such as carbohydrates or fatty acids, are attached to particular proteins during their import and maturation.

9. *Does a Polypeptide Imported in Vitro Become Functional inside the Mitochondria?*

This question is the most difficult to answer. In fact, it has not yet been answered for any polypeptide imported into mitochondria, although a positive result must be obtained before one can conclude that correct import has been achieved *in vitro*. Promising techniques include immunoprecipitation of oligomeric enzymes by antisera directed against subunits other than those whose import is being studied, chemical cross-linking to adjacent subunits, and examination of changes in proteolytic sensitivity of hemoproteins as a result of oxidation or reduction.[18]

[18] G. Basile, C. Dibello, and H. Taniuchi, *J. Biol. Chem.* **255,** 7181 (1980).

[24] Molecular Cloning of Middle-Abundant mRNAs from *Neurospora crassa*

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In exponentially growing hyphae of *Neurospora crassa* three abundancy classes of mRNA have been found,[1] similar to those in other cells. Many nuclear-coded mitochondrial proteins, e.g., the adenine nucleotide translocator, as well as subunits of the ATP synthase, the cytochrome oxidase, and the bc_1 complex, represent a sizable portion of total cellular protein in *Neurospora*.[2-4] The mRNAs of these proteins most likely belong to the middle-abundancy class, and accordingly should occur at a frequency range of around 1 per 1000. After cloning total polyadenylated RNA as cDNA plasmids in *E. coli* there is a high probability of finding one of these middle-abundant species if a few thousand clones are analyzed. Screening such a number of clones based entirely on the translation of hybridization-selected mRNA appears to be feasible. Cell-free translation of mRNA selected by hybridization to immobilized DNA represents an established

[1] L.-J. Wong and G. A. Marzluf, *Biochim. Biophys. Acta* **607,** 122 (1980).
[2] W. Sebald and G. Wild, this series, Vol. 55, p. 344.
[3] H. Weiss and W. Sebald, this series, Vol. 53, p. 66.
[4] H. Weiss, B. Juchs, and B. Ziganke, this series, Vol. 53, p. 99.

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technique for the identification of coding sequences in DNA fragments.[5,6] Hybridization to cDNA plasmids immobilized on nitrocellulose filters has been used to isolate cDNAs complementary to, e.g., the mRNA of interferon[7] and of histocompatibility antigen.[8] Large numbers of cDNA plasmids can be screened in the described experiments, since pooled cDNA plasmids are used for the selection of mRNA.

Principle

The procedure for the identification of a certain cDNA involves four steps: (*a*) the preparation of an ordered cDNA clone bank from total polyadenylated RNA; (*b*) the construction of a cDNA-plasmid bank consisting of DNA covalently coupled to diazobenzoxymethyl paper; (*c*) the isolation of mRNA by hybridization; and (*d*) cell-free translation of selected mRNA, immunoprecipitation of translation products with antibodies against a certain protein, and analysis of translation products and immunoprecipitates by SDS–gel electrophoresis.

The hybridization selection of mRNA is specific enough, and cell-free translation of mRNA is sensitive enough so that many cDNA plasmids can be combined, and bound together to one paper. Thus, hundreds of clones can be analyzed in one experiment. If a plasmid pool has been identified containing a cDNA complementary to the desired mRNA, individual clones can be analyzed. Once a bank of paper-bound cDNA plasmids has been prepared, it can be used several times for the screening for different mRNAs. In the following a protocol is described that allowed the isolation of cDNA encoding the major part of the mRNA of the proteolipid subunit of the mitochondrial ATP synthase from *Neurospora crassa*.[9]

Procedures

Construction of a cDNA Clone Bank

Isolation of Polyadenylated RNA. Hyphae of *Neurospora crassa* SL 74A (FGSC stock No. 987) are grown under aeration in Vogel's minimal me-

[5] J. G. Williams, *in* "Genetic Engineering" (R. Williamson, ed.), p. 1. Academic Press, New York, 1981.

[6] J. R. Parnes, B. Velan, A. Felsenfeld, L. Ramanathan, U. Ferrini, E. Appella, and J. G. Seidman, *Proc. Natl. Acad. Sci. U.S.A.* **78,** 2253 (1981).

[7] S. Nagata, H. Taira, A. Hall, L. Johnsrud, M. Stenli, J. Ecsödi, W. Boll, K. Cantell, and C. Weissmann, *Nature (London)* **284,** 316 (1980).

[8] S. Kvist, F. Bregegere, L. Rask, B. Carni, H. Garoff, F. Daniel, K. Wiman, D. Larhammar, J. P. Abastado, G. Gachelin, P. A. Peterson, B. Dobberstein, and P. Kourilsky, *Proc. Natl. Acad. Sci. U.S.A.* **78,** 2772 (1981).

[9] A. Viebrock, A. Perz, and W. Sebald, *EMBO J.* **1,** 565 (1982).

dium plus 2% sucrose at 30° to a wet weight of 10–15 g/liter.[10] They are harvested by filtration, washed with water, immediately frozen in liquid nitrogen, and pulverized under liquid nitrogen in a Waring blender. From 100 g wet weight of cells, about 750 mg of total RNA are extracted using a phenol medium.[11] After chromatography on poly(U)-cellulose,[12] about 3 mg of enriched polyadenylated RNA are obtained, which are stored in 70% ethanol at −20°.

Preparation of cDNA Recombinant Plasmids. Single-stranded cDNA is synthesized from total polyadenylated RNA according to Friedman and Rosbash[13] omitting actinomycin D and using 1 unit of reverse transcriptase (avian myeloblastosis virus) per microgram of RNA. Yields of cDNA are between 3 and 5%. The second strand is synthesized at 25° for 4 hr in the presence of 1 m*M* of each of the four dNTPs.[14] Thereafter, the DNA is treated with S1 nuclease with a recovery of about 40% of the input cDNA. The double-stranded cDNA is tailed with dC.[15] Plasmid pBR322 is cleaved with *Pst*I, and the linearized form is purified by CsCl gradient centrifugation. After tailing with dG[16] the plasmid DNA is annealed with dC-tailed double-stranded cDNA at a weight ratio of 5 : 1. Transformation of *E. coli* 5K is performed according to Dagert and Ehrlich.[17] Employing 12 ng of the annealed DNAs per 0.1 ml of competent *E. coli* cells, 800–1600 tetracycline-resistant cells are obtained after plating directly on nitrocellulose filters. This corresponds to 400–800 colonies per nanogram of input cDNA. The tailed plasmid annealed in the absence of cDNA yields 10–20 tetracycline-resistant cells, i.e., <2%. Cells of single colonies are inoculated in 50 μl of LB medium (1% tryptone, 0.5% yeast extract, 1% NaCl) plus tetracycline in the 96 wells of a Microtiter plate. After overnight growth, 25 μl of glycerol are added, and the sealed plates are stored at −20°. Analysis of a random sample of 96 clones shows that the cloned cDNA inserts range in size from about 100 bp to 1400 bp.

cDNA-Plasmid Bank

Preparation of Plasmid DNA. Single colonies from the clone bank are inoculated individually into 50 ml of LB medium plus tetracycline (5 μg/ml) and grown overnight at 37° on a rotatory shaker. Each time twelve cultures

[10] W. Sebald, W. Neupert, and H. Weiss, this series, Vol. 55, p. 144.
[11] R. Michel, E. Wachter, and W. Sebald, *FEBS Lett.* **101,** 373 (1979).
[12] R. Sheldon, C. Jurale, and J. Kates, *Proc. Natl. Acad. Sci. U.S.A.* **69,** 417 (1972).
[13] E. G. Friedman and M. Rosbash, *Nucleic Acids Res.* **4,** 3455 (1977).
[14] D. Woods, J. Crampton, B. Clarke, and R. Williamson, *Nucleic Acids Res.* **8,** 5157 (1980).
[15] H. Land, M. Grez, H. Hauser, W. Lindenmaier, and G. Schütz, *Nucleic Acids Res.* **9,** 2251 (1981).
[16] J. H. J. Hoeijmakers, P. Borst, J. Van den Burg, C. Weissmann, and G. A. M. Cross, *Gene* **8,** 391 (1980).
[17] M. Dagert and S. D. Ehrlich, *Gene* **6,** 23 (1979).

are combined, supercoiled plasmid DNA is extracted following a modified protocol[18] of Birnboim and Doly,[19] and is purified by banding once in an 11-ml CsCl gradient. Yields from twelve 50-ml cultures are 50–200 μg of DNA that is slightly contaminated by RNA.

Plasmid DNA (50–100 μg) is partially depurinated by an incubation for 10 min at 25° in 200 μl of 50 m*M* HCl. The DNA is broken and dissociated by adding 0.2 ml of 1 *N* NaOH for 30 min at 37°. The DNA is precipitated with 1 ml of ethanol plus 0.08 ml of 3 *M* sodium acetate, pH 4.8. After a 70% ethanol wash, the sediment is desiccated and dissolved in 10 μl of water. The solution is heated for 2 min at 100°. Then 40 μl of dimethyl sulfoxide plus 0.5 μl of 3 *M* sodium acetate, pH 4.8, are added. The solution is added to two 1 cm^2 circles of activated diazobenzyloxymethyl paper[20] that has been equilibrated with 60 m*M* sodium acetate, pH 4.8, in 80% dimethyl sulfoxide. After incubation at room temperature overnight, the loaded papers are washed once with water and four times for 30 min with 0.5 *N* NaOH at 37°. The papers are rinsed with water until the pH is neutral and stored at 4° in hybridization buffer [50% formamide, 5× SSC = 750 m*M* NaCl plus 75 m*M* sodium citrate, 0.1% SDS, 100 μg of poly(A), and 100 μg of yeast tRNA/ml].

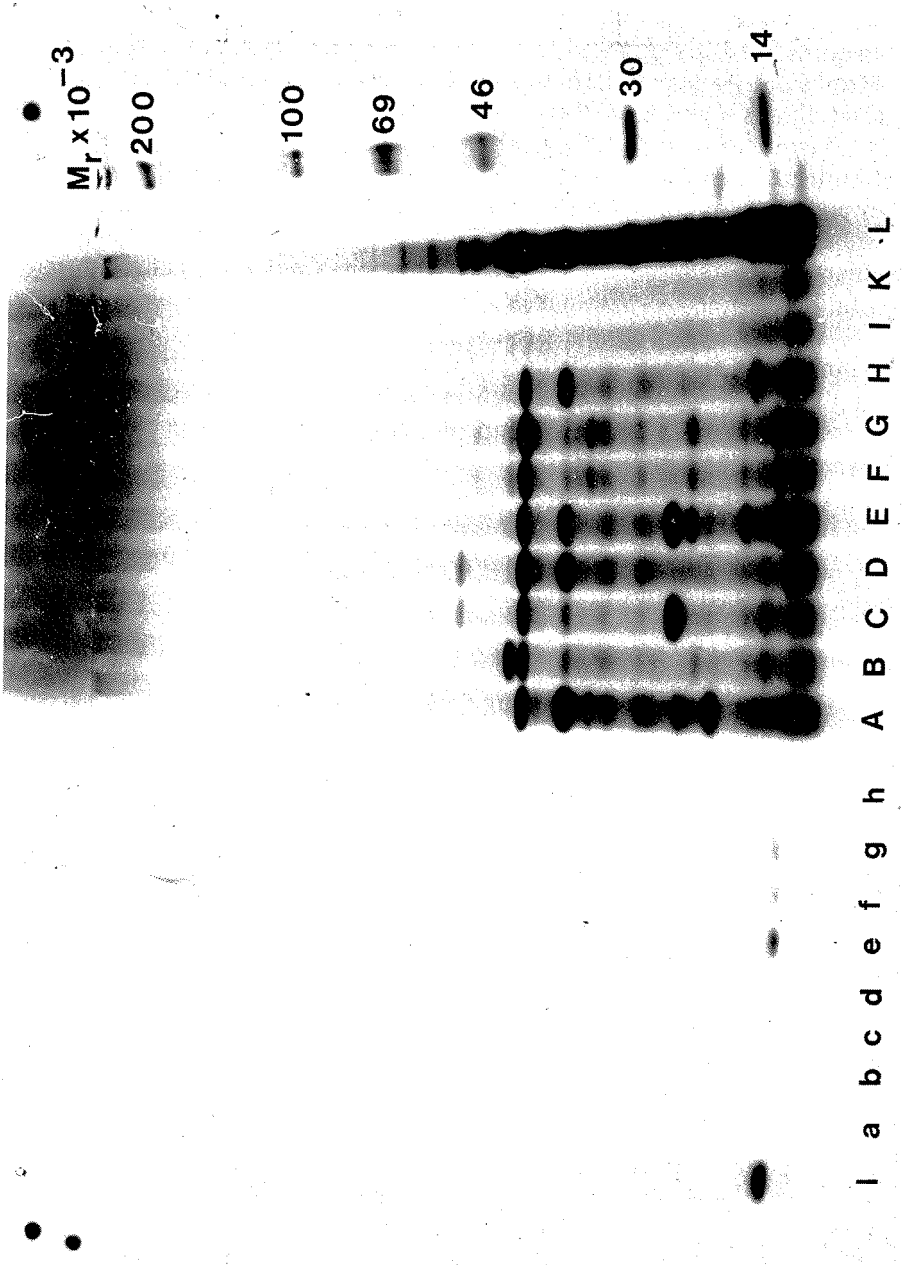
Selections of mRNA by Hybridization

Two circles of DNA paper are soaked with 80 μl of 1.25-fold concentrated hybridization buffer, and 50 μg of polyadenylated RNA dissolved in 20 μl of H_2O are added. Hybridization proceeds for 15 hr at 37°. The papers are then washed twice with hybridization buffer (5 ml), twice with 50% formamide, 0.2× SSC, 30 m*M* sodium phosphate, pH 7.3, 0.1% SDS at 37°. The bound RNA is eluted at 65° with four times 0.1 ml of 98% formamide, containing 5 μg of yeast tRNA per milliliter, 1 m*M* EDTA, 0.1% SDS, and 10 m*M* Tris-HCl, pH 7.5. Particles—mainly disintegrated paper—are removed by centrifugation for 5 min at 18,000 *g*. RNA is precipitated with 1 ml of ethanol after addition of 40 μl of 2 *M* sodium acetate, pH 6. RNA is reprecipitated from 400 μl of water and dissolved in 10 μl of water: 5-μl aliquots are analyzed in a 15-μl wheat germ assay.

Cell-Free Translation of mRNA and Immunoprecipitation

A cell-free protein-synthesizing system is prepared from commercial wheat embryos[21] using an acetate medium.[22] Assay conditions are as de-

[18] F. G. Grosveld, H. M. Dahl, E. de Boer, and R. A. Flavell, *Gene* **13**, 227 (1981).
[19] A. C. Birnboim and J. Doly, *Nucleic Acids Res.* **7**, 1513 (1979).
[20] M. L. Goldberg, R. P. Lifton, G. R. Stark, and J. G. Williams, this series, Vol. 68, p. 206.
[21] B. E. Roberts and B. M. Paterson, *Proc. Natl. Acad. Sci. U.S.A.* **70**, 2330 (1973).
[22] J. W. Davies, A. M. J. Aalbers, E. J. Stuik, and A. Van Kammen, *FEBS Lett.* **77**, 265 (1977).


$M_r \times 10^{-3}$
200
100
69
46
30
14
A B C D E F G H I K L
l a b c d e f g h

scribed[11] employing 1 mCi of [^{35}S]methionine per milliliter. Using 15-μl assays, the system corresponds linearly to added polyadenylated RNA up to 100 μg/ml. Incorporation into hot trichloroacetic acid-insoluble material is 1 to 3 $\times$ 10^6 cpm per microgram of added RNA corresponding to a 10- to 30-fold stimulation over endogenous incorporation.

Protein synthesis is stopped by adding 15 μl of 2% SDS and by heating the mixture for 2 min at 100°. Aliquots (about 10%) are removed for analysis of total translation products. The rest is diluted with 0.5 ml of 0.5% Triton X-100, 0.15 *M* NaCl, 10 m*M* sodium phosphate, pH 7.5. Specific immunoglobulins are added for 1–4 hr at 0°. Immunoglobulins are adsorbed to protein A-Sepharose CL-4B for another 1–15 hr. After washing twice with 1 ml of 0.5% Triton X-100, 0.15 *M* NaCl, 10 m*M* sodium phosphate, pH 7.5, twice with 0.15 *M* NaCl, 10 m*M* sodium phosphate, pH 7.5, and once with 10 m*M* Tris-HCl, pH 7.5, the adsorbed protein is released by boiling for 1 min in 62.5 m*M* Tris-HCl, pH 6.8, 20% glycerol, 2% SDS, 2% 2-mercaptoethanol. Immunoadsorbed proteins and total translation products are analyzed electrophoretically on 12 to 20% concave gradients of SDS–polyacrylamide gels,[23] and radioactivity is visualized by fluorography.[24]

Results

Figure 1 shows an SDS-gel of the total translation products synthesized in response to 8 mRNA populations selected by hybridization to DNA papers each containing 12 pooled cDNA plasmids. The corresponding immunoprecipitates obtained with antibodies to the proteolipid subunit of the mitochondrial ATP synthase from *Neurospora crassa* have been separated on the same gel.

The mRNA from three pools directed the synthesis of a protein that reacted with proteolipid antibodies and exhibited a molecular weight similar to that of the preproteolipid. The twelve clones comprising the positive pool Pl-E have been grown up individually, and single cDNA plasmids have been analyzed as described above. Proteolipid mRNA is specifically selected by

[23] U. K. Laemmli, *Nature* (*London*) **227**, 680 (1970).
[24] W. M. Bonner and R. A. Laskey, *Eur. J. Biochem.* **46**, 83 (1974).

FIG. 1. Translation of mRNAs selected by hybridization to 12 pooled cDNA plasmids. A fluorography of a sodium dodecyl sulfate gel is shown after electrophoretic separation of total cell-free translation products (lanes A–L) and of immunoprecipitates (lanes 1, a–h) obtained with antibodies against the ATP synthase proteolipid subunit. The [^{35}S]methionine-labeled proteins were synthesized in response to total polyadenylated RNA (lanes L, 1), to water (lane K), and to mRNAs selected by 8 pools of cDNA plasmids Pl-A to Pl-H (lanes A–H, and a–h). Each pool contained 12 cDNA plasmids. Lane I shows the translation products synthesized in response to mRNA selected by a paper containing only plasmid pBR322.

the cDNA in clone Pl-E2.[9] (Similarly, one positive clone could be identified among the positive pool Pl-G. This clone, as well as the positive pool Pl-F, have not been further analyzed.) The cDNA insert from clone Pl-E2 represents a copy of about 150 bp of the 3′ end of the proteolipid mRNA including a short poly(dA) tail. Using this insert as a probe, further cDNA clones encoding proteolipid mRNA were identified by colony filter hybridization at a frequency of about 0.2%. One isolated cDNA represented the major part of the proteolipid mRNA. The nucleotide sequence of this cDNA showed 243 bases corresponding to the known amino acid sequence of the mature proteolipid and, in addition, 178 bases coding for an amino-terminal presequence. Noncoding sequences of 48 bases at the 5′ end and of 358 bases at the 3′ end plus a poly(A) tail were determined.[9] The long presequence of 66 amino acids is very polar, in contrast to the lipophilic mature proteolipid, and includes 12 basic and no acidic side chains. It is suggested that the presequence is specifically designed to solubilize the proteolipid for posttranslational import into the mitochondria.

Prospects

The identification of a cloned mRNA by techniques based on the translation of hybridization-selected mRNA depends on few prerequisites, provided that an immunological or another specific test for a protein is available. Thus, this method can be applied in systems where enrichment of mRNA or of cDNA clones is impossible, and where, as in yeast, genetic complementation of defined mutants by cloned genomic DNA cannot be applied. The screening of large numbers of cDNA clones appears to be possible. Several thousand cDNA plasmids in groups of 96 pooled clones were covalently bound to paper, and in several of these pools a cDNA complementary to the mRNA of the adenine nucleotide translocator from *Neurospora* was identified (H. Arends and W. Sebald, unpublished results). The adenine nucleotide translocator[25] and the ATP synthase proteolipid are on a molar basis the most abundant mitochondrial proteins in *Neurospora*. The cloned mRNA of these two proteins is therefore most easily isolated. It is a distinct possibility, however, that the cloned mRNAs of other mitochondrial or cellular proteins occurring at a molar ratio up to 10-fold lower can be identified by the described approach. It is hoped that once a bank of paper-bound cDNA plasmids has been prepared, it can be utilized for several rounds of hybridization for various proteins.

[25] H. Hackenberg, P. Riccio, and M. Klingenberg, *Eur. J. Biochem.* **88,** 373 (1978).