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

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Neurospora crassa

Abstract
Preparation of a cell-free translation system from a wild type strain of *Neurospora crassa*
mid or transforming DNA inserts the wild type gene into a resident, indispensable locus. (Alternatively, one tries to purify this strain, but the transformed nucleus will be maintained in the transformant as the source of the indispensable function. As disruptive integration by a second DNA molecule could occur.) The nucleus in which this happens will be viable as a homokaryon, but will contribute the selected function to a heterokaryotic transformant. The transformed nucleus will be maintained in the transformant as the source of the indispensable function. As one tries to purify this strain, it will keep throwing off the untransformed mutant, and will appear to be an "unstable transformant." This might be interpreted as a plasmid that cannot be maintained, owing to poor replication or to excision. Crosses of the heterokaryotic transformant to wild type or the untransformed mutant will yield no transformants as ascospores in the cases in which transforming DNA is embedded in a disrupted, indispensable gene.

This is almost certainly a banal lesson to those familiar with the biology of Neurospora. However, those unfamiliar with the complications of heterokaryosis should not have to rediscover them first hand. Because this situation has no counterpart in transformation work in yeast, this description is offered to those who might rely unduly on yeast as a paradigm.

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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 investigations 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 chronic 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 0.625 mM ATP, 0.250 mM GTP, 25 mM creatine phosphate, 2.5 µg creatine phosphokinase, 100 µM spermine, 25 µM 0.625 mM Hepes buffer -- 100 mM potassium acetate -- 2 mM dithiothreitol, pH 7.4, with acld-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, equalized 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 - 19 amino acids, 1-2 µl of [35S] 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 µl of the lysate was treated with 12 µg of micrococcal nuclease (Boehringer-Mannheim) in the presence of 1.2 mM CaCl2 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 [35S]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 [35S]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 [35S]methionine.

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A Neurospora crassa mutant which overaccumulates carotenoid pigments.

Previously it was demonstrated that photoinduced carotenoid biosynthesis in Neurospora crassa mycelia shows an unusual temperature dependence (R.W. Harding. 1974 Plant Physiol. 54: 142-147). The primary light reaction is independent of temperature as expected, but the amount of pigment which subsequently accumulates in the dark is temperature dependent, and surprisingly the optimum temperature is 6°C. We have isolated mutants which produce more pigment than the wild-type strain at temperatures above 6°C but about the same amount at 6°C. We have designated this type of mutant as ovc (overaccumulator of carotenoid ds). One of these ovc mutants (S20-16), isolated after UV mutagenesis of cot-4 (70007c, FGSC #1177), has been characterized, and the results are presented here. The ovc locus was then put into a wild-type background by a series of four backcrosses with Em5297a (FGSC #352).

Fig.1 presents the absorption spectra of neutral and acidic carotenoid extracts obtained from dark or light treated wild-type and ovc (S20-16) strains. Treatments were given at 6 vs. 25°C as described in the text. The Em5297a wild-type strain is designated as Em in the figure. The volume of each extract was adjusted to 30 ml hexane/g dry weight of mycelia extracted before absorption spectra were determined.

Figure 1.-- Absorption spectra of neutral and acidic carotenoid extracts (in hexane) obtained from dark or light treated wild-type and ovc (S20-16) strains. Treatments were given at 6 vs. 25°C as described in the text. The Em5297a wild-type strain is designated as Em in the figure. The volume of each extract was adjusted to 30 ml hexane/g dry weight of mycelia extracted before absorption spectra were determined.