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M Devchand

M. Kapoor

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Abstract

Preparation of a cell-free translation system from a wild type strain of *Neurospora crassa*

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Devchand, M. and M. Kapoor

Preparation of a cell-free translation system from a wild type strain of *Neurospora crassa*.

Cell-free translation systems have proved to be invaluable tools in investiaations aimed at the molecular mechanism of protein synthesis and control of gene expression. Unfortunately, the commercially available eukaryotic in vitro translation systems, derived from wheat germ and rabbit reticulocytes are not suitable for translation of all eukaryotic messengers. We found it impractical to use them for some Neurospora messengers hence the development of an efficient in vitro translation system (IVTS) of fungal origin appeared warranted.

An IVTS based on extracts of the cell-wall deficient ("slime") mutant of *N. crassa* has been reported by Szczesna-Skorupa et al. (1981, Eur. J, Biochem 121: 163). Although this strain offers the advantage of a complete absence of a rigid cell wall and facile lysis, difficulties are experienced in achieving uniform growth and reproducible cell densities on account of a heterogeneous population of cells in liquid 'cultures. An IVTS from a wild type strain should be potentially more useful as those and other artifacts originating from unrelated mutations are avoided.

During the course of a study on the isolation and cloning of the gene for Neurospora pyruvate kinase (PK) it was necessary to maximize the translation of the messenger RNA for this protein. Therefore we developed a method for preparation of a lysate from wild type *N. crassa* cells that was effective in supporting protein synthesis with homologous and heterologous messengers. We obtained efficient in vitro translation of the pyruvate kinase-specific messenger RNA as well as globin mRNA using this system In principle this method should be readily applicable toward formulation of in vitro translation systems from other filamentous fungi in general.

Preparation of cell-free extract and in vitro protein synthesis

All of the solutions used in the preparation of the lysate were autoclaved and the glassware was washed in chromic acid and treated with diethylpyrocarbonate just before use to minimize nuclease activity. Wild type *N. crassa* (FGSC #262) was grown in Vogel's minimal medium + 2% sucrose at 28° C, for 16 h with shaking. The mycelium was harvested on filter paper washed thoroughly and homogenized in 2.5 ml/g cells (wet wt.) of 30 mM Hepes buffer -- 100 mM potassium acetate -- 2 mM dithiothreitol, pH 7.4, with acid-washed sand in a cold mortar. The homogenate was centrifuged for 15 min at 23,000 g at 4° C and upper two-thirds of the supernatant was collected avoiding the top lipid layer, passed through a Sephadex-G25 coarse column, equilibrated with the same buffer. The fractions following the column void volume, with the highest absorbance at 260 nm, were pooled and frozen at -76° C in 200 µl aliquots.

For studying endogenous protein synthesis, a 20 µl volume contained the cell lysate supplemented with 0.625 mM ATP, 0.250 mM GTP, 25 mM creatine phosphate, 2.5 µg creatine phosphokinase, 100 µM spermine, 25 µM each of- I9 amino acids, 1-2 µl of [^{35}S] methionine (Amersham 10-13 µCi/µl), 0.4 mM Mg acetate and 100 mM K-acetate. Samples were incubated at 21° C for 60 to 90 min., and incorporation of the label into acid-insoluble material was determined by spotting 2-5 µl on filter paper discs.

For translation of exogenously added messenger RNA, 1 ml of the lysate was treated with 12 µg of micrococcal nuclease (Boehringer-Mannheim) in the presence of 1.2 mM $CaCl_2$ by incubation at 20° C for 5 min. The reaction was stopped by addition of EGTA to a final concentration of 2.5 mM This nuclease-treated lysate was used for protein synthesis with 1 to 5 µg RNA and optimized Mg- and K-acetate (0.35 mM and 20 mM, respectively for the RNA enriched in PK specific message) in a final volume of 25 µl as described for the endogenous protein synthesis.

Results: Using this system the incorporation of [^{35}S]methionine into acid-insoluble material increased linearly for 40-60 min. in the endogenous system. The N. crassa lysate subjected to micrococcal nuclease treatment did not support any translation; no increase in incorporation of the label into protein fraction was observed as a function of incubation time. On priming this lysate with Neurospora RNA translation proceeded for about 60 min. At optimal levels of exogenously added RNA, the incorporation of the label approached the level of endogenous translation observed in untreated lysates. Translation of both poly (A)-containing and poly (A-) RNA fractions of Neurospora was supported by this system. Analysis of in vitro translation products of endogenous messengers and exogenously supplied Neurospora mRNA on SDS-polyacrylamide gels revealed a protein profile identical to that demonstrated in extracts of cells pulse-labelled in vivo with [^{35}S]methionine. Polypeptides of up to 200,000 daltons were synthesized in vitro. Pyruvate kinase was detected in the translation products by immunoprecipitation using monospecific antibodies as well as by immunoadsorption on Affigel columns coupled to anti-PK antibody.

It is necessary to establish the optimum K- and Mg-acetate concentration for each type of mRNA as efficiency of translation and its dependence on these salts may vary from message to message. Whereas optimal Mg-acetate requirement for both endogenous and exogenous translation was 0.35 to 0.40 mM, endogenous protein synthesis exhibited a sharp K-acetate optimum at 80 mM but the exogenous PK-RNA enriched fraction was translated more efficiently with 20-50 mM.

Another factor that influenced translation was GTP concentration. With 0.125 mM GTP both endogenous exogenously primed protein synthesis proceeded efficiently for only ~10 min. On the other hand with 0.25 mM GTP it was observed to proceed linearly for at least 40 min. This could be due to the stabilization of initiation factors by higher levels of GTP. Heterologous RNA, such as globin mRNA (BRL) was translated efficiently in this system, the optimum K- and Mg-acetate concentrations being 250 mM and 4 mM, respectively. The translation of exogenous RNA was comparable to that supported by the commercial rabbit reticulocyte lysate (BRL) as witnessed by a similar incorporation of [^{35}S]methionine. - - - Department of Biology, University of Calgary, Calgary, Alberta, Canada, T2N 1N4.