An efficient isolation method for polyadenylated messenger ribonucleic acid from Neurospora mycelia

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Abstract
An efficient isolation method for polyadenylated messenger ribonucleic acid from N. crassa.
Nine variants and four derivatives of variants were genetically analysed. The determinants of deterioration acted as single genes; all of them were recessive in the diploid. They were found in all linkage groups, with the exception of groups V and VIII (Figure 1). In Figure 1, linkage maps are not drawn to scale and are approximate. The symbol | designates a deletion. The symbol \^ designates the determinant of deterioration while a square designates the genetic marker of the "Master" strain. The broken line represents the duplicate segment of linkage group I terminally attached to linkage group II. Variants allocated only to their linkage group are shown at the right. Centromeres are represented by open circles. The symbol /---\ indicates that the determinant of deterioration of the variant is linked to the genetic marker and may be located in any of the sides.

To perform the allelism tests, besides the variants isolated in this work, some deteriorated variants obtained by other authors were used. When the two determinants of deterioration are not alleles, we find normal and deteriorated colonies. If the determinants are alleles we find only deteriorated colonies. The determinants of deterioration were alleles in only two out of sixteen crosses performed. These results indicate that the determinants of deterioration must be located at random in the linkage groups, although there are spots in certain linkage groups in which an expressive number of determinants of deterioration is located. Supported by PIG/CNPq. – – – ^1 Dept. de Genetica, I.B., UFRJ, Caixa Postal 68.011, Rio de Janeiro, Brazil; ^2Inst. de Genetica, ESALQ/USP, Caixa Postal 83, Sao Paula, Brazil

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We have analyzed orthophosphate repressible enzymes produced in culture medium of wild type (74A) hyphae grown in low phosphate medium. Protein and enzyme relationships of alkaline phosphatase, 5'-nucleotidase, acid and alkaline nuclease, cyclic phosphodiesterase (cPDase), endonuclease and ribonuclease N1 were established using SDS-polyacrylamide gel electrophoresis and two dimensional gel electrophoresis (Furukawa, Hasunuma and Shinohara, submitted).

To analyze the regulation of these enzymes at the transcriptional level and to clone cDNAs for these genes, the development of an efficient method for the preparation of polyadenylated messenger ribonucleic acid (mRNA) was essential. In addition to the above nucleases, wild type hyphae grown in low phosphate medium contain numerous nuclease activities (K. Hasunuma, 1978, Molec. Gen. Genet. 160:259-265). To block these nuclease activities, the isolation method for mRNA using 6 M urea and 3 M LiCl (J.A.A. Chambers and V.E.A. Russo, 1986, Fungal Genet. Newsl. 33:25-26) is insufficient. For our method, we modified the phenol based procedure for mRNA isolation (W.R. Reinert, V.B. Patel and N.H. Giles, 1981, Molec. Cell. Biol. 1:829-835). The procedure results in a good yield of RNA (1.1 mg/g fresh wt.) compared with the other procedure (0.5-1 mg RNA/g fresh wt. with 6 M urea and 3 M LiCl). The procedure is also suitable for mini-preparations and for radioisotopic labeling. The procedure is as follows:

A conidial suspension (10^6 cells/ml; 10 ml) was inoculated into 1 l of low phosphate (1/20 Pi) Fries minimal medium in a Sakaguchi flask (2 l). The culture was shaken at 25° C for 24 hr. Fifteen minutes before harvesting hyphae, cycloheximide (final concentration, 0.5 mg/ml) was added. The hyphal pads were collected by filtration and stored frozen at -80° C.

Frozen hyphae (10 g) were powdered with a pestle in a mortar containing liquid nitrogen. Five volumes of 0.1 M Tris (pH 9.0)/1 mM EDTA/1% SDS were added and the same volume of 80% phenol equilibrated with the above buffer was added. The mixture was well ground in the mortar and further homogenized using a Polytron homogenizer (Kinematica type PT10/35) at full speed to 4 to 5 set (method II). In the original procedure we did not use a Polytron homogenizer (method I). (The Polytron homogenizer could also be used for minipreparation of RNA and samples labeled with a radioisotope). A high-pH extraction buffer (pH 9.0) was used for efficient solubilization of RNA (C. Auffrang and F. Rougeon, 1980, Eur. J. Biochem. 107:303-314) and to avoid degradation of RNA by nucleases since most of the nucleases show pH optima of around 6.
The resulting homogenate was shaken for 10 min and then centrifuged at 3,000 rpm for 15 min at 15° C. The aqueous phase was taken and an equal volume of phenol:chloroform:isoamyl alcohol; 49:49:2 was added. After shaking for 10 min, the mixture was centrifuged as above and the aqueous phase taken. Phenol was removed by four extractions with ether. To the RNA solution 2.5 volumes of cold absolute ethanol (-20° C) was added and the resulting mixture was stored at -80° C for at least 30 min. The RNA precipitate was collected by centrifugation, washed with cold absolute ethanol and then dried under a flow of N2 gas. The precipitate was dissolved in 5 ml of buffer containing 0.1 M sodium acetate (pH 5.0)/1 mM EDTA/1% SDS (buffer A).

The crude RNA extract was subjected to gel filtration through a Sephadex G-100 column (2x32 cm) equilibrated with buffer A and fractionated into 2 ml fractions. RNA fractions 6 to 11 were pooled and the solution was diluted 4-fold with buffer A. About 450 times as much RNA was recovered using method II compared with that recovered with method I. The gel filtration is useful to remove small RNAs and to remove free radiolisotope when labeling of RNA was performed. To the RNA solution 2.5 volumes of cold absolute ethanol was added and the resulting mixture was stored at -80° C for at least 30 min. The ethanol precipitate was dissolved in 10 ml of 0.2 M NaCl and 25 ml of cold absolute ethanol added. After collecting the precipitate by centrifugation, it was dissolved in 10 ml of 10 mM Tris (pH 7.5)/0.5 M KCl. The RNA solution was loaded to an oligo (dT)-cellulose (Pharmacia, type 7) column. RNAs were eluted stepwise with buffers containing 10 mM Tris (pH 7.5)/0.5 M KCl, 10 mM Tris (pH 7.5)/0.1 M KCl and 10 mM Tris (pH 7.5). The polyadenylated mRNA fraction eluted with 10 mM Tris (pH 7.5) was pooled and loaded to an oligo (dT)-cellulose column. Polyadenylated mRNA was purified by 2 or 3 cycles of oligo (dT)-cellulose chromatography. Total amounts of RNA isolated by method II were 670-fold larger than those by method I, and polyadenylated mRNAs isolated by method II were 150-fold greater in amounts than those by method I. The ratio of polyadenylated mRNA to total RNA was 1.0%; this value is very similar to those reported previously (M.C. Lucas et al. 1977, J. Bacteriol. 130:1192-1198).

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Reissig and Kinney reported the role of Ca^+2 in the induction of apical branching in N. crassa STL74A (Reissig and Kinney 1983 J. Bact. 154:1397-1402). Slayman et al. (1976 Biochim. Biophys. Acta 426:732-744) had suggested that spontaneous localized depolarization events could lead to localized Ca^+2 entry and hence branching. In the present study, attempts were made to determine the influence of calcium (Ca^+2) on carbohydrate metabolism and carotenogenesis in N. crassa.

N. crassa (wild type, carotenogenic) obtained from the Division of Mycology and Plant Pathology, Indian Agricultural Research Institute, New Delhi, India was grown as described earlier (Nair and Chhatpar 1983 Neurospora Newsletter 30:11). Ca^+2 was added to the synthetic medium devoid of Ca^+2 as CaCl2 (anhydrous) at the desired concentration. Calcium deficient, calcium optimal and calcium supraoptimal conditions indicate no addition, addition of 10 ug/ml and 100 ug/ml or above to the growth medium, respectively. Methods for the preparation of cell-free extract, assay of FDP aldolase, isocitrate lyase, G6P dehydrogenase and protein were the same as described earlier (Savant et al. 1982 Experientia 38:310-311). Amylase was assayed according to the method of Bernfeld (Bernfeld, P. Meth. Enzymol. 1:149). Thin layer chromatography was carried out on 0.25 mm silica gel G plates (Ranboxy Co.). The solvent system used was 20% ethyl acetate in methylene chloride. Carotenoids were estimated according to the method of Davies (B.H. Davies, In: Chemistry and Biochemistry of Plant Pigments (T.W. Goodwin, Ed.) Academic Press. pg. 389)

N. crassa grown under calcium deficient condition showed lower activity of extracellular amylase as compared to calcium optimal and supraoptimal conditions (Table 1). Calcium deficient cultures however showed higher activities of FDP aldolase and isocitrate lyase as compared to calcium optimal and supraoptimal conditions. No significant change was observed in the activity of FDP aldolase from calcium optimal to supraoptimal conditions. However, in the case of isocitrate lyase, the activity was found to be decreased in supraoptimal as compared to optimal conditions.