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

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. Pate1 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-l 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 1 of low phosphate (1/20 Pi) Fries minimal medium in a Sakaguchi flask (2 1). 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)/l mM EDTA/l% 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 (Kinemetica 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 mixture was placed in a freezer 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 to 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 radioisotope 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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