



PROPERTIES OF A POLYNUCLEOTIDE SYNTHESIZED BY STRAIN 74A OF *NEUROSPORA CRASSA*

ELEONORA C. CARMONA,* SANG W. HAN,* MARIO S. PALMA† and ANTONIO ROSSI‡§

*Departamento de Bioquímica e Microbiologia, IB – UNESP, 13506-900 Rio Claro, SP, Brazil; †Departamento de Biologia, IB-UNESP, 13506-900 Rio Claro, SP, Brazil; ‡Departamento de Química, FFCLRP-USP, 14040-901 Ribeirão Preto, SP, Brazil

(Received in revised form 5 June 1995)

Key Word Index: *Neurospora crassa*; fungi; polynucleotide; alkaline phosphatase; RNA-binding proteins.

Abstract—A polynucleotide (or a fragment of RNA) was purified to apparent homogeneity by HPLC from mycelium of the wild strain 74A of the mould *Neurospora crassa*, after growth on sucrose and in the presence of saturating amounts of inorganic phosphate (Pi) for 72 hr at 30°. The M_r was ca 20 000 as determined by HPLC at pH 6.8. Polynucleotide synthesis ranged from 4.0 to 6.5 µg polynucleotide per mg dry mycelium in mycelium of the wild strain 74A and the various phosphorus regulatory and structural mutant strains of the mould *N. crassa*. Kinetic data showed that the polynucleotide interacts with mycelial Pi-repressible alkaline phosphatase by inhibiting its *p*-nitrophenylphosphatase activity and by protecting the enzyme against thermal inactivation in the presence of high concentrations of ammonium sulphate.

INTRODUCTION

Several proteins of known enzymatic function that are apparently unrelated to RNA metabolism have been found to possess RNA-binding activities [1]. Although their binding specificity and physiological relevance are not well defined, it is possible that RNA-enzyme interactions participate in either post-transcriptional (auto)regulation or metabolic regulation mechanisms involving oligoribonucleotide or a fragment of RNA [1, 2]. RNA-enzyme interaction could also be involved in regulating the localization of an exportable enzyme or its interaction with membranes [3, 4].

In the present paper we report the purification of a polynucleotide (or a fragment of RNA) which is constitutively synthesized by the wild strain 74A of the mould *Neurospora crassa*. Some properties of the purified polynucleotide such as the M_r and the effect on mycelial inorganic phosphate (Pi)-repressible alkaline phosphatase synthesized by *N. crassa* were also determined.

RESULTS AND DISCUSSION

The results presented in Fig. 1 show that the mould *Neurospora crassa* synthesizes a factor that protects Pi-repressible alkaline phosphatase against thermal inactivation at 50° in the presence of high concentrations of

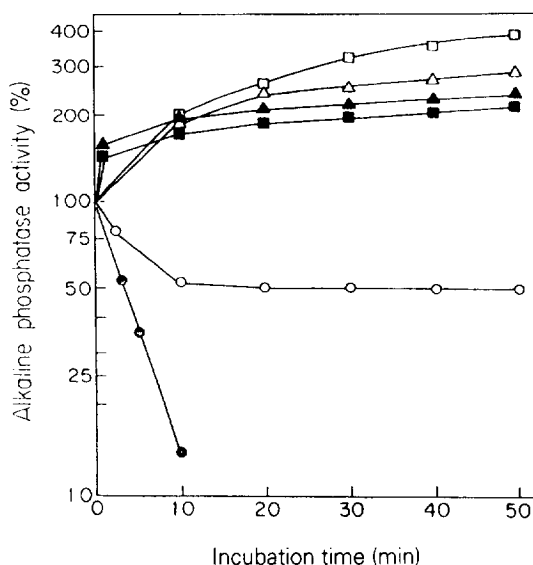


Fig. 1. Thermal stability of purified alkaline phosphatase synthesized by mycelium of *N. crassa* grown in 50 µM Pi for 72 hr by standing at 30°. The purified enzyme was incubated for the time indicated at 50° in 0.2 M Tris-HCl buffer, pH 9. □ and ■ represent velocities of the enzyme synthesized at pH 5.4 and pH 7.8, respectively, with no further additions. ○ and ● represent velocities of the enzyme synthesized at pH 5.4 and pH 7.8, respectively, in the presence of 4.2 M $(\text{NH}_4)_2\text{SO}_4$. △ and ▲ represent velocities of the enzyme synthesized at pH 5.4 and pH 7.8, respectively, in the presence of the supernatant recovered in the 95% $(\text{NH}_4)_2\text{SO}_4$ saturation, and obtained by salt fractionation of the crude extract.

§Author to whom correspondence should be addressed.

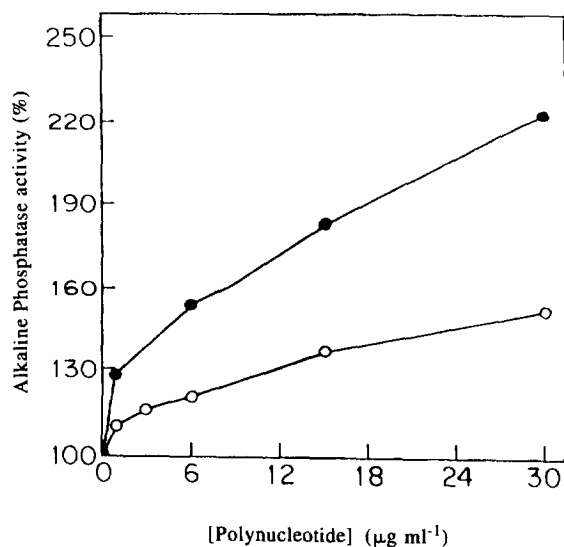


Fig. 2. Effect of concentration of the polynucleotide on the *p*-nitrophenylphosphatase activity of purified alkaline phosphatase synthesized by mycelium of *N. crassa* grown in 50 μ M Pi, pH 5.4. The purified enzyme was incubated for 1 hr at 50° in 0.2 M Tris-HCl buffer, pH 9, containing 1.3 M $(\text{NH}_4)_2\text{SO}_4$ (final concentration) in the absence and presence of various concentrations of polynucleotide. Curves represent the effect of polynucleotide purified from mycelium grown on high (●) and low (○) Pi medium, respectively.

ammonium sulphate. The enzyme stabilizing factor was detected in the supernatant of mycelial extracts precipitated with ammonium sulphate up to 95% salt saturation and was characterized as a polynucleotide because it did not reveal colorimetric reaction for proteins and deoxyribose, its ultraviolet absorption spectrum showed maximum absorbance at 257 nm, and the A_{260}/A_{280} ratio was *ca* 1.98. A molar ratio of 1:1:1 was found for Pi/ribose/nitrogen bases.

Mycelial specific activities for polynucleotide synthesis (values ranging from 4.0 to 6.5 μ g polynucleotide per mg dry weight mycelium) showed that *N. crassa* synthesizes this polynucleotide constitutively, i.e. regardless of the extracellular pH and Pi concentrations. Almost the same specific activities for polynucleotide synthesis were observed in mycelium of the various phosphorus regulatory and structural mutant strains grown on different media (*nuc-1A*, *nuc-2A*, *preg^c*, *pcon^c*, *pho-2A* and *pho-3A*).

The procedure described here provided good conditions for the purification of the major polynucleotide synthesized by mycelium grown on both 50 μ M and 5 mM Pi that protects Pi-repressible alkaline phosphatase against inactivation. All preparations (at least four independent preparations of each growth condition) appeared to be homogeneous by HPLC at pH 6.8, with the sharp peak having a retention time of *ca* 16 min and representing at least 98% of the total polynucleotide injected. The M_r of the purified polynucleotide was *ca* 20 000 as determined by HPLC, indicating that it may be composed of about 60 ribonucleotide residues. Although

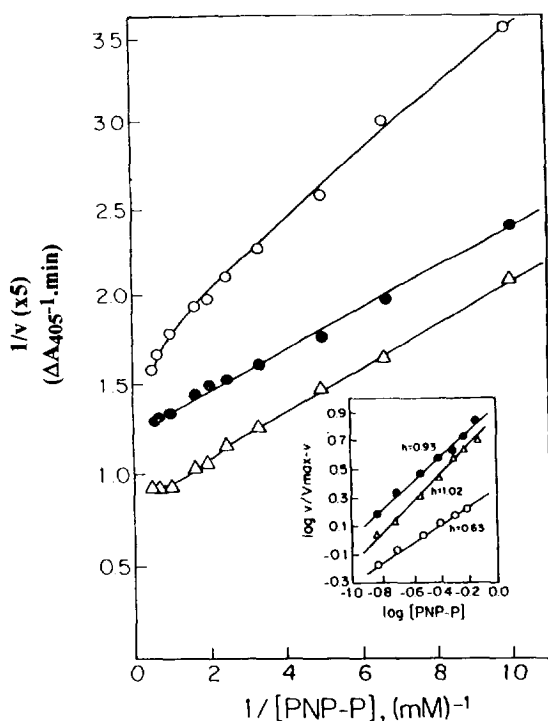


Fig. 3. Double reciprocal plots of velocity of repressible alkaline phosphatase purified from mycelium grown at pH 5.4, as a function of PNP-P concentration at pH 9. Each tube contained 0.6 μ g of purified enzyme. ● and ○ represent the velocities in the absence and presence of polynucleotide, respectively. Δ represents the velocities after incubation of the enzyme at pH 9 and at 50°, in the presence of polynucleotide. Each tube contained 1.2 μ g of polynucleotide purified from mycelium grown in high Pi medium, at pH 5.4. Insert: Hill plots of $\log v/(V_{\max}-v)$ versus $\log$ PNP-P concentration. ● and ○ represent the velocities in the absence and presence of polynucleotide. Δ represents the velocities measured after incubation of the enzyme at pH 9 and 50°, in the presence of polynucleotide.

the data allow only a crude estimate of its molecular size, the polynucleotide is possibly smaller than other small RNAs such as transfer and ribosomal RNAs.

Figure 2 shows that the polynucleotide not only protects but also apparently activates the Pi-repressible alkaline phosphatase when the enzyme is heated in the presence of high concentrations of ammonium sulphate. This effect is dependent on the concentration of polynucleotide and was lower when it was purified from mycelium grown on low-Pi medium. The probable action of the RNases synthesized on low Pi medium may have decreased the size of the polynucleotide and changed its interaction with the enzyme. The dissociation promoted by heating Pi-repressible alkaline phosphatase at 50°, pH 9, changes the steady state kinetic properties of the enzyme, i.e. alkaline phosphatase synthesized at pH 5.4 is of the Michaelis type when assayed in the dimeric form ($K_m = 0.9 \times 10^{-4}$ M, $n = 0.93$) (Fig. 3) and deviates from Michaelis kinetics when assayed after dissociation [5]. These and other results suggest the existence of an equilibrium between forms when the enzyme synthesized at

pH 5.4. is monomerized and assayed at pH 9.0 [5]. Heating the enzyme in the presence of the purified polynucleotide did not change its Michaelis kinetics (Fig. 3), but activation of the enzyme occurred with increasing K_m value for substrate hydrolysis ($K_m = 1.4 \times 10^{-4} \text{ M}$, $n = 1.02$) probably due to stabilization of one of the monomeric enzyme forms [5]. However, the purified polynucleotide inhibits the dimeric form of Pi-repressible alkaline phosphatase synthesized by *N. crassa* grown at pH 5.4, by affecting its Michaelis kinetics (Fig. 3), i.e. the enzyme assay at pH 9 and at 37° shows negative cooperativity ($K_{0.5} = 3.3 \times 10^{-4} \text{ M}$, $n = 0.63$). A Pi-repressible acid phosphatase synthesized by *N. crassa* grown at pH 5.4 [6] was not affected by the purified polynucleotide (results not shown).

The present results show the constitutive expression of a polynucleotide (or a fragment of RNA), which interacts with Pi-repressible alkaline phosphatase. Although speculative, it is possible that this polynucleotide-enzyme interaction is involved in the chaperonage of alkaline phosphatase through its excretion pathway. The probable constitutive synthesis of Pi-repressible phosphatases supports this hypothesis [6].

EXPERIMENTAL

Strains and culture conditions. The following strains of *N. crassa*, obtained from the Fungal Genetic Stock Center (University of Kansas Medical Center, Kansas City, Kansas, USA), were used: wild-type strain St. L. 74A, *nuc-1A* mutant (FGSC 1995), *nuc-2A* mutant (FGSC 1996), *pcon^c* mutant (FGSC 2534), *preg^c* mutant (FGSC 2532), *pho-2A* mutant (FGSC 3061) and *pho-3A* mutant (FGSC 3051). Both *nuc-1A* and *nuc-2A* mutant strains were selected for their inability to utilize RNA or DNA as their sole phosphorus source [7, 8]. The *preg^c* mutant strain was selected for its ability to produce Pi-repressible alkaline phosphatase and to secrete acid phosphatase constitutively [9]. The *pcon^c* mutant strains are constitutive for repressible alkaline phosphatase and for a high affinity, high pH phosphate permease [7]. The *pho-2A* and *pho-3A* mutant strains were identified as carrying mutations in the structural genes for Pi-repressible alkaline phosphatase [10, 11] and for acid phosphatase [12], respectively. Stock cultures were maintained on slants of Vogel's medium (1.6% agar) [13]. Conidial suspensions (0.5 ml containing $ca 10^8 \text{ cell ml}^{-1}$) of each strain were grown for 72 hr at 30°, and without shaking on Petri dishes (14 cm diam.) containing 50 ml of high (7 mM) or low (50 or 500 μM) Pi medium adjusted to pH 5.4 (non-buffered) or pH 7.8 (buffered with 50 mM Tris-HCl), supplemented with 44 mM sucrose as carbon source and prepd as described previously [14, 15].

Prepn of extracts and assays. The mycelium harvested from each strain after growth on both low and high Pi medium at pH 5.4 or pH 7.8 was extracted with sand and 50 mM NaOAc buffer containing 1% SDS, pH 5 (30 ml buffer g^{-1} mycelium) and centrifuged for 20 min at 8000 g at 4°. The supernatant (crude extract) was col-

lected by centrifugation and brought to 95% (w/v) saturation by the addition of solid $(\text{NH}_4)_2\text{SO}_4$. The mixture was stirred for 15 min, left standing for 2 hr at 4° and then centrifuged 15 min at 12000 g . The ppt was discarded and samples of the supernatant were mixed vigorously with an equal vol. of phenol- CHCl_3 -3-methyl-1-butanol (50:50:1) [16]. The emulsion was centrifuged for 10 min at 5000 g and the upper aq. phase was used for quantitative determinations of polynucleotide, after 5-fold dilution. Mycelial specific activities for polynucleotide synthesis are reported as μg polynucleotide per mg dry wt mycelium. After growth for 72 hr, mycelium was harvested by filtration, washed with H_2O , blotted to remove excess liquid, dried for 24 hr at 98° and weighed (dry wt mycelium). M_r were measured by HPLC on an aquapore SEC column, model OH-300 (4.6 mm $\times$ 25 cm, CG Instruments, Brazil), using appropriate RNA markers. RNA and polynucleotide samples (10 μl) were injected into a HPLC column (CG Instruments) previously equilibrated with 50 mM NH_4OAc buffer, pH 6.8. Elution was performed with this same buffer at a flow rate of 250 $\mu\text{l min}^{-1}$. A at 254 nm was monitored.

Total polynucleotide or ribose was measured by the orcinol method, as modified in Ref. [17] using *E. coli* RNA or ribose as standards, respectively. Control experiments established that the orcinol reaction was unaffected in the presence of $(\text{NH}_4)_2\text{SO}_4$ up to 50% satn. Polynucleotide hydrolysis was carried out 0.1 M HCl for 60 min at 108° and the Pi liberated was measured by the method of Ref. [18]. Total nitrogen bases were measured by the method of Ref. [19] using a mixture containing equimolar concns of uracil, adenine, cytosine and guanine in 10 mM HCl as standard, and A was measured at 260 nm ($E = 10150 \text{ l.mol.cm}$). DNA was measured by the diphenylamine colorimetric method as proposed in Ref. [17] using 2-deoxyribose as standard. Protein was measured by the method of Ref. [20] using bovine serum albumin as standard.

The alkaline phosphatase assay as described in Ref. [21] was carried out in 0.3 M glycine buffer (pH 9) containing 1 mM EDTA using 2 ml of 6 mM *p*-nitrophenylphosphate (PNP-P) as substrate. One unit of alkaline phosphatase activity was defined as 1 μmol substrate hydrolysed per min, at 37°. Sp. acts are expressed as units (mg protein^{-1}).

Thermal stability. Relative heat stability was determined in the absence and presence of $(\text{NH}_4)_2\text{SO}_4$ or purified polynucleotide by incubating the enzyme diluted conveniently in 0.2 M Tris at pH 9, at 50°, in the same experiment. At appropriate times, samples (0.1 ml) were taken to measure *p*-nitrophenylphosphatase activity, at pH 9, as described under *Prepn of extracts and assays*.

Kinetic studies. Maximum velocity (V_{max}) and K_m were determined by plotting initial velocities as described in Ref. [22]. Interaction constants for the substrate (n) were determined by the Hill procedure as described in Ref. [23]. The kinetic constants given in this paper were obtained from linear-square analysis.

Enzyme purification. Mycelial Pi-repressible alkaline phosphatase synthesized by the wild-type strain of the

mould *N. crassa* grown at pH 5.4 or pH 7.8 was purified to apparent homogeneity by 7.5% PAGE [21,24] as described in Ref. [5].

Polynucleotide purification. The supernatant recovered at 95% $(\text{NH}_4)_2\text{SO}_4$ satn, and obtained by salt fractionation of the crude extract as described under *Prepn of extracts and assays*, was treated twice with an equal vol. of phenol- CHCl_3 -3-methyl-1-butanol (50:50:1) as described in Ref. [16]. Phenol present in the upper aq. phase was removed by 4 successive extractions with Et_2O , which in turn was removed by evaporation for 2 hr at 50° [25]. Small samples (*ca* 4 ml) with no further treatment were chromatographed on a Sephadex G-25 column (1.1×56 cm) previously equilibrated with 50 mM NaOAc buffer, pH 5. Elution was performed with this same buffer at a flow rate of 15 ml hr^{-1} (1.5 ml fractions). Polynucleotide elution was detected by measuring both *A* at 257 nm and the protective effect against inactivation of mycelial Pi-repressible alkaline phosphatase in the presence of 3.4 M $(\text{NH}_4)_2\text{SO}_4$, at 50° , as follows: an aliquot (0.1 ml) of each effluent fraction was mixed with *ca* 0.8 enzyme units in 0.2 M Tris-HCl, pH 9, containing 4 M $(\text{NH}_4)_2\text{SO}_4$. (The enzyme used in this assay was that synthesized at pH 7.8 and recovered in the 60–95% $(\text{NH}_4)_2\text{SO}_4$ satn as described in Ref. [5]). After incubation for 40 min at 50° , *p*-nitrophenylphosphatase activity was measured at pH 9 as described under *Prepn of extracts and assays*. The tubes representing the polynucleotide peak were pooled, freeze-dried, dissolved in a small vol. of 0.2 M Tris-HCl pH 8.9, and stored at 4° .

Acknowledgements—This work was supported by grants from FAPESP, CNPq, IDB-USP and FUNDUNESP. We thank Mrs. Elettra Greene for revising the English and Newton R. Alves for technical assistance.

REFERENCES

- Hentze, M. W. (1994) *Trends Biochem. Sci.* **19**, 101.
- Jonsson, I. (1981) *Biochem. Biophys. Acta* **660**, 204.
- Walter, P. and Blobel, G. (1982) *Nature* **299**, 691.
- Mattaj, I. W. (1993) *Cell* **73**, 837.
- Palma, M. S., Han, S. W. and Rossi, A. (1989) *Phytochemistry* **28**, 3281.
- Han, S. W., Michelin, M. A., Barbosa, J. E. and Rossi, A. (1994) *Phytochemistry* **35**, 1131.
- Metzenberg, R. L. (1979) *Microbiol. Rev.* **43**, 361.
- Ishikawa, T., Toh-e, A., Uno, I. and Hasunuma, K. (1969) *Genetics* **63**, 75.
- Metzenberg, R. L., Gleason, M. K. and Littlewood, B. S. (1974) *Genetics* **77**, 25.
- Lehman, J. F. and Metzenberg, R. L. (1976) *Genetics* **84**, 175.
- Han, S. W., Maccheroni Jr, W. and Rossi, A. (1992) *Brazilian J. Med. Biol. Res.* **25**, 441.
- Nelson, R. E., Lehman, J. F. and Metzenberg, R. L. (1976) *Genetics* **84**, 183.
- Vogel, H. J. (1956) *Microbiol. Genet. Bull.* **13**, 42.
- Crocken, B. and Nyc, J. F. (1963) *Can. J. Microbiol.* **9**, 689.
- Nahas, E., Terenzi, H. F. and Rossi, A. (1982) *J. Gen. Microbiol.* **128**, 2017.
- Clemens, M. J. (1984) in *Transcription and Translation: A Practical Approach* (Hames, B. D. and Higgins, S. J., eds). IRL Press Ltd, Oxford.
- Ashwell, G. (1957) in *Methods in Enzymology* (Vol. III) (Colowick, S. P. and Kaplan, N. O., eds). Academic Press, New York.
- Gomori, G. (1942) *J. Lab. Clin. Med.* **27**, 955.
- Bendich, A. (1957) in *Methods in Enzymology* (Vol. III) (Colowick, S. P. and Kaplan, N. O., eds). Academic Press, New York.
- Hartree, E. F. (1972) *Anal. Biochem.* **48**, 422.
- Nahas, E. and Rossi, A. (1984) *Phytochemistry* **23**, 507.
- Lineweaver, H. and Burk, D. (1934) *J. Am. Chem. Soc.* **56**, 658.
- Koshland, Jr, D. E. (1970) in *The Enzymes* (Vol. I) (Boyer, P. D., ed.). Academic Press, New York.
- Davis, B. J. (1964) *Ann. NY Acad. Sci. USA* **121**, 407.
- Furukawa, K., Hasunuma, K. and Hamada, Y. (1987) *Fungal Genet. Newsl.* **34**, 30.