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Antibodies against synthetic oligopeptides allow identification of the mRNA-maturase encoded by the second intron of the yeast *cob-box* gene

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Genetic and biochemical evidence has strongly suggested that several introns located in yeast mitochondrial genes specifying apocytochrome b or cytochrome oxidase encode *trans*-acting proteins (termed mRNA-maturases) responsible for splicing the cognate intron and maturation of the mRNA. We have chemically synthesized three oligopeptides, predicted from the DNA sequence of the open reading frame (ORF) present in the second intron of the *cob-box* gene, and raised antibodies against them. These antibodies have allowed us to identify a protein of 42 kd as the product translated from the ORF of the wild-type intron. In two splicing-deficient mutants this protein is replaced by shorter polypeptides whose lengths and antigenic properties are in full agreement with the positions of TAA codons established by the DNA sequence of the intron's ORF.

Key words: mitochondria, mRNA-maturase/yeast/antibodies/synthetic oligopeptides

Introduction

A salient feature of the organisation of many mitochondrial genes in fungi is their discontinuous structure. More than 25 introns have already been identified in four genes of various fungal species (see 'Mitochondria 1983' for recent overviews). In particular, the *Saccharomyces cerevisiae cob-box* gene which codes for apocytochrome b displays a mosaic organisation comprising, in the strain 777-3A, six exons and five introns (Lazowska *et al.*, 1980). Although they were discovered 6 years ago, the biological function, if any, of introns is still unknown. From genetic analysis of numerous splicing-deficient intron mutants, it has been inferred that three introns of the *cob-box* gene direct the synthesis of specific diffusible molecules whose presence is required for the proper functioning of the splicing machinery (Slonimski *et al.*, 1978; Church *et al.*, 1979; Haid *et al.*, 1979; Lamouroux *et al.*, 1980). In addition, physiological (Dujardin *et al.*, 1980; Groudinsky *et al.*, 1981; Jacq *et al.*, 1982; Kruszewska, 1982) as well as biochemical evidence (Lazowska *et al.*, 1980, 1981 and in preparation; Bechmann *et al.*, 1981; De la Salle *et al.*, 1982; Weiss-Brunner *et al.*, 1982; Anziano *et al.*, 1982) has strongly suggested that the diffusible product is a protein which has been named mRNA-maturase. However, no direct evidence exists at present showing that introns are indeed translated into proteins.

To identify this putative protein we used antibodies raised against synthetic peptides whose amino acid sequences were predicted from the known second cytochrome b intron DNA sequence. We show that two 'anti-peptide' antisera specifically immunoprecipitate a mitochondrially synthesized protein of 42 kd in extracts of ^{35}S-labelled mitochondria. In two splicing-deficient mutants this protein is replaced by shorter polypeptides whose lengths and antigenic properties are in full agreement with the position of TAA codons established by DNA sequence analysis of the second intron open reading frame (ORF).

Results

The choice of peptides to be synthesized and used as antigens

The *cob-box* gene of the *S. cerevisiae* strain 777-3A consists of six exons and five introns. The DNA sequence of intron bi2, has been determined by Lazowska *et al.* (1980, 1983 and in preparation). This 1406-bp intron contains an ORF 840 bp long in phase with the upstream exon, followed by a 566-bp blocked frame. Using the yeast mitochondrial genetic code (which differs from the standard genetic code, cf. Hudspeth *et al.*, 1982) the amino acid sequence of the corresponding protein could be deduced. This sequence corresponds to a strongly basic protein with many hydrophobic regions. Since antigenic determinants are usually found amongst hydrophilic domains of a protein (Hopp and Woods, 1981), we analyzed the hydrophilicity of the amino acid sequence according to these authors. As shown on Figure 1 the putative intron-encoded protein comprises in its second half several hydrophilic peptides separated by long hydrophobic stretches. These hydrophilic regions could be used as antigenic probes for the putative protein. As a further constraint we selected peptide sequences which would be identical using either mitochondrial or the standard genetic code. From this we programmed the chemical synthesis of three peptides which were used as haptens for the immunization of rabbits. Their sequence and position (see Figure 1) are:

Peptide I: Glu-Lys-Arg-Lys-Gly-Gly-Lys-Gly-Thr-Arg position 709–738 bp
Peptide II: Arg-Leu-Gly-Lys-Lys-Gly-Lys-Ile-Arg-Gln-Tyr position 844–876 bp
Peptide III: Phe-Ser-Tyr-Lys-Asp-Val-Gln-Tyr position 1075–1098 bp

The choice of mutants to be analysed

According to the model proposed by Lazowska *et al.* (1980), mRNA-maturases should be found, in very low amounts, in wild-type strains. Indeed, their mechanism of action assumes that they destroy their own mRNA by the splicing and maturation of cytochrome b pre-mRNA. This autogenous regulation implies that they should appear in only catalytic amounts in the mitochondrial compartment. However, the model predicts the existence of conditions where the maturases should be overproduced. The intron upon which they act is not only the template for their synthesis but also the substrate for their activity. It should be possible to dissociate the two functions by mutations, i.e., to obtain mutants retaining the template activity but losing the substrate properties. The mutant G5046 is a case in point. It fails to excise the intron bi2 (Halbreich *et al.*, 1980) but it re-

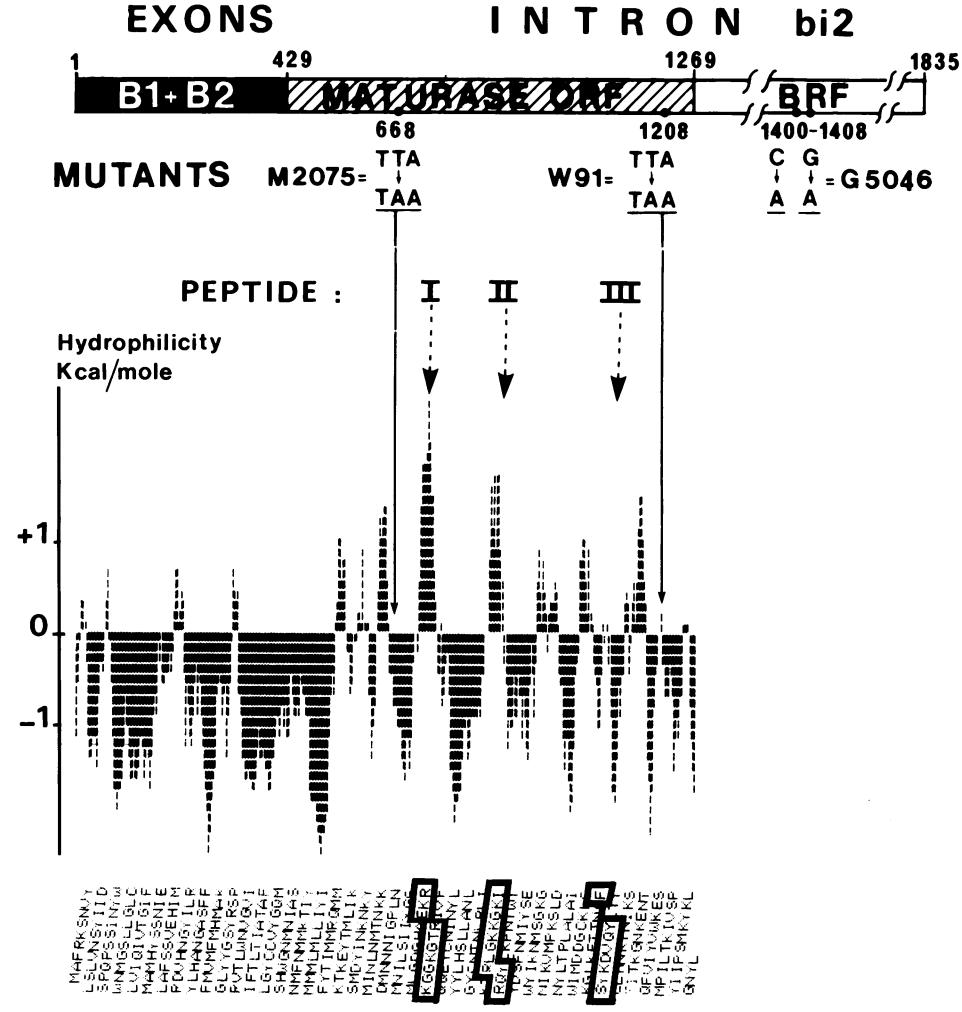

Fig. 1. The upper bar represents schematically the primary sequence of the putative wild-type bi2 mRNA-maturase encoded, after splicing of the first bi1 intron, by the first two exons (B1 and B2, black area positions 1–429 bp) and the ORF of the second intron (hatched area, positions 430–1269 bp) (Lazowska *et al.*, 1980, 1983 and in preparation). The position and nature of the three mutants analysed is indicated below the bar: two of them are *trans*-recessive and located in the ORF; as a result of nonsense mutations they accumulate a truncated maturase (arrows). The third mutant is *cis*-dominant and located in the blocked reading frame (BRF) of the intron; as a result of structural modifications of the RNA substrate it accumulates a full-length maturase. The middle panel represents local hydrophilicity of the maturase sequence, as deduced from the DNA sequence, and computed according to Hopp and Woods (1981). At each position of the sequence the hydrophilicity was averaged over the nearest seven amino acids with the help of a computer program developed by A.Henaut. The position of the three peptides which have been synthesized are shown by dotted arrows. The entire, 423 amino acid sequence of maturase is shown in the bottom panel and the peptides used as antigenic probes are encircled. Peptides I and II correspond to the most hydrophilic parts of the sequence.

tains the ability to complement in *trans* mutants defective in intron bi2 encoded splicing activity (Lamouroux *et al.*, 1980). This implies that the mutant is able to synthesize the intron-encoded diffusible product. DNA sequence established by J.Lazowska (Jacq *et al.*, 1982; Lazowska *et al.*, in preparation) shows that in the mutant G5046 several base substitutions have modified the sequence of the non-translatable 3′ part of the intron (Figure 1), presumably preventing the productive folding of the intron necessary for splicing to occur. We have used this mutant as an overproducer of an active maturase. Two other mutants should, according to the model, also overproduce an inactive maturase. They are W91, in which an ochre codon has replaced a leucine codon at the position 1208 of the putative maturase (Lazowska *et al.*, 1980), and M2075 which also creates a stop codon upstream in the sequence (see Figure 1, details of the establishment of its sequence are given in Material and methods). Both mutants fail to excise the intron bi2 (Halbreich *et al.*, 1980; Schmelzer *et al.*, 1981) and are complemented in *trans* by the mutant G5046 (Lamouroux *et al.*, 1980). These mutants have been used as overproducers of truncated translates of the intron.

Immunoprecipitation of mitochondrially synthesized proteins

We have labelled the mitochondrially synthesized proteins in the presence of cycloheximide and asked whether antisera raised against the three synthetic peptides would immunoprecipitate the active or truncated maturases produced by the mutants G5046, W91 and M2075. As can be seen in Figure 2 antisera raised against peptide I and peptide II were clearly able to immunoprecipitate a protein of 42 kd in the mutant G5046 (lanes J and K) and a protein of 38 kd in the mutant W91 (lanes F and G). Antisera collected before immunization (lanes A and I, for E see below), as well as the antiserum raised against peptide III (data not shown) failed to precipitate labelled proteins. Furthermore, no precipitation was observed with labelled proteins from the mutant M2075 (lanes B and C). These results are consistent with the predicted position of

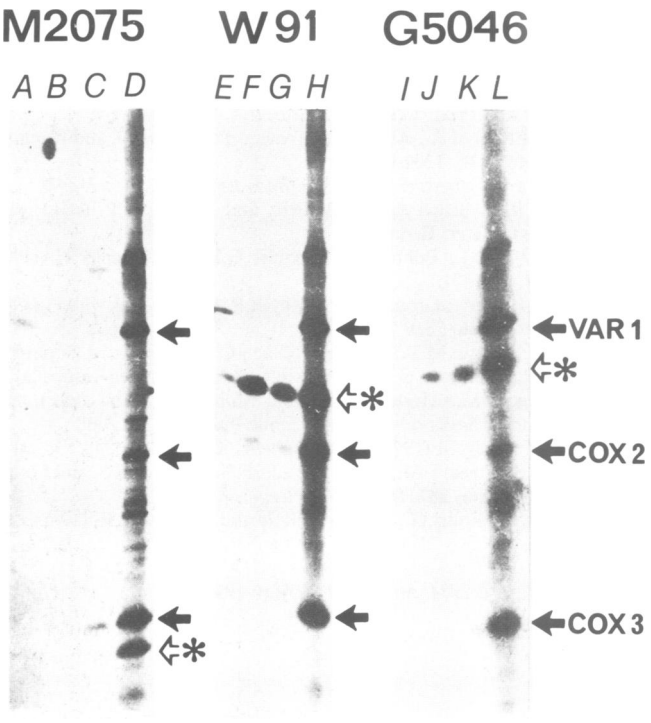

Fig. 2. Electrophoretic analysis of mitochondrial proteins immunoprecipitated with antisera raised against synthetic peptides. Mitochondria were labeled in the presence of cycloheximide under conditions selective for mitochondrial protein synthesis (see Materials and methods). Mitochondrial lysates were immunoprecipitated either with the control, pre-immune rabbit serum (**lanes A, E** and **I**) or with sera raised against peptide I (**lanes B, F** and **J**) or peptide II (**lanes C, G** and **K**). Total, non-precipitated mitochondrial lysates were electrophoresed in **lanes D, H** and **L**. The nature and position of mutations in the three mutants analysed: M2075 (**lanes A – D**), W91 (**lanes E – H**) and G5046 (**lanes I – L**) are shown in Figure 1. The asterisks indicate in each case the position of the mRNA-maturase. Position of the ribosomal protein Var1 and of the cytochrome oxidase subunits II and III are also shown.

the termination codon in this mutant (upstream of the sequences used for each of the peptides, see Figure 1). For reasons which are unclear, in some experiments and not in others, some immunoprecipitations of proteins corresponding to Var1, subunit III of cytochrome oxidase or to the 38-kd protein are observed with pre-immune serum. These immunoprecipitations were always very weak in comparison with the immunoprecipitation of the 42-kd or 38-kd proteins observed with immune sera and therefore considered non-specific. Solioz and Schatz (1979) have already reported similar unspecific precipitations with some rabbit sera.

Discussion

Starting from a mitochondrial intron sequence, we could raise antibodies against the putative translation product of this sequence by synthesizing specific peptides predicted by the sequence. These antibodies were able to immunoprecipitate a 42-kd protein in a mutant overproducing the active maturase. Moreover, truncated and inactive maturases having translation termination mutations located downstream from the sequence of selected immunogenic peptides were also immunoprecipitated by the anti-peptide sera, whereas shorter polypeptides terminating upstream from these antigenic peptides were not (compare mutants W91 and M2075, Figures 1 and 2). Thus the genetic, nucleotide sequence and immunological results agree perfectly. Previous work has shown that the position of chain-terminating mutations in the intron bi2 ORF could be correlated with the size of mitochondrially encoded proteins, that these proteins were recognized by the cytochrome b antisera and that they shared extensive fingerprint homologies between themselves and only limited structural homologies with apocytochrome b (Lazowska *et al.*, 1980; Bechmann *et al.*, 1981; Jacq *et al.*, 1982). Taken together with the data of the present work, the overall results demonstrate that the upstream cytochrome b exons and the downstream intron are translated in mitochondria into a chimaeric protein and identify this protein with the mRNA-maturase required for splicing of the second intron of the gene. The technique we have used, raising antibodies against peptides predicted from a DNA sequence, has been also used with success by others (Bhatnagar *et al.*, 1982; Lerner *et al.*, 1981; Mariottini *et al.*, 1983; Sutcliffe *et al.*, 1980). The fact that, in our case, the most hydrophilic peptides were antigenic, while a more hydrophobic one was not, corroborates the validity of the method (cf. Hopp and Woods, 1981). More than 20 ORFs have already been found in numerous mitochondrial introns (cf. 'Mitochondria 1983' for reviews). Although it is generally believed that they encode proteins, in no single instance has proof been adduced. The method we have applied in the present work may be helpful in identifying the production of other intron-encoded proteins. An alternative method is described in Jacq *et al.* (1984). The availability of antibodies recognizing specific proteins involved in splicing should be useful in unravelling the biochemical mechanism of this process.

Materials and methods

Synthesis of peptides

Synthetic deca (I), undeca (II) and octapeptides (III) (see Figure 1) were prepared according to the liquid-phase, mixed anhydride method (Beyerman, 1972). They appeared to be homogeneous on t.l.c. and were characterized by elemental microchemical and amino acid analysis.

Coupling of the synthetic peptides to carrier protein and immunization procedures

All peptides were coupled to keyhole limpet hemocyanin (KLH) using glutaraldehyde. 1 mg of peptide and 10 mg of KLH were dissolved in 2 ml of sodium phosphate buffer (0.1 M, pH 7). 1 ml of glutaraldehyde (2.5%) was added dropwise over 1 h at room temperature. The reaction was stopped by addition of 0.2 ml of 1.5 M lysine. Rabbits received 200 μg of peptide-coupled KLH in complete Freund's adjuvant in 20 intradermal injections in the back. Two months later, the rabbits were boosted with the same emulsion. At varying time intervals beginning 1 week after the booster injections the rabbits were bled. The antiserum was stored at 4°C in the presence of 0.1% sodium azide. The anti-peptide antisera were tested using an indirect ELISA assay as described by Pfaff *et al.* (1982) with the following modifications: the coating of the plates with the antigen was performed at 4°C overnight in 0.1 M sodium carbonate pH 9.4. All subsequent washings were done with sodium phosphate buffer containing 0.1% Tween 20 (Merck, Darmstadt). In order to detect bound antibodies, we used a sheep anti-rabbit antibody coupled to horse radish peroxidase (I.P.P. Paris).

Labelling and immunoprecipitation of mitochondrial translation products in various yeast strains

The labelling of proteins translated from the mitochondrial genome was performed as described by Douglas *et al.* (1979) modified according to Claisse *et al.* (1977). For immunoprecipitation studies, labelled mitochondria were gently stirred in phosphate-buffered saline containing 0.5% Nonidet P-40 (NP-40), 0.5% sodium deoxycholate and 1% SDS at 0°C. After centrifugation (10 min, 15 000 *g*, 4°C) ~50% of the radioactivity was recovered in the supernatant. This supernatant was diluted with buffer containing 0.5% NP-40 and 0.5% sodium deoxycholate to adjust the SDS concentration to 0.1%, then incubated with normal or anti-peptide rabbit serum (1/25 v/v) at 0°C for 15 h. Complexes were collected by centrifugation after an incubation for 30 min at 0°C with 5 mg of protein A Sepharose (Pharmacia). The pellet was washed five times with the incubation buffer and loaded on an SDS-polyacrylamide gel. Gels were treated for fluorography, dried and exposed to pre-

fogged film at −70°C. All yeast strains were isogenic except for the *cob-box*$^-$ mutation involved. They were obtained by crossing the original mutants derived from strain 777-3A by a *rho*^{o} strain KL14-4A/60 (for details see Lazowska *et al.*, 1980).

DNA sequence of intron mutants

The sequence of the mutant W91 has been reported previously (Lazowska *et al.*, 1980), that of G5046 has been established by J.Lazowska (Jacq *et al.*, 1982; Lazowska *et al.*, in preparation) and that of M2075 has been established in this work. For this purpose the mtDNA from strain 777-3A/M2075 (Bechmann *et al.*, 1981) was purified according to De la Salle *et al.* (1982), restricted with *Bam*HI + *Bgl*II endonucleases and inserted into pBR322 cut with *Bam*HI. After transformation, A^RT^S recombinant plasmids were screened by colony hybridization using a specific intron bi2 probe. The DNA sequence was established by the Maxam and Gilbert (1980) procedure from the unique *Ava*II site localised in the bi2 intron. Only one change, with respect to the wild-type, was found in a stretch of 310 bp analyzed: a T→A transversion transforming, in the ORF of the intron, a Leu codon into ochre at position 239 of the intron (see Figure 1). This change is the one responsible for cytochrome b deficiency as shown by restoration tests with discriminating *rho*$^-$ mutants which allocate M2075 to the interval 11 of the the *box3* locus (see Lazowska *et al.*, 1980 for further details). Thus, genetic and sequence data agree perfectly.

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