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Particular RNA primer from growth medium differentially stimulates in vitro DNA synthesis and in vivo cell growth of *Neurospora crassa* and its slime mutant

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Summary. Purine rich small "RNA-primer" molecules (about 10–12 nucleotides), secreted into the growth medium of 3-h germinated conidia of *N. crassa*, strongly stimulated a concentration-dependent in vitro DNA synthesis of *N. crassa* slime mutant as well as DNAs from the human cancer cells but did not affect that from normal cells. These "RNA-primer" molecules stimulated also in vivo cell growth of *N. crassa* slime mutant, but not of the *N. crassa* wild type. Our studies suggest that DNAs from the slime mutant of *N. crassa* as well as DNAs from human cancerous cells provide increased sites for enhanced in vitro and in vivo replication of DNAs. "RNA-primer" molecules can be hydrolyzed by T1 RNase but not by pancreatic RNase.

Key words: RNA primer – Purine rich small RNA molecules – *Neusrospora crassa* slime mutant – in vitro and in vivo DNA synthesis

Introduction

Several investigators have shown that replication of DNA by DNA dependent DNA polymerase I is the first necessary step in cell division (Fox et al. 1973; Schekman et al. 1972). Naturally produced short chain RNA primers must participate in this process to initiate a new DNA chain synthesis and thus make cell mulitplication possible. RNA primers have been found in living cells and have also been used for in vitro DNA synthesis (Plawecki and Beljanski 1974; Roven and Komberg 1978). With different types of RNase (RNase preparations generally contain phosphatase activity), one may obtain a family of RNA pieces through cleavage of ribosomal RNAs (Beljanski et al. 1983). Such molecules differ in their sizes and base compositions. Some of them act as primers for DNA in vitro synthesis, while others are used by DNA dependent DNA polymerase for the synthesis of particular DNAs, such as viral DNAs (Beljanski et al. 1983). These observations have been recently confirmed using retroviral RNA and pancreatic RNase (Champoux et al. 1984; Smith et al. 1984). On the other hand, at a given stage of growth some cells or tissues synthesize a larger excess of ribosomal RNAs which, as in the case of *Neurospora crassa* (Dutta et al. 1983; Mukhopadhyay and Dutta 1979), disappear as soon as cell division starts.

In this paper we report that in vitro DNA synthesis of the slime mutant cells (which, like malignant cells have lost the ability to differentiate) differs markedly from that in the wild type strain of *N. crassa* 74A in response to the addition of exogenous RNA primer. In addition, data are presented to show that germinated conidia (3-h cells) of the normal strain of *N. crassa*, which show excessive rRNA transcription, excrete small (10–12 bp) RNAs in the growth medium which are biologically active.

Materials and methods

Chemicals. The sources of chemicals and enzymes were as follows: deoxyribonucleoside-5'-triphosphates (dnTP), Miles Laboratories, USA; ^{3}H-labelled TTP (specific activity 17.5 Ci/mol), Amersham, England; Pancreatic RNase, RNase T1, Worthington Co., USA; DNA polymerase 1 (EC 2.7.7.7) originated from *Escherichia coli* (Belajanski and Beljanski 1974).

Cultures. The wild type 74A strain of *N. crassa* (FGSC #987) and the slime mutant (FGSC #1118) were obtained from the Fungal Genetics Stock Center, Kansas City, MO, USA. The wild

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type *N. crassa* 74A shows fluffy aerial growth, orange colored on agar medium, and a mycelia growth like mat of compact cells. The slime mutant (Emerson 1963) of *N. crassa* (fz; sq; cs-1) requires nutrient broth, yeast extract, and constant subculturing after every week. It lacks a cell wall and has lost cell contact ability. It is sensitive to osmotic shock and physical disturbance. The colonies grow like slimy yeast cells instead of having a mycelial mat-like growth (Chaudhuri et al. 1973).

DNA isolation from different sources for in vitro template studies. Basically similar procedures were applied to all cells. In case of Neurospora mycelia, cells were collected from liquid cultures by straining through 4-fold cheese cloth. *N. crassa* slime mutant cells were collected by centrifugation at 5,000 g. Cells from mice spleen and lung, and human breast and cancer tissues obtained by excision of tissues followed by freezing at −20 °C. All of those cell mass or frozen sliced tissues were homogenized with 30 ml of buffer containing 100 mM Tris (pH 7.5), 10 mM EDTA, and 1% trition X-100 for 15 min, centrifuged for 12,000 g for 10 min, and the supernatant was saved. To the supernatant was added the same volume of water-saturated phenol (v/v); the mixture was shaken for 10 min and again centrifuged. The process was repeated three times more. The upper phase was treated with chloroform and the process repeated twice more. To the aqueous phase chilled 95% ethyl alcohol (v/v) with 0.1 m KCl was added and the mixture left overnight at 4 °C. After centrifugation at 12,000 x g for 10 min the pellet was dissolved in 2SSC (1SSC: 0.15 M NaCl + 0.015 M sodium citrate) solution and centrifuged at 1,000 x g for 15 min. The supernatant containing DNA was dialysed against 2SSC solution for overnight at 4 °C. Optical densities were taken at 260 and 280 nm to check ratios of 260/280 = 2 as an indication of purity. DNA in 2SSC solution was then treated with DNase free RNase (25 μg/ml of DNA solution) for 40 min at 37 °C. RNase was then removed by 3 chloroform treatments each of which was followed by centrifugation (5,000 x g for 10 min) at 4 °C. DNA was precipitated with 3 volume of 95% alcohol, dissolved in 2SSC solution and dialysed against this solution overnight at 4 °C.

Purified DNA (absorbance at 260/280 = 2.0–2.1) was stored at −20 °C without losing its polymerized form. DNAs were characterized by physical means (Beljanski et al. 1981) using Cs_2SO_4 buoyant density gradient and thermal denaturation curves. The hyperchromic shift in the presence of alkali was between 35% and 45%.

Isolation of RNA primer from N. crassa. The RNA-primer molecules were isolated from 3 to 4 L of liquid growth medium of *N. crassa* wild type cells grown for 1, 3, and 10 h and from 48-h mycelia and from the growth medium of the slime mutant cells. Growth media were separated from the cultures by centrifugation at 2,000 g. The procedure of RNA isolation involved addition of twice the volume of ethyl alcohol + 0.1 M KCl for overnight at 4 °C followed by centrifugation at 8,000 g for 30 min. The supernatant was discarded. The pellet was dissolved in sterile distilled water followed by centrifugation at 500 g for 15 min. The supernatant was treated twice with chloroform and dialysed against distilled water. The supernatant was then checked for spectrophotometric tests (optical density ratio of 260/280 = 2.0).

RNA molecules were further purified by several treatments with chloroform. The final aqueous phase containing nucleic acid material was mixed with two volumes of chilled 95% alcohol + 0.1 M KCl (30 min in ice box). The precipitate, collected by centrifugation at 10,000 rpm in Sorvall Centrifuge, was dialysed against distilled water for 6 h at 4 °C. The amount of UV absorbing material was determined at 260, 280 and 230 nm. This material was tested as primer for in vitro DNA synthesis with *N. crassa* slime mutant as well as from the wild type strain.

The dialysed material was fractionated on Sephadex G-25 column (60 x 1.5 cm) equilibrated with 10^{-2} M Tris buffer, pH 7.5. Two ml fractions were collected. On the basis of UV absorbance at 260 nm, three peaks (A, B, C) were characterized (Fig. 4). Peak B was predominant. However, some yellow pigment was present in each of these fractions. Each of these peak materials was rechromatographed (after concentration of eluate in vacuum). Orcinol reactions were positive with the concentrated eluate.

Second chromatography column. UV absorbing materials from peaks A, B, and C were filtered on a Sephadex G-25 superfine column (46 x 1.5 cm) according to Andrew's procedure (Andrews 1965). Oligo $d(pT)_{12}$ and Poly A = 40 nucleotides (mW 14,000), were used as markers. Two ml fractions were collected. UV absorbance at 260 nm is illustrated in results. Material from peak A gave peak A_1 and peak A_2. Fractions were collected and lyophilized. The resulting material was tested for priming DNA synthesis and analysed for base composition. Material from peak A_1 contains about 20 nucleotides and peak A_2 10 nucleotides. Peak B from the first Sephadex G-25 column was rechromatographed and analysed fractions showed a similar elution pattern to those found for fraction A. Two peaks, B_1 which is predominant and peak B_2 present in small amounts were obtained. After passage of peak C through Sephadex G-25 column, analysed fractions revealed the presence of only one peak determined by UV absorbance at 260 nm. After lyophilization, the UV absorbing material was analysed for primer activity in DNA synthesis and for base ratios.

Base ratio analysis of UV absorbing material: A_1, A_2, B_1, B_2 and C eluted from second sephadex G-25 column. Each material (100 μg) was hydrolysed with 1 N HCl (100 °C for 1 h) and chromatographed on an Ecteola cellulose plate with the following solvents: N°1: N-butanol/H_2O (86:14 + NH_3). N°2: isopropanol/H_2O/HCl concentrated (170:43:30) (Bjork and Svenson 1967). Only two UV absorbing spots for each fraction were localized and eluted with 0.1 N HCl for 16 h and their amount measured (Hori 1967) (Table 1). Only A and G nucleotides were found. Unhydrolysed material from each fraction did not move on an Ecteola cellulose plate chromatographed under the same conditions as hydrolysed material.

In vitro DNA synthesis. The incubation conditions for in vitro DNA synthesis have been described elsewhere (Beljanski et al. 1981). The reaction mixture per 0.15 ml contains all necessary components for DNA synthesis, such as Tris-HCl buffer (pH 7.6) 25 μmoles; $MgCl_2$, 2 μmoles; 4 dNTPs, each 5 nanomoles. ^{3}H-TTP (50,000 cpm) was used as a mrker for detecting the amount of newly synthesized DNA. The incubation time was 10 min at 36 °C. 0.5 μg of purified DNA; 0.45 μg/assay "RNA-primer" molecules and 0.6 μg of DNA dependent DNA polymerase 1 were used. The amount of acid precipitable ^{3}H-labelled DNA (TCA, 5% solution) was determined in the absence and presence of each tested compound. The acid precipitable product was filtered on a millipore glass filter, washed, and dried. Its radioactivity was then measured with a Packard liquid scintillation counter.

Partially purified DNA dependent DNA polymerase 1 (EC 2.7.7.7) orginated from *Escherichia coli* (Beljanski and Beljanski 1974).

In vivo studies on cell growth. RNA primer molecules isolated from the growth media of 3-h cells and 48-h mycelia of the nor-

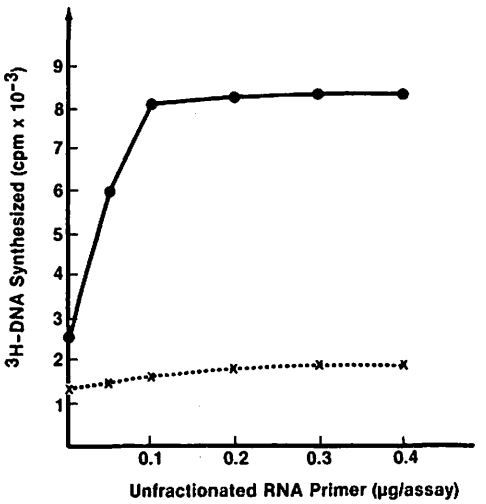


Fig. 1. In vitro DNA synthesis with increasing concentration of the 3-h *N. crassa* wild type unfractionated RNA primers when reacted with (•——•) slime DNA; and when reacted with (x – – x) *N. crassa* wild strain DNA

Table 1. In vitro DNA synthesis in the presence of unfractionated RNA primer found in *Neurospora crassa* culture media grown for different hours

Origin of RNA primer	Amount of DNA (cpm) synthesized in 10-min at 36 °C	
	With template DNA from	
	Wild type	Slime mutant
1-h sprout culture medium	1260	1301
3-h sprout culture medium	1272	7204
3-h sprout culture medium (after hydrolysis)	1272	1423
10-h sprout culture medium	1269	1362
24-h mycelial culture medium	1266	1298

Details of reaction conditions etc. are given in the text

mal strain and from the growth medium of the slime mutant were used. RNA-primer molecules were added in the normal growth medium (i.e. minimal medium containing sucrose and all of the needed salts) and the slime mutant were grown in the special nutrient broth medium, as described by Dutta et al. (1976). The amount of RNA primers was adjusted according to 50 ml of growth media.

Both the normal *N. crassa* cells and the slime mutant were grown under constant shaking at 25 to 26 °C for 48 h, when optimum growth of the cultures were obtained. The yield per flask for the normal mycelia was taken in gm after straining through 4-fold cheese cloth. The growth of the slime mutant was measured by optical density at 600 nm.

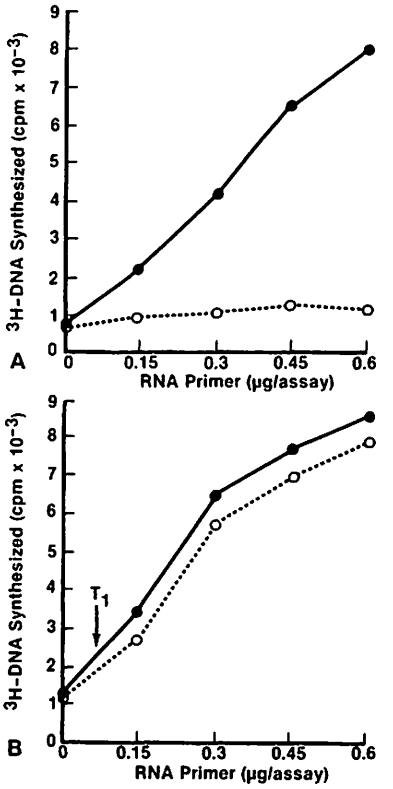


Fig. 2A, B. Effect of RNase T1. When RNA primer of 3-h *N. crassa* wild strain reacted with slime mutant DNA. A at the initiation of the reaction: •——• with no RNase T1, and o – – o with RNase T1, B when RNase T1 treated one hour after the reaction started: •——• not treated, and o – – o when T1 treated

Results

Effects of the N. crassa (3-h) and the slime mutant (48-h) unfractionated RNA primers on in vitro DNA synthesis

RNA-primer molecules from 3-h germinated conidia (also referred as sprouts) stimulated in vitro slime DNA synthesis but those from *N. crassa* wild type DNA did not. The increase of DNA synthesis was dependent on the concenration of this small particular "RNA-molecule" (Fig. 1). Such RNA molecules were not found in growth medium from 10 h sprouts (Table 1). The RNA primer molecules isolated from 48 h slime growth medium were used for in vitro DNA synthesis tests. When the slime RNA primer was used, in vitro synthesis of slime DNA was stimulated, but this slime RNA-primer was only poorly active on the wild type DNA isolated from 24-h mycelia.

In vitro DNA synthesis was not abolished by pancreatic ribonuclease added either at 0 time of incubation or later. In contrast, RNase T1 strongly depressed DNA in vitro synthesis only when this enzyme was added to the incubation mixture at 0 h time (Fig. 2) of incubation

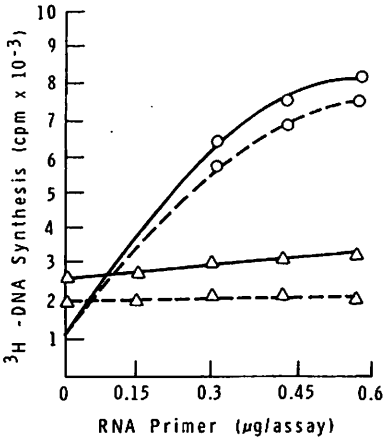


Fig. 3. Effect of *N. crassa* wild strain 3-h RNA primer on the in vitro synthesis of DNAs from normal mice spleen (△——△) and lung cells (△ – – △); and from human lung cancer DNA (o——o) and human breast cancer (o – – o) DNA

or when RNA primer was preincubated with it. Thus the integrity of RNA primer molecule was necessary for DNA replication. Analysis of the particular RNA primer molecules from 3-h *N. crassa* cells showed that they contained 90% of G for only 10% of A nucleotides, thus suggesting that some initiation site of *Neurospora crassa* DNA replication should contain complementary bases (C and T).

In the TLC-plates, 3-h RNA primers were seen in significantly higher quantities than in from 1-, 10-, and 48-h growth cultures.

Effect of N. crassa wild strain 3-h unfractionated RNA primer on the synthesis of DNA from animal tissues

DNA from mouse spleen and lung as well as from human breast and lung cancer DNA have been used as templates for DNA synthesis in the absence and presence of

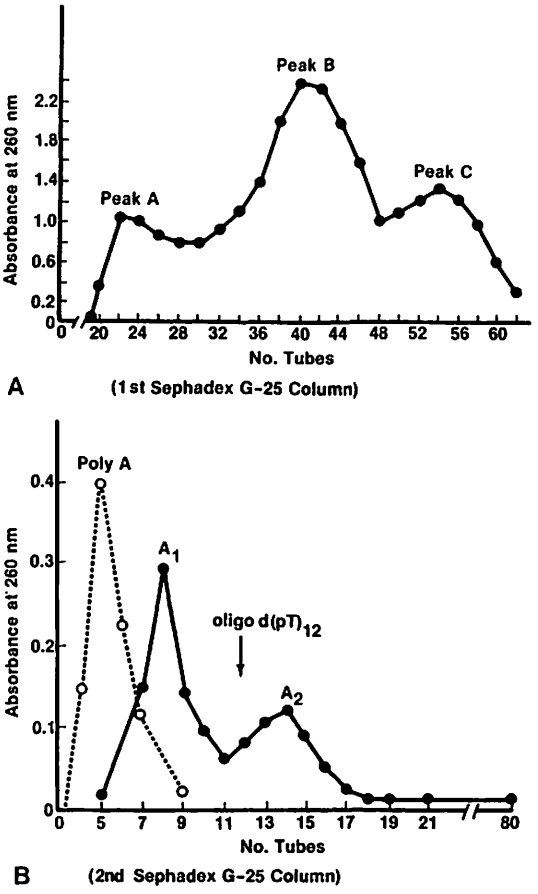


Fig. 4A, B. Composite data upon Sephadex G-25 fractionation of 3-h RNA primer. A First fractionation, B Second fractionation of peak A from the first fractionation. Details are given in the text under Materials and methods

N. crassa 3-h wild strain "RNA primer". Figure 3 shows that RNA primer used at increasing concentrations strongly stimulates in vitro DNA synthesis of human lung and breast cancer cell DNAs but not of normal mice lung and spleen DNA.

Table 2. Base analysis and priming in vitro activities of RNA primers isolated from 3-h sprouts

Fractions from 3-h sprout	Moles for 100 moles of analysed nucleotides				In vitro DNA synthesis (cpm) template DNA	
					Slime mutant	Wild type
	A	G	C	U	2060 no primer	1268
Fraction A_1 (0.4 µg)	36	64	0	0	7400	1416
Fraction A_2 (0.4 µg)	24	76	0	0	13906	1610
Fraction B_1 (1.2 µg)	23	77	0	0	7430	Not done
Fraction B_2 (0.08 µg)	12	88	0	0	5800	1400
Fraction C (3.7 µg)	2	98	0	0	5900	1385

CPM (counts per minute) shown here are those measured in the presence of indicated concentrations which lead to maximal DNA synthesis (plateau)

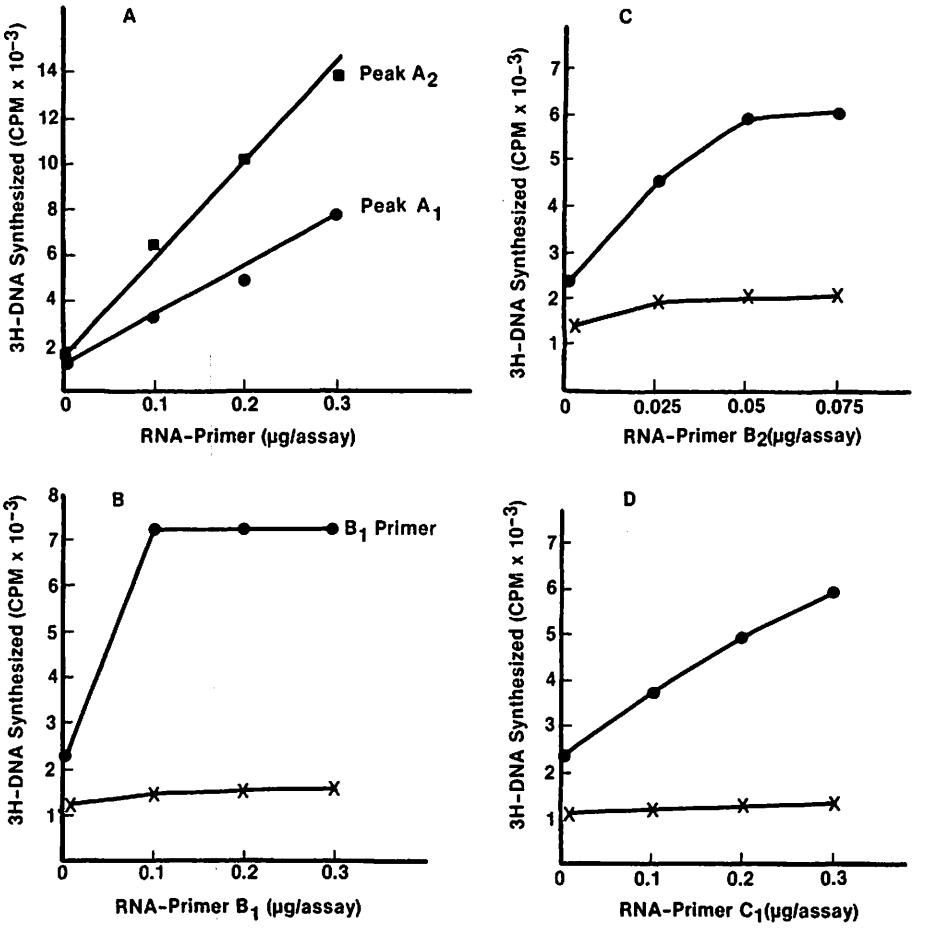
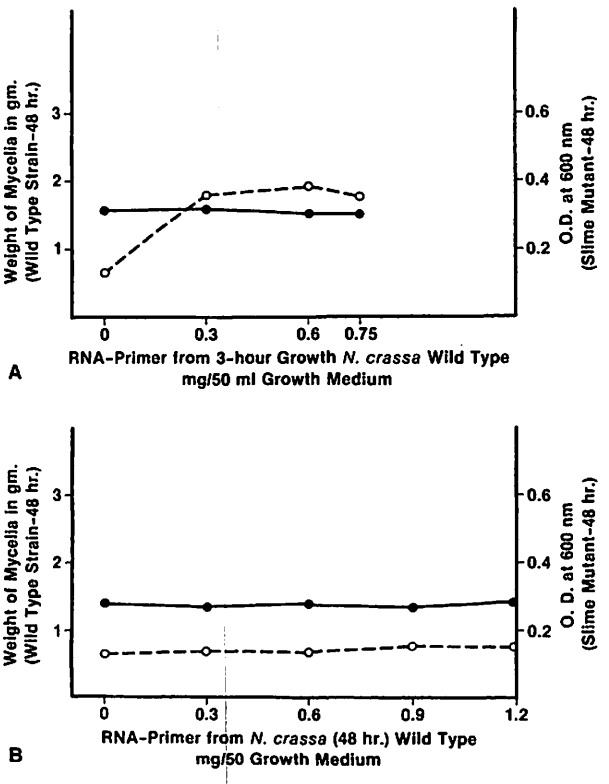


Fig. 5A–D. Concentration dependent effects of various fractions of *N. crassa* wild strain (3-h) RNA primers on in vitro synthesis of DNAs from the *N. crassa* wild type and the *N. crassa* wild type and the *N. crassa* slime mutant. A when peak A_1 (•——•) and A_2 (▪——▪) were used on slime DNA, B when B_1 fraction was used on *N. crassa* slime DNA (•——•) and on *N. crassa* wild DNA (x——x), C when B_2 fraction was used on slime DNA (•——•) and *N. crassa* wild strain DNA (x——x), D when fraction C RNA-primer was used on slime DNA (•——•) and on wild DNA (x——x)



◀ Fig. 6. A Effect of *N. crassa* wild strain 3-h RNA primer on the in vivo synthesis of DNAs from *N. crassa* slime mutant DNA (o – – o); and on *N. crassa* wild strain DNA (•——•). B When 48-h mycelia RNA primer used with (o – – o) slime DNA; and (•——•) wild DNA

Effects of N. crassa (3-h) fractionated RNA-primer on in vitro synthesis

The results given in Figs. 4A–4B, 5A–5D, and Table 2 summarize ribonucleotide analyses of various fractions of RNA isolated from 3-h sprout and show that excessively G-rich small RNAs (10–20 nbp) were excreted into the 3-h culture medium of wild type strain of *N. crassa*. These RNA: A_1, A_2, B_1, B_2, and C were shown to be excellent primers for in vitro synthesis for *N. crassa* slime mutant DNA.

Differential effects of RNA primers on in vivo cell growths

Figure 6 shows in vivo growths when RNA primers from the 3-h sprouts (Fig. 6A) and from the 48-h mycelia (Fig. 6B) were added exogeneously in the culture me-

Various organisms allow an excessive transcription on certain genes such as those coding for ribosomal or other types of RNAs. These RNAs might undergo cleavage by nucleases. Some degradation products are excreted into the culture medium, as shown in this work. It was reported, many years ago, that small transforming RNA may be excreted into the culture medium by the *E. coli* show-domycin resistant strain (Beljanski et al. 1972). This RNA is purine rich and possesses quite interesting biological properties (Beljanski et al. 1972; Beljanski and Le Goff 1986).

Figures 6 and 7 clearly demonstrate the biological properties of particular RNA-primers on differential cell growth. The data shown in vitro DNA synthesis are confirmed in actual growth of cells. The in vitro and in vivo data provide evidence supporting the model of Beljanski (1983): that particular RNA primers can act more efficiently on destabilized DNA which are the characteristic of cancer or cells of the *N. crassa* slime mutant.

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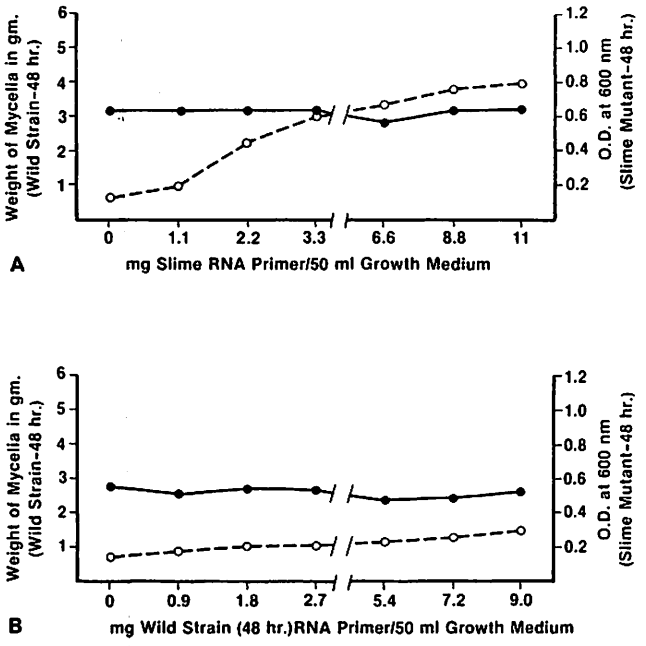

Fig. 7A, B. Differential effects of RNA primers on the in vivo growth of *N. crassa* slime mutant and the normal cells. **A** when RNA primer is isolated from the *N. crassa* slime mutant and used in the growing medium of the slime mutant (o – – o) and wild type strain (•——•); and **B** when RNA primer was isolated from the *N. crassa* wild type

dium. Three-hour RNA primer can stimulate slime growth but not the normal mycelia growth. Figure 7A shows data on enhancement of in vivo growth of the slime mutant by slime mutant RNA primer. Figure 7B shows that RNA primer isolated from the culture medium of the *N. crassa* (48-h) mycelia cannot stimulate the growth of the slime mutant or growth of normal mycelia.

Discussion

Data presented thus far clearly show that purine (G) rich RNA molecules obtained from growth medium of 3-h *N. crassa* wild strain were excellent primers for in vitro synthesis of given DNAs. RNA primer isolated from the slime mutant culture medium was highly active with mutant DNA used as a template but was poorly active with wild strain DNA in vitro synthesis. The fact that, in the absence of exogeneously added primer, DNA from the mutant strain was slightly better template than wild type DNA, suggested some differences in the DNA initiation sites, as has already been shown with wild and mutant strains of *Salmonella typhimurium* (see Beljanski 1983). For this reason, we attempted here to demonstrate the differential behavior of these two types of DNAs. The fact that DNAs from the slime mutant strain and those from human cancer cells were better templates for in vitro DNA synthesis (Figs. 1, 2 and 5) and in vivo growth (Figs. 6 and 7) than *N. crassa* wild type DNAs suggests that the former DNAs might have more free initiation sites for DNA replication, some of which are easily accessible to RNA primer from the 3-h growth medium.

It appears now well established that DNAs from human cancer tissues, plant cancer DNA (Beljanski and Le Goff 1979), and bacterial and fungal mutant have a common destabilized (or relaxed) secondary structure, making these DNAs susceptible to exogeneous chemical compounds. Thus one may conceive that "destabilized DNAs" of various cells or tissues, which for some reason have lost physiological control mechanisms, allow an excessive transcription of certain genes such as those coding for ribosomal or other types of RNAs. RNAs accumulated in excess for a given time might undergo cleavages by different nucleases, thus furnishing oligoribonucleotides which participate either in DNA replication as primers or act as regulators of the translation process (Lee-Juang et al. 1977). Some degradation products were excreted into the culture medium as shown in this work. Along these lines, it should be recalled that a small size transforming (purine rich) RNA is excreted into the culture medium by the *E. coli* showdomycin-resistant strain. This RNA possesses quite interesting biological properties, which have been recently reviewed and discussed by one of us (Beljanski 1983).

These RNA-primer molecules, when non-hydrolysed, strongly stimulated slime mutant *Neurospora crassa* in vitro DNA synthesis. The increase of DNA synthesis was dependent on the concentration of this small particular "RNA molecule". In contrast, such a molecule was not found in 1- and 10-h grown cells or in culture medium of mycelia (24-h). The fact that RNA primer molecule has been found in 3-h sprout culture medium suggests that this material originates from extra copies of ribosomal RNAs which accumulated during the 3-h period and then disappeared immediately when cell division started. Tyler and Giles (1985) have also demonstrated the maximum in vitro rRNA transcription at 3 h.

RNase T1 destroyed priming activity, while RNase A was without effect. This finding confirms that RNase A cleaves the ribonucleotide chains at pyrimidine bases, while RNase T1 exhibits its action on guanine nucleotides in the RNA molecules, thus explaining the selective hydrolysing effect of RNase T1 on RNA primers. The absence of RNase T1 activity on RNA primer which has been engaged in DNA chain synthesis might be explained by the fact that, once fixed on DNA, RNA primer was not attainable by T1 nuclease. Similar observations have been reported in other systems (Schekman et al. 1972; Fox et al. 1973). It should be pointed out that primers A_1, A_2, B_1, B_2, and C active in slime mutant DNA synthesis were without effect on wild type DNA in vitro synthesis (Table 2).