Isolation and Characterization of a Double-Stranded Ribonucleic Acid from *Penicillium chrysogenum*

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Double-stranded ribonucleic acid has been obtained from cells of the fungus *Penicillium chrysogenum*. This ribonucleic acid appears to be associated with mycophage and is an efficient inducer of interferon. Its extraction and partial purification are discussed, and evidence for its double-stranded and ribosomal nature is reviewed. The implications of viral nucleic acid in the life processes of fungi are considered.

Evidence for the occurrence of virus in species of *Penicillium* has been obtained independently in several laboratories (2-4, 11, 19). There is now considerable evidence that the component nucleic acid of these viruses is double-stranded ribonucleic acid (dsRNA), a compound of potential therapeutic value recognized for its ability to induce interferon (3, 20, 22).

In an attempt to isolate deoxyribonucleic acid (DNA) from mycelia of *P. chrysogenum* Thom, we inadvertently obtained a highly polymerized RNA. This material appears to be double-stranded because it resists ribonuclease treatment, contains equal molar ratios of purine to pyrimidine, and exhibits thermal melting and an increased absorbancy ($\lambda_{\max}$, 256 nm) after heating and rapid cooling.

This nucleic acid may well be associated with the virus particles reported in *P. chrysogenum* (2), for it induces interferon activity in mice. Our yields of this RNA species have been appreciable, approximately 100 μ g per g of cells, and depend upon treatment of mycelial macerates with protease.

MATERIALS AND METHODS

Growth medium. *P. chrysogenum* (NRRL 1951) was grown in a minimal medium containing: purified lactose, 36 g; glucose, 27 g; $(\text{NH}_4)_2\text{SO}_4$, 7.5 g; KH_2PO_4 , 0.15 g; K_2HPO_4 , 0.21 g; CaCO_3 , 10 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 90 mg; $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$, 20 mg; CaCl_2 , 30 mg; $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 4 mg; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 30 mg; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 2 mg; and distilled water, 1,000 ml. Mycelia were harvested after 5 days of growth at 25 C from a spore inoculum of 10^6 cells per 100 ml of medium.

Ribonucleic acids were labeled with tritium by the addition of uracil-5-T (Amersham/Searle, 1,000 mCi/mmol) to basal medium at a concentration of 5 μ Ci/ml. Incorporation of label was determined with a Packard Tri-Carb scintillation spectrometer (model

3380) at about 20% counting efficiency. The scintillation medium was 700 ml of toluene, 300 ml of ethoxy-ethyl alcohol, 4 g of 2,4-diphenyloxazole, and 0.6 g of 1,4-bis-[2-(4-methyl-5-phenyloxazolyl)]-benzene.

Preparation of mycelial macerates. Cells (50 g) were washed twice with distilled water and once each with standard saline citrate (SSC; 0.15 M NaCl plus 0.015 M trisodium citrate, pH 7.0 to 7.2) and saline ethylenedinitrilotetraacetic acid [EDTA; 0.15 M NaCl plus 0.1 M EDTA (tetrasodium), pH 8]. Cells were washed successively in 95% ethyl alcohol, acetone-ethyl alcohol (1:1), and acetone. Acetone-washed cells were dried in vacuo and then ground in a pre-cooled mortar for 5 min in the presence of a 5-cm cube of dry ice and a teaspoonful of 0.2-mm glass beads.

Extraction of ribonuclease-resistant nucleic acids. Macerates were suspended in 15 ml of saline-EDTA with 750 μ g of protease (Sigma Chemical Co.; type V) and were incubated for 1 hr at 42 C. Subsequent procedure followed closely that of Marmur (23) for the extraction of DNA, but final precipitation with isopropanol, suggested by Marmur's method, was omitted. Ribonuclease treatment (Sigma Chemical Co.; type IA) was repeated before final deproteinization and extraction. Nucleic acid was precipitated with 95% ethyl alcohol and dried in vacuo.

Purification of dsRNA. Procedures for fractionation of nucleic acids in salt of high molarity have been outlined recently (14). These procedures are selective and can be used to enrich for dsRNA. Accordingly, ribonuclease-resistant nucleic acids from *Penicillium* were dissolved in 1 M NaCl, and this solution [optical density at 260 nm (OD_{260} , 100)] was frozen at -20 C. The solution was slowly thawed at 1 C, and any precipitate that formed was removed by centrifugation and discarded. The remaining nucleic acids, soluble in 1 M NaCl, were precipitated with two volumes of ethyl alcohol and examined for RNA content.

Frequently, minor amounts of DNA were recovered in addition to RNA from *Penicillium* cells. DNA was removed from a sample by overnight treatment with

100 μ g of deoxyribonuclease per ml (Sigma Chemical Co.; type I) at 25 C in 0.01 M magnesium acetate. Reprecipitation of RNA was improved by adjusting the molarity of the deoxyribonuclease-treated sample to that of SSC with concentrated SSC ($10\times$ SSC).

Samples of RNA, after ribonuclease treatment and deproteination, were equilibrated by dialysis to 0.2 M NaCl (pH 7) and applied to a column (1 by 7 cm) of benzooylated diethylaminoethyl (DEAE) cellulose (26) to separate the oligonucleotides of hydrolyzed RNA from ribonuclease-resistant RNA. The column was eluted by means of a linear gradient from 0.2 to 1.0 M NaCl.

Determination of double-strandedness: thermal denaturation. Nucleic acids were heated from ambient temperature to 100 C and then quenched either by rapid cooling or with 1.5% formaldehyde (12). Critical thermal melting, T_m , was determined according to the method of Marmur and Doty (24) in a thermostatically controlled Beckman spectrophotometer.

Determination of double-strandedness: ribonuclease resistance. Samples of salmon sperm DNA (Calbiochem; 2620) and yeast RNA (Calbiochem; 55714), as well as nucleic acid from *P. chrysogenum*, were treated with the following commercial nucleases: deoxyribonuclease type I (2,400 Klett units/mg) and ribonuclease-A (bovine pancreatic, 90 Klett units/mg) from Sigma Chemical Co.; ribonuclease RAF (3,000 units/mg), ribonuclease T_1 (300,000 units/mg), and micrococcal nuclease (6000 units/mg) from Worthington Biochemical Corp. Enzymatic activity was determined spectrophotometrically at 260 nm. Treatment with deoxyribonuclease was in 0.1 M acetate buffer (pH 5) plus 0.001 M $MgSO_4$ at 25 C. Ribonuclease treatment was in either SSC or dilute SSC (0.1 SSC) at 37 C.

Analysis for base composition. Bases were hydrolyzed from nucleic acids with 70% perchloric acid at 100 C for 60 min. Hydrolysates were developed overnight on cellulose thin-layer chromatography plates (E. Merck; 5757) in isopropanol-HCl-water (65:16:19) and were examined directly for ultraviolet absorbance. Zones corresponding to standard bases were eluted into 0.1 N HCl and quantified spectrophotometrically with a Beckman DB-G spectrophotometer according to methods discussed by Bendich (5).

Guanine composition of nucleic acids was determined after selective depurination with 0.1 N HCl and dialysis according to a procedure developed by Huang and Rosenberg (17) for DNA analysis. Their method and formula [$x_G = (13.1 - 5.0R)/(5.8 + 1.9R)$, where $R = A_{265}/A_{280}$] are assumed to be applicable to an analysis of dsRNA.

Determination of the sugar moiety. Nucleic acids were examined for differential staining with orcinol (25) and diphenylamine (9). Further identification of sugars was attempted by thin-layer chromatography. Nucleic acids were autoclaved for 15 min in 1 N HCl. Hydrolysates were chromatographed on thin-layer chromatography plates in isopropanol-acetic acid-water (3:1:1) and were assayed for a ribose or deoxyribose content with aniline-diphenylamine reagent (6, 27).

Assay for interferon induction. Nucleic acids from *P. chrysogenum* and other commercially prepared samples of nucleic acid were evaluated according to procedures developed for assay of antiviral activity by statolon (21). Mice were injected intraperitoneally with graded concentrations of nucleic acid and sacrificed 18 hr thereafter. Interferon titers in sera were determined by a plaque-reduction method in murine tissue cultures infected with vesicular stomatitis virus.

RESULTS

The RNA characterized in this paper was obtained by use of Marmur's (23) classical procedure for the extraction of DNA. Yields of the nucleic acid were enhanced by certain modifications of Marmur's basic method. Two principal modifications were cell disruption by grinding acetone-dried mycelia in the presence of dry ice and preincubation of mycelial macerates with protease. As much as 0.1 mg per g of cells or 0.01% of the mycelia of *P. chrysogenum* has assayed as a dsRNA.

This nucleic acid has an absorption maximum of 256 nm and a ratio of extinction, $E_{260/280}$, of 2.39 (Fig. 1). It is fibrous and white, and superficially it resembles highly polymerized DNA. To date, the most satisfactory preparations have indicated 1 unit OD₂₆₀ per 70 μ g of material.

Samples examined for sedimentation coefficient at 200,000 $\times$ g centrifugation had an estimated average molecular weight of 10^6 daltons, with an approximate $S_{25,SSC}^0$ of 12. The dsRNA banded with a buoyant density of 1.61 after Cs_2SO_4 density gradient centrifugation. The RNA prepared in this manner was examined for ultrastructure

FIG. 1. Absorption profile of double-stranded RNA from *P. chrysogenum* in standard saline-citrate.

according to the procedure of Kleinschmidt and Zahn (18; Fig. 2).

DNA has been effectively removed from preparations of the dsRNA by deoxyribonuclease treatment. Absence of DNA has been indicated by the diphenylamine assay.

The dsRNA from *Penicillium* is subject to thermal denaturation. The critical T_m of this material in SSC was in excess of 100 C and in 0.1 SSC was 92 C (Fig. 3). The nucleic acid, when heated at 100 C and then cold quenched, elicited hyperchromicity ranging from 11 to 25% (Fig. 4).

Formaldehyde is known to react with nucleic acids, presumably with the free amino groups of individual strands (12). This reaction results in increased absorbancy at 260 nm and concomitantly shifts maximal absorption to a longer wavelength. Yeast RNA incubated with formaldehyde at ambient temperature exhibits such changes in absorption, but dsRNA from *Penicillium* was relatively nonreactive with formaldehyde at room temperature (Fig. 5). The dsRNA reacted with formaldehyde when the mixture was heated, and the kinetics of this reaction suggested thermal melting (Fig. 6). The formaldehyde

quenching effectively lowered the temperature of denaturation of the RNA by approximately 20 C.

Resistance of the dsRNA to pancreatic ribonuclease was first indicated after extraction. Resistance to various other commercial nucleases has also been demonstrated (Table 1). Ribonu-

FIG. 3. Thermal denaturation curves for double-stranded RNA in standard saline-citrate (●) and dilute saline-citrate (○).

FIG. 2. Molecules of the double-stranded RNA estimated to weigh in the order of 10^6 daltons obtained after isopycnic and density-gradient (Cs_2SO_4) centrifugation (44,000 rev/min). Molecules have an average contour length of 0.5 μm . $\times 40,000$. (Courtesy of L. F. Ellis.)

FIG. 4. Hyperchromicity of double-stranded RNA in dilute saline-citrate heated for 20 min at 100 C and rapidly cooled. (○) Initial sample; (●) treated sample.

FIG. 5. Formaldehyde-RNA complexing at ambient temperature (32 C). (○) Initial sample in 0.01 M NaCl, 0.005 M MgCl₂ and tris(hydroxymethyl)-aminomethane buffer (0.01 M, pH 7.0); (●) sample incubated 4 hr in presence of 1.5% formaldehyde; (▲) sample incubated 8 hr in presence of 1.5% formaldehyde.

lease resistance at an enzyme concentration of 1 μg/ml at 25 C in SSC proved to be below the limits of detection, even after 16 hr of treatment. Deoxyribonuclease also failed to act on this nucleic acid.

Base ratio analyses indicated the presence of four bases, including uracil (λ_{max} , 259 nm). Perchloric acid hydrolysates assayed for molar excesses of cytosine and guanine (Table 2). Specific analysis for guanine composition, based on selective depurination (17), indicated approximately 30% guanine.

The dsRNA, when hydrolyzed in the presence of orcinol and diphenylamine reagents, was significantly orcinol-positive and diphenylamine-negative (Table 3). Mild acid hydrolysates were

FIG. 6. Effect of formaldehyde on thermal denaturation of double-stranded nucleic acids. (▲) DNA in standard saline-citrate; (●) double-stranded RNA in standard saline-citrate; (○) double-stranded RNA in dilute saline-citrate.

TABLE 1. Effect of selected nucleases on double-stranded RNA from *Penicillium chrysogenum*

Nuclease	Hyperchromicity (OD ₂₆₀) ^a					
	Yeast RNA		Salmon sperm DNA		Double-stranded RNA	
	0.1 SSC	SSC	0.1 SSC	SSC	0.1 SSC	SSC
Ribonuclease (bovine pancreatic), 10 μg/ml, 37 C, 4 min	0.207	0.059	0	0	0.068	0
Ribonuclease T ₁ , 1 μg/ml, 37 C, 15 min	0.400	0.082	0.290	0.147	0.170	0
Micrococcal nuclease ^b , 10 μg/ml, 37 C, 30 min	>1.0		>1.0		0	
Deoxyribonuclease (bovine, pancreatic) ^c , 100 Klett units/ml, 25 C, 10 min	0.019		0.360		0	

^a DNA substrate represents an OD₂₆₀ of 5.0/ml of reaction mixture; RNA and double-stranded RNA substrates represent an OD₂₆₀ of 1.0/ml of reaction mixture.

^b Enzyme and substrate combined in 0.005 M CaCl plus 0.05 M borate buffer, pH 8.8.

^c Enzyme and substrate combined in 0.005 M MgSO₄ plus 0.1 M acetate buffer, pH 5.0.

TABLE 2. *Base composition of double-stranded RNA from Penicillium chrysogenum*

Fraction	Mole percentage of			
	Uracil	Cytosine	Adenine	Guanine
I	21.5	30.3	21.7	26.5
II	20.8	26.2	23.6	29.4
Average	21.2	28.2	22.6	28.0

chromatographed, and a sugar corresponding to ribose was detected with aniline-diphenylamine reagent.

Alkaline hydrolysis of the double-stranded nucleic acid indicated a polyribonucleotide (Fig. 7).

The dsRNA is a potent inducer of interferon. Interferon titers in excess of 1,000 units were observed with a 10 μ g (OD₂₆₀ = 0.2/ml) dosage of nucleic acid per mouse. Based on these data, the dsRNA from *P. chrysogenum* would appear to be at least equivalent in potency to other recognized inducers of interferon (3, 21, 22). As a control, commercial samples of DNA and yeast RNA were tested at a concentration of 60 μ g/ml for stimulation of interferon, but these nucleic acids were inactive.

The dsRNA can be inactivated as an inducer of interferon by excessive treatment with ribonuclease; i.e., 50 μ g/ml concentration of enzyme in 0.1 SSC and incubation at 37 C for 18 hr.

Ribonucleic acids from *Penicillium* treated with 10 μ g of ribonuclease per ml at 25 C for 1 hr were loaded onto a column of benzoylated DEAE cellulose and eluted with a salt gradient (Fig. 8). A series of 12 fractions (3 ml) were collected. These were evaluated for absorption at 260 nm, content of ³H-uracil, and potential for interferon induction. A major absorption peak, fractions 5-8, coincided with the uracil label and potential for interferon induction. Fraction 6 of this peak was examined for thermal denaturation (Fig. 3). A 10- μ g dosage of RNA from this fraction induced 2,000 units of interferon.

DISCUSSION

The RNA isolated from *P. chrysogenum* and characterized in this paper is of presumed viral origin and is thereby regarded as a nonessential

co-metabolite. This nucleic acid has been characterized as a highly polymerized dsRNA, a type of nucleic acid not recognized as a normal constituent of the eukaryotic cell. The distribution of dsRNA is limited to very few phage genomes, and various *Penicillium* species have been shown to harbor such phages (2-4, 11, 19).

The significance of mycophage and of dsRNA in *P. chrysogenum* is not now apparent. The nucleic acid is present in large quantity and would constitute extrachromosomal genetic material. Accordingly, the presence of virus might be ex-

FIG. 7. Basic hydrolysis of RNA (37 C, 18 hr) in standard saline-citrate adjusted from pH 8 to 12 with NaOH. (○) Yeast RNA; (●) double-stranded RNA.

FIG. 8. Benzoylated DEAE cellulose chromatography of double-stranded RNA from *Penicillium* after ribonuclease treatment.

TABLE 3. *Differential staining of double-stranded RNA from Penicillium chrysogenum with orcinol and diphenylamine reagents*

Nucleic acid	Orcinol index (OD ₆₆₀ /OD ₂₆₀) [*]	Diphenylamine index (OD ₆₆₀ /OD ₂₆₀)
Salmon sperm DNA.....	0.032/0.110 = 0.290	0.292/0.520 = 0.562
Yeast RNA.....	0.173/0.180 = 0.963	0.011/1.360 = 0.0081
Double-stranded RNA.....	0.304/0.240 = 1.27	0.040/1.600 = 0.025

pected to influence profoundly the biology of *Penicillium*.

Strains of *P. chrysogenum* have been recognized as unstable (1). Strains sector for growth rate, sporulation efficiency, spore coloration, and antibiotic productivity, but generalized or predictable lysis of cultures has not been observed. Initial detection of mycophage in *Penicillium* was indirect and dependent upon dsRNA content of statolon (11, 20).

Viruses may eventually prove to be widely distributed among the fungi, but their detection in the absence of lysis will remain difficult. Knowledge of mycophages is admittedly limited (16). Several fungi have been implicated as vectors in the transmission of viruses pathogenic to higher plants (13). Among nonpathogenic fungi, the evidence for virus has been principally electron microscopic (11, 15). Special emphasis has been given to the viruslike particles of *Penicillium* species. These have been isolated, and dsRNA has been obtained from them (3, 20). To date, neither transmission nor replication of a fungal phage has been demonstrated, and little is known regarding the influence of virus on fungal metabolism.

At this time, it seems appropriate to review the subject and make certain predictions concerning an association between fungus and phage. First, and foremost, the phages of *Penicillium* do not appear to be virulent, but they are well formed, with a morphology not indicative of prophage. L. F. Ellis of the Lilly Laboratories examined virus from *P. chrysogenum* (Fig. 9) and estimated that 10^8 particles are present per g of mycelia. These particles are isometric, polygonal,

35 nm in dimension. The viral particles of *P. chrysogenum* are somewhat larger but otherwise comparable in morphology to those of *P. stoloniferum* (11, 19).

Second, mycophages appear to be more symbiotic than lytic, a feature that might be expected of a fungal phage. Extensive lysis would not be required for viral transmission in fungi. The fungi, including many imperfect fungi, exhibit heterokaryosis. Formation of the heterokaryon involves localized lysis between adjacent and compatible cells. Specific mycophages might determine the frequency and specificity of this heterokaryotic process. The viruses from different species of *Penicillium* have now been shown to be antigenically specific (3). Their specificity in transmission remains to be demonstrated.

Finally, the large amount of dsRNA isolated from *P. chrysogenum* suggests that a sizable, extraparticulate pool of this nucleic acid is present in the mold. This pool probably represents the replicative form of viral nucleic acid. Such nucleic acid, present in the homologous cytoplasm of the mold, might further excode for transcription and ultimately the synthesis of secondary metabolites. Accordingly, antibiotic synthesis in *P. chrysogenum* could be related to or influenced by the presence of mycophage. We have, thus far, been unable to obtain a strain of *P. chrysogenum* cured of virus, although such strains have been reported in other species of *Penicillium* (3, 4). Strains of *P. stoloniferum* lacking virus are apparently deficient for galactosamine content of cell wall (8).

Further characterization of the ribonuclease-resistant RNA from *Penicillium* is needed, as

FIG. 9. Virus particles from mycelia of *P. chrysogenum*. $\times 150,000$. (Courtesy of L. E. Day and L. F. Ellis.)

conclusive evidence for its double-stranded and helical configuration will require data based on secondary structure. Ribonucleic acids with secondary structure other than that of double-strandedness have been recognized in the fungi (10, 28). To the extent that such nucleic acid resists ribonuclease treatment and remains soluble in high salt, it might be expected to contaminate the dsRNA prepared from *Penicillium*.

Initial studies have indicated that a dsRNA can be obtained from a common industrial mold. This molecule has unusual biological properties. It is a potent inducer of interferon and a potential antiviral compound. Perhaps its most exciting feature is its resistance to native nucleases. It is a nucleic acid resistant to the enzymes which degrade other nucleic acids. Accordingly, it is inherently stable and a model compound to modify chemically and assay for regulation of specific biological functions. Specialized and nuclease-resistant nucleic acids, such as chromosomal RNA-DNA complexes, have been implicated in the regulation of higher organisms (7). The synthesis of specific compounds able to influence the genetic regulation of eukaryotic organisms seems inevitable if not imminent.

P. chrysogenum, the serendipitous mold, may dominate another generation of industrial microbiology.

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