full-length ssRNA transcripts of the dsRNA components of 3b1a-B and 3b1a-C viruses were detected by gel electrophoresis and fluorography after a 2·5 h reaction and by electron microscopy after a 15 min reaction. For all viruses the time course of the polymerase reactions (e.g. Fig. 1) showed that the initial rate of reaction diminished rapidly and this was probably due to failure of transcripts to be released and/or failure of transcription to re-initiate in many particles.

The detection of transcribing particles with looped RNA molecules, which had a continuous range of lengths from very short to near full-length transcripts (Fig. 6b, c), suggests that a loop of ssRNA, rather than the 5′ end, may initiate the extrusion of the transcript from the virion. Similar loops have been observed by electron microscopy of

transcribing cores of reovirus and it has been suggested that the 5′ end of the transcript may remain associated with a site in the internal region of the spike, through which it is released, and that the first end to be set free outside the particle might be the 3′ OH end (Bartlett *et al.*, 1974). A similar mechanism could operate with the *G. graminis* viruses.

The absence of detectable RNA polymerase activity *in vitro* in either of the two group III viruses could indicate that *in vivo* these viruses may utilize the RNA polymerases encoded by the group I and II viruses (3b1a-C and 3b1a-B and F6-C and F6-B) which are found together with viruses 3b1a-A and F6-A in the fungal isolates 3b1a and F6 respectively. However, since the genomes of the group III viruses are larger than those of the group I and group II viruses, it is more likely that these viruses do indeed encode their own RNA polymerases and that these become inactivated during the virus purification procedure.

An interesting feature of all the polymerase reactions of the *G. graminis* group I and II viruses is that [^{3}H]UMP was incorporated into the template dsRNA components as well as into the ssRNA transcripts. In the case of 38-4-A virus this [^{3}H]UMP incorporation corresponded to about 40% of one strand per dsRNA molecule (or one complete strand in 40% of the dsRNA molecules). Analysis of polymerase reaction products of the other viruses by gel electrophoresis and fluorography indicated similar levels of [^{3}H]UMP incorporation into dsRNA. It is clear that these substantial levels of UMP incorporation cannot be accounted for by 'filling up' of ssRNA tails on predominantly dsRNA molecules by a ss→dsRNA polymerase. The gel electrophoretic mobilities of the dsRNA components of all the *G. graminis* viruses studied were unchanged following incubation with ribonuclease A under conditions where ssRNA is degraded, indicating the absence of all except possibly very short ssRNA tails. Two other possibilities which could explain the [^{3}H]UMP incorporation into dsRNA by the *G. graminis* virion-associated polymerases are as follows. (i) Transcription could occur by a semi-conservative mechanism, so that the ssRNA strand released represents a displaced strand of the parental dsRNA and the newly synthesized strand becomes part of the dsRNA duplex. Such a mechanism has been shown to operate in the case of RNA polymerases associated with virions of an *Aspergillus foetidus* virus, virus AfV-S (Ratti & Buck, 1978, 1979) and of bacteriophage ϕ6 (Van Etten *et al.*, 1980). (ii) Semi-conservative replication of dsRNA within a proportion of the virions could occur giving rise to diploid virions, as has been shown for *Penicillium stoloniferum* virus PsV-S (Buck, 1975, 1978).

The substantial incorporation of [^{3}H]UMP into dsRNA by the virion-associated RNA polymerases of the *G. graminis* group 1 and II viruses clearly distinguishes these polymerases, like those of AfV-S and phage ϕ6, from the virion-associated RNA polymerase of reovirus, which catalyses conservative transcription of dsRNA into which no detectable incorporation of labelled nucleotide precursor occurs during the reaction (Banerjee & Shatkin, 1970). It is possible that the *G. graminis* virus polymerase reactions could also be different from the transcription reaction, catalysed by an RNA polymerase associated with *Saccharomyces cerevisiae* virus ScV, during which little or no incorporation of labelled precursors into dsRNA occurs (Herring & Bevan, 1977; Welsh & Leibowitz, 1980; Bruenn *et al.*, 1980). The dsRNA mycoviruses occur in great variety and it is likely that several virus families will be needed in order to accommodate them all. Mycovirus dsRNA mol. wt. range from $0 \cdot 27 \times 10^6$ to $6 \cdot 3 \times 10^6$, polypeptide mol. wt. range from 25 000 to 130 000 and dsRNA components may be monocistronic or polycistronic (Buck, 1980). It would not therefore be surprising to find differences in the virion-associated RNA polymerases of individual dsRNA mycoviruses.

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