EXPERIMENTAL MYCOLOGY 1, 173–182 (1977)

Endogenous RNA-Bound RNA-Dependent DNA Polymerase Activity in *Neurospora crassa*

SISIR K. DUTTA,* MIRKO BELJANSKI,† AND PIERRE BOURGAREL †

*Botany Department, Howard University, Washington, D.C. 20059, and
†Department of Biochimie Cellulaire, Institut Pasteur,
Paris, 75015, France

Received August 9, 1976; revised January 20, 1977

DUTTA, S. K., BELJANSKI, M., AND BOURGAREL, P. 1977. Endogenous RNA-bound RNA-dependent DNA polymerase activity in *Neurospora crassa*. *Experimental Mycology* 1, 173–182. Evidence indicating the presence of a RNA-bound RNA-dependent DNA polymerase (RDDP) in the eukaryotic fungus *Neurospora crassa* is presented. This enzyme, free of DNA-dependent DNA polymerase, is capable of synthesizing DNA only in the presence of all four deoxyribonucleoside 5′-triphosphates and Mg^{2+}. The DNA polymerase activity is decreased considerably if the RNA bound to the enzyme is eliminated. The activity can be restored by addition of some particular RNA but not by addition of DNA. Both template RNA and the synthesized DNA product sedimented on sucrose density gradients at approximately 6 S. RNA-bound RNA-dependent DNA polymerase partially purified from extracts of *N. crassa* was distinguishable from known DNA-dependent DNA polymerases. DNA synthesized by RDDP was acid-precipitable and complementary to enzyme-bound RNA.

INDEX DESCRIPTORS: *Neurospora crassa*; RNA-bound RNA-dependent DNA polymerase; DNA product; template RNA; RNase-sensitive DNA polymerase.

The enzyme RNA-dependent DNA polymerase ("reverse transcriptase") is of considerable interest not only in studies of oncogenic viruses (Baltimore, 1970; Temin and Mizutani, 1970; Spiegelman *et al.*, 1970; Gallo, 1971) but also for the role that this enzyme may play in normal cell development, differentiation, and antibody formation (Kang and Temin, 1972). These investigations and further observations made by many authors (see review by Wu and Gallo, 1975) have, however, been conducted essentially with RNA-free RNA-dependent DNA polymerase. Evidence for the presence of an RNA-bound RNA-dependent DNA polymerase in a prokaryotic system, *Escherichia coli*, has been provided by Beljanski (1972, 1973) and Beljanski and Beljanski (1974). Our interest in looking for RNA-directed DNA polymerase in *Neurospora crassa* Dodge was suggested by the fact that RNA can mediate the genetic transformation of *N. crassa* mutants (Mishra *et al.*, 1975). This idea was strengthened by several reports that this fungus and others contain RNA viruses (Tuveson and Peterson, 1972; Kuntzel *et al.*, 1973; Wood *et al.*, 1971). In the present report we describe a procedure for the isolation and characterization of an RNA-bound RNA-dependent DNA polymerase (RDDP) [1] found in extracts of *N. crassa*.

[1] Abbreviations used: RDDP, RNA-bound RNA-dependent DNA polymerase; dNTP, deoxyribonucleotide 5′-triphosphate; DEAE-, diethylaminoethyl-; SLS, sodium lauryl sulfate; DTT, dithiothreitol; PB, phosphate buffer; TCA, trichloroacetic acid.

To the best of our knowledge, our results constitute the first report of the occurrence of this enzyme in a nonvertebrate eukaryotic organism.

MATERIALS AND METHODS

Unlabeled deoxyribonucleotide 5'-triphosphates (dNTP) were obtained from Sigma Chemical Company, St. Louis, Missouri; poly(rA) oligo(dT)$_{12-18}$ and poly(dA) oligo(dT)$_{12-18}$, from P-L Biochemicals, Milwaukee, Wisconsin; tritium-labeled dNTPs from ICN Company, Irvine, California; Triton X-100 from Beckman Company, Rockville, Maryland; rifampicin from Dr. R. Cricchio, Gruppo Lepetit, Milan, Italy; DEAE-cellulose (DE-52) from H. Reeve Angel Inc., Clifton, New Jersey; and Sephadex G-100 and G-200 from Pharmacia Company, Uppsala, Sweden. Purified RNase T_1, RNase A, and DNase were obtained from Worthington Biochemicals, Freehold, New Jersey; actinomycin D and Pronase were obtained from Calbiochem, San Diego, California.

N. crassa (Fungal Genetics Stock Center No. 987) 74 A wild-type strain was used throughout this investigation. Mycelial cells grown in liquid medium at 30°C were harvested at mid-log phase as described by Dutta *et al.* (1967).

Detection of RNA-bound RNA-dependent DNA polymerase. The general procedure used for the detection of RNA-bound RNA-dependent activity in *N. crassa* extract is described by Beljanski and Beljanski (1974). Certain modifications made by us were as follows: Mycelial cells at their mid-log phase were collected by filtration, washed with .05 M Tris-HCl buffer (pH-7.4) containing 0.2 M sucrose and 10^{-4} M $MgCl_2$, and ground with seasand using an ice-cold mortar and pestle. The ground material was suspended in the same buffer and centrifuged at 10,000g for 30 min to sediment unbroken cells, cell walls, nuclei, and mitochondria. The protein concentration of the supernatant was adjusted to 10 mg of protein/ml with 10^{-2} M phosphate buffer (pH7.4) + 0.2 M sucrose. The supernatant was then progressively mixed with solid $(NH_4)_2SO_4$ to 40% saturation while the pH was maintained at 7.4. The precipitate, collected by centrifugation for 15 min at 12,000 rpm, was discarded and the supernatant was kept. The supernatant was mixed with $(NH_4)_2SO_4$ (A.S.) to 70% saturation and the pH was maintained at 7.4. The precipitate, collected by centrifugation, was dialyzed overnight against 10^{-2} M phosphate buffer (pH 7.4). Approximately 4 to 5 g of dialyzed proteins were then adsorbed onto a DEAE-cellulose column (40 × 3 cm) preequilibrated with the above buffer. Proteins were eluted with a linear gradient: 10^{-2} M Tris-HCl (pH 7.4), 1000 ml-10^{-2} M Tris-HCl (pH 7.4) plus 0.7 M KCl, 1000 ml. Five-milliliter fractions were collected and tested for ability to polymerize the dNTP in the absence and in the presence of exogeneously added template RNA or DNA. Active fractions were collected and progressively mixed with solid $(NH_4)_2SO_4$ to 50% of saturation (pH maintained at 7.4). The precipitate was collected by centrifugation and dissolved in 10^{-2} M Tris-HCl (pH 7.4) containing 0.2 M sucrose. The dialyzed fraction was then layered onto a Sephadex G-200 column (30 × 2 cm) preequilibrated with buffer containing 10^{-2} M Tris-HCl (pH 7.4) and 0.2 M sucrose. Fractions of 3 ml were collected and RNA-bound reverse transcriptase activity was detected in those fractions which eluted immediately before the bulk of the proteins. This procedure gave an approximately 10-fold purification of the *Neurospora* RNA-dependent DNA polymerase; the isolated enzyme was stable at −20°C for 1 to 2 weeks in 0.2 M sucrose dissolved in 10^{-2} M Tris-HCl buffer (pH 7.4).

RNA bound to reverse transcriptase was removed by treatment with pancreatic

RNase (20 μg of RNase for each reaction volume of 0.2 ml for 25 min at 36°C) and the enzyme fraction was passed through a Sephadex G-200 column a second time. The activity was tested in the absence and presence of RNA and DNA templates as described in the legend to Table 3.

Analysis of template RNA and ^{3}H-labeled DNA product. The postmitochondrial supernatant was centrifuged at 105,000*g* for 90 min. This 105,000*g* pellet of *N. crassa* mycelia was an excellent source for obtaining a large quantity of purified (free from any detectable quantity of DNA) template RNA which can be transcribed into DNA. The microsomal pellet was treated with 2% SLS (sodium lauryl sulfate), 10^{-4} M $MgCl_2$ in 10^{-2} M Tris–HCl at pH 7.4, and the RNA was extracted using phenol and chloroform. After centrifugation at 12,000*g* for 10 min, the upper aqueous phase, containing RNA, was mixed with 2 vol of alcohol. The precipitate was dialyzed against 10^{-2} M Tris–HCl overnight at 4°C. This RNA preparation was found to be free from any DNA contamination. RNA molecules were further characterized by gel electrophoresis followed by ultraviolet densitometer tracings (Beljanski, 1973), Cs_2SO_4 density gradients, and nucleotide analysis as described by Beljanski and Beljanski (1974).

^{3}H-labeled DNA product synthesized under optimal conditions in the presence of *N. crassa* RNA was separated from the enzyme by chloroform, followed by treatment with 0.3 N KOH at 80°C for 20 min to hybridize RNA from ^{3}H-labeled DNA:RNA complex. Further characterization of enzymatically and nonenzymatically synthesized ^{3}H-labeled DNA:RNA hybrids before KOH treatment and ^{3}H-labeled DNA product after KOH treatment was achieved using Cs_2SO_4 equilibrium density gradients as described by Beljanski and Beljanski (1974).

Hybridization of ^{3}H-labeled DNA product with template RNA. The reaction mixture, for synthesis of ^{3}H-labeled DNA product, contained 30 μM [^{3}H]dATP, 24 μM [^{3}H]dCTP, 27 μM [^{3}H]dGTP, 50 μM TTP (unlabeled), 5 mM $Mg^{2+}Cl_2$, 0.2 μM $Mn^{2+}Cl_2$, 50 mM Tris–HCl (pH 7.9), 5 mM DTT (dithiothreitol), 5 mM NaF, 0.05% Triton X-100, 50 μM Actinomycin D, and the *N. crassa* 105,000*g* pellet suspension. After incubation for 10 min at 36°C the reaction mixture was treated two or three times with phenol and the purified ^{3}H-labeled DNA (still ^{3}H-labeled DNA:RNA hybrid) was extracted. This ^{3}H-labeled DNA:RNA hybrid was treated with 0.4 M KOH at 37°C for 24 h, neutralized with HCl, and then adsorbed on a hydroxyapatite column which was previously equilibrated with 0.002 M phosphate buffer (PB). All nonincorporated counts were washed out at this molarity. The ^{3}H-labeled DNA single strands were then hybridized with *N. crassa* RNA isolated from the same 105,000*g* pellet, as described by Dutta (1973). ^{3}H-labeled DNA:RNA hybrids were eluted at 0.10 M. PB and unhybridized ^{3}H-labeled DNA (single stranded) were eluted at 0.05 M PB. The percentage ^{3}H-labeled DNA:RNA hybridization was measured as described by Dutta (1973).

RESULTS

Activity of RNA-Bound RNA-Dependent DNA Polymerase

Table 1 summarizes the data regarding endogenous activity of the RNA-bound RNA-dependent DNA polymerase under different incubation conditions. It is evident that DNA synthesis takes place only when all four dNTPs and Mg^{2+} are present. If any one of the dNTPs is missing, the amount of DNA synthesis is decreased by about 91%. Under optimal conditions the amount of ^{3}H-labeled DNA increases as a function of enzyme concentration up to 200 μg of protein (Fig. 1).

TABLE 1

Endogenous RNA-Bound RNA-Dependent DNA Polymerase Activity under Different Incubation Conditions[a]

Reactions	Picomoles of [^{3}H]dATP incorporated per 100 μg of protein	Percentage inhibition
Complete	250	0
−dCTP, dGTP, dTTP	10	96
−Mg^{2+}	7	97
−dCTP	20	92
−dGTP	22	91
−dTTP	15	94
+RNase A (20 μg preincubated)	60	96
+RNase T_1 (10 μg preincubated)	100	60
+DNase (50 μg)	6	98
+Salmon DNA (3 μg)	255	0
+Thymus DNA (3 μg)	245	0
+*Alcaligenes fecalis* RNA (4 μg)	300	0
+Enzyme heated at 100°C for 10 min	1	100

[a] Incubation mixture per 0.2 ml: Tris-HCl, pH 7.4, 25 μmol; $MgCl_2$, 2 μmole; 5 nmol each of dNTP, [^{3}H]dATP (50,000 cpm); 0.05 ml of partially purified RNA-bound RNA-dependent DNP polymerase from *N. crassa* (40–60 μg of protein from Sephadex G-200 column), incubation at 36°C for 10 min. In certain cases, the enzyme preparation was preincubated in the presence of RNase A or T_1. The reaction was stopped with TCA (5%); the acid-precipitable material was filtered on Millipore filters, washed, and dried, and its radioactivity was measured in a Packard spectrometer. The data presented here are calculated from one set of reactions and expressed for activity of 100 μg of protein for uniformity. Each of these reactions were, however, repeated three times or more and experimental variations were within ±2% of the data presented here. Similar repeatability was obtained with respect to the data presented in the other tables.

The amount of ^{3}H-labeled DNA synthesized increases with time up to 15 min (Fig. 2). The enzyme shows optimal activity at pH 7.4. The ^{3}H product is almost undetectable in the presence of DNase. RNase (20 μg in preincubation treatment) leads to a considerable decrease of the ^{3}H product. Activity is completely lost when the enzyme is heated at 100°C for 10 min.

Table 3 summarizes the activity of the enzyme when the enzyme-bound RNA was partially separated. Thymus DNA or salmon DNA do not affect the activity of the enzyme. However, these two DNAs serve as active templates with *Escherichia*

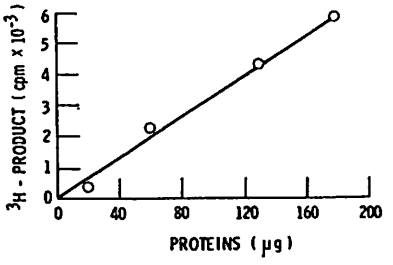

FIG. 1. Dependence of enzyme activity on enzyme concentration.

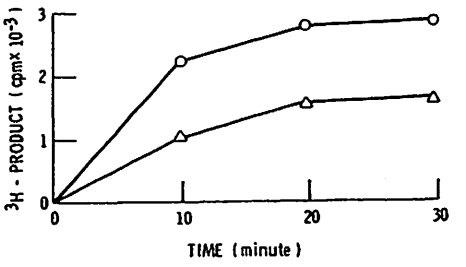

FIG. 2. Time course of enzyme activity. Absence of RNase A, ○; presence of RNase, △.

TABLE 2

Estimation of the Possible Presence of DNA in the RNA Isolated from *N. crassa* RNA-Bound RNA-Dependent DNA Polymerase[a]

Amount of protein per milliliter	21 mg
280/260 nm absorbance ratio	0.85
Total nucleic acid isolated as estimated by uv absorption	1060 μg
Quantity of RNA estimated by orcinol reaction in 70 μg of nucleic acid (uv absorption)	69 μg
Diphenylamine positive component in 700 μg of nucleic acid by uv absorption	None

[a] The amount of nucleic acid in the total protein was determined by the 280/260 nm absorbance ratio (Warburg and Christian, 1942) which correlated with the amount actually isolated. Protein estimation was made at 540 nm by the biuret method (Gornal *et al.*, 1949). Ribose (RNA) determination was done at 670 nm by the orcinol reaction (Beljanski, 1949) and DNA estimation was done by the diphenylamine method (Burton, 1956). This reaction is positive with 2 μg of purified thymus DNA (in a volume of 0.2 ml).

coli DNA polymerase. Poly(dA) oligo(dT)$_{12-18}$ has no effect on RNA-dependent DNA polymerase of *N. crassa*. The effect of poly(rA) oligo(dT)$_{12-18}$ in the reaction is evident.

Evidence That Only RNA was Bound to the Enzyme

The enzyme from either the DEAE–cellulose or the Sephadex G-200 column had a 280/260 nm absorbance ratio of 0.85 to 0.90, which corresponds to 4 to 6% nucleic acid content (Warburg and Christian, 1942). This nucleic acid, when separated from the enzyme by phenol and chloroform, was found to be entirely RNA (Table 2).

Similarly, when the RNA was separated from the RNA-bound enzyme by treatment with RNase and filtration on Sephadex G-200, the activity of the free protein fraction was strongly increased by the addition of exogenous RNA, but not with DNA. The DNA preparation tested, however, was an excellent template for *E. coli* DNA-dependent DNA polymerase (Table 3). The diphenylamine reaction used to test the presence of DNA in a large amount of RNA

TABLE 3

Activity of Protein Fraction after Removal of RNA from RNA-Dependent DNA Polymerase of *N. crassa*[a]

	Picomoles of [^{3}H]dATP incorporated in 10 min at 36°C	
	N. crassa RNA-dependent DNA polymerase	*E. coli* DNA-dependent DNA polymerase
Protein fraction (containing 0.5–1% RNA)	40	5
+65 A.S. RNA from *N. crassa* (4 μg)	400	8
+65 A.S. RNA from *A. fecalis* (3 μg)	350	7
+Thymus DNA (2 μg)	50	298
+Salmon DNA (3 μg)	45	376
+Poly(rA) oligo(dt)$_{12-18}$ (3 μg)	400	—
+Poly(dA) oligo(dt)$_{12-18}$ (3 μg)	50	—

[a] RNA-bound RNA-dependent DNA polymerase was treated at 30°C with pancreatic RNase A (20 μg of RNase per 0.2-ml reaction volume for 25 min) and passed through a Sephadex G-200 column. Approximately 30 μg of protein was used for each reaction. Purified 65 A.S. RNA was isolated from total ribosomal RNA at 65% $(NH_4)_2SO_4$ saturation (Green and Hughes, 1955). *E. coli* DNA-dependent polymerase I (fraction DEAE, 60 μg) was isolated using the procedure described by Beljanski and Beljanski (1974)).

TABLE 4

Activity of RNA-Bound RNA-Dependent DNA Polymerase in the Presence of Inhibitors[a]

	Picomoles of [^{3}H]dATP incorporated per 60 μg of protein	Percentage inhibition
Complete	150	0
+Actinomycin D (3 μg)	101	36
+Actinomycin D (6 μg)	70	53
+Rifampicin (10 μg)	140	7
+Rifampicin (20 μg)	105	30
+Rifampicin (40 μg)	65	57
+0.1 M KCl	108	28
+0.2 M KCl	75	50
+0.4 M KCl	60	60

[a] Incubation conditions are as given in footnote a to Table 1. All compounds were added to the incubation mixture prior to the addition of the enzyme.

(700 μg) was negative, although it was positive with 2 μg of thymus DNA (Table 2). All of these observations indicate that RNA-dependent DNA polymerase is bound to RNA but not to DNA. This is in agreement with the observation that DNA synthesis by RDDP is considerably decreased in the presence of RNase (Table 1). The fact that KOH destroys the RNA bound to ^{3}H-labeled DNA (RNA:^{3}H-labeled DNA hybrid) also suggests that the template for RDDP is RNA but not DNA.

The Effect of Inhibitors on RNA-Bound RNA-Dependent DNA Polymerase

In the presence of various concentrations of actinomycin D, rifampicin, or KCl (Table 4), a partial inhibition of the activity of the RNA-bound RNA-dependent DNA polymerase was observed. Rifampicin does not have an inhibitory effect on *E. coli* DNA-dependent DNA polymerase I (Riva and Silvestri, 1972).

Size of RNA Bound to RNA-Dependent DNA Polymerase and ^{3}H-Labeled DNA Product

The size of the template RNA separated from RNA-bound RNA-dependent DNA polymerase preparation was determined by two different methods. First, the total RNA isolated from ribosomal pellet of *Neurospora crassa* was centrifuged in a linear sucrose density gradient (5–20%). Fractions were collected and incubated with all the components required for DNA synthesis. For convenience, *E. coli* RNA-free RNA-dependent DNA polymerase was used to determine the amount of ^{3}H-labeled DNA synthesized on RNA present in each fraction of the gradient. Figure 3 shows profiles of sucrose density gradients showing the size of the ^{3}H-labeled DNA product and template RNAs. It is apparent that the peak representing synthesized DNA is located at around 6 S. This observation shows that ribosomal RNA preparation from *N. crassa* contains a small RNA (possibly messenger-like RNA) which is transcribed into DNA. Second, total *N. crassa* RNA was fractionated by ammonium sulfate and dialyzed as described by Beljanski (1973). RNA, which precipitates with $(NH_4)_2SO_4$ to 65% of saturation (Green and Hughes, 1955), acts as a template for

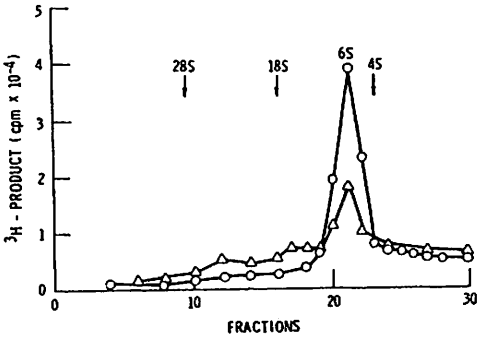

FIG. 3. Profiles of two separate sucrose density gradients, superimposed, showing the size of the ^{3}H-labeled DNA product (○) and template RNA (△). Details are given in the text. ^{3}H-labeled DNA product was synthesized *in vitro* on *N. crassa* 65% $(NH_4)_2SO_4$ RNA as template and separated from RNA as described in the text. Ribosomal RNA (600 μg) of *N. crassa* was centrifuged (see Materials and Methods) and each fraction of the gradient was tested for template RNA capacity in the presence of *E. coli* RNA-dependent DNA polymerase.

E. coli RNA-free RNA-dependent DNA polymerase (Table 3). The latter enzyme and 65% $(NH_4)_2SO_4$ RNA were incubated under optimal conditions for DNA synthesis, using [^{3}H]dCTP as marker. The ^{3}H product was separated from the enzyme by phenol and chloroform treatments. The resulting extract contained template RNA and ^{3}H product. From this duplex, RNA was eliminated by incubation with 0.3 M KOH for 20 min at 80°C and the ^{3}H product was centrifuged on a 5–20% sucrose gradient (in 10^{-2} M Tris–HCl, pH 7.3). Figure 3 shows that the ^{3}H-labeled DNA product sediments at the position of the transcribing RNA. At present we cannot exclude the possibility that some RNA (in *N. crassa*) of bigger or smaller size than 6 S RNA could be transcribed into DNA.

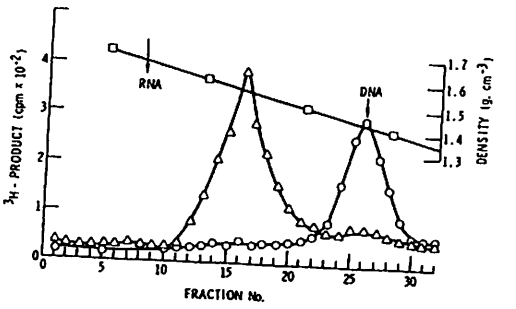

FIG. 4. Cs_2SO_4 density gradient analysis of ^{3}H-labeled DNA product synthesized on template RNA of *N. crassa*, with *E. coli* RNA-free RNA-dependent DNA polymerase. Enzyme was removed by chloroform. One part of the aqueous solution containing the ^{3}H product (4000–7000 cpm) was mixed with Cs_2SO_4 (1.8 g; pH 7.3; final volume, 3 ml) and centrifuged (see Materials and Methods). Another fraction of the ^{3}H product was treated with 0.3 N KOH at 80°C for 20 min and centrifuged in a separate tube. ^{3}H product untreated, △; ^{3}H product treated with alkali, ○.

^{3}H-Labeled DNA:RNA Hybrid Synthesized during Enzymatic Reaction

$(NH_4)_2SO_4$ RNA (65%) for *N. crassa* was incubated with *E. coli* RNA-free RNA-dependent DNA polymerase in the presence of the four [^{3}H]dNTPs under optimal conditions. The enzyme was eliminated by treatment of the incubation mixture with phenol and then chloroform. The upper aqueous phase, containing template RNA and ^{3}H product, was submitted to equilibrium sedimentation in Cs_2SO_4 density gradient (Beljanski and Beljanski, 1974), and the TCA-precipitable radioactivity of each fraction was determined. Profiles of ^{3}H-labeled DNA products are shown in Fig. 4. The peak of radioactive material was found at a density between that of RNA and DNA. After treatment with alkali, the radioactivity sediments at the density of DNA. These observations suggest that during enzymatic reaction ^{3}H-labeled DNA is associated with template RNA.

Similarly, ^{3}H-labeled DNA:RNA was synthesized using the 105,000*g* *N. crassa* RNA (65% A.S.) (i.e., RNA isolated from the *N. crassa* 105,000*g* pellet and fractionated with $(NH_4)_2SO_4$) and the four [^{3}H]-dNTPs. The ^{3}H-labeled DNA product, after treatment with alkali, hybridized with the same RNA to 50% as analyzed by the hydroxyapatite chromatography procedure (Dutta, 1973). This ^{3}H product did not hybridize with the poly(A) obtained from Sigma Chemical Company.

DISCUSSION

Several workers (Crippa and Tocchini-Valentini, 1971; Ficq and Brachet, 1971; Brown and Tocchini-Valentini, 1972) have reported the presence of RNA-dependent DNA polymerase in microsomal pellets of *Xenopus laevis* and correlated it with gene amplification. Bobrow *et al.* (1972) showed the existence of RNase-sensitive DNA polymerase in normal human blood lymphocytes and demonstrated that the enzyme was different from the RNA-dependent DNA polymerase (reverse transcriptase) found in oncogenic RNA viruses and human leukemic cells. RNase-sensitive DNA polymerase was also found in normal human cells

(Penner *et al.*, 1971). Similar results were obtained by Reitz *et al.* (1974), showing that RNase-sensitive DNA synthesis in microsomal pellets of normal human lymphocytes was different from the endogenous RNase-sensitive DNA synthesis in type-C RNA tumor viruses and in related cytoplasmic particles from human leukemic cells.

We have presented evidence that the normal strain of *N. crassa* shows RNA-bound RNA-dependent DNA polymerase activity. Several criteria to support this fact include: (1) This enzyme requires all four dNTPs and Mg^{2+} ions for activity; (2) the activity decreases when the enzyme is preincubated with RNase (20 μg); (3) the activity is stimulated by the addition of RNA to the enzyme from which the bound RNA had previously been removed; (4) using chemical and biological tests, we have shown that the nucleic acid fraction of the enzyme is exclusively RNA, having a sedimentation coefficient of about 6 S; (5) ^{3}H-labeled DNA synthesis requires a particular RNA template and the ^{3}H product is TCA-precipitable; (6) this ^{3}H-labeled DNA is sensitive to DNase and resistant to RNase and alkali treatment; (7) ^{3}H-labeled DNA product has the characteristic sedimentation properties of DNA (Fig. 4) in the Cs_2SO_4 density gradient. In the Cs_2SO_4 density gradient the DNA: RNA hybrid sediments between DNA and RNA (Fig. 4). This ^{3}H-labeled DNA, when made single-stranded by alkali treatment, is complementary to RNA as judged by hybridization data, which show that RNA was utilized as a template by RNA-dependent DNA polymerase.

Our data, presented here, show that RNA-dependent DNA polymerase from *N. crassa* is not like DNA-dependent DNA polymerases I, II, and III as demonstrated in bacteria (Kornberg and Gefter, 1971). Partially purified RNA-dependent DNA polymerase from *N. crassa* is free from DNA-dependent DNA polymerase and from DNA (Tables 2 and 3).

When a protein fraction of the enzyme complex was separated from RNA, the decreased activity was restored only by addition of template RNA, which sedimented at about 6 S. Neither ribosomal RNA (28 S + 18 S), transfer RNA (4 S), nor DNA stimulated the reaction. Rifampicin and actinomycin-D inhibited the activity of RNA-bound RNA-dependent DNA polymerase up to 40 to 60%. Rifampicin has no effect at all on DNA-dependent DNA polymerase from bacteria (Beljanski and Beljanski, 1974), while actinomycin D almost completely inhibits the activity of this enzyme.

The activity of RNA-dependent DNA polymerase was inhibited from *N. crassa* to about 50% only by 0.2 M KCl; similar results were obtained with the *E. coli* extracts. Also, it should be remembered that 0.2 M KCl has no effect on DNA polymerase I and virtually abolishes the activity of DNA polymerase II and III. Furthermore, 50% or more hybridization of ^{3}H-labeled DNA product was obtained with template RNA and none with poly(A). All the data taken together show that *N. crassa* contains an RNA-bound RNA-dependent DNA polymerase.

Although several workers have reported the presence of virus-like particles in *N. crassa* mutants, we failed to detect a difference in RDDP activities in several wild-type and mutant strains of *N. crassa* irrespective of the reported presence or absence of virus-like particles. It has been shown by Sarangadharan *et al.* (1972), that the RNase-sensitive DNA polymerase of RNA tumor viruses differs from normal (without any tumor) RNase-sensitive DNA polymerase. It will be interesting to see whether RDDP of "poky" mutant (Kuntzel *et al.*, 1973) of *N. crassa*, which has virus-like particles is biochemically and/or immunologically different from RDDP strains

of *N. crassa* in which no viruses have yet been reported.

ACKNOWLEDGMENTS

This research was supported in part by Contract No. N00014-73-A-0346-0001 with the U. S. Department of Naval Research and contract No. E(40-1) 4182 with the U. S. Energy Research and Development Administration.

REFERENCES

BALTIMORE, D. 1970. RNA-dependent DNA polymerase in virions of RNA tumor viruses. *Nature (London)* 277: 1209–1211.

BELJANSKI, M. 1949. A propos du microdosage du ribose dans les acides nucleiques et leurs derivés. *Ann. Inst. Pasteur* 76: 651–652.

BELJANSKI, M. 1972. Synthese *in vitro* de l'ADN sur une matrice d' ARN par une transcriptase d' *E. coli*. *Compt. Rend. Acad. Sci. Paris Ser. D* 274: 2801–2803.

BELJANSKI, M. 1973. Separation de la transcriptase inverse de l'ADN polymerase ADN dependante. Analyse de l' ADN synthetisé sur le modèle de l' ADN transformant. *C R. Acad. Sci. Paris Ser. D* 276: 1625–1628.

BELJANSKI, M., AND BELJANSKI, M. 1974. RNA-bound reverse transcriptase in *E. coli* and *in vitro* synthesis of a complimentary DNA. *Biochem. Genetics* 12: 163–180.

BOBROW, S. N., SMITH, R. G., REITZ, M. S., AND GALLO, R. C. 1972. Stimulated normal human lymphocytes contain a RNase-sensitive DNA polymerase distinct from viral RNA-directed DNA polymerase. *Proc. Nat. Acad. Sci. USA* 69: 3228–3232.

BROWN, R. D., AND TOCCHINI-VALENTINI, G. P. 1972. On the role of RNA in gene amplification. *Proc. Nat. Acad. Sci. USA* 69: 1746–1748.

BURTON, K. 1956. A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation and DNA. *Biochem. J.* 62: 315–322.

CRIPPA, M., AND TOCCHINI-VALENTINI, G. P. 1971. Synthesis of amplified DNA that codes for ribosomal RNA. *Proc. Nat. Acad. Sci. USA* 68: 2769–2773.

DUTTA, S. K., 1973. Transcription of non-repeated DNA in *N. crassa*. *Biophys. Biochim. Acta.* 324: 482–487.

DUTTA, S. K., RICHMAN, N., WOODWARD, V. W., AND MANDEL, M. 1967. Relatedness among species of fungi and higher plants measured by DNA hybridization and base ratios. *Genetics* 57: 719–727.

FICQ, A., AND BRACHET, J. 1971. RNA-dependent DNA polymerase: Possible role in the amplification of ribosomal DNA in *Xenopus* oocytes. *Proc. Nat. Acad. Sci. USA* 68: 2774–2776.

GALLO, R. C. 1971. Reverse transcriptase, the DNA polymerase of oncogenic RNA viruses. *Nature (London)* 234: 194–198.

GORNAL, A. G., BARDAWILL, C. J., AND DAVID, M. M. 1949. Determination of serum proteins by means of the biuret reaction. *J. Biol. Chem.* 177: 751–766.

GREEN, A. A., AND HUGHES, W. L. 1955. In *Methods in Enzymology* (S. P. Colowick and N. O. Kaplan, Eds.), Vol. 1, pp. 67–90. Academic Press, New York.

KANG, C.-Y., AND TEMIN, H. M. 1972. Endogenous RNA-directed DNA polymerase activity in uninfected chicken embryos. *Proc. Nat. Acad. Sci. USA* 69: 1550–1554.

KORNBERG, T., AND GEFTER, M. L. 1971. Purification and DNA synthesis in cell-free extracts: Properties of DNA polymerase II. *Proc. Nat. Acad. Sci. USA* 68: 761–764.

KUNTZEL, H., BARATH, A., ALI, I., KIND, J., AND ALTHAUS, H. 1973. Virus-like particles in an extranuclear mutant of *Neurospora crassa*. *Proc. Nat. Acad. Sci. USA* 70: 1574–1578.

MISHRA, N. C., NIU, M. C., AND TATUM, E. L. 1975. Induction by RNA of inositol independence in *Neurospora crassa*. *Proc. Nat. Acad. Sci. USA* 72: 642–645.

PENNER, P. E., COHEN, L. H., AND LOEB, L. A. 1971. RNA-dependent DNA polymerase: Presence in normal human cells. *Biophys. Biochem. Res. Commun.* 42: 1228–1234.

REITZ, M. S., JR., SMITH, R. G., ROSEBERRY, E. A., AND GALLO, R. C. 1974. DNA-directed and RNA-primed DNA synthesis in microsomal and mitochondrial fractions of normal human lymphocytes. *Biophys. Biochem. Res. Commun.* 57: 934–948.

RIVA, S., AND SILVESTRI, L. G. 1972. Rifamycins: A general view. *Ann. Rev. Microbiol.* 26: 199–224.

SARANGADHARAN, M. G., SARIN, P. S., REITZ, M. S., AND GALLO, R. C. 1972. Reverse transcriptase activity of human acute leukemic cells: Purification of the enzyme, response to AMV 70S RNA, and characterization of the DNA product. *Nature New Biol.* 231: 67–72.

SPIEGELMAN, S., BURNY, A., DAS, M. R. KEYDAR, J., SCHLOM, J., TRAVNICEK, M., AND WATSON, K. 1970. Characterization of the product of RNA-directed DNA polymerase in oncogenic RNA viruses. *Nature (London)* 227: 563–567.

TEMIN, H. M., AND MIZUTANI, M. S. 1970. RNA-dependent DNA polymerase in virions of Rous sarcoma virions. *Nature (London)* 226: 1211–1213.

TUVESON, R. W., AND PETERSON, J. F. 1972. Virus-like particles in certain slow-growing strains of *N. crassa*. *Virology* 47: 527–531.

WARBURG, O., AND CHRISTIAN, W. 1942. Isolierung und Kristallalisatio der Garumgsferments Enolase. *Biochem. J.* 310: 384–421.

WOOD, H. A., BOZARTH, R. F., AND MISLEVEC, P. B. 1971. Virus-like particles associated with an isolate of *Penicillium brivi-compactum*. *Virology* 44: 592–598.

WU, A. M., AND GALLO, R. C. 1975. Reverse transcriptase. *Crit. Rev. Biochem.* 289–347.